

Original Contributions

Apolipoprotein(a) Kringle IV Repeat Number Predicts Risk for Coronary Heart Disease

H.G. Kraft, A. Lingenhel, S. Köchl, F. Hoppichler, F. Kronenberg, A. Abe, V. Mühlberger, D. Schönitzer, G. Utermann

Abstract A high plasma concentration of lipoprotein(a) [Lp(a)] has been suggested as a risk factor for coronary heart disease (CHD), but some recent prospective studies have questioned the significance of Lp(a). Lp(a) concentrations are determined to a large extent by the hypervariable apo(a) gene locus on chromosome 6q2.7, which contains a variable number of identical tandemly arranged transcribed kringle IV type 2 repeats. The number of these repeats correlates inversely with plasma Lp(a) concentration. We analyzed whether apo(a) gene variation (kringle IV repeat number) is associated with CHD. Apo(a) genotypes were determined by pulsed-field gel electrophoresis/genomic blotting in CHD patients who had undergone angiography (n=69) and control subjects matched for age, sex, and ethnicity (n=69) and were related to Lp(a) concentration,

apo(a) isoform in plasma, and disease status. Apo(a) alleles with a low kringle IV copy number (<22) and high Lp(a) concentration were significantly more frequent in the CHD group ($P<.001$), whereas large nonexpressed alleles were more frequent in control subjects. The odds ratio for CHD increased continuously with a decreasing number of kringle IV repeats and ranged from 0.3 in individuals with >25 kringle IV repeats on both alleles to 4.6 in those with <20 repeats on at least one allele. This provides direct genetic evidence that variation at the apo(a) gene locus, which determines Lp(a) levels, is also a determinant of CHD risk. (*Arterioscler Thromb Vasc Biol.* 1996; 16:713-719.)

Key Words • kringle IV • lipoprotein(a) • coronary heart disease • apolipoprotein(a) • genetic risk factors

The analysis of quantitative genetic traits and their relation to complex disease, eg, CHD, still pose a major challenge. Lp(a) represents a quantitative trait in human plasma,¹ but the relation of Lp(a) to CHD is still controversial.¹ Plasma Lp(a) is assembled from LDL and the high-molecular-weight glycoprotein apo(a).¹ The plasma concentration of Lp(a) shows extreme variation (>1000-fold) among subjects from all human populations studied. Lp(a) levels have been reported to range from almost undetectable (<0.1 mg/dL) to >300 mg/dL. The distribution of Lp(a) concentrations is highly skewed to low levels in Caucasian and Asian populations.² Mean (median) concentrations also differ significantly between ethnic groups. In a given healthy individual, however, Lp(a) concentration is a rather stable genetic marker and almost unaffected by sex, age, diet, or physical exercise.¹

The discovery of a genetic size polymorphism [apo(a)] and the inverse association of apo(a) size with Lp(a) concentration¹ identified the structural gene for apo(a)³ as the major gene that regulates Lp(a) levels in human plasma. The apo(a) gene is highly homologous to the plasminogen gene.³ One motif in this gene, which codes

for a plasminogen-like kringle IV structure, is repeated severalfold in the apo(a) gene.³ The number of kringle IV repeats varies among subjects and ranges from 11 to >50,^{4,5} which explains the apo(a) size polymorphism. Several alleles are expressed at levels too low to be detected by immunoblotting.^{4,6}

Numerous but not all retrospective case-control studies have found an association between high Lp(a) concentration and premature CHD (for reviews, see References 1 and 2), but it was unclear whether this was primary or secondary to disease. Prospective studies have provided contradictory results. Whereas four identify Lp(a) as a risk factor for myocardial infarction,^{4,7-9} two do not.^{10,11} In a multipopulation study¹² apo(a) isoforms that were associated with high Lp(a) concentrations were shown to be more frequent in CHD patients than in control subjects in each of six ethnic groups. Protein isotyping of apo(a), however, fails to detect all apo(a) size alleles. Studies relating apo(a) isoforms to CHD are therefore necessarily biased.

In this study we carried the analysis one step further by studying the association of apo(a) alleles defined by DNA typing with the risk for CHD and observed a significant negative correlation between the number of kringle IV repeats in the apo(a) gene and CHD risk.

Methods

Subjects

CHD patients were selected from Tyrolean patients referred to the Department of Internal Medicine, University of Innsbruck, for the evaluation of suspected CHD by coronary angiography during the summer and fall of 1992. In none of the patients were Lp(a) levels known before angiography. The

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From the Institute for Medical Biology and Human Genetics (H.G.K., A.L., S.K., F.K., A.A., G.U.) and the Departments of Internal Medicine (F.H., V.M.) and Blood Transfusion (D.S.), University of Innsbruck, Innsbruck, Austria.

Correspondence to Prof Dr G. Utermann, Institute for Medical Biology and Human Genetics, Schöpfstr 41, A-6020 Innsbruck, Austria.

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Selected Abbreviations and Acronyms

CHD	= coronary heart disease
HDL-C	= HDL cholesterol
Lp(a)	= lipoprotein(a)
OR	= odds ratio
TC	= total cholesterol

inclusion criterion into this study ($\geq 50\%$ obstruction of one of the great coronary arteries) was met by 124 individuals. Blood (10 mL) was drawn into EDTA-containing tubes after an overnight fast. Plasma and white blood cells were separated from these specimens to perform the laboratory tests and to isolate the genomic DNA, respectively.

The control group was recruited from 224 unrelated voluntary blood donors from the same geographical area and the same ethnicity as the patients. All these individuals were healthy according to physical examination, determination of blood pressure, and a series of laboratory tests.¹² They were therefore considered a healthy population sample. Because the control subjects were considerably younger than the patients, subgroups of age- and sex-matched patients and control subjects were drawn at random. For each successive CHD patient the first control subject who matched for sex and age was drawn from the computer file. This resulted in 69 matched pairs. No control subject counterparts were obtained for 55 patients; this group was therefore not included in the further analysis. Mean (median) Lp(a) concentration and KpnI allele frequencies were not different between the 69 patients who were included in the study and the 55 excluded patients. The 69 control subjects can be considered as true representatives of the "normal" Tyrolean population. The mean age for each group was 51.0 ± 8.1 years (range, 29 to 66 years), and each group consisted of 55 men and 14 women. Forty-one of the CHD patients had suffered a myocardial infarction.

Laboratory Procedures

TC and HDL-C were assayed with commercial enzymatic test kits (Boehringer-Mannheim). Plasma levels of Lp(a) protein were measured by a sandwich enzyme-linked immunosorbent assay with a polyclonal, affinity-purified rabbit anti-Lp(a) antibody for coating and the horseradish peroxidase-labeled monoclonal anti-apo(a) antibody 1A² for detection.¹³

Apo(a) Protein and DNA Phenotyping

Apo(a) isoforms were determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting exactly as described⁴ with the monoclonal anti-apo(a) antibody 1A². The sensitivity of the immunoblotting procedure was determined by applying serial dilutions of plasma with only one expressed isoform. All apo(a) isoforms that had a concentration >0.6 mg apo(a) glycoprotein/dL were detected.

The determination of the apo(a) KpnI alleles by pulsed-field gel electrophoresis/Southern blotting was performed exactly as described.⁴ Apo(a) alleles were designated according to their number of kringle IV repeats.

Statistical Analysis

The nonparametric Wilcoxon rank sum test was applied to compare Lp(a) levels between patients and control subjects. TC and HDL-C concentrations were compared by one-way ANOVA.

Apo(a) KpnI allele frequencies were compared between case and control subjects by Pearson's χ^2 statistic. Because of the high number of apo(a) KpnI alleles, the individual cells contained very low numbers. To account for this problem and also

to compare the data with those previously obtained by protein phenotyping,¹² KpnI alleles were binned into four groups corresponding to the originally described protein isoforms.¹⁴ Group 1 includes three apo(a) size alleles with 17 to 19 kringle IV repeats corresponding to S1 type isoforms. Accordingly, groups 2 and 3 each include three size alleles corresponding to S2 and S3 isoforms, respectively, and group 4 encompasses all apo(a) alleles with >25 kringle IV repeats (S4 isoform). Additionally, the comparison of KpnI allele frequencies was done with a Monte Carlo simulation method to derive probability values for Fisher's exact test.¹⁵ The significance values that were obtained with this method did not differ from those employing the χ^2 statistic.

The Spearman rank correlation coefficient was calculated to estimate the correlation between apo(a) KpnI alleles and Lp(a) levels.

To estimate the fraction of the increase in Lp(a) concentration that could be explained by the difference in the allele frequencies, the following procedure was used. The mean apo(a) size allele-associated Lp(a) concentration was calculated from a Tyrolean population sample ($n=224$) exactly as described.¹⁶ With the use of these values and the allele frequencies in the patients, an "expected" mean Lp(a) concentration was calculated for the patients' group. The difference between this value and the Lp(a) concentration observed in the control subjects defines the fraction of the increase in Lp(a) that is explained by differences in allele frequencies.

The contribution of apo(a) gene variation to the variance of Lp(a) levels in CHD patients and control subjects was estimated by R^2 statistics of the ANOVA.

To estimate the risk imposed by small KpnI alleles (kringle IV repeats 11 to 22, corresponding to isoforms F, B, S1, and S2⁴) or by high Lp(a) plasma concentrations (>30 mg/dL), ORs were calculated from 2×2 contingency tables. Stepwise logistic regression analysis was performed to estimate the relationship between disease status and the variables apo(a) KpnI allele numbers, the sum of the allele numbers, and cholesterol, HDL-C, and Lp(a) concentrations. For all calculations SPSS for Windows, version 5.0 (SPSS Inc) was used.

Results

Distribution of Lp(a) Concentration in CHD Patients and Control Subjects

TC, HDL-C, and Lp(a) concentrations were compared between CHD patients who had undergone angiography (defined by $>50\%$ stenosis of one large vessel) and healthy control subjects matched for age and sex and identical in ethnicity. The mean \pm SD cholesterol levels in CHD patients and the control group were 225 ± 54.1 and 211 ± 42.4 mg/dL, respectively (NS). This difference was further reduced when the contribution that Lp(a)-cholesterol adds to the TC level was subtracted (data not shown). HDL-C levels did not differ between the two groups (patients, 47.6 mg/dL; control subjects, 47.8 mg/dL).

Mean and median Lp(a) levels were significantly increased in the patient group (Fig 1), and the distribution of Lp(a) concentration was also significantly different between CHD patients and healthy control subjects ($P=.015$). In the control subjects the distribution was highly skewed toward low concentrations, which is characteristic of Caucasian populations (skewness=1.536). In the patient group the distribution was much less skewed (skewness=0.960). Only patients had Lp(a) levels >100 mg/dL, and the frequency of subjects with

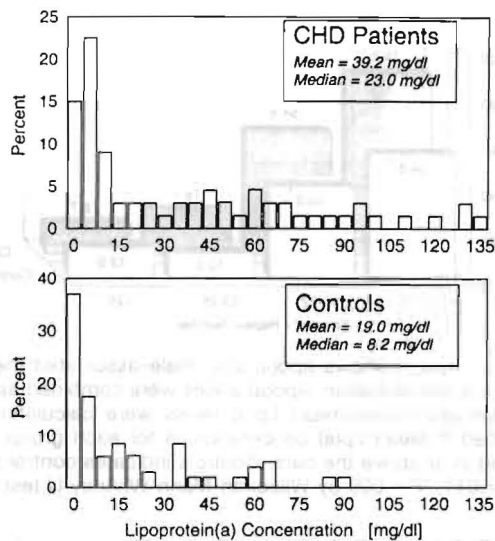


FIG 1. Bar graphs show distribution of Lp(a) plasma concentrations in CHD patients and control subjects (Controls).

Lp(a) levels >30 mg/dL was significantly higher in the patient (44.9%) than in the control (23.2%; $P<.01$) group.

TABLE 1. Apo(a) Allele Frequencies

K-IV Repeats, n	CHD Patients		Control Subjects	
	All†	Expressed‡	All†	Expressed‡
17	1	1	0	0
18	2	2	0	0
19	9	8	3	3
20	11	11	5	4
21	9	8	4	3
22	6	5	3	3
Sum of small alleles	38	35	15	13
23	11	8	8	5
24	7	4	6	3
25	5	5	5	4
26	5	4	5	4
27	6	3	9	5
28	7	6	19	11
29	12	8	15	8
30	6	2	11	3
31	10	5	10	6
32	14	6	9	5
33	4	2	7	4
34	8	3	7	1
35	2	1	8	2
36	2	0	0	0
37	0	0	0	0
38	0	0	3	1
39	1	0	0	0
42	0	0	1	0
Sum of large alleles	100	57	123	62
Total	138	92	138	75

*Kpn I allele was determined by pulsed-field gel electrophoresis/Southern blotting.

†Apo(a) isoform was detected in plasma by immunoblotting.

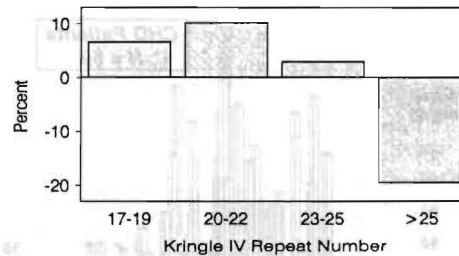


FIG 2. Histogram demonstrating the excess of apo(a) size allele frequencies in CHD patients. Apo(a) size alleles were categorized into four groups corresponding to the original apo(a) isoforms (see "Methods").

Apo(a) KpnI Allele and Isoform Frequencies in CHD Patients and Control Subjects

Apo(a) KpnI alleles were determined in the two groups (Table 1). A total of 23 apo(a) size alleles were present with kringle IV repeat numbers ranging from 17 to 42. In accordance with previous studies and with the high number of alleles, 93% of the subjects were heterozygous for apo(a) size alleles and only 7% were homozygotes.

For statistical evaluation and also to compare with the protein isoforms from immunoblots, KpnI alleles were binned (see "Methods"). The frequencies of KpnI alleles with 17 to 22 kringle IV repeats were significantly higher in the patient group, whereas KpnI alleles with >25 kringle IV repeats were significantly less frequent in the patients ($P<.001$ by χ^2 test). The excess of apo(a) KpnI allele frequencies in CHD patients is illustrated graphically in Fig 2.

Apo(a) isoforms were determined by sodium dodecyl sulfate electrophoresis followed by immunoblotting with monoclonal antibody 1A² in all study subjects. The frequencies of the apo(a) protein isoforms are given in Table 1. The number of subjects with no detectable apo(a) isoform in plasma was higher in the control subjects ($n=11$) than in the patients ($n=7$). The simultaneous determination of apo(a) DNA types and protein isoforms establishes whether the apo(a) allele is expressed. Ninety-two of the 138 apo(a) alleles analyzed in each group were expressed in the CHD patients and 75 were expressed in the control subjects. The percentage of detectable alleles was significantly ($P<.01$ by χ^2 test) higher in patients (66.7%) than control subjects (54.3%). In both groups >90% of the nonexpressed alleles belonged to "large" apo(a) alleles (>22 kringle IV repeats), which are associated with low Lp(a) levels. Thus, large and nonexpressed apo(a) alleles were less frequent in CHD patients.

Apo(a) Type-Associated Lp(a) Concentration in Patients and Control Subjects

As in previous studies, a significant inverse association between Lp(a) plasma levels and apo(a) isoform/fragment size was noted.^{1,4,5,6} A graphical representation of this correlation is given by three-dimensional graphs in Fig 3. The R^2 value from the ANOVA, which quantifies this association, was .5 for the patients and .3 for the healthy control subjects. The rank correlation coefficient between Lp(a) concentration and the number of kringle

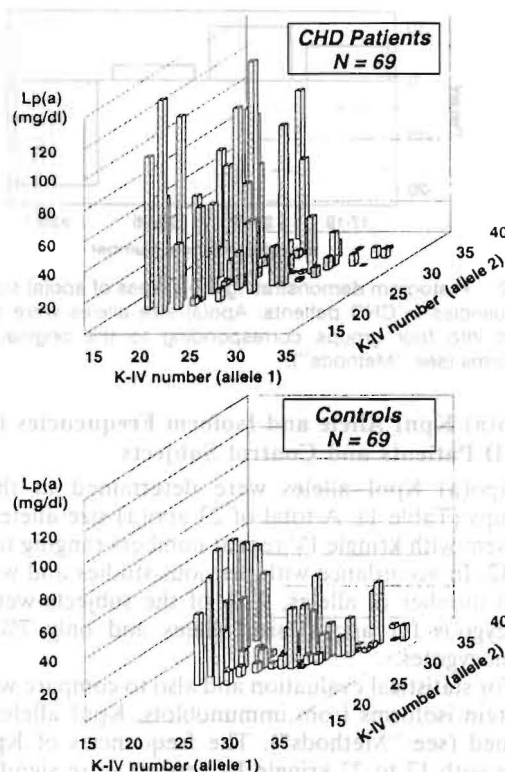


FIG 3. Three-dimensional graph illustrates the negative correlation between the number of kringle IV repeats in the apo(a) alleles (x and y axes) and Lp(a) plasma concentration (z axis) in the groups of CHD patients and control subjects (Controls).

IV repeats in the smaller apo(a) allele of an individual was $-.671$ ($P<.0001$) and $-.419$ ($P<.001$) for the patients and control subjects, respectively.

The increased frequency of small apo(a) KpnI alleles (kringle IV repeats 17 to 22) and decreased frequency of large apo(a) alleles (kringle IV repeats 23 to 42) in the patients is in accord with their higher Lp(a) concentrations, but it fails to explain the entire increase in the mean Lp(a) plasma concentration in CHD patients. Using the allele frequencies from the patient group and the mean allele-associated concentration estimated from a control population ($n=224$), we calculated a mean expected concentration of 28.3 mg/dL for the patient group. This is less than the 39.2 mg/dL observed. The difference in apo(a) size allele frequencies between case and control subjects thus explained only about half (46%) of the increase in plasma Lp(a) levels in the case subjects. Consistent with this finding, the mean Lp(a) levels were higher in the CHD patients over the whole range of apo(a) size classes except the largest (Fig 4). The mean Lp(a) levels were significantly higher in phenotypes with apo(a) alleles kringle IV 17 to 22 and nonsignificantly higher in phenotypes with apo(a) alleles 23 to 25 kringle IV repeats. Individuals with phenotypes containing large apo(a) alleles (>25 kringle IV repeats) had nearly identical Lp(a) concentrations among both CHD patients and control subjects (Fig 4). Hence, not all phenotypic groups contributed equally to the increase.

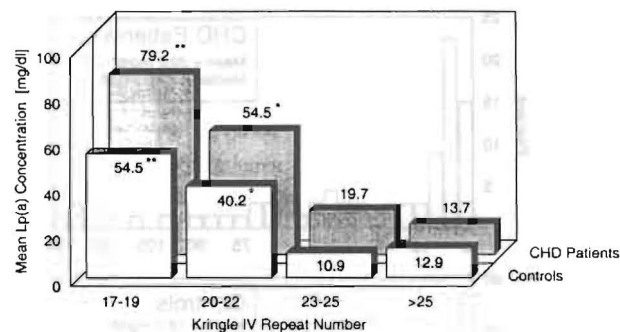


FIG 4. Graph shows apo(a) size allele-associated mean Lp(a) plasma concentration. Apo(a) alleles were combined as in Fig 2. Apo(a)-associated mean Lp(a) levels were calculated as described.¹⁶ Mean Lp(a) concentrations for each group are indicated in or above the bars. Controls indicates control subjects. ** $P=.011$, * $P=.009$ by Wilcoxon-Mann-Whitney U test.

CHD Risk in Relation to Lp(a) Concentration and Apo(a) KpnI Allele Frequencies

We next calculated the ORs for individuals to be in the group of CHD patients on the basis of the presence of smaller or larger apo(a) KpnI alleles (Table 2). The OR decreased steadily with increasing numbers of kringle IV repeats in both alleles of the apo(a) gene. Significant ORs were calculated for very small and very large apo(a) alleles. Apo(a) alleles with <20 kringle IV repeats were 4.63 times more likely to be found in the patient group ($P=.0112$), whereas very large apo(a) alleles (>25 kringle IV repeats) were three times more likely to be in the control group ($P<.001$). The same results were obtained when only expressed apo(a) alleles were used for this calculation (data not shown). Accordingly, ORs were also calculated for quartiles of Lp(a) concentration. A significant OR ($P=.016$) was estimated only for the highest quartile [Lp(a) >25.7 mg/dL]. Individuals with Lp(a) levels in the highest quartile had

TABLE 2. Relative Risk (OR) for CHD Depending on Lp(a) Plasma Concentration and Kringle IV Repeat Number of Apo(a) Alleles

	OR for CHD Risk (95% Confidence Bounds)
Lp(a) range, mg/dL	
>25.7	2.31* (1.129-4.733)
8.2-25.7	0.71 (0.338-1.750)
3.2-8.2	0.84 (0.436-2.292)
0-3.2	0.48 (0.203-1.134)
Kringle IV smaller apo(a) alleles, n	
17-19	4.630* (1.240-17.23)
20-22	2.08 (0.928-4.655)
23-25	1.087 (0.488-2.418)
>25	0.314† (0.155-0.635)
Kringle IV larger apo(a) alleles, n	
17-19	...
20-22	2.078* (1.742-2.479)
23-25	1.837 (0.518-6.578)
>25	0.292* (0.089-0.957)

* $P<.05$, † $P<.001$.

a 2.31 times higher risk of belonging to the patient group. This value is comparable to the risk imposed by cholesterol levels >90th percentile ($OR=2.59$, $P=.017$). When both Lp(a) and cholesterol levels were >90th percentile the OR increased to 7.05 ($P<.005$).

Stepwise logistic regression analysis revealed that either the Lp(a) plasma concentration or the size of the smaller apo(a) allele made a significant contribution to the risk for CHD. The highest predictive value was reached by the apo(a) kringle IV number of an individual's smaller apo(a) allele, followed by the Lp(a) plasma concentration. The TC level reached significance as a predictor only when both Lp(a) level and apo(a) allele number were eliminated from the model. HDL-C values were not significant as predictors at all.

Discussion

We used apo(a) DNA phenotyping of genomic DNA to determine whether variation at the apo(a) locus is a risk factor for CHD and to estimate its contribution to the risk. An impressive inverse correlation of the number of kringle IV repeats in apo(a) alleles with the risk for CHD was demonstrated. When expressed as ORs, the relative risk for CHD was 14-fold higher for subjects with a low kringle IV repeat number (<22 kringle IV repeats) in at least one allele than in those with high repeat numbers (>25 kringle IV repeats) in both alleles.

Association studies have related Lp(a) plasma levels or apo(a) isoform size (defined by immunoblotting) to the risk for CHD, myocardial infarction, stroke, or peripheral vascular disease (for review, see Reference 2). With only a few exceptions,¹⁷ all published retrospective case-control studies report an association of high Lp(a) with atherosclerotic vascular disease.^{1,2} Further evidence for a significant role of Lp(a) in CHD came from offspring studies that demonstrated higher Lp(a) levels in offspring of a parent with myocardial infarction than in those without such a parent.¹⁸ Animal models have also suggested that Lp(a) or possibly even apo(a) is a risk factor for atherothrombotic vascular disease.

This uniform trend has not continued in more recent prospective studies.^{4,7-11} Two large studies, the Helsinki Heart study¹⁰ and the Physicians Health study,¹¹ did not find an association of high Lp(a) levels with CHD. As a consequence, the role of Lp(a) has been questioned in some editorials with provocative titles ("Has lipoprotein 'little' (a) shrunk?"¹⁹). It is interesting to note that the Physicians Health study concluded that genetic markers should be used for Lp(a) analysis in further studies,¹¹ as studies using apo(a) isoforms as genetic markers had already been published¹² and results had supported the view that high Lp(a) is a risk factor. This conclusion was based on the finding that small apo(a) isoforms [which are associated with high Lp(a) levels in plasma] were more frequent in patients than in control subjects in each of six different ethnic groups.^{12,20} Although this finding has not been reproduced in all subsequent studies,²¹ none of the studies considering apo(a) isoforms have ever observed a lower frequency of low-molecular-weight isoforms in patients than in control subjects, which would be expected if there were no association with disease, and differences resulted from random deviations.

In the present study the frequency of low-molecular-weight isoforms was again higher in CHD patients than in control subjects, confirming the results from a previous analysis of the same Tyrolean population in an independent sample. This supports the notion that Lp(a) is a risk factor. Nonetheless, studies based on apo(a) isoforms may also be biased due to the peculiar nature of the apo(a)/Lp(a) system.^{1,2} There exists a strong negative correlation of apo(a) isoform size and apo(a) concentration in plasma, and not all apo(a) alleles are expressed when determined by immunoblotting.^{4,6} In studies based on apo(a) isoforms, only those alleles are considered that produce apo(a) at concentrations above the detection limit of the immunoblot. More importantly, homozygotes cannot be distinguished from heterozygotes in subjects with only one isoform. Thus, the calculated allele frequencies and differences in frequencies may be influenced by the Lp(a) concentration in the sample. The present study allowed a direct comparison of results from DNA and protein phenotyping and demonstrates that apo(a) allele frequencies calculated from apo(a) isoform data are indeed biased towards small-sized alleles. Although this trend was the same in both patients and control subjects, the magnitude of the effect [ie, deviation of the frequency of small versus large apo(a) alleles determined by protein versus DNA phenotyping] was larger in the patient (10.5%) than in the control (6.4%) group (Table 1). Thus, protein isotyping may in fact have introduced some bias into the previous association studies. Nonetheless, as supported from the DNA data presented here, the general conclusions from these studies were correct.

In contrast to protein phenotyping, DNA phenotyping allows the identification of both apo(a) alleles in each individual and distinguishes homozygotes from heterozygotes. Apo(a) DNA phenotypes [defined by kringle IV repeat number in apo(a) alleles] do not, however, allow the prediction of an individual's Lp(a) concentration. Although there is a strong inverse correlation between the number of kringle IV repeats in apo(a) and Lp(a) plasma concentration, there is also a wide variation of Lp(a) concentrations within a given apo(a) allele size. This variation reflects largely unknown sequence variations in apo(a) genes of identical size.²² Isoforms of the same size (ie, same kringle IV repeat number) may segregate with very different Lp(a) concentrations. If high Lp(a) is indeed a risk factor for CHD, then this relation predicts two results. First, it is expected that alleles with low kringle IV numbers are associated with disease. Second, it is expected that Lp(a) concentrations are higher within apo(a) size categories in the CHD patients due to enrichment with apo(a) sequence variants that are associated with high Lp(a). Precisely this was observed in the present study (and also in our previous analysis based on protein isoforms¹²). The data are consistent with the interpretation that apo(a) alleles associated with high Lp(a) are enriched in CHD patients.

It might be argued that the higher kringle IV-associated Lp(a) concentrations in CHD patients reflect the action of other genes and/or environmental factors rather than sequence variation in apo(a) or may even be a consequence of the disease process itself. Although we cannot exclude on the basis of our data that such factors

operate, these possibilities seem unlikely for several reasons. First, the association of apo(a) size with Lp(a) concentration is also present in CHD patients (see Fig 3 and Reference 12).

In fact, the Spearman rank correlation coefficient and the R^2 value from the ANOVA show that the association is stronger in CHD patients than control subjects. This is likely to result from less sequence variation within alleles of identical size. Second, Lp(a) concentrations are almost entirely controlled by the apo(a) locus not only in healthy individuals^{4,23} but also in multiplex families of myocardial infarction probands with high Lp(a) levels.²⁴ Third, no environmental factors have yet been identified that affect Lp(a) levels to any significant extent. The only well-documented nongenetic effects are sex hormones²⁵ and renal disease.¹³ Thus, there is no reason to speculate that high Lp(a) in CHD is caused by anything except inherited apo(a) alleles. Rigorous proof of this has to await the identification of all sequence variations in apo(a) that affect Lp(a) levels.

Association studies that link genetic markers to complex disorders are prone to several kinds of bias, the most important of which is stratification bias, which may result in false positive associations. Stratification bias may result from differences, eg, in age, sex, or ethnic composition, between case and control groups if allele frequencies are affected by these variables. In the present study patients and control subjects were all from the same small geographic area and from a homogeneous population (Tyroleans from Austria) and were carefully matched for sex, age, and ethnicity, which makes stratification bias unlikely. Apo(a) gene frequency differences and ORs for small apo(a) isoforms and high Lp(a) levels remained significant, however, when we analyzed all our data (ie, when we included unmatched patient [n=124] and control [n=224] subjects; data not shown). Indirect evidence also supports the argument that the observed association is neither a chance finding nor due to other bias. Our observations are consistent with studies that have considered Lp(a) levels and/or apo(a) isoforms¹²; there are plausible pathogenetic mechanisms explaining the disease association^{2,26}; and results from animal models, eg, transgenic mice, support the findings in humans.^{27,28}

The strongest argument against a chance finding is the impressive stepwise increase in risk with decreasing kringle IV repeat number. One may wonder why such a strong association of Lp(a) levels and apo(a) gene variation with CHD is apparent in a small case-control study of only 69 case-control pairs and not in some much larger prospective studies encompassing >200 case and control subjects. We reason that those studies that failed to detect an association are likely to be biased. In one negative study,¹⁰ which was originally designed as an intervention trial, all patients with previous myocardial infarction were excluded. Thus, all patients with early complications and presumably a strong genetic background were removed. A US study¹¹ included patients defined as "predominantly white," which in the United States would represent a broad range of ethnicities descended from all over Europe and elsewhere. Studies of apo(a) isoforms and other genes (eg, cystic fibrosis mutations²⁹) have revealed strong frequency gradients in Europe, eg, from south to north. Lumping all these

ethnicities together may well result in severe stratification bias, but our study design allowed us to avoid such a bias. Additionally, the mean age of our patients (51 years) was low compared with some other studies. Together, these differences may have contributed to the clear result of our study, which for the first time directly demonstrates that apo(a) gene variation relates to CHD in humans.

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