Coronary Artery Disease Is Associated With Increased Lipoprotein(a) Concentrations Independent of the Size of Circulating Apolipoprotein(a) Isoforms

Martyn Farrer, Frances L. Game, Catherine J. Albers, H. Andrew W. Neil, Peter H. Winocour, Mike F. Laker, Philip C. Adams, K. George M.M. Alberti

Abstract Lipoprotein(a) [Lp(a)] concentration and apolipoprotein(a) [apo(a)] isoforms (identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE] and Western blotting) were determined in a group of 508 asymptomatic Caucasian members of the community and in 318 Caucasian patients with angiographically defined coronary artery disease (CAD). Conventional risk factors for CAD were also measured. Lp(a) concentration was almost twice as high in subjects with CAD (geometric mean, 152 mg/L [geometric SD, 10 to 1398 mg/L]) as in asymptomatic control subjects (geometric mean, 84 mg/L [geometric SD, 21 to 334 mg/L]). Asymptomatic women had higher concentrations of Lp(a) than asymptomatic men. Patients with CAD were older and were more likely to have smoked and to have a first-degree relative with premature CAD (<55 years of age), and a higher proportion were male. Patients with CAD had higher concentrations of Lp(a) independently of the number of isoform bands expressed. When apo(a) isoforms were allocated to 1 of 10 classes on the basis of their molecular size (R, versus apoB in SDS-PAGE), patients with CAD did not express an excess of low-molecular-mass (higher concentration) isoforms but did express a higher proportion of double-band phenotypes with fewer "null" phenotypes. The relationship between the two isoform bands in a double-band phenotype was the same in both populations. Isoform mobility was defined as a continuous variable equal to the mobility of a single isoform band (single-band phenotypes) or the mean of the two isoforms in a double-band phenotype. Two variables, isoform mobility and the number of isoform bands expressed, were used to summarize the large range of isoform patterns (at least 45) that could be identified. Isoform mobility, the number of isoform bands expressed, and the presence of CAD were the three most important independent predictors of Lp(a) concentration (descending order). Only sex and LDL cholesterol were additional independent predictors of Lp(a) concentration in stepwise regression models including a wide range of demographic factors and lipid and glycemic risk factors. We conclude that Lp(a) concentration is associated with CAD independently of the isoform pattern expressed. The apo(a) gene locus exerts a strong control over circulating Lp(a) concentration, and a better understanding of the control of expression of the apo(a) gene will be essential to understand the relationship between Lp(a) and CAD. (Arterioscler Thromb. 1994;14:1272-1283.)

Key Words: • lipoprotein(a) • atherosclerosis • coronary artery disease • apolipoprotein(a) isoforms • aortocoronary bypass surgery

Lipoprotein(a) was first described in 1963.1 It is a lipid-rich particle similar to low-density lipoprotein (LDL) but containing a unique glycoprotein, apolipoprotein(a). The latter is linked by disulfide bonds to an apolipoprotein B-100 molecule indistinguishable from that of LDL24 with a stoichiometry of 1:1.5 Lipoprotein(a) [apo(a)] concentration is increasingly recognized to be associated quantitatively with coronary artery disease (CAD).6-14 It is also predictive of cardiac events15,16 and is therefore a true risk factor for CAD. The mechanism by which lipoprotein(a) concentration and cardiac events are related is not known. The DNA and protein sequence homology with plasminogen17-18 suggest that lipoprotein(a) may interfere with plasminogen-mediated thrombolysis in vitro.19-21 Alternatively, the structural similarities between lipoprotein(a) and LDL22-24 suggest that it may be a risk factor because of its cholesterol content. In support of this, lipoprotein(a) has been demonstrated in coronary artery and vein graft atherosclerotic lesions by immunohistochemical methods.25,26

Concentrations of lipoprotein(a) are largely determined by genetic factors,6,27-30 and the circulating concentration of lipoprotein(a) has recently been shown to be dependent on the size of the isoforms of its specific glycoprotein, apolipoprotein(a).27-29 The strong inverse relationship between molecular size of apolipoprotein(a) isoform and circulating concentration of lipoprotein(a) is now widely accepted,27-29,31 and a clear relationship between the size of allelic DNA (and number of kringle 4–encoding repeats in the gene) and apolipoprotein(a) isoform size has been established.31,32 Whether particular isoforms of lipoprotein(a) are atherogenic or the circulating concentration of lipoprotein(a) determines the risk of CAD is not known and is the subject of the present report.

Methods

Subjects

Control subjects were recruited from a cohort of 700 men and women who attended a voluntary health screening program based at a primary health care center in Newcastle upon

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Tyne, a city in the north of England. The prevalences of cardiovascular risk factors in this cohort were diabetes mellitus 1.4%, hypertension (systolic blood pressure ≥160 mm Hg and/or diastolic blood pressure ≥95 mm Hg) 10%, smoking 15%, and hypercholesterolemia (>6.5 mmol/L) 27%, similar to previous UK population studies. Symptoms of vascular disease (intermittent claudication, previous myocardial infarction, and angina, by the World Health Organization–Rose questionnaire) were present in 4%, and the prevalence of electrocardiographic abnormalities compatible with ischemic heart disease (Minnesota codes 1-1, 1-2, 4-1, 5-1, 8-1, 8-3) was 3% in a representative sample of 447 subjects. Of the 700 screened between September 1988 and March 1990, 508 Caucasian individuals (256 male) were selected for study on the basis of the availability of both apolipoprotein(a) phenotype and plasma lipoprotein(a) concentration together with freedom from symptoms suggestive of vascular disease (previous myocardial infarction, angina, intermittent claudication, transient ischemic attacks, previous stroke, or congestive cardiac failure). CAD subjects were recruited between October 1988 and December 1989: 353 consecutive patients (298 male) with angiographically defined severe symptomatic CAD were screened. All were to undergo elective coronary artery bypass graft (CABG) surgery from a geographically defined area of the northern region. They were studied at the time of admission before surgery. Apolipoprotein(a) phenotype and serum lipoprotein(a) concentration data were available in 318 individuals (90% of cohort), who were all Caucasian. All subjects gave informed consent, and the relevant approval from our Institutional Review Board was obtained.

Procedures

Subjects attended after a 10- to 12-hour overnight fast. Body mass index was calculated (weight in kilograms divided by the square of height in meters). Blood pressure was measured with a mercury-in-glass sphygmomanometer. The mean of two measurements, made at intervals of 5 minutes after subjects had been seated for more than 30 minutes, was used in subsequent analyses. Total cholesterol, triglycerides, insulin, apolipoprotein AI, and apolipoprotein B concentrations were determined in serum. High-density lipoprotein (HDL) cholesterol concentration was measured in EDTA/plasma and glucose concentration in fluoride oxalate/plasma. Venous plasma glucose concentration was measured with a continuous-flow autoanalyzer (Technicon AA2, Technicon Instruments; interassay coefficient of variation [CV]; 1% to 3%). Serum insulin was determined by a standard radioimmunoassay. Standard enzymatic methods were used to measure serum cholesterol (cholesterol oxidase; interassay CV, 1.5% to 2.2%) and triglycerides (lipase–glycerol kinase; interassay CV, 3.0%). HDL cholesterol was isolated after precipitation of apolipoprotein B–containing lipoproteins with heparin and manganese and assayed by the cholesterol oxidase method (interassay CV, 3.6%). Apolipoprotein AI and apolipoprotein B were measured by an end-point immunonephelometric assay (Hoechst UK Ltd; CV, 4.9% to 5.6%) for control subjects and a rate immunonephelometric assay (Technicon DPA; interassay CV, 3.6% to 8%) for CAD patients. The regression equations were, for apolipoprotein AI, rate immunonephelometric method: \(0.78\times\text{end-point immunonephelometric method} + 0.20\) and for apolipoprotein B, rate immunonephelometric method: \(0.80\times\text{end-point immunonephelometric method} - 0.06\). These equations were applied to adjust values obtained from the end-point immunonephelometric methods to be equivalent to those from the rate immunonephelometric method. HbA1 was determined by an electrophoresis method (CV, 8.1%).

Lipoprotein(a) Measurement

Samples were frozen (−70°C) for later assay of lipoprotein(a) by an enzyme-linked immunosorbent assay (ELISA; Biopool) with a between-batch CV of 3% to 8%. The lower limit of detection was 10 mg/L, which is the maximum cross-reactivity between the polyclonal anti-human lipoprotein(a) antibody used and plasminogen at a concentration of 200 mg/L. [plasminogen standards known to be free of lipoprotein(a) were not available, so this may be an overestimate of antibody cross-reactivity). Appropriate reference samples of each known pure apolipoprotein(a) isoform class were used as described below. All samples were measured with kits with a single batch of antibody whose performance against the various isoforms of apolipoprotein(a) is known to be satisfactory. Lipoprotein(a) Stability

Lipoprotein(a) concentrations were measured in citrated plasma (control subjects) or serum (CAD patients) after storage at −70°C. Both populations were studied over a similar period with equivalent storage times. Freeze-thaw stability was confirmed, but samples were assayed only within 8 hours of a single thawing. A small number of blood samples were split, and one aliquot was stored as citrated plasma, the other being stored as serum. There was no difference between lipoprotein(a) concentrations in citrated plasma and serum [serum Lp(a) mg/L = 1.04 citrate plasma Lp(a) mg/L; \(R^2 = 0.976; n=6\)]. The stability of lipoprotein(a) samples stored at −70°C was determined from a serum sample containing a single apolipoprotein(a) band of isoform class 1.0 that was divided into aliquots and frozen immediately after the blood was drawn and separated. Freshly thawed aliquots (mean, 612 mg/L; CV, 4.4%; \(n=30\)) were used for quality assurance in the lipoprotein(a) assay, and a stable concentration was recorded for assays performed over a period of 9 months. Lipoprotein(a) stability was also examined in both study populations in samples from individuals in which only a single isoform was detectable. Lipoprotein(a) concentrations were analyzed for each isoform class separately, dividing the period of collection and storage of samples into 3-month periods. For a given apolipoprotein(a) isoform, lipoprotein(a) concentrations (geometric mean values) were no different in samples stored for the longest time (25 months) compared with the shortest time (13 months) for plasma or serum samples.

Apolipoprotein(a) Isoforms

Individuals are known to express one or two different-size apolipoprotein(a) isoforms in the circulation at the same time. A wide range of molecular size of the individual isoforms is expressed, which may complicate the assay of lipoprotein(a) quantity. Only if the binding affinity of the ELISA capture antibody is measured by a standard method in the same sample will quantification of both be reliable. In addition, it is not possible to determine isoform size in a small number of samples in which lipoprotein(a) is detectable by ELISA. These samples and samples in which lipoprotein(a) is undetectable by ELISA are defined as having a “null” phenotype. Lipoprotein(a) isoforms were demonstrated by immunoblotting after separation with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. This technique gives good separation of isoforms with excellent reproducibility of relative mobility (CV, 0.9% to 6.4%). Isoforms were classified according to their relative mobility in SDS-PAGE against that of apolipoprotein B-100 (Rl versus apoB). A standard with the same mobility as apolipoprotein B-100 was run at three points on every gel (both ends and the middle) for reference. A small number of duplicate samples were assayed on different occasions to confirm that for lipoprotein(a), storage as citrate plasma or serum had no effect on the isoforms demonstrated. Ten isoform classes of apolipoprotein(a) were recorded by the phenotyping method as previously reported. Isoform standards for Utermann classes S3, S2, S1, and F were obtained from Immuno GmbH.

Statistics

Before analysis, triglycerides, glucose, insulin, and lipoprotein(a) distributions were normalized by logarithmic transfor-
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TABLE 1. Characteristics of Study Groups

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<thead>
<tr>
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<th>Control Subjects</th>
<th>CAD Patients</th>
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<tbody>
<tr>
<td>n</td>
<td>508</td>
<td>318</td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>256:252</td>
<td>273:45</td>
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<tr>
<td>Family history of IHD, %</td>
<td>26</td>
<td>35</td>
<td>†</td>
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<td>Lipoprotein(a) isoforms, null/single/double bands, %</td>
<td>9/60/31</td>
<td>1/58/41</td>
<td>†</td>
</tr>
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<td>Smoking, current/previous/never, %</td>
<td>16/35/49</td>
<td>2/84/14</td>
<td>†</td>
</tr>
<tr>
<td>Age, y</td>
<td>46 (11)</td>
<td>57 (8)</td>
<td>*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.8 (4.6)</td>
<td>25.7 (2.6)</td>
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<tr>
<td>Systolic BP, mm Hg</td>
<td>121 (17)</td>
<td>127 (19)</td>
<td>*</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>77 (10)</td>
<td>76 (10)</td>
<td>*</td>
</tr>
<tr>
<td>Lipoprotein(a), mg/L</td>
<td>84 (21-334)</td>
<td>152 (10-1398)</td>
<td>*</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.9 (1.3)</td>
<td>6.5 (0.9)</td>
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<td>Triglycerides, mmol/L</td>
<td>1.26 (1.00-1.58)</td>
<td>1.91 (1.48-2.45)</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.43 (0.37)</td>
<td>1.01 (0.18)</td>
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<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.77 (0.69)</td>
<td>4.54 (0.98)</td>
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<tr>
<td>Apolipoprotein AI, g/L</td>
<td>1.40 (0.34)</td>
<td>1.03 (0.18)</td>
<td>*</td>
</tr>
<tr>
<td>Apolipoprotein B, g/L</td>
<td>0.92 (0.35)</td>
<td>1.11 (0.17)</td>
<td>*</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>6.0 (0.3)</td>
<td>6.7 (0.8)</td>
<td>*</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>5.1 (4.7-5.5)</td>
<td>5.3 (4.1-6.8)</td>
<td>*</td>
</tr>
<tr>
<td>Fasting insulin, µU/mL</td>
<td>6 (2-19)</td>
<td>8 (2-36)</td>
<td>*</td>
</tr>
</tbody>
</table>

*P<.001; †P<.01.
Geometric mean (geometric SD). Other values are mean (SD) or percent.

Results

The characteristics of the two subject groups studied are shown in Table 1. Patients with CAD were older and had higher body mass index and higher systolic blood pressure than the asymptomatic control subjects. More patients with CAD were male, a higher proportion had a family history of premature ischemic heart disease (at least one relative having ischemic heart disease at age ≤55 years), and more had smoked. Patients with CAD had higher total cholesterol, LDL cholesterol, triglycerides, apolipoprotein B, HbA1c, fasting glucose, and fasting insulin levels but lower concentrations of HDL cholesterol and apolipoprotein AI. Lipoprotein(a) concentration in the CAD group was almost double that in asymptomatic control subjects (GM, 152 mg/L; SD, 10 to 1398 mg/L for CAD versus 84 mg/L; geometric SD, 21 to 334 mg/L for control subjects; P<0.001).

Performance of the Quantitative Lipoprotein(a) Assay to Different Apolipoprotein(a) Isoforms (Fig 1)

The ability of the lipoprotein(a) assay to quantify these different isoforms in a mixture is demonstrated in Fig 1. Three samples, each containing a pure single-band isoform (isoform classes 0.5, 0.8, and 1.0), were used to produce linear standard curves (Fig 1A) and curves of observed versus expected concentrations for a varying amount of one pure isoform added to a constant concentration of another pure isoform (Fig 1B, 1C, and 1D). Slopes of the recovery plots lay between 0.88 and 0.97 for all possible combinations. When added to 551±22 mg/L of isoform 0.5, recovery slopes were 0.93, R²=.992 (for isoform 0.8) and 0.88, R²=.995 (for isoform 1.0). When added to 390±20 mg/L of isoform 0.8, recovery slopes were 0.97, R²=.990 (isoform 0.5) and 0.90, R²=.995 (isoform 1.0). When added to 572±15 mg/L of isoform 0.8, recovery slopes were 0.97, R²=.990 (isoform 0.5) and 0.90, R²=.995 (isoform 0.5). When added to 390±20 mg/L of isoform 1.0, recovery slopes were 0.97, R²=.996 (isoform 0.8) and 0.97, R²=.998 (isoform 0.5).

Demographic Variables and Lipoprotein(a) Concentration (Table 2)

Lipoprotein(a) concentration was higher in women than in men in the group of asymptomatic control subjects.
Lipoprotein(a) Concentration in Single- and Double-Band Phenotypes

The concentration of lipoprotein(a) was higher in double-band phenotypes than in single-band phenotypes and similarly higher in single-band phenotypes than in null phenotypes (ie, those in which no isoform bands could be detected; Table 2; P<.0001 by ANOVA). Concentrations of lipoprotein(a) were higher in patients with CAD than in asymptomatic control subjects, independent of the number of bands expressed (controlling for the effects of sex, family history of premature CAD, and smoking by ANOVA). For both CAD patients and control subjects, there was no difference between men and women in the proportion of each having null, single-band, or double-band phenotypes or a family history of premature ischemic heart disease (by χ² analysis).

Apolipoprotein(a) Isoform Size and Lipoprotein(a) Concentration

Apolipoprotein(a) isoform can either be described as a continuous variable according to its mobility in SDS-PAGE or be categorized into classes of mobility. Concentrations of lipoprotein(a) were compared for each class of isoform in patients with CAD and asymptomatic control subjects (Fig 2). For the single-band phenotypes, larger isoforms had a slower mobility (a smaller Rf value versus apoB value) and had a lower circulating concentration of lipoprotein(a) than smaller isoforms, which had a faster mobility. This relationship between isoform class and concentration of lipoprotein(a) was seen in both subject groups and is in good agreement with previously published data.7-29,31 This relationship is more easily seen when lipoprotein(a) concentration is plotted against isoform mobility (analyzed as a continuous variable, Fig 3). For asymptomatic control subjects, log lipoprotein(a) concentration = 1.03 + 1.42 (Rf versus B), R² = .171; for severe CAD, log lipoprotein(a) concentration = 1.27 + 1.30 (Rf versus B), R² = .137. When single-band phenotypes were analyzed by class of isoform mobility (categorical variable), lipoprotein(a) concentration was lower for isoforms of low Rf versus B compared with those of high Rf versus B for both control subjects and patients with CAD (Figs 2 and 3).

Analysis of individuals with double-band phenotypes was more complex. With 10 classes of apolipoprotein(a) isoform mobility, there are 55 possible different phenotypes, of which 45 are double-band phenotypes. In the two subject populations studied, isoform mobility (continuous variable) had approximately normal distributions in single-band phenotypes and for both slower- and faster-mobility bands in double-band phenotypes. In Fig 4, the relationship between slow isoform mobility and fast isoform mobility in double-band phenotypes is shown for both subject groups in which the relationship is linear and identical. This relationship allowed comparison of lipoprotein(a) concentration between subject groups for double-band phenotypes. As in single-band phenotypes, total circulating lipoprotein(a) concentrations were higher in CAD patients than in control...
subjects for all classes of the double-band phenotype (Fig 2). The similar relationship between faster and slower bands of double-band phenotypes allowed a mean isoform mobility (of the two bands) to be used to summarize a double-band phenotype. This is a continuous variable and is defined as isoform mobility. The relationship between log lipoprotein(a) and isoform mobility is shown in Fig 3 for the double-band phenotypes. Log lipoprotein(a) concentration was higher in patients with CAD than in control subjects throughout the range of isoform mobility and isoform class of the faster and slower isoform in the double-band phenotypes (Figs 2 and 3).

### Comparison of Isoform Frequency in CAD and Control Subjects (Fig 5)

There was no increase in the proportion of faster-mobility isoforms (whether as a single band or one of a double isoform band) in patients with CAD compared with normal subjects. Patients with CAD had a small excess of slower isoforms (classes 0.35 and 0.50, $P < .0001$, characteristically present in lower concentrations), and asymptomatic control subjects had a small excess of faster isoforms (class 0.90, $P < .05$, characteristically present in higher concentrations).

### Univariate Predictors of Lipoprotein(a) Concentration

Factors predictive of lipoprotein(a) concentration were sought in both subject groups (Table 3). Factors most strongly correlated with log lipoprotein(a) concentration in both subject groups were isoform mobility (the mobility [$R_f$ versus $B$] of the single-band phenotypes and the mean $R_f$ versus $B$ of the two isoforms in double-band phenotypes) and the number of isoform bands expressed. The only other factors with significant correlations were, in CAD patients, LDL cholesterol [calculated with the Friedewald formula corrected for lipoprotein(a) contribution$^9$], total cholesterol, log triglycerides (a negative correlation, $r = -.13$, $R^2 = .018$, $P < .02$), and fasting insulin and in asymptomatic subjects, fasting blood glucose (a negative correlation, $r = .20$, $R^2 = .016$, $P < .005$). Analysis of single- and double-band phenotypes separately and analysis of double-band phenotypes by faster or slower band mobility revealed no difference in the covariates, with significant linear relationship to log lipoprotein(a) concentration for each subject group (data not presented).

### Multivariate Predictors of Lipoprotein(a) Concentration

Multivariate associations were sought initially within and then between the two subject groups (Table 4). For CAD patients, only isoform mobility, number of isoform bands, LDL cholesterol, and apolipoprotein B were independently related to log lipoprotein(a) concentration. For control subjects, isoform mobility, number of isoform bands, fasting plasma glucose, fasting serum insulin, age, sex, and body mass index were independently related to log lipoprotein(a) concentration. Re-
Fig 2. Bar graphs showing frequency of apolipoprotein(a) isoforms and concentration of lipoprotein(a). The frequencies of each class of isoform mobility are shown for asymptomatic control subjects and patients with coronary artery disease (CAD) for single- and double-band phenotypes. Geometric mean lipoprotein(a) concentration is shown above the bar for each class of isoform.
subjects. This is in clear agreement with several previous studies.\(^6\)–\(^16\) Explanation of this association remains incomplete, and the cellular events that allow lipoprotein(a) to become incorporated into atheroma\(^25\)–\(^26\) are not understood, although specific receptors may be involved.\(^40\)–\(^41\) The very polymorphic nature of lipoprotein(a) complicates its study, and mechanisms controlling the circulating concentration of lipoprotein(a) in Caucasians and explaining the difference in median lipoprotein(a) concentration between Caucasians and other races, eg, American blacks,\(^42\)–\(^46\) are also not fully understood. This report sought to study whether particular isoforms of lipoprotein(a) are atherogenic or the circulating concentration of lipoprotein(a) determines the risk of CAD.

**Apolipoprotein(a) Classification**

A unifying classification of apolipoprotein(a) isoforms and phenotypes is lacking, the number of isoform classes varying from 6\(^22\) to 12\(^29\) or 34,\(^47\)\(^48\) in this study, a sensitive method was used to separate and classify apolipoprotein(a) isoforms into 10 categories of isoform mobility.\(^38\) These categories are compatible with existing classifications of isoforms and have been related to the original 6 Utermann isoform classes (Table 5). The use of very sensitive methods\(^47\)\(^48\) allows resolution of 34 isoforms \([in agreement with 34 different-sized allelic DNA isoforms at the apolipoprotein(a) locus]\(^9\)). The technique requires resolution of 1-mm steps between isoforms on agarose gels,\(^47\)\(^48\) and we estimate that a between-gel CV of \(<1\%\) would be required to place 68\% of consecutive samples with the same isoform in the same isoform class. We believe the use of isoform

**Discussion**

Lipoprotein(a) concentration is almost twice as high in subjects with severe CAD compared with normal subjects. This is in clear agreement with several previous
molecular weight of each kringle glycoprotein subunit of apolipoprotein(a) has been estimated to be approximately 13 kD.49 The range of isoform sizes encompassed by the isotyping method in this article is therefore consistent with the 34 different-sized apolipoprotein(a) gene products. When the Kamboh method was used in our own laboratory, the relative mobility (Rf versus B) of standards S3, S2, S1, F, and a range of patient samples were the same for both methods. These findings, like those of Gaubatz et al,29 suggest that the three most sensitive isotyping methods identify the full range of isoform sizes but that the limit of detection of a low-concentration isoform differs between them.

Isoform Frequencies

The proportion of double-band phenotypes expressed (31% for control subjects, 41% for CAD patients) is less than expected from genotyping data,31 in which the distribution of alleles approximates to Hardy-Weinberg equilibrium with many more heterozygotes than are identified by phenotype analysis by SDS-PAGE. When methods are used that identify all 34 isoforms,47 a much higher proportion of double-band phenotypes is observed (80%) and a >90% heterozygosity index calculated. Approximately 19% of true heterozygotes are not appropriately identified because the concentration of the subordinate isoform in the heterozygote is present at a lower concentration than the detection limit of the technique. The detection of more double-band isoforms and fewer null phenotypes in the CABG group (Table 2) is almost certainly due to the higher circulating lipoprotein(a) concentrations in the CAD group. There is no evidence that an excess of lower-molecular-weight isoform classes explains the increased concentration of lipoprotein(a) in patients with severe CAD but rather that higher-molecular-weight isoforms are better detected because they are present in higher concentrations in patients with CAD (Fig 5).

Classification of Double-Band Phenotypes

The double-band phenotypes present a particular challenge for analysis. The six isoforms identified by Utermann et al27 produce 15 different double-band phenotypes. With the classification in this report38 there are 45 different double-band phenotypes, with that of Gaubatz et al29 there are 55, and with that of Kamboh there are 253 (n=23 isoforms)47 or 561 (n=34 isoforms).48 A large proportion of ostensibly single-band phenotypes possess an unidentified subordinate band that contributes only a small amount to lipoprotein(a) concentration, while in double-band phenotypes both isoforms contribute a significant amount to the total concentration of lipoprotein(a). The detection limit of the isotyping technique in this study is 0.02 µg, from which it is possible to estimate that the maximum concentration of a circulating lipoprotein(a) isoform when an apolipoprotein(a) isoform is not detected is approximately 10 mg/L. This represents approximately 10% of the mean circulating concentration in the subjects with ostensibly single-band phenotypes. This theoretical limit was confirmed by the loading of gels with varying concentration mixtures of two different ostensibly single-band isoforms and scanning of blots by laser densitometry. It is not possible from the present or previous large epidemiological studies to determine the proportion of total lipoprotein(a) concentration attributable to each isoform in a circulating pair.

One approach to analyzing the large number of potential phenotypes is to limit isoform recognition to the six isoform classes described by Utermann et al.27 This, however, does not reflect the diversity known to be present at the level of the gene and in the circulating gene product. We have therefore sought alternative summary measures for the large range of double-band phenotypes that may exist. We have chosen the mean isoform mobility as a summary measure for three reasons: (1) The relationship between the mobility of the two isoforms in double-band phenotypes can be described by an identical linear correlation in both our subject populations (Fig 4). The
Apolipoprotein(a) polymorphism is in Hardy-Weinberg equilibrium, and therefore, the relationship between isoform mobilities will be the same for all subjects in which two isoforms are expressed, including those with single bands in which a very-low-concentration subordinate band is present [only 1% of individuals are true homozygotes, and a similar proportion do not express any lipoprotein(a)]. (2) The relationship between lipoprotein(a) concentration and mean isoform mobility of the two isoforms in a double-band phenotype is linear and has a similar slope in both subject groups (Fig 3). (3) The relationship between mean isoform mobility and lipoprotein(a) concentration is unaffected by the size of the difference in isoform mobility between the two isoform bands (data not presented). The linear correlation between lipoprotein(a) concentration and isoform mobility is different for single-band phenotypes compared with double-band phenotypes (Fig 3). We have defined isoform mobility as the isoform mobility (R, versus B) of the single isoform in single-band phenotypes and the mean of the two isoform relative mobilities for double-band phenotypes.

### TABLE 4. Regression Analysis of the Variables Linearly Related to the Log Circulating Concentration of Lipoprotein(a)

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<tr>
<th></th>
<th>CAD patients</th>
<th>Control subjects</th>
<th>CAD and control subjects (CAD unidentified)</th>
<th>CAD and control subjects (CAD identified)</th>
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<tbody>
<tr>
<td></td>
<td>R²</td>
<td>Regression SSq</td>
<td>Residual SSq</td>
<td>F</td>
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<tr>
<td>Isoform mobility*</td>
<td>0.12</td>
<td>8.4</td>
<td>60.9</td>
<td>31.9</td>
</tr>
<tr>
<td>Number of isoform bands</td>
<td>0.18</td>
<td>12.4</td>
<td>56.8</td>
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<tr>
<td>LDL cholesterol</td>
<td>0.21</td>
<td>14.5</td>
<td>54.7</td>
<td>20.4</td>
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<tr>
<td>Apo B</td>
<td>0.23</td>
<td>16.0</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>82.6</td>
<td>30.1</td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; LDL, low-density lipoprotein; Apo, apolipoprotein; and BMI, body mass index. R² is calculated for the model after each variable is added, as are the sum of squares (SSq) attributable to the regression equation and the residuals and the corresponding F value. Values of the partial regression coefficient (β) are calculated after the last significant variable is added and represent the partial regression coefficients adjusted for all other variables in the model. T values and corresponding P values are calculated for the β values shown.

*Isoform mobility is the mobility (R, versus B) of the single isoform in single-band phenotypes and the mean of the two isoform relative mobilities for double-band phenotypes.
TABLE 5. Apolipoprotein(a) Isoform Classification

<table>
<thead>
<tr>
<th>Isoform mobility relative to apo B</th>
<th>Faster</th>
<th>Same</th>
<th>Slower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoform mass*</td>
<td>400-500 kD</td>
<td>500-550 kD</td>
<td>550-1000 kD</td>
</tr>
<tr>
<td>Utermann†</td>
<td>F</td>
<td>B</td>
<td>S1</td>
</tr>
<tr>
<td>Farrer</td>
<td>&gt;1.15</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Gaubatz</td>
<td></td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.35</td>
<td></td>
</tr>
</tbody>
</table>

*Apparent molecular mass of apolipoprotein(a) isoforms in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
†Isoform mobility in the SDS-PAGE system used in this report: F = 1.10±0.01, S1 = 0.92±0.02, S2 = 0.78±0.02, S3 = 0.66±0.02, S4 not defined.
Additional mobility class identified but not defined.

recognized.6-10 Recent reports have identified a higher proportion of small-molecular-mass isoforms in patients with CAD in some populations.51,52 While this may be partly responsible for differences in lipoprotein(a) concentration between patients and control subjects, higher lipoprotein(a) concentrations were observed in CAD for each of 21 isoform patterns definable by the phenotyping methods employed.51,52 This observation was attributed to the effect of putative nongenetic influences raising lipoprotein(a) concentration in CAD.52 There are large differences between our two study populations with respect to conventional risk factors as well as lipoprotein(a) concentration. We were able to investigate the importance of these in determining higher lipoprotein(a) concentrations in the CAD group. We used univariate linear regression analysis (Table 3) and multiple regression analysis (Table 4) between covariates and log lipoprotein(a) concentration in the two study populations analyzed separately. Multivariate associations between the covariates and log lipoprotein(a) concentration were also investigated in the pooled data from both study groups, excluding and then including identification of which group (CAD patients or control subjects) the subjects belonged to (Table 4). In this analysis, isoform mobility was included as a single continuous variable. By these methods, the most important independent determinants of circulating lipoprotein(a) concentration for all subjects were isoform mobility (P < .0001), number of isoform bands present (P < .0001), presence of CAD (P < .0001), sex (P < .005), and LDL cholesterol (P < .01) [corrected for the contribution of lipoprotein(a) to LDL29]. Separate analysis of single- and double-band phenotypes produced identical findings. Some covariates affecting the susceptibility to CAD may appear to be related to lipoprotein(a) concentration when the subject group is not identified, but they would not be independent predictors of lipoprotein(a) concentration when subject group (CAD or control) is identified. This probably explains the relationship between age and apolipoprotein AI concentration and lipoprotein(a) concentration, which is not independent of the presence of CAD.

Apolipoprotein(a) Genetics and Circulating Lipoprotein(a) Concentration

The number of kringle 4 repeats in the circulating apolipoprotein(a) isoform is directly related to the number of kringle 4-encoding DNA repeats at the apolipoprotein(a) gene locus.31,32 Apolipoprotein(a) size is strongly associated with circulating lipoprotein(a) concentration, explaining as much as 40% to 60% of the variability in circulating concentration of lipoprotein(a).28,29,53 Widely differing concentrations of lipoprotein(a) have, however, been observed in individuals with the same circulating apolipoprotein(a) isoforms.53 A recent study demonstrates that in families without familial hypercholesterolemia, circulating lipoprotein(a) concentrations are at least 90% explained by alleles at the apolipoprotein(a) gene locus.53 A study of monozygotic and dizygotic women twins supports this observation with a heritability estimate of

![Fig 5. Bar graph showing overall isoform frequencies in asymptomatic control subjects and patients with coronary artery disease (CAD). The proportions of subjects expressing each class of isoform either as a single-band or one of a double-band phenotype are shown.](http://atvb.ahajournals.org/)
circulating lipoprotein(a) concentration of 94%. More than 65% of the variability in lipoprotein(a) concentration is estimated to be due to the kringle 4-encoding region of the apolipoprotein(a) gene, but a significant proportion of the control of circulating lipoprotein(a) concentration (>25%) is attributable to cis-acting sequences separate from the apolipoprotein(a) kringle 4-encoding sequences. If these sequences are in linkage disequilibrium with kringle 4-encoding DNA at the apolipoprotein(a) gene locus, we may overestimate the contribution of kringle 4-encoding sequences to lipoprotein(a) concentration and underestimate the role of these as yet undefined cis-acting sequences.

Our findings are consistent with the hypothesis that an additional factor related to CAD is responsible for the higher lipoprotein(a) concentration for any class of apolipoprotein(a) isoform expressed. This is consistent with the genetic data and is in agreement with observations in asymptomatic carotid atherosclerosis. Other genes may still be important, and an apparent interaction of the gene loci for familial hypercholesterolemia and apolipoprotein(a) in determining circulating concentrations of lipoprotein(a) has been reported. We cannot discriminate between these mechanisms, but the concentration of lipoprotein(a) has been shown to be higher in individuals who had familial hypercholesterolemia and CAD compared with individuals with familial hypercholesterolemia who did not have CAD for each phenotype class recognized. Under such circumstances, the effect of the LDL receptor gene is not responsible for the differences in lipoprotein(a) between the subject groups. Genetic influence from the non–kringle 4-encoding gene sequences at the apolipoprotein(a) locus is one possible explanation of these observations. When a group of subjects with the same size of apolipoprotein(a) isoform but different lipoprotein(a) concentration was studied, a wide range of circulating lipoprotein(a) concentrations was observed, and these correlated strongly with the rate of lipoprotein(a) production rather than lipoprotein(a) catabolism. An insight into the mechanism responsible for this observation may follow from the observation that in vivo apolipoprotein(a) activity varies in a concordant manner with the circulating lipoprotein(a) concentration in those individuals. There are new insights into the relationship between the size polymorphism and posttranslational controls. In human liver, the size of mRNA molecules does not correlate with plasma lipoprotein(a) concentrations. The implication that posttranscriptional steps are also regulatory has been confirmed in the primary baboon hepatocyte model in which some null isoforms are identified at which the precursor apolipoprotein(a) molecule does not leave the endoplasmic reticulum. Confirmation that lipoprotein(a) concentrations in patients with severe CAD are under genetic control related to the apolipoprotein(a) locus is the necessary prerequisite before candidate gene sequences controlling the increased lipoprotein(a) concentrations in CAD are sought.

We conclude that CAD is associated with increased lipoprotein(a) concentrations independently of the size of circulating apolipoprotein(a) isoforms. We believe that a clearer understanding of the factors controlling apolipoprotein(a) gene expression along with important information concerning the regulation of posttranslational modification will give important insights into the role of lipoprotein(a) in CAD.

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