Glutamine/Hisotidine Polymorphism in Apo A-IV Affects Plasma Concentrations of Lipoprotein(a) and Fibrin Split Products in Coronary Heart Disease Patients

Arnold von Eckardstein, Jürgen Heinrich, Harald Funke, Helmut Schulte, Rainer Schönfeld, Ekkehart Köhler, Armin Steinmetz, and Gerd Assmann

A glutamine/histidine polymorphism at residue 360 in apolipoprotein (apo) A-IV that generates two electrophoretically detectable isoforms, apo A-IV-1 and apo A-IV-2, affects the plasma concentration of lipoprotein(a) (Lp[a]) in a healthy population. To verify this unexpected association we analyzed the effect of the apo A-IV polymorphism on Lp(a) serum concentrations in 275 male coronary heart disease patients. Allele frequencies of apo A-IV-1 and apo A-IV-2 were 0.917 and 0.083, respectively. In addition, apo A-IV-1/2 heterozygotes showed a 30% lower geometric mean concentration of Lp(a) than apo A-IV-1/1 homozygotes in this study. The relative frequency of Lp(a) concentrations >20 mg/dl was significantly increased by a factor of 2.25 in apo A-IV-1/1 homozygotes. Other lipid parameters were not significantly affected by this apo A-IV polymorphism. Because of the relations between Lp(a) and the fibrinolytic system, we also analyzed the effect of the apo A-IV polymorphism on hemostatic variables. Apo A-IV-1/2 heterozygosity was associated with a 70% higher geometric mean plasma concentration of D-dimer, i.e., proteolytic fragments of cross-linked fibrin. Plasma concentrations of prothrombin fragments F1+F2, fibrinogen, plasminogen, and plasminogen activator inhibitor-1 were unaffected. In conclusion, our results indicate a hitherto unappreciated role of the apo A-IV gene or a closely linked locus for the regulation of Lp(a) metabolism and hemostasis and also possibly for atherosclerosis and thrombosis.

(Key Words: apo A-IV polymorphism • lipoprotein(a) • d-dimer • fibrinolysis • hemostasis • thrombosis • atherosclerosis)

Secreted by enterocytes, apolipoprotein (apo) A-IV is a structural component of chylomicrons and some high density lipoproteins (HDLs). Its physiological role is unknown. In vitro, apo A-IV activates lecithin:cholesterol acyltransferase, modulates the activation of lipoprotein lipase by apo C-II, and promotes cholesterol efflux from various cells by binding to putative cell surface receptors for HDL. Therefore, apo A-IV plays some role in reverse cholesterol transport and the metabolism of triglyceride-rich lipoproteins.

The apo A-IV primary structure is encoded by three exons on chromosome 11. In the Caucasian population the apo A-IV gene shows three nonsynonymous polymorphisms in codons −8, 347, and 360 and additional variation with rare mutant alleles. The histidine-for-glutamine replacement in position 360 of the mature protein can be detected by isoelectric focusing (IEF). Reported allele frequencies for the isoforms apo A-IV-1 (apo A-IV<sup>His<sub>360</sub></sup>) and apo A-IV-2 (apo A-IV<sup>Glu<sub>360</sub></sup>) were 0.91–0.93 and 0.07–0.09, respectively. The impact of this apo A-IV polymorphism on lipid metabolism is controversially discussed. In three screening studies, apo A-IV-1/2 heterozygotes showed significantly higher levels of HDL cholesterol and lower triglycerides compared with apo A-IV-1/1 homozygotes. Other studies, including one from our laboratory, did not show these relations. However, in our study we observed 30% lower median concentrations of lipoprotein(a) (Lp[a]) in healthy apo A-IV-1/2 heterozygotes of both sexes compared with apo A-IV-1/1 homozygotes. This observation was unexpected because there is no apparent relation between the metabolic pathways of lipoproteins that contain apo A-IV and Lp(a).
Lp(a) is synthesized and secreted by hepatocytes as an apo B–containing lipoprotein to which a glycoprotein (apo[a]) is covalently attached. The catabolic fate of Lp(a) is unknown but has been suggested to include its removal from the circulation by LDL receptors. The apo(a) gene is localized on the long arm of chromosome 6(q26-q27) in a linkage group with the plasminogen gene. Apo(a) contains three motifs that are also present in plasminogen: a protease domain with 94% homology to plasminogen’s protease domain, a single plasminogen-like kringle-5 domain, and multiple plasminogen-like kringle-4 domains. The number of kringle-4 domains is genetically determined and generates a size polymorphism with various isoforms with a molecular mass inversely related to the Lp(a) plasma concentration. The effect of the apo(a) size polymorphism on the Lp(a) concentration has been estimated to amount to 40–70%. Because Lp(a) plasma concentrations are minimally influenced by environmental factors, genes other than that of apo(a) apparently contribute to the large interindividual variability of Lp(a) plasma concentrations in the population. Presently, only rare mutations in the LDL receptor gene that cause familial hypercholesterolemia have been found to affect Lp(a) plasma concentrations. Therefore, the apo A-IV polymorphism would be the first instance of a genetic variable that quantitatively affects Lp(a) serum concentrations, which is frequent in the population and distinct from the apo(a) gene.

To verify the relation between the apo A-IV polymorphism and Lp(a) serum concentrations, we performed the population and distinct from the apo(a) gene.

**Methods**

**Patients and Samples**

Two hundred seventy-five male CHD patients were recruited from a cardiological rehabilitation center. Baseline data of the patients are described in Table 1. Venous blood samples were drawn at least 4 weeks after myocardial infarction or cardiac surgery. Sera and plasma were immediately frozen after removal of cells by centrifugation. Quantification of both lipid and hemostatic parameters was performed centrally and as a series within 3 months after sample collection.

**Quantitative Analyses**

Serum was used for quantification of lipids, apolipoproteins, and Lp(a); citrate plasma was used for the measurement of hemostatic parameters. Serum concentrations of triglycerides and cholesterol were quantified with an autoanalyzer (Hitachi/Boehringer, Mannheim, FRG). HDL cholesterol concentrations were measured after precipitation with phosphotungstic acid/MgCl2 (Boehringer). LDL cholesterol was calculated by the Friedewald formula. Concentrations of apo A-I and apo B were determined with a modified, commercially available turbidimetric assay (Boehringer). Lp(a) concentrations were determined by electroimmunodiffusion with the use of antiserum and standards from Immuno (Vienna). Prothrombin time; partial thromboplastin time; and plasma concentrations of fibrinogen (according to Clauss), plasminogen, and prothrombin fragments F1 + F2 were quantified by assays from Behringwerke (Marburg, FRG). The concentration of d-dimers, which are proteolytic fragments of cross-linked fibrin, were determined by an enzyme-linked immunosorbent assay from Boehringer.

**Phenotyping of Apo A-IV Polymorphism**

The apo A-IV polymorphism was phenotyped by IEF of human plasma and subsequent immunoblotting with a rabbit anti-human apo A-IV antiserum raised in the laboratory of Dr. Steinmetz (Marburg, FRG), a biotinylated donkey anti-rabbit antiserum (Amersham), and streptavidin–horseradish peroxidase. Statistical analyses were done with the SPSS package. All concentrations are represented by mean±SD. Because not all variables were normally distributed, non-parametric statistical tests (Mann-Whitney U test and Spearman's rank correlation) were used.

**Results**

Figure 1 demonstrates the separation of the two frequent, genetically determined apo A-IV isoforms by IEF of human plasma and subsequent immunoblotting with anti-human apo A-IV antiserum. The anodic, more
frequent polymorph is called apo A-IV-1 and the more cathodic one apo A-IV-2. In the present study the allele frequencies of apo A-IV-1 and apo A-IV-2 were 0.917 and 0.083, respectively. Because only three apo A-IV-2/2 homozygotes were found, only data from 232 apo A-IV-1/1 homozygotes and 40 apo A-IV-1/2 heterozygotes were used to estimate the impact of this apo A-IV polymorphism on lipid parameters and hemostatic variables. Baseline parameters of patients from both groups were not different (Table 1).

Table 2 compares the plasma concentrations of lipids, lipoproteins, and apolipoproteins in apo A-IV-1/1 homozygotes, apo A-IV-1/2 heterozygotes, and apo A-IV-2/2 homozygotes. Statistically significant differences were observed only for serum concentrations of Lp(a), which were lower in apo A-IV-1/2 heterozygotes than apo A-IV-1/1 homozygotes (p<0.025, Mann-Whitney U test). The arithmetic mean was decreased by 50% (11.4 versus 21.6 mg/dl), the geometric mean by 30% (9.6 versus 13.6 mg/dl), and the median by 10% (8 versus 9 mg/dl). Although it was not significant, the geometric mean concentration of Lp(a) was also lower in the three apo A-IV-2/2 homozygotes compared with apo A-IV-1/2 heterozygotes (5.8 versus 9.6 mg/dl).

In Caucasian populations, the frequency distribution of Lp(a) plasma concentrations that are shifted to the lower values is well known. Remarkably, in apo A-IV-1/2 heterozygotes this shift seemed even more pronounced when compared with apo A-IV-1/1 homozygotes because of the lack of apo A-IV-1/2 heterozygotes with Lp(a) concentrations >50 mg/dl (Figure 2). Our laboratory has recently suggested 20 mg/dl as a threshold Lp(a) value to define patients at risk for myocardial infarction.

Although the differences were not significant, it is noteworthy that in a converse manner the mean concentrations of LDL cholesterol and apo B and the concentrations of HDL cholesterol and apo A-I showed opposite trends in apo A-IV-1/2 heterozygotes and apo A-IV-1/1 homozygotes and differed by 3-5% (Table 2). Because of the putative interrelations between Lp(a) and fibrinolysis, we analyzed whether the apo A-IV polymorphism also affects hemostatic parameters (Table 3). We found highly significant differences for the plasma concentrations of D-dimers, which are proteolytic fragments of cross-linked fibrin (p<0.0025, Mann-Whitney U test).

\[
\begin{array}{cccc}
\text{Table 2. Impact of Apolipoprotein A-IV Polymorphism on Lipid Values} \\
\hline
& 1/1 (n=232) & 1/2 (n=40) & 2/2 (n=3) \\
\text{Cholesterol (mg/dl)} & 227.8±40.7 & 221.2±36.4 & 265.3±74.1 \\
\text{Triglycerides (mg/dl)} & 79.8<124.0<192.5 & 69.4<115.6<192.5 & 97.5<141.2<204.4 \\
\text{HDL cholesterol (mg/dl)} & 34.9±8.4 & 36.9±8.1 & 32.7±10.9 \\
\text{LDL cholesterol (mg/dl)} & 164.3±34.8 & 157.9±33.5 & 197.0±36.3 \\
\text{Lp(a) (mg/dl)*} & 4.8<13.6<39.3 & 4.5<9.7<20.9 & 1.3<5.8<26.6 \\
\text{Apo A-I (mg/dl)} & 112.7±19.5 & 116.2±15.5 & 106.3±13.1 \\
\text{Apo B (mg/dl)} & 89.2±16.9 & 86.4±17.9 & 102.3±44.7 \\
\hline
\end{array}
\]

HDL, high density lipoprotein; LDL, low density lipoprotein; Lp(a), lipoprotein(a); apo, apolipoprotein.

\*p<0.025 by comparison of values from apo A-IV-1/1 homozygotes and apo A-IV-1/2 heterozygotes by Mann-Whitney U test. All other differences were not significant. Concentrations of triglycerides and Lp(a) are presented as \(-1SD<.C< +1SD\) ranges as obtained by retransformation of ln(triglycerides) and ln(Lp(a)), respectively.
TABLE 3. Impact of the Apolipoprotein A-IV Polymorphism on Hemostatic Variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>1/1 (n=232)</th>
<th>1/2 (n=40)</th>
<th>2/2 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin time (%)</td>
<td>79.2±38.4</td>
<td>80.8±40.9</td>
<td>ND</td>
</tr>
<tr>
<td>PTT (seconds)</td>
<td>41.9±10.4</td>
<td>40.8±9.2</td>
<td>ND</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.22±0.81</td>
<td>3.15±0.69</td>
<td>4.11±1.45</td>
</tr>
<tr>
<td>Plasminogen (%)</td>
<td>97.0±19.1</td>
<td>93.8±22.2</td>
<td>118.4±17.9</td>
</tr>
<tr>
<td>PAI-1 (units/ml)</td>
<td>0.90&lt;2.12&lt;5.00</td>
<td>0.90&lt;2.05&lt;5.16</td>
<td>1.11&lt;1.93&lt;3.39</td>
</tr>
<tr>
<td>F1+F2 (nmol/l)</td>
<td>0.36&lt;0.68&lt;1.27</td>
<td>0.39&lt;0.76&lt;1.48</td>
<td>0.37&lt;0.60&lt;0.98</td>
</tr>
<tr>
<td>D-Dimer (/tg/l)*</td>
<td>137.0&lt;287.1&lt;601.1</td>
<td>249.6&lt;483.0&lt;934.5</td>
<td>210.6&lt;395.4&lt;742.5</td>
</tr>
</tbody>
</table>

PTT, partial thromboplastin time; PAI-1, plasminogen activator inhibitor type 1; F1+F2, prothrombin fragments F1 and F2.

*p<0.0025 by comparing values from apolipoprotein (apo) A-IV-1/1 homozygotes and apo A-IV-1/2 heterozygotes by Mann-Whitney U test. All other differences were not significant. Concentrations of PAI-1, F1+F2, and D-dimer do not exhibit a Gaussian frequency distribution; therefore, their mean values are presented as -1SD<x<+1SD ranges as obtained by retransformation of ln(PAI-1), ln(F1+F2), and ln(D-dimer), respectively.

Whitney U test). Apo A-IV-1/2 heterozygotes showed a 50% higher arithmetic mean (574.8 versus 372.5 µg/l) and a 70% higher geometric mean concentration of D-dimer (480 versus 287 µg/l). Median concentrations of D-dimer differed by 88% (516 versus 274 µg/l). Figure 3 shows that in apo A-IV-1/1 homozygotes the frequency distribution of plasma concentrations of D-dimer is shifted to the lower values, whereas it almost shows a Gaussian frequency distribution in apo A-IV-1/2 heterozygotes. Other hemostatic variables were not different between the two groups.

Table 4 summarizes univariate correlations between the natural logarithm of Lp(a) and the natural logarithm of D-dimer and various lipid and hemostatic parameters. In univariate regression analyses values for Lp(a) and D-dimer did not correlate.

Discussion

This study verified the unexpected effect of the glutamine/histidine polymorphism in apo A-IV on the serum concentration of Lp(a) that we had previously observed in both male and female students.24 In CHD patients the frequency distribution of Lp(a) serum concentrations was shifted to higher values compared with the student population, with a 30% higher geometric mean and median of Lp(a). Nevertheless, male apo A-IV-1/2 heterozygotes of both healthy and diseased populations showed geometric mean serum concentrations of Lp(a) that were 30% lower than apo A-IV-1/1 homozygotes from the same populations. Altogether, these findings indicate a constant effect of the apo A-IV gene or a closely linked gene on Lp(a) serum concentrations in the population. In consideration of the heterozygosity frequency of 13-16% for apo A-IV-1/2 in Caucasians,24-30 the apo A-IV polymorphism thus affects the variation of Lp(a) serum concentrations in the population by 4-5%. Lp(a) levels are otherwise minimally influenced by environmental but maximally by genetic factors, especially by the apo(a) gene.32-34 First estimations of the genetic effect of apo(a) on Lp(a) plasma concentrations were made by studies in which the apo(a) polymorphism was characterized as phenotypes on the mobility of six different apo(a) isoforms in sodium dodecyl sulfate-gel electrophoresis. These population studies suggested that the effect of the apo(a) gene on Lp(a) levels amounts to 40%.32 More recent family studies analyzed the apo(a) polymorphism as genotypes by the use of pulse-field electrophoresis of Kpn I restriction fragments of the apo(a) gene. This method helped to separate 19 apo(a) alleles that are

![Figure 3. Bar graph showing effect of apolipoprotein (apo) A-IV polymorphism on frequency distribution of plasma concentrations of D-dimer. Note left-shifted frequency distribution of D-dimer in apo A-IV-1/1 homozygotes and near-Gaussian frequency distribution in apo A-IV-1/2 heterozygotes.](http://atvb.ahajournals.org/content/bibliography/5/12/243/F5.large.jpg)
TABLE 4. Spearman Correlation Coefficients Between Lp(a), D-Dimer, Age, Body Mass Index, Lipids, and Hemostatic Variables

<table>
<thead>
<tr>
<th></th>
<th>Lp(a)</th>
<th>D-Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.097</td>
<td>0.485†</td>
</tr>
<tr>
<td>Body mass index</td>
<td>-0.124*</td>
<td>0.033</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.136*</td>
<td>-0.139*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.006</td>
<td>-0.258†</td>
</tr>
<tr>
<td>HDL chol</td>
<td>0.055</td>
<td>0.061</td>
</tr>
<tr>
<td>LDL chol</td>
<td>0.137*</td>
<td>0.070</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.000</td>
<td>-0.042</td>
</tr>
<tr>
<td>Apo A-1</td>
<td>-0.009</td>
<td>-0.037</td>
</tr>
<tr>
<td>Apo B</td>
<td>0.106*</td>
<td>-0.085</td>
</tr>
<tr>
<td>Prothrombin time</td>
<td>-0.122*</td>
<td>-0.146*</td>
</tr>
<tr>
<td>PTT</td>
<td>0.106*</td>
<td>0.042</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-0.024</td>
<td>0.543†</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>0.059</td>
<td>0.088</td>
</tr>
<tr>
<td>PAI-1</td>
<td>-0.079</td>
<td>-0.179*</td>
</tr>
<tr>
<td>F1+F2</td>
<td>-0.009</td>
<td>0.258†</td>
</tr>
<tr>
<td>D-Dimer</td>
<td>-0.042</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Lp(a), lipoprotein(a); HDL, high density lipoprotein; LDL, low density lipoprotein; apo, apolipoprotein; PTT, partial thromboplastin time; PAI-1, plasminogen activator inhibitor type 1; F1+F2, prothrombin fragments F1+F2. *p<0.05, †p<0.001.

supposed to differ from each other by the number of kringle-4 repeat coding regions. By this method, a recent study in siblings with zero, one, or two identical apo(a) alleles suggested that the apo(a) gene accounts for 91% of the variance in Lp(a) plasma concentrations. The effect of the number of kringle-4 repeats was estimated to account for 69% and the effect of presently undefined, regulatory, cis-acting sequences for 22%. Consequently, medians of Lp(a) concentrations that differ by 30% between apo A-IV-1/1 homozygotes and apo A-IV-1/2 heterozygotes, as well as the calculated 5% contribution of the apo A-IV polymorphism to the variation of Lp(a) serum concentrations in the population, seem considerably high. Because the genes for apo A-IV and apo(a) reside on distinct chromosomes, the effect of the apo A-IV gene must be considered independent of the apo(a) gene.

Presently, genetic variation of only one other gene, the LDL receptor gene, has been shown to influence Lp(a) plasma concentrations. Heterozygotes for familial hypercholesterolemia had twofold to threefold higher Lp(a) concentrations than unaffected members of the same families. In an individual that is heterozygous for familial hypercholesterolemia, genetic variation in the LDL receptor gene significantly contributes to the serum Lp(a) concentration and therefore possibly to the risk for CHD. However, because heterozygosity for familial hypercholesterolemia is present in the population at a relatively low frequency (one out of 500), underlying mutations in the LDL receptor gene contribute only 0.4–0.5% to the variation of Lp(a) levels in the population. Therefore, the effect that is exerted through the apo A-IV polymorphism in the population is only 10%.

Because of the putative interrelation of Lp(a) and the fibrinolytic system, we also analyzed whether the apo A-IV polymorphism would influence hemostatic variables. Interestingly, it was associated with pronounced changes in the plasma concentrations of d-dimer. Apo A-IV-1/2 heterozygotes showed 70% higher median concentrations of d-dimer, which are proteolytic, cross-linked fibrin fragments that are ultimately produced by plasmin. Also of interest, Lp(a) has been hypothesized by many authors to exert its atherogenic effect through interference with fibrinolysis. First, apo(a) is highly homologous to plasminogen. Second, Lp(a) competes with plasminogen for binding to endothelial cells, monocytes, fibrinogen, and fibrin. Last, Lp(a) prevents the inhibition of tissue plasminogen activator by plasminogen activator inhibitor-1. In subjects with high Lp(a) levels, it has recently been observed that Lp(a) is bound to fibrin and that the generation of plasmin at the plasm-in-fibrin interface is decreased. These observations together suggest that Lp(a) inhibits plasmin-mediated fibrinolysis. Therefore, although in our study, plasma concentrations of Lp(a) and d-dimer did not correlate, it is intriguing to speculate whether the higher median concentrations of d-dimer in apo A-IV-1/2 heterozygotes reflect facilitated fibrinolysis because of the lower Lp(a) concentration in this group.

Numerous studies demonstrated an association between elevated Lp(a) levels and the risk for CHD, stroke, and vessel reocclusion after aortocoronary bypass surgery. Therefore, the association of the apo A-IV-2 allele with lower plasma concentrations of Lp(a) addresses the question of whether carriers of this allele are at a decreased risk for myocardial infarction. The allele frequency of apo A-IV-2 in the heretofore-described CHD patients was 0.083 and consequently in the range that has been described for Caucasian populations. The mean age of apo A-IV-1/2 heterozygotes was slightly higher. Coronary angiographic scores were not different between the two groups (not shown). Therefore, a tremendous antiatherogenic effect of the apo A-IV-2 allele can apparently be ruled out, although the question should be read- dressed in more comprehensive studies.

In summary, our results demonstrate a highly significant effect of the glutamine/histidine polymorphism at residue 360 in apo A-IV on the plasma concentrations of Lp(a) and d-dimer. Although the metabolic basis for our findings is presently unknown, these associations shed some important light on the previously unappreciated role of the apo A-IV gene or a closely linked locus in the regulation of Lp(a) and hemostasis.

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