Apolipoprotein E Polymorphism and Coronary Artery Disease

Hans-Jürgen Menzel, Rolf-Georg Kladetzky, and Gerd Assmann

Lipid status and apolipoprotein E phenotypes were tested in 1000 patients who underwent coronary angiography. The same number of factory employees was chosen as a control group. We distinguished between six different apolipoprotein E phenotypes and determined their frequencies in all groups. For the three homozygous phenotypes E3/3, E4/4, and E2/2, the percentage distribution in the group of factory employees was 62.7%, 2.3%, and 0.8%, respectively; for the three heterozygous phenotypes E4/3, E3/2, and E4/2, we determined frequencies of 20.3%, 11.0%, and 3.0%, respectively. In the group of patients with and without signs of coronary atherosclerosis, we observed almost the same frequencies except that heterozygotes (E3/2) occurred significantly more frequently in the group of coronary angiography patients unaffected by coronary sclerosis. Cholesterol and triglyceride values were significantly elevated in patients with coronary artery disease, whereas high density lipoprotein cholesterol levels were not significantly different. The data further suggest that apolipoprotein E2/2 homozygosity, despite the presence of β-very low density lipoproteins in the plasma of these patients, cannot be considered a biochemical indicator of an increased risk of coronary atherosclerosis. On the other hand, apolipoprotein E3/2 heterozygosity may have a protective effect on the development of early atherosclerosis. (Arteriosclerosis 3:310–315, July/August 1983)

Since Type III hyperlipoproteinemia was described by Fredrickson et al.,1 individuals with this disease have been shown to be at very high risk for atherosclerosis.2 In these patients (about one in 10,000 individuals), an abnormal lipoprotein, beta very low density lipoprotein (β-VLDL) accumulates in the plasma. This has been linked to the storage of cholesteryl esters in macrophages and possibly atheroma formation.3–5 The biochemical defect of Type III hyperlipoproteinemia, a polygenic disorder, has been described as the absence of apo E-3 and apo E-4 and the presence of apo E-2 together with a manifestation of primary or secondary hyperlipoproteinemia.6–9

It is at present unknown whether patients who are homozygous for apolipoprotein E-2 (1 in 100 persons), and do not show the typical clinical signs of Type III hyperlipoproteinemia (tuberoeruptive xanthoma, peripheral vascular disease, and coronary heart disease) are susceptible to atherosclerosis. Homozygosity for apo E-2 is always accompanied by the presence of β-VLDL and, provided total cholesterol is in the normal range, also by low density lipoprotein (LDL) cholesterol values.9 However, it is not known whether the normally protective effect of a low serum cholesterol is balanced, or even outweighed, by the atherogenic effect of β-VLDL. The intention of this investigation was to provide further insight concerning possible connections between coronary artery disease, apolipoprotein E phenotypes, and lipid values.

Methods

Patients

Serum was collected from about 1000 fasting patients who underwent coronary angiography at the Department of Medicine, Universities of Düsseldorf and Münster. All patients gave informed consent and the procedure was in accord with institutional guidelines. Indications for coronary angiography were chest pain, pathological stress EKG, heart valve failure (preoperative diagnosis), cardiomyopathy, and cardiac arrhythmias. Sera from the control group were gathered from factory employees in connection with an epidemiological study, the Münster Prospective Cardiovascular Study.10

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Isolation of Apo VLDL

We centrifuged 1 ml of serum if triglycerides were greater than 100 mg/dl, 2 ml of serum if triglycerides were less than 100 mg/dl, for 24 hours (4°C) at 25,000 rpm in a type 25 Beckman rotor. The top 300 µl were siphoned and 0.9% NaCl and 0.02% NaN₃ were added and ultracentrifuged under the same conditions. The washed VLDL was either frozen at -20°C or directly delipidated with ethanol/ether (3:1). The apolipoproteins were dissolved in 50 µl 0.01 M Tris-HCl (pH 8.2) containing 1% decylsulfate (Kodak), 2% ampholytes (pH 3-7), 10% β-mercaptoethanol, and 13% sucrose, and were applied to the focusing gels.

Polyacrylamide Gel Isoelectric Focusing

Isoelectric focusing was done according to the method of Pagnan et al.12 with certain modifications. We used the slab gel system Model 220 (Bio Rad, Richmond, Virginia), with ampholytes pH 3-5 (Serva, Heidelberg), pH 5-7 (Serva), and pH 5-7 (LKB) mixed 2:1:1. The gel solution was poured into the cassettes of a Bio Rad Model 220 electrophoresis cell with 2 mm spacers. A 16-well comb was used to prepare the individual slots. After polymerization, the slots were filled with the samples and these were layered with 2% ampholytes and 8% sucrose solution. The electrophoresis was run overnight (17 hours) at 200 V starting with power limited to 3 W per plate, and at 600 V in the morning for 1 hour. For staining we used a modification of the method of Malik and Berrie.13 With this method about 30 samples could be handled each day by one technician.

Modification of VLDL

Isolated VLDL was treated with cysteamine (Sigma Chemical Company, St. Louis, Missouri) for 16 hours at 37°C14 for the charge modification of cysteine. β-mercaptoethanol was then omitted in isoelectric focusing. Neuraminic acid was cleaved with 0.01 U neuraminidase (Sigma), using 100 µg of VLDL protein in 0.5 ml of 0.05 M sodium acetate (pH 5.5) for 1 hour at 37°C. After modification, the VLDL was delipidated and used for isoelectric focusing.

2-D Gel Electrophoresis

After isoelectric focusing, strips from the focusing gels were cut and immersed in 0.002 M ethylmorpholine-HCl (pH 8.5), 0.2% sodium dodecylsulfate (SDS), 0.1% β-mercaptoethanol, bromophenol blue solution, and 4% sucrose for 15 minutes at room temperature, and applied for electrophoresis in the second dimension on an SDS gel. The SDS system was prepared according to Neville15 with 15% acrylamide concentration.

Total cholesterol, triglycerides, and high density lipoprotein (HDL) cholesterol were enzymatically determined with a commercially available test kit (Boehringer, Mannheim).

Coronary Angiography

Coronary angiography was performed as described previously.16 Individuals who had chest pain and a greater than 50% reduction of lumen size in one or more coronary vessels were classed as coronary artery disease (CAD +); all other coronary angiography patients were classed as CAD -.

Statistical Methods

Apolipoprotein E frequencies were compared by a χ² test and lipid values by the method of Mann and Whitney.17

Results

Isoelectric focusing of human apo VLDL in a pH gradient from 3 to 7 revealed several bands (Figure 1) which have been identified by 2-D techniques as the apolipoprotein C, apolipoprotein E, and apolipoprotein A-I groups (not shown). The nature of the polymorphic forms in the different groups has been established by modification treatment with neuraminidase and cysteamine (not shown) and is in agreement with the previously published results.6 12 14 18 -20

In Figure 1, the most common genetically determined polymorphic forms of apolipoprotein E are presented. In Lanes C to G, only apo E-3 and the
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In Figure 1, the most common genetically determined polymorphic forms of apolipoprotein E are presented. In Lanes C to G, only apo E-3 and the

Figure 1. Isoelectric focusing of apo VLDL in a pH gradient from 3 to 7. Lane A, Individual with phenotype E3/2. Lane B, Individual with phenotype E4/3. Lanes C-G, Individuals with phenotype E3/3.

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sialated form of apo E-3 (migrating to a more acidic position) are visible in the apolipoprotein E region. These individual patients were considered to have the phenotype E3/3. Lane A and Lane B demonstrate the two heterozygous forms; Lane A shows apo E-3 plus apo E-2 and Lane B shows apo E-3 plus apo E-4. The apo E-2 band is slightly more basic than the sialated form of apo E-3, thus permitting easy identification of the phenotype E3/2. The bands slightly above apolipoprotein A-I-1 can be removed by neuraminidase treatment of VLDL and thus represent sialated forms of either apo E-2 or E-3.

The rare apo E phenotypes are shown in Figure 2. For comparison, we include in Lanes E, F, and J apo VLDL derived from individuals with phenotype E3/3 and in Lanes D, H, and K apo VLDL from patients with the phenotype E3/2. Apo VLDL from probands homozygous for apo E-2 (phenotype E2/2) is shown in Lane G and from probands homozygous for apo E-4 (phenotype E4/4) in Lane B. The combination of apo E-2 and apo E-4 (phenotype E4/2) is seen in Lanes A and C.

After establishing the identity of the six apolipoprotein E phenotypes by the screening procedure described here, the technique was applied in connection with a large prospective epidemiological study of factory employees in the area of Westphalia and in a group of patients who underwent coronary angiography.

Patients with signs of coronary atherosclerosis (CAD + ) exhibit almost the same phenotype frequency as the control group of factory employees (Table 1). However, patients with phenotype E3/2 occurred significantly more frequently in the group of coronary angiography patients where no coronary artery disease was detected (CAD - ). This difference was even more pronounced taking only males into account (Table 2), whereas for females no significant variations in frequencies were observed (Table 3).

The lipid values for the six apo E phenotypes in the three groups (A, B, and C) are shown in Table 4. Patients with coronary atherosclerosis had higher (p < 0.001) mean cholesterol (236 ± 50 mg/dl) and triglyceride (189 ± 110 mg/dl) values than patients in both control groups (Group A: cholesterol 196 ± 35 mg/dl, triglycerides 128 ± 69 mg/dl; Group C: cholesterol 214 ± 42 mg/dl, triglycerides 151 ± 93 mg/dl). When lipid values were separately analyzed for the six E phenotypes, patients with the e2 allele in all three groups had lower (p < 0.01) serum choles-

![Figure 2. Isoelectric focusing of apo VLDL in a pH gradient from 3 to 7. Lanes A and C. Phenotypes E4/2.](image-url)

![Figure 3. Table 1. Apo E Polymorphism and Coronary Artery Disease](image-url)

<table>
<thead>
<tr>
<th>Apo E Polymorphism</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 1000)</td>
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<tr>
<td>e3,e3</td>
<td>62.7</td>
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<tr>
<td>e2,e2</td>
<td>0.8</td>
</tr>
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<td>e4,e4</td>
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<tr>
<td>e3,e2</td>
<td>11.0†</td>
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<td>e4,e3</td>
<td>20.2</td>
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</table>

*p < 0.1.
†p < 0.025.
CAD + = patients with coronary artery disease.
CAD - = patients without coronary artery disease.

![Table 2. Apo E Polymorphism and Coronary Artery Disease in Men](image-url)

<table>
<thead>
<tr>
<th>Apo E Polymorphism</th>
<th>Frequency (%)</th>
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<tr>
<td></td>
<td>Control (n = 626)</td>
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<td>e3,e3</td>
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<tr>
<td>e3,e2</td>
<td>10.8†</td>
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<tr>
<td>e4,e3</td>
<td>19.9</td>
</tr>
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*p < 0.025.
†p < 0.01.
CAD + = patients with coronary artery disease.
CAD - = patients without coronary artery disease.
Table 3. Apo E Polymorphism and Coronary Artery Disease In Females

<table>
<thead>
<tr>
<th>Apo E Polymorphism</th>
<th>Genotype</th>
<th>Frequency (%)</th>
<th>Control (n = 374)</th>
<th>CAD+ (n = 90)</th>
<th>CAD- (n = 255)</th>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>E3/E3</td>
<td>E3/3</td>
<td>62.6</td>
<td>63.3</td>
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<tr>
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<td>0.5</td>
<td>1.1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>E4/E4</td>
<td>E4/4</td>
<td>2.4</td>
<td>1.1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>e4/e2</td>
<td>E4/2</td>
<td>2.7</td>
<td>3.3</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>E3/E2</td>
<td>E3/2</td>
<td>11.2</td>
<td>12.2</td>
<td>13.7</td>
<td></td>
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<tr>
<td>E4/E3</td>
<td>E4/3</td>
<td>20.6</td>
<td>18.9</td>
<td>18.0</td>
<td></td>
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</tbody>
</table>

CAD+ = patients with coronary artery disease.  
CAD- = patients without coronary artery disease.

Cholesterol values (199 ± 41 mg/dl) than patients without the e2 allele (211 ± 45 mg/dl), but higher (p < 0.01) triglyceride values (155 ± 89 mg/dl vs 142 ± 86 mg/dl). There were no differences in HDL cholesterol values.

Differences in lipid values were also observed between males and females. Triglycerides were higher (p < 0.001) in males (155 ± 91 mg/dl vs 126 ± 61 mg/dl); HDL cholesterol was lower (p < 0.001) in males (41 ± 10 mg/dl vs 49 ± 13 mg/dl); there were no differences in total cholesterol. When patients with coronary heart disease were divided into male and female groups (Groups E and H) and compared with the respective controls (Groups D, F, G, and J), it was evident that those with coronary heart disease...
had higher \( (p < 0.001) \) total cholesterol and triglyceride values (Tables 5 and 6). Females with coronary atherosclerosis had lower \( (p < 0.001) \) HDL cholesterol values than the control group \( \text{Group H showed } 42 \pm 12 \text{ mg/dl vs Group J which showed } 47 \pm 15 \text{ mg/dl} \). No significant difference was observed in males.

**Discussion**

The development of a screening technique for determination of the apolipoprotein E phenotypes permits investigation of large populations. In conjunction with coronary angiography, it is possible to evaluate the existence of a specific coronary risk for individuals with one of the six different apolipoprotein E phenotypes.

Our results demonstrate similar frequencies of the six apolipoprotein E phenotypes, comparing the group of patients affected by coronary atherosclerosis with two control groups (factory employees and patients without signs of coronary atherosclerosis as evaluated by coronary angiography). The significant difference in the frequencies of heterozygotes \( \text{E3/2} \) between the patient groups with and without CAD \( (11.2\% \text{ and } 16.4\%, \text{ respectively}) \) is of potential interest (Table 1). In males, this difference is even more pronounced \( (19\% \text{ vs } 11\%) \) (Table 2).

The origin of this observation may relate to the effect of the \( \text{e2} \) allele on serum cholesterol. Coronary angiography patients with phenotype \( \text{E3/2} \) (in both CAD+ and CAD− groups) have lower serum cholesterol values compared to patients with other apoE phenotypes. The lowest cholesterol concentrations were observed in \( \text{E3/2} \) patients unaffected by coronary artery disease. It is, therefore, possible that \( \text{E3/2} \) heterozygosity, through its hypocholesterolemic effect, may be considered a negative risk factor for CAD.

We cannot explain the discrepancy in the prevalence of the phenotype \( \text{E3/2} \) among the control groups \( (11\% \text{ in factory employees}, \ 16.4\% \text{ in patients without signs of coronary atherosclerosis}) \), but this may relate to differences in mean age between the patients \( (54 \text{ years}) \) and factory employees \( (37 \text{ years}) \). When only CAD patients younger than 45 years were considered, the frequency of the \( \text{E3/2} \) phenotype in the control group was intermediate between the frequencies of the two patient groups \( (\text{CAD+} = 8.5\%, \text{control group} = 11\%, \text{and} \text{CAD−} = 17.3\%) \).

It was of particular interest to establish that apoE-2 homozygosity occurs with almost the same frequency \( (p < 0.7) \) among patients affected versus those unaffected by coronary atherosclerosis. As already demonstrated by Utermann et al., and confirmed here, individuals homozygous for apolipoprotein \( \text{E-2} \) exhibit \( \beta\)-VLDL in their plasma, and on the average have lower serum cholesterol values than patients homozygous for either apo E-3 or apo E-4. Apparently, the occurrence of \( \beta\)-VLDL in these patients is not necessarily associated with an increased risk for coronary atherosclerosis, and only extreme elevation of \( \beta\)-VLDL in plasma, as occurs in Type III hyperlipoproteinemia, is linked to premature atherosclerosis. Rall et al. have recently demonstrated that persons with the apolipoprotein E-2 phenotype represent a genetically heterogeneous group. These authors were able to show that Arg-Cys replacements occur at different sites in apolipoprotein E, affecting the functional properties of apolipoprotein E-2 (e.g., binding to apo B, E receptor cells) to a different degree. It is, therefore, possible that apolipoprotein E-2 homozygosity, depending on the precise site of the mutation, affects the metabolism of the apolipoprotein E-containing lipoproteins to a more severe degree in some persons than in others. Further investigation is required to establish in which individuals such metabolic abnormalities are associated with an increased risk for coronary artery disease.

The results of this investigation suggest that apoE-2 homozygosity or heterozygosity, in the absence of hypercholesterolemia, cannot be considered a coronary risk factor. By contrast, as demonstrated by the higher prevalence of \( \text{E3/2} \) heterozygosity in CAD− patients, the \( \text{e2} \) allele may have a protective effect on the development of coronary atherosclerosis.

**Acknowledgments**

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**References**

17. Mann HB, Whitney DP. On a test of whether one of two random variables is stochastically larger than the other. Ann Math Statist 1974;18:50–60
18. Zannis VI, Breslow JL. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modifications. Biochemistry 1979;20:1033–1041

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