Effects of a Frequent Apolipoprotein E Isoform, ApoE4Freiburg
(Leu28→Pro), on Lipoproteins and the Prevalence of Coronary Artery Disease in Whites

Matthias Orth, Wei Weng, Harald Funke, Armin Steinmetz, Gerd Assmann, Matthias Nauck,
Jutta Dierkes, Andreas Ambrosch, Karl H. Weisgraber, Robert W. Mahley,
Heinrich Wieland, Claus Luley

Abstract—Different isoforms of apoE modulate the concentrations of plasma lipoproteins and the risk for atherosclerosis. A novel apoE isoform, apoE4Freiburg, was detected in plasma by isoelectric focusing because its isoelectric point is slightly more acidic than that of apoE4. apoE4Freiburg results from a base exchange in the APOE gene that causes the replacement of a leucine by a proline at position 28. Analysis of the allelic frequencies in whites in southwestern Germany revealed that this isoform is frequent among control subjects (10:4264 alleles) and is even more frequent in patients with coronary artery disease (21:2874 alleles; \( P=0.004; \) adjusted odds ratio, 3.09; 95% confidence interval, 1.20 to 7.97). ApoE4Freiburg affects serum lipoproteins by lowering cholesterol, apoB, and apoA-I compared with apoE4 (\( P<0.05 \)). Our 4 apoE4Freiburg homozygotes suffered from various phenotypes of hyperlipoproteinemia (types IIa, IIb, IV, and V). In vitro binding studies excluded a binding defect of apoE4Freiburg, and in vivo studies excluded an abnormal accumulation of chylomicron remnants. ApoE4Freiburg and apoE4 accumulated to a similar extent in triglyceride-rich lipoproteins. HDLs, however, contained about 40% less apoE4Freiburg than apoE4. In conclusion, our data indicate that apoE4Freiburg exerts its possible atherogenic properties by affecting the metabolism of triglyceride-rich lipoproteins and HDL. (Arterioscler Thromb Vasc Biol. 1999;19:1306-1315.)

Key Words: apoE polymorphism • mutation • atherosclerosis • isoelectric focusing

Apolipoprotein E is a constituent of all human lipoproteins except LDL1 and occurs in 3 common isoforms (E2, E3, and E4) that are encoded by 3 codominant alleles (\( e2, e3, \) and \( e4 \)) of the APOE gene.2 ApoE plays a pivotal role in triglyceride and cholesterol metabolism by mediating the sequestration and removal of remnants of triglyceride-rich lipoproteins via the heparan sulfate proteoglycan-LDL receptor-related protein (LRP) and LDL receptor pathways.3–5 The apoE isoforms have different affinities for these pathways.3–5 The APOE alleles modulate the risk for coronary artery disease (CAD),6,9,10 cerebral atherosclerosis,11 and Alzheimer’s disease.12,13 In addition to the common apoE isoforms, several rare apoE variants have been identified in hyperlipidemic patients and their kindreds. Most of the variants are characterized by replacements of one14–22 or more23,24 charged amino acids by uncharged amino acids or vice versa. Some replacements in the LDL receptor-binding region (positions 136 to 150) cause defective binding to the LDL receptor and are associated with the recessive form of type III hyperlipoproteinemia, and some replacements of basic amino acids with neutral or acidic amino acids, leading to defective heparan sulfate proteoglycan (HSPG) binding, were associated with the dominant form of type III hyperlipoproteinemia.3,18 Variants with a basic amino acid at amino acid residue 3 display increased binding to LDL receptors and were associated with increased LDL cholesterol.25,26 The total number of patients with mutant APOE alleles identified by studying hyperlipidemic patients and their families, however, is very low.

The common isoforms of apoE and most of the known rare variants of apoE have been determined on the basis of different isoelectric points caused by replacements of charged amino acids by uncharged species (or vice versa). Replacements involving only uncharged amino acids should result either in no or in only small changes of the isoelectric point of the newly formed isoproteins. (The total charge of a protein is determined by the charges of the side chains of its amino acids and, to a lesser extent, by the 3-dimensional arrange-

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From the Institut für Klinische Chemie und Pathobiochemie, Universität Magdeburg (M.O., J.D., A.A., C.L.), Institut für Klinische Chemie und Laboratoriumsmedizin, Universität Münster (W.W., H.F., G.A.), Abteilung für Endokrinologie und Stoffwechsel, Universität Marburg (A.S.), and Abteilung für Klinische Chemie, Universität Freiburg, Germany (M.O., M.N., H.W., C.L.); Gladstone Institute of Cardiovascular Disease, San Francisco (M.O., K.H.W., R.W.M.), Cardiovascular Research Institute (M.O., K.H.W., R.W.M.), and Departments of Medicine (R.W.M.) and Pathology (K.H.W., R.W.M.), University of California, San Francisco. Current address for Wei Wang Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, New York, NY 10021-6399.
Correspondence to Dr Matthias Orth, Universitätsklinikum Benjamin Franklin, Freie Universität, Institut für Klinische Chemie und Pathobiochemie (WE 13), Hindenburgdamm 30, D-12220 Berlin, Germany. E-mail orth@ukbf-fu-berlin.de
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ment of the side chains.) These small changes of the isoelectric point are difficult to detect by isoelectric focusing in polyacrylamide gels and subsequent immunoblotting. Therefore, we developed a more sensitive protocol for these small charge differences using isoelectric focusing in agarose gels and immunofixation, combining specificity with improved resolution.27 Using this protocol, we detected a frequent novel isoform, apoE4_{Freiburg} that migrates to a slightly more acidic position than apoE4 after isoelectric focusing. The aims of this study were to elucidate the underlying molecular basis of the apoE4_{Freiburg} isoform, to compare its allelic frequencies among healthy control subjects and CAD patients in southwestern Germany, and to study its effects on fasting and postprandial plasma lipoprotein and apolipoprotein concentrations, receptor-binding activity, and accumulation in the various lipoprotein classes.

Methods
The protocol for all studies involving human subjects was approved by the local ethics committee, and all participants gave written informed consent for their participation in this study.

Control Subjects and Patients of the Epidemiological and the Family Studies
Subjects for the biochemical studies, the analysis of allelic frequencies, the analysis of the effects of e4_{rare} and other APOE alleles on plasma lipids and apolipoproteins, and cosegregation analysis came from 3 study groups (control subject group, patient group, and family study group). All subjects were whites residing in the region surrounding Freiburg in southwestern Germany. The control group consisted of 1589 healthy blood donors (1180 men, 409 women), who were recruited in 60 different towns, and 543 healthy female employees of a large factory. All control subjects had been screened for the absence of clinically overt CAD. The patient group originated from the same geographical area and consisted of 1437 patients (548 men, 889 women) from a cardiological hospital and from the cardiology Department of the Freiburg University Hospital. All patients had a clinical diagnosis of CAD or had survived a myocardial infarction. In addition, 991 had undergone coronary angiography, and of these, 794 patients (80%) displayed 1 or more stenoses occluding >50% of the vessel lumen. The control subjects were younger than the patients (mean±SD: 39.3±12.4 versus 56.6±11.8 years). The family study group consisted of 7 healthy apoE4_{rare} blood donors and 56 of their relatives. The 63 subjects in this group were studied to determine heritability and cosegregation of phenotype and genotype of the new apoE4 isoform. These relatives were not included in the analysis of allelic frequencies.

Blood was drawn by a standard procedure. Cholesterol, apoB, apoA-I, and triglycerides were measured in serum by enzymatic and turbidimetric tests (Boehringer Mannheim), as described.28 Genomic DNA was isolated from blood leukocytes of subjects of the family study by the alkaline lysis method.29 At the time of the blood sampling, the patients, the relatives of the blood donors in the family study, and the factory workers had been fasting overnight. Blood donors were in an undefined postprandial state and were therefore excluded from analysis of triglycerides. All study subjects were included in the statistical analysis of serum cholesterol, apoB, and triglycerides. The apoE4_{rare} allele was not analyzed.

Distribution of ApoE with Different Lipoproteins
Plasma from fasting apoE4/apoE4 Freiburg heterozygotes was separated by gel filtration (Sephadex 6B-CL, Pharmacia) as described.30 Gel filtration was used instead of ultracentrifugation to avoid the high centrifugal forces thatstrip apolipoproteins from lipoproteins during separation.31 Fractions containing VLDL, IDL, and HDL peaks were dialyzed to eliminate buffer.

Isolelectric Focusing of ApoE
In all subjects, phenotyping of apoE by isoelectric focusing was performed as described.27,32 Briefly, 12 µL of a delipidation solution (76 mMol/mL urea [Merck], 19.2 mMol/mL DTT [Sigma], 10 mMol/mL Tris [Merck], and 23% [vol/vol] Tween 20 [Sigma]) was added to 50 µL of serum. After 30 minutes, the samples were applied to a horizontal 2% agarose gel containing 3 mol/L urea and 4% (vol/vol) of an ampholyte mixture (pH 3 to 10 and pH 5 to 6, 1:1; Serva) and subjected to isoelectric focusing. After focusing, the apoE isoforms underwent immunofixation by incubating the gel with goat antiserum against apoE (Greiner) and staining the immunofixed bands with Coomassie blue.

APOE Gene Sequencing
Genomic DNA was amplified by PCR for sequencing. Primers (21 to 24 bases long) were placed approximately 30 bases upstream of the 5’ end of the gene segments to be sequenced. The PCR reaction was performed in 100 µL of PCR buffer (Beckman) with 10% DMSO (Sigma), containing 0.5 to 1 µg genomic DNA, and final concentrations of 200 µmol/L dNTPs (Pharmacia) and 0.1 µmol/L of each primer. Initial denaturation at 100°C for 10 minutes was followed by the addition of 2 to 5 U Taq polymerase (Beckman) and 30 incubation cycles of 96°C (90 s), 60°C (1 minute), and 70°C (1 minute). For the sequencing of the G/C-rich exon 4 of apoE, 75% of the dGTP content in the reaction mix was replaced with 7-deaza-dGTP (Pharmacia), and temperature cycles were changed to 96°C (80 s), 55°C (1 minute), and 71°C (70 s) and repeated for 40 cycles. The product was purified by electrophoresis in 2% NuSieve agarose (FMC); DNA of the expected size was cut out of the gel and electroeluted in 0.5× Tris-acetate/EDTA for 75 minutes at 200 V. The DNA was desalted and concentrated to 70 µL by ultrafiltration in Centricon X-100 tubes (Amicon).

Isoelectric Focusing of ApoE
Isoelectric focusing was performed by the Gyllensten and Erlich method34 using the same scheme as above. Primer concentration was 0.1 µmol/L; a second primer was not used. Single-strand templates containing exon 4 were synthesized with 7-deaza-dGTP at the same concentration used for double-strand production. Sequencing was done from single-strand templates using T7 polymerase (Pharmacia) and 32P-dNTP (Amersham) following the supplier’s instructions. Reaction products were run on a sequencing gel, dried, and visualized by autoradiography.

Receptor-Binding Studies
Human apoE4_{rare}, apoE3, and apoE4 were isolated from d<1.006 kg/L lipoproteins of homozygotes, as described previously,35 and checked for purity by SDS-PAGE. Human LDL were isolated from plasma of normal fasting subjects by sequential ultracentrifugation36 and radiolabeled by the iodine monochloride method (NEN).37 The various isoforms of apoE and dimyristoylphosphatidylcholine (DMPC, Sigma) were mixed at a ratio of 1:3.75 (wt/wt, protein:DMPC), and complexes were isolated by density gradient ultracentrifugation.38 All of the apoE·DMPC complexes contained apoE as the only protein moiety. Normal human fibroblasts were plated at 3.5×10³ cells/dish 1 week before the experiment. On day 5, the cells were switched to medium (DMEM, Life Technologies) containing 10% human lipoprotein-deficient serum. On day 7, the cells were incubated at 4°C in medium containing 2.0 µg/mL ^125I-LDL and increasing concentrations of apoE·DMPC complexes. The competitive binding of these complexes against human ^125I-LDL was determined.38
by isoelectric focusing of serum, the apoE4 Freiburg isoform was detected in combination with the common apoE isoforms E3, E4, and E2 (Figure 1A) and as the only apoE isoform (Figure 1B). It is evident from these gels that apoE4 Freiburg is easily detected in serum of apoE4/apoE4 Freiburg heterozygotes because it focuses in a slightly more acidic position than apoE4, and a pronounced double band of similar intensity is easily discernible (Figure 1A). To detect apoE4 Freiburg in apoE2/apoE4 Freiburg heterozygotes, apoE3/apoE4 Freiburg heterozygotes, or homozygotes, samples containing apoE4 were run in an adjacent lane to detect the slight acidic shift of apoE4 Freiburg versus apoE4.

**DNA Sequencing and Segregation Analysis**

The molecular basis of this isoform was identified by sequencing all exons, 80 bases of 5' sequence, and the consensus splice donor and acceptor sites of the APOE gene of 4 independent apoE4 Freiburg carriers. A single mutation was detected in all carriers studied. This mutation, a T-to-C mutation in position 3100 of the APOE4 gene (GenBank accession M10065), changes CTG of codon 28, coding for leucine, to CCG, coding for proline (Figure 2).

The DNA mutation underlying the Leu → Pro replacement leads to the formation of an MspI restriction site (C CGG). In the absence of the mutation, the 271-bp fragment of genomic PCR-amplified DNA is cleaved into 2 fragments of 191 and 80 bp. In the presence of the mutation, the 191-bp fragment is cleaved into fragments of 136 and 55 bp (Figure 2).

Heritability and cosegregation of genotype and phenotype were studied in 7 study participants (blood donors) and 56 of their relatives. Genotype (ie, presence of a specific MspI restriction site of PCR-amplified genomic DNA) and phenotype (ie, apoE4 Freiburg band in isoelectric focusing) were congruent in all families studied indicating cosegregation of phenotype and genotype. The CCG mutation was only observed on e4 chromosomes and not on e2 or e3 chromosomes. Screening of the 56 relatives (30 men, 26 women) identified 33 clinically healthy heterozygous and homozy-
Figure 2. The molecular basis of apoE4 Freiburg. A, Representative DNA sequences from wild-type (left) and heterozygous apoE4 Freiburg subjects (right). The arrow indicates the T → C exchange at position 3100 of the apoE gene and the amino acid exchange at position 28 of the polypeptide chain. B, The location of the apoE4 Freiburg mutation within the apoE gene. C, An example of apoE4 Freiburg carrier detection by restriction length fragment polymorphism after restriction digest of PCR-amplified DNA with MspI. Lane 1, apoE2/E2, 3 heterozygotes with e2, 21 with e3, and 7 with e4. Both homozygotes (subjects 2 and 3) were hyperlipidemic but did not suffer from CAD (Table 1). Screening for common apoE phenotypes revealed 1 subject with apoE2/E3, 16 subjects with apoE3/E4, and 6 subjects with apoE3/E4 and apoE3/E4 among these relatives.

### Allele Frequencies of ApoE4 Freiburg

The allelic frequencies of apoE4 Freiburg were studied by phenotyping 2132 clinically healthy subjects (control group) and 1437 CAD patients (patient group). The absolute and relative frequencies of the common and apoE4 Freiburg phenotypes are given in Table 2. A total of 30 carriers of apoE4 Freiburg were detected in these 3569 subjects. One CAD patient was homozygous and 29 subjects were heterozygous for apoE4 Freiburg. The frequencies of the apoE4 Freiburg allele were 1:242 (10:4264 alleles) in the control group (with similar frequencies among blood donors and factory workers, data not shown) and significantly more common, 1:137 (21:2874) in the CAD patient group (P = 0.004). The age- and sex-adjusted OR for CAD was 3.09 (95% CI, 1.20 to 7.97) in carriers of apoE4 Freiburg relative to noncarriers. The most common heterozygous combination of apoE4 Freiburg, the combination with apoE3, was also more frequent among patients than among control subjects (1.1%, 16 of 1437 versus 0.2%, 4 of 2132; P = 0.0001). Restriction of the analysis to patients in whom CAD was diagnosed by angiography revealed an allelic frequency similar to that of all CAD patients (1.7%, 1:61, 13 of 794).

The allelic frequencies of the common APOE alleles (Table 2) in our white population were very similar to those reported previously. The apoE4 frequency was 0.1326 in patients and 0.1266 in control subjects (P = 0.320) (Table 2).

### Binding to the LDL Receptor

ApoE4 Freiburg protein was isolated from apoE4 Freiburg homozygotes, complexed with DMPC, and used for competition experiments with 125I-LDL. The in vitro binding properties of the apoE4 Freiburg·DMPC complexes were compared with those of complexes containing apoE3 or apoE4. This analysis revealed similar dose-response curves for these apoE isoforms and excluded a binding defect of apoE4 Freiburg·DMPC complexes with LDL receptors.

### Effects of ApoE4 Freiburg on Lipids and Apolipoproteins

Three homozygotes from 2 unrelated families were identified in this study, and one homozygote was identified among the patients of our lipid clinics. All homozygotes had various types of hyperlipoproteinemia (type Ia, type IIb, type IV, and type V) and 2, both with hypertriglyceridemia, had CAD (Table 1).

The phenotypes of the homozygotes led us to study the effects of the common APOE alleles (e2, e3, and e4) and of apoE4 Freiburg on lipoprotein concentrations in all subjects. The effects of the different common APOE alleles were analyzed by comparing the wild-type apoE3/E3 with apoE2/E2, apoE4/E4, and apoE3/E4 (Figure 3) because these phenotypes modulate concentrations of lipids and apolipoproteins. The effects of apoE4 Freiburg on lipids and apolipoproteins were studied in 2 steps. First, all carriers with 1 or 2 alleles of apoE4 Freiburg (n = 63) were compared with all noncarriers of apoE4 Freiburg (n = 3562) with the aim of including as many apoE4 Freiburg individuals as possible (Figure 4). The caveat of this analysis is that the results are easily confounded by known (opposite) effects of the other apoE isoforms. Second, only the most frequent combinations of apoE4 Freiburg (ie, the combinations apoE3/E4 Freiburg and apoE4/E4 Freiburg) were compared with apoE3/E4 and apoE4/E4, respectively. Although fewer subjects were analyzed than in the first analysis, this analysis allows the effects of apoE4 Freiburg to be clearly differentiated from those of e4 (Figure 3 and Table 3).

Analysis of cholesterol and apoB concentrations revealed, as expected, that e2 and e4 had opposite effects on lipid and apolipoprotein concentrations. Cholesterol and apoB were lower in apoE2/E2 and higher in apoE4/E4 and in apoE3/E4 than in apoE3/E3 (Figure 3A and 3B). No

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### TABLE 1. Lipids and Apolipoprotein Concentrations and Risk Factors for CAD in 4 Homozygous apoE4 Freiburg Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>8.368</td>
<td>5.217</td>
<td>6.475</td>
<td>18.828</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.914</td>
<td>3.060</td>
<td>0.764</td>
<td>53.257</td>
</tr>
<tr>
<td>VLDL cholesterol, mmol/L</td>
<td>0.439</td>
<td>1.524</td>
<td>0.285</td>
<td>16.968</td>
</tr>
<tr>
<td>VLDL triglycerides, mmol/L</td>
<td>1.912</td>
<td>2.981</td>
<td>0.342</td>
<td>52.818</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>6.224</td>
<td>2.763</td>
<td>4.644</td>
<td>1.059</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.705</td>
<td>0.930</td>
<td>1.554</td>
<td>0.801</td>
</tr>
<tr>
<td>ApoA-1, mg/dL</td>
<td>158</td>
<td>126</td>
<td>164</td>
<td>112</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>181</td>
<td>105</td>
<td>99</td>
<td>162</td>
</tr>
<tr>
<td>Age, y/sex</td>
<td>55/M</td>
<td>51/F</td>
<td>52/M</td>
<td>57/M</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>31</td>
<td>28</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>150/100</td>
<td>135/90</td>
<td>130/85</td>
<td>165/105</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

$^*$This patient was identified among the patients attending the lipid clinics of Magdeburg and not included in the evaluation of allelic frequencies or the effects of apoE4 Freiburg on lipoproteins.
effects of the e4\textsubscript{Freiburg} allele on cholesterol and apoB were detected when all subjects were analyzed (Figure 4A and 4B). When the opposite effects of the e2 and e4 alleles were excluded by the direct comparison of apoE3/E4\textsubscript{Freiburg} with apoE3/E4, however, this analysis revealed that apoE3/E4\textsubscript{Freiburg} subjects had a 0.524 mmol/L lower cholesterol (Figure 3A), and the comparison of apoE4/E4\textsubscript{Freiburg} with apoE4/E4 revealed that apoE4/apoE4\textsubscript{Freiburg} heterozygotes had 20.7 mg/dL lower apoB (Figure 3B). ApoB was not significantly different between apoE3/E4\textsubscript{Freiburg} and apoE3/E4 (Figure 3B), and cholesterol was not significantly different between apoE4/E4\textsubscript{Freiburg} and apoE4/E4 (Figure 3A; for CIs, see Table 3).

ApoA-I concentrations were lower in apoE4/E4 and in apoE3/E4 than in apoE3/E3, but no differences were observed in apoE2/E2 (Figure 3C). The e4\textsubscript{Freiburg} allele had a profound effect on apoA-I concentration even when all subjects were analyzed (Figure 4C). Mean apoA-I concentrations were 17.2 mg/dL lower in carriers of e4\textsubscript{Freiburg} than in noncarriers (95% CI, 10.2 to 24.0 mg/dL; \(P<0.000\); mean in noncarriers, 153.4 mg/dL); this relationship also held true after adjustment for age and sex (21.1 mg/dL lower in subjects with the apoE4\textsubscript{Freiburg} isoform; \(P<0.003\)).

ApoA-I was 15.9 mg/dL lower in apoE3/E4\textsubscript{Freiburg} compared with apoE3/E4 but did not reach statistical significance between apoE4/E4\textsubscript{Freiburg} and apoE4/E4 (Figure 3C and Table 3).

### Table 2. Observed and Calculated Phenotype Frequencies of the Common ApoE Isoforms and of ApoE4\textsubscript{Freiburg}

<table>
<thead>
<tr>
<th>Pheno</th>
<th>E2/E2</th>
<th>E2/E3</th>
<th>E2/E4</th>
<th>E2/E4\textsubscript{Freiburg}</th>
<th>E3/E3</th>
<th>E3/E4</th>
<th>E3/E4\textsubscript{Freiburg}</th>
<th>E4/E4</th>
<th>E4/E4\textsubscript{Freiburg}</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>21 (1.0%)</td>
<td>241 (11.3%)</td>
<td>52 (2.4%)</td>
<td>3 (0.1%)</td>
<td>1356 (63.6%)</td>
<td>419 (19.7%)</td>
<td>4 (0.2%)</td>
<td>33 (1.6%)</td>
<td>3 (0.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CAD patients</td>
<td>16 (1.1%)</td>
<td>149 (10.4%)</td>
<td>21 (1.5%)</td>
<td>2 (0.1%)</td>
<td>890 (61.9%)</td>
<td>323 (22.5%)</td>
<td>16 (1.1%)</td>
<td>18 (1.3%)</td>
<td>1 (0.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>All subjects</td>
<td>37 (1.0%)</td>
<td>390 (10.9%)</td>
<td>73 (2.4%)</td>
<td>5 (0.1%)</td>
<td>2246 (62.5%)</td>
<td>742 (20.8%)</td>
<td>20 (0.6%)</td>
<td>51 (1.4%)</td>
<td>1 (0.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Estimated number of subjects*</td>
<td>21 (0.6%)</td>
<td>429 (12.0%)</td>
<td>70 (2.0%)</td>
<td>2 (0.1%)</td>
<td>2231 (62.5%)</td>
<td>728 (20.4%)</td>
<td>25 (0.7%)</td>
<td>59 (1.7%)</td>
<td>4 (0.1%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Allelic frequencies for e2, e3, e4, and e4\textsubscript{Freiburg} were 0.0759, 0.7907, 0.1290, and 0.0043, respectively. The estimated number of all subjects was calculated from these allelic frequencies. Note that the 56 participants of the family study were not included in this analysis of the apoE phenotype frequencies.

![Figure 3](http://atvb.ahajournals.org/)

Figure 3. ApoE phenotype-specific box-and-whisker plots for cholesterol (A), apoB (B), apoA-I (C), and triglycerides (D). The box represents the interquartile range, which contains 50% of the values. The whiskers extend from the box to the highest and lowest values, excluding outliers. The line across the box indicates the median. Differences from the values of apoE3/E3 (wild-type) were calculated to identify the effects of the common apoE phenotypes and between apoE4/E4 and apoE4/E4\textsubscript{Freiburg} as well as between apoE3/E4 and apoE3/E4\textsubscript{Freiburg} to identify the effects of E4\textsubscript{Freiburg}. For confidence intervals, see Table 3. Fewer subjects were included in the analysis of triglycerides because it was restricted to subjects with fasting triglycerides <7.874 mmol/L. Subjects with apoE2/E3, apoE2/E4, apoE2/E4\textsubscript{Freiburg}, and apoE4\textsubscript{Freiburg}/E4\textsubscript{Freiburg} are not shown.
The analysis of the effects of different APOE alleles on fasting triglycerides was restricted to the study subjects in whom the blood samples were obtained under fasting conditions (ie, all patients, all control subjects from the occupational health clinics, and all participants of the family study, n=2036). Fasting triglycerides were <7.874 mmol/L in 99.4% of all subjects, but the variability of triglyceride concentrations (as indicated by standard deviation and skewness) in all different apoE phenotypes studied exceeded by far the variability of cholesterol, apoB, and apoA-I concentrations (Figures 3 and 4). This higher variability was an obstacle for the statistical analysis. Nevertheless, triglycerides were higher in apoE2/E2, apoE4/E4, and apoE3/E4 than in apoE3/E3. Although the number of subjects examined in our study was quite large, the only difference that reached statistical significance was between apoE3/E3 and apoE2/E2 (Figure 3D). When the effects of the apoE4Freiburg isoform on triglycerides were studied in all carriers of E4Freiburg, triglyceride concentrations were higher by 0.402 mmol/L in subjects with apoE4/E4Freiburg (n=56) than in noncarriers (n=1968) (95% CI, 0.032 to 0.837 mmol/L; P=0.035; mean in apoE4/E4Freiburg, 2.359 mmol/L) (Figure 4D), and this difference was still present after adjustment for sex (P=0.006). Differences between apoE4 and apoE4Freiburg did not reach statistical significance in the comparison of certain phenotypes. Triglycerides were 0.187 mmol/L higher in apoE3/E4/E4Freiburg (n=39) than in apoE3/E4 (n=419) and 0.462 mmol/L higher in apoE4/E4/E4Freiburg (n=8) than in apoE4/E4 (n=31) (Figure 3D; for CIs, see Table 3).

In summary, the subjects with apoE4Freiburg had significantly lower cholesterol (versus E4/E4), lower apoB (versus E3/E4), and lower apoA-I, and, possibly, higher fasting triglycerides than those with apoE4.

**Distribution of ApoE4Freiburg and ApoE4 with Different Lipoproteins**

Analysis by gel filtration of lipoproteins in 2 apoE4/apoE4Freiburg subjects on 2 independent occasions (n=4) revealed that most of the plasma apoE is present in VLDL and IDL and only very little is in HDL (Figure 5). To test whether the apoE isoforms differ in their accumulation in VLDL and HDL, as has been observed for apoE3 and apoE4,9,43 the amounts of apoE4 and apoE4Freiburg present in VLDL, IDL, and HDL were measured in these apoE4/apoE4Freiburg heterozygotes. Both isoforms showed a very similar accumulation in VLDL (ratio apoE4Freiburg to apoE4, 0.99) and IDL (ratio, 0.95), but much less apoE4Freiburg than apoE4 (ratio, 0.67) accumulated in HDL (Figure 5).

**Effects of ApoE4Freiburg on Postprandial Lipoproteins**

We also analyzed the effects of the apoE4Freiburg isoform on postprandial lipoproteins because the epidemiological data...

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**TABLE 3. Effects of ApoE4Freiburg on Lipid and Apolipoprotein Concentrations**

<table>
<thead>
<tr>
<th>Lipid/Polipoprotein</th>
<th>ApoE3/E4Freiburg (n=41) vs ApoE3/E4 (n=747)</th>
<th>ApoE4/apoE4Freiburg (n=11) vs ApoE4/E4 (n=51)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Difference</td>
<td>95% CI</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>--------</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>-0.524</td>
<td>-0.945 to -0.108</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>-4.26</td>
<td>-16.9 to 8.4</td>
</tr>
<tr>
<td>ApoA-I, mg/dL</td>
<td>-15.9</td>
<td>-24.3 to -7.4</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.187</td>
<td>-0.325 to 0.533</td>
</tr>
</tbody>
</table>

*Analysis of triglyceride concentrations was restricted to fasting subjects with triglycerides <7.874 mmol/L (apoE3/E4Freiburg, n=39; apoE3/E4, n=419; apoE4/E4Freiburg, n=8; apoE4/E4, n=31).*

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revealed several effects of the apoE4 Freiburg isoform on fasting lipoproteins and because carriers of many rare apoE variants display a delayed clearance of postprandial lipoproteins. A standard fatty meal containing retinyl palmitate was given to 3 apoE4 Freiburg homozygotes (for clinical characteristics, see subjects 1, 2, and 3 in Table 1), and the different lipoprotein classes were monitored for 10 hours. These data were compared with those from 2 control groups of normolipemic subjects, 1 group of 29 subjects (with the apoE phenotypes E2/E3, n = 8; E3/E3, n = 8; E3/E4, n = 7; E4/4, n = 6) designated non-apoE2/E2, and 8 subjects with the phenotype E2/E2. Although all 3 apoE4 Freiburg homozygotes displayed different forms of hyperlipoproteinemia in the fasting state, their postprandial increases of chylomicrons, chylomicron remnants, and VLDL were moderate and did not differ from those obtained in non-apoE2/E2 normolipemic control subjects (Figure 6A through 6C).

We next examined in detail the composition of HDL after a fatty meal. Postprandial concentrations of HDL triglycerides, an indicator for both high cholesteryl ester transfer protein activity and the presence of high concentrations of triglyceride-rich lipoproteins, increased in response to the postprandial lipoprotein surge in all 3 groups (Figure 6D). There were, however, differences between the groups. The total triglyceride enrichment, as measured by the area under the curve of HDL triglycerides, tended to be lowest in (hyperlipidemic) apoE4 Freiburg homozygotes, followed by normolipemic non-apoE2/E2 subjects, and was significantly higher in normolipemic apoE2/E2 homozygotes (0.834, 1.129, 1.390 mmol·L⁻¹·h⁻¹, respectively; P = 0.038 for apoE4 Freiburg versus apoE2/E2). The ratio of apoA-I to HDL cholesterol (Figure 6F) and the molar ratio of HDL cholesterol to phospholipids (apoE4 Freiburg, 0.86 ± 0.05; non-apoE2/E2, 0.94 ± 0.02; apoE2/E2, 0.92 ± 0.04) were similar among these 3 groups. Both ratios did not change postprandially in homozygous apoE4 Freiburg patients or in normolipemic control subjects. In summary, these studies revealed a normal clearance of postprandial lipoproteins and a slightly lower enrichment of HDL with

Figure 5. Examples of the distribution of cholesterol (■), triglycerides (○), and apoE (*) among different lipoproteins. Plasma (5 mL) from a fasting male apoE4/E4 Freiburg heterozygote was chromatographed on a Sepharose 6B-CL column, and the concentrations of cholesterol, triglycerides, and apoE were determined in each eluate fraction. Aliquots of VLDL, IDL, and HDL were subjected to isoelectric focusing and immunofixation. The inset shows the relative amounts of apoE4 and apoE4 Freiburg in different lipoproteins.

Figure 6. Effects of a fatty meal on lipoproteins in 3 apoE4 Freiburg homozygotes. The fatty meal was ingested just after a fasting blood sample was drawn at 0 hours. Data are mean and SD. Time course of chylomicron triglycerides (A), chylomicron remnant retinyl esters (B), VLDL triglycerides (C), HDL triglycerides (D), molar HDL cholesterol to triglyceride ratio (E), and apoA-I to HDL cholesterol ratio (F) are shown. Results from 29 normolipemic non-apoE2/E2 subjects and 8 apoE2/E2 are given for comparison.
Discussion
The molecular basis of the novel apoE isoform apoE4Freiburg is a thymidine-to-cytidine substitution at position 3100 of APOE4, which changes leucine to proline at position 28 of the polypeptide chain. ApoE4Freiburg differs from most other known apoE variants in that the amino acid replacement involves 2 neutral amino acids and therefore does not change the net charge of the isoprotein. Denaturing conditions present at isoelectric focusing allow the detection of a slightly more acidic net charge of the isoform, especially in apoE4/ apoE4Freiburg heterozygotes (Figure 1A). In this case, isoelectric focusing with subsequent immunofixation leads to a doublet at the apoE4 position with the additional band migrating to a position about a fifth net charge more acidic than apoE4. The leucine-to-proline substitution in codon 28 is not located within the receptor-binding region (residues 136 to 150), the HSPG-binding sites (142 to 147), or the lipid-binding domain in the carboxyl terminus (residues 244 to 272).35,46 and the predicted normal functions of these regions could be demonstrated. In fact, normal receptor-binding function could be demonstrated by comparing the binding properties of apoE4FreiburgDMPC complexes with those of DMPC complexes with apoE3 or apoE4. Defective HSPG-binding is very unlikely because of the phenotype of heterozygotes, which in cases of defective HSPG-binding resembles autosomal dominant type III hyperlipoproteinemia.3 A similar binding to triglyceride-rich lipoproteins, apoA-I, and HDL triglycerides. The effects of different APOE alleles on triglycerides are secondary to the effects on LDL. Our interpretation is that the effects of APOE on apoA-I concentrations are secondary to the effects of modulating triglyceride concentrations. The much higher intradividual variability of triglycerides (compared with apoA-I),56 however, makes it more difficult to detect these modulating effects on triglycerides.

The effects of apoE4Freiburg on the concentration of fasting lipids and apolipoproteins were significantly different from those of apoE4/ apoE4, lowered cholesterol (ε3/ε3Freiburg versus ε3/ε4), apoB (ε4/ε4Freiburg versus ε4/ε4), and apoA-I. The mechanism of the effect of apoE4Freiburg on lipoproteins is unknown, but the epidemiological data, together with data from the postprandial studies, indicate a connection of apoE4Freiburg with triglyceride-rich lipoproteins, apoA-I, and HDL triglycerides. The effects of apoE4Freiburg on cholesterol and apoB concentrations can be explained by the known metabolic link between high triglycerides and low LDL cholesterol.57 To get some insight into the molecular mechanism of apoE4Freiburg in lipoprotein metabolism, we characterized the receptor-mediated clearance of lipoproteins containing apoE4Freiburg with 2 approaches.

First, the in vitro clearance by LDL receptors was analyzed by receptor-binding studies of apoE4Freiburg and other apoE isoforms complexed with small DMPC disks. These receptor-binding studies revealed no binding defect of apoE4Freiburg. This is consistent with the observation that none of the apoE4Freiburg homozygotes presented with type III hyperlipoproteinemia, which is associated with LDL receptor-binding-defective isoforms of apoE.2

Second, the in vivo clearance of postprandial lipoproteins from 3 apoE4Freiburg homozygotes was unremarkable and did not differ from the clearance of triglyceride-rich lipoproteins in
normalipemic control subjects \(^{28}\) despite higher fasting triglycerides in the \(e_4^{Freiburg}\) homozygotes. Surprisingly, the postprandial accumulation of triglycerides in HDL was even less pronounced than in the normalipemic control subjects. Higher fasting and postprandial triglycerides have been shown to increase triglycerides in HDL.\(^{28}\)

A possible clue for the molecular mechanism comes from the studies of the association of apoE4 and apoE4\(_{Freiburg}\) with different lipoproteins. Although the majority of each isoform accumulated in triglyceride-rich lipoproteins (Figure 5), marked differences were observed in their accumulation in HDL. Unequivocally less apoE4\(_{Freiburg}\) than apoE4 was associated with HDL in the patients studied. These data do not exclude a lower HDL preference of apoE4\(_{Freiburg}\) caused by certain lipid-binding characteristics.\(^{28}\) However, certain lipoprotein particles (Lp) in HDL containing apoE (ie, triglyceride-rich LpE:A-I) may be removed more rapidly in carriers of apoE4\(_{Freiburg}\) than in carriers of the common apoE isoforms. Rapid removal of LpE4\(_{Freiburg}:A-I\) also reduces apoA-I concentrations. The rapid removal of LpE4\(_{Freiburg}:A-I\) might partially be responsible for the lower apoA-I concentrations in carriers of \(e_4^{Freiburg}\) the lower postprandial triglyceride-enrichment of HDL in \(e_4^{Freiburg}\) homozygotes observed after a fatty meal, and the higher allelic frequency of \(e_4^{Freiburg}\) in CAD patients. Because of the low number of homozygotes available and the multifactorial modulation of HDL concentrations, we cannot calculate the quantity of HDL cholesterol and HDL triglycerides removed after a fatty meal. However, the preferential uptake of LpE4\(_{Freiburg}:A-I\) might take place at all times and not only after a fatty meal and might decrease HDL long-term. We further speculate that these LpE4\(_{Freiburg}:A-I\) might interfere with the catabolism of triglyceride-rich lipoproteins and subsequently decrease HDL. Elevated concentrations of triglyceride-rich lipoproteins decrease HDL, presumably by the activation of cholesteryl ester transfer protein–mediated lipid transfer.\(^{45}\) High concentrations of triglyceride-rich lipoproteins and low concentrations of HDL promote CAD.\(^{55,56}\) In this context, it is intriguing to see a 3-fold higher allelic frequency of \(e_4^{Freiburg}\) among CAD patients than among control subjects. The receptor-binding studies with apoE4\(_{Freiburg}\) DMPC cannot address the effects of different apoE isoforms on the clearance of LpE:A-I. Because of their high homogeneity and good reproducibility between different preparations, these DMPC complexes have been successfully used for numerous receptor-binding studies. In alternative methods (eg, use of native lipoproteins obtained from different subjects), differences in protein and lipid composition can confound the results. An alternative hypothesis for the differences in the accumulation of apoE4\(_{Freiburg}\) in HDL is that the effects result from differences in its lipoprotein preference, either directly by stabilization of the long helices at the expense of short helices, which might cause a low affinity for HDL,\(^{59}\) or indirectly by its effects on the arginine-61 side chain.\(^{58}\)

In summary, apoE4\(_{Freiburg}\) is a frequent apoE mutation and is significantly more common among CAD patients. The observed effects of this isoform on plasma lipids and apolipoproteins under fasting and postprandial conditions, the phenotype of homozygous and heterozygous carriers, receptor-binding studies, and the distribution of apoE4\(_{Freiburg}\) among different lipoproteins imply that this isoform exerts its atherogenic properties by modulating the metabolism of triglyceride-rich lipoproteins and HDL.

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**References**


Effects of a Frequent Apolipoprotein E Isoform, ApoE4_Freiburg (Leu28→Pro), on Lipoproteins and the Prevalence of Coronary Artery Disease in Whites
Matthias Orth, Wei Weng, Harald Funke, Armin Steinmetz, Gerd Assmann, Matthias Nauck, Jutta Dierkes, Andreas Ambrosch, Karl H. Weisgraber, Robert W. Mahley, Heinrich Wieland and Claus Luley

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