Apolipoprotein Isoform E4 Does Not Increase Coronary Heart Disease Risk in Carriers of Low-Density Lipoprotein Receptor Mutations

Running title: Versmissen et al.; No CHD risk increase by ApoE4 when LDLR mutated

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Abstract:

**Background** - In humans, the E4 allele of the apolipoprotein E gene (APOE) is associated with increased coronary heart disease (CHD) risk. Surprisingly, in rodents, ApoE4 only accelerates the atherosclerotic process when transgenic for the human low-density lipoprotein (LDL) receptor protein. We therefore investigated whether the LDLR locus interacted with APOE genotype on CHD risk in patients clinically diagnosed with familial hypercholesterolemia (FH) with and without LDLR mutation. We investigated whether the presence of an LDLR mutation diminishing LDL receptor function was protective in E4/E4 carriers.

**Methods and Results** - In a cohort of 2,400 patients clinically diagnosed with FH, we found an LDLR gene mutation in 1,383 patients whereas in 1,013 patients such mutation was not present. In 92 patients homozygous for APOE4, the presence of an LDLR mutation conferred lower CHD risk (hazard ratio HR 0.16; 95% confidence interval (CI) 0.05-0.58; p=0.005). Mirroring these results, the APOE4/E4 genotype was also associated with lower CHD risk in FH patients with an LDLR mutation (HR 0.26 HR 0.08-0.80; p=0.02).

**Conclusions** - LDL receptor function is key to the detrimental effects of ApoE4 in humans. Kinetic studies in humans are now required to study the consequences of our observation for prevention of both CHD and Alzheimer’s disease.

**Key words:** familial hypercholesterolemia; Apolipoprotein E; coronary heart disease
Background

Complications of atherosclerotic vascular disease are the most common causes of death and morbidity in the Western world, with coronary heart disease (CHD) as its most prominent manifestation. Hereditary predisposition plays an important role in the pathobiology of atherosclerosis. One of the genes best known for its association with CHD risk is the one coding for the low-density lipoprotein receptor (LDLR) protein. Mutations in this gene cause an autosomal dominant disorder called familial hypercholesterolemia (FH), which is characterized by severe hypercholesterolemia and premature CHD. Another important gene known to influence CHD risk is Apolipoprotein E (APOE). Mice completely lacking the ApoE protein are severely hypercholesterolemic and develop extensive atherosclerotic lesions, a process which is accelerated when they are fed a high-fat diet. Homozygous deficiency of the APOE gene in humans is extremely rare and is also characterized by atherogenic lipid abnormalities and premature CHD.

In humans, three main haplotypes of the APOE gene have been identified: APOE2, APOE3 and APOE4. The encoded ApoE2, ApoE3 and ApoE4 proteins differ in their amino acid sequences at positions 112 and 158. Although these differences are not located in the LDL receptor binding domain, affinity to the LDL receptor differs between genotypes: ApoE2 has the lowest affinity for the LDL receptor while ApoE4 has the highest. This leads to lower LDL-cholesterol levels in APOE2 carriers due to up-regulation of LDL receptors and higher LDL-cholesterol levels in APOE4 carriers due to down-regulation of LDL receptors. While the APOE4 allele is best known for its strong association with Alzheimer’s disease, it is also consistently associated with a 26-42% higher CHD risk. This increased CHD risk in APOE4 carriers cannot merely be explained by small differences in LDL-cholesterol levels. The LDL receptor-APOE interaction is, however, central to the increased CHD risk associated with ApoE4: atherosclerosis resulting from...
specific APOE genotypes can be replicated in rodents, but only when human LDL receptors are also abundant. Notably, mice expressing human ApoE4 develop no substantial atherosclerosis, but they display fulminant disease when the human LDL receptor is also introduced.

To date, the interaction between ApoE and the LDL receptor has not directly been examined in humans. A suitable group of patients for investigating this interaction would consist of patients who have a genetic defect in the LDLR gene that results in lower residual function of the LDL receptor protein. In fact, in two earlier studies of modest study size in FH patients, the APOE4 allele was not associated with CHD risk. We therefore hypothesized that carriers of the E4/E4 genotype might benefit from a reduction in functional LDL receptors. In a large population of persons clinically diagnosed with FH, we investigated whether the presence of an LDLR mutation that reduces LDL receptor function was protective in E4/E4 carriers.

Materials and methods

FH Cohort

During 1989-2002, we recruited a cohort of 2,400 patients with severe hypercholesterolemia from 27 lipid clinics as described in detail previously. We selected 2,400 unrelated subjects who fulfilled the internationally established FH diagnostic criteria. A well-trained team of 13 data collectors reviewed medical records to establish extensive phenotypic data including CHD events. Total cholesterol, high-density lipoprotein cholesterol (HDL) and triglyceride levels were measured by standard methods in fasting patients who had been withdrawn from lipid-lowering medication at least 6 weeks before blood collection. LDL-cholesterol concentration was calculated with the Friedewald formula. The promoter region and all exons (including exon-intron boundaries) of the
*LDLR* gene were sequenced in all patients and multiplex ligation-dependent probe amplification (MLPA) technique was used to identify large re-arrangements. Exon 26 and 29 of the APOB gene, encoding the major LDL receptor binding sites, were also sequenced. All known mutations were tested in duplo and sequencing and MLPA was performed twice when no previously known mutation was identified. Therefore, false negative results for *LDLR* mutation assessment were unlikely (around 0.06%). Genetic variants without clear effect on LDL receptor function were filtered using pedigree data from the Dutch screening program as published earlier: if a potential mutation did not segregate with hypercholesterolemia, pathogenicity of this variant is questionable. In the current study, such variants were not considered as LDLR mutation. Carriers of such mutations were considered to have no *LDLR* mutation.\(^{21}\) In a later stage, *PCSK9* was sequenced in samples in which no mutation was identified but this analysis did reveal only a limited number of mutations in patients with an FH phenotype in general and none in this cohort.\(^{22}\) The DNA of 2145 unrelated patients was available for *APOE* genotyping, performed in a multilocus genotyping assay.\(^{23}\) All patients gave informed consent, and the ethics institutional review board of each hospital approved the protocol.

**CHD outcome measures**

CHD was defined as the presence of at least one of the following: (i) myocardial infarction, proved by at least two of the following: (a) classical symptoms (>15 minutes), (b) specific ECG abnormalities, and (c) elevated cardiac enzymes (>2x upper limit of normal); (ii) percutaneous coronary intervention or other invasive procedures; (iii) coronary artery bypass grafting; (iv) angina pectoris, diagnosed as classical symptoms in combination with at least one unequivocal result of one of the following: (a) exercise test, (b) nuclear
scintigram, (c) dobutamine stress ultrasound, or (d) more than 70% stenosis on a coronary angiogram.

**Statistical analysis**

General characteristics were compared using analysis of variance (ANOVA) for continuous variables (Statistical analyses of substantially skewed data were performed after logarithmic transformation) and X² test for categorical variables. Variables with low frequencies (hypertension, diabetes, CHD) were analyzed using the Fisher’s exact test comparing each genotype separately with APOE3/E3. In Table 1, p-values are given when comparing E3/E3, E3/E4 and E4/E4, while in the supplemental Table 2 overall p-values comparing all genotypes are given. Firstly, we investigated the effect of having an LDLR mutation on CHD risk in this FH study population using Cox proportional hazards models adjusted for year of birth, sex and smoking as the Cox proportional hazards modelling seems most powerful in genetic studies. Follow-up started at birth and ended at the first occurrence of CHD. Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to causes other than CHD. The proportional hazards assumption was tested by drawing log minus log plots of the survival function and was met for all Cox proportional hazard models used. Next, we stratified by APOE genotype. We also studied the effect of APOE genotypes on CHD risk in the whole study population as well as stratified according to presence of an LDLR mutation. Interaction was tested by introducing an interaction term in Cox regression analyses. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 15.0.

**Results**
In 1,383 patients an LDLR mutation was identified while in 1,017 patients no pathogenic mutation in the LDLR or APOB gene was found by sequencing the complete LDLR gene or by using the multiplex ligation-dependent probe amplification technique. Comparison of individuals with and without LDLR mutation in this cohort showed significant differences as published earlier by Van Aalst-Cohen et al.: FH patients without LDLR mutation had higher triglyceride levels (1.71 vs 1.39 mmol/l), body mass index (25.6 vs 24.7 kg/m²) and systolic blood pressure (137 vs 133 mm Hg) and were more likely to have ever smoked (79.5 vs 68.7%), while FH patients with LDLR mutation had higher LDL-C levels (8.18 vs 6.61 mmol/l).\textsuperscript{25} Successfully genotyping for APOE was assessed in 2,061 patients of whom 1,150 had an LDLR mutation. The polymorphisms at positions 112 and 158 were in Hardy Weinberg equilibrium both in the whole population as in the group with an LDLR mutation. Genotype frequencies are shown in supplemental Table 1. General characteristics are shown in Table 1. For the sake of clarity, we only list APOE2/E3, E3/E4 and E4/E4 and p-values given are for this comparison: patients carrying genotypes consisting of one or two APOE2 alleles were not significantly different except that triglyceride levels appeared to be increased by the APOE2 allele in the group without LDLR mutation (supplemental Table 2; p-values comparing all genotypes). HDL-C levels also appeared significantly different in the group without LDLR mutation but this association disappeared after adjustment for triglyceride levels.

In the total cohort successfully genotyped for APOE, 603 CHD events occurred of which 547 were in the E3/E3, E3/E4 and E4/E4 genotyped individuals (Table 1) Classical risk factors and lipid levels were not significantly different between subjects carrying the different APOE genotypes except Lp(a) levels in the analysis restricted to FH patients with an LDLR mutation: E4/E4 genotyped FH patients with an LDLR mutation displayed the lowest Lp(a) levels while E3/E4 genotyped FH patients with a mutation were intermediate between E3/E3
and $E4/E4$. The most striking difference was the prevalence of coronary heart disease: this was lowest in the $APOE4/E4$ genotyped patients, but only in the group with an $LDLR$ mutation ($E4/E4$ 6.1% vs 25.1% in $E3/E3$; $p=0.009$; group without $LDLR$ mutation 36.4% vs 37.1%; $p=0.61$).

**LDL receptor mutation protective in patients expressing ApoE4**

The effect of the presence of an $LDLR$ mutation on CHD risk in these FH patients is shown in Table 2. Within the entire cohort, the presence of an $LDLR$ mutation was not significantly associated with CHD (HR 0.88; 95% CI 0.76-1.02; $p=0.10$). The borderline significant protective effect of having an $LDLR$ mutation appeared to be due to the larger burden of classical risk factors such as smoking and hypertension in FH patients without $LDLR$ mutation; as published earlier, when adjusting for these additional risk factors the apparent difference in CHD risk difference did no longer exist. However, in $E4/E4$ genotyped FH patients a strong protective effect of an $LDLR$ mutation was observed (HR 0.16; 95% CI 0.05-0.58; $p=0.005$). Since the groups with and without $LDLR$ mutation differed with regard to classical risk factors such as smoking and hypertension, we added different covariates to the analyses. None did influence the protective effect of the $LDLR$ mutation in $APOE4/E4$ genotyped individuals in this cohort of individuals with the clinical diagnosis of FH.

**$APOE4/E4$ protective in the presence of an $LDLR$ mutation?**

Since it appeared that the lowest number of events occurred in $E4/E4$ genotyped patients with an $LDLR$ mutation (Table 1), we tested whether the $APOE4$ allele was associated with a lower CHD risk using a Cox proportional hazards model (Table 3). In the complete study population, the $APOE4/E4$ genotype was not significantly related to CHD ($E4/E4$ vs $E3/E3$
HR 0.66; 95% CI 0.42-1.05; p=0.94). However, separate analyses of patients with and without LDLR mutation showed that a CHD protective effect was restricted to APOE4/E4 homozygotes who had an LDLR mutation (no LDLR mutation: HR 1.16; 95% CI 0.69-1.95; p=0.57; with LDLR mutation: HR 0.26 HR 0.08-0.80; p=0.02). We confirmed the interaction by analyzing LDLR mutation status, APOE genotype and interaction terms for LDLR mutation with APOE genotype. Indeed, the interaction term APOE4/E4*LDLR mutation was highly significant (HR 0.22; 95% CI 0.06-0.76; p=0.016). CHD risk in carriers of APOE2 containing genotypes was not significantly different from CHD risk in APOE3/E3 genotyped FH patients (data not shown).

Discussion

We show for the first time in humans that the LDL receptor plays a key role in the detrimental consequences of Apolipoprotein E4 carrierness that is known to lead to the development of CHD. Our data reveal a protective role for LDLR mutations in FH subjects carrying the APOE4/E4 genotype; in fact, this genotype seems to even reduce CHD risk in FH patients, in contrast to the increased risk that ApoE4 confers in the general population.11 It should be stressed that having an LDLR mutation is detrimental for CHD risk in the first place, and since our study is restricted to analyses within a severely hypercholesterolemic population we cannot say that CHD risk in FH patients with an LDLR mutation is normalized by the APOE4/E4 genotype; most likely CHD risk is still increased but to a lesser extent in comparison with other APOE genotypes. This CHD risk reduction might partly be explained by lower Lp(a) levels, but in all likelihood this cannot be directly deduced from our study.

ApoE3 and ApoE4 differ by only one amino acid at position 112, but the effects on the risk of CHD and Alzheimer’s disease show that the consequences of such a change can be
immense. In humans, the interaction between ApoE and the LDL receptor has been studied only indirectly. Three small studies have previously shown that APOE4/E4 is not a genetic risk factor for CHD in FH patients despite the observed increased risk in APOE4/E4 carriers in the general population.\(^{16,17,26}\) Similarly, two polymorphisms in the LDLR gene were linked to Alzheimer’s disease, but only in patients carrying at least one APOE4 allele.\(^{27}\) To the best of our knowledge, a role of the ApoE-LDL receptor interaction in the etiology of atherosclerosis in human subjects is a novel concept.

Most studies investigating the interaction between the LDL receptor and ApoE have been performed in murine models in which the ldlr gene was either knocked out or overexpressed by replacing the mouse ldlr gene by the human LDLR gene. Ldlr knockout mice homozygous for the human APOE4 haplotype (Ldlr\(^{-/-}\) Apoe\(^{4/4}\)) display less atherosclerosis in response to a Western-type diet than both Apoe\(^{4/4}\) mice over-expressing the human LDL receptor and Apoe\(^{3/3}\) mice with physiological murine ldlr receptor levels.\(^{14,15}\) Mice homozygous for the APOE3 isoform did not show different responses to variations in LDL receptor expression.

We recognize a number of weaknesses of our study. Firstly, all patients in our cohort had hypercholesterolemia, which means that patients in this cohort without an LDLR mutation cannot be considered as healthy controls; in fact, they are individuals having another primary lipid disorder, most likely familial combined hyperlipidemia (FCH).\(^{25}\) On the other hand, this fact implies that hypercholesterolemia per se cannot be responsible for the interaction. The fact that in our study the APOE4/E4 genotype was not associated with CHD in patients without an LDLR mutation, in contrast to a strong and consistent association in earlier studies and meta-analyses, suggests that the contribution of the APOE4/E4 genotype in patients with severe dyslipidemia is small.\(^{11,12}\) If the control group would have consisted of persons without hypercholesterolemia, the APOE4/E4 genotyped
individuals would most likely have been identified as having an increased CHD risk.\textsuperscript{11,12} The distributions of the different \textit{LDLR} mutations were similar among \textit{APOE} genotypes. However, we did not measure residual LDL receptor activity in all individuals.

A number of artefacts could underlie our remarkable findings. Survival bias was not likely in view of the Hardy Weinberg equilibrium and similar ages of the different genotype groups. We confirmed the \textit{APOE} genotype distribution in an additional cohort of FH patients but the number was too small to consider this as a true replication (n=464; data not shown). Classical CHD risk factors, most importantly smoking and untreated total and LDL-cholesterol levels, did not differ between \textit{APOE} genotypes with or without \textit{LDLR} mutation. Earlier studies suggested an interaction between \textit{APOE4} and smoking.\textsuperscript{28,29} Adjustment for smoking did not change our results. Differences in risk profiles, especially untreated lipid levels, might have led to earlier statin treatment and consequent risk reduction. However, as expected from the lack of differences in terms of classical risk factors, age of starting statins was similar between different \textit{APOE} genotypes with or without \textit{LDLR} mutation.

The substitution at position 112 of a cystein in ApoE3 by an arginin in ApoE4 causes structural variations. Firstly, the interaction between the N-terminal and C-terminal domain leads to a more compact structure with lower thermal and chemical stability making ApoE4 more prone to aggregation and the formation of molten globules and subsequently to increased degradation in the unfolded protein pathway.\textsuperscript{30-32} Secondly, ApoE4 displays altered preference for lipoproteins and increased affinity to the LDL receptor.\textsuperscript{8,9,33-36} There is substantial evidence that the higher binding affinity of ApoE4 to the LDL receptor leads to unfavourable “trapping” of ApoE, enhancing sequestration of VLDL at the hepatocyte surface, which consequently delays internalization leading to an increased conversion at the
hepatocyte surface of VLDL and intestine-derived chylomicrons into atherogenic remnants.\textsuperscript{13,14,37}

Our findings might therefore be explained by the interaction between ApoE4 and less functional LDL receptors. Alternatively, increased binding to other receptors, such as related LDLR family members or proteoglycans, could also explain why the presence of an LDLR mutation might be beneficial for \textit{APOE4/E4} genotyped persons.\textsuperscript{38-42} Many of these receptors display favourable effects on atherosclerosis, which might be more pronounced if more ApoE is able to bind to these receptors.\textsuperscript{38-43}

In conclusion, we show that an \textit{LDLR} mutation is protective in FH patients with the \textit{APOE4/E4} genotype, and that this genotype even seems to reduce CHD risk in FH patients with an \textit{LDLR} mutation instead of increasing it as observed in the general population. This risk reduction might involve reduced \textit{Lp(a)} levels. Further studies are needed to unravel the biological basis of our finding and to find therapeutic approaches using this interaction in the prevention of CHD. These results might be extrapolated to an important role of this interaction in Alzheimer disease as well.

\textbf{Acknowledgments:} The authors would like to thank Steve Humphries for helpful suggestions to improve the manuscript

\textbf{Funding Source:} This work was funded by the Netherlands Heart Foundation (2006B190; Menno Hoekstra 2008T070)

\textbf{Conflict of Interest Disclosures:} Dr. Kastelein has received research funding from, served as a consultant for, and received honoraria for lectures from AstraZeneca, Genzyme, ISIS, Merck, Novartis, Pfizer, Roche, Schering Plough and Sankyo, but this was all not related to the current topic. Dr. Sijbrands has received not-drug elated research funding from Pfizer and Merck, also not related to the current topic. All authors declare they are independent from funders.

\textbf{References:}


Table 1. General characteristics per genotype with and without LDLR mutation

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<th>No LDLR mutation</th>
<th>LDLR mutation present</th>
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<tbody>
<tr>
<td>Male (%)</td>
<td>258 (51.4)</td>
<td>157 (54.3)</td>
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<tr>
<td>Age first visit lipid clinic (years)</td>
<td>48.9±11.8</td>
<td>48.2±11.7</td>
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<tr>
<td>Follow-up time (years)</td>
<td>50.0±12.0</td>
<td>49.7±11.7</td>
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<tr>
<td>Smoking ever (%)</td>
<td>363 (80.7)</td>
<td>212 (80.6)</td>
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<tr>
<td>Hypertension (%)</td>
<td>51 (10.2)</td>
<td>40 (14.2)</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>35 (7.0)</td>
<td>16 (5.5)</td>
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<td>Coronary heart disease (%)</td>
<td>186 (37.1)</td>
<td>97 (33.6)</td>
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<td>Statin at baseline (%)</td>
<td>118 (23.5)</td>
<td>75 (26)</td>
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<td>Age statin started (years)</td>
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<td>46.2±11.0</td>
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<td>BMI (kg/m2)</td>
<td>25.3±3.4</td>
<td>25.5±3.3</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
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<td>8.56±1.36</td>
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<td>LDL-cholesterol (mmol/l)</td>
<td>6.26±1.16</td>
<td>6.31±1.21</td>
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<td>HDL-cholesterol (mmol/l)</td>
<td>1.21±0.32</td>
<td>1.18±0.32</td>
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<td>Triglycerides (mmol/l)</td>
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<tr>
<td>Lp(a) (mg/l)</td>
<td>193±257</td>
<td>166±215</td>
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<tr>
<td>Lp(a)&gt;3mg/dl</td>
<td>166(43.6)</td>
<td>85 (37)</td>
</tr>
</tbody>
</table>

BMI: Body mass index; CHD: Coronary heart disease; NS: non-significant; *: APOE4/E4 vs APOE3/E3; Values are mean±standard deviation; Follow-up time from birth to event or censoring.
Table 2. Protective effect of an LDLR mutation on CHD risk in APOE4/E4 FH patients

<table>
<thead>
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<th>Model I</th>
<th>Model II</th>
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<tr>
<td></td>
<td>N</td>
<td>HR</td>
</tr>
<tr>
<td>Overall</td>
<td>2400</td>
<td>0.88</td>
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<tr>
<td>APOE3/E3</td>
<td>1178</td>
<td>0.84</td>
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<tr>
<td>APOE3/E4</td>
<td>580</td>
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<tr>
<td>APOE4/E4</td>
<td>93</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*Model I Adjusted for gender, year of birth; Model II additionally adjusted for smoking*

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Table 3. Protective effect APOE4/E4 genotype in FH patients with an LDLR mutation

<table>
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<tr>
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<th>With LDLR mutation</th>
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<td>0.83</td>
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<tr>
<td>APOE4/E4</td>
<td>38</td>
<td>1.16</td>
</tr>
</tbody>
</table>

*Adjusted gender, birth year, smoking*
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_Circ Cardiovasc Genet._ published online October 18, 2011;
_Circulation: Cardiovascular Genetics_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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“Apolipoprotein isoform E4 does not increase coronary heart disease risk in carriers of low-density lipoprotein receptor mutations”

-Supplemental Table 1
-Supplemental Table 2
-Summary for clinicians
-Acknowledgment permission
### Supplemental Table 1. Genotype frequencies

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>No (LDLR) mutation</th>
<th>(LDLR) mutation present</th>
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<tr>
<td>APOE3/E3</td>
<td>1178 (57.2)</td>
<td>502 (55.1)</td>
<td>676 (58.8)</td>
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<td>APOE2/E3</td>
<td>140 (6.8)</td>
<td>48 (5.3)</td>
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<tr>
<td>APOE3/E4</td>
<td>580 (28.1)</td>
<td>289 (31.7)</td>
<td>291 (25.3)</td>
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<td>APOE2/E4</td>
<td>61 (3.0)</td>
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<td>7 (0.8)</td>
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<tr>
<td>APOE4/E4</td>
<td>93 (4.5)</td>
<td>44 (4.8)</td>
<td>49 (4.3)</td>
</tr>
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</table>

*Values are count (percentage)*