Low-Density Lipoprotein Receptor Gene Mutations and Cardiovascular Risk in a Large Genetic Cascade Screening Population

Marina A.W. Umans-Eckenhausen, MD; Eric J.G. Sijbrands, MD, PhD; John J.P. Kastelein, MD, PhD; Joep C. Defesche, PhD

Background—A large cohort of patients with familial hypercholesterolemia (FH), free from selection for cardiovascular disease (CVD), and their unaffected relatives was collected by genetic cascade screening and examined for the influence of different mutations of the LDL receptor gene on lipoprotein levels and the risk of CVD. Multivariate analyses with adjustment for age, sex, and specific family ties were performed.

Methods and Results—Significant variation of LDL levels was observed among 399 patients with FH with different mutations. Null alleles were associated with more severely elevated LDL cholesterol, whereas the frequent N543H/2393del9 mutation led to less elevated LDL cholesterol. The type of mutation did not influence HDL cholesterol levels. Patients with FH had CVD 8.5 times more often compared with their unaffected relatives (RR, 8.54; 95% CI, 5.29 to 13.80). The N543H/2393del9 mutation was associated with a smaller increase of risk compared with other mutations \(p<0.0001\). After exclusion of families with the N543H/2393del9 mutation, null alleles and other allele mutations no longer differed with regard to LDL cholesterol levels and CVD risk.

Conclusions—LDL receptor mutations only partly contributed to the variation of LDL cholesterol levels and cardiovascular burden in FH. Additional, so far unidentified, familial risk factors must underlie the differences of CVD risk, most likely independent of lipids and lipoproteins. (Circulation. 2002;106:3031-3036.)

Key Words: atherosclerosis | cardiovascular diseases | genetics | lipoproteins | risk factors

Familial hypercholesterolemia (FH) is a common disorder of lipoprotein metabolism, leading to severe hypercholesterolemia and an increased risk of cardiovascular disease (CVD). The disorder is caused by mutations in the LDL receptor gene. To date, >700 DNA sequence variations have been identified and categorized in 6 different functional classes. The effects of these mutation classes on plasma lipoprotein levels and on risk of CVD have been studied extensively.

In these studies, however, patient populations were small and invariably selected through lipid clinics. Thus, other risk factors for CVD were likely to determine the excess mortality rates from FH, whereas the type of mutation had seemingly little or no relevant contribution. In effect, we recently reported the presence of additional dyslipidemias in some FH families. Hence, in previous studies, it was not clear whether variance of lipoprotein levels and CVD risk could be attributed to variation at the LDL receptor locus or to other familial factors. Therefore, the exact relation between the different LDL receptor mutation classes, lipoprotein levels, and the risk of CVD is still unclear.

The Dutch national screening program for FH provided a large-population, free-form selection for CVD after exclusion of probands. This study assessed the effect of different LDL receptor gene mutations on plasma lipoproteins and risk of CVD in this cohort after adjustment for familial risk factors.

Methods

Screening Program

The national genetic testing program for FH was executed as described before. Patients with FH were identified according to a uniform diagnostic protocol. DNA samples of these patients were analyzed for the presence of an LDL receptor gene mutation and, once identified, such a patient was referred to as an index case. The mutations were described previously and known to be pathogenic. Relatives of the index patient were then actively contacted, and DNA testing was performed for the presence of the mutation. This genetic testing program did not allow for extensive data collection or measurement of many biochemical parameters, thereby limiting a full-scale risk assessment.

Study Population and Patient Selection

Only relatives of index cases were included in this study, and the participation rate among relatives was 90%. Selection was based...
solely on the mendelian inheritance pattern of the LDL receptor mutations and not on referral type. Therefore, analysis of genotype-phenotype relations without selection on additional risk factors for CVD was possible. To avoid ascertainment bias, index cases were excluded from all analyses, since a proportion could have been referred based on the onset of CVD and this would lead to overestimation of the risk from FH. On the other hand, index cases were selected on being alive, which could also introduce a selection bias leading to underestimation of the risk from the disorder. Individuals receiving any form of cholesterol-lowering medication were excluded from analyses of the lipid and lipoprotein parameters.

Laboratory Methods
Plasma concentrations of total cholesterol, HDL cholesterol, and triglycerides were measured by commercially available kits (Boehringer Mannheim) in fasting blood samples. LDL cholesterol concentrations were calculated by the Friedewald formula only if the triglyceride concentration was <398 mg/dL.14 Genomic DNA was isolated from the leukocyte fraction of 10 mL freshly collected blood. Mutational analyses were performed with the use of polymerase chain reaction and restriction enzyme analysis as described previously.12

Cardiovascular Disease
Cardiovascular disease was defined as fatal myocardial infarction or onset of nonfatal symptoms. The latter included the (1) onset of angina pectoris assessed with electrocardiographic exercise testing, (2) 70% stenosis as assessed by coronary angiography, (3) myocardial infarction documented by electrocardiography, or a deep and wide Q-wave as an electrocardiographic manifestation of an old infarction or CPK-MB monitoring during the acute phase, and (4) performance of coronary bypass or PTCA.

Statistical Analysis
Four frequent mutations allowed for separate analyses, as did receptor-negative (null-alleles W23X and 1359-1) and receptor-defective (other alleles) mutations.

All data were analyzed with the use of SPSS software (version 9.0, SPSS). To estimate the effect of mutational heterogeneity at the LDL receptor locus on lipoprotein levels, two different methods were used: (1) comparison of lipid levels of carriers of the mutations by ANOVA and multiple linear regression with adjustment for age and sex; (2) calculation of mean differences in lipoprotein levels, SEM, and 95% CIs of lipoprotein levels between relatives with and without a mutation, using a matched multiple regression model. Each case was matched with the mean value of the unaffected relatives with and without adjustment for age, sex, and specific family ties. In multivariate analyses, difference in age was entered as a continuous covariable and family and degree of relative as dichotomous dummies. Cumulative survival was analyzed by means of the Kaplan-Meier method and Cox regression. In effect, the population was stratified by family, degree of relative, sex, and age. Generation and date of birth were correlated, and separate analyses yielded similar results. Date of birth was used in the Cox regression to adjust for differences in life expectancy over calendar periods.

Moreover, in all multivariate analyses we have compared the proportion of shared genome with the nearest proband with the degree of relative code. These techniques yielded identical results. We chose for the more neutral codes of the dummy variables instead of the proportions to avoid putting weight on relations without being able to consider shared environmental factors. In this way, estimation were made in the multivariate model and were not influenced by our interpretation of the relations.

Results
Patient Characteristics
A total of 1695 relatives of 66 consecutive index patients were screened for the LDL receptor gene mutation transmitted in the respective families. Figure 1 gives a breakdown of

![Figure 1](image-url)

**Figure 1.** Patient selection. “Without medication” means that individuals were not taking cholesterol-lowering medication. Index cases and their first-degree relatives were not included.

the study population with regard to carrier and treatment status. The mean age differed significantly between treated and untreated carriers: 47 ± 0.9 years versus 31 ± 1.1 years, respectively (P < 0.001), whereas the proportion of men was similar in these two groups: 47% ± 3% and 49% ± 3%, respectively (P = 0.7).

In Table 1, the characteristics of the carriers of null and other alleles and of unaffected relatives are presented. In the following analyses, additional adjustment for significantly different risk factors did not change the outcome. The number of patients with hypertension and type 2 diabetes was too small for meaningful analyses.

Mutations and Lipoproteins
Four mutations were present in substantial numbers. Unadjusted mean lipid values (±SD) of untreated male and female patients with FH and unaffected relatives are shown in Table 1. The N543H/2393del9 mutation, which is a combination of a point mutation in exon 11 and a deletion of 9 base pairs in exon 9 linked on the same allele, was present in 185 patients. V408M was found in 51 patients. Two splice defects at positions 313 +1 or 2 in intron 3 and 1359-1 in intron 9 were found in 39 and 38 patients, respectively. Five other mutations, Leiden-3, W23X, E207K, P664L, and S285L, were found in 39 and 38 patients, respectively. Other alleles and of unaffected relatives are presented. In the following analyses, additional adjustment for significantly different risk factors did not change the outcome. The number of patients with hypertension and type 2 diabetes was too small for meaningful analyses.

![Table 1](table-url)

**Table 1.** The characteristics of the carriers of null and other alleles and of unaffected relatives.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Carriers without medication</th>
<th>Carriers</th>
<th>Non-carriers</th>
<th>Non-carriers without medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>N543H/2393del9</td>
<td>205 (65.6%)</td>
<td>148 (96.3%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
<tr>
<td>V408M</td>
<td>31 (5.0%)</td>
<td>206 (94.9%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
<tr>
<td>W23X</td>
<td>20 (5.0%)</td>
<td>206 (94.9%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
<tr>
<td>E207K</td>
<td>20 (5.0%)</td>
<td>206 (94.9%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
<tr>
<td>P664L</td>
<td>19 (5.0%)</td>
<td>207 (95.0%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
<tr>
<td>S285L</td>
<td>19 (5.0%)</td>
<td>207 (95.0%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
<tr>
<td>Leiden-3</td>
<td>4 (1.3%)</td>
<td>211 (98.7%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
<tr>
<td>Other alleles</td>
<td>33 (9.7%)</td>
<td>335 (90.3%)</td>
<td>1648 (96.4%)</td>
<td></td>
</tr>
</tbody>
</table>

In Table 1, the characteristics of the carriers of null and other alleles and of unaffected relatives are presented. In the following analyses, additional adjustment for significantly different risk factors did not change the outcome. The number of patients with hypertension and type 2 diabetes was too small for meaningful analyses.

Mutations and Lipoproteins
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![Figure 1](image-url)

**Figure 1.** Patient selection. “Without medication” means that individuals were not taking cholesterol-lowering medication. Index cases and their first-degree relatives were not included.
3% in those without dyslipidemia. Exclusion of these relatives did not change the results of the risk analyses.

Correction for familial factors, not related to the LDL receptor gene locus, was also performed. The mean differences in lipoprotein levels between mutation carriers and their unaffected relatives were estimated with the multiple linear regression method, adjusted for age, sex, and family ties. Figure 2 shows mean differences in plasma LDL and HDL cholesterol between carriers of different types of LDL receptor mutations and their unaffected relatives adjusted for differences in age, sex, and family ties. Compared with unaffected relatives, patients with FH had increased plasma concentrations of total cholesterol (difference, 83 mg/dL; 95% CI, 77 to 89; P<0.001) and LDL cholesterol (difference, 88 mg/dL; 95% CI, 83 to 93; P<0.001). HDL cholesterol was decreased by 4 mg/dL (95% CI, −5 to −2; P<0.001). Additional adjustment for triglycerides did not change this finding. Plasma triglyceride levels were not significantly different between patients and unaffected relatives (mean difference, −11 mg/dL; 95% CI, −21 to −5, P=0.06). LDL cholesterol levels of carriers of the N543H/2393del9 mutation were significantly less elevated compared with three other mutations (Figure 2, P<0.001). Differences in HDL cholesterol levels between carriers and noncarriers were similar for the different mutation carriers. Carriers of null alleles had significantly higher LDL levels compared with the carriers of other alleles. However, after exclusion of the families with the N543H/2393del9 mutation, the mean difference in LDL level was 113 mg/dL (95% CI, 100 to 125) for carriers of null alleles versus 109 mg/dL (95% CI, 101 to 117) for carriers of the other alleles (Figure 2).

### TABLE 1. Characteristics of Carriers of Null Alleles, Carriers of Other Alleles, and Unaffected Relatives

<table>
<thead>
<tr>
<th></th>
<th>Null Alleles</th>
<th>Other Alleles</th>
<th>Unaffected Relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (total)</td>
<td>124</td>
<td>484</td>
<td>1087</td>
</tr>
<tr>
<td>Mean age, y±SEM</td>
<td>34.8±1.6</td>
<td>36.8±0.9</td>
<td>40.7±0.6</td>
</tr>
<tr>
<td>Age range, y</td>
<td>16–80</td>
<td>16–84</td>
<td>16–96</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>61/63</td>
<td>233/251</td>
<td>511/576</td>
</tr>
<tr>
<td>Risk factors, No. (%±SEM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>9 (7±2%)</td>
<td>17 (4±1%)</td>
<td>62 (6±0%)</td>
</tr>
<tr>
<td>Diabetes type II</td>
<td>0 (0±0%)</td>
<td>2 (0±0%)</td>
<td>26 (2±1%)</td>
</tr>
<tr>
<td>Former smokers</td>
<td>29 (23±4%)</td>
<td>77 (16±2%)*</td>
<td>197 (18±1%)</td>
</tr>
<tr>
<td>Current smokers</td>
<td>32 (26±4%)</td>
<td>93 (19±2%)†</td>
<td>284 (26±1%)</td>
</tr>
<tr>
<td>n (untreated)</td>
<td>26 (F)</td>
<td>28 (M)</td>
<td>178 (F)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>328 (67)</td>
<td>291 (48)</td>
<td>289 (63)</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>258 (59)</td>
<td>229 (44)</td>
<td>217 (59)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>39 (8)</td>
<td>37 (10)</td>
<td>45 (14)</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>153 (78)</td>
<td>128 (73)</td>
<td>126 (79)</td>
</tr>
</tbody>
</table>

Lipoprotein values are mean values (±SD).

*In comparison with the other two groups, P=0.2; in comparison with the null alleles group, P=0.07.
†Significantly lower than in the other two groups (P=0.03).
this model, the differences in HDL cholesterol levels between carriers of null and other alleles could not be reproduced.

**Cardiovascular Disease**

The numbers of events were 20 in 241 for the N543H/2393del9bp mutation, 53 in 367 for other mutations, and 37 in 1087 for unaffected relatives. In our population, 27% (95% CI, 21% to 33%) started treatment after a CVD event. Four percent (95% CI, 2% to 6%) of the untreated patients with FH had CVD 3 times more often than those who were not taking medication (RR, 3.04; 95% CI, 1.55 to 5.94; \( P < 0.001 \)). This illustrates that medication was mostly prescribed as secondary prevention. In Table 2, the CVD risk of mutation carriers relative to unaffected relatives, adjusted for date of birth and sex, is shown. In the total group, patients had a 4-times-higher risk of CVD relative to all unaffected relatives. However, after adjustment for family ties, the patients had an 8.5-times-higher CVD risk compared with their unaffected relatives. Additional adjustment for the ratio of total cholesterol to HDL cholesterol did not change this risk (data not shown). The carriers of the N543H/2393del9bp mutation had lower CVD risk compared with the other three mutations (RR, 0.30; 95% CI, 0.18 to 0.52; \( P < 0.001 \)). After adjustment for family ties, the risk of the N543H/2393del9bp and the V408M mutation became similar albeit significantly different from the other two mutations. Additional adjustment for the ratio of total cholesterol to HDL cholesterol did not change the results. The carriers of null alleles had a 6-times-higher CVD risk compared with unaffected relatives, and this risk tended to higher values after adjustment for family ties and lipid profile. The analyses of coronary artery disease risk with and without the N543H/2393del9bp mutation families yielded virtually identical results (Table 2). In Figure 3, Kaplan-Meier curves represent the CVD-free survival of carriers of the N543H/2393del9bp mutation, carriers of all other mutations, and unaffected relatives. The numbers of censored persons were, respectively, 221 (92%), 314 (86%), and 1044 (97%). The log rank test was 112, \( df = 2 \) (\( P < 0.0001 \)).

**Discussion**

Since the elucidation of the cholesterol pathway by Brown and Goldstein, many studies have addressed the relation between mutations causing FH and the resulting phenotypic expression of the disorder. Gudnason and Humphries found that patients with FH with null alleles had significantly higher LDL cholesterol levels but were not able to establish a relation with CVD risk. This may be a result of recruiting patients from a lipid clinic population in which CVD was almost omnipresent. Two studies in French-Canadian patients revealed that LDL cholesterol levels as well as severity of

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**TABLE 2. Risk of Coronary Artery Disease in Patients With FH Relative to Unaffected Relatives, Adjusted for Age and Sex**

<table>
<thead>
<tr>
<th></th>
<th>Not Adjusted for Family Ties</th>
<th>Family Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n RR* 95% CI</td>
<td>n RR* 95% CI</td>
</tr>
<tr>
<td>All mutations</td>
<td>608/1087 4.00 2.83–5.65</td>
<td>124/236 6.03‡ 3.58–10.16</td>
</tr>
<tr>
<td>N543H/2393del9bp</td>
<td>241/401 2.15† 1.30–3.57</td>
<td>1359 7.39 4.35–12.56</td>
</tr>
<tr>
<td>V408M</td>
<td>77/181 5.19 2.82–9.54</td>
<td>1359 7.01 2.18–22.59</td>
</tr>
<tr>
<td>1359–1</td>
<td>102/176 7.39 4.35–12.56</td>
<td>1359 15.95 5.52–46.14</td>
</tr>
<tr>
<td>Null alleles</td>
<td>124/236 6.03‡ 3.58–10.16</td>
<td>124/236 10.43 4.08–26.69‡</td>
</tr>
<tr>
<td>Other alleles, N543H/2393del9 included</td>
<td>484/851 3.58 2.47–5.19</td>
<td>Other alleles, N543H/2393del9 excluded</td>
</tr>
<tr>
<td>Other alleles, N543H/2393del9 excluded</td>
<td>243/450 5.53 3.61–8.46</td>
<td>243/450 7.53 3.84–14.76</td>
</tr>
</tbody>
</table>

\( n \) indicates No. of carriers/No. of noncarriers.

\*Relative risk differed significantly (\( P < 0.003 \)) compared with unaffected relatives.

†In comparison with the other 3 mutations, the risk of coronary artery disease was 0.30 (95% CI, 0.18 to 0.52, \( P < 0.0001 \)).

‡Not significantly different compared with other alleles.
CVD was higher in patients carrying null alleles, findings confirmed by a third study in homozygous patients with FH.\(^{7,8,15}\) However, the results of these studies may have been influenced by unknown ethnic or familial genetic factors. Sun et al\(^{16}\) examined a small cohort of 12 patients from 7 families with the 313+1 (G to A) splice-site mutation in intron 3 and concluded that this mutation leads to a relatively severe clinical phenotype. Although in our patients with mutations in the 313+1/2 cluster the LDL levels were not extreme, we found relatively frequent CVD among the carriers of these mutations, in agreement with Sun et al.

We also show that LDL cholesterol levels and CVD risk significantly varies within and between carriers of different LDL receptor gene mutations. In particular, carriers of the N543H/2393del9 mutation had less abnormal lipid profiles and accordingly lower CVD risk, compared with carriers of other mutations. The allelic combination of the N543H and 2393del9 mutations has been described as a functional class 2 defect (transport deficient),\(^{17}\) but its mild phenotype was first noticed in this study.

Carriers of null alleles had a worse lipoprotein profile when compared with carriers of other alleles. However, after exclusion of families with the N543H/2393del9 mutation, lipoproteins nor CVD risk no longer differed.

The lipoprotein profile, however, poorly explained the differences of cardiovascular risk between mutations. Together, our findings suggest a relevant genotype-phenotype effect on lipids and cardiovascular burden but accentuate, on the other hand, the importance of other, probably not lipid-related, risk factors for CVD in FH.

Assessment of cardiovascular burden in family analyses, free from selection for CVD, represents the strength of our study. Kindreds of large series of index cases with a molecular diagnosis of FH were recruited, and these index cases were excluded from our analyses. This study design clearly contrasts with others that actually selected on CVD. Peculiar to the present study was the ability to compare association and family studies, whereas others neglected the influence of family ties. Unfortunately, the family analyses had less power as a result of smaller groups. Nonetheless, these analyses showed more severely increased point estimates of the CVD risk compared with the analyses without considering the family ties. This phenomenon was a result of the relatively high prevalence of CVD among the relatives without FH, reflecting the high prevalence of CVD in our general population. Restricting analyses to families revealed that although the relatives without FH often had CVD, patients with FH had onset of CVD symptoms at a much younger age. Therefore, we argue that the point risk estimates in families described the cardiovascular burden of FH better than those without considering family ties. A possible limitation of our study was the possibility of underestimating cardiovascular burden as a result of premature death among relatives with FH. In a recent family tree mortality study, however, we showed that exclusion of such “severe” heterozygous cases did not occur.\(^{18}\) Death from heterozygous FH occurs in middle-aged men and elderly women, and it is therefore unlikely that premature death had a relevant influence on the present analyses.\(^{9}\)

Our older observations and those of others may have been biased by the presence of additional (familial) lipoprotein disorders, because in previous studies it was not possible to perform family analyses as in the present study.\(^{9,18}\) The analyses of CVD risk with and without the N543H/2393del9 mutation families yielded virtually identical results.

We included the patients who were taking medication in all CVD risk analyses. Excluding patients taking medication would certainly lead to further underestimation of risk by excluding a proportion of patients with CVD. The latter were on medication mostly in the framework of secondary prevention and belong to the highest risk category. In our analyses, we cannot adjust for the effect of medication without also adjusting for the relatively high a priori risk of the majority of patients taking medication. Therefore, our study is not able to estimate the effect of medication on CVD risk.

In conclusion, clear genotype-phenotype relations exist between specific LDL receptor mutations, plasma lipids, and CVD burden. However, independent of lipoprotein profiles, additional familial risk factors contributed to the CVD burden of FH.

The combination of genetic testing and assessment of cardiovascular risk by IMT measurement may lead to a strategy for an individual approach in preventive medicine.\(^{9,19}\) More aggressive treatment of elevated LDL cholesterol levels in FH has shown that the modalities for optimal patient identification and treatment are now available for these patients.\(^{20}\)

**Acknowledgments**

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