Assessment of Carotid Atherosclerosis in Normocholesterolemic Individuals With Proven Mutations in the Low-Density Lipoprotein Receptor or Apolipoprotein B Genes

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Background—Genetic cascade screening for heterozygous familial hypercholesterolemia (FH) revealed that 15% of individuals given this diagnosis do not exhibit elevated low-density lipoprotein cholesterol (LDL-C) levels. We assessed whether cardiovascular risk for these individuals differs from that of hypercholesterolemic FH heterozygotes and unaffected relatives.

Methods and Results—Individuals aged 18 to 55 years were recruited within 18 months after genetic screening. Three groups were studied: subjects given a molecular diagnosis of FH and with LDL-C levels at genetic screening below the 75th percentile (FH-low), subjects with FH and an LDL-C level above the 90th percentile (FH-high), and subjects without FH (no-FH). We measured carotid intima-media thickness (IMT) by ultrasonography. Differences in carotid IMT between groups were assessed using multivariate linear regression analyses. Mean carotid IMT of 114 subjects in the FH-low group (0.628 mm; 95% CI, 0.613 to 0.642 mm) was significantly smaller than that of 162 subjects in the FH-high group (0.664 mm; 95% CI, 0.648 to 0.679 mm; P<0.001) and did not significantly differ from the mean carotid IMT of 145 subjects in the no-FH group (0.628 mm; 95% CI, 0.613 to 0.642 mm; P=0.67).

Conclusions—Our findings suggest that the risk of cardiovascular disease in patients with FH to a large extent is related to LDL-C levels and not to the presence of a mutation per se. Consequently, this study cautiously suggests that individuals with an FH genotype without expression of hypercholesterolemia may not require a pharmaceutical intervention that is as aggressive as the standard for subjects with FH. (Circ Cardiovasc Genet. 2011;4:413-417.)

Key Words: hyperlipoproteinemia type II ■ diagnosis ■ arteriosclerosis ■ phenotype ■ genetic screening ■ familial hypercholesterolemia

Familial hypercholesterolemia (FH) is a condition that meets key criteria for genetic screening.1,2 It is a prevalent inherited disorder of lipoprotein metabolism characterized by markedly elevated low-density lipoprotein cholesterol (LDL-C) levels and, if left untreated, premature coronary artery disease.1 In fact, cholesterol-lowering treatment has been shown to dramatically reduce coronary artery disease risk in patients with FH.3,4 Subsequently, first-degree relatives are offered DNA analysis for the presence of the specific FH-causing mutation, and cascade screening is extended to distant relatives by using the inheritance pattern across the pedigree.6

Within this cohort, ≈15% of patients with FH do not exhibit severely elevated LDL-C levels at diagnosis.5,7 In current practice, mutation carriers without elevated LDL-C levels are likely to remain untreated.7 It is unknown, however, whether it is justified to withhold treatment for these individuals. The purpose of the present study, therefore, was to assess whether cardiovascular risk differs between carriers of an LDLR or carriers of an APOB mutation without or with hypercholesterolemia and their unaffected relatives.

Clinical Perspective on p 417

Nationwide genetic screening for FH has been ongoing in the Netherlands since 1994, and ≈20 000 individuals given a molecular diagnosis of FH have been identified to date. To diagnose FH in these individuals, DNA samples from patients with clinically suspected FH are analyzed for the presence of a mutation in the LDL receptor LDLR or apolipoprotein B APOB gene.5 A patient is then considered a proband for family screening when a pathogenic mutation is identified.

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lands. This screening program was approved and funded by the Dutch government. The current study was approved by the ethics committee.

Men and women aged 18 to 55 years were eligible if they met the following criteria: having had a genetic test for FH between January 2007 and January 2010 and having a known lipid profile. Individuals were excluded if they used cholesterol-lowering medication before or at the time of the screening and if they were not able to plan a study visit within 18 months after the genetic test. Probands also were excluded.

We classified subjects based on the presence of a pathogenic LDLR or APOB mutation and on their age and gender-specific percentiles of LDL-C.8 The lipids were measured with the LDX Analyzer (Cholestech Corporation; Hayward, CA) during blood withdrawal for the genetic test.9 LDL-C levels were subsequently estimated with the Friedewald formula.10 Age- and sex-specific percentiles of LDL-C were calculated using the reference values of a western population.11

We identified 3 groups of subjects: those given a molecular diagnosis of FH and LDL-C levels at genetic screening below the 75th percentile (FH-low), those with FH and an LDL-C level above the 90th percentile (FH-high), and those without FH from families participating in the cascade screening program (no-FH). Subjects in the selected groups (ie, FH-low, FH-high, no-FH) were invited to participate in the current study through surface mail. We sent batches of 200 invitation letters until >240 mutation carriers and >120 no-FH group subjects were recruited. If prospective subjects returned a reply card to the researchers indicating their interest in participation, they were phoned by the study physician for further information. If approved for participation, a study visit was planned at our facility.

Study Visit
Fasting blood samples were obtained for analysis of lipid measures. The medical history was recorded, and a physical examination was performed. All participants underwent ultrasonography of the carotid arteries.

Carotid Intima-Media Thickness
Carotid ultrasound measurements of intima-media thickness (IMT) were performed according to a standardized and validated methodology as described in detail before.12 Two experienced and certified sonographers performed the scans using an Acuson Sequoia with a linear array vascular transducer (L7) (Siemens; Erlangen, Germany). Six predefined carotid segments, the common carotid, carotid bulb, and internal carotid were imaged bilaterally in all subjects. Still images were saved as DICOM files. One certified image analyst analyzed these images off line. Both the sonographers and the image analyst were blinded to clinical genetic and laboratory data. A per-subject carotid IMT aggregate over all available segments was calculated as the primary outcome measure.

Statistical Analysis
Assuming an SD of 0.12 mm, 120 patients were required in each study group to detect a difference of 0.044 mm in carotid IMT among the study groups with a power of 80% and a 2-sided α=0.05. For comparison, the difference in carotid IMT between children with FH and unaffected siblings reached 0.033 mm at age 14 years.13 Differences in demographic and baseline characteristics among the 3 groups (FH-low, FH-high, no-FH) were evaluated using linear or logistic regression analysis. Multivariate linear regression analysis was applied to evaluate the association between carotid IMT and the different groups. We adjusted for potential confounders by means of stepwise backward elimination. All analyses were performed using the generalized estimating equation method to account for correlations within families. The exchangeable correlation structure was used for these models. Subgroup analyses were performed in subjects who remained untreated until the study visit.

Variables with a skewed distribution were log-transformed before statistical analyses. A P<0.05 was considered statistically significant. Data were analyzed with SPSS for Windows version 16.0.2 (SPSS Inc; Chicago, IL).

Results
Study Population
Among the screened population, 2016 individuals met the inclusion criteria. Recruitment was discontinued when sufficient numbers of individuals with and without genetic FH were enrolled. A total of 421 individuals provided written informed consent to participate in this study. These subjects originated from 257 different families in which at least 1 individual with a pathogenic LDLR or APOB mutation had been identified and in which genetic cascade screening for molecular FH was initiated. In total, 256 participants were related to at least 1 other participant in varying degrees.

Participants were enrolled after a median period of 11 months (interquartile range, 8 to 14 months) since the genetic test for FH. Demographic and clinical characteristics of the 3 groups are summarized in Table 1. In general, subjects with FH were younger than those without FH. Mean LDL-C levels, adjusted for age and sex, were comparable between FH-low and no-FH, whereas LDL-C levels were much higher in FH-high individuals. As expected, mutation carriers more often used statin treatment after diagnosis than did those without FH. Among subjects given a molecular diagnosis of FH, the FH-high group used statins more often than the FH-low group.

Table 2 lists the distribution between the FH-low and FH-high groups of the 16 most prevalent LDLR mutations in The Netherlands and the p.R3527Q mutation in APOB.8 These 17 mutations accounted for 60% of all mutation carriers in the FH-low group and 70% in the FH-high group. Most of these 17 mutations were present in both groups. Table 3 lists the mutation class distribution among mutation carriers. Class 1 mutations were more prevalent in the FH-high group than in the FH-low group, whereas the proportion of class 2B mutations was higher in the FH-low group. All individuals with FH carried a mutation that is hitherto assumed to be pathogenic.

Carotid IMT
Online Only Data Supplement Figure 1 presents the actual distributions of carotid IMT plotted against age and shows that carotid IMT is positively correlated with age. Figure exhibits for the 3 study groups the mean carotid IMT values adjusted for age, sex, smoking, body mass index, and systolic blood pressure. Mean carotid IMT of the FH-low group (0.623 mm; 95% CI, 0.609 to 0.638 mm) was significantly smaller than that of the FH-high group (0.664 mm; 95% CI, 0.648 to 0.679 mm; P<0.001) but did not significantly differ from mean carotid IMT values in the no-FH group (0.628 mm; 95% CI, 0.613 to 0.642 mm; P=0.67).

Because of the substantial differences between groups in the proportion of individuals who had initiated statin treatment in the period after the genetic test, a subgroup analysis was performed in the population that had remained untreated until the study visit (n=280). The adjusted mean carotid IMT of 89 untreated individuals in the FH-low group (0.622 mm; 95% CI, 0.606 to 0.639 mm) was significantly smaller than
that of 51 untreated subjects in the FH-high group (0.673 mm; 95% CI, 0.648 to 0.698 mm; P = 0.001) and did not significantly differ from the adjusted carotid IMT in the 140 untreated participants in the no-FH group (0.634 mm; 95% CI, 0.620 to 0.649 mm; P = 0.28).

Table 2. Distribution of Mutations Carried by the Subjects

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Class</th>
<th>FH-Low Group</th>
<th>FH-High Group</th>
<th>P*</th>
<th>P†</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.R3527Q (APOB, exon 26)</td>
<td>3</td>
<td>18 (16)</td>
<td>34 (21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1359-1 (intron 9)</td>
<td>1</td>
<td>1 (1)</td>
<td>3 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.313+1/2 (intron 3)</td>
<td>1</td>
<td>...</td>
<td>3 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.W44X (exon 2)</td>
<td>1</td>
<td>1 (1)</td>
<td>6 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.S306L (exon 6)</td>
<td>2B</td>
<td>3 (3)</td>
<td>9 (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.E228K (exon 4)</td>
<td>2B</td>
<td>...</td>
<td>1 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 kb deletion exon 7 en 8 (Cape Town-2)</td>
<td>1</td>
<td>...</td>
<td>1 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.191-2 (intron 2)</td>
<td>1</td>
<td>...</td>
<td>2 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.G207G (exon 4)</td>
<td>3</td>
<td>2 (2)</td>
<td>7 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.V429M mol/L (exon 9)</td>
<td>5</td>
<td>1 (1)</td>
<td>...</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>p.G343S (exon 7)</td>
<td>2B</td>
<td>8 (7)</td>
<td>4 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.A705P (exon 14)</td>
<td>2B</td>
<td>2 (2)</td>
<td>3 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.R81C (exon 3)</td>
<td>2B</td>
<td>8 (7)</td>
<td>5 (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.A431T (exon 9)</td>
<td>5</td>
<td>2 (2)</td>
<td>3 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.C255R (exon 5)</td>
<td>2B</td>
<td>4 (4)</td>
<td>1 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.P699L (exon 14)</td>
<td>2B</td>
<td>4 (4)</td>
<td>5 (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other mutations</td>
<td>...</td>
<td>46 (40)*</td>
<td>49 (30)†</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD, n (%), or median (interquartile range). FH indicates familial hypercholesterolemia; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; pLDL, percentile LDL-C for age and sex; TC, total cholesterol.

Discussion

The present data show that individuals who carry pathogenic mutations in LDLR or APOB but do not exhibit the severely elevated LDL-C phenotype possess carotid arterial walls of similar thickness as their unaffected relatives. To our knowledge, this study is the first to use a surrogate marker for atherosclerotic burden in a cohort of patients with FH solely identified by cascade screening and, thus, are in essence free of referral bias. Nevertheless, several prior studies have assessed the contribution of LDL-C levels to IMT in cohorts of patients with heterozygous FH, and these studies have

Table 3. Mutation Class Distribution

<table>
<thead>
<tr>
<th>Mutation Class</th>
<th>FH-Low Group</th>
<th>FH-High Group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 (2)</td>
<td>24 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2A</td>
<td>7 (6)</td>
<td>8 (5)</td>
<td>0.79</td>
</tr>
<tr>
<td>2B</td>
<td>71 (62)</td>
<td>76 (47)</td>
<td>0.014</td>
</tr>
<tr>
<td>3</td>
<td>26 (23)</td>
<td>45 (28)</td>
<td>0.40</td>
</tr>
<tr>
<td>4</td>
<td>0 (0)</td>
<td>4 (3)</td>
<td>0.15</td>
</tr>
<tr>
<td>5</td>
<td>3 (2)</td>
<td>3 (2)</td>
<td>0.69</td>
</tr>
<tr>
<td>Undetermined</td>
<td>5 (4)</td>
<td>2 (1)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

*Data are presented as n (%). Mutation classes are described based on Goldstein et al.: class 1, LDL-receptor (LDLR) is not synthesized at all; class 2, LDLR is not properly transported from the endoplasmic reticulum to the Golgi apparatus for expression on the cell surface; class 2A, transport from the endoplasmic reticulum to the cell surface is delayed; class 3, LDLR does not properly bind LDL-C on the cell surface; class 4, LDLR bound to LDL-C does not properly cluster in the clathrin-coated pits for receptor-mediated endocytosis; and class 5, LDLR is not recycled back to the cell surface. Abbreviations as in Table 1.
consistently shown that the higher the LDL-C levels are, the more carotid IMT was increased.14–16 As such, those findings agree with the present study results, which show that FH heterozygotes without elevated LDL-C exhibited less-pronounced carotid atherosclerosis than patients with FH with hypercholesterolemia. The novelty of the current study, however, lies in the fact that we specifically recruited a sizable group of FH mutation carriers without hypercholesterolemia, which enabled us to also compare their atherosclerotic burden to that of unaffected relatives. Because mean carotid IMT did not differ between these groups, we might conclude that individuals with an FH genotype but without elevated LDL-C levels are not at increased risk of coronary artery disease.

The reasons why a proportion of LDLR and APOB mutation carriers do not express a severe dyslipidemia phenotype are not fully understood. LDL-C levels per se in FH are influenced by variation in several genes, such as those coding for apoB, PCSK9 (proprotein convertase subtilisin/kexin type 9), and apoE.17–21 In a recently performed analysis, our group tested 77 individuals with heterozygosity for a pathogenic LDLR or APOB mutation who lacked the hypercholesterolemia phenotype and showed this lack of phenotype in 6 (8%) cases because of concomitant other mutations with a hypocholesterolemic effect in APOB or PCSK9 (unpublished data).20 In addition to genetic variation in other genes influencing LDL-C levels, lifestyle factors contribute to the expression of dyslipidemia in patients with FH.22,23 The most likely explanation for the observed large variation in lipid phenotype, however, is the difference in functionality of the 61 specific mutations that we identified in the study participants. For example, several individuals from the FH-low group carried mutations that are associated with a milder FH phenotype, such as the p.G343S and p.R81C mutations. These receptor-defective mutations generally lead to only modest LDL-C elevations and were more prevalent in the FH-low than in the FH-high group.8

Conversely, the severe class 1 mutations where the LDLR protein is not synthesized at all were underrepresented in the FH-low group.

Some aspects of the present study merit discussion. For logistical reasons, there was a lag time between molecular screening and actual participation in the study. During that time, 142 (33%) of the 434 participants initiated statin treatment, often on the basis of the dyslipidemia communicated at the time of screening. Such treatment could, in theory, have biased carotid IMT comparisons among the study groups. Statin treatment has been shown to slow progression of atherosclerosis or even to result in regression in several carotid IMT studies in patients with FH.24–26 As a result, the measured carotid IMTs may have underestimated the atherosclerotic burden not only in the FH-high group, but also in the FH-low group. One could argue, however, that the impact on carotid IMT values of 10 months of statin treatment in 25 treated individuals in the FH-low group was modest because a subgroup analysis of all 229 participants who were untreated revealed no difference between the carotid IMT values in the FH-low versus the no-FH group.

In conclusion, the present findings show yet again that carotid atherosclerosis is increased in patients with molecularly proven FH with severely elevated LDL-C levels. Pharmaceutical management of these FH heterozygotes is urgently required. Evidently, genetic testing for FH within families remains an efficient manner to detect individuals at risk for cardiovascular disease. However, 1 consequence of genetic testing within families is that individuals who carry LDLR or APOB mutations but exhibit normal to slightly elevated LDL-C levels are identified. Those individuals are not necessarily at increased cardiovascular risk. Therefore, the present findings suggest that the risk of cardiovascular disease is mostly related to LDL-C levels and not to the presence of mutations per se. Consequently, this study provides the cautious suggestion that pharmaceutical intervention in individuals with an FH genotype who do not express hypercholesterolemia does not need to be as aggressive as is the standard in FH per se. Future longitudinal studies are needed to determine whether those subjects are indeed at similar cardiovascular risk as the general population. Until then, we recommend that these individuals are carefully monitored.

Acknowledgments
We thank the participants for their cooperation. In addition, we thank Judith Meester, Mia Muller, Dees Klappe, Johan Gort, Gaby van der Biezen, and Theo Postma for their excellent assistance.

Disclosures
Dr Kastelein has been involved in several pharmaceutical companies in the field of lipid lowering.

References
Patients with familial hypercholesterolemia (FH) generally are at severely increased risk of coronary artery disease. For that reason, genetic screening for FH within families is ongoing within several countries. Because of such genetic screening, a minority of FH mutation carriers are identified who lack a hypercholesterolemia phenotype. For clinicians, it is unknown whether the individuals from this subgroup should be treated because it is unknown whether those carriers have an atherosclerotic phenotype.

### CLINICAL PERSPECTIVE

Patients with familial hypercholesterolemia (FH) generally are at severely increased risk of coronary artery disease. For that reason, genetic screening for FH within families is ongoing within several countries. Because of such genetic screening, a minority of FH mutation carriers are identified who lack a hypercholesterolemia phenotype. For clinicians, it is unknown whether the individuals from this subgroup should be treated because it is unknown whether those carriers have an atherosclerotic phenotype.

In a recent study, patients with FH were classified into FH-high and FH-low groups based on the number of FH mutations. The FH-high group had a significantly higher carotid intima-media thickness than the FH-low group. In the FH-high group, the carotid intima-media thickness was significantly associated with the number of FH mutations. In contrast, in the FH-low group, the carotid intima-media thickness was not significantly associated with the number of FH mutations.

These findings suggest that the risk of cardiovascular disease in patients with FH is to a large extent related to low-density lipoprotein cholesterol levels and not to the presence of a mutation per se. Consequently, the present study cautiously suggests that individuals with an FH genotype without expression of hypercholesterolemia may not require a pharmaceutical intervention that is as aggressive as the standard for individuals with FH. Until then, we recommend that these individuals are carefully monitored.
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SUPPLEMENTAL MATERIAL

Supplemental figure 1: Distribution of carotid intima-media thickness and age

The groups were categorized based on genetic FH mutation status: one group where mutation was absent (No-FH, ○) and two groups of mutation carriers in whom the untreated LDL-cholesterol percentile was: either below 75\textsuperscript{th} (FH-low, △) or above 90\textsuperscript{th} percentile for age and gender (FH-high, ⊙). The unadjusted mean carotid IMT with standard deviation was 0.66 ± 0.12 mm in the No-FH group, and 0.62 ± 0.10 mm in the FH-low group, and 0.64 ± 0.13 mm in the FH-high group. The regression lines are depicted for each group separately.