Familial hypercholesterolemia: the Dutch approach

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Assessment of carotid atherosclerosis in normocholesterolemic individuals with proven mutations in the LDL-receptor or apolipoprotein B genes

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ABSTRACT

Background: Genetic cascade screening for heterozygous familial hypercholesterolemia (FH) revealed that fifteen percent 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: 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 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 the groups were assessed using multivariate linear regression analyses.

Results: Mean carotid IMT of 114 subjects in the FH-low group (0.623 mm 95%CI: 0.609 to 0.638 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 in 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 is to a large extent 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.
INTRODUCTION

Familial hypercholesterolemia (FH) is a condition that meets key criteria for genetic screening. 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 (CAD). In fact, cholesterol-lowering treatment has been shown to dramatically reduce CAD risk in patients with FH.5, 6

Nationwide genetic screening for FH has been ongoing in the Netherlands since 1994 and approximately 27,000 individuals with a molecular diagnosis of FH have been identified to date. In order to diagnose FH in these individuals, DNA samples from clinically suspected FH patients are analyzed for the presence of a mutation in the LDL receptor (LDLR) or apolipoprotein B (APOB) gene. A patient is then considered a proband for family screening when a pathogenic mutation is identified. 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, approximately 15% of the FH patients do not exhibit severely elevated LDL-C levels at diagnosis.6, 7 In current practice, mutation carriers, but 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 unaffected relatives. Here we present our results.

METHODS

Study population
In this single-center cross-sectional study, we recruited subjects from the database of the screening organization for FH in the Netherlands.6 This screening program was approved and financed 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 \textit{LDLR} or \textit{APOB} mutation and on their age and gender specific percentiles of LDL-C.\textsuperscript{8} The lipids were measured with the LDX-Analyser (Cholestech corporation, Hayward, USA) during blood withdrawal for the genetic test.\textsuperscript{9} LDL-C levels were subsequently estimated with the Friedewald formula.\textsuperscript{10} Age and sex specific percentiles of LDL-C were calculated using the reference values of a western population.\textsuperscript{11}

We identified three groups of subjects: those given a molecular diagnosis of FH and LDL-C levels at genetic screening below the 75\textsuperscript{th} percentile (FH-low), those with FH and an LDL-C level above the 90\textsuperscript{th} percentile (FH-high) and those without FH from families participating in the cascade screening program (No-FH).

The selected groups – i.e. FH-low, FH-high, and No-FH - were invited to participate in the current study through surface mail. We sent batches of 200 invitation letters until more than 240 mutation carriers and more than 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.

\textbf{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.

\textbf{Carotid Intima-Media Thickness}

Carotid ultrasound measurements of IMT were performed according to standardized and validated methodology as described in detail before.\textsuperscript{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 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 primary outcome measure.
Statistical Analysis

Assuming a standard deviation 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 two-sided alpha of 0.05. For comparison, the difference in carotid IMT between FH children and unaffected siblings reached 0.033 mm around the age of 14 years.\(^{13}\)

Differences in demographic and baseline characteristics between the three 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 equations (GEE)-method to account for correlations within families. The exchangeable correlation structure was used for these models. Subgroup analyses were performed in subjects that remained untreated until the study visit.

Variables with a skewed distribution were log-transformed before statistical analyses. A \(p\)-value < 0.05 was considered statistically significant. Data were analyzed with SPSS for Windows 16.0.2 (Chicago, IL, USA).

RESULTS

Study population

Among the screened population, 2,016 individuals met 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 421 subjects originated from 257 different families in which at least in one individual 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 one other participant in varying degrees.

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

<table>
<thead>
<tr>
<th></th>
<th>No-FH</th>
<th>FH-low</th>
<th>FH-high</th>
<th>No-FH vs FH-low</th>
<th>No-FH vs FH-high</th>
<th>FH-low vs FH-high</th>
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<tbody>
<tr>
<td></td>
<td>n=145</td>
<td>n=114</td>
<td>n=162</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>69 (48)</td>
<td>52 (46)</td>
<td>68 (42)</td>
<td>0.75</td>
<td>0.32</td>
<td>0.55</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42.3 ± 8.7</td>
<td>37.5 ± 8.5</td>
<td>35.2 ± 8.7</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.032</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>14 (10)</td>
<td>8 (7)</td>
<td>10 (6)</td>
<td>0.57</td>
<td>0.40</td>
<td>0.83</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>1 (1)</td>
<td>-</td>
<td>1 (1)</td>
<td>0.32</td>
<td>0.96</td>
<td>0.32</td>
</tr>
<tr>
<td>Smoker ever, n (%)</td>
<td>73 (50)</td>
<td>51 (45)</td>
<td>66 (41)</td>
<td>0.37</td>
<td>0.090</td>
<td>0.51</td>
</tr>
<tr>
<td>Statin use*, n (%)</td>
<td>5 (3)</td>
<td>25 (22)</td>
<td>111 (69)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.7 ± 4.2</td>
<td>25.6 ± 5.1</td>
<td>25.0 ± 4.4</td>
<td>0.93</td>
<td>0.16</td>
<td>0.28</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128 ± 14</td>
<td>124 ± 12</td>
<td>124 ± 13</td>
<td>0.005</td>
<td>0.003</td>
<td>0.92</td>
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### Lipid profile (mmol/L)

#### At molecular screening

<table>
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<tr>
<th></th>
<th>LDL-C</th>
<th>pLDL</th>
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<tbody>
<tr>
<td></td>
<td>3.2 ± 1.0</td>
<td>40 (21-68)</td>
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#### At study visit

<table>
<thead>
<tr>
<th></th>
<th>LDL-C</th>
<th>pLDL</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3.4 ± 1.0</td>
<td>3.4 ± 1.0</td>
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</table>

Continuous variables were depicted as mean ± standard deviation in case of normal distribution, and median (interquartile range) for pLDL and triglycerides because these variables had a skewed distribution. *Statin use initiated after genetic testing. Abbreviations: LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; pLDL, percentile LDL-C for age and gender.
Table 2 lists the distribution among the FH-low and FH-high groups of the 16 most prevalent LDLR mutations in the Netherlands and the p.R3527Q mutation in APOB. These 17 mutations accounted for 60% of all mutation carriers in the FH-low group and 70% of 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 present in the FH-high 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.

Table 2: Distribution of mutations carried by the participants

| Mutation                                      | Class | FH-low n (%) | | FH-high n (%) | |
|-----------------------------------------------|-------|--------------||----------------|---|
| p.R3527Q (APOB, exon 26)                      | 3     | 18           | | 34             | 21 |
| p.N564H exon 11/c.2393_2401del exon 17        | 2B    | 14           | | 26             | 16 |
| c.1359-1 (intron 9)                           | 1     | 1            | | 3              | 2  |
| c.313+1/2 (intron 3)                          | 1     | -            | | 3              | 2  |
| p.W44X (exon 2)                               | 1     | 1            | | 6              | 4  |
| p.S306L (exon 6)                              | 2B    | 3            | | 9              | 6  |
| p.E228K (exon 4)                              | 2B    | -            | | 1              | 1  |
| 2.5 kb deletion exon 7 en 8 (Cape Town-2)     | 1     | -            | | 1              | 1  |
| c.191-2 (intron 2)                            | 1     | -            | | 2              | 1  |
| p.G207G (exon 4)                              |      | 2            | | 7              | 4  |
| p.V429M (exon 9)                              | 5     | 1            | | -              | -   |
| p.G343S (exon 7)                              | 2B    | 8            | | 4              | 2  |
| p.A705P (exon 14)                             | 2B    | 2            | | 3              | 2  |
| p.R81C (exon 3)                               | 2B    | 8            | | 5              | 3  |
| p.A431T (exon 9)                              | 5     | 2            | | 3              | 2  |
| p.C255R (exon 5)                              | 2B    | 4            | | 1              | 1  |
| p.P699L (exon 14)                             | 2B    | 4            | | 5              | 3  |
| Other mutations                               | -     | 46*          | | 49†            | 30 |

Mutations are ordered based on prevalence in the Netherlands. Prevalence and nomenclature were described in: Huijgen et al Human Mutation 2010. 31 (6): 752-760. 29 different other mutations; 35 different other mutations. Mutation classes were described by Goldstein, Hobbs and Brown, in The metabolic and molecular bases of inherited disease 2001. 2863-2913.
Table 3: Mutation class distribution

| Mutation class | FH-low n (%) | FH-high n (%) | p  
|----------------|--------------|---------------|---
| 1              | 2 (2)        | 24 (15)       | <0.001  
| 2A             | 7 (6)        | 8 (5)         | 0.79  
| 2B             | 71 (62)      | 76 (47)       | 0.014  
| 3              | 26 (23)      | 45 (28)       | 0.40  
| 4              | 0 (0)        | 4 (3)         | 0.15  
| 5              | 3 (2)        | 3 (2)         | 0.69  
| Undetermined   | 5 (4)        | 2 (1)         | 0.13  

Mutation classes were described by Goldstein, Hobbs and Brown. Class 1: LDL-receptor (LDLR) is not synthesized at all; Class 2: LDLR is not properly transported from the endoplasmatic reticulum to the Golgi apparatus for expression on the cell surface: Blocked (2A) or delayed (2B) transport from the endoplasmic reticulum to the cell surface; Class 3: LDLR does not properly bind LDL-cholesterol on the cell surface because of a defect in either apolipoprotein B100 or in LDLR; Class 4: LDLR bound to LDL-cholesterol does not properly cluster in the clathrin-coated pits for receptor-mediated endocytosis. Class 5: LDLR is not recycled back to the cell surface.

Carotid Intima-Media Thickness

Carotid IMT is positively correlated with age (data not shown). Figure 1 exhibits mean carotid IMT values for the three subgroups, 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 that 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 as compared with the mean carotid IMT 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).
Figure 1: Mean carotid intima-media thickness for the three predefined groups

The groups were categorized based on genetic FH mutation status - group where mutation was absent (No-FH) - and for mutation carriers the untreated LDL-cholesterol percentile: either below 75th (FH-low) and above 90th percentile for age and gender (FH-high). Mean carotid intima-media thickness (IMT) was adjusted for age, sex, smoking, body mass index and systolic blood pressure.

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 FH patients solely identified by cascade screening, thus, are in essence free of referral bias. Nevertheless, several prior studies have assessed the contribution of LDL-C levels to intima-media thickening in cohorts of heterozygous FH patients and these studies have consistently shown that the higher the LDL-C levels were, the more carotid IMT was increased. 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 FH patients 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 that enabled us to also compare their atherosclerotic burden to that of unaffected relatives. Since 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, proprotein convertase subtilisin/kexin type 9 (PCSK9) and apolipoprotein E. In a recently performed analysis, our group tested 75 individuals with heterozygosity for a pathogenic LDLR or APOB mutation, who lacked the hypercholesterolemia phenotype, and showed that this lack of phenotype was in 5 cases (7%) due to concomitant other mutations with a hypocholesterolemic effect in APOB. In addition to genetic variation in other genes influencing LDL-C levels, lifestyle factors contribute to the expression of dyslipidemia in patients with FH. 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 our 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. The latter two mutations are both class 2B mutations (see Table 3), which implies that the mutated allele results in a LDLR protein that does not function as effective as that produced by the wild type allele. 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. 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 our study merit discussion. For logistical reasons, there is 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 between the study groups. Statin treatment has been shown to slow progression of atherosclerosis or even to result in regression in several carotid IMT studies with FH patients. As a result, the measured carotid IMTs may have underestimated the atherosclerotic burden foremost in the FH-high group, but also in the FH-low group. One could argue, however, that the impact of ten months of statin treatment in the 25 treated individuals on carotid IMT values 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, our findings show yet again that carotid atherosclerosis is increased in molecularly proven FH patients 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, one consequence of genetic testing within families is that individuals who carry \textit{LDLR} or \textit{APOB} mutations but exhibit normal to slightly elevated LDL-C levels are identified. Those individuals are not necessarily at increased cardiovascular risk. Therefore, our 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 familial hypercholesterolemia per se. Future longitudinal studies are needed to determine if those subjects are indeed at similar cardiovascular risk as the general population. Until then, we recommend that these individuals are carefully monitored.
REFERENCES


