Femoral Atherosclerosis In Heterozygous Familial Hypercholesterolemia
Influence Of The Genetic Defect

Mireia Junyent, Rosa Gilabert, Daniel Zambón, Miguel Pocoví, Miguel Mallén, Montserrat Cofán, Isabel Núñez, Fernando Civeira, Diego Tejedor, Emilio Ros

Objective—The purpose of this study was to assess femoral atherosclerosis by ultrasound in patients with molecularly defined heterozygous familial hypercholesterolemia (FH) in comparison with matched control subjects and in relation to mutational class in the LDL receptor and apolipoprotein B (APOB) genes.

Methods and Results—Femoral intima-media thickness (IMT) and plaque were evaluated in 146 FH patients carrying null alleles (n=48), defective-receptor alleles (n=62), undetermined-function alleles (n=25), or APOB defects (n=11) and in 193 healthy subjects. Twenty-three patients had coronary heart disease (CHD). The frequency of both tendon xanthisomas and CHD was ~2-fold higher and average LDL cholesterol was 30 mg/dL higher in null-allele genotype compared with receptor-defective mutations. All femoral measurements were increased in FH patients versus controls (P<0.001), and null-allele mutations showed higher age-, sex-, and LDL cholesterol-adjusted maximum IMT than receptor-defective or APOB defects (P for trend, 0.001). By multivariate analysis, independent associations of mean IMT, a measure of early atherosclerosis, were age, LDL cholesterol, sex, and systolic blood pressure. Age, null-allele genotype, sex, and smoking explained 42% of the variability of maximum IMT, a measure of advanced atherosclerosis.

Conclusions—FH patients have increased femoral IMT in relation to mutational class. The findings support the usefulness of genetic testing in FH beyond securing the diagnosis. (Arterioscler Thromb Vasc Biol. 2008;28:580-586)

Key Words: familial hypercholesterolemia ■ femoral atherosclerosis ■ intima-media thickness ■ atheroma plaque ■ low-density lipoprotein receptor mutations ■ apolipoprotein B mutations

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by lifelong elevation of low-density lipoprotein (LDL) cholesterol levels, tendon xanthomas, and early-onset coronary heart disease (CHD).1 The commonest underlying molecular defects are mutant alleles of the LDL receptor gene (LDLR) or of the gene encoding apolipoprotein (apo) B, the ligand for the LDLR in LDL particles.2 Recently, defects at a third locus causing monogenic hypercholesterolemia, protein convertase subtilisin/kexin type 9 (PCSK9), have been identified.3 Despite the use of stringent clinical criteria, only the detection of molecular defects provides an unequivocal diagnosis of FH.4

Heterozygosity for functional mutations of LDLR is the most frequent and best characterized cause of FH.1,5 More than 900 LDLR mutations have been reported to date (http://www.ucl.ac.uk/ldlr/Current), and specific defects with a differential effect on residual receptor function affect both the lipid phenotype and CHD risk.6,7 In contrast, only a small number of functional mutations have been identified in APOB, which are usually associated with a milder lipid phenotype.8 If the type of genetic defect influences the risk for CHD, it will also affect atherosclerosis in other arterial beds that can be evaluated by imaging techniques.

B-mode ultrasound is a noninvasive imaging technique useful in the assessment of atherosclerosis in large arteries, such as the carotid and femoral arteries.9 The pathogenic associations and predictive power of carotid intima-media thickness (IMT) have received much attention, and carotid IMT is an accepted surrogate marker for atherosclerotic disease.10 Although less investigated than carotid IMT, femoral IMT is also associated with cardiovascular risk factors in different populations9,11-13 and can serve as an intermediate phenotype for cardiovascular risk14,15 and a surrogate end point for the efficacy of intervention on the arterial wall.16 In a recent prospective study,17 femoral plaque, an advanced stage of increased IMT, had predictive value for future cardiovascular events.

There is a paucity of data on sonographic measurements of femoral atherosclerosis in FH. Femoral IMT was increased in...
FH patients when compared with healthy controls\textsuperscript{18,19} or to subjects with non-FH hypercholesterolemia,\textsuperscript{12,20} and was a better predictor of CHD severity than carotid IMT.\textsuperscript{15} Also, statin treatment influences femoral IMT to a greater extent than carotid IMT in FH.\textsuperscript{16} One study categorized femoral IMT by the type of \textit{LDLR} and \textit{APOB} defects and found no differences among groups with mutations of different severity.\textsuperscript{20} Plaque burden was not evaluated in this study. To gain more insight into the pathogenic significance of femoral atherosclerosis in FH and the relevance of genetic screening, we performed femoral sonography for evaluation of both IMT and plaque burden in 146 FH patients who were classified according to the severity of the molecular defect and compared the findings with those of a control population.

**Methods**

**Subjects**

From March 1998 to April 2006, we assessed 146 consecutive adults in whom a molecular diagnosis of FH was obtained. All patients had been referred by primary care physicians to the Lipid Clinic of Hospital Clinic in Barcelona for diagnosis of severe hypercholesterolemia or because of alleged refractoriness to treatment. Within 2 to 6 weeks of the first visit, subjects underwent sonographic assessment of femoral atherosclerosis according to a protocol approved by the local review board and provided informed consent.

Subjects with clinical FH are recruited into the Spanish FH Register and submitted to DNA testing for identification of \textit{LDLR} and \textit{APOB} mutations following a standard protocol.\textsuperscript{21} During the same period, we evaluated 193 healthy control subjects, who were recruited from hospital personnel and lists of primary health physicians and were matched for sex and age to FH patients.

**Clinical and Laboratory Characteristics**

All subjects were assessed for family history of early-onset CHD, clinical history, medication use, demographic characteristics, cardiovascular risk factors, and presence of tendon xanthomas. In asymptomatic subjects, fasting blood for baseline biochemical profiles was drawn after at least 4 weeks without hypolipidemic drug treatment. In patients with prior CHD, baseline lipid values were obtained from clinical records. For details in the procedures, please see the supplemental materials (available online at http://atvb.ahajournals.org).

**Molecular Testing**

\textit{LDLR} gene mutations and the presence of the R350Q mutation within the putative receptor-binding region of the \textit{APOB} gene were assessed in genomic DNA by standard methods. For details, please see the supplemental materials.

Functional \textit{LDLR} mutations may be classified into different types based on biosynthetic and functional studies of fibroblast cell strains.\textsuperscript{1} For the purpose of this study, we classified mutations into 3 groups: (1) null alleles, namely, disruptions of the promoter sequence, large rearrangements, nonsense, frameshift, or mutations resulting in a deletion of the translation initiation signal and early stop codons, which result in no protein synthesis; (2) receptor-defective alleles, that is, transcription and missense defects that do not completely suppress the function of the protein, which has residual receptor activity; and (3) undetermined receptor activity alleles, which are splicing defects with an unknown effect on protein function. \textit{APOB} mutations were analyzed separately of \textit{LDLR} defects because they are known to produce a less severe phenotype,\textsuperscript{22} which has also been shown in Spanish FH cohorts.\textsuperscript{23} The identified mutations are listed in supplemental Table I. A total of 60 different mutations were detected in 146 subjects, resulting in 48 null alleles, 62 receptor-defective alleles, 11 \textit{APOB} mutations, and 25 defects with undetermined residual function. We included 2 missense defects (C176 years and G528D) among null alleles because evidence were provided of equivalent and negligible residual receptor activity (http://www.ucl.ac.uk/ldlr/Current).

**Femoral Ultrasonography**

A standardized imaging protocol was used for the IMT measurements. The primary variable was mean common femoral IMT and secondary variables were maximum femoral IMT, plaque presence, and plaque score. For description of the technique, please see the supplemental materials.

**Clinical Features and Lipid Profiles**

Of the 146 FH subjects, 123 were asymptomatic and 23 had a history of CHD, confirmed in all cases by review of medical records (16 myocardial infarction and 7 angina). No patient had symptoms suggestive of peripheral arterial disease. Table 1 shows the clinical characteristics and lipid levels of the FH and control groups. FH subjects with CHD differed from asymptomatic FH subjects in that they were older, were predominantly male, and had a higher frequency of hypertension. Lipid profiles were similar between groups except for lower HDL cholesterol in patients with CHD. As expected from study design, FH subjects showed a higher number and potency of cardiovascular risk factors than healthy controls.

At the time of referral to our Lipid Clinic, 73 subjects were treatment-naïve and 73 subjects had been treated previously with lipid-lowering drugs, including all 23 patients with prior

### Table 1. Clinical Characteristics and Lipid Profiles in Patients With a Molecular Diagnosis of FH With and Without CHD and in Healthy Control Subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Asymptomatic FH (n=123)</th>
<th>FH With CHD (n=23)</th>
<th>Control Subjects (n=193)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (range)</td>
<td>40.7 (20–76)</td>
<td>50.7 (28–70)*</td>
<td>49.5 (25–80)</td>
</tr>
<tr>
<td>Men/women, n/n</td>
<td>56/67</td>
<td>18/5*</td>
<td>85/108</td>
</tr>
<tr>
<td>Family history of CHD, n (%)</td>
<td>76 (62)</td>
<td>12 (52)</td>
<td>19 (10)</td>
</tr>
<tr>
<td>Ever smoked, n (%)</td>
<td>38 (31)</td>
<td>12 (52)</td>
<td>54 (28)</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>24.7±3.7</td>
<td>25.8±3.3</td>
<td>25.2±3.7</td>
</tr>
<tr>
<td>Arterial hypertension, n (%)</td>
<td>9 (7)</td>
<td>7 (30)*</td>
<td>9 (5)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>0</td>
<td>1 (4)</td>
<td>0</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>387±84</td>
<td>379±68</td>
<td>202±29</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>305±83</td>
<td>300±74</td>
<td>128±24</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>55±15</td>
<td>49±15*</td>
<td>56±15</td>
</tr>
<tr>
<td>Cholesterol/HDL ratio</td>
<td>7.7±2.9</td>
<td>7.9±3.1</td>
<td>3.8±0.9</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>108 (71–154)</td>
<td>137 (95–181)</td>
<td>92 (64–112)</td>
</tr>
</tbody>
</table>

Values are mean±SD except for triglycerides (median and interquartile range). CHD indicates coronary artery disease. *P<0.05 vs asymptomatic FH.
Table 2. Clinical Characteristics and Lipid Profiles of FH Patients Depending on Mutational Class

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Null Alleles (n=48)</th>
<th>Defective Alleles (n=62)</th>
<th>Undetermined Alleles (n=25)</th>
<th>ApoB 3500 (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (range)</td>
<td>42 (21–76)</td>
<td>43 (20–67)</td>
<td>42 (21–64)</td>
<td>43 (25–63)</td>
</tr>
<tr>
<td>Men/women, n/n</td>
<td>23/25</td>
<td>33/29</td>
<td>12/13</td>
<td>5/6</td>
</tr>
<tr>
<td>Prior hyperlipidemic treatment, n (%)</td>
<td>22 (46)</td>
<td>32 (52)</td>
<td>15 (60)</td>
<td>4 (36)</td>
</tr>
<tr>
<td>Familial early-onset CHD, n (%)</td>
<td>26 (54)</td>
<td>40 (65)</td>
<td>18 (72)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>Prior CHD, n (%)</td>
<td>13 (27)</td>
<td>8 (13)</td>
<td>1 (4)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Ever smoked, n (%)</td>
<td>16 (36)</td>
<td>32 (52)</td>
<td>7 (28)</td>
<td>4 (36)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.6±3.5</td>
<td>25.2±3.9</td>
<td>23.8±3.2</td>
<td>24.5±3.3</td>
</tr>
<tr>
<td>Tendon xanthomas, n (%)</td>
<td>29 (66)</td>
<td>22 (35)</td>
<td>13 (52)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>128±19</td>
<td>123±11</td>
<td>122±14</td>
<td>122±19</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>77±13</td>
<td>76±8</td>
<td>75±10</td>
<td>75±10</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>89±8</td>
<td>92±11</td>
<td>88±13</td>
<td>91±12</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>404±86</td>
<td>379±78</td>
<td>389±89</td>
<td>349±67</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>326±84</td>
<td>296±79</td>
<td>309±85</td>
<td>263±61</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL‡</td>
<td>51±16</td>
<td>55±14</td>
<td>61±19</td>
<td>59±14‡</td>
</tr>
<tr>
<td>Total cholesterol/HDL ratio‡</td>
<td>8.6±3.3‡</td>
<td>7.4±2.6‡</td>
<td>6.9±2.3‡</td>
<td>6.6±3.4‡</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>116 (77–151)</td>
<td>118 (81–167)</td>
<td>100 (82–140)</td>
<td>95 (71–133)</td>
</tr>
<tr>
<td>Apolipoprotein B, g/L‡</td>
<td>2.13±0.58‡</td>
<td>1.95±0.44</td>
<td>1.94±0.47</td>
<td>1.66±0.48‡</td>
</tr>
<tr>
<td>Apolipoprotein A1, g/L</td>
<td>1.36±0.26</td>
<td>1.39±0.25</td>
<td>1.40±0.25</td>
<td>1.49±0.29</td>
</tr>
<tr>
<td>Lipoprotein(a), mg/dL</td>
<td>27 (13–54)</td>
<td>19 (7–53)</td>
<td>29 (11–74)</td>
<td>29 (13–47)</td>
</tr>
</tbody>
</table>

Values are means±SD except for triglycerides and lipoprotein(a) (medians and interquartile ranges). CHD indicates coronary heart disease.

*P<0.001 and †P<0.003 by chi-square tests. ‡P<0.05 by ANOVA statistics.
Values within a row with different superscript letters are significantly different (P<0.05, Bonferroni post-hoc test).

CHD (51 statins, 10 fibrates, 4 resins, and 8 combined treatments), for a median period of 36 months (range, 6 to 72 months). Those treated with statins had received an average daily dose with potency equivalent to 30 mg simvastatin (range, 10 to 80 mg). Such small average doses of statins indicate ineffective cholesterol-lowering treatment.

The clinical features of FH groups by mutational class are shown in Table 2. Subjects with the R3500Q APOB mutation showed the least frequency of xanthomas and the mildest lipid phenotype, but there were no significant differences compared with those with defective or undetermined alleles. The frequency of both prior CHD and Achilles tendon xanthomas was ~2-fold higher in subjects with null alleles than in those with defective alleles. The null-allele genotype showed a more severe lipid phenotype than the defective allele group, without significant differences. Subjects with undetermined alleles and APOB mutations had higher HDL cholesterol and lower cholesterol/HDL ratios than those with null alleles. Because of the unknown residual LDLR function in subjects with undetermined alleles, they were excluded from analyses in which the severity of the genetic defect was the variable under consideration.

 Associations of Femoral IMT and Plaque
Among both FH subjects and controls, highly significant correlations existed between mean IMT and age for either sex, with r-values ranging from 0.275 to 0.545 (P<0.001; all). Therefore, sonographic measurements of femoral atherosclerosis were adjusted for sex and age (Table 3). FH patients with or without CHD had higher IMT and plaque values than controls, and those with CHD had the highest values. There were also differences in adjusted mean IMT between smokers and nonsmokers (P=0.033). Adjusted mean IMT and median plaque score were 1.08 mm and 2.30 mm in patients treated with hypolipidemic drugs and 0.88 mm and 2.69 mm in those who were treatment-naïve, respectively (P>0.1; both).

Additional significant (P<0.001) correlates of mean IMT in FH were systolic (r=0.419) and diastolic blood pressure (r=0.288), glucose (r=0.310), total cholesterol (r=0.301), LDL cholesterol (r=0.283), apolipoprotein B (r=0.405), and the cholesterol/HDL ratio (r=0.281). For all these variables, the correlations were somewhat attenuated after IMT was corrected for age and sex (data not shown).

Table 3. Age- and Sex-Adjusted Femoral IMT and Plaque Scores in Patients With Molecularly Diagnosed FH and Healthy Subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Asymptomatic FH Controls</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>Mean IMT, mm</td>
<td>0.99±0.71</td>
</tr>
<tr>
<td>Maximum IMT, mm</td>
<td>1.50±1.02</td>
</tr>
<tr>
<td>Presence of plaques, n (%)</td>
<td>53 (43)</td>
</tr>
<tr>
<td>Plaque score, mm</td>
<td>1.1 (0.6–3.1)</td>
</tr>
</tbody>
</table>

Values are means±SD except for plaque score (median and interquartile ranges). CHD indicates coronary heart disease. †P<0.001 vs the 2 FH groups; *P<0.05 and †P<0.001 vs asymptomatic FH.
Predictors of Femoral IMT by Multivariate Analyses

After adjustment for various confounders, independent associations of mean IMT by stepwise multiple regression analysis in 121 FH patients (undetermined alleles excluded) were age, LDL cholesterol, systolic blood pressure, and gender (negative for women) in this order (adjusted \( R^2 = 0.311 \); Table 4). When maximum IMT was the dependent variable, the factors independently associated were age, presence of null allele, gender, and smoking, explaining together 42% of its variability. Finally, age was the sole independent factor associated with plaque score. When the apoB level was allowed into the model, this variable replaced LDL cholesterol as an association of mean IMT and slightly increased the overall information (adjusted \( R^2 = 0.347 \)). Also, apoB was associated with maximum IMT and smoking fell off the equation, with adjusted \( R^2 = 0.437 \). Finally, apoB was associated with plaque score and increased the information of the model (\( R^2 = 0.272 \)). Entering the cholesterol/HDL or ApoB/ApoAI ratios into the regression analysis added no more overall information. The results of multivariate analysis did not materially change when CHD patients were excluded.

Discussion

The major novel finding of this study is that patients with a molecular diagnosis of FH characterized by null allele (receptor-negative) mutations of the LDLR gene show more advanced femoral atherosclerosis than those with receptor-defective mutations or APOB defects, independently of gender, age, and untreated LDL cholesterol levels. Inasmuch as femoral atherosclerosis is an intermediate marker of CHD risk, \(^9\) \(14\) – \(16\) that has predictive value of future CHD events, \(^17\) these findings have implications for the diagnosis and management of FH. They also support the utility of obtaining a molecular diagnosis in patients with clinical FH.

Several studies in FH cohorts genetically heterogeneous like the present one have shown that LDLR-null allele variants are associated with more severe lipid phenotypes, increased prevalence of xanthomas, and higher CHD risk than receptor-defective mutations. \(^5\) \(7\) \(23\) – \(25\) Few investigations have dealt with the differential effect of LDLR mutational class on carotid IMT in sizeable FH cohorts. \(^20\) \(26\) \(27\) The studies of Descamps et al \(^20\) and Tonstad et al \(^26\) found a tendency to higher unadjusted carotid IMT in adult FH patients with null alleles compared with those with defective alleles, whereas the study of Koeijvoets et al \(^27\) with higher statistical power, found significant differences in mean carotid IMT between FH children carriers of null alleles and receptor-defective
Lipid Clinic with a severe lipid phenotype, but not of those still undiagnosed within their families or the general population, who may have milder lipid phenotypes and a lower risk for atherosclerosis.32 Another limitation is that the third genetic locus involved in causing the FH phenotype, PCSK9, was not screened for. However, PCSK9 mutations are a rare cause of FH in genetically heterogeneous European populations.5,33 Finally, our classification of mutations within the functional categories may be subjected to error when no studies of residual receptor function are available, as the prediction based on specific

<table>
<thead>
<tr>
<th>Table 4. Independent Associations of Femoral IMT by Stepwise Multiple Regression Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH With All Mutations Except Undetermined Alleles (n=121)</td>
</tr>
<tr>
<td>Dependent Variable</td>
</tr>
<tr>
<td>Mean IMT</td>
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<tr>
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<tr>
<td>Maximum IMT</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>Plaque score</td>
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</tbody>
</table>

Variables allowed to enter the model were age, gender, previous cholesterol-lowering treatment, tendon xanthomas, family history of early-onset CHD, smoking habit, body mass index, systolic and diastolic blood pressure, glucose, LDL cholesterol, HDL cholesterol, log triglycerides, log lipoprotein(a), and mutational class.

mutations, independently of the LDL cholesterol level. Descamps et al20 also evaluated allele-specific associations of mean femoral IMT and again found a tendency to higher IMT in null alleles, but only in women. We found a tendency to higher adjusted mean femoral IMT and a significantly higher maximum femoral IMT (namely, worse advanced atherosclerosis) in carriers of null alleles versus receptor-defective or APOB defects. Adjusted plaque scores, a measure of the overall atherosclerotic burden not previously investigated in FH, tended to be higher in null alleles. Multivariate analyses with inclusion of LDL cholesterol and apoB levels as confounders also showed that the null-allele genotype was independently associated with advanced femoral atherosclerosis, as assessed by maximum IMT. Although this finding should be confirmed in studies of larger FH cohorts with more statistical power, it suggests that factors beyond a higher load of atherogenic lipoprotein particles are implicated in atherogenesis in this population. Patients with null alleles had nonsignificantly lower HDL cholesterol than those with defective alleles, but the differences persisted after IMT was adjusted for this variable. Possibly lifelong, severely elevated LDL cholesterol levels trigger other self-sustaining atherogenic mechanisms, such as inflammation and immune activation, which begin early in life28 and are enhanced in patients with more severe clinical phenotypes.29,30 This is certainly an avenue of research worth pursuing.

The markedly increased femoral IMT and plaque burden observed in asymptomatic FH subjects in comparison to healthy controls is in line with the enhanced risk for CHD of the FH phenotype.1,6,7 In proof, FH patients with prior CHD had significantly higher IMT and plaque scores than asymptomatic ones (Table 3). As shown in Figure 1, the annual progression rate of mean femoral IMT in FH subjects with null and defective alleles was nearly 4-fold and 3-fold, respectively, that of sex- and age-matched control subjects. From the slopes of the regression lines of IMT versus age, it can be estimated that on average healthy controls reach an IMT of 1.0 mm at 65 years of age, whereas FH subjects with null alleles, defective alleles, and APOB defects attain this value at ages 30, 40, and 50 years, respectively. De Groot et al19 also reported that the annual progression rate of averaged carotid and femoral IMT in pooled FH subjects doubled that of healthy controls. As expected, age was a strong independent association of all measurements of femoral atherosclerosis at multivariate analysis. Interestingly, when the apoB level was allowed into the equations in lieu of the LDL cholesterol value it had a strong multivariate association with all femoral IMT measurements and increased the predictive value of each model. This supports the potential of apoB to provide an overall measure of all atherogenic particles. Smoking, a strong risk factor for femoral atherosclerosis,31 was weakly associated with maximum IMT but not with mean IMT or the plaque score. A likely reason is that there were few smokers among asymptomatic subjects, thus reducing statistical power. Predictably from the well known delay in onset of CHD in FH women compared with men,1,6 the female gender was independently and inversely associated with IMT.

Our study has the strengths of a cross-sectional design in a large series of molecularly defined FH patients not receiving effective hypolipidemic drug treatment that could have influenced IMT. A limitation of the study is that our cohort is representative of patients with FH as they present to a Lipid Clinic with a severe lipid phenotype, but not of those still undiagnosed within their families or the general population, who may have milder lipid phenotypes and a lower risk for atherosclerosis.32 Another limitation is that the third genetic locus involved in causing the FH phenotype, PCSK9, was not screened for. However, PCSK9 mutations are a rare cause of FH in genetically heterogeneous European populations.5,33 Finally, our classification of mutations within the functional categories may be subjected to error when no studies of residual receptor function are available, as the prediction based on specific
disruption of the LDLR protein does not necessarily follow a canonical rule.34 Still, the allocation of genetic defects to functional classes in supplemental Table I is updated to present knowledge (http://www.ucl.ac.uk/ldlr/Current).

Several groups have reported the clinical utility of molecular diagnosis in patients with FH.7,25,26,35 An important question is whether the genetic diagnosis of FH could help in the selection of patients at greater CHD risk among clinically indistinguishable patients, who therefore deserve more aggressive intervention for risk factor reduction. In agreement with previous data,27 our results suggest that selection of null alleles might identify FH subjects with the highest CHD risk who may then be considered for more aggressive cholesterol-lowering treatment.

In conclusion, FH patients have more femoral atherosclerosis than control subjects in strong association with the presence of null allele mutations. These findings support the usefulness of genetic testing in FH beyond securing the diagnosis.

Acknowledgments
We thank Emili Corbella for expert statistical advice.

Sources of Funding
This work was supported by grants from the Spanish Ministry of Health (RTIC G03/181, PI05/0075, PI05/0134, and PI06/0365) and Fundació Privada Catalana de Nutrició i Lípidos, Barcelona, Spain.

Disclosures
Diego Tejedor is employed by Progenika Biopharma, the company that commercializes the microarray (Lipochip) for genetic diagnosis of FH in Spain. None of the other authors has any conflict of interest.

References


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Arterioscler Thromb Vasc Biol. 2008;28:580-586; originally published online December 20, 2007;
doi: 10.1161/ATVBAHA.107.153841
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplementary material to:

FEMORAL ATHEROSCLEROSIS IN HETEROZYGOUS FAMILIAL HYPERCHOLESTEROLEMIA. INFLUENCE OF THE GENETIC DEFECT.

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METHODS

Clinical and laboratory characteristics. Cardiovascular risk factors and clinical chemistry variables were determined by standard methods. History of smoking was established if the subject reported smoking ever ≥3 cigarettes per day for at least 5 years. Weight and height were measured with calibrated scales and a wall-mounted stadiometer, respectively. Body mass index was calculated as weight in kilograms divided by the square of height in centimeters. Blood pressure was measured in duplicate with a validated semi-automatic oscillometer (Omron HEM-705CP). Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or current use of antihypertensive medication. Diabetes mellitus was defined as fasting glucose level ≥126 mg/dL or treatment with antidiabetic agents.

Blood glucose was measured by the glucose-oxidase method. Cholesterol and triglycerides were determined by standard enzymatic methods. HDL cholesterol was measured by a precipitation technique. LDL cholesterol was estimated with the Friedewald equation. ApoAI, apoB and lipoprotein(a) were determined by using immunoturbidimetry (Unimate 3, Roche, Basel, Switzerland).

Femoral ultrasonography. B-mode ultrasound imaging of the right and left femoral arteries was performed by using Powervision and Applio instruments (Toshiba, Nasu, Japan) equipped with 7-10 MHz broadband linear array transducers. Mean common femoral IMT was defined as the average of 4 to 8 distances between the far wall lumen–intima and media–adventitia ultrasound interfaces taken bilaterally in the 1-cm segment proximal to the bifurcation. Maximum IMT was defined as the highest distance in the same 1-cm segments at any of the two sites. Plaques were sought by using B-mode and color Doppler examinations in both longitudinal and transverse planes to take into consideration circumferential asymmetry and were defined as focal intrusions into the lumen ≥1.2 mm thick. A plaque score was constructed as the sum of maximum heights of all plaques present or maximum IMT when there were no plaques. IMT and plaque height were measured online with electronic calipers by two experienced sonographers (RG and IN). Interobserver variability was examined in 15 subjects. The CVs of paired readings of mean IMT, maximum IMT, and plaque score were 5.6%, 7.1%, and 9.5%, respectively.
Molecular testing
The methods used to screen genomic DNA for LDLR gene mutations and the R3500Q mutation in the APOB gene have been described previously.\textsuperscript{1,2} For assessing LDLR gene mutations, the promoter region, the translated exon sequences, the exon-intron boundaries, and short proximal intronic sequences of the LDLR gene were individually amplified by PCR, and subsequently analyzed by SSCP. Purified PCR products from DNA samples with abnormal SSCP patterns were directly sequenced. Mutation nomenclature followed present HGSV recommendations (http://www.hgvs.org). Nucleotide numbering was based on the cDNA sequence, with position +1 being the A of the ATG translation initiation codon and aminoacid numbering with position +1 being the translation initiator methionine. Large rearrangements in the LDLR gene were analyzed using a method based on quantitative fluorescent multiplex PCR.

Since early 2004, DNA was screened for LDLR and APOB gene defects by using a microrarray (Lipochip®, Progenika Biopharma, Derio, Spain) designed to detect the more prevalent mutations in the Spanish population, which is periodically updated as new mutations are identified.\textsuperscript{3} When no mutations were detected, the LDLR gene was sequenced as described above to search for mutations not included in the microarray design.

Statistical analyses
Data are presented as means (±SD) for continuous variables (medians and interquartile ranges for variables with a skewed distribution) and as frequencies or percentages for categorical variables. Differences in mean values were assessed using analyses of variance and t-tests. Categorical variables were compared using chi-square tests. Pearson correlation coefficients were constructed to test for relationships between continuous variables. Because predictably IMT was strongly related to age and showed sex differences, age and sex-adjusted IMT values were used when examining associations with other variables. ANOVA statistics were used to test adjusted femoral measurements depending on the type of mutation, using post-hoc Bonferroni testing for assessment of group differences. We used stepwise multiple regression analyses to test the associations of various cofactors with IMT and plaque scores. Two-sided P<0.05 was considered significant. SPSS software (version 12.0) was used.

REFERENCES
<table>
<thead>
<tr>
<th>Location</th>
<th>cDNA</th>
<th>Mutation name</th>
<th>Usual Name</th>
<th>Type</th>
<th>Previously described</th>
<th>n</th>
</tr>
</thead>
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**Null alleles**

- exon 1: c.1-?_67+?del4kb del4Kb rearrangement No 3
- exon 2: c.910T>G p.Glu333X Q12X nonsense Yes 2
- exon 3: c.214delG p.Asp727fsX132 211delG frameshift Yes 1
- exon 4: c.304C>T p.Gln102X Q81X nonsense Yes 1
- exon 4: c.480C>T p.Gln154X Q133X nonsense Yes 1
- exon 4: c.518delG p.Cys173SerfsX31 518delG frameshift Yes 2
- exon 4: c.596G>A p.Cys197Tyr C176Y missense Yes 1
- exon 5: c.730AT p.Arg240HisX19 732delAT frameshift No 1
- exon 6: c.884delT p.Val295IlefsX73 884delAT frameshift Yes 1
- exon 7: c.1045delC p.Gln349SerfsX19 1045delC frameshift Yes 14
- exon 7: c.1045_1046insA p.Gln349ThrfsX7 1045insA frameshift No 1
- exon 7: c.1054_1060+4del11 small rearrangement Yes 1
- exon 8: c.1176C>A p.Cys392X C371X nonsense Yes 3
- exon 9: c.1200C>A p.Tyr400X Y379X nonsense Yes 1
- exon 14: c.2085del19 p.Cys696SerfsX6 2085del19 frameshift Yes 1
- exon 15: c.2183delG p.Arg728SerfsX1 2183delG frameshift Yes 2
- exon 15: c.2307_2299insT p.Val736ValfsX44 2270insT frameshift Yes 1

**Receptor-defective mutations**

- c.1942C>T (+49) C/T transcription Yes 4
- c.1365G>T (+42) C/G transcription Yes 1
- c.284G>A p.Cys95X C74X missense No 3
- c.301G>A p.Glu101Lys E80K missense Yes 1
- c.464G>A p.Cys155X C134X missense No 1
- c.530C>T p.Tyr177Leu S156L missense Yes 8
- c.664G>T p.Cys218X C196R missense Yes 2
- c.654_666delTGG p.Gly186_Gly219del in-frame deletion No 1
- c.689G>T p.Glu233X E216X missense Yes 1
- c.705G>T p.Ala235X A217X missense No 7
- c.930A>G p.Asp310X D291X missense No 1
- c.1070G>T p.Glu323X G207X missense No 2
- c.1136G>A p.Cys379X C358X missense Yes 1
- c.1310G>A p.Thr434X T413X missense Yes 1
- c.1641T>C p.Leu547Pro L534P missense Yes 3
- c.1800G>C p.Glu600Stop E597X missense Yes 1
- c.1907T>C p.Phe636Leu F614L missense Yes 2
- c.2054T>C p.Pro685Leu P664L missense Yes 1
- c.2375T>C p.Ile792Thr I771T missense No 2
- c.2470G>T p.Asp823Stop N801X missense Yes 1
- c.274C>T[+1](313-10+C) p.[Glu92(+)17] Q76+13+10=C missense + splicing Yes 6
- c.1690A>C[+1](233_240insA) p.[Asp564His(+)11] N543H + 233_240insA frameshift + missense Yes 2
- c.850T>C[+1](931-30+C) p.[Ser283Pro(+)9] S262P + 931-30+C frameshift Yes 1
- c.850T>C[+1](1104delC) p.[Gly288Asp(+)5] 1045delC frameshift + missense Yes 1

**Splicing mutations. Undetermined receptor activity**

- c.896-3G>C splicing Yes 3
- c.1188+5G>A splicing No 1
- c.1338+10A>G splicing Yes 2
- c.1706-100G>A splicing Yes 1
- c.1845+10C>G splicing Yes 4
- c.2140+5G>A splicing Yes 2
- c.2311+7G>T splicing No 2
- c.2312-47G>A splicing No 3
- c.2389+8C>T splicing No 1
- c.2390-10C>G splicing Yes 6

**APOB 3500 mutations**

- p.Arg527Gln R527Q missense Yes 11

**Total**

146