Familial hypercholesterolemia: the Dutch approach

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Chapter 10

Plasma Levels of Proprotein Convertase Subtilisin Kexin type 9 (PCSK9) and Phenotypic Variability in Familial Hypercholesterolemia

Roeland Huijgen, Sigrid W. Fouchier, Michael Denoun, Barbara A. Hutten, Maud N. Vissers, Gilles Lambert and John J.P. Kastelein

Chapter 10

ABSTRACT

Background: The extent of hypercholesterolemia varies considerably in patients with familial hypercholesterolemia (FH). We hypothesized that the variability of the FH phenotype might be partly explained by variation in proprotein convertase subtilisin kexin type 9 (PCSK9) activity.

Methods: Individuals between 18 and 53 years of age who had been tested for a pathogenic LDLR or APOB mutation were eligible. Mutation carriers with a LDL-C level below the 75th percentile were selected (‘FH-low’), as well as those with LDL-C above the 90th percentile (‘FH-high’). Relatives who tested negative for the mutation were the ‘Controls’.

Results: PCSK9 plasma levels were assessed in 267 individuals who did not receive cholesterol-lowering treatment at the time of the study. Mean PCSK9 plasma levels (95%CI) were lower in the FH-low group compared with the FH-high group (152 [137 to 167] ng/ml vs. 186 [165 to 207] ng/ml, p=0.010) and the Control group (177 [164 to 190] ng/ml, p=0.013). Mean PCSK9 levels did not statistically differ between the FH-high and Control group (p=0.50).

Conclusions: Plasma PCSK9 levels are positively associated with LDL-C levels in FH patients and might contribute to the phenotypic severity in this disorder. Therefore, the results of pharmaceutical inhibition of PCSK9 in FH patients are eagerly awaited.
INTRODUCTION

Familial hypercholesterolemia (FH, MIM #143890) is a frequent autosomal co-dominant disorder of lipoprotein metabolism. It is clinically characterized by elevated levels of total (TC) and low-density lipoprotein cholesterol (LDL-C) levels, the presence of tendon xanthomas and premature atherosclerosis. Defects in genes that code for proteins involved in the hepatic clearance of low-density lipoprotein (LDL) cholesterol (LDL-C) underlie this hereditary disorder. In fact, more than a 1000 different mutations in the genes coding for the LDL-receptor (LDLR, MIM +606945), apolipoprotein B (APOB, MIM +107730) and proprotein convertase subtilisin/kexin type 9 (PCSK9, MIM +607786) are now known to cause FH.1-3 If left untreated, the risk of cardiovascular disease is severely increased,4 but the prognosis of FH can be improved substantially with cholesterol-lowering treatment.5

The identification of a mutation that underlies FH in a particular kindred enables genetic testing of family members for the presence of the same mutation and makes it possible to initiate effective medical management before the cardiovascular consequences of FH become clinically manifest.4 This notion has led to the implementation of a nationwide genetic cascade screening program for FH in the Netherlands and since 1994, approximately 27,000 subjects with FH have been found and treated.6

However, molecularly diagnosed FH patients not always exhibit a hypercholesterolemic phenotype. In fact, fifteen percent of the heterozygous mutation carriers, identified by our national screening program, show pre-treatment LDL-C levels below the 75th percentile for age and gender.7 The reasons as to why some individuals with a confirmed FH genotype lack the hypercholesterolemia phenotype are largely unknown.

We hypothesized that such non-penetrance of an FH mutation could, in part, be explained by variation in PCSK9 activity. PCSK9 is a natural inhibitor of the LDLR: it binds to the hepatic LDLR and thereby directs it towards lysosomal degradation rather than to recycling to the cell membrane.8, 9 The presence of a specific gain-of-function mutation in PCSK9 aggravates the hypercholesterolemia phenotype exerted by a concurrent pathogenic LDLR mutation.10, 11 Conversely, low activity of PCSK9 could lead to increased presence of LDLR at the hepatic cell surface and consequently to increased clearance of plasma LDL-C. This would theoretically reduce the extent of cholesterol elevation caused by an LDLR mutation.

To test this hypothesis, we measured plasma PCSK9 levels in individuals who underwent DNA testing for genetic FH.12
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METHODS

Study population and design
The study population derived from participants of a previous single centre cross-sectional study, described in detail elsewhere. In short, we recruited individuals from the database of the national screening program for autosomal dominant hypercholesterolemia. Men and women between 18 and 55 years of age were eligible if they met the following criteria: genetically tested for the specific pathogenic LDLR or APOB mutation residing within their family between January 2007 and January 2010 and a known lipid profile. Subjects were excluded if they were unable to participate within 18 months after the genetic test. Individuals using cholesterol-lowering medication before genetic testing and probands, who were primarily clinically diagnosed, were excluded. Subjects of whom we failed to obtain plasma, required for PCSK9 measurements, were also excluded.

Individuals who had been identified with a pathogenic mutation were categorized based on their untreated LDL-C level at genetic diagnosis. Mutation carriers with a LDL-C level below the age and sex specific 75th percentile were selected and referred to as 'FH-low', those with LDL-C above the 90th percentile were referred to as 'FH-high'. A third group, consisting of first degree relatives negative for the familial LDLR or APOB mutation, was referred to as 'controls'.

The selected individuals who consented, made a single study visit to the Academic Medical Center in Amsterdam within 18 months after the genetic test. The study was approved by the local Ethics Committee.

Biochemical analyses and cIMT assessment
Blood samples were obtained for analysis of lipid measures and spare plasma between 8 AM and 10 AM, after an overnight fast. These samples were collected in 7 ml EDTA Vacutainer® (BD Vacutainer Systems, K3E 15% 0.084 ml, Plymouth, UK) venous blood collection tubes using standard phlebotomy practices. Immediately after collection, tubes were gently inverted 5 times, and centrifuged at 1500 – 2000g for 15 min and the supernatant plasma was centrifuged again in similar fashion. The plasma was transferred into 2 ml freezer vials, in 0.5 ml aliquots. The samples were frozen at -80°C and shipped on dry ice. PCSK9 concentrations were measured in triplicate using the CY-8079 ELISA kit (Cyclex, Nagano, Japan), according to the manufacturer’s protocol.
The medical history was recorded and physical examination performed according to a standardized procedure. Carotid arteries were examined with ultrasound to assess intima-media thickness (cIMT), using methodology as described in detail before.

**Statistical Analysis**

Differences in demographic and clinical characteristics between the three predefined groups (FH-low, FH-high, control) were evaluated using linear or logistic regression analysis. Linear regression analysis was applied to evaluate the association between PCSK9 and patient characteristics, LDL-C or cIMT and to assess differences in plasma PCSK9 levels between the three predefined groups. Multivariable regression models were applied to adjust for potential confounders. Inclusion in a final model was determined by backward stepwise 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. The main study outcome pertains to the subjects that remained untreated until the study visit. For transparency, we also analyzed the entire population of participants, including the patients who initiated statin treatment after genetic diagnosis.

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 for the original study. 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 the original study. Among these 421 subjects, 378 individuals were included for the sub-analysis of PCSK9 levels: 13 individuals without FH were excluded because they were older than 53 years and for 30 subjects we did not have spare plasma to measure PCSK9 in. The median period between genetic testing was 11 (IQR: 8 to 14) months.

As expected, carriers were more often treated with statins after diagnosis than non-carriers, and those from the FH-high group had initiated statin treatment more often than the individuals from the FH-low group (data not shown). In total 267 individuals (71%) were still untreated at the time of the study visit. Clinical
characteristics of the untreated participants, subdivided into the three groups, are summarized in Table 1. LDL-C levels were comparable between the individuals from the FH-low and control groups, whereas levels were significantly higher in the FH-high group. Accordingly, the mean IMT of the three segments of the left and right carotid arteries was greater in the FH-high group than in the FH-low group and the control group.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>FH low</th>
<th>FH high</th>
<th>vs. low</th>
<th>vs. high</th>
<th>vs. high</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male gender n (%)</strong></td>
<td>54 (48)</td>
<td>37 (39)</td>
<td>25 (40)</td>
<td>0.20</td>
<td>0.36</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>Age years</strong></td>
<td>40.7 ± 8.1</td>
<td>36.7 ± 8.4</td>
<td>33.9 ± 8.6</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.054</td>
</tr>
<tr>
<td><strong>Hypertension n (%)</strong></td>
<td>10 (9)</td>
<td>5 (5)</td>
<td>4 (7)</td>
<td>0.33</td>
<td>0.63</td>
<td>0.72</td>
</tr>
<tr>
<td><strong>Diabetes n (%)</strong></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td></td>
<td>0.17</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Current smoker n (%)</strong></td>
<td>23 (21)</td>
<td>16 (17)</td>
<td>18 (30)</td>
<td>0.52</td>
<td>0.19</td>
<td>0.066</td>
</tr>
<tr>
<td><strong>Body mass index kg/m²</strong></td>
<td>25.6 ± 4.1</td>
<td>25.6 ± 5.3</td>
<td>24.3 ± 4.8</td>
<td>0.95</td>
<td>0.098</td>
<td>0.097</td>
</tr>
<tr>
<td><strong>Systolic blood pressure mmHg</strong></td>
<td>128 ± 15</td>
<td>124 ± 13</td>
<td>122 ± 14</td>
<td>0.038</td>
<td>0.014</td>
<td>0.054</td>
</tr>
<tr>
<td><strong>Lipid profile mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLDL (IQR)</td>
<td>40 (19-65)</td>
<td>45 (20-63)</td>
<td>97 (95-98)</td>
<td>0.89</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>At study visit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>5.3 ± 1.1</td>
<td>5.4 ± 1.1</td>
<td>7.1 ± 1.2</td>
<td>0.24</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C</td>
<td>3.3 ± 0.9</td>
<td>3.5 ± 1.0</td>
<td>5.3 ± 1.1</td>
<td>0.11</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.5 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>0.65</td>
<td>0.85</td>
<td>0.55</td>
</tr>
<tr>
<td>Triglycerides (IQR)</td>
<td>0.9 (0.6-1.4)</td>
<td>0.7 (0.5-1.1)</td>
<td>0.7 (0.5-1.1)</td>
<td>0.024</td>
<td>0.012</td>
<td>0.69</td>
</tr>
<tr>
<td>Mean cIMT* (SE) mm</td>
<td>0.63 ± 0.008</td>
<td>0.62 ± 0.009</td>
<td>0.67 ± 0.013</td>
<td>0.38</td>
<td>0.009</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, body mass index, systolic blood pressure and smoking. Abbreviations: cIMT, carotid intima-media thickness; IQR, Borders of quartiles; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; pLDL, percentile LDL-C for age and gender; SE, standard error; TC, total cholesterol.

Plasma PCSK9 levels in the predefined groups and the association with LDL-C

Because of the substantial effect of statin treatment on PCSK9 levels, our main study outcome was based on the untreated individuals. Subjects from the FH-low group (N=94) had significantly lower mean PCSK9 levels 152 (137 to 167) ng/ml than the untreated individuals from the other two groups, i.e compared with FH-high (N=61); 186 (165 to 207) ng/ml, p=0.010, and compared with controls (N=112) 177 (164 to 190) ng/ml, p=0.013 (Figure 1). PCSK9 levels are associated with several patient characteristics which were not equally represented among the three predefined
PCSK9 plasma levels and variable FH phenotype

groups. Therefore, we adjusted for these characteristics, i.e. age, sex, body mass index and systolic blood pressure, by means of a multiple linear regression analysis.

On treatment, mean PCSK9 levels (95%CI) were significantly lower for the 16 subjects from the FH-low group as compared to the 92 FH patients from the FH-high group (167 (135-199) ng/ml vs. 219 (201-238) ng/ml, \( p = 0.006 \)). For the entire cohort of both treated and untreated subjects, PCSK9 plasma levels were yet again significantly lower in the FH-low group compared to the FH-high (\( p = 0.001 \)) and No FH group (\( p = 0.004 \)), and levels did not statistically differ between the FH-high and control group (\( p = 0.52 \)): Mean PCSK9 plasma levels (95%CI) were 183 (169 to 197) ng/ml, 154 (141 to 168) ng/ml and 204 (189 to 219) ng/ml for the Non-FH, FH-low and FH-high groups, respectively.

Figure 1: Mean PCSK9 levels in the three groups of untreated subjects

![Graph showing mean PCSK9 levels in three groups](image)

The groups were categorized based on genetic FH mutation status and LDL-cholesterol level: The group where mutation was absent (controls) and for FH heterozygotes, the untreated LDL-cholesterol percentile: either below the 75th (FH low) or above the 90th percentile for age and gender (FH high). The presented data were based on a model adjusted for age, sex, body mass index and systolic blood pressure.

We also associated plasma PCSK9 levels with LDL-C levels at the study visit for the 155 untreated individuals with genetic FH. The multivariable analysis revealed that the mean percentile LDL-C for each mutation and PCSK9 levels were the only two variables that remained independently associated with LDL-C levels after backward elimination (Table 2). A separate analysis for association between PCSK9 and LDL-C plasma levels between the different mutation classes was performed. However, this did not lead to additional insight, due to little numbers of subjects in most mutation classes (data not shown). Thus, low LDL-C levels in untreated
individuals with genetic FH were primarily observed in those who carried a \textit{LDLR} or \textit{APOB} gene mutation that is generally associated with mild hypercholesterolemia, and/or in those who had low plasma levels of PCSK9.

\textbf{Table 2:} Association between LDL-C levels at the study visit and clinical characteristics in the 155 untreated subjects with genetic FH

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
<th>Multivariable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>95%CI</td>
</tr>
<tr>
<td>Age years</td>
<td>0.006</td>
<td>-0.019 to 0.031</td>
</tr>
<tr>
<td>Male gender</td>
<td>-0.013</td>
<td>-0.448 to 0.421</td>
</tr>
<tr>
<td>Body mass index kg/m(^2)</td>
<td>-0.008</td>
<td>-0.049 to 0.034</td>
</tr>
<tr>
<td>Mean pLDL specific mutation*</td>
<td>0.045</td>
<td>0.033 to 0.056</td>
</tr>
<tr>
<td>PCSK9 pg/mL</td>
<td>4.56</td>
<td>1.93 to 7.19</td>
</tr>
</tbody>
</table>

Abbreviations: 95\%CI: 95\% confidence interval; B: unstandardized coefficient of regression model.
*Average percentile LDL-C for age and sex observed for all untreated individuals carrying a specific FH or FDB mutation, to estimate the severity of that specific mutation, as described in detail before.\textsuperscript{13}

\textbf{Association between plasma PCSK9 levels and cIMT in non-FH subjects}

\textbf{Table 3} depicts the association between mean cIMT and clinical characteristics in the 112 untreated individuals without FH. In the multivariable regression analysis, PCSK9 levels remained statistically significant associated with cIMT after backward elimination. In contrast, the individual components of the lipid profile did not remain statistically significant associated in the multivariable model. Thus, plasma PCSK9 levels were positively associated with cIMT even after adjustment for the lipid profile and other traditional cardiovascular risk factors.
Table 3: Association between carotid intima-media thickness and clinical characteristics for untreated controls

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
<th></th>
<th></th>
<th>Multivariable</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B 95% CI</td>
<td>( p )</td>
<td>B 95% CI</td>
<td>( p )</td>
<td></td>
</tr>
<tr>
<td>Age years</td>
<td>0.008</td>
<td>0.006 to 0.010</td>
<td>&lt;0.001</td>
<td>0.007</td>
<td>0.005 to 0.009</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.057</td>
<td>0.014 to 0.100</td>
<td>0.010</td>
<td>0.062</td>
<td>0.027 to 0.098</td>
<td>0.001</td>
</tr>
<tr>
<td>Body mass index kg/m²</td>
<td>0.099</td>
<td>0.004 to 0.014</td>
<td>0.001</td>
<td>0.005</td>
<td>0.000 to 0.009</td>
<td>0.039</td>
</tr>
<tr>
<td>Systolic blood pressure mmHg</td>
<td>0.003</td>
<td>0.001 to 0.004</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tobacco use pack years</td>
<td>0.003</td>
<td>0.001 to 0.005</td>
<td>0.008</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDL-cholesterol mmol/L</td>
<td>0.044</td>
<td>0.022 to 0.067</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HDL-cholesterol mmol/L</td>
<td>-0.026</td>
<td>-0.120 to -0.008</td>
<td>0.026</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triglycerides*</td>
<td>0.043</td>
<td>0.003 to 0.082</td>
<td>0.036</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCSK9 pg/mL</td>
<td>0.46</td>
<td>0.17 to 0.75</td>
<td>0.002</td>
<td>0.28</td>
<td>0.034 – 0.53</td>
<td>0.026</td>
</tr>
</tbody>
</table>

*Log transformed before analysis. Abbreviations: 95% CI: 95% confidence interval; B: unstandardized coefficient of regression model.

DISCUSSION

In the present study, PCSK9 levels were measured in a cross-sectional study of individuals who had genetic FH, with or without severely elevated LDL-C levels, and controls. Our findings demonstrate that PCSK9 levels were significantly lower in normcholesterolemic FH patients than in the two other groups. Moreover, PCSK9 levels were closely associated with LDL-C levels across all groups. Consequently, a reasonable assumption would be that low plasma PCSK9 activity might lead to lower LDL-C levels in heterozygous FH.

To our knowledge, this study is the first to compare plasma levels of PCSK9 between FH patients with and FH patients without severely elevated LDL-C levels. However, several groups have reported on the effect of genetic variation in the PCSK9 gene on the phenotype of FH. Abifadel and colleagues showed that individuals who co-inherited pathogenic mutations in both PCSK9 and LDLR had higher LDL-C levels than their relatives with either mutation alone. Conversely, Strom and colleagues studied the effect of a loss-of-function PCSK9 mutation, R46L, in FH. They screened 1130 FH patients and identified the R46L mutation in 30 individuals, who had a 6% lower total cholesterol levels than those without. These results of the association between genetic variation in PCSK9 and LDL-C levels support our findings, since we observed that FH patients with low levels of PCSK9 also have low LDL-C levels.

A crucial question is what causes this variation in PCSK9 levels. Loss-of-function mutations in PCSK9 might be a likely explanation. We recently genotyped PCSK9...
in a cohort of 77 heterozygous FH patients who were selected for low LDL-C levels. Just like Strom and colleagues,\textsuperscript{15} we found the R46L variant in \textit{PCSK9} in one (1.3\%) of those patients.\textsuperscript{16} Thus, genetic variation in PCSK9 does contribute to a variable FH phenotype, but the explained percentage remains disappointingly low.\textsuperscript{10, 15} In addition to genetic variation, other factors may also affect plasma levels of PCSK9, of which again only a fraction has been identified.\textsuperscript{17}

We also showed that high PCSK9 levels are associated with more pronounced carotid atherosclerosis, apparently independent of lipid levels. Moreover, we recently showed that plasma levels of PCSK9 were positively associated with recurrent coronary event in patients with stable coronary heart disease treated with a low dose atorvastatin in a nested case control study in the Treating to New Targets trial.\textsuperscript{18} These findings combined with the fact that decreased PCSK9 activity is associated with lower LDL-C levels and a reduced risk of coronary heart disease,\textsuperscript{19} supports the inhibition of PCSK9 as a target of great significance. In fact, several agents are already being investigated in humans at different stages of development.\textsuperscript{20-25}

Several limitations of our study merit discussion. First, this is an observational study and therefore a causal relationship between low plasma PCSK9 levels and lack of a hypercholesterolemia phenotype cannot be proved. Second, a substantial number of participants initiated statin treatment between genetic FH diagnosis and study visit. Because statin treatment results in increased PCSK9 levels, this hinders the interpretation of our findings.\textsuperscript{26-28} Nevertheless, we could demonstrate that PCSK9 levels were lower in FH patients with low LDL-C levels than in those with hypercholesterolemia, both in treated and untreated individuals. This supports the notion that the differences in plasma PCSK9 levels between groups are not solely due to the effect of statin treatment. Last, our cohort of FH patients consisted of carriers of a myriad of pathogenic \textit{LDLR} and \textit{APOB} mutations. As a consequence we were unable to perform meaningful statistics on the effect of specific type of \textit{LDLR} mutations. The PCSK9 plasma levels remained a predictor of plasma LDL-C levels, however, after adjustment for the mean percentile of LDL-C, as induced by specific mutations. Thus, the severity of the FH mutation cannot be the only explanation for the association that we observed between plasma PCSK9 levels and LDL-C levels. In line, plasma PCSK9 levels were observed to be strongly and positively associated with LDL-C levels in a cohort of 260 non-treated FH heterozygotes from South Africa carrying one single \textit{LDLR} mutation (Lambert et al., manuscript in preparation).

In conclusion, plasma PCSK9 levels likely contribute to low LDL-C levels in FH heterozygotes. Therefore, the results of pharmaceutical inhibition of PCSK9 in FH patients are eagerly awaited.
References


