Healthy Individuals Carrying the PCSK9 p.R46L Variant and Familial Hypercholesterolemia Patients Carrying PCSK9 p.D374Y Exhibit Lower Plasma Concentrations of PCSK9

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BACKGROUND: We measured plasma PCSK9 concentrations in healthy men with a PCSK9 (proprotein convertase subtilisin/kexin type 9) loss-of-function variant (p.R46L), in statin-treated patients with a clinical diagnosis of familial hypercholesterolemia (FH) and carrying a PCSK9 gain-of-function mutation (p.D374Y), and in statin-treated patients with FH due to different genetic causes.

METHODS: PCSK9 was measured with a previously described ELISA.

RESULTS: In 81 healthy middle-aged Caucasian men, the PCSK9 concentration was significantly associated with the concentrations of total cholesterol (r = 0.42; P < 0.0001), LDL cholesterol (r = 0.34; P = 0.01), and triglycerides (r = 0.25; P = 0.02). In p.R46L carriers, mean (SD) concentrations of PCSK9 were 15% lower than in RR individuals [65.5 μg/L (21.6 μg/L) vs 77.5 μg/L (18.2 μg/L); P = 0.03]. In patients with the p.D374Y variant (n = 7), the mean PCSK9 concentration was significantly lower than in the combined group of patients with an LDLR (low density lipoprotein receptor) mutation (n = 25), an APOB (apolipoprotein B (including Ag(x) antigen)) variant encoding p.R3527Q (n = 6), or no detectable mutation (n = 14) [96.4 μg/L (42.5 μg/L) vs 151.6 μg/L (69.6 μg/L); P = 0.02]. Two of the 14 patients with no mutation had PCSK9 concentrations below the mean for p.D374Y carriers; sequencing of the PCSK9 gene and promoter revealed no mutations. Among 409 FH patients, we identified 6 carriers of the promoter variant −287G>A (1.5%), a frequency similar to that (1.0%) previously reported for 2772 healthy men in the UK. In neither group was the −287G>A variant associated with differences in lipid traits.

CONCLUSIONS: The loss-of-function p.R46L variant is associated with the expected lower concentrations of circulating PCSK9; the gain-of-function p.D374Y mutation is also associated with lower concentrations, presumably because of the higher affinity of this variant for the LDL receptor and its more rapid clearance. In treated FH patients, a low plasma PCSK9 concentration does not appear to be a useful screening tool for identifying novel PCSK9 mutations.

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The PCSK9 gene (proprotein convertase subtilisin/kexin type 9) encodes an enzyme that is involved in regulating the degradation of the LDL receptor protein in the lysosome of the cell, preventing it from being recycled to the cell surface (1, 2). PCSK9 is synthesized as an inactive proprotein, which then undergoes autocatalytic cleavage in the endoplasmic reticulum to produce an enzyme with the prodomain noncovalently bound to the catalytic site, preventing further enzyme action. When PCSK9 is secreted into the blood, mostly from the liver, it binds to the LDL receptor, thereby directing the receptor to the lysosome for degradation rather than being recycled to the cell membrane (3). PCSK9 may also bind to LDL receptor molecules intracellularly and target them for degradation (4). Research has identified loss-of-function PCSK9 mutations that inactivate the enzyme via several different mechanisms and lead to less degradation of the LDL.
receptor and thus higher receptor levels on the cell surface (5). The most common of these variants is caused by a c.137G>T change in exon 1 that leads to the replacement of an Arg residue by a Leu residue at position 46 (p.R46L). This variant enhances the clearance of LDL cholesterol (LDL-C) from the plasma, thereby lowering the LDL-C concentration and the risk of coronary heart disease (CHD) (6–8). In the UK, approximately 3% of individuals in the general population are carriers of p.R46L (9).

By contrast, gain-of-function mutations in PCSK9 that increase activity increase LDL receptor degradation, reducing the numbers of receptors on cell surfaces (1, 2, 10). Hence, autosomal dominant hypercholesterolemia (ADH) is clinically indistinguishable from classic familial hypercholesterolemia (FH) (11). The only common PCSK9 variant in the UK is p.D374Y, which occurs in about 2% of patients with a clinical diagnosis of FH (12). This variant is associated with particularly high pretreatment LDL-C concentrations and a high risk of developing premature CHD, compared with FH patients with a mutation in the LDLR gene (low density lipoprotein receptor) (13). Interestingly, the PCSK9 promoter contains sterol-responsive elements (14), and cellular and plasma PCSK9 concentrations increase after statin treatment (15, 16). To some extent, this effect of statin therapy on the PCSK9 concentration will counter statin-induced LDL receptor production and reduce the lipid-lowering effect. This countering effect is likely to be particularly striking in FH patients who carry a gain-of-function PCSK9 variant such as p.D374Y and probably explains why the LDL-C concentration responds poorly to statin treatment in these patients (12, 13).

In patients with the strongest clinical suspicion of ADH/FH, particularly those with tendinous xanthomas, a causative mutation can be found in 70%–80% of cases (12, 17). Most commonly, the mutation is in the LDLR gene, but mutations have also been found in the APOB [apolipoprotein B (including Ag(x) antigen)] and PCSK9 genes (18). The molecular cause still has not been identified in the remaining patients, however, leading to speculations that additional genetic loci must be involved (18). We therefore hypothesized that measurement of plasma PCSK9 in this “no-mutation detected” FH patient group might identify individuals for whom further molecular testing of the PCSK9 gene might be useful. A recent report (19) described 2 PCSK9 promoter variants in ADH patients (c.−288G>A and c.−332C>A) and presented evidence that the c.−332A allele increased promoter strength such that higher plasma PCSK9 protein concentrations would be expected to cause the hypercholesterolemia; however, plasma PCSK9 concentrations were not reported. Several methods have recently been developed for rapid and accurate measurement of PCSK9 concentrations in plasma samples (16, 20–22). As expected, the plasma PCSK9 concentration is strongly correlated with the LDL-C concentration in healthy individuals (20–22). The aim of the present study was to measure PCSK9 concentrations in healthy carriers and noncarriers of the p.R46L variant and in statin-treated patients with FH caused by mutations in the PCSK9 gene (p.D374Y). We also intended to compare these results with PCSK9 concentrations in similarly treated FH patients with mutations in the LDLR or APOB gene (specifically p.R3527Q, previously known as R3500Q), as well as in a group of patients for whom no molecular cause has been identified.

Materials and Methods

STUDY PARTICIPANTS

We selected 81 healthy individuals from the Second Northwick Park Heart Study (NPHS-II) according to their previously determined PCSK9 p.R46L genotype (9). Forty individuals were homozygous for the p.R46 allele (genotype RR), and 41 were carriers of the p.R46L allele (genotype RL). The characteristics and study design of the NPHS have previously been described (23, 24). In brief, at baseline all of the participants were free of a history of unstable angina, myocardial infarction, coronary surgery, anticoagulant therapy (including aspirin), malignancy, cardiovascular disease, and any other condition precluding informed consent. Ethics approval was granted by the local institutional review committee, and patients provided written informed consent. Participants were in a nonfasting state when they were examined and had avoided smoking, vigorous exercise, and heavy meals from midnight before the examination. A 5-mL sample of venous blood was drawn by the Vacutainer technique (Becton Dickinson) into citrated tubes, and routine lipid profiles were obtained with standard methods, as previously described (24). The LDL-C concentration was calculated according to the Friedewald formula. PCSK9 was measured in samples that had been stored at −80 °C under identical conditions for approximately 15 years. Four hundred nine patients with clinically-defined definite FH (12, 25) were recruited for the Simon Broome British Heart Foundation study, which is a cross-sectional comparison of Caucasian patients ≥18 years of age with treated heterozygous FH, with and without clinically documented CHD (25). Recruitment meth-
MEASUREMENT OF PLASMA PCSK9 PROTEIN

PCSK9 concentrations in plasma samples were measured with a recently described PCSK9 dual-monoclonal antibody sandwich ELISA (16, 21). In brief, wells were coated overnight with anti-PCSK9 capture monoclonal antibody (1 g/L horseradish peroxidase–labeled anti-PCSK9 monoclonal antibody diluted with 1000 volumes of 0.67 mol/L phosphoric acid, and the plates were then aspirated and washed 3 times with Tris-buffered Saline (TBS) (Tris-buffered Saline) (Pierce; Thermo Fisher Scientific). Next, 100 μL of recombinant non–His-tagged PCSK9 calibrators (varying concentrations of recombinant protein in assay buffer) were added to the wells to produce a calibration curve. Plasma samples were then diluted with 20 volumes of assay buffer, added to their respective wells in the ELISA plate, and incubated for 2 h at room temperature. The wells were then aspirated and washed 3 times with assay buffer, and 100 μL of diluted conjugate antibody (1 g/L horseradish peroxidase–labeled anti-PCSK9 monoclonal antibody diluted to 1000 volumes of assay buffer) was added to the wells and incubated for 1 h with Blocker Casein in TBS (Tris-buffered Saline) (Pierce; Thermo Fisher Scientific). After the last TBS aspiration, we added 100 μL of 3,3',5,5'-tetramethylbenzidine development substrate (TMB Substrate Solution; Pierce; Thermo Fisher Scientific) to the wells and incubated the wells for 30 min at room temperature. The reaction was stopped with an equal volume of 0.67 mol/L phosphoric acid, and the plates were read at 450 nm. SigmaPlot software (version 8.0; Systat Software) was used to fit the calibration curves.

MOLECULAR GENETIC ANALYSIS

Standard methods were used to isolate genomic DNA from samples of whole blood (12). Genotyping for exon 1 c.137G>T (p.R46L), exon 9 c.1420A>G (p.L474V), and exon 12 c.2009A>G (p.V661G) variants were carried out with PCR and RFLP methods and conditions as previously described (9). The molecular methods used to screen for mutations in the LDLR gene and to test for specific mutations in APOB (p.R3527Q) and PCSK9 (p.D374Y) have previously been described (12). The LDLR mutations present were c.–191C>A, c.58G>C (p.G20R), c.6deG (n = 3), c.118delA (p.J40fs), del ex2–18, c.301G>T (p.E101K; n = 2), c.666G>A (p.C89Y; n = 3), c.513delC, c.662A>G (p.D221G), del ex5, c.1029C>T (p.C313X), c.1048C>T (p.R350X), c.1150C>T/1158C>G (p.Q384X/D386E; n = 3), c.1436T>C (p.L479P), c.1567G>A (p.V523M), c.1436T>C (p.L479P), dup ex11–12, c.2043C>A (p.C681X), and c.2054C>T (p.P685L).

A high-resolution melting (HRM) method was used to screen the PCSK9 promoter (c.–250 to c.–437) for any detectable sequence changes (26, 27). In brief, the following primers were used to amplify the region: 5’-CCCTCTCCGGGTAATCTGAC-3’ and 5’-GGCTCAGACCTGAACCTGAA-3’. PCR and HRM analyses were conducted in the Rotor-Gene™ 6000 5-Plex (Corbett Life Science/Qiagen). The PCR conditions were 95 °C for 2 min and 45 cycles of 95 °C for 10 s and 57 °C for 90 s; the PCR was followed by HRM from 85 °C to 95 °C. Samples with a pattern suggestive of a base change were sequenced with either the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on the ABI 377 DNA Sequencer (Applied Biosystems) or with the DYEnamic™ ET Dye Terminator Kit (Amersham/GE Healthcare Life Sciences) on the MegaBACE 1000 capillary sequencer (GE Healthcare Life Sciences). Genotyping for the PCSK9 c.–287G>A promoter variant was carried out with the same primers as for the HRM analysis. The 20-μL PCR reaction contained the following: 50 mmol/L KCl; 10 mmol/L Tris-HCl, pH 8.3; 10 mg/L gelatin; 4.0 mmol/L MgCl2; 0.2 mmol/L each of dATP, dGTP, dTTP, and dCTP; 8 pmol of each primer; 0.4 U Taq polymerase (Invtrogen); and 30 ng genomic DNA. The PCR protocol consisted of 94 °C for 3 min; 34 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min; and 16 °C for 3 min. Restriction enzyme digestion was carried out with 3 U AatII (New England Biolabs) per digest for 4 h at 37 °C. AatII does not cut wild-type sequence (GG), which yields a band size of 227 bp, whereas the enzyme cuts heterozygote sequence (GA) to produce band sizes of 227 bp, 174 bp, and 53 bp. The TT homoyzgote produces band sizes of 174 bp and 53 bp. Products were separated on 75 g/L polyacrylamide (19:1, acrylamide:bisacrylamide) microplate-array diagonal-gel electrophoresis gel (28) and visualized by staining with ethidium bromide. All gels included a control status. Any discrepancies were reanalyzed by repeat PCR and digestion.

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STATISTICAL ANALYSIS

All statistical analyses were carried out with STATA software (Intercooled Stata 9.2; Stata Corporation). Spearman rank correlation analysis was used to assess the association with lipids. PCSK9 concentrations were log-transformed to normalize their distribution. Concentrations were compared by genotype or mutation group with ANOVA, or with analysis of covariance if adjustment was made for lipids. Results are presented as the geometric mean and approximate SD. Throughout, a $P$ value $\leq 0.05$ was considered to indicate statistical significance. In the comparison of PCSK9 concentrations in NPHS-II men of different p.I474V and p.E670G genotypes, not all individuals had these data available because of random dropout caused by the RFLP method used [described in (9)].

Results

HEALTHY MEN IN NPHS-II

Table 1 shows mean (SD) values for baseline characteristics, including lipid concentrations, for NPHS-II men according to PCSK9 p.R46L genotype. As previously reported (9), p.R46L carriers had significantly lower mean values for total cholesterol, apolipoprotein B (apo B), and body mass index, but other traits were not significantly different. Mean PCSK9 concentrations were 15% lower in p.R46L carriers than in RR men ($P = 0.03$); Fig. 1 shows a scattergram of the data. The PCSK9 concentration was not significantly associated with p.E670G genotype [EE (n = 60), 72.6 $\mu$g/L (26.2 $\mu$g/L); EG (n = 9), 72.8 $\mu$g/L (21.0 $\mu$g/L); $P = 0.99$] or with p.I474V genotype [II (n = 40), 68.8 $\mu$g/L (25.8 $\mu$g/L); IV + VV (n = 31), 73.6 $\mu$g/L (26.1 $\mu$g/L); $P = 0.45$].

Table 2 shows the correlation of PCSK9 concentration with measured plasma lipid traits in the healthy individuals. As expected, PCSK9 concentration was significantly correlated with the plasma concentration of total cholesterol and the calculated LDL-C concentration. A weaker correlation was noted with apo B

<table>
<thead>
<tr>
<th>Variable</th>
<th>RR (n = 40)</th>
<th>RL (n = 41)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>56.0 (3.6)</td>
<td>56.1 (3.6)</td>
<td>0.88</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>142.5 (18.6)</td>
<td>143.7 (22.0)</td>
<td>0.80</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>86.3 (11.6)</td>
<td>86.1 (12.9)</td>
<td>0.92</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
<td>27.0 (3.6)</td>
<td>25.2 (2.9)</td>
<td>0.01</td>
</tr>
<tr>
<td>Smoking, % (n)</td>
<td>20.0 (8)</td>
<td>36.6 (15)</td>
<td>0.10</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.82 (0.99)</td>
<td>5.27 (1.04)</td>
<td>0.02</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.92 (1.12)</td>
<td>1.86 (0.97)</td>
<td>0.79</td>
</tr>
<tr>
<td>apo A, g/L</td>
<td>1.62 (0.31)</td>
<td>1.58 (0.36)</td>
<td>0.68</td>
</tr>
<tr>
<td>apo B, g/L</td>
<td>0.92 (0.25)</td>
<td>0.75 (0.27)</td>
<td>0.01</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>0.79 (0.23)</td>
<td>0.85 (0.20)</td>
<td>0.28</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>4.07 (0.86)</td>
<td>3.62 (0.97)</td>
<td>0.08</td>
</tr>
<tr>
<td>PCSK9, g/L</td>
<td>77.5 (18.2)</td>
<td>65.5 (21.6)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$^a$ SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index.
$^b$ Data are expressed as the geometric mean for log-transformed data (approximate SD).

Fig. 1. PCSK9 concentrations in healthy individuals and FH patients.

(A), PCSK9 concentrations in healthy individuals by R46L genotype. (B), PCSK9 concentrations in FH patients with no identified mutation and with different molecular causes. Geometric mean (approximate SD): None (n = 14), 151.6 $\mu$g/L (69.6 $\mu$g/L); LDLR mutation (n = 25), 143.2 $\mu$g/L (63.1 $\mu$g/L); APOB mutation (n = 6), 147.5 $\mu$g/L (64.1 $\mu$g/L); PCSK9 mutation (n = 7), 96.4 $\mu$g/L (42.5 $\mu$g/L); overall $P = 0.16$. PCSK9 concentrations are significantly lower in patients with the PCSK9 p.D374Y variant than in the other 3 groups combined ($P = 0.02$, Mann–Whitney U-test).
concentration. For none of these correlations was there significant evidence of a heterogeneity of effect between RR with RL individuals (data not shown). There was also a statistically significant positive correlation with plasma triglycerides. There was no association with age, blood pressure, or body mass index, however, and there was no difference between smokers and non-smokers (P = 0.88; data not shown).

Table 3 shows lipid concentrations at baseline and during treatment, statin-treatment data, and PCSK9 concentrations by variant type in the FH patients. As previously reported (12), untreated individuals and patients on treatment who had the PCSK9 p.D374Y variant had significantly higher total cholesterol and LDL-C concentrations than the other FH patients, although there were no significant differences between patients with different molecular defects in the proportions of the patients who were on statin treatment or with respect to the durations of treatment. Overall, PCSK9 concentrations were 1.94-fold higher in the FH patients than in the healthy individuals (P = 0.0001), an effect that remained after adjusting for the plasma concentration of total cholesterol (P = 0.0001). PCSK9 concentrations in individuals for whom no molecular cause had been identified were not significantly differ-

## Table 2. Correlation of plasma PCSK9 concentration with baseline characteristics and lipid concentrations in NPHS-II men.

<table>
<thead>
<tr>
<th>Variable</th>
<th>NPHS-II men (n = 81)</th>
<th>FH patients (n = 72)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>Age</td>
<td>0.08</td>
<td>0.47</td>
</tr>
<tr>
<td>SBP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.76</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.01</td>
<td>0.90</td>
</tr>
<tr>
<td>Total cholesterol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.34</td>
<td>0.01</td>
</tr>
<tr>
<td>HDL</td>
<td>0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>apo A-I</td>
<td>0.12</td>
<td>0.34</td>
</tr>
<tr>
<td>apo B&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.21</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<sup>a</sup> SBP, systolic blood pressure; BMI, body mass index.

<sup>b</sup> Not significantly different by p.R46L genotype (P = 0.49): RR men, r = 0.29 (P = 0.07); RL men, r = 0.49 (P = 0.001).

<sup>c</sup> Not significantly different by p.R46L genotype (P = 0.75).

<sup>d</sup> Not significantly different by p.R46L genotype (P = 0.29).

## Table 3. Pretreatment and posttreatment lipid concentrations, statin use, and PCSK9 concentrations by mutation type in FH patients with different molecular causes.

<table>
<thead>
<tr>
<th>Trait</th>
<th>None (n = 14)</th>
<th>LDLR mutation (n = 25)</th>
<th>APOB p.R3527Q (n = 6)</th>
<th>PCSK9 p.D374Y (n = 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals on statin therapy, % (n)</td>
<td>92.9 (13)</td>
<td>80.0 (20)</td>
<td>100.0 (6)</td>
<td>100.0 (7)</td>
<td>0.50</td>
</tr>
<tr>
<td>Duration of statin therapy, years&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.1 (1.4)</td>
<td>4.0 (2.5)</td>
<td>5.1 (4.4)</td>
<td>6.3 (3.0)</td>
<td>0.13</td>
</tr>
<tr>
<td>Pretreatment cholesterol, mmol/L&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.05 (1.46)</td>
<td>10.17 (2.15)</td>
<td>8.67 (1.09)</td>
<td>13.12 (1.69)</td>
<td>0.007</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.88 (1.05)</td>
<td>7.23 (1.40)</td>
<td>6.39 (0.86)</td>
<td>8.54 (2.04)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Percent change in cholesterol (95% CI)</td>
<td>−35.1 (−42.5 to −26.8)</td>
<td>−29.1 (−36.5 to −20.9)</td>
<td>−30.4 (−41.8 to −16.8)</td>
<td>−41.3 (−57.3 to −19.4)</td>
<td>0.36</td>
</tr>
<tr>
<td>LDL-C, mmol/L&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.56 (0.92)</td>
<td>5.40 (1.59)</td>
<td>4.44 (0.93)</td>
<td>6.77 (1.82)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PCSK9, μg/L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>151.6 (69.6)</td>
<td>143.2 (63.1)</td>
<td>147.5 (64.1)</td>
<td>96.4 (42.5)</td>
<td>0.16</td>
</tr>
<tr>
<td>Adjusted PCSK9, μg/L&lt;sup&gt;e&lt;/sup&gt;</td>
<td>160.2 (71.7)</td>
<td>138.0 (61.9)</td>
<td>149.4 (59.8)</td>
<td>87.1 (36.2)</td>
<td>0.11</td>
</tr>
<tr>
<td>PCSK9, μg/L&lt;sup&gt;f&lt;/sup&gt;</td>
<td>163.2 (123–219)</td>
<td>138.5 (105–229)</td>
<td>137.3 (101–205)</td>
<td>83.6 (79.6–153)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are presented as the mean (SD).

<sup>b</sup> Data are presented as the geometric mean (approximate SD). Sample sizes: None, n = 11; LDLR, n = 20; APOB, n = 4; PCSK9, n = 4.

<sup>c</sup> Data are presented as the geometric mean (approximate SD).

<sup>d</sup> PCSK9 concentrations are significantly lower in individuals with the PCSK9 p.D374Y mutation than in the other 3 groups combined (P = 0.02, Mann–Whitney U-test).

<sup>e</sup> Adjusted PCSK9 concentrations are significantly lower in individuals with the PCSK9 p.D374Y mutation than in the other groups combined (87.1 μg/L [36.2 μg/L] vs 146.1 μg/L [64.0 μg/L]; P = 0.02).

<sup>f</sup> Data are presented as the median (interquartile range).
ent from the concentrations in individuals with a defined \( \text{LDLR} \) or \( \text{APOB} \) mutation; however, PCSK9 concentrations in individuals with the \( \text{PCSK9} \) p.D374Y variant were significantly lower than in the other FH groups combined \( (P = 0.02) \). This difference was maintained after adjustment for on-treatment LDL-C concentration \( [87.1 \mu g/L (36.2 \mu g/L)] \) vs \( 146.1 \mu g/L (64.0 \mu g/L); P = 0.02 \). The results of these comparisons are presented graphically in Fig. 1B.

The correlations between PCSK9 concentration and concurrently measured plasma lipid concentrations in the FH patients are presented in Table 2. Surprisingly, there were no significant correlations with total cholesterol, LDL-C, or apo B concentration, although the significant correlation with plasma triglyceride concentration observed in the healthy individuals was also seen in this group. When we analyzed PCSK9 concentrations in patients with an \( \text{LDLR} \) mutation (adjusted for LDL-C concentration and mutation group), there was no evidence of a correlation with the LDL-C concentration in the mutation-positive group \( (n = 25; r = -0.04; P = 0.86) \), whereas the expected positive correlation was observed in the other FH patients combined \( (n = 27; r = 0.36; P = 0.08) \).

The data in Fig. 1B indicate that several individuals with FH and no identified molecular cause for their disease had PCSK9 concentrations as low as the individuals with the p.D374Y variant. All individuals in this group had previously been screened for mutations in the \( \text{PCSK9} \) coding region and at the intron–exon junctions \( (12) \), and no mutations had been identified. A recent report indicated, however, that mutations in the promoter of this gene may also cause hypercholesterolemia \( (19) \). Therefore, we used an HRM method to screen the promoter region between c.\(-250\) and c.\(-437\) for sequence changes in all 52 FH patients, and we measured the PCSK9 concentrations. Fig. 2 presents an example of the HRM plot; each run included a DNA sample from a carrier of the c.\(-287\)A variant. No variants were identified in any of the 14 individuals with no mutations; however, 1 patient who carried an \( \text{LDLR} \) mutation (p.C60Y) was a carrier for a previously reported nonfunctional variant \( \text{c.}-287\text{G} \Rightarrow \text{A} \) \( (19) \).

To take this analysis further, we used the HRM method to screen the \( \text{PCSK9} \) promoter in all 409 individuals in the FH patient group. No novel variants were identified, but 6 patients carried the c.\(-287\text{G} \Rightarrow \text{A} \) variant (all of whom also had an \( \text{LDLR} \) mutation; frequency of carriers, 1.5%). All of these changes were confirmed by sequencing (data not shown). To determine whether this variant also occurred in non-FH individuals, we genotyped 2775 of the NPHS-II men for this variant and found 56 carriers and 1 homozygous individual (rare allele frequency, 1.0%). The mean (SD) LDL-C concentration in these 57 individuals was not different from that for noncarriers \( [2.99 \text{mmol/L} (1.02 \text{mmol/L})] \) vs \( 3.10 \text{mmol/L} (1.01 \text{mmol/L}; P = 0.46) \). See Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue12.

**Discussion**

The availability of an ELISA assay for measuring plasma PCSK9 concentration has allowed us to address 3 questions.

**PLASMA PCSK9 AND LIPID CONCENTRATIONS IN HEALTHY INDIVIDUALS AND FH PATIENTS**

Healthy men showed the expected strong correlation between plasma PCSK9 concentration and the concentrations of total cholesterol and LDL-C, along with a nonsignificant correlation with apo B concentration. Similar values have previously been reported for healthy men \( (21) \). At recruitment, any individual taking lipid-lowering medication was excluded \( (23) \), so statin use does not confound this correlation. Interestingly, a statistically significant, albeit weak, correlation was observed with plasma triglyceride concentration, a result that has also recently been reported \( (22) \). PCSK9 has been reported to also interact with the VLDL and apo E receptors \( (29) \), so this protein may be having an effect on triglyceride concentrations by influencing the recycling of these receptors. All of the NPHS-II participants were male, so we cannot comment on the sex differences in PCSK9 concentration that have been reported \( (20, 22) \).

By contrast, the FH patients, who were all being treated with high doses of statins \( (24) \), had, as ex-
ected, overall PCSK9 concentrations that were 2-fold higher, but we observed no correlation between the PCSK9 concentration and the on-treatment total cholesterol or LDL-C concentration, although we did confirm the correlation with the triglyceride concentration previously observed in healthy men and found it to be of a similar magnitude. Although this lack of correlation may seem unexpected, a small clinical trial of 40 mg atorvastatin per day in individuals with high LDL-C concentrations recently found that PCSK9 concentration was correlated with LDL-C concentration before but not after treatment (16). The investigators proposed that the statin treatment had caused a sterol-regulatory element binding protein–mediated increase in PCSK9 of 34% but a decrease in LDL-C concentration (via up-regulation of the LDL receptors). Statin treatment thus had broken down the expected metabolic relationship between PCSK9 and LDL-C concentrations.

**PLASMA PCSK9 CONCENTRATIONS AND PCSK9 GENOTYPE**

In the healthy men, PCSK9 concentrations were 15% lower in p.46L carriers than in RR individuals. This result agrees with the results reported for a family carrying the p.R46L variant (20) and with a recent US study of 23 p.R46L carriers and 843 noncarriers (23) in which PCSK9 concentrations in carriers were 22% lower. Taken together with the data we have presented, these results convincingly demonstrate that the lower LDL-C concentrations seen in p.R46L carriers [11% in NPHS-II (10)] can largely be attributed to the lower plasma concentration of the protein. The mechanism of the p.R46L effect has been partially elucidated (30). Experiments with hepatoma cells have demonstrated that the p.R46L variant has a greater susceptibility to proteolysis, possibly due to decreased phosphorylation of the serine residue at position 47, although the p.A53V variant, which is not associated with a difference in plasma lipid concentrations, also caused reduced phosphorylation in this study. Bicarae studies, however, have demonstrated that the PCSK9 p.46L variant has a 50% lower affinity for the LDL receptor than PCSK9 p.46R (31), suggesting that a decreased affinity is the main mechanism of the effect. This lower affinity is unlikely to hinder the uptake of PCSK9 p.46R secreted in heterozygous individuals. If this is the case, there would be more available LDL receptor sites for uptake of the wild-type PCSK9 protein. We can rule out assay or antibody-binding complications because both antibodies used in the sandwich-format ELISA recognize regions that are far removed from the p.R46L mutation site. Although our study was not sufficiently powered to detect a small effect, it is worth noting that we found no evidence of differences in the plasma PCSK9 concentrations in carriers of the 2 other common missense variants, p.E670G and p.I474V, confirming previously reported results (22).

In the FH patients carrying the p.D374Y gain-of-function mutation, PCSK9 concentrations were 40% lower than in the patients with other molecular causes of FH; the latter groups of patients (e.g., those with an LDLR mutation or the APOB p.3527Q variant) had similar PCSK9 concentrations. One of the limitations of this study is that because these patients were not recruited for a clinical trial, we do not have any data on the doses or type of the statins being used for these individual patients. In addition, pretreatment concentrations were missing for some patients because treatment had commenced before recruitment. The treating physicians were unaware of their patients’ genotypes (which were determined some years after recruitment), however, so it is reasonable to assume that the increased lipid concentrations in these different groups of FH patients were at least being treated similarly and that the patients most likely were receiving higher statin doses in light of their higher risk for CHD (12, 13). The PCSK9 p.374Y carriers experienced the greatest decrease (41%) in the on-treatment concentration of total cholesterol, but the mean LDL-C concentration, at 6.77 mmol/L, remained among the highest concentrations. Because statins increase the PCSK9 concentration (16), as we discussed above, the particularly striking lower mean PCSK9 concentration observed for PCSK9 p.374Y carriers strongly suggests that the lower PCSK9 concentrations seen in this group are due to the patients’ genotype and not to confounding by lower statin doses. These data are in agreement with the mechanism proposed for the effect of this mutation, which has been shown to increase the strength of the interaction with the LDL receptor (32). This mutation would increase the binding of plasma PCSK9 and enhance its clearance and thus the PCSK9-mediated degradation of the LDL receptor. This mechanism may also lead to intracellular binding of PCSK9 to the LDL receptor and thus decrease PCSK9 secretion from the liver (33). Both effects—the increased binding of PCSK9 in the plasma and its decreased secretion—would lower the plasma concentration of PCSK9 protein.

**PCSK9 CONCENTRATIONS IN NO-MUTATION FH PATIENTS**

Although the most common PCSK9 mutation in UK patients is that encoding the p.D374Y variant (12), at least 12 other missense mutations causing high LDL-C concentrations have been reported to date (2). In addition, mutations in the gene promoter that cause increased transcription (especially if they influence the sterol responsiveness of the promoter) may also be im-
portant, and one such mutation has been reported (19). We hypothesized that one way to identify such individuals for molecular analysis would be to select those with plasma PCSK9 concentrations that are either particularly high or, as exemplified by p.D374Y, particularly low compared with their cholesterol concentrations. All 409 FH patients had previously been screened for mutations in the PCSK9 coding region and intron–exon junctions (12), and none had been identified. When we examined the promoter region of the gene in the 409 FH patients, we identified 6 patients (1.5%) as carriers of the previously reported c.–287G>A variant (19), all of whom were also carriers of an identified LDLR mutation. In vitro experiments with a reporter construct have shown that this variant does not affect the transcription rate of the PCSK9 promoter (19). As expected for a nonfunctional variant, its frequency was similar in >2700 healthy men in NPHS-II, with the lipid concentrations of carriers and noncarriers being the same.

Overall, these data suggest that measurement of plasma PCSK9 in statin-treated FH patients is unlikely to be a useful way to identify individuals with novel mutations in the PCSK9 gene, although we cannot rule out the possibility that such measurements in newly diagnosed, treatment-naive patients may provide more insight.

References

Plasma PCSK9 Concentrations and PCSK9 Genotypes