Genetic Variation at the Proprotein Convertase Subtilisin/Kexin Type 5 Gene Modulates High-Density Lipoprotein Cholesterol Levels

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Background—A low level of plasma high-density lipoprotein cholesterol (HDL-C) is a risk factor for cardiovascular disease. HDL particles are modulated by a variety of lipases, including endothelial lipase, a phospholipase present on vascular endothelial cells. The proprotein convertase subtilisin/kexin type 5 (PCSK5) gene product is known to directly inactivate endothelial lipase and indirectly cleave and activate angiopoetin-like protein 3, a natural inhibitor of endothelial lipase. We therefore investigated the effect of human PCSK5 genetic variants on plasma HDL-C levels.

Methods and Results—Haplotypes at the PCSK5 locus were examined in 9 multigenerational families that included 60 individuals with HDL-C <10th percentile. Segregation with low HDL-C in 1 family was found. Sequencing of the PCSK5 gene in 12 probands with HDL-C <5th percentile identified 7 novel variants. Using a 2-stage design, we first genotyped these single-nucleotide polymorphisms (SNPs) along with 163 tagSNPs and 12 additional SNPs (n=182 total) in 457 individuals with documented coronary artery disease. We identified 9 SNPs associated with HDL-C (P<0.05), with the strongest results for rs11144782 and rs11144766 (P=0.002 and P=0.005, respectively). The SNP rs11144782 was also associated with very low-density lipoprotein (P=0.039), triglycerides (P=0.049), and total apolipoprotein levels (P=0.022). In stage 2, we replicated the association of rs11144766 with HDL-C (P=0.014) in an independent sample of Finnish low HDL-C families. In a combined analysis of both stages (n=883), region-wide significance of rs11144766 and low HDL-C was observed (unadjusted P=1.86×10^{-4} and Bonferroni-adjusted P=0.031).

Conclusions—We conclude that variability at the PCSK5 locus influences HDL-C levels, possibly through the inactivation of endothelial lipase activity, and, consequently, atherosclerotic cardiovascular disease risk. (Circ Cardiovasc Genet. 2009;2:467-475.)

Key Words: cholesterol ■ coronary disease ■ genetics ■ lipids ■ lipoproteins

Clinical Perspective on p 475

The metabolism of HDL is complex and involves a carefully orchestrated interplay between the biogenesis of nascent HDL particles,3 the continual exchange of lipid and protein moieties of HDL in plasma, and the modulation of HDL particles by a variety of enzymes, especially lipases.4 Endothelial lipase (EL), discovered by Rader and colleagues,5 is a phospholipase present on vascular endothelial cells. It can be inactivated by angiopoetin-like protein 3 (ANGPTL3)6 or by secretory proprotein convertases of the subtilisin/kexin type, such as Furin, PCS5/6, and PACE4.7 The mammalian proprotein convertases comprise a family of 9 members related to bacterial subtilisin and yeast kexin-like serine proteinases, critically involved in the activation/inactivation of various physiological and pathological processes, including those implicated in regulation of vascular events. These include PC1/3, PC2, Furin, PC4, PC5.
Although PCSK9 has been found to play a critical role in regulating lipid levels by enhancing low-density lipoprotein (LDL) receptor degradation,² proof of in vivo functions of the proprotein convertase subtilisin/kexin type 5 (PCSK5) (Online Mendelian Inheritance in Man: 600488) and its protein, PCS5/6, in dyslipidemia and cardiovascular pathologies, has yet to be established. Murine Pcsk5 is localized on chromosome 19 and encodes 2 alternatively spliced isoforms, soluble PCS5A (915 amino acids; 21 exons) and membrane-bound PCS5B (1877 amino acids; 38 exons).¹⁰ Although devoid of a second isoform in most tissues (87% to 100%), and only the intestine and kidney show a predominance of PCS5B (74% to 92%).¹²,¹³ In humans, PCSK5 is located on chromosome 9q21.13, and while 2 alternatively spliced transcripts are described for this gene,¹⁴,¹⁵ only one, generating a 21-exon isoform and typically referred to as PCSK5, has its full length nature known (NM_006200.3).

There is strong biological plausibility for the involvement of PCSK5 in lipoprotein metabolism. Jin et al.⁷ showed that PCS5A inactivates ex vivo EL and lipoprotein lipase (LPL), with both lipases being present at the vascular endothelial surface. High expression of PCS5/6 in enterocytes also suggests a possible role in processing protein substrates that could regulate food and/or sterol/lipid absorption.¹⁰ In addition, recent data from a genome-wide scan reports a region close to PCSK5 on chromosome 9, implicated in lipid regulation,¹⁶ but its link to HDL deficiency remains to be explored. In light of this, there is compelling evidence that suggests that PCS5/6 may be a good candidate proteinase implicated in the control of circulating HDL-C levels and mutations affecting its function may influence lipoprotein metabolism and HDL subspecies more specifically. Therefore, we undertook a study to determine whether genetic variation within the PCSK5 gene contributes to variation in HDL-C levels, which consequently, could influence atherosclerotic cardiovascular disease risk.

**Methods**

**French Canadian Family Subjects**

A total of 9 multigenerational French Canadian families consisting of 175 genotyped members were examined and sampled in the Preventive Cardiology/Lipid Clinic of the McGill University Health Centre (MUHC). The selection criterion for probands was HDL-C <5th percentile (age and gender matched), based on the Lipid Research Clinics Population Studies Data Book.¹⁷ All subjects provided informed consent for plasma and DNA sampling, isolation, and storage. The research protocol was reviewed and approved by the Research Ethics Board of the MUHC.

**Stages 1 and 2 Association Study Samples**

**French Canadian Subjects**

For stage 1 analysis, unrelated patients (n=457) of French Canadian descent were selected from the Cardiology Clinic of the Clinical Research Institute of Montreal that were <60 years of age and had angiographically documented coronary artery disease.¹⁸ Individuals had HDL-C values ranging from HDL-C <5th percentile to HDL-C >95th percentile (age and gender-matched).¹⁷ We excluded patients with known causes of HDL deficiency (severe hypotriglyceridemia defined as plasma triglycerides (TG) >10 mmol/L, cellular phospholipid or cholesterol efflux defect or previously identified mutations in genes associated with HDL deficiency).¹⁹,²⁰ Demographic and clinical information, medications, and lipoprotein profiles were determined on all participating subjects as previously described.²⁰ The research protocol was approved by the Research Ethics Board of the MUHC.

**Finnish Family Subjects**

The stage 2 study sample consisted of 39 Finnish low HDL-C families (426 genotyped individuals) recruited at the Helsinki and Turku central hospitals, as previously described.²¹ Each subject involved in this study provided written informed consent. The study design was approved by the ethics committees of the participating institutions. Inclusion criteria for the probands were age 30 to 60 years, at least 50% stenosis in 1 or more coronary arteries, HDL-C level below the Finnish age- and sex-specific 10th population percentile (subjects coded as affected), total cholesterol (TC) <6.3 mmol/L for males and <6.0 mmol/L for females, and TG <2.3 mmol/L for both genders.²¹ Serum lipid and glucose parameters were measured as previously.²² Exclusion criteria for the probands were type 1 and 2 diabetes mellitus, severe hepatic or renal disease, or body mass index >30.²¹

**Haplotyping**

Microsatellite genotypes were determined by deCODE Genetics (Reykjavik, Iceland) at markers D9S1777, D9S1876, D9S175, and D9S1843 spanning 11.3 Mb and flanking the PCSK5 gene on chromosome 9q21.13. Haplotypes were constructed using Cyrillic version 2.1.3 (Cherwell Scientific Publishing Ltd, Oxford, United Kingdom) to examine the segregation of the PCSK5 locus with the low HDL-C trait in 9 families with HDL-C deficiency.

**Sequencing**

Sequencing of the 21 exons and exon-intron boundaries of the PCSK5 gene was performed in 12 unrelated individuals with HDL-C <5th percentile. Exon-specific oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, Iowa) and designed using the Primer3 software (http://frodo.wi.mit.edu/) to include at least 22 bp of intronic sequence at each intron-exon boundary. Polymerase chain reaction (PCR)-amplified fragments were purified using the Millipore purification plate (Multiscreen PCR) and directly sequenced at the McGill University and Genome Quebec Innovation Centre. Sequencing Platform using the Applied Biosystems 3730xl DNA Analyzer system. The data were analyzed by Sequencing Analysis version 5.2 and Mutation Surveyor version 2.41 (SoftGenetics, State College, Pa).

**Single-Nucleotide Polymorphisms Selection**

To select the most informative single-nucleotide polymorphism (SNPs) for the first-stage genotyping of PCSK5, we used a tagSNP strategy using HapMap Utah Residents with Northern and Western European Ancestry (CEU) spanning 304 714 bp of genomic DNA and including 2 kb upstream of PCSK5 (HapMap Rel27 PhaseII+III; Haploviev version 4.0)²³. We used a minor allele frequency (MAF) >0.05 and r² threshold of 0.80. In addition, we included novel variants identified through sequencing (n=7), and SNPs selected from public genetic databases (NCBI, UCSC, SeattleSNPs, and Human SNP [Broad Institute]) (n=12), for a total of 182 SNPs to be genotyped in stage 1.
In the second stage, of 9 SNPs that provided significant evidence of association with \( P < 0.05 \), we selected 2 SNPs with \( P \leq 0.01 \) for stage 2 genotyping.

**Genotyping**

Stage 1 genotyping of 182 SNPs was performed using the Sequenom MassARRAY Assay Design 3.1 software. DNA was amplified in a multiplex PCR and labeled using a single base extension reaction. The products were desalted and transferred to a 384-element SpectroCHIP array. Allele detection was performed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Mass spectrometers and clusters were analyzed by the TYPER 3.4 software. Ehrich et al. have previously provided details of the procedure. Before association analysis, quality control-check was performed by assessing integrity of genotypic data. We obtained a 96% success rate for the SNPs, and 99% of subjects (451 individuals) were successfully genotyped. For the remaining SNPs, a genotyping call rate >98% was obtained. After frequency and gender pruning, there were 169 SNPs that were analyzed.

Genotyping of the 2 second-stage replication SNPs was performed in the Finnish low HDL families using the pyrosequencing technique on the PSQHS96A platform with a >94% genotyping call rate. Both SNPs were in Hardy-Weinberg Equilibrium in the unrelated founders (\( P > 0.5 \)). The Pedcheck program was used to detect Mendelian errors in the families.

**Statistical Analyses**

Statistical analyses for the French Canadian association study were performed with PLINK version 1.04 software (http://pngu.mgh.harvard.edu/purcell/plink/) and the SAS package version 9.1.3 performed with PLINK version 1.04 software (http://pngu.mgh.harvard.edu/purcell/plink/). Statistical analyses for the French Canadian association study were weighted by the square root of the sample size to calculate the corresponding combined family-based study were weighted by the square root of the sample size to calculate the corresponding combined family component.

Association analysis in the Finnish family cohort was performed using the quantitative transmission disequilibrium test approach is robust to population stratification and has been recognized as a powerful method that corrects for multiple testing. We adjusted for 169 SNPs tested in stage 1 and for the 2 SNPs tested in stage 2, resulting in a Bonferroni correction for 171 independent tests in the overall combined analysis.

**Results**

**Familial Segregation Analyses**

We first investigated whether genetic variability at the PCSK5 locus was associated with HDL-C in a Mendelian fashion. We examined the segregation of PCSK5 haplotypes with a severe HDL-C deficiency trait (HDL-C <10th percentile) in 9 unrelated multigenerational families of French Canadian descent (175 subjects, mean number per kindred 17). Our study included 71 men and 104 women of which 29 and 31 were affected (HDL-C <10th percentile), respectively. Mean age was 50±16 years for men and 48±19 years for women; the mean HDL-C level in affected men was 0.64±0.08 mmol/L compared with 1.12±0.22 mmol/L in nonaffected men. Similarly, HDL-C levels in affected women were 0.87±0.15 mmol/L versus 1.39±0.26 mmol/L in nonaffected. Four microsatellites, D9S1777, D9S1876, D9S175, and D9S1843, located upstream and downstream of the PCSK5 locus and spanning 11.3 Mb within the 9q21.13 region, were used to construct haplotypes at the PCSK5 gene. Only, 1 small (n=7 subjects) kindred was observed to display perfect segregation with HDL-C levels (data not shown). Thus, cumulatively, we did not find unambiguous segregation of this locus with the low HDL-C trait using a dominant model of inheritance, suggesting that PCSK5 does not exert a Mendelian monogenic effect on HDL-C in these families.

**Sequencing**

We undertook a thorough examination of the PCSK5 gene locus for coding and noncoding variants. Sequencing at the PCSK5 locus was performed on all 21 exons and exon-intron boundaries using 22 pairs of primers for a total of 7455 bps in 12 unrelated individuals with low HDL-C levels (<5th percentile). We identified a total of 19 polymorphisms, 7 of which were novel noncoding variants (Table 1, Figure 1). Of the 12 previously characterized SNPs, we observed 4 synonymous polymorphisms (rs7040769, rs7020560, rs2297342, and rs10521468), 1 SNP in the 5’ untranslated region (rs12005073), and 7 intronic. Two of the novel variants were insertions: 1 in intron 19 (IVS19-71insTAAAA) and the other in the 5’ untranslated region (c.385insGAGCTGCGGCGGCCCGGGC). We also found a deletion in intron 20 (IVS20-50delTACTTTCA-GGACTAAT), a variant in intron 4 (IVS4-3016T>A), and 3 polymorphisms in the 5’ and 3’ untranslated regions (c.125C>A, c.72C>T, c.323G>A, respectively) (Table 1).

**Association Studies**

To investigate whether common genetic variants at the PCSK5 locus influence HDL-C levels and therefore explain some of the interindividual variation of HDL-C plasma concentrations, we conducted a quantitative association analysis using a 2-stage approach. In stage 1, we genotyped 169 tagSNPs in 457 unrelated subjects of French Canadian descent to screen for associations. In stage 2, we genotyped significant signals (\( P < 0.01 \)) in Finnish low HDL families, and subsequently performed a combined analysis of the 2 stages to identify variants of region-wide significance. Skol et al. originally introduced this approach to reduce the cost of genotyping in stage 1 while maintaining the overall power of the study.

In stage 1, a total of 169 SNPs (Figure 2) were tested for association in 457 French Canadian individuals. Using an additive model and adjusting for age and sex as covariates, we identified 9 SNPs significantly associated with HDL-C (\( P < 0.05 \)), with the strongest results being rs11144782 and rs11144766 (MAF 0.164, \( \beta = -0.076 \) mmol/L, \( P = 0.002 \); MAF 0.197, \( \beta = -0.063 \) mmol/L, \( P = 0.005 \); respectively; Table 2, Figure 1). The rare G-allele of rs11144782 decreased HDL-C levels by 0.076 mmol/L on average per allele,
whereas the A risk allele (A) of rs11144766 decreased plasma HDL-C levels by 0.063 mmol/L. The effect of these minor alleles on HDL-C are displayed in Table 3. In addition, of the 9 polymorphisms identified, 3 other SNPs were shown to be significantly associated with decreased plasma HDL-C levels (rs11144688, rs11144690, and rs1338746), whereas 4 others were associated with an increase in HDL-C (rs1339246, rs1331384, rs4745522, and rs2050833; Table 2). These 9 variants were all found in noncoding regions and were not in linkage disequilibrium (LD).

We next tested our 2 most significant polymorphisms (P<0.01), rs11144782 and rs11144766, for association with other lipoprotein traits and, adjusting for age and sex under an additive model, observed a significant positive effect on plasma TG (P=0.049), very low-density lipoprotein cholesterol (VLDL-C; P=0.039), and apolipoprotein B (apoB; P=0.022) levels (Table 4) for rs11144782, suggesting that it modulates several aspects of lipid metabolism. We also conducted a stepwise conditional regression analysis in the presence of rs11144782 and age and sex as covariates. Using this approach, we reidentified rs11144766 (P<0.001) and 2 other nonredundant SNPs (rs2050833, P<0.036; rs4745488, P<0.038) that contributed to the variability of HDL-C (Table 5), independently of one another, providing further evidence for the role of PCSK5 in HDL-C metabolism.

In stage 2, we followed up rs11144782 and rs11144766, which provided the most significant associations (P≤0.01) in the French Canadian cohort, for replication in an independent sample of 39 low HDL-C Finnish dyslipidemic families (n=426).21 We tested for association between these variants and low HDL-C using the quantitative transmission disequilibrium test with age and sex as covariates. Although we did not observe an association with rs11144782, rs11144766 was found to be significantly associated with HDL-C in the same direction as in the French Canadians (P=0.014).

Next, we performed a combined analysis of the stages 1 and 2 unrelated and family samples (n=883) for the 2 SNPs by combining the Z statistics, as described in Materials and Methods. We observed a strong association between rs11144766 and low HDL-C (P=1.86×10⁻⁴) for the additive

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**Table 1. PCSK5 Polymorphisms Identified by Sequencing**

<table>
<thead>
<tr>
<th>Functional Class</th>
<th>Location</th>
<th>Validated SNPs*</th>
<th>Novel Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exonic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synonymous</td>
<td>Exon 1</td>
<td>rs7040769</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 1</td>
<td>rs7020560</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 12</td>
<td>rs2297342</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 18</td>
<td>rs10521468</td>
<td></td>
</tr>
<tr>
<td>5’UTR</td>
<td>rs12005073</td>
<td>c.125C&gt;A</td>
<td>c.72C&gt;T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.385insGAGCTGGGCGGCCGGCTGC</td>
<td></td>
</tr>
<tr>
<td>3’UTR</td>
<td>Int.4</td>
<td></td>
<td>c.323G&gt;A</td>
</tr>
<tr>
<td></td>
<td>Int.7</td>
<td>rs2297344</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Int.8</td>
<td>rs1418547</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Int.11</td>
<td>rs3824474</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Int.16</td>
<td>rs2270570</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Int.17</td>
<td>rs1537183</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Int.19</td>
<td>rs3830384</td>
<td>IVS19-7InsAAA</td>
</tr>
<tr>
<td></td>
<td>Int.20</td>
<td>rs10869726</td>
<td>IVS20-50delTAATTCAGACTAAT</td>
</tr>
</tbody>
</table>

UTR indicates untranslated region; Int, intron.

*Validated SNPs correspond to variants identifying by sequencing, but previously characterized.

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![Figure 1](http://circgenetics.ahajournals.org/)

**Figure 1.** SNP locations in the PCSK5 gene. Schematic representation of the human PCSK5 gene locus showing the exon structure and the location of the 19 polymorphisms (bottom panel) discovered through sequencing and the 9 genetic variants associated with HDL-C (upper panel) identified by genotyping. SNPs in bold are associated with HDL-C with P<0.01. Locations are based on RefSeq NM_006200.3.
model and the same A risk allele. This result is region-wide significant: it surpasses the Bonferroni correction for all 171 tests performed (169 SNPs tested in stage 1 and 2 SNPs in stage 2 [Bonferroni adjusted $P<0.031$]). Thus, the association between rs11144766 and HDL-C in these Finnish dyslipidemic families is consistent with the results from the French Canadian unrelated individuals and provides strong evidence for the influence of rs11144766 on HDL-C levels.

**Discussion**

The investigation of the molecular genetics and pathophysiology of HDL-C deficiency has been an area of fertile research. Despite a large body of information identifying HDL-C as a potent antiatherosclerosis lipoprotein, the fundamental mechanisms underlying the genetic regulation of the HDL-C metabolic pathway remain complex and poorly understood.

In this study, we have demonstrated that genetic variability at the PCSK5 gene modulates HDL-C levels. By sequencing the gene, we identified 7 novel noncoding variants in patients with HDL-C deficiency (Figure 1). Although none of these newly identified variants represent a missense, frameshift, or nonsense polymorphism with obvious functional consequences, the possibility of their regulatory role cannot be excluded and further studies to delineate their mechanistic effects are needed. We next performed an association study of PCSK5 SNPs with HDL-C through a 2-stage study design. This approach was an efficient way to optimize the power to detect true associations, while minimizing the overall amount of genotypes required for sufficient regional coverage.31

**Table 2. Association Results of Stage 1 Analysis for the HDL-C Trait**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Effect ($\beta$)</th>
<th>$P$</th>
<th>MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11144782*</td>
<td>-0.076</td>
<td>0.002</td>
<td>0.164</td>
</tr>
<tr>
<td>rs11144766*</td>
<td>-0.063</td>
<td>0.005</td>
<td>0.197</td>
</tr>
<tr>
<td>rs1339246</td>
<td>0.056</td>
<td>0.018</td>
<td>0.176</td>
</tr>
<tr>
<td>rs1331384</td>
<td>0.037</td>
<td>0.038</td>
<td>0.485</td>
</tr>
<tr>
<td>rs11144688</td>
<td>-0.053</td>
<td>0.039</td>
<td>0.137</td>
</tr>
<tr>
<td>rs11144690</td>
<td>-0.093</td>
<td>0.040</td>
<td>0.040</td>
</tr>
<tr>
<td>rs1338746</td>
<td>-0.036</td>
<td>0.044</td>
<td>0.424</td>
</tr>
<tr>
<td>rs4745522</td>
<td>0.051</td>
<td>0.045</td>
<td>0.143</td>
</tr>
<tr>
<td>rs2050833</td>
<td>0.045</td>
<td>0.045</td>
<td>0.199</td>
</tr>
</tbody>
</table>

Quantitative association analysis for HDL-C in French Canadians was performed using linear regression, after adjustments for age and sex, under an additive model. $\beta$ is the linear regression coefficient corresponding to the effect size per copy of the minor allele.

*Significant variants ($P<0.01$) selected for stage 2 analysis.
stage 1, we investigated the association of PCSK5 SNPs with HDL-C levels in 457 unrelated subjects of French Canadian descent, using quantitative analyses. We identified 9 significant SNPs (P < 0.05, Figure 1, Table 1), 5 of them being associated with a decrease and 4 with an increase in HDL-C plasma concentration. The strongest signals, rs11144782 and rs11144766 (MAF 0.164, β = −0.076, P = 0.002; MAF 0.197, β = −0.63, P = 0.005, respectively), were found to be negative modulators of HDL-C levels, displaying an allele dosage effect (Table 3): the minor allele (G) of rs11144782 was observed to contribute to a decrease of 8% in plasma HDL-C levels, whereas the minor allele (A) for rs11144766 lowered HDL-C serum concentration by 6% (Tables 2 and 3). Interestingly, the MAF of rs11144782 and rs11144766 in the HapMap-CEU samples (MAF 0.156 and 0.142) was in concordance with both the French Canadian (MAF 0.164 and 0.197) and the Finnish study cohorts (MAF 0.168 and 0.197).

In stage 2, we followed up significant markers in the low HDL Finnish families to confirm the observed associations. By means of a combined analysis of both stages (n = 883), region-wide significance between rs11144766 and HDL-C was observed (unadjusted P = 1.86 × 10−4). Using the Bonferroni correction, rs11144766 remained significant (P = 0.031) after adjusting for multiple testing (n = 171), providing sound and consistent evidence for its role in HDL-C metabolism.

Furthermore, in conditional regression analysis, we observed 2 additional SNPs putatively contributing to HDL-C (P < 0.05), independent of the effects of rs11144782 and rs11144766 (Table 5). As a result, in this study, we have identified four signals at the PCSK5 locus, independent of one another. Interestingly, in stage 1 analysis, the rs11144782 variant was also associated with other lipid traits including VLDL-C (P = 0.039), TG (P = 0.049), and total apoB (P = 0.022) levels (Table 4), translating in an absolute increase in VLDL-C (0.169 mmol/L), TG (0.502 mmol/L), and apoB (10.72 g/L) per allele, respectively, suggesting that PCSK5 genetic variability may influence other aspects of lipoprotein metabolism and not solely HDL-C.

The 2 polymorphisms identified in this study, rs11144766 and rs11144782, are both located in introns of PCSK5. rs11144766 is found in the 10th intron, between exons coding for a region of the catalytic domain of PCS5/6, whereas rs11144782 occurs in the 15th intron, between exons encoding the cysteine-rich domain of PCS5/6 (Figure 1). Despite their intronic localization, we show here that both of these SNPs are important regulators of HDL-C levels, potentially mediating PCS5/6 activity.

There are several possible explanations for such effects. These SNPs may be involved in regulating splicing of the PCSK5 transcript, to either enhance or suppress proper intron removal. To explore this possibility, we used the ACESCAN2 Web Server (http://genes.mit.edu/acescan2/index.html) to scan for possible sites that may affect splicing. We identified a specific GTGTGG sequence present in the rare A-allele of rs11144766 as an intronic splicing enhancer. This suggests that individuals carrying this variant may have modified splicing of the PCSK5 gene, which could impact PCS5/6 activity. The catalytic domain of PCSK5 is crucial for its proteolytic convertase function, necessary for EL cleavage. Indeed, deleting exon 4 resulted in embryonic lethality,12,13 stressing the importance of this domain. In contrast, although no intronic splicing enhancers were found to be associated with rs11144782, it may have other effects on splicing, which could alter PCS5/6 function. The cysteine-rich domain of the latter is involved in regulating PCS5/6A localization, which, in combination with tissue inhibitors of metalloproteases, binds to HSPG at the cell surface.32 Data reveal that the cysteine-rich domain confers protein-protein interactions, cell surface tethering and is essential for the efficacious processing of the human proEL precursor, likely due to a proximity effect resulting from close juxtaposition of the convertase and EL

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**Table 3. Distribution of Genotypic Classes for rs11144782 and rs11144766 in Stage 1 French Canadian Subjects**

<table>
<thead>
<tr>
<th>Genotypes (n)</th>
<th>rs11144782</th>
<th>rs11144766</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (n=301)</td>
<td>GC (n=123)</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.69</td>
<td>0.28</td>
</tr>
<tr>
<td>Mean HDL-C*, mmol/L</td>
<td>0.98±0.29</td>
<td>0.89±0.24</td>
</tr>
</tbody>
</table>

Analyses were performed using PLINK version 1.04 with age and sex as covariates, under an additive model.

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**Table 4. Lipid Traits Associated With rs11144782**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Trait</th>
<th>Effect (β)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11144782</td>
<td>HDL, mmol/L</td>
<td>−0.076</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>TG, mmol/L</td>
<td>0.502</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>VLDL, mmol/L</td>
<td>0.169</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>apoBtot, mg/dL</td>
<td>10.720</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Analyses were performed using PLINK version 1.04 with age and sex as covariates, under an additive model. β is the linear regression coefficient, corresponding to the effect size per copy of the minor allele. apoBtot indicates total apolipoprotein B.

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**Table 5. Independent Signals Found at the PCSK5 Locus for the HDL-C Trait**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Effect (β)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11144782</td>
<td>−0.076</td>
<td>0.002</td>
</tr>
<tr>
<td>rs11144766</td>
<td>−0.072</td>
<td>0.001</td>
</tr>
<tr>
<td>rs2050833</td>
<td>0.047</td>
<td>0.036</td>
</tr>
<tr>
<td>rs4745488</td>
<td>−0.039</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Conditional analyses were performed using stepwise linear regression adjusting for age, sex, rs11144782, rs11144766, and rs2050833, respectively. β is the linear regression coefficient, corresponding to the effect size per copy of the minor allele.
through interactions with cell-surface HSPG. Therefore, altering either the cysteine-rich motif or the catalytic domain would likely impair PCS5/6 activity and, indirectly, overexpress EL. Subsequently, this might explain the observed decrease in HDL-C levels in individuals carrying these SNPs. Beyond the possibility of modulating splicing, these variants may be located in important binding sites for unknown factors, such as microRNAs. Furthermore, it is also possible that they are in LD with other ungenotyped SNPs.

Though we have highlighted the importance of rs11144782 and rs11144766 in this study, much work remains to be done to better elucidate their functional mechanisms and effects on PCS5/6 activity. Despite this, our findings suggest an in vivo conceptual mechanism for how rs11144766 and rs11144782 could potentially affect HDL-C metabolism (Figure 3). We propose that by altering PCS5/6 function, these variants could prevent the internal cleavage of the HSPG-bound EL by PCS5/6 or its inhibition by activated ANGPTL3. As a result, EL will be fully active and its effect on HDL unopposed, resulting in a pronounced phospholipase activity and consequently, hydrolysis of HDL phospholipids that will produce smaller HDL particles. This will reduce plasma HDL-C levels, in concordance with studies in which mice overexpressing human EL revealed a marked depletion in HDL-C levels. The EL-mediated reduction of HDL phospholipids can also alter the lipid composition and physical properties of HDL, resulting in a diminished ability of HDL to mediate scavenger receptor class B type I-dependent cholesterol efflux. In addition, association of rs11144782 with increased VLDL-C, TG, and apoB levels implicates the effects of other lipases, such as LPL and hepatic lipase. Further studies are needed, however, to elucidate their exact physiological role on plasma lipoprotein metabolism in the presence of PCS5 variants affecting PCS5/6 function. Indeed, a PCS5/6 loss of activity could allow the activation of the heparin-like glycosaminoglycans-bound LPL and its triglyceride hydrolyse function on chylomicrons and VLDL, subsequently decreasing them. Similarly, ANGPTL3, a liver-derived member of the vascular endothelial growth factor family, shown to be an endogenous inhibitor of EL in cell-free systems, plays a potential role in regulating HDL levels. In line with the study done by Shimizu-gawa et al in KK/San mice resulted in a marked increase of TG-enriched VLDL levels through the inhibition of LPL activity, loss of hepatic PCS5/6 activity under pathophysiological, genetic, or diseased conditions could increase EL and LPL activities, resulting in reduced plasma HDL-C, VLDL-C, and TG concentrations. Thus, there are several venues by which PCS5 variants could impact lipoprotein metabolism. The relative contribution of these pathways on PCS5/6 inactivation is still unknown, and further work is required to determine the overall importance of each component in the system.

Given the significance of our results, a clearer understanding of the molecular interactions between the PCS5/6-EL system and HDL structure, as well as the direct impact of HDL remodeling by PCS5/6-EL on the reverse cholesterol transport process and endothelial function, should be the focus of future scientific studies. It would also be essential to replicate our findings in larger and more diverse study samples and functionally characterize these variants. Accordingly, unraveling the in vivo and in vitro effects of the PCS5/6-EL system could refine our comprehension of the complex HDL metabolic pathway and provide novel insights into the human atheroprotective system in health and disease.

In conclusion, we observed an association of region-wide significance between rs11144766 and HDL-C under an additive model in an unrelated and family-based sample (n=883). These results support the contribution of PCS5 to HDL-C levels and its pivotal role in HDL-C metabolism. Although previous work by Cao et al identified 2 silent SNPs in PCS5 varying in frequency among ethnic groups, no other studies thus far have analyzed the genetic variability at the PCS5 gene locus and its contribution to HDL-C levels. This report is therefore the first comprehensive examination of such genetic variation, implicating PCS5 as an important and influential modulator of HDL-C serum levels in humans. These findings can thus firmly place PCS5 on the list of genes associated with HDL-C and emphasize the need to investigate PCS5 and its related substrates for identification of specific therapeutic targets for treatment of cardiovascular disease.
Acknowledgments

We thank the French Canadian and Finnish families who participated in this study. Leena Peltonen is thanked for sample collection and E. Nikkola and M. Lupsakko for their laboratory technical assistance. We also thank the Montreal Genome Centre staff for their sequencing and genotyping services.

Sources of Funding

This work was supported by the Canadian Institutes of Health Research (MOP 62834, MOP 44363, and CTP 82946) and the Heart and Stroke Foundation of Quebec. Dr Genest holds the McGill Novartis Chair in Cardiology at McGill University. Dr Iatan is a recipient of a Doctoral Research Award from the Fonds de la Recherche en Santé du Québec and a Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award from the Canadian Institutes of Health Research. Dr Dastani is a recipient of a Doctoral Research Award from the Heart and Stroke Foundation of Canada, and Dr Do holds a Canada Graduate Scholarship Doctoral Award from Canadian Institutes of Health Research. This study was also funded by the National Institutes of Health (HL-28481 and HL082762 to P.P.); the Clinical Research Institute Helsinki University Central Hospital Ltd (to M.-R.T.); and the Finnish Heart Foundation (to M.-R.T.). Drs Wessig, Volkov and Huertas-Vazquez are supported by the National Human Genome Research Institute (T32 HG02536) and the American Heart Association (0725232Y), respectively.

Disclosures

None.

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CLINICAL PERSPECTIVE

The metabolism of high-density lipoproteins is complex and only partially understood. The understanding of the genetic basis for lipoproteins disorders has paved the way for novel therapeutic approaches. In this study, we have demonstrated that genetic variation at the proprotein convertase subtilisin/kexin type 5 (PCSK5) gene regulates plasma high-density lipoprotein cholesterol (HDL-C) levels. Using a 2-stage association study, we investigated the association of PCSK5 SNPs with HDL-C levels in 457 unrelated subjects of French Canadian descent, and then we replicated the most significant (associated with HDL-C) in an independent study sample consisting of Finnish families with low HDL-C (n=426). In a combined analysis of both stages (n=883), we observed an association of region-wide significance between significant genetic markers and low HDL-C, surpassing the Bonferroni threshold. We further observed an association of significant SNPs with other lipoprotein traits, suggesting that PCSK5 modulates several aspects of lipid metabolism and, consequently, atherosclerotic cardiovascular disease risk. Thus, our findings place PCSK5 on the list of genes associated with HDL-C, likely through the modulation of vascular endothelial lipases, implicating it as an important and influential modulator of HDL-C serum levels in humans. These observations emphasize the need to investigate proprotein convertase 5 and its related substrates further for identification of specific therapeutic targets for treatment of cardiovascular disease. Unraveling the in vivo and in vitro effects of the PCS5/6-EL system could refine our understanding of the complex HDL metabolic pathway.
Genetic Variation at the Proprotein Convertase Subtilisin/Kexin Type 5 Gene Modulates High-Density Lipoprotein Cholesterol Levels


Circ Cardiovasc Genet. 2009;2:467-475; originally published online August 22, 2009; doi: 10.1161/CIRCGENETICS.109.877811

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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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