Conclusions—Even though the R46L variant was present in 3% of our FH population, carriers of this polymorphism showed attenuated effect of the low density lipoprotein receptor mutation on parameters, such as low density lipoprotein cholesterol, apolipoprotein B, total cholesterol, and non-high density lipoprotein. More importantly, this mutation is associated with a significant lower risk of cardiovascular disease compared with noncarriers. It is therefore likely that targeting PCSK9 in FH patients with novel anti-PCSK9 therapies will be useful in reducing cardiovascular risk in affected subjects. (Arterioscler Thromb Vasc Biol. 2014;34:2700-2705.)

Key Words: cardiovascular □ FH □ hypercholesterolemia □ LDL-C □ PCSK9 □ xanthomas

Clearance of plasma low density lipoprotein cholesterol (LDL-C) is mediated by the low density lipoprotein receptor (LDLR). Familial hypercholesterolemia (FH) is a frequent autosomal codominant disease with a prevalence estimated at 1 in 500 individuals. This disease is characterized by high plasma levels of LDL-C and is typically associated with ectopic cholesterol deposition in tendons (xanthomas), in the eyelids (xanthelasmas), and at the level of the cornea (corneal arcus) as well as in arteries, leading to premature cardiovascular disease (CVD). Mutations of the LDLR, APOB (apolipoprotein B), and PCSK9 (proprotein convertase subtilisin-kexin type 9) genes were identified as causing FH.1–5

PCSK9 binds the LDLR and targets it for late endosomal/lysosomal degradation. Mutations increasing this interaction are known as gain of function mutations, leading to higher plasma LDL-C concentrations. Mutations causing a defective PCSK9 (LOF, loss of function) have been shown to be beneficial by decreasing LDL-C concentrations and the risk of CVD.6

Clinical studies in non-FH populations showed that carriers of heterozygous LOF polymorphisms in PCSK9 were associated with a 15% to 49% reduction in LDL-C levels compared with noncarriers.7,8 Among the PCSK9 LOF variants, R46L is the most common occurring in exon 1 of the PCSK9 gene, affecting ≈1/50 individuals, with an average reduction in LDL-C of ≈14% for heterozygous carriers.9–14 Interestingly, the R46L variant is found more frequently (mean value ≈14% for heterozygous carriers).9–14 R46L carriers showed significantly lower plasma PCSK9 concentration compared with noncarriers,16–21 thereby supporting the fact the PCSK9 R46L protein is secreted less efficiently. Moreover, Cohen et al showed significant associations between the presence of the PCSK9 R46L genetic variant and reduced levels of
plasma LDL-C, total cholesterol and, importantly, showed also a decrease in coronary heart disease. Additionally, important observations from meta-analysis have highlighted the association of the R46L carriers to a reduced risk of ischemic heart disease and of early-onset myocardial infarction. Presently, statins are the first line medications used to lower LDL-C and the risk of CVD. Unfortunately, statins also upregulate the PCSK9 gene expression, therefore, compromising their LDL lowering effect. Nevertheless, a previous report observed that compound carriers of the PCSK9 R46L variation and of a LDLR mutation associated with FH responded much better to statin therapy compared with controls. A decade of scientific research on PCSK9 resulted in the development of neutralizing molecules, such as monoclonal antibodies. These molecules are currently undergoing phase II/III clinical trials and have been shown to lower LDL-C efficiently by ≈50% to 60%. Outcome trials are underway to test the ability of these drugs to reduce cardiovascular events, but results are not expected before 2018 to 2020.

Taken together all this evidence suggests that PCSK9 could be an important modifier gene of the FH phenotype. We hypothesize that the presence of the R46L mutation among FH subjects would counteract the damaging effects of the inherited LDLR mutation and thus be associated with lower plasma LDL-C concentration and confer protection against CVD. The aim of our study is to screen a large cohort of FH patients for the PCSK9 R46L genetic variant and analyze its effect on their lipid profile, the prevalence of xanthomas, and on the occurrence of cardiovascular events.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement

**Results**

**Frequency of R46L Among an FH Population**

The structure of this observational cross-sectional cohort study is shown in Figure 1. Out of 20434 patients followed at the IRCCM (Institut de Recherches Cliniques de Montréal) lipid clinic, 2654 individuals presenting a clinical phenotype compatible with FH were identified. Of these, 1387 patients were excluded from this study because they lacked DNA samples. Of the patients for whom DNA was available, 582 carried an FH causing LDLR mutation. The frequency of each LDLR mutation is summarized in Figure 2A. The most frequent mutation (70.4%) observed was the deletion of >15 kb (del 15 kb) followed by the exon 3 (W87G), accounting for 16.7% of FH patients. The balance of FH-causing mutations was explained by mutations in exon 4 (C667Y, 2.9%) and deletion of 5 kb (del 5 kb, 2.1%) and exon10 (Y489X, 0.1%). Overall, 3% of these FH patients also carried the R46L PCSK9 genetic variant (Figure 2B).

We compared 46L carriers to noncarriers for classic cardiovascular risk factors. Patient characteristics were obtained at baseline and are presented in Table 1 where the average age in both groups was 37 and 36 years and did not differ statistically. Both groups were composed of similar proportions of males (59%) and females (41%). Subjects in the 2 groups had a normal average body mass index of ≈25 kg/m². A significantly higher proportion of patients with hypertension were identified in the R46L carrier group compared with noncarriers (33% versus 13%, respectively; P=0.02). Average systolic (130 versus 121 mm Hg) and diastolic (82 versus 72 mm Hg) blood pressure was slightly higher in the R46L group, but these differences were not statistically significant. The noncarriers and R46L-carrier groups did not differ significantly in the frequency of diabetes mellitus (2% versus 6%, respectively) and the average fasting blood glucose was normal in both groups (5.0 versus 4.8 mmol/L, respectively). No significant differences were observed in the percentage of smokers (previous and current) or previous statin use between the 2 groups. Therefore, R46L carriers did not present any significant difference in the prevalence of nonlipid, major cardiovascular risk factors compared with the noncarriers except for a slightly higher prevalence of hypertension.

**Lower Atherogenic Lipoproteins in R46L FH Carriers**

Comparison of the lipoprotein profiles of noncarriers and PCSK9 R46L carriers (Table 2) revealed that there was no statistically significant differences for HDL-C (1.01 versus 1.10 mmol/L), triglycerides (1.69 versus 0.99 mmol/L), VLDL-C (very low-density lipoprotein cholesterol; 1.01 versus 0.84 mmol/L), or Lp(a) (lipoprotein [a]; 16 versus 23 mg/L). However, PCSK9 R46L polymorphism carriers presented significant lower concentrations of LDL-C by 11% (6.74 versus 7.58 mmol/L; P<0.001), total cholesterol by 9% (8.69 versus 9.53 mmol/L, P=0.007), ApoB by 10% (214 versus 233 mg/L; P=0.037), and non-HDL concentration by 12% (7.59 versus 8.59 mmol/L; P<0.001). These differences are illustrated in Figure 3.

**Protective Effect of R46L on Xanthoma and Cardiovascular Events**

FH is often associated with the presence of tendinous xanthoma and premature CVD, but it has been recognized that not all FH patients will present these clinical features. Carriers of the R46L PCSK9 variant tend to have a lower risk of presenting ≥1 tendinous xanthoma compared with noncarriers, although this difference was not statistically significant (0.28 versus 0.48, respectively; P=0.09; Figure 4A). Nonetheless, a statistically significant difference was observed in the average number of tendinous xanthoma per patient. R46L carriers presented less than half the number of xanthomas per patient compared with noncarriers (0.33 versus 0.76; P<0.0001). The effect of the R46L genetic variation on CVD is presented in Figure 5. The CVD events in our FH cohort come from all events throughout patients’ history and are presented in Table I in the online-only Data Supplement. The prevalence

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tbody>
<tr>
<td>APOB</td>
</tr>
<tr>
<td>CVD</td>
</tr>
<tr>
<td>FH</td>
</tr>
<tr>
<td>LOF</td>
</tr>
<tr>
<td>LDL-C</td>
</tr>
<tr>
<td>LDLR</td>
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<tr>
<td>PCSK9</td>
</tr>
</tbody>
</table>
of presenting ≥1 cardiovascular event in carriers of the R46L variant was 11% compared with 33% to controls, and this difference was statistically significant (P=0.05; Figure 5A). Furthermore, the average number of cardiovascular events per patient (Figure 5B) was significantly lower in R46L carriers compared with noncarriers (0.22 versus 0.70, respectively; P<0.0001), indicating that these patients presented less severe CVD and less frequent recurrent cardiovascular events.

**Discussion**

The objectives of our observational cross-sectional cohort study were 3-fold: to investigate the effect of the frequent LOF R46L PCSK9 genetic variant on the level of LDL-C, on the frequency of xanthomata, and importantly, on the risk of atherosclerotic events in an FH population. We selected patient who presented a clinical phenotype compatible with FH (LDL-C above the 95th percentile for age and sex, autosomal familial inheritance of hypercholesterolemia, and premature CAD). This represented 13% of the 20434 patient from the lipid clinic. Of these, 1267 patients had consented to participate in research, and DNA was available. Roughly half of these patients (46%) had a mutation of the LDLR known to cause FH. This frequency of genotype-positive FH is in line with previously published observations.25,29 The relative frequency of FH causing LDLR mutation in our population is similar to the frequency observed in other French Canadian cohorts.30 Thus, all patients in our study were found positive for an FH-causing LDLR gene mutation and were classified as having a definite diagnosis of FH according to the Dutch Lipid Clinic Network and the Simon Broome Registry criteria.

In our cohort, 3% of FH subjects carried the R46L PCSK9 variation. This observed frequency is similar to the frequency previously reported in a non-FH population of American Caucasians and in a Scandinavian FH cohort (1.7% and 2.7%, respectively).10,13,29 A large genome-wide association study investigating genes associated with cardiovascular risk reported a minor allelic frequency of 1.54% for the R46L mutation in an American population of European origin.31 We have previously showed that the R46L PCSK9 variant was more prevalent in the French Canadian population with a frequency of 4.76% that could reach 12% in certain regions.21 The difference of prevalence of the R46L in our study could be because of the population studied. Indeed, the IRCM clinic is a referral center for dyslipidemia and few normolipidemic or hypolipidemic patients are present in our Biobank.

The frequency of cardiovascular risk factors in our FH cohort is similar to the one observed in other populations.32 There were no significant differences in atherosclerotic risk factors between R46L carriers and noncarriers. FH carriers of the PCSK9 R46L genetic variant were not at higher risk of diabetes mellitus compared with the noncarriers, but we did observe a statistically significant increase in the prevalence of hypertension and a higher mean baseline systolic blood pressure in this group. Interestingly, one in vitro study showed that PCSK9 is able to regulate the epithelial sodium channel trafficking in the biosynthetic pathway. Authors suggest that by reducing epithelial sodium channel number, PCSK9 could decrease the epithelial sodium absorption.33 Thus, it could be conceivable that a PCSK9 loss-of-function variant, such as R46L, could induce less degradation

<table>
<thead>
<tr>
<th>Baseline Patient Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Sex, n (women)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
</tr>
<tr>
<td>Weight, kg</td>
</tr>
<tr>
<td>Hypertension, n</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
</tr>
<tr>
<td>Diabetes mellitus, n</td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
</tr>
<tr>
<td>Smoking, n</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; DBP, diastolic blood pressure; and SBP, systolic blood pressure.
Table 2. Lipid Profile of the FH Subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Noncarriers (n=538)</th>
<th>R46L-Carriers (n=18)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C, mmol/L</td>
<td>7.58±1.73</td>
<td>6.74±0.96</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>9.53±1.97</td>
<td>8.69±1.14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.01±0.3</td>
<td>1.10±0.28</td>
<td>0.20</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.43 (1.08–2.00)</td>
<td>1.39 (1.10–1.97)</td>
<td>0.90</td>
</tr>
<tr>
<td>VLDL-C, mmol/L</td>
<td>1.01±0.74</td>
<td>0.84±0.43</td>
<td>0.14</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>233.01±53.42</td>
<td>214.49±33.87</td>
<td>0.04</td>
</tr>
<tr>
<td>Lp(a), mg/dL</td>
<td>16 (6–35)</td>
<td>25 (6–36)</td>
<td>0.72</td>
</tr>
<tr>
<td>Non-HDL, mmol/L</td>
<td>8.59±1.95</td>
<td>7.59±1.04</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data were collected at baseline without any lipid lowering medication. Values for Lp(a) and TG are shown as medians (interquartile range). Values for LDL-C, TC, HDL-C, VLDL, and non-HDL are shown as average values±SD.

ApoB indicates apolipoprotein B; FH, familial hypercholesterolemia; HDL, high density lipoprotein; HDL-C, HDL cholesterol; LDL-C, low density lipoprotein cholesterol; lipoprotein (a); TC, total cholesterol; TG, triglycerides; and VLDL-C, very low density lipoprotein cholesterol.

Figure 4. Xanthoma in familial hypercholesterolemia (FH) patients. Physical stigmata of dyslipidemia observed in FH patients carrying or not the proprotein convertase subtilisin-kexin type 9 (PCSK9) R46L genetic variant included corneal arcus, xanthelasma, and xanthoma (tendinous xanthoma of the fingers, Achilles tendon, plantar, patellar triceps, and tuberous xanthoma). A, Analysis of FH subjects presenting ≥1 xanthoma/patient did not show a significant difference (P=0.09). B, However, FH patients carrying the R46L variant revealed statistically significant lower numbers of xanthoma/patient compared with noncarriers (P<0.0001).

Figure 3. Significant lipid variations in the familial hypercholesterolemia (FH) cohort. Comparison of FH patients carrying a proprotein convertase subtilisin-kexin type 9 (PCSK9) R46L genetic variant with noncarriers revealed significant reduction in circulating low density lipoprotein cholesterol (LDL-C; A, ↓11%), non-high density lipoprotein (HDL; B, ↓12%), apolipoprotein B (ApoB; C, ↓10%), and total cholesterol (TC; D, ↓9%) parameters.

of the sodium channels, resulting in a higher availability of epithelial sodium channel for renal sodium absorption and, finally, leading to an increased risk for hypertension.

Our study has shown that the PCSK9 R46L genetic variation is associated with a significant 12% lower plasma LDL-C concentration compared with noncarriers in FH patients. This difference is similar to the one observed in a non-FH cohort that showed a 15% lower LDL-C concentration in R46L carriers compared with noncarriers.11 Previous studies investigating the effect of the R46L variant on total cholesterol concentration (LDL-C values were not available) in FH patients failed to show a statistically significant difference between the carriers and noncarriers. The authors of this study concluded that inhibiting PCSK9 in FH patients may not be an effective therapeutic approach.29 In this previous Scandinavian study, ≥136 distinct LDLR mutations were present in the cohort, leading to a high genetic heterogeneity. It has been previously well documented that different LDLR mutations have been associated with varying levels of LDL-C concentration, and a population presenting a large heterogeneity in LDLR mutation will also present a larger variability in LDL-C concentration.34 Indeed, some LDLR mutations, such as the class 3 mutations that result in a reduced binding to the LDL, present a phenotype that is less severe than the class 1 mutations, which compromises the precursor production of the protein.35 Because of a genetic founder effect in the French Canadian population,36 our cohort has the distinct advantage of being composed of carriers of a limited number of FH causing mutations, of which 2 (del >15 kb and W66G exon 3) account for 87% of our FH population (Figure 2). This drastically diminishes the effect of the heterogeneity associated with individual FH mutations in our cohort and may explain the differences observed between the 2 studies. To further lower variability associated with LDL-C measurement, baseline lipid profiles from FH patients were obtained without lipid lowering therapy (all lipid lowering medication were washed out for ≥4 weeks before measurements) and the method used to measure LDL-C was the National Heart, Lung, and Blood Institute reference method rather than the more common calculation using the Friedwald formula.

We have also shown that a PCSK9 LOF genetic variation was associated with a decrease in the burden of ectopic cholesterol deposition in patients affected with FH. Tendinous xanthomata is a physical finding that is almost pathognomonic for FH. A previous study in the prestatin era has shown that roughly 70% to 90% of adult FH patients presented tendinous xanthomas.37 In our study, 48% of the control patients presented ≥1 xanthoma compared with only 28% of R46L carriers, although this difference did not reach statistical significance (P=0.09). Nonetheless, a significant difference was observed in the average number of xanthoma per patient between these 2 groups. Indeed, the total number of xanthoma/patient was 0.33 in the R46L group compared with 0.76 in the noncarrier group (P<0.0001). No differences were observed for other physical findings that can be associated with FH although less specific, such as corneal arcus and xanthelasma. Because
ecptic cholesterol deposition (as witnessed by the presence of xanthoma) is associated with a higher risk of atherosclerotic vascular disease.

Our study is the first to show that a PCSK9 LOF genetic variant is associated with a lower LDL-C concentration and a lower risk of atherosclerotic events in patients with FH. Our analysis show that 33% of the FH patients who did not carry the R46L variant had ≥1 vascular event compared with only 11% in the R46L carrier group. Carriers of the R46L PCSK9 variant had an 86% lower risk of presenting ≥1 atherosclerotic event compared with noncarriers (odds ratio, 0.14; 95% confidence interval, 0.032–0.63; P = 0.001). Previous statin intervention trials have shown that a 1 mmol/L reduction in LDL-C is associated with a 21% reduction in the incidence of major cardiovascular events after 5 years. The R46L genetic variant is associated with a 0.84 mmol/L difference compared with noncarriers, and this would translate into an 18% risk reduction. The important difference between the observed and predicted risk reduction is likely explained by the lifelong reduction in LDL-C observed in the R46L carriers because these patients are predicted to have a lower cholesterol-year score compared with noncarriers. The average number of cardiovascular events per individual was also significantly lower in FH subjects who carried the R46L (0.22 compared with 0.70 in noncarriers). These results indicate that the severity or the frequency of recurring events was lower in the R46L group. Taken together, these findings are extremely important for the future treatment of FH patients with therapies targeting PCSK9 using monoclonal antibodies or antisense mRNAs. Our results tend to indicate that inhibiting PCSK9 in an FH population should be at least as efficient to reduce atherosclerotic vascular events as in non-FH patients.

One limitation of our study resides in the low number of R46L variant group among our FH population. Considering that this variant is associated with lower LDL, lower prevalence of xanthoma, and lower prevalence of CVD and that these variables were measured independently, it is unlikely that the observed effects are simply because of statistical chance. Another limitation is caused by a referral bias of our cohort. Indeed, our subjects were selected from a lipid clinic population, and FH patients with low LDL-C were therefore less likely to be referred to our clinic. This could lead to an underestimation of the true prevalence of the R46L polymorphism among FH patients and perhaps underestimation of the effect on LDL-C and cardiovascular risk.

In conclusion, the PCSK9 R46L genetic variation is an important modifier of the FH phenotype. Indeed, our study is the first to show that FH carriers of the R46L LOF PCSK9 variant had significantly lower levels of LDL-C compared with noncarriers. More importantly, our study also showed that carriers of this mutation presented a significantly lower cardiovascular risk even with an FH background. The use of monoclonal antibodies aimed at PCSK9 has successfully led to an average 67% decrease in LDL-C in FH patients with no significant increase in adverse events. Taken together, these results tend to indicate that FH patients could benefit greatly from PCSK9 lowering therapies.

Sources of Funding
This work was supported by the Fondation Leducq Transatlantic Network of Excellence in Cardiovascular Research Program and a start-up grant from the McGill University Hospital Research Center.

Disclosures
Dr Davignon is a consultant or scientific advisor for Amgen, Acasti Pharma, Anthera, Genzyme, Pfizer, Merck, and Valeant and participates in clinical trials for Acasti Pharma, Amgen, Genzyme, Novartis, and Sanofi. Dr Dufour is a consultant or scientific advisor for Amgen, Regeneron, Sanofi, and Valeant and participates in clinical trials for Acasti, Amgen, Pfizer, Isis, Novartis, Orthera, Regeneron, and Sanofi. Dr Baess received research grants from Merck and Astra-Zeneca and participates in clinical trials for Amgen, Pfizer, Isis, Regeneron, and Sanofi.

References
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of the FH phenotype and that future treatment of FH patients with anti-PCSK9 drugs should be effective to lower the cardiovascular disease risk.

Finally, these findings suggest that PCSK9 is an important modifier gene and also lower average number of tendinous xanthoma/patient compared with noncarriers. Importantly, FH patients carrying the PCSK9 R46L variant showed a significant 86% lower risk of cardiovascular disease. Therefore, these findings suggest that PCSK9 is an important modifier gene of the FH phenotype and that future treatment of FH patients with anti-PCSK9 drugs should be effective to lower the cardiovascular disease risk.

Arterioscler Thromb Vasc Biol

Regional distribution and metabolic effect of PCSK9 insLEU and R46L gene mutations and apoE genotype.


Benjamin S, Ryuhs D, Hamelin J, Nossary N, Seidah NG. The pro-protein convertase (PC) PCSK9 is inactivated by furin and/or PC56A: functional consequences of natural mutations and post-translational modifications.


Significance

Familial hypercholesterolemia (FH) is a frequent monogenic disease associated with extremely low low density lipoprotein cholesterol plasma concentrations, ectopic cholesterol deposition (xanthoma), and premature cardiovascular disease. In the last decade, PCSK9 (proprotein convertase subtilisin/kexin type 9) has been shown to be an important regulator of the low density lipoprotein receptor and plasma low density lipoprotein cholesterol concentration. This study analyzed the effect of the PCSK9 R46L genetic variation in a cohort of FH patients with known mutations in the low density lipoprotein receptor gene. R46L carriers presented significant lower circulating concentrations of low density lipoprotein cholesterol (11%) and also lower average number of tendonous xanthoma/patient compared with noncarriers. Importantly, FH patients carrying the PCSK9 R46L variant showed a significant 86% lower risk of cardiovascular disease. Finally, these findings suggest that PCSK9 is an important modifier gene of the FH phenotype and that future treatment of FH patients with anti-PCSK9 drugs should be effective to lower the cardiovascular disease risk.
PCSK9 R46L, Lower LDL, and Cardiovascular Disease Risk in Familial Hypercholesterolemia: A Cross-Sectional Cohort Study
Yascara Grisel Luna Saavedra, Robert Dufour, Jean Davignon and Alexis Baass

Arterioscler Thromb Vasc Biol. 2014;34:2700-2705; originally published online October 2, 2014;
doi: 10.1161/ATVBAHA.114.304406
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Materials and methods

Study population

Among 20434 subjects treated at the IRCM (Institut de Recherches Cliniques de Montréal) lipid clinic for hyperlipidemia, 2654 subjects had a clinical phenotype compatible with familial hypercholesterolemia: LDL-C plasma concentrations above the 95th percentile for age and sex, autosomal familial inheritance of hypercholesterolemia and premature CAD. All patients in our study were found positive for a FH-causing LDLR gene mutation and were classified as having a definite diagnosis of FH according to the Dutch Lipid Clinic Network and the Simon Broome Registry criteria. Blood was obtained from 1267 subjects to be genetically screened for FH causing mutations. Informed consent, approved by the IRCM institutional review board and ethical committees, was obtained from all participants. Data was collected for a fifteen year period. In all cases, medical records provided information on patient characteristics such as age, sex, weight, height, blood pressure, diabetes, glucose, past and current smoking habits, hypertension and statin use.

DNA analysis

DNA was extracted from white blood cells using an automated 340A DNA extractor (Applied Biosystems, Foster City, CA) or QIAMP Blood Maxi Kit (Qiagen, Missisauga, Ont.). A method based upon the polymerase chain reaction (PCR) was developed to analyze LDLR gene mutations and was previously described. Briefly, the screening of mutations were assessed by the use of one set of primer pairs for each selective PCR amplifications of exon 3, 4, 10 and 14; followed by sequencing and specific fragment digestions. The PCR product containing the T → G nucleotide substitution resulting in the mutation Trp87 → Gly (Exon 3) was digested with BslI (New England Biolabs). The C → T substitution resulting in the mutation Glu228 → Lys (Exon 4) was similarly determined by digestion of exon 4 with Mn1I (New England Biolabs). The Y → X (Tyr489 → X) nonsense mutation (Exon 10) was determined by sequencing and PCR fragments from exon 10 indicate a cytosine to guanine transversion converting codon 489 (TAC) encoding for a tyrosine into a TAG-stop codon. The G → A substitution causing a mutation Cys667 → Tyr (Exon 14) was analyzed by competitive oligonucleotide priming by using fluorescent labeled primers. Quantification of fluorescence was analyzed by automated DNA sequencer (AB applied biosystems, Life Technologies) and the relative quantities of the mutant or wild-type genotypes were calculated by integration of fluorescence peaks using the GeneScanner software. The presence of the French-Canadian LDLR of >15 Kb (del 15 Kb) and 5 Kb (del 5 Kb) gene deletions was determined by Southern Blot or alternatively by semi-quantitative PCR assay. Briefly, for southern blotting, DNA was digested with EcoRV and XbaI, digestion fragments separated and probed with a radiolabeled cDNA fragment spanning exons 2 to 5 of the LDLR gene to detect the >15 Kb and the 5 Kb deletions. For semi-quantitative PCR, 200-500 ng of genomic DNA was PCR amplified with two set primers corresponding to the promoter and to exon 3 of the LDLR and one of each set was fluorescently labeled. Relative quantities of amplified DNA corresponding to the promoter and to exon 3 were calculated and compared with the mean of that in three subjects known not to carry either of these mutation. For detection of the PCSK9 R46L missense genetic variant, quantitative PCR was performed using the TaqMan® SNP Genotyping Assay for amplification and detection of specific SNP alleles (AB applied biosystems, Life Technologies).
Technologies). Purified genomic DNA samples were used for amplification of fragments on PCSK9 exon 1 with predesigned primers and two different fluorescent probes were used for detection of the two possible variants being R46 (WT) or the L46 (Missense loss of function mutant). Results analyses performed with the ViiA 7 software v 1.2.2 (AB applied biosystems, Life Technologies).

Lipid and lipoprotein determination

Blood was collected from subjects who had fasted for 12 h overnight. It was drawn under vacuum from an arm vein into tubes containing EDTA (final concentration: 1.5 mg/mL). LDL-C, VLDL-C and HDL-C were assayed by ultracentrifugation at \( d =1.006 \) g/mL according to the reference method described by the NHLBI ⁵. Plasma, lipoprotein cholesterol and triglyceride concentrations were determined enzymatically on an automated analyzer (Abbott Biochromatic Analyzer model 100, Abbott Laboratories, Pasadena, CA). Lp(a) was measured in total plasma with a commercial ELISA kit (Macra EIA Kit, Strategic Diagnostics Industries, Inc., Newark). All treated patients were required to stop all lipid lowering medications for a period of at least 4 weeks prior to lipid measurements. All lipid measurements were performed in the same IRCM laboratory.

Cardiovascular events and xanthoma

Patients were screened for physical stigmata of dyslipidemia such as corneal arcus, xanthelasmas and xanthoma. The later included tendinous xanthoma of the fingers, Achilles tendon, plantar, patellar triceps as well as tuberous xanthoma. Cardiovascular events grouped the following atherosclerotic manifestations: angina, myocardial infarction, coronary angioplasty, coronary artery bypass surgery, claudication, peripheral arterial angioplasty, peripheral artery bypass surgery, stroke, transient ischemic attack and carotid endarterectomy. Data concerning physical stigmata of dyslipidemia and cardiovascular events was obtained in a transverse fashion simultaneously to the lipid profiles.

Statistical analysis

Continuous normally distributed variables were summarized as means +/- SD. Whereas, continuous logarithmic variables were summarized as median +/- interquartile range (e.g. Lp(a), TG). Discrete variables were presented as percentages. Comparisons of risk factor levels between R46L carriers and non-carriers were performed with t-tests for continuous variables and z-tests for discrete variables. Odds ratio and the 95 percent confidence interval were calculated for the risk of presenting at least one cardiovascular event. Cardiovascular events were not adjusted for age, sex, smoking, or statin use because no statistical differences were observed between R46L carriers and non-carriers. Because of the small size of the R46L population sample, no correction was performed for hypertension. Since hypertension is a known cardiovascular risk factor, correcting for this factor would only strengthen the association between the PCSK9 R46L and lower cardiovascular risk.
References


### Supplemental Table I: Cardiovascular events in the FH cohort

<table>
<thead>
<tr>
<th>Event</th>
<th>Non-carriers (n)</th>
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