A new method for measurement of total plasma PCSK9: clinical applications

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Abstract The proprotein convertase subtilisin kexin-9 (PCSK9) circulates in plasma as mature and furin-cleaved forms. A polyclonal antibody against human PCSK9 was used to develop an ELISA that measures total plasma PCSK9 rather than only the mature form. A cross-sectional study evaluated plasma levels in normal (n = 254) and hypercholesterolemic (n = 200) subjects treated or untreated with statins or statin plus ezetimibe. In controls, mean plasma PCSK9 (89.5 ± 31.9 ng/ml) correlated positively with age, total cholesterol, LDL-cholesterol (LDL-C), triglycerides, and fasting glucose. Sequencing PCSK9 from individuals at the extremes of the normal PCSK9 distribution identified a new loss-of-function R434W variant associated with lower PCSK9 values than non-FH (147.01 ± 42.5 vs 127.2 ± 40.8 ng/ml, P < 0.005) and increased in proportion to the statin dose, combined or not with ezetimibe. In treated patients (n = 139), those with familial hypercholesterolemia (FH; due to LDL receptor gene mutations) had higher PCSK9 values than non-FH (147.01 ± 42.5 vs 127.2 ± 40.8 ng/ml, P < 0.005), but LDL-C reduction correlated positively with achieved plasma PCSK9 levels to a similar extent in both subsets (r = 0.316, P < 0.02 in FH and r = 0.275, P < 0.009 in non-FH). The detection of circulating PCSK9 in both FH and non-FH subjects means that this assay could be used to monitor response to therapy in a wide range of patients.—Dubuc, G., M. Tremblay, G. Paré, H. Jacques, J. Hamelin, S. Benjannet, L. Boulet, J. Genest, L. Bernier, N. G. Seidah, and J. Davignon. A new method for measurement of total plasma PSCK9: clinical applications. J. Lipid Res. 2010, 51: 140–149.

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The familial hypercholesterolemia (FH) phenotype is characterized by elevated plasma LDL-cholesterol (LDL-C) levels, xanthomas, and premature atherosclerosis associated with increased risk of coronary artery disease (CAD). FH is caused primarily by loss-of-function (LOF) mutations in the LDL receptor gene (LDLR) responsible for the removal of plasma cholesterol, which is mainly found in LDL particles (1) or in the apolipoprotein B gene (2), the main protein component of LDL. In 2003, Abifadel et al. (3) identified another protein associated with this phenotype, the proprotein convertase subtilisin/kexin-9 (PCSK9) (4).

The human PCSK9 gene (PCSK9) located on chromosome 1p32.3 is ~22 kb long and comprises 12 exons encoding a 692 amino acid protein (5). PCSK9 is expressed mainly in the liver, small intestine, and kidney (4) and is thought to accelerate the degradation of hepatic LDLR in endosomes/lysosomes (6) by direct binding to the epidermal growth factor-like repeat A (EGF-A) domain of the LDLR (7, 8). PCSK9 overexpression in cell lines and mice has been shown to reduce LDLR levels and increase plasma LDL-C (9–12). Similarly, transgenic overexpression of mouse (13) and human PCSK9 (14) in hepatocytes or human PCSK9 in kidney (15) also results in a dramatic reduction of hepatic...
Conversely, inactivation of the mouse Saxon mutation, D374Y, total cholesterol (TC) values to severe hypercholesterolemia. In the most severe Anglo- associated with low LDL-C. GOF mutations result in mild changes are classified as gain-of-function (GOF) mutations when they are associated with high levels of LDL-C and as LOF mutations when associated with low LDL-C. GOF mutations result in mild to severe hypercholesterolemia. In the most severe Anglo-Saxon mutation, D374Y, total cholesterol (TC) values reach as high as 13.1 mmol/L (21). The onset of CAD in patients with D374Y may be 10 years sooner than in heterozygous FH patients with severe LDLR mutations (22). On the other hand, a retrospective study has shown a significantly reduced risk of CAD in carriers of PCSK9 LOF variants R46L (partial LOF) and Y142× or C679× (complete LOF). Together, the latter two nonsense mutations were associated with a 28% reduction of plasma LDL-C and an 88% reduction in the frequency of coronary events (23). While that study supported the cardio-protective role of long-term reduction of cholesterol levels, a direct protective effect of reduced PCSK9 was not excluded. Furthermore, a compound heterozygote for two inactivating mutations (Y142× and R97) in PCSK9 had a strikingly low plasma level of LDL-C (0.36 mmol/L) and no immunodetectable circulating PCSK9 (18). Another individual homozygous for the C679× mutation had a plasma LDL-C of 0.41 mmol/L (24). All these findings support the hypothesis that levels and/or higher activity of plasma PCSK9 modulate the levels of LDL-C and TG, suggesting that long-term lowering of PCSK9 might be beneficial in reducing the incidence of CAD (25).

PCSK9, like the LDLR, is regulated by sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor involved in activation of many genes implicated in cholesterol metabolism (26, 27). This finding is supported by our previous work, in which we showed that in HepG2 cells (a human hepatoma cell line) and human primary hepatocytes, PCSK9 mRNA levels were increased by statins, likely via SREBP-2 (28). Preliminary data on the response of PCSK9 to cholesterol-lowering therapy revealed that statins and fibrates can significantly modify plasma PCSK9 levels (29–31).

In the present study, we measured plasma PCSK9 by ELISA in 254 volunteers and 200 hypercholesterolemic patients. We demonstrated that plasma PCSK9 levels are correlated significantly with age and with levels of TC, LDL-C, triglycerides (TG), and fasting glucose. Moreover, we show that PCSK9 levels are markedly higher in hypercholesterolemic patients than in controls and higher still in patients receiving cholesterol-lowering therapy. Finally, we identified a novel LOF R434W mutant exhibiting lower plasma levels of LDL-C and PCSK9. The mechanism behind such observations is shown to be related to a 3-fold lower secretion rate of PCSK9-R434W from cells and ~70% LOF on its effect on cell surface LDLR.

MATERIALS AND METHODS

Production and purification of anti-PCSK9 antibodies

Recombinant truncated human PCSK9 (rPCSK9; Met-amino acids 31–454) was produced in bacteria and purified as described (6). It was injected into two rabbits by a standard protocol to generate a polyclonal antibody to human PCSK9 (hPCSK9-Ab). The antibodies were first prepurified by precipitation with ammonium sulfate (50% final concentration). After solubilization and dialysis of the precipitate, the antibodies were purified by affinity chromatography using a CNBr-activated Sepharose 4B column (GE Healthcare Bio-Sciences AB, Sweden) coupled with the purified antigen (rPCSK9). A fraction of this purified antibody was conjugated with horseradish peroxidase (hPCSK9-Ab-HRP) using the EZ-Link™ Plus Activated Peroxidase protocol from Pierce (Rockford, IL). Finally, the conjugated antibody was purified from excess free HRP using the FreeZeYme Conjugate Purification Kit (Pierce prod no. 44920).

Immunoprecipitation and immunoblotting

Immunoprecipitations were carried out as previously described (17) using the hPCSK9-Ab and activated agarose beads coupled with goat anti-rabbit IgG (Trueblot™ eBioscience, San Diego, CA) according to the manufacturer’s instructions. Immunoprecipitated proteins were separated on 4–20% gradient acrylamide gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P™, Millipore, Billerica, MA). For immunoblotting, hPCSK9-Ab-HRP was used at a dilution of 1:500. The blots were revealed by chemiluminescence with Pierce SuperSignal™ West Dura on Amersham Hyperfilm™ ECL (GE Healthcare Limited, UK). HepG2 and HuH7 cells were cultured as previously described (17, 28).

Subjects, sample handling, and sequencing

Blood was collected into EDTA-Vacutainer™ tubes after a 12 h fast. Samples were taken from 254 healthy volunteers over 18 years of age who were not taking any medication for hyperlipidemia, hypertension, or diabetes, and from 200 hyperlipidemic patients attending our lipid clinic. Plasma and blood leukocytes were obtained by centrifugation at 3,000 rpm for 15 min at 4°C. Total and lipoprotein cholesterol and TGs were quantitated at the laboratory of the Centre Hospitalier de l’Université de Montréal using a standard enzymatic method on a Bayer Advia multi-analyzer. LDL-C was calculated using the Friedewald equation, except if TG were >4.5 mmol/L. (n = 6) (32). All subjects gave informed written consent and the Institut de recherches cliniques de Montréal (IRCM) ethics committee approved this protocol. DNA was extracted from white blood cells using QIAmp Blood Maxi kit (Qiagen, Missisauga, ON) according to the manufacturer’s instructions. The sequences of the primers used for amplifying all 12 exons were obtained from NCBI at http://www.ncbi.nlm.nih.gov/genome/probe/using the resequencing amplicons for PCSK9. The amplified fragments were purified from agarose gels using QIAquick Gel Extraction kit (Qiagen, Missisauga, ON) and sequenced on a 3130 XL Genetic analyzer from ABI (Applied Biosystems, Foster City, CA) using M13 sequencing primers. Sequences were

Human plasma PCSK9, its natural mutants, and cholesterol-lowering drugs
ELISA assay

LumiNunc Maxisorp white assay plates (Nunc, Denmark) were coated with 0.5 μg/well of hPCSK9-Ab by incubation at 37°C for 3 h in PBS (NaPO₄ 10 mM, NaCl 0.15 M, pH 7.4) containing NaN₃ (1 g/L) then stored at 4°C. The plates were washed six times before use with PBS containing Tween 20 (0.5 mL/L) and then incubated for 1 h at room temperature with blocking buffer (PBS, casein 0.1%, merthiolate 0.01%). Calibrators were prepared using serial dilutions of rPCSK9 in dilution buffer (PBS, urea 1.8 M, BSA 0.25%, Tween 20 0.5 mL/L, and merthiolate 0.01%). Samples were diluted 1:20 in dilution buffer without BSA. Calibrators and samples were incubated for 30 min in a water bath at 37°C prior to plate addition (100 μL) in duplicate. The plates were incubated overnight at 37°C with shaking. After washing, 100 μL of hPCSK9-Ab-HRP diluted 1:750 was added for 3 h at 37°C with shaking. Finally, after washing, 100 μL of substrate (SuperSignal™ ELISA Femto Substrate, Pierce) was applied to each well. Chemiluminescence was quantitated on a PerkinElmer luminometer (BMG Labtech).

Statistical analysis

Spearman correlation coefficients (r) were used to assess the relationship between variables. Data were analyzed with GraphPad Prism software and significance defined as P < 0.05 (two-sided). Stepwise regression analysis was performed by the statistical department of the Université de Montréal using SPSS software, version 15. ANOVA was used to determine drug dosage relationship between variables. Data were analyzed with GraphPad Prism software and significance defined as P < 0.05 (two-sided).

Functional analysis of the novel natural mutant R434W

HEK293 cells were transiently transfected with pIRES-cDNAs coding for WT PCSK9, or the novel variant R434W, tagged at the C terminus with a V5-antigen (4). Forty-eight hours post-transfection, the cells were pulsed for 4 h with 35S-(Cys+Met). Cell lysates and media were then immunoprecipitated with a V5 mAb and the precipitates separated by SDS-PAGE (8%) and analyzed by autoradiography, as reported (4, 10). To define the ability of the R434W mutant to degrade LDLR, 24 h spent media were prepared from HEK293 cells transiently transfected with each construct. An ELISA assay of the media defined the amount of PCSK9 secreted for each construct. The media were then incubated with HuH7 cells for 1 h or overnight and the cells were washed, detached in 0.5 mM EDTA (Versene, Gibco), and subjected to fluorescence-activated cell sorting (FACS) analysis using an LDLR-specific monoclonal antibody (C-7 mAb, 1:100 dilution, Santa-Cruz, CA), thus quantitating the levels of cell-surface LDLR.

RESULTS

Anti-PCSK9 antibody recognizes both mature and furin-cleaved PCSK9 in human plasma

Using affinity-purified hPCSK9-Ab, Western blot analysis of human PCSK9 in the culture media of HEK293 cells overexpressing hPCSK9 revealed the mature (~60 kDa) and furin-cleaved PCSK9 (PCSK9-ΔN216, ~53 kDa) forms (17), none of which were observed in control cells expressing an empty pIRES vector. Similar immunoreactive PCSK9 forms are also observed in the plasma of three different individuals (Fig. 1).

Validation of the ELISA to measure PCSK9 in human plasma

We designed an ELISA to measure circulating levels of PCSK9 in human plasma samples using an affinity-purified hPCSK9-Ab polyclonal antibody. Part of this antibody was conjugated to HRP and excess HRP was removed by affinity chromatography. A linear standard curve was established with culture media and serial 1:2 dilutions of the media of HEK293 cells overexpressing recombinant human PCSK9 (r² = 0.997). This culture medium was calibrated with respect to purified rPCSK9 characterized by quantitative amino acid analysis. Intra- and inter-assay variation coefficients of plasma samples were 1.6% (n = 33) and 7.5% (n = 48), respectively. A spike and recovery assessment was performed in two different plasma samples containing very low endogenous PCSK9. Five different quantities of recombinant PCSK9 (1–10 ng) were added to plasma and mean recoveries were 90, 88, 94, and 98%, respectively. A plasma sample having a high PCSK9 concentration presented a 10% variation within a dilution range of 1:20 to 1:80. Three different plasma samples were subjected to three freeze-thaw cycles (~80°C to room temperature) and their PCSK9 concentration varied by ~3%, which is within the variability range of the assay. Interestingly, no detectable immunoreactive PCSK9 was present in urine or saliva.

Fig. 1. Specificity of hPCSK9-Ab for native PCSK9 in culture medium and plasma. Immunoprecipitation was carried out with polyclonal hPCSK9-Ab against culture media of HEK293 cells respectively transfected with vector alone (pIRES), PCSK9, PCSK9, and furin, and against plasma from three different individuals. Immunoprecipitates were separated by a 4-20% polyacrylamide gradient SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and revealed with hPCSK9-Ab-HRP. PCSK9-ΔN216 represents PCSK9 cleaved by furin (17).
ELISA assay of PCSK9: identification of a novel R434W LOF variant

We collected plasma from 254 healthy volunteers, 117 males and 137 females. Clinical characteristics, fasting plasma lipids, and PCSK9 levels are shown in Table 1. There was no significant difference in plasma PCSK9 levels or in any other variables measured between men and women in this sample (Mann Whitney U test). Distribution of plasma PCSK9 levels measured by ELISA was skewed toward higher values in both men and women (supplementary Fig. 1). The mean concentration was 89 ± 32 ng/ml (range, 35–225 ng/ml), and it did not differ significantly between men and women (85 ± 27 ng/ml and 93 ± 35 ng/ml, respectively) (supplementary Fig. 1). Combining both genders, we sequenced PCSK9 in subjects whose plasma values were at the extreme of the frequency distribution, i.e., values < 60 ng/ml or > 150 ng/ml (Fig. 2). This revealed that 5/37 persons exhibiting low PCSK9 levels also showed either a known hypocholesterolemic variation/polymorphism [R46L (3, 19) and double A53V + L10 (19, 34)] or a new, previously unreported variant R434W with no other mutations in the sequence (Fig. 2).

Biosynthetic analysis using a 4 h pulse with 35S-[Met+Cys] of HEK293 cells overexpressing the R434W mutant or the WT sequence revealed that the autocatalytic zymogen cleavage of proPCSK9 to PCSK9 is slightly reduced and the PCSK9 secretion was lower in the R434W mutant (Fig. 3A). Calculations revealed an ~2.5-fold lower level of PCSK9 in the 4 h medium. These data were further confirmed by ELISA assays, which showed at steady state a 3-fold lower level of secreted PCSK9-R434W (0.60 µg/ml) compared with the WT protein (1.82 µg/ml) (Fig. 3B). To compare the ability of PCSK9 to that of the R434W variant to degrade LDLR, we incubated overnight human hepatic HuH7 cells revealed that compared with WT PCSK9, the R434W variant had ~30% of the LDLR-lowering activity, suggesting a partial LOF (Fig. 3C). Thus, the heterozygote variant PCSK9-R434W results in ~2-fold lower circulating levels of PCSK9 (Fig. 2), rationalized by a ~3-fold lower secretion rate of the protein (Fig. 3B) and a ~40% lower LDL-C (Fig. 2) likely related to its LOF on LDLR degradation (Fig. 3C).

Plasma PCSK9 levels correlate with cholesterol

Statistical analysis showed significant correlations between PCSK9 levels and TC (r = 0.382, P < 0.001), LDL-C (r = 0.351, P < 0.001), TG (r = 0.356, P < 0.001), fasting glucose (r = 0.354, P < 0.001), age (r = 0.376, P < 0.001), and body mass index (BMI) (r = 0.264, P < 0.001) (Fig. 4). No significant correlation was observed between PCSK9 levels and HDL-cholesterol (HDL-C) (r = −0.074, P = 0.239). Hormone treatment such as oral contraceptives (n = 19) or hormone replacement therapy (n = 7) had no detectable effect on plasma PCSK9 levels (not shown).

Stepwise regression: a model to predict PCSK9 value

Because many of the parameters studied are colinear, such as TC, LDL-C, and HDL-C, we performed a multiple stepwise regression using SPSS software. This stepwise regression selects variables to include in a regression model for the purpose of identifying an optimal subset of predictors. The best model to predict PCSK9 levels showed that TC (β = 8.84), fasting glucose (β = 11.58), TG (β = 9.19), gender (β = −10.14 for men), and age (β = 0.18) were parameters that had a significant influence on PCSK9 levels in this multiple regression model. This model explains ~27% of the PCSK9 variability among individuals in our sample.

| TABLE 1. Clinical characteristics, fasting plasma lipids, and PCSK9 levels of 254 healthy volunteers (mean ± SD) |
|---------------------------------------------------------------|-------------------|-------------------|
| All Subjects (min–max) | Men (min–max) | Women (min–max) |
| Age (years) | 42 ± 13 (20–77) | 41 ± 13 (20–77) | 43 ± 12 (21–69) |
| BMI (kg/m²) | 24.5 ± 4.3 (16.2–41.6) | 25.6 ± 4.0 (17.6–38.8) | 23.4 ± 4.4 (16.2–41.6) |
| PCSK9 (ng/ml) | 89.3 ± 31.9 (35.3–225.2) | 85.5 ± 26.9 (35.3–172.1) | 93.1 ± 35.4 (47.4–225.2) |
| TC (mmol/L) | 4.80 ± 0.90 (2.83–7.30) | 4.90 ± 0.89 (3.11–7.05) | 4.83 ± 0.91 (3.13–7.30) |
| TG (mmol/L) | 1.17 ± 0.62 (0.34–4.30) | 1.22 ± 0.64 (0.45–4.30) | 1.12 ± 0.59 (0.34–3.86) |
| LDL-C (mmol/L) | 2.84 ± 0.80 (1.17–4.72) | 3.01 ± 0.80 (1.17–4.72) | 2.60 ± 0.78 (1.36–4.59) |
| HDL-C (mmol/L) | 1.50 ± 0.38 (0.80–2.60) | 1.33 ± 0.32 (0.80–2.36) | 1.64 ± 0.36 (0.83–2.60) |
| TC-HDL-C | 3.44 ± 1.02 (1.70–6.79) | 3.85 ± 1.03 (2.00–6.79) | 3.08 ± 0.88 (1.70–6.19) |
| Glucose (mmol/L) | 4.84 ± 0.61 (3.60–6.70) | 4.89 ± 0.63 (3.60–6.70) | 4.70 ± 0.58 (3.60–6.70) |
Statins and ezetimibe upregulate plasma PCSK9

We measured plasma PCSK9 in 200 patients attending the lipid clinic at the IRCM, a tertiary care lipidology reference center. Fifty-nine patients were not on medication, 98 were on statin treatment (55 on atorvastatin, 27 on rosuvastatin, 14 on simvastatin, and 2 on pravastatin), 39 were on a statin-ezetimibe combination, and 4 were on ezetimibe treatment only. All patients had been treated for at least 3 months. There was no significant difference in the correlation between PCSK9 and LDL-C changes according to statin type, nor was there a gender difference in the relationship between PCSK9 and the reduction in LDL-C. However, a significant correlation between plasma PCSK9 and the percent reduction of LDL-C from baseline (before drug treatment) was observed ($r = 0.341, P < 0.0001$) (Fig. 5). A positive correlation between PCSK9 plasma levels and change in LDL-C was also seen when we looked at each statin separately ($r = 0.21, 0.28$, and $0.4$ for atorvastatin, rosuvastatin, and simvastatin, respectively) (not shown). For the two statins (atorvastatin and rosuvastatin) for which we had a sufficient number of patients, we observed a significant increase in PCSK9 with increasing statin dose in the absence of ezetimibe. When atorvastatin was increased from 5 to 80 mg, plasma PCSK9 levels increased from $109 \pm 33$ to $142 \pm 35$ ng/ml ($n = 53, P$ for trend $= 0.0001$ by ANOVA) and for rosuvastatin from 5 to 40 mg, plasma PCSK9 increased from $123 \pm 23$ to $168 \pm 84$ ng/ml ($n = 28, P$ for trend $= 0.0001$ by ANOVA). In treated patients ($n = 139$) FH ($n = 51$) subjects had higher PCSK9 values than non-FH ($n = 88, 147 \pm 42$ vs. $127 \pm 41$ ng/ml, $P < 0.005$). However, plasma PCSK9 levels and LDL-C reduction correlated positively to a similar extent in both subsets ($r = 0.316, P < 0.02$ in FH and $r = 0.275, P < 0.009$ in non-FH). These data support the hypothesis that treat-
patients on treatments to lower LDL-C levels exhibited even higher plasma PCSK9 levels. In view of the upregulation of LDL-C by PCSK9, this could potentially result in an otherwise dampened response to these treatments.

**DISCUSSION**

PCSK9 is now recognized as an important contributor in cholesterol homeostasis and has become a promising target for cholesterol-lowering therapy and CAD prevention (25, 35, 36). Herein, we report a new sandwich ELISA to measure human plasma PCSK9 concentrations using a polyclonal antibody. With this method, plasma PCSK9 levels in a healthy sample of population average ~90 ng/ml (ranging from 35.3 to 225.2 ng/ml) (Fig. 2) and are significantly correlated with both TC and LDL-C levels (Fig. 4A, B).

The significant correlation with TGs, though intriguing, agrees with a very recent report on a large multieth-
To glucose, it seems that factors that contribute to insulin resistance such as TGs and BMI are associated with higher circulating levels PCSK9 (37). Furthermore, it was also recently reported that in mice, PCSK9 deficiency reduces postprandial triglyceridemia and enhances the hepatic clearance rate of chylomicrons (38). The underlying mechanism(s) requires more extensive studies. Three other groups also developed ELISAs for PCSK9 using different approaches, species, and/or antigens to produce the polyclonal antibodies (14, 30, 39). One report presented a mean value of ~200 ng/ml in plasma with a range of 50–600 ng/ml (14), another a range of 11–115 ng/ml in serum (no mean value given) (39), and the third, a mean value of ~4 µg/ml and a range of 0.1–9.3 µg/ml (30). Using a different method involving immunoprecipitation, immunoblotting, and densitometry, Mayne et al. (40) obtained a mean concentration of plasma PCSK9 of 6.1 µg/ml, which is about 50 times the value found in this study but similar to that reported by Lambert et al. (30). The differences are likely due to sample, methodology, antibody specificities, and the standard used for the absolute quantitation of PCSK9. We used purified full-length human PCSK9 as our reference standard; this was confirmed by amino acid sequence analysis and mass spectrometry and quantitated by amino acid composition of a known weighed sample. Only our assay recognizes both active and furin-inactivated forms of PCSK9 (Fig. 1), offering the potential to measure the ratio of both forms in relation to drug effects and in response to physiological modulators.

While Mayne et al. (40) found significant correlations between PCSK9 and cholesterol levels in men only within a cohort of 182 individuals, we did not confirm this finding in our cohort of 254 individuals, because we found a significant correlation with cholesterol levels in both men ($P < 0.001$) and women ($P < 0.001$) (Table 2). This difference may be in part related to the age of the population sampled [mean age of 53 (40) or 42 in the present study]. However, in a much larger cohort comprising 3,138 individuals, Lakoski et al. (37) reported that circulating PCSK9 levels are slightly higher in premenopausal women than in postmenopausal women and that it is ~15% higher in women versus men. Given the physiological role of PCSK9 in degrading LDLR, the observed correlation with LDL-C was expected. Cholesterol metabolism is profoundly modified during aging, and LDL-C increases by ~40% from 20 to 60 years of age (41). Other physiological changes occur with age, such as reduced physical activity, redistribution of body fat with a relative increase in visceral adipose mass, decreased insulin sensitivity, and increased blood pressure. Thus, a correlation between PCSK9 and age may explain, in part, correlations with other parameters modulated by age.

This study first demonstrated that measurements of plasma PCSK9 levels can help in the identification of novel PCSK9 variants using our strategy. Indeed, we identified a new variant, R434W (Fig. 2). Biosynthetic analysis of this variant revealed that it results in a slightly reduced zymogen activation of proPCSK9, a lower secretion rate by ~50%, and a ~70% lower activity of the PCSK9-enhanced degradation of LDLR in HuH7 cells (Fig. 3). The ~3-fold lower secretion rate observed in HEK293 cells (Fig. 3A, B) correlate with the ~50% lower levels of circulating PCSK9-R434W in this heterozygote subject, which is 51 ng/ml versus the mean value of $89 \pm 32$ ng/ml in normal subjects (Fig. 2). The LOF of the PCSK9-R434W mutation is intriguing, as this residue does not seem to be implicated in the direct interaction of the catalytic domain of PCSK9 with the EGF-A domain of the LDLR (8). The R434W variant occurs in a loop structure occurring in a hinge region between the catalytic domain and the Cys-His rich domain of PCSK9 (42) (supplementary Fig. II). However, because the Cys-His rich domain is critical for the sorting of the complex LDLR-PCSK9 toward endosomes/lysosomes for degradation (7), the R434W mutation may hamper such a process and hence result in a LOF (supplementary Fig. II).

The Leu insertion in the signal peptide of PCSK9 (p. L15ins1L or L10) is often associated with another variation, namely A53V, and this double variant has been reported to be associated with low plasma PCSK9 levels, a finding that supports the hypothesis that a modification in the signal peptide could subtly interfere with protein folding, processing, and/or secretion (43). A high level of PCSK9 (222 ng/ml) was observed in an hypercholesterolemic patient of African descent exhibiting the single variant A443T. Although rare in the Caucasian population, the A443T mutation is relatively frequent in African-Americans, where the heterozygous condition is associated with a wide range of cholesterol values (19). The PCSK9 mutation A443T has been reported in a case of mild and variable hypercholesterolemia (GOF), which is sensitive to diet, but the mutation did not segregate with the phenotype in the

| TABLE 2. Relationship between plasma PCSK9 and individual variables in men and women |
|----------------------------------|-----------|-----------|-----------|
|                                 | All Subjects (n = 254) | Men (n = 117) | Women (n = 137) |
| Age (years)                     | 0.376     | < 0.001   | 0.276     | 0.003    | 0.448     | < 0.001   |
| BMI (kg/m²)                     | 0.264     | < 0.001   | 0.280     | 0.002    | 0.347     | < 0.001   |
| TC (mmol/L)                     | 0.382     | < 0.001   | 0.404     | < 0.001  | 0.381     | < 0.001   |
| TG (mmol/L)                     | 0.356     | < 0.001   | 0.304     | < 0.001  | 0.411     | < 0.001   |
| LDL-C (mmol/L)                  | 0.351     | < 0.001   | 0.370     | < 0.001  | 0.372     | < 0.001   |
| HDL-C (mmol/L)                  | −0.074    | 0.239     | −0.037    | 0.691    | −0.174    | 0.042     |
| TC-HDL-C (mmol/L)               | 0.273     | < 0.001   | 0.303     | < 0.001  | 0.422     | < 0.001   |
| Glucose (mmol/L)                | 0.354     | < 0.001   | 0.230     | 0.013    | 0.466     | < 0.001   |
family (44). However, in homozygotes, it is associated with low levels of LDL-C (19, 44). Indeed, Benjannet et al. (17) showed in vitro that the A435T mutation resulted in an Oglycosylated protein that was more susceptible to furin cleavage, suggesting a LOF.

One of the main findings in this study was the high levels of PCSK9 in patients treated with two widely used cholesterol-lowering agents, statins and ezetimibe. We have already demonstrated that PCSK9 mRNA is upregulated in vitro by statins (28) via a pathway involving reduced intracellular cholesterol concentration and increased SREBP-2 levels (26, 27, 31). The present work supports the hypothesis of the in vivo upregulation of PCSK9 protein in plasma in response to statins. This is in line with the observation of Careskey et al. (31) that plasma PCSK9 increases as LDL-C goes down during 16 weeks of atorvastatin therapy. We also showed that plasma PCSK9 is even higher when ezetimibe and statin are used in combination (Fig. 6).

We surmise that the correlation between PCSK9 and the percent reduction in LDL-C is driven by the inhibition of cholesterol synthesis, as seen in vitro. One group reported the effect of statins when the PCSK9 gene was deleted in the mouse (16) and another when the gene was disrupted by a LOF mutation in humans (45). Both groups observed increased sensitivity to statin treatment when PCSK9 was either not expressed or expressed at low levels. Accordingly, to counteract the effect of cholesterol-lowering agents on PCSK9, it is likely that a potent inhibitor of PCSK9 used in combination with statins would greatly enhance LDL-C lowering.

Addition of ezetimibe, an inhibitor of intestinal cholesterol absorption, was associated with still higher PCSK9 levels (Fig. 6). Although PCSK9 mRNA is expressed in the small intestine (4), nothing is yet known of the function of PCSK9 in this tissue, except that, at least in the mouse, it is not a major contributor to circulating PCSK9 (13). Therefore, we cannot determine whether the high levels of plasma PCSK9 associated with ezetimibe treatment arise from increased production in the intestine or in the liver, the main PCSK9 producer. One possibility would be to test this drug on tissue-specific knockout mice lacking PCSK9 expression specifically in hepatocytes (13). Another group measured PCSK9 mRNA in peripheral blood mononuclear cells in 24 subjects and found a 5.5% increase after 2 weeks of combination treatment with 40 mg simvastatin and 10 mg ezetimibe versus baseline (46). In accordance with this observation, four of our patients who were on ezetimibe alone had a mean PCSK9 concentration of 105 ng/ml, equivalent to that of untreated hypercholesterolemic patients. Finally, it was recently shown that monotherapy with fibrates also resulted in a significant downregulation of plasma PCSK9 by ~8.5%, paralleling the efficacy of fibrates in decreasing LDL-C and TG (29).

It was also suggested that treatment with fibrates can suppress the induction of PCSK9 by statins while still resulting in lower LDL-C (47).

In conclusion, we report for the first time an ELISA method that measures total PCSK9 in plasma, which also allows the estimation of the relative proportion of the mature protein and the fragment cut by furin by Western blot. This method allowed the identification of a novel R434W variant exhibiting a partial LOF on LDLR degradation. This report is also the first demonstration that ezetimibe administration in combination with a statin is associated with a markedly high PCSK9 level, consistent with the ability of ezetimibe to significantly enhance the statin effect on LDL-C, as evidenced by a meta-analysis (48). If the high LDL-C concentrations still observed in patients during treatment reflect an increase in PCSK9 levels, drugs aimed at reducing PCSK9 expression would be expected to greatly enhance current cholesterol-lowering therapies. With the rapid pace of discoveries in the field, it is hoped that within a few years lead molecules reducing the level and/or activity of PCSK9 will be uncovered and that these will emerge for therapeutic use after clinical trials to assess their potency and safety. In that context, it was recently shown that administration of a monoclonal antibody that blocks the PCSK9-LDLR interaction to either mice or cynomolgus monkeys results in an ~80% drop in LDL-C lasting 10 days and that in mice, such an effect enhances the LDL-C lowering by the statin mevinolin (49).

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