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Serum Proprotein Convertase Subtilisin Kexin Type 9 Is Correlated Directly with Serum LDL Cholesterol

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Background: **Proprotein convertase subtilisin kexin type 9 (PCSK9) is gaining attention as a key regulator of serum LDL-cholesterol (LDLC). This novel serine protease causes the degradation of hepatic LDL receptors by an unknown mechanism. In humans, gain-of-function mutations in the *PCSK9* gene cause a form of familial hypercholesterolemia, whereas loss-of-function mutations result in significantly decreased LDLC and decreased cardiovascular risk. Relatively little is known about PCSK9 in human serum.**

Methods: **We used recombinant human PCSK9 protein and 2 different anti-PCSK9 monoclonal antibodies to build a sandwich ELISA. We measured PCSK9 and lipids in 55 human serum samples and correlated the results. We used the anti-PCSK9 antibodies to assay lipoprotein particle fractions separated by sequential flotation ultracentrifugation.**

Results: **Serum concentrations of PCSK9 ranged from 11 to 115 μg/L and were directly correlated with serum concentrations of LDLC ($r = 0.45$, $P = 0.001$) and total cholesterol ($r = 0.50$, $P = 0.0003$), but not with triglycerides ($r = 0.15$, $P = 0.28$) or HDL cholesterol concentrations ($r = 0.13$, $P = 0.36$). PCSK9 was not detectable in any lipoprotein particle fraction, including LDL.**

Conclusions: **PCSK9 is present in human serum, likely not associated with specific lipoprotein particles. The circulating concentrations of human PCSK9 are directly correlated with LDL and total cholesterol concentrations.**

The novel serum protease proprotein convertase subtilisin kexin type 9 (PCSK9)[1] is rapidly gaining attention as a potential key regulator of serum LDL cholesterol (LDLC). [For recent reviews see Refs. *(1–3)*.] PCSK9 is a protease made by the liver and thought to degrade hepatic LDL receptors (LDLR) *(4–10)*. The mechanism by which PCSK9 degrades LDLR is not completely understood and is likely to be complex *(1–10)*. When LDLR concentrations are decreased, the liver is less able to bind LDL from the circulation and serum LDLC increases. As a result, gain-of-function and loss-of-function mutations in the *PCSK9*[2] gene have dramatic effects on serum LDLC concentrations in humans.

Patients with activating mutations of *PCSK9* have severe familial hypercholesterolemia and accompanying increased cardiovascular risk *(11–14)*. These mutations in *PCSK9* account for the approximately 10%–25% of familial dominant hypercholesterolemia cases that cannot be explained by mutations affecting the LDLR or apolipoprotein B *(11–14)*. In contrast, heterozygous patients with loss-of-function mutations in *PCSK9* have significantly decreased concentrations of LDLC in serum and dramatically decreased cardiovascular risk *(15–18)*. Approximately 2% of African Americans carry such mutations, with an accompanying 80%–90% decreased risk of serious cardiovascular events *(19)*. Recently the first compound heterozygote for *PCSK9* loss-of-function mutations was described. This patient, a healthy 32 year-old woman, had an extremely low serum LDLC of 140 mg/L (14 mg/dL) and no detectable PCSK9 protein as assessed by immunoprecipitation and Western blotting *(17)*.

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[1] Nonstandard abbreviations: PCSK9, proprotein convertase subtilisin kexin type 9; LDLC, LDL cholesterol; LDLR, LDL receptor; HRP, horseradish peroxidase; TBST, Tris-buffered saline-casein blocking buffer containing 1 mL Tween 20/L.

[2] Human gene: *PCSK9*, proprotein convertase subtilisin/kexin type 9.

These remarkable findings highlight the need for a robust and easy-to-perform immunoassay for measuring PCSK9 in human serum. Such an assay would allow comparison of LDLC and PCSK9 concentrations, shedding light on how important PCSK9 is in regulation of LDLC. Such an assay might also allow the prediction of which patients might best respond to a PCSK9 inhibitor, if one were to become available. In this report, we describe in detail a dual monoclonal antibody sandwich ELISA to measure PCSK9 in human serum and correlate the measured PCSK9 concentrations with LDL and total cholesterol concentrations.

Materials and Methods

SERUM SAMPLES, ROUTINE CLINICAL CHEMISTRY ANALYSIS, AND LIPOPROTEIN FRACTIONS

From Bioreclamation, we obtained 55 human serum samples from otherwise healthy donors (26 from males and 29 from females; age range 22–86 years, mean 45 years) whose total and LDLC concentrations happened to span a relatively broad range. These samples were received frozen on dry ice and stored at −70 °C. We used a Hitachi Chemistry System (Roche Diagnostics) to measure serum triglycerides and total cholesterol concentrations and HDL cholesterol, which was measured by use of a direct homogeneous assay. These results were used to calculate serum LDLC. Highly purified lipoprotein fractions were obtained from Athens Research and Technology, with VLDL collected from 0.95–1.006 kg/L and LDL from 1.02–1.063 kg/L.

RECOMBINANT PCSK9 GENERATION, PRODUCTION OF ANTI-PCSK9 ANTIBODIES, AND LABELING OF ANTIBODIES

Recombinant human PCSK9 protein was expressed and purified by use of previously described methods *(18)*. Briefly, human PCSK9 was cloned from a human liver cDNA library with a resulting construct (with a C-terminal HIS tag) used to generate an HEK293 stable cell line overexpressing PCSK9. The PCSK9 protein was purified using Ni-nitriloacetic acid followed by size-exclusion chromatography. Identity of the protein was confirmed by N-terminal sequencing, and purity was judged to be >95% based on SDS-PAGE followed by Coomassie blue staining. Once purified, the recombinant protein was divided into aliquots and stored at −70 °C. Rabbit polyclonal antibody against recombinant full-length PCSK9 protein for use in Western blotting was produced by injecting rabbits with purified recombinant human PCSK9 protein and affinity-purifying the antisera obtained against the full-length immunogen. In addition, 3 different monoclonal anti-PCSK9 antibodies (anti-PCSK9-1, anti-PCSK9-2, and anti-PCSK9-3) were produced by immunizing mice with purified recombinant human PCSK9 protein. All antibodies were evaluated for their ability to immunoprecipitate recombinant and endogenous PCSK9 protein from human serum. It is not known at this time which epitopes of PCSK9 these antibodies recognize. Approximately 1 mg of each antibody was horseradish peroxidase (HRP)-labeled using a Pierce assay for use in Western blotting and ELISA experiments. After the labeling was performed, HRP-labeled antibodies were diluted in 50% glycerol and stored at −20 °C.

IMMUNOPRECIPITATION OF PCSK9

For each immunoprecipitation, 100–500 μL of serum or lipoprotein fraction were added to 900–4500 μL of immunoprecipitation buffer (50 mmol/L HEPES, pH 7.40, 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 mmol/L EDTA, 5 mmol/L EGTA). PCSK9 was immunoprecipitated overnight with 1 μg of anti-PCSK9-3 antibody coupled to protein G beads. Afterward, beads were washed twice with immunoprecipitation buffer, and 40 μL of 2× sample buffer (100 mmol/L Tris, pH 6.80, 40 g/L SDS, 200 mL/L glycerol, 20 mg/L bromphenol blue, 15 g/L dithiothreitol) were added to each tube. Samples were vortex-mixed, boiled for 5 min, and stored at −20 °C before analysis.

WESTERN BLOTTING

Samples were loaded onto SDS-polyacrylamide gels. Colored molecular weight markers (Invitrogen) were run on each gel. Proteins were separated for 1 h at 175 V at room temperature and transferred to ECL nitrocellulose paper (Amersham) for 1 h (100 V, 4 °C). Nitrocellulose blots were blocked for 1 h at room temperature in Tris-buffered saline-casein blocking buffer (Pierce) containing 1 mL Tween 20/L (TBST). After blocking, blots were probed with polyclonal HRP-labeled anti-PCSK9 antibody in blocking buffer for 1 h at room temperature. Blots were washed 3 times (10 min each) with TBST (10 mmol/L Tris, pH 7.40, 150 mmol/L NaCl with 1 mL Tween 20/L). After washing, blots were developed with ECL reagent (Amersham). After air-drying, blots were exposed to Bio-Max x-ray film (Kodak).

ELISA DESIGN

An ELISA was constructed using the reagents described above. Briefly, wells were coated overnight (Pierce carbonate-bicarbonate coating buffer, pH 9.40) with anti-PCSK9-3 antibody at a concentration of 5 mg/L. The following day, wells were aspirated, washed 3 times with TBST, and blocked for 1 h with TBS-casein blocking buffer (Pierce). Next, 100 μL of recombinant PCSK9 standards (varying concentrations of recombinant protein in assay buffer consisting of 50 mmol/L HEPES, pH 7.40, 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 mmol/L EDTA, and 5 mmol/L EGTA) were added to the wells to generate a calibration curve. Afterward, serum samples were diluted 1:15 in assay buffer, added to their respective wells, and the ELISA plate was allowed to incubate for 2 h at room temperature. Following aspiration, wells were washed 3 times with TBST, and 100 μL of a 1:1000 dilution of conjugate antibody (HRP-labeled anti-PCSK9-1 antibody, 1 mg/mL) were added to the wells for a 1-h

incubation at room temperature. Following aspiration, wells were washed 3 times with TBST. After the last aspiration of TBST, 100 μL of 3,3′,5,5′-tetramethylbenzidine development substrate (Pierce) was added to the wells and allowed to incubate for 30 min at room temperature. The reaction was stopped with an equal volume of 2 N phosphoric acid, and plates were read at 450 nm. In all ELISA experiments, SigmaPlot version 8.0 was used for fitting of the calibration curves. PCSK9 ELISA dilution curves for the recombinant standard and human serum samples were determined to be parallel, and the ELISA also demonstrated excellent dilutional linearity for serum samples over a range of 1:10 to 1:80.

DATA ANALYSIS

SigmaPlot version 8.0 was used for fitting of the calibration curves for the PCSK9 ELISA. Data were plotted using the program FigP (Biosoft). Statistical analysis was performed using the same program.

Results

The overall strategy for characterizing endogenous and recombinant PCSK9 protein is depicted in Fig. 1, which shows that the anti-PCSK9 monoclonal antibodies were able to immunoprecipitate recombinant PCSK9 added to 500 μL of human serum. In addition, endogenous PCSK9 was also successfully immunoprecipitated from 500 μL of normal human serum. Interestingly, for the recombinant PCSK9, all 3 antibodies performed about equally for protein recognition, but for endogenous PCSK9 recognition, anti-PCSK9-3, and anti-PCSK9-1 antibodies were somewhat superior to anti-PCSK9-2 antibody.

In light of the role PCSK9 plays in degradation of the hepatic LDLR, we examined if PCSK9 targets the LDLR by being a component of LDL itself. To test this idea, we determined the lipoprotein particle distribution of PCSK9 by immunoprecipitating highly purified VLDL, HDL, LDL, intermediate-density lipoprotein, and chylomicron particle fractions or unfractionated serum with anti-PCSK9-3 antibody. In these experiments, minimal PCSK9 was detected by subsequent Western blotting in any of the lipid fractions (data not shown), suggesting that PCSK9 is not LDL-associated. To further address this question, fast-protein liquid chromatography experiments were performed and the LDL fractions were analyzed for PCSK9. Again, minimal PCSK9 could be detected in the LDL fractions (data not shown), further suggesting that PCSK9 is not LDL-associated. These results are consistent with the idea that although PCSK9 is clearly able to bind the LDLR, its structural motifs do not indicate a special avidity for binding lipoprotein particles.

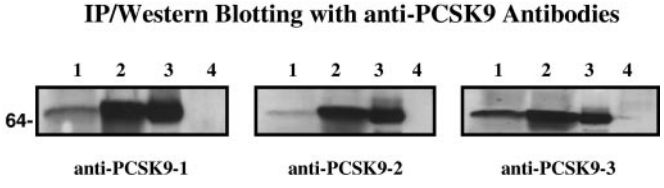

Fig. 1. Characterization of recombinant and endogenous human PCSK9 and anti-PCSK9 monoclonal antibodies.

Recombinant PCSK9 (0.1 μg) was added to 500 μL of human serum and immunoprecipitated with 1 μg of anti-PCSK9 monoclonal antibodies (anti-PCSK9-1, anti-PCSK9-2, and anti-PCSK9-3, respectively) (*lane 2* of each blot). Immunoprecipitation was also carried out with 500 μL of human serum in the absence of any added recombinant PCSK9 (*lane 1* of each blot). Recombinant PCSK9 (0.1 μg) was also analyzed directly (*lane 3* of each blot). Beads incubated with buffer only (negative control) were also analyzed (*lane 4* of each blot). Afterward, immunoprecipitates were separated electrophoretically and transferred to nitrocellulose for subsequent Western blotting with polyclonal anti-PCSK9 antibody.

Next, the above data with the 3 monoclonal antibodies were taken into account to design an ELISA capable of quantifying human serum concentrations of PCSK9. During development of the assay, the preferred orientation of the antibodies was found to be anti-PCSK9-3 as the capture antibody and anti-PCSK9-1 as the conjugate antibody (Fig. 2, A and B). Other configurations using anti-PCSK9-2 were less optimal. The ELISA format shown in Fig. 2B was chosen based on the fact that it appeared to be slightly more sensitive than the configuration shown in Fig. 2A. Although the differences were slight, the Fig. 2B configuration consistently produced an A_{450} of approximately 2.0 at a PCSK9 concentration of 8 μg/L, vs the Fig. 2A configuration, which produced an A_{450} of approximately 1.0 at 8 μg/L of PCSK9. With the use of ELISA assays performed with this orientation of antibodies, the detection limit of the assay (limit of the blank, mean + 3 SD of the zero calibrator) was 1 μg/L.

Next, freeze-thaw stability was evaluated by testing 5 different serum samples. These results showed excellent freeze-thaw stability with >90% recovery even after 4 freeze-thaw cycles. Individual results for the 4 freeze-thaw cycles were as follows: sample A–74, 74, 68, and 68 μg/L, respectively; sample B–39, 38, 39, and 39 μg/L; sample C–38, 36, 38, 37 μg/L; sample D–69, 64, 64, and 64 μg/L; and sample E–27, 26, 27, and 26 μg/L.

Precision of the ELISA was next assessed using serum samples containing 11, 27, and 77 μg/L of endogenous PCSK9. Intraassay (n = 20) imprecision (CVs) were 3.9%, 7.6%, and 8.9%, respectively. To determine recovery of added recombinant PCSK9 protein in human serum, recombinant PCSK9 protein was added to 3 different human serum samples (each containing very low concentrations of endogenous PCSK9), at concentrations of 250, 125, 62, and 31 μg/L, and these samples were analyzed using the ELISA. Mean (SD) results were 213 (32) μg/L, 105 (4) μg/L, 52 (2) μg/L, and 28 (4) μg/L, respectively, corresponding to recoveries of 85%, 84%, 84%, and 90%, respectively.

To correlate human serum PCSK9 concentrations using the ELISA with those obtained via immunoprecipitation and Western blotting, a calibration curve of recombinant PCSK9 was created by diluting the protein to a concentration of 250 μg/L and making serial 1:2 dilutions. Afterward, 8 human serum samples were run on this final ELISA format and were also assessed via immunoprecipi-

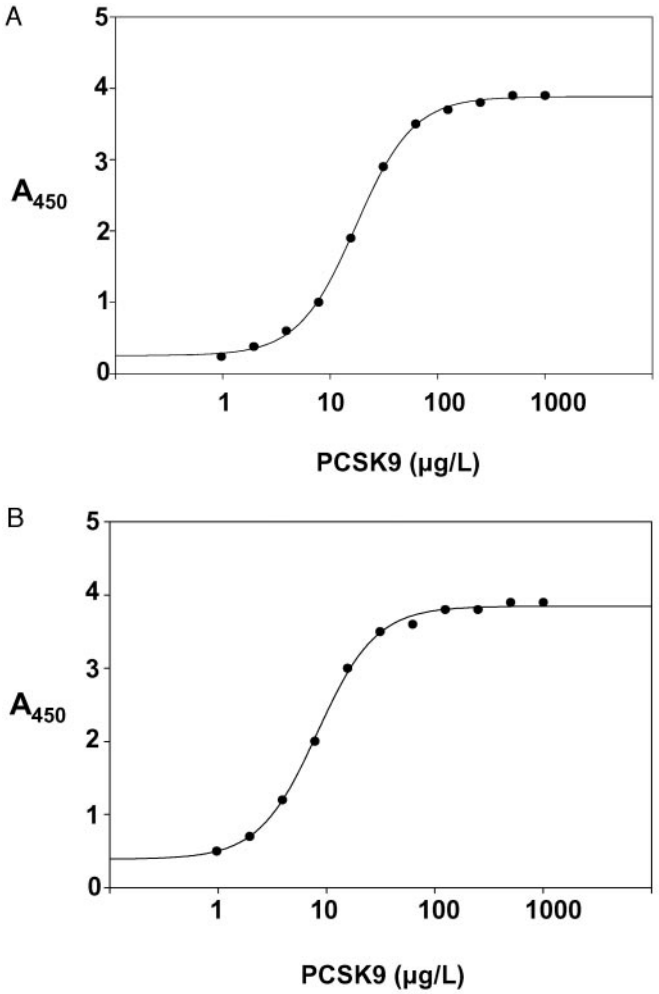

Fig. 2. Anti-PCSK9 antibody orientation is important in ELISA design.

(*A*), a dual-antibody sandwich ELISA was developed to measure PCSK9. Recombinant PCSK9 was used to generate a calibration curve starting at a concentration of 1000 μg/L (1000 ng/mL) with serial 1:2 dilutions. Anti-PCSK9 antibodies were oriented as anti-PCSK9-1-capture, anti-PCSK9-3-conjugate. (*B*), an alternative dual-antibody sandwich ELISA for PCSK9 was developed as described above except that the antibody orientation was anti-PCSK9-3-capture, anti-PCSK9-1-conjugate.

A

Sample #	1	2	3	4	5	6	7	8
IP/Western Value ($x10^7$)	6.14	2.51	3.80	1.78	1.12	4.09	2.05	4.91
IP/Western Result								
A_{450}	1.39	1.26	1.36	0.63	0.49	1.37	0.84	1.47
PCSK9 (μg/L)	83	76	82	36	27	82	49	86

B

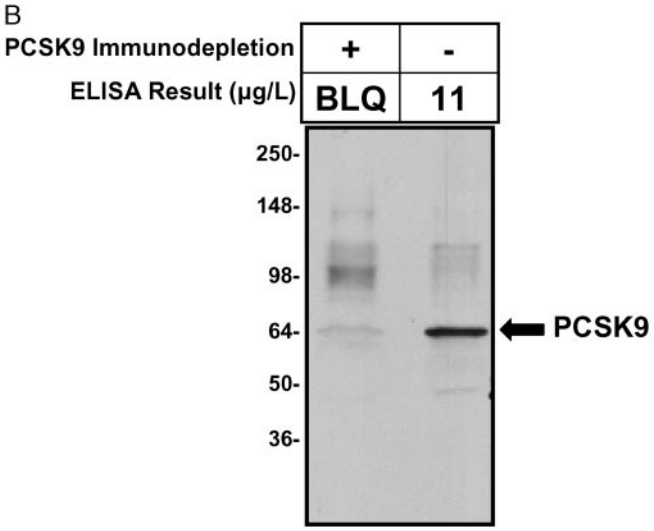

Fig. 3. Correlation of PCSK9 ELISA with immunoprecipitation and Western blotting.

(*A*), based on the above results, the antibody orientation of anti-PCSK9-3-capture, anti-PCSK9-1-conjugate was chosen, and the ELISA calibration curve range was modified appropriately. Eight human serum samples were analyzed on the ELISA at a 1:15 dilution with absorbances plotted on the curve. At the same time, PCSK9 was immunoprecipitated from 100 μL of each of these same 8 human serum samples. Afterward, immunoprecipitates were separated electrophoretically and transferred to nitrocellulose for subsequent Western blotting with polyclonal anti-PCSK9 antibody. A direct comparison between the Western blotting results and the ELISA results is shown. (*B*), a human serum sample was divided into aliquots, and PCSK9 was immunoprecipitated from 500 μL as in Fig. 1*A* (*right lane*). A separate 500-μL aliquot underwent immunodepletion of PCSK9, and was then processed identically to the first aliquot (*left lane*). Each of these samples was also analyzed on the ELISA at a final 1:15 dilution (BLQ, below the limit of quantification). Results are representative of those obtained with 3 independent human serum samples.

tation with anti-PCSK9-3 antibody followed by Western blotting with polyclonal anti-PCSK9 antibody. At a dilution of 1:15, all human serum samples were found to be on the calibration curve of the ELISA. Fig. 3A shows a direct comparison for the serum samples between the Western blotting results (including scanned densities of the bands), the A_{450} from the ELISA, and the calculated serum concentration from the ELISA. These data confirmed variation in serum PCSK9 concentrations from person to person and indicated that the ELISA results compared favorably with the immunoprecipitation and Western blotting results. It is worth noting, however, that the PCSK9 concentrations generated by the ELISA were dependent on equal recognition of recombinant and endogenous PCSK9 by the antibodies used in the ELISA. Anti-PCSK9-1 antibody, although it recognized recombinant PCSK9 as well as anti-PCSK9-3 antibody, was somewhat less adept at recognizing endogenous PCSK9 (Fig. 1). Therefore, the possibility that concentrations of PCSK9 in serum may be somewhat greater than those extrapolated from the PCSK9 ELISA calibration curve cannot be ruled out.

During the course of these experiments, as well as those shown in Fig. 1, the antibody used in the Western blotting, in addition to recognizing the prominent PCSK9 band, also recognized several much fainter bands thought to be nonspecific and to not contribute to the ELISA signal. To confirm these suppositions, we used both Western blotting and ELISA to analyze 3 different human serum samples before and after specific immunodepletion

of PCSK9. The results (Fig. 3B) confirmed that the nonspecific bands present in the Western blotting do not contribute to the ELISA signal.

The above data suggested that concentrations of PCSK9 in serum vary considerably and are in the microgram per liter range, lower than those of most apolipoproteins such as apolipoprotein B. In light of the fact that PCSK9 degrades LDLR, resulting in less LDL being cleared from the circulation by the liver, we hypothesized that serum PCSK9 concentrations might be directly related to serum LDLC concentrations. To test this hypothesis, we performed additional ELISA experiments to quantify PCSK9 in 55 otherwise healthy subjects who happened to span a broad range of LDLC. Serum PCSK9 ranged from 11 to 115 μg/L and was directly correlated with serum LDLC ($r = 0.45$, $P = 0.001$) and total cholesterol concentrations ($r = 0.50$, $P = 0.0003$). In contrast, there was no statistically significant correlation of serum PCSK9 concentrations with serum triglycerides ($r = 0.15$, $P = 0.28$) or HDL cholesterol ($r = 0.13$, $P = 0.36$).

Discussion

The above results demonstrate that PCSK9 is present in human serum. The observed serum concentrations covered a 10-fold range and correlated directly with LDL and total cholesterol concentrations. We were unable to be certain that the study participants had not taken statins. In light of recent observations that statins up-regulate *PCSK9* mRNA while lowering LDLC concentrations, and the fact that *PCSK9* mutations may confer hypersensitivity to statins, the correlation between serum PCSK9 concentrations and LDLC may be higher in confirmed statin-naive individuals *(20, 21)*.

We were unable to demonstrate that purified human LDL lipoprotein particles (or any other lipoprotein particle fraction) contained PCSK9, suggesting that the protein circulates free in the plasma or at least not in association with any subclass of lipoprotein particles. In light of these observations and the fact that PCSK9 in the circulation may cause the degradation of hepatic LDLR in the liver, PCSK9 would seem to be an attractive drug target for lowering LDLC. Nature has already performed this experiment, with the result that patients heterozygous for inactivating mutations of *PCSK9* have significantly decreased LDLC and cardiovascular disease *(15–19)*. Furthermore, a compound heterozygote for *PCSK9*-inactivating mutations was reported to be otherwise healthy and to have an LDLC concentration of 140 mg/L (14 mg/dL) *(17)*.

This possibility of pharmacologic intervention is even more attractive because statins may actually up-regulate PCSK9 expression *(20, 21)*. The *PCSK9* promoter contains a sterol regulatory element, and statins increase the expression of sterol regulatory element-binding protein-2, a transcription factor that activates both the LDLR and *PCSK9* genes *(12, 20, 21)*. Based on the assumption that this mRNA up-regulation translates into increased circulating PCSK9 protein, adding a PCSK9 inhibitor to statin therapy presents the possibility of further lowering LDLC to recommended concentrations in patients unable to attain desired LDLC on statin therapy alone. In such cases, a serum PCSK9 protein assay to predict which patients might best respond to PCSK9-inhibitor therapy may be particularly important. Our assay provides a means to tailor PCSK9-inhibitor therapy to patients most likely to respond to and benefit from pharmacologic inhibition of PCSK9.

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