

# Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9

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**Abstract** Proprotein convertase subtilisin/kexin type 9 (PCSK9) has gained attention as a key regulator of serum low density lipoprotein cholesterol (LDL-C) levels. This novel protease causes the degradation of hepatic low density lipoprotein receptors. In humans, gain-of-function mutations in PCSK9 cause a form of familial hypercholesterolemia, whereas loss-of-function mutations result in significantly decreased LDL-C levels and cardiovascular risk. Previous studies have demonstrated that statins upregulate PCSK9 mRNA expression in cultured cells and animal models. In light of these observations, we studied the effect of atorvastatin on circulating PCSK9 protein levels in humans using a sandwich ELISA to quantitate serum PCSK9 levels in patients treated with atorvastatin or placebo for 16 weeks. We observed that atorvastatin (40 mg/day) significantly increased circulating PCSK9 levels by 34% compared with baseline and placebo and decreased LDL-C levels by 42%. **Figure** These results suggest that the addition of a PCSK9 inhibitor to statin therapy may result in even further LDL-C decreases.—Careskey, H. E., R. A. Davis, W. E. Alborn, J. S. Troutt, G. Cao, and R. J. Konrad. **Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9.** *J. Lipid Res.* 2008. 49: 394–398.

**Supplementary key words** low density lipoprotein • low density lipoprotein cholesterol • low density lipoprotein receptor

The serum protease proprotein convertase subtilisin/kexin type 9 (PCSK9) has gained tremendous attention as a potential key regulator of serum low density lipoprotein cholesterol (LDL-C) levels (1–3). PCSK9 is a protease made by the liver that acts to degrade hepatic low density lipoprotein receptors (LDLRs) (4–10). The mechanism by which PCSK9 degrades LDLRs is extremely complex and is only beginning to be understood. It was recently suggested that the protease itself does not have to be proteolytically active to cause degradation of the LDLR but rather binds to the LDLR and subsequently targets it for intracellular

destruction within the hepatocyte (11–13). Regardless of the exact mechanism, the result of LDLR levels being decreased is that the liver has a decreased ability to bind LDL from the circulation and serum LDL-C levels increase. Therefore, mutations in PCSK9 can have dramatic effects on serum LDL-C levels in humans.

Patients with gain-of-function mutations of PCSK9 manifest severe familial hypercholesterolemia and accompanying increased cardiovascular risk (14–17). These mutations in PCSK9 account for ~10–25% of familial dominant hypercholesterolemia cases that could not be explained by mutations in either the LDLR or apolipoprotein B (apoB) (14–17). In contrast, heterozygous subjects with loss-of-function mutations in PCSK9, including mutations that prevent the self-cleavage and secretion of the protein itself, have significantly decreased levels of serum LDL-C and dramatically decreased cardiovascular risk (18–20). Approximately 2% of African-Americans carry such mutations, with an accompanying 80–90% decreased risk of serious cardiovascular events (18). Recently, the first compound heterozygote for PCSK9 loss-of-function mutations was described. This subject, a healthy 32 year old female, had an extremely low serum LDL-C level of 14 mg/dl (20).

Interestingly, statins have been shown to increase the activity/nuclear translocation of sterol-regulatory element binding protein-2 (SREBP-2), a transcription factor that activates both the LDLR and PCSK9 genes (15, 21, 22). As a result, statins have been reported to upregulate PCSK9 mRNA expression (15, 21, 22). Therefore, we used our recently developed PCSK9 ELISA (23) to investigate the effect of atorvastatin treatment for 16 weeks on human serum PCSK9 protein levels.

Abbreviations: apoB, apolipoprotein B; LDL-C, low density lipoprotein cholesterol; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP-2, sterol-regulatory element binding protein-2.

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## MATERIALS AND METHODS

### Serum samples

Human serum samples were obtained from patients in a recently described phase 2 clinical trial who gave permission for their serum samples to be banked for future exploratory lipid analysis (24). After obtaining protocol approval from an Institutional Review Board and the proper informed consent from each patient, these samples were collected, banked, and deidentified to protect patient privacy, so that only lipid data and dose group could be linked to the PCSK9 ELISA results. The specific clinical trial studied the effect of a peroxisome proliferator-activated receptor  $\alpha$  agonist added to atorvastatin therapy in patients with high LDL-C levels, and the results were thoroughly disclosed in a recent publication (24). For this study, only patients who received placebo or statin therapy alone were studied, and only patients who contributed a complete set of baseline and end point banked samples were analyzed for PCSK9 levels. This resulted in 12 patients in the placebo group who received placebo only for 16 weeks and 12 patients in the atorvastatin group who received 40 mg/day atorvastatin only for 16 weeks. Serum samples were shipped on dry ice and were stored at  $-70^{\circ}\text{C}$  before subsequent analysis.

### Routine lipid analysis

Concentrations of serum triglycerides, total cholesterol, and apoB were measured using Hitachi Chemistry Systems (Roche Diagnostics, Indianapolis, IN). HDL-C was measured via a dextran sulfate precipitation method in which a 10 g/l dextran sulfate (molecular weight, 50,000)  $\text{Mg}^{2+}$  solution was used to precipitate apoB-containing particles. After centrifugation, the cholesterol content of the HDL-C-containing supernatant was measured on a Roche Hitachi 717 analyzer. LDL-C was measured via a  $\beta$ -quant ultracentrifugation assay in which 3 ml of serum was centrifuged using a Beckman L8-70M ultracentrifuge at 25,000 g for 20.25 h. Afterward, a Beckman centrifuge slicer was used to remove the bottom fraction of the tube. After volume correction, the cholesterol content of the bottom fraction of the tube was measured on a Roche Hitachi 717 analyzer. LDL-C was then determined as cholesterol present in the bottom fraction minus HDL-C.

### PCSK9 levels

PCSK9 levels in the serum samples were measured using our recently described PCSK9 dual monoclonal antibody sandwich ELISA (23, 25). Briefly, wells were coated overnight (Pierce carbonate-bicarbonate coating buffer, pH 9.40) with anti-PCSK9-3 monoclonal antibody at a concentration of 5  $\mu\text{g}/\text{ml}$ . The next day, wells were aspirated, washed three times with assay buffer (50 mM HEPES, pH 7.40, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 5 mM EGTA), and blocked for 1 h with TBS-casein blocking buffer (Pierce). Next, 100  $\mu\text{l}$  of recombinant PCSK9 standards (varying concentrations of recombinant protein in assay buffer) were added to the wells as a standard curve. Afterward, serum samples were diluted 1:20 in assay buffer and added to their respective wells, and the ELISA plate was allowed to incubate for 2 h at room temperature. After aspiration, wells were washed three times with assay buffer, and 100  $\mu\text{l}$  of a 1:1,000 dilution of conjugate antibody (HRP-labeled anti-PCSK9-1 monoclonal antibody, 1 mg/ml) was added to the wells for a 1 h incubation at room temperature. After aspiration, wells were washed three times with TBS + Tween. After the last aspiration of TBS + Tween, 100  $\mu\text{l}$  of 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 the plates were read at 450 nm. SigmaPlot, version 8.0, was used for fitting of the calibration curves.

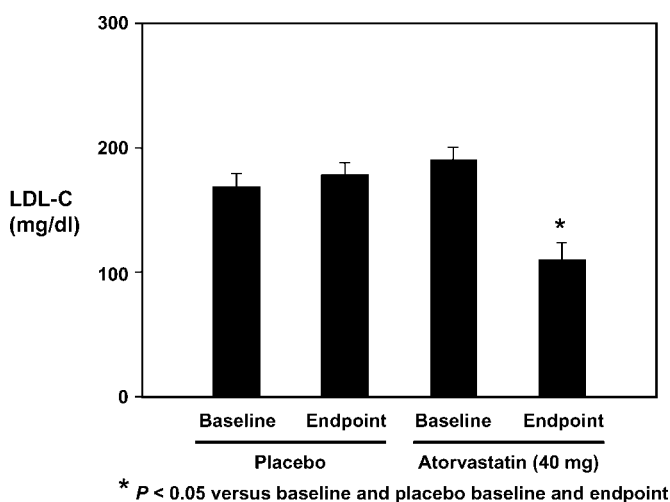
### Data analysis

SigmaPlot, version 8.0, was used for fitting of the calibration curves for the PCSK9 ELISA. Data were analyzed and plotted using the program FigP (Biosoft, St. Louis, MO). Statistical analysis was performed using the same program.  $P < 0.05$  was considered to indicate statistical significance.

## RESULTS

**Figure 1** demonstrates the effect of atorvastatin, 40 mg/day, versus placebo on serum LDL-C levels. In the placebo group, there was no significant change in LDL-C from baseline to end point. At baseline in the placebo group, LDL-C levels were  $169 \pm 11$  mg/dl. After 16 weeks of placebo-only treatment, there was no significant change, with end point LDL levels being  $178 \pm 10$  mg/dl. In contrast, in the atorvastatin 40 mg/day group, there was a significant decrease in LDL-C from baseline to end point. At baseline in the atorvastatin 40 mg/day group, LDL-C levels were  $190 \pm 10$  mg/dl. After 16 weeks of atorvastatin treatment at a dose of 40 mg/day, there was a significant 42% decrease, with end point LDL levels of  $110 \pm 14$  mg/dl ( $P < 0.05$  vs. baseline and vs. placebo baseline and end point). Similar results were also observed for total cholesterol levels, with a nonsignificant 3% increase observed in the placebo group from baseline to end point versus a significant 33% decrease in the atorvastatin 40 mg/day group from baseline to end point (data not shown).

Recent observations that statins upregulate PCSK9 mRNA levels while decreasing LDL-C levels and that

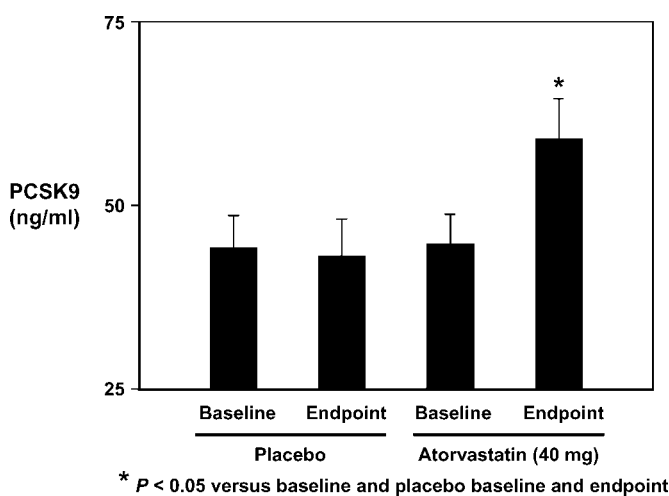


**Fig. 1.** Effect of atorvastatin on serum low density lipoprotein cholesterol (LDL-C) levels. Baseline and end point samples from patients receiving placebo only for 16 weeks or atorvastatin only (40 mg/day) for 16 weeks were analyzed for LDL-C levels using  $\beta$ -quant ultracentrifugation. LDL-C levels at baseline and end point were plotted for each group. Data are expressed as means  $\pm$  SEM. Atorvastatin 40 mg/day caused a significant 42% decrease in serum LDL-C.

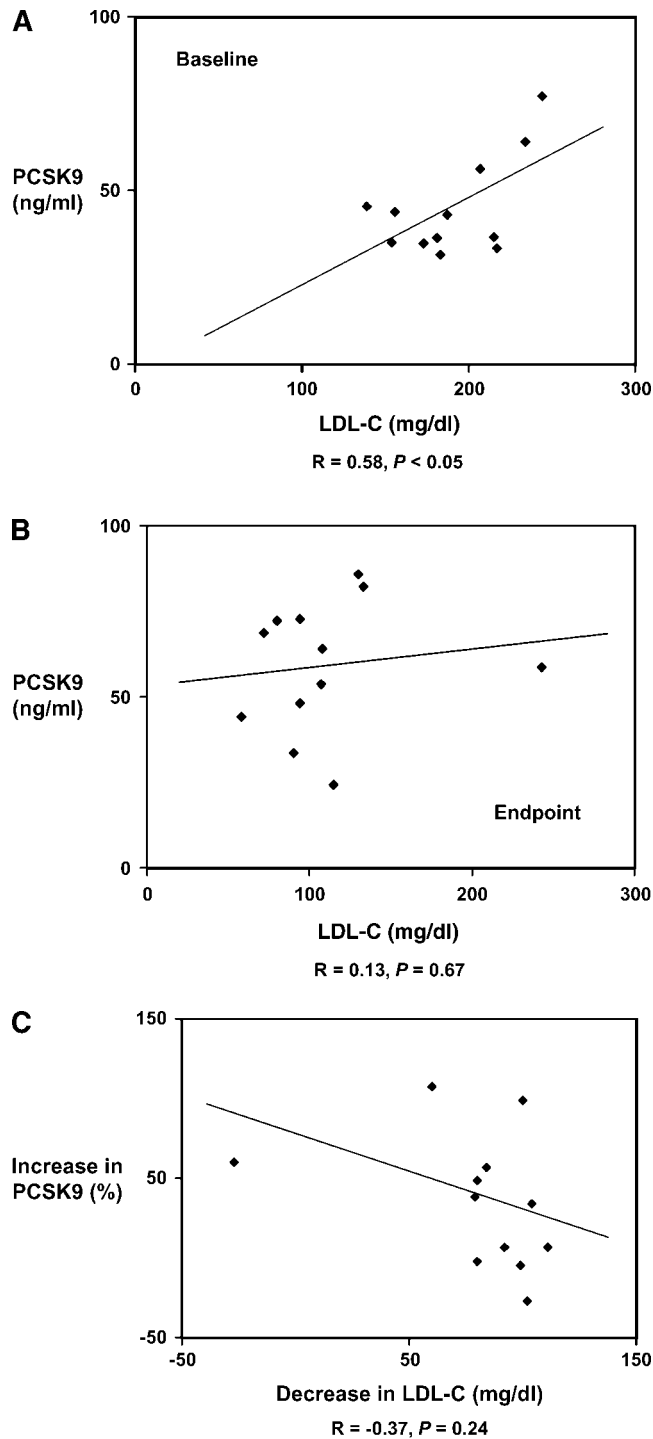
PCSK9 mutations may confer hypersensitivity to statins (15, 21, 22) led us to investigate the effect of atorvastatin on PCSK9 levels. We hypothesized that atorvastatin treatment might increase circulating PCSK9 protein levels, because statins increase the activity/nuclear translocation of SREBP-2, a transcription factor that activates both the LDLR and PCSK9 genes (15, 21, 22). To test this hypothesis, we used our recently developed PCSK9 dual monoclonal antibody sandwich ELISA (23) to measure serum PCSK9 levels.

**Figure 2** demonstrates the effect of atorvastatin, 40 mg/day, versus placebo on serum PCSK9 levels. In the placebo group, there was no significant change in PCSK9 from baseline to end point. At baseline in the placebo group, PCSK9 levels were  $44 \pm 4$  ng/ml. After 16 weeks of placebo-only treatment, there was no significant change, with end point PCSK9 levels being  $43 \pm 4$  ng/ml. In contrast, in the atorvastatin 40 mg/day group, there was a significant increase in PCSK9 serum protein levels from baseline to end point. At baseline in the atorvastatin 40 mg/day group, PCSK9 levels were  $44 \pm 4$  ng/ml. After 16 weeks of atorvastatin treatment at a dose of 40 mg/day, there was a significant increase, with end point PCSK9 levels of  $59 \pm 5$  ng/ml ( $P < 0.05$  vs. baseline and vs. placebo baseline and end point). This represented a significant 34% increase in serum PCSK9 in response to atorvastatin 40 mg/day.

In light of these observations, we performed additional analyses on the 40 mg/day atorvastatin group by examining the LDL-C decrease and the increase in PCSK9 across the group. **Figure 3A** shows the correlation between LDL-C and PCSK9 at baseline for the 40 mg/day atorvastatin group. At baseline, there was a direct correlation between PCSK9 levels and LDL-C ( $r = 0.58$ ,  $P < 0.05$ ). **Figure 3B**



**Fig. 2.** Effect of atorvastatin on serum proprotein convertase subtilisin/kexin type 9 (PCSK9) levels. The same baseline and end point samples from the patients described for Fig. 1 were analyzed for PCSK9 levels using a PCSK9 dual monoclonal antibody sandwich ELISA as described in Materials and Methods. Serum PCSK9 levels at baseline and end point were plotted for each group. Data are expressed as means  $\pm$  SEM. Atorvastatin 40 mg/day caused a significant 34% increase in serum PCSK9.



**Fig. 3.** Relationship of LDL-C to serum PCSK9 levels in the 40 mg/day atorvastatin group. A: Correlation between LDL-C and PCSK9 at baseline for the 40 mg/day atorvastatin group ( $r = 0.58$ ,  $P < 0.05$ ). B: Correlation between LDL-C and PCSK9 at end point after 4 months of treatment for the 40 mg/day atorvastatin group ( $r = 0.13$ ,  $P = 0.67$ ). C: Relationship between increases in PCSK9 levels and observed LDL-C decreases at end point after 4 months of treatment for the 40 mg/day atorvastatin group ( $r = -0.37$ ,  $P = 0.24$ ).

shows the relationship between LDL-C and PCSK9 at end point after 4 months of treatment for the 40 mg/day atorvastatin group. At end point, there was less correlation between PCSK9 levels and LDL-C ( $r = 0.13$ ,  $P = 0.67$ ) as a

result of the fact that atorvastatin increased PCSK9 levels while decreasing LDL-C.

Figure 3C shows the relationship between the percentage increases in PCSK9 levels and the observed LDL-C decrease. This figure demonstrates that although the correlation did not reach statistical significance ( $r = -0.37$ ,  $P = 0.24$ ), there was a trend toward a  $P$  value of 0.2. During this analysis, it was noted that one patient had an unexpected increase in LDL-C. This is the patient indicated by the data point to the far left in Fig. 3C. If this patient had been excluded from the analysis, the correlation would have been greater ( $r = -0.50$ ,  $P = 0.11$ ), although still not reaching statistical significance. It should be noted that Fig. 3C shows the percentage change in PCSK9 versus the absolute change in LDL-C, because the variability in baseline PCSK9 levels observed in this study was relatively greater than that for LDL-C. The results from comparing absolute changes in PCSK9 levels with absolute changes in LDL-C levels were similar ( $r = -0.31$ ,  $P = 0.33$ ). Likewise, if the same patient with the increase in LDL-C had been excluded from the analysis, the correlation would have been greater ( $r = -0.41$ ,  $P = 0.21$ ), although still not becoming statistically significant.

We also measured apoB levels and performed similar analyses on the 40 mg/day atorvastatin group. At baseline, there was a trend toward direct correlation between PCSK9 levels and apoB, although it did not reach statistical significance ( $r = 0.37$ ,  $P = 0.23$ ). At end point, there was less correlation between PCSK9 levels and apoB ( $r = 0.19$ ,  $P = 0.53$ ), attributable to the fact that atorvastatin increased PCSK9 levels while decreasing apoB levels. The comparison between percentage increases in PCSK9 levels and apoB decreases was performed next. As with LDL-C, a trend was observed ( $r = -0.15$ ,  $P = 0.64$ ), although this was not significant and was not as strong as was the case for PCSK9 and LDL-C. One reason for this observation may be that the banked patient samples used in this study might have been drawn from patients while in the nonfasting state, resulting in apoB-48 contributing to the apoB measurements, thus diminishing the correlation.

In light of these data, we also examined banked samples from patients in the same study who had received 10 mg/day atorvastatin for 16 weeks. Unfortunately, this analysis was somewhat limited by the smaller number of these patients completing the study and contributing complete baseline and end point sample sets ( $n = 7$ ). Interestingly, in patients receiving 10 mg/day atorvastatin, although there was a significant 30% decrease in LDL-C levels after 16 weeks of treatment, there was no real change (1% decrease) in PCSK9 serum levels (data not shown).

## DISCUSSION

The results described above demonstrate that atorvastatin treatment (40 mg/day) decreases LDL-C levels in humans while at the same time increasing serum PCSK9 protein levels. Recent observations that statins upregulate PCSK9 mRNA levels while decreasing LDL-C levels and that PCSK9 mutations may confer hypersensitivity to statins in

animal models make these results at the protein level in human serum particularly important (20, 21).

Interestingly, we did not observe any increase in PCSK9 levels in patients who had been treated with 10 mg/day atorvastatin, despite the fact that this dose of atorvastatin reduced LDL-C levels by 30% compared with a 42% decrease observed with atorvastatin 40 mg/day. It should be remembered, however, that interpretation of the data from the 10 mg/day atorvastatin group may be hindered by the relatively smaller number of patient samples available for analysis. Nevertheless, it is tempting to speculate that statin-induced increases in circulating PCSK9 may diminish the LDL-decreasing effect of increasing doses of statins.

The fact that 10 mg/day atorvastatin decreased LDL-C almost as much as 40 mg/day while not affecting PCSK9 levels was at first perplexing, especially because statins increase the activity/nuclear translocation of SREBP-2, a transcription factor that activates both the LDLR and PCSK9 genes (15, 21, 22). It is possible, however, that at this dose of atorvastatin, any increased circulating PCSK9 protein might be negated by the increased hepatic LDLRs that would act to bind the PCSK9 in the circulation. In contrast, at a higher dose of atorvastatin (40 mg/day), further increased expression of PCSK9 protein might exceed LDLR binding, resulting in increased circulating levels of PCSK9 protein and only a modest additional decrease in LDL-C.

Our observations with regard to LDL-C decreases in this study were consistent with the rule of 6% for statins, which states that each doubling of the statin dose results in an ~6% further decrease in LDL-C. In this study, we observed a 30% LDL-C reduction and no increase in serum PCSK9 levels at 10 mg/day atorvastatin. At a dose of atorvastatin four times higher (40 mg/day), there was a further 12% reduction in LDL-C (for a total decrease of 42%) but also a 34% increase in serum PCSK9 levels. These data indicate that increasing PCSK9 might reduce efficacy across the entire range of statins.

Taking these data together, PCSK9 would seem to be an attractive drug target for decreasing LDL-C levels. Our current study, although preliminary, suggests that the addition of a PCSK9 inhibitor to statin therapy presents the possibility of further decreasing LDL-C levels in patients currently unable to attain desired LDL-C levels on statin therapy alone. **FIG**

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