Plasma PCSK9 Concentrations Correlate with LDL and Total Cholesterol in Diabetic Patients and Are Decreased by Fenofibrate Treatment

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BACKGROUND: Proprotein convertase subtilisin/kexin type 9 (PCSK9) promotes the degradation of the LDL receptor (LDLr) in hepatocytes, and its expression in mouse liver has been shown to decrease with fenofibrate treatment.

METHODS: We developed a sandwich ELISA using recombinant human PCSK9 protein and 2 affinity-purified polyclonal antibodies directed against human PCSK9. We measured circulating PCSK9 concentrations in 115 diabetic patients from the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study before and after fenofibrate treatment.

RESULTS: We found that plasma PCSK9 concentrations correlate with total (r = 0.45, P = 0.006) and LDL (r = 0.54, P = 0.001) cholesterol but not with triglycerides or HDL cholesterol concentrations in that cohort. After 6 weeks of treatment with comicronized fenofibrate (200 mg/day), plasma PCSK9 concentrations decreased by 8.5% (P = 0.041 vs pretreatment). This decrease correlated with the efficacy of fenofibrate, as judged by a parallel reduction in plasma triglycerides (r = 0.31, P = 0.015) and LDL cholesterol concentrations (r = 0.27, P = 0.048).

CONCLUSIONS: We conclude that this decrease in PCSK9 explains at least in part the LDL cholesterol–lowering effects of fenofibrate. Fenofibrate might be of interest to further reduce cardiovascular risk in patients already treated with a statin.

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BACKGROUND: Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a key regulator of plasma LDL cholesterol concentrations by virtue of its ability to target the LDL receptor (LDLr) for degradation in the lysosome (1, 2). Patients carrying heterozygous gain-of-function mutations on PCSK9 present with familial hypercholesterolemia and premature cardiovascular events (2, 3), whereas carriers of PCSK9 loss-of-function mutations have low plasma LDL cholesterol concentrations and reduced incidence of coronary heart disease (1). PCSK9 has emerged as a potential therapeutic target to decrease plasma LDL cholesterol concentrations, as shown by PCSK9 knockout and knockdown studies (4, 5).

The molecular mechanism by which PCSK9 targets hepatic LDLr for degradation is quite complex. It was first envisaged that PCSK9 acted on the LDLr before it reached the basolateral surface of the hepatocyte (6). But PCSK9 is secreted, and when exogenously added to cells, it requires LDLr-mediated internalization to inhibit LDLr expression (7–10). Direct binding of PCSK9 to the extracellular domain of the LDLr appears to target the LDLr for lysosomal degradation rather than recycling to the plasma membrane (11–14). PCSK9 enzymatic activity is not required in that process, indicating that PCSK9 acts as a chaperone to promote LDLr degradation (15, 16).

These studies indicate that the function of PCSK9 as a secreted factor is physiologically significant. Therefore it is relevant to measure circulating concentrations of PCSK9 from a clinical perspective in humans and to study pharmacological factors affecting its secretion. It is well established that PCSK9 gene expression is positively regulated by statins (5, 17). We also recently showed that PCSK9 expression is downregulated in...
mouse livers by the peroxisome proliferator–activated receptor α (PPARα) agonist fenofibrate (18), a lipid-lowering drug used to decrease plasma triglycerides (TG), particularly in patients with clinical features of the metabolic syndrome or type 2 diabetes (19–22).

The aim of this study was to determine whether fenofibrate modulates plasma PCSK9 concentrations in humans. We developed a sandwich ELISA and measured circulating PCSK9 concentrations in a cohort of 115 diabetic individuals (22) before and after fenofibrate treatment. We found that circulating PCSK9 correlates with plasma total cholesterol and LDL cholesterol and that fenofibrate treatment decreases plasma PCSK9 in this cohort.

Materials and Methods

PATIENTS AND PLASMA SAMPLES
We collected plasma samples from 115 diabetic patients enrolled in the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study and followed in the Royal Prince Alfred Hospital, Sydney, (a) after a 6-week placebo run-in period (a capsule daily at breakfast; no lipid-lowering medication) and (b) after a 6-week active run-in period (200 mg/day comirionized fenofibrate) (22) between December 1999 and July 2000. The samples were divided into aliquots and frozen at −80 °C until use (see Supplemental Data Fig. 1). Plasma samples from 8 healthy young individuals were used to establish the ELISA. Serum total cholesterol, TG, and HDL cholesterol were measured in accredited clinical laboratories using routine clinical methods. LDL cholesterol was calculated using the Friedewald equation. The study was approved by the Royal Prince Alfred Hospital ethics committee, and all patients and healthy individuals gave written informed consent.

PCSK9 PROTEIN EXPRESSION, PURIFICATION, AND ACTIVITY
A human PCSK9 (hPCSK9) cDNA construct encoding the 692-residue natural sequence fused to a V5 histidine tag (2, 3) was stably transfected into HEK293 cells. Using cell conditioned medium, we purified self-processed secreted PCSK9 protein by immobilized nickel affinity chromatography (Macherey-Nagel). We assessed the purity of the recombinant PCSK9 preparations using the SDS-PAGE PhastGel® system (GE Healthcare) followed by silver staining. We assessed the activity of the recombinant protein by measuring PCSK9 binding to soluble LDLr (sLDLr) extracellular domain (R&D Systems) using electrochemiluminescence technology as per the manufacturer’s instructions. Briefly, MSD plates (Meso Scale Discovery) were coated with 0.03 pmol sLDLr per well and incubated with different concentrations of recombinant PSCK9 protein. We measured PCSK9 binding to the sLDLr on a Sector Imager 2400 (Meso Scale Discovery) using an anti-V5 epitope primary antibody and a ruthenium-coupled antimouse IgG secondary antibody (Invitrogen).

WESTERN BLOTS
We resolved purified PCSK9 on Nu-PAGE 4%–12% Bis-Tris gels in MES-SDS buffer (Invitrogen) under reducing and nonreducing conditions. Proteins were transferred onto a Hybond-ECL nitrocellulose membrane (GE Healthcare) and probed with affinity purified polyclonal antibodies [rabbit anti-hPCSK9 #10007185 (Cayman), goat antirabbit IgG-HRP #sc-2030 (Santa-Cruz Biotechnology), goat anti-hPCSK9 #ab28770 (Abcam), rabbit antigom IgG-HRP #ab6741 (Abcam)] using ECL Plus (GE Healthcare).

CELL CULTURE AND IMMUNOPRECIPITATION
We plated human hepatoma cells (HuH-7) were plated on 6-well plates in DMEM containing 10% fetal bovine serum at a density of 10⁶ cells. The next day, we placed the cells in serum-free DMEM treated with 200 μmol/L fenofibric acid (ABCR GmbH) dissolved in dimethyl sulfoxide (Sigma). Two days later, media were collected, centrifuged at 10 000g to get rid of cell debris, and immunoprecipitated using the goat anti-hPCSK9 antibody (1:200). We resolved immunoprecipitates by Western blot using the rabbit anti-hPCSK9 antibody. The experiment was performed 3 times in duplicate.

HUMAN PCSK9 ELISA
We coated 96-well ELISA plates (Iwaki) with 100 μL of 1× PBS containing 1 μg goat anti-hPCSK9 antibody per well and incubated them overnight at 4 °C. The wells were washed 3 times with washing buffer (1× PBS containing 0.05% Tween-20) using a Bio-Rad autowasher and blocked for 1 h at 37 °C with 200 μL of 1× PBS containing 1 g/L casein. We diluted recombinant PCSK9 in dilution buffer (1× PBS containing 1 g/L casein and 0.05% Tween-20) at final concentrations ranging from 0 to 3 mg/L PCSK9, and we diluted plasma samples 5-fold in dilution buffer. Between each of the following steps, the wells were washed 3 times as described above. (a) We added 100 μL of the PCSK9 standards and sample dilutions in duplicate to the wells and incubated them overnight at 37 °C. (b) We added 100 μL of 1× PBS containing 0.4 μg rabbit anti-hPCSK9 antibody to each well and incubated them for 3 h at 37 °C. (c) We added 100 μL of 1× PBS containing goat antirabbit IgG-HRP antibody diluted 1:5000 to each well and incubated them for 3 h at 37 °C. (d) We added 100 μL azino-bis-ethylbenzazoline sulfonic acid peroxidase substrate system A&B solution (KPL) to each well and incubated them for 15 min at room temperature, then added 100 μL of 2.5 mol/L...
H$_2$SO$_4$. The absorbance was read at 407 nm. During incubations, the plates were covered with a sealing film to avoid evaporation. We assessed PCSK9 twice in duplicate in each plasma sample.

**STATISTICAL ANALYSIS**

Values are expressed as mean (SD). We made comparisons between groups using Student t test for independent samples (2-tailed). Using Prism4®, we determined Spearman correlation coefficients ($r$) to establish the relationship between the concentrations of PCSK9 and plasma lipids. Statistical significance was defined as $P < 0.05$.

**Results**

**VALIDATION OF THE ELISA TO MEASURE PCSK9 IN HUMAN PLASMA**

To measure the circulating concentrations of PCSK9 in human plasma samples, we developed an ELISA using 2 commercially available affinity-purified anti-hPCSK9 antibodies raised in goat and rabbit, respectively, and we produced recombinant hPCSK9 to use as a standard in our assay. We assessed the purity of the hPCSK9 standard by SDS-PAGE in nonreducing conditions followed by silver staining and found it to be >99% as judged by the single PCSK9 band detected above the 60-kDa molecular weight marker (Fig. 1A). We also performed Western blot analysis of our hPCSK9 standard (5 and 30 ng per well) under reducing and nonreducing conditions, using both the goat and the rabbit anti-hPCSK9 antibodies to perform the ELISA. Only a single band of apparent molecular weight above 60 kDa could be detected under both reducing and nonreducing conditions, and the intensities of the bands were directly proportional to the amount of recombinant hPCSK9 loaded onto each well (Fig. 1B). To ascertain the integrity of the recombinant PCSK9 protein preparation, we measured its binding affinity toward the extracellular domain of the LDLr (Fig. 1C). PCSK9 demonstrated an affinity against the coated soluble LDLr of $K_d$ 300 nM, in agreement with published values (11–14).

We designed the ELISA and found that the optimal orientation of the antibodies was goat anti-hPCSK9 as the capture antibody and rabbit anti-hPCSK9 as the detection antibody, at optimal concentrations and assay conditions described in “Materials and Methods.” We created a standard curve of hPCSK9 using serial dilutions of the recombinant protein from a 3 mg/L stock solution, and found that the assay was linear ($r^2 = 0.997$) within this concentration range (Fig. 1D). We assessed 8 human serum samples from young, healthy individuals by ELISA and found them to be within the standard curve range at a 1:5 dilution, with final plasma PCSK9 concent-
Concentrations of 4.14 (2.47) mg/L. Using 2 samples with the lowest (0.8 mg/L) and the highest (7.9 mg/L) PCSK9 content, we determined the assay intra- and interplate variability to be <5% and 7% (n = 5), respectively. PCSK9 concentrations of 2 samples with the highest PCSK9 content were found to be within the assay variability range when measured in 1:5, 1:10, 1:15, and 1:20 dilutions. To ensure that there was no interference in the assay, we also spiked the plasma sample having the lowest PCSK9 concentration with increasing amounts of recombinant hPCSK9 and found that the increases in A (absorbance) in plasma were similar to those obtained in buffer (not shown). Plasma samples with PCSK9 concentrations of 2.6 and 4.2 mg/L were subjected to 3 freeze-thaw cycles (−80 °C/room temperature), and their PCSK9 concentrations were reassessed and found to be within 2.3–2.7 and 3.9–4.5 mg/L, respectively—that is, within assay variability range.

**CORRELATION OF CIRCULATING PCSK9 WITH LIPOPROTEIN CONCENTRATIONS**

We next assessed by ELISA circulating PCSK9 concentrations in samples from 115 diabetic patients before and after fenofibrate treatment. Pretreatment PCSK9 concentrations ranged from 0.1 to 9.3 mg/L and had a nearly bell-shaped distribution (Fig. 1D) around a mean of 4.08 (2.16) mg/L, comparable to that measured in 8 healthy individuals at 4.14 (2.47) mg/L. The clinical characteristics of our patients as well as their plasma lipids before and after a 6-week course of fenofibrate treatment are displayed in Table 1.

We performed a Spearman correlation analysis between the patients’ plasma total cholesterol (range 3–6.5 mmol/L), HDL cholesterol (range 0.6–2.1 mmol/L), TG (range 0.7–4.5 mmol/L), and LDL cholesterol (range 1.2–4.6 mmol/L) and their circulating PCSK9 concentrations. PCSK9 correlated positively and significantly with both total cholesterol (r = 0.45, P = 0.006) and LDL cholesterol (r = 0.54, P = 0.001), but not with HDL cholesterol (r = 0.07, NS) or TG (r = 0.11, NS) (Fig. 2). PCSK9 concentrations did not correlate significantly with body mass index (BMI), systolic blood pressure, waist circumference, diabetes duration, or age (data not shown).

**EFFECT OF FENOFIBRATE TREATMENT ON PLASMA PCSK9 CONCENTRATIONS**

The patients received 6 weeks of treatment with a single daily dose of 200 mg comircized fenofibrate (22). Plasma isolated before and after the treatment period was analyzed for lipids (Table 1) and PCSK9 concentrations. To minimize assay variability, pre- and post-treatment samples were analyzed for PCSK9 on the same ELISA plate. Treatment with fenofibrate decreased PCSK9 concentrations by 8.5% at 3.73 (1.98) mg/L (P = 0.041 vs pretreatment). The relationship of pre- and posttreatment PCSK9 concentrations is shown in Fig. 3A. Of 115 patients, 28 had an increase or no change in circulating PCSK9 concentrations after treatment with fenofibrate, 30 had slightly decreased (<7%) concentrations, 29 had moderately decreased (8%–20%) concentrations, and 28 had concentrations that decreased by >20%. The fenofibrate-induced changes in PCSK9 correlated positively and significantly with the changes in plasma TG (r = 0.31, P = 0.015) (Fig. 3B). We also found a significant and positive correlation between changes in plasma PCSK9 and LDL cholesterol upon fenofibrate treatment (r = 0.27, P = 0.048) (Fig. 3C).

To gain mechanistic insight into the role of fenofibrate in lowering plasma PCSK9, we plated human hepatoma cells in cholesterol-deprived culture conditions to induce PCSK9 gene expression with or without fenofibric acid, the active metabolite of fenofibrate (Fig. 4). We found that PCSK9 secretion in the culture medium was significantly reduced (by 50%) when the

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**Table 1. Clinical characteristics and plasma lipids before and after fenofibrate treatment in a cohort of 115 diabetic patients.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Before Treatment</th>
<th>After Treatment</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>62.8 (9.0)</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>30.5 (5.2)</td>
<td></td>
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<tr>
<td>Sex, M/F</td>
<td>90/25</td>
<td></td>
</tr>
<tr>
<td>Prior cardiovascular disease, %</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>132 (12)</td>
<td></td>
</tr>
<tr>
<td>Diastolic</td>
<td>77 (7)</td>
<td></td>
</tr>
<tr>
<td>Waist circumference &gt;88 cm in women</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>or &gt;102 cm in men</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diabetes diagnosis, years</td>
<td>56.0 (7.9)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>4.93 (0.78)</td>
<td></td>
</tr>
<tr>
<td>After fenofibrate treatment (200 mg/day)</td>
<td>4.43 (0.74)*</td>
<td></td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>1.12 (0.32)</td>
<td></td>
</tr>
<tr>
<td>After fenofibrate treatment (200 mg/day)</td>
<td>1.20 (0.29)</td>
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</tr>
<tr>
<td>TG, mmol/L</td>
<td></td>
<td></td>
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<tr>
<td>Pretreatment</td>
<td>1.84 (0.69)</td>
<td></td>
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<tr>
<td>After fenofibrate treatment (200 mg/day)</td>
<td>1.39 (0.63)*</td>
<td></td>
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<tr>
<td>LDL cholesterol, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>2.98 (0.65)</td>
<td></td>
</tr>
<tr>
<td>After fenofibrate treatment (200 mg/day)</td>
<td>2.60 (0.64)*</td>
<td></td>
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</table>

Data are mean (SD) unless stated otherwise.

* P < 0.0001.
Discussion

We assessed the effect of fenofibrate on circulating PCSK9 concentrations in a cohort of 115 diabetic patients. Our results clearly indicate that diabetic and nondiabetic subjects have, on average, similar plasma PCSK9 concentrations and that these concentrations correlate with plasma total cholesterol and LDL cholesterol concentrations. We also report for the first time that fenofibrate decreases the concentrations of circulating PCSK9 in humans, suggesting that fenofibrate might reduce the residual cardiovascular risk in patients already on statins.

In this study, we measured PCSK9 concentrations in the plasma of 8 healthy young individuals and found them to be within the 0.8–7.9 mg/L range. In the cohort of 115 diabetic patients, PCSK9 concentrations were within the 0.1–9.3 mg/L range. Plasma PCSK9 concentrations have been reported in the 0.05–0.6 mg/L range in a population of 72 volunteers (8), in the 0.011–0.115 mg/L range in a population of 55 healthy donors (23), and in the 0.42–13.39 mg/L range in a population of 182 normolipemic individuals (24). The discrepancy between these 4 studies in terms of absolute PCSK9 concentrations may result from the use of monoclonal antibodies as capture antibodies in both studies with low absolute PCSK9 concentrations (8, 23) vs polyclonal antibodies in our study and in the study with the highest PCSK9 absolute concentrations (24). It is noteworthy, in the latter study, that an immunoprecipitation procedure followed by immunoblotting was used to measure circulating PCSK9 concentrations, rather than an ELISA. More likely, the discrepant absolute PCSK9 concentrations obtained in these studies may result from variable ability of the antibodies used to recognize epitopes on

Fig. 2. Correlation between plasma PCSK9 and total cholesterol, LDL cholesterol, HDL cholesterol, and TG concentrations in 115 nontreated diabetic patients.

Spearman correlation coefficients (r) and statistical significance (P) are indicated. TC, total cholesterol.
recombinant and endogenous PCSK9, as suggested elsewhere (23).

PCSK9 concentrations correlated positively with LDL cholesterol but not with HDL cholesterol or TG in our population of diabetic subjects, a result similar to what has been reported in healthy donors (23). Such a correlation was confined to males in another study (24). The \( r^2 \) values correlating plasma concentrations of PCSK9 (as measured by ELISA) and LDL cholesterol were 0.20 in healthy donors (23) and 0.29 in our study, consistent with a proposition that secreted PCSK9 accounts for about 20%–30% of the variability in plasma LDL cholesterol concentrations in these groups. In contrast, when PCSK9 concentrations were assayed by immunoprecipitation and Western blotting, the \( r^2 \) value relating these variables was 0.08 in males (23). Thus, it seems that plasma PCSK9 concentrations are not altered significantly in diabetes, nor is PCSK9’s function as a circulating inhibitor of the LDLr. We cannot rule out, however, that intracellularly, diabetes may have an effect on PCSK9 expression, as suggested by the effect that insulin exerts on PCSK9 expression in rodent primary hepatocytes (25).

We previously showed in mice that fenofibrate decreases the hepatic expression of PCSK9 (18). It is well known that activation of mouse and human PPAR\(\alpha\) often results in opposite physiological effects in the liver, as recently shown in humanized PPAR\(\alpha\) knock-in mice (26). In the current study, however, circulating plasma PCSK9 concentrations also decreased with fenofibrate. In addition, this decrease correlated with the efficacy of fenofibrate, since we found a positive correlation between changes in plasma PCSK9 and variations in TG, before and after treatment. The molecular mechanisms by which fenofibrate decreases plasma TG are enhancement of plasma lipolytic activity (an increase in lipoprotein lipase and a decrease of its in-

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**Fig. 3.** Assessment of 6-week fenofibrate monotherapy on circulating PCSK9 concentrations in 115 diabetic patients.

Posttreatment plasma PCSK9 concentrations are plotted against pretreatment values (A). The dotted line separates the 28 patients in whom PCSK9 concentrations were increased or not changed (above) from the 87 patients in whom PCSK9 concentrations were decreased (below) upon fenofibrate treatment. Variations of plasma TG (B) and LDL cholesterol (C) are plotted against variation in circulating PCSK9 concentrations before and after fenofibrate treatment. The patients in whom both PCSK9 and TG/LDL cholesterol concentrations decreased upon fenofibrate treatment are in the bottom left quadrant. \( \Delta \), change.

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**Fig. 4.** Fenofibric acid decreases PCSK9 secretion from human hepatoma HuH-7 cells.

Cells were grown in serum-free medium with 200 \( \mu \)mol/L fenofibric acid (Fen Ac) or DMSO (vehicle). PCSK9 secreted in the culture medium was immunoprecipitated, resolved by Western blot, and quantified by densitometric analysis. The figure displays a representative experiment (\( n = 3 \)). *\( P < 0.02 \) compared with vehicle treatment.
hibitor apolipoprotein C-III) and hepatic fatty acid oxidation (resulting in decreased TG-rich lipoprotein hepatic production) (20). Considering that the primary target of fenofibrate is to decrease plasma TG, it is not surprising that, if fenofibrate also decreases circulating PCSK9 concentrations, we found a positive correlation between changes in both PCSK9 and TG after fenofibrate treatment. We propose that the correlation that we observed between changes in TG and PCSK9 concentrations results from 2 distinct actions of fenofibrate and that they are not causatively linked.

We also found a positive correlation between changes in circulating PCSK9 (−8.5%) and changes in its ultimate target, LDL cholesterol (−13%), before and after fenofibrate treatment. This strongly suggests that the LDL cholesterol–lowering effect of fenofibrate is mediated by PCSK9. This study does not, however, rule out that the decrease observed in LDL cholesterol upon fenofibrate treatment in diabetic patients may also be mediated in part independently of PCSK9. In our study, the decrease in circulating PCSK9 concentrations (−8.5%) upon fenofibrate was modest compared to that observed in the livers of mice treated with the same drug (−60%) (18). Notably, we found that PCSK9 secretion was reduced by 50% from human hepatoma cells treated with fenofibric acid. It can be speculated that reduced clearance of circulating PCSK9 from the plasma compartment by extrahaepatic organs may compensate to some extent for the decrease in hepatic output in patients treated with fenofibrate, but the mechanism by which fenofibrate inhibits PCSK9 concentrations is not known. It could result from increased intrahepatic cholesterol concentrations and subsequently reduced SREBP2 (sterol regulatory element–binding protein 2) activity on PCSK9 gene transcription (5, 17). However, fenofibrate has been shown in mice to reduce the intestinal absorption of dietary cholesterol, which actually depletes intrahepatic cholesterol stores (27). Together with our results showing reduced PCSK9 secretion from fenofibric acid–treated hepatoma cells, this strongly suggests that fenofibrate negatively modulates PCSK9 expression and/or secretion directly at the hepatocyte level. We therefore propose that this negative modulation of PCSK9 explains at least in part the LDL cholesterol–lowering effect of fenofibrate.

This mode of action sheds new light on the use of fenofibrate to reduce the residual risk in patients already on statins, since the efficacy of statins on cardiovascular events has been clearly demonstrated in all patients groups investigated to date. Interestingly, while our manuscript was in revision, atorvastatin was shown to increase plasma PCSK9 concentrations by 34% (28). We found similar results in 6 newly diagnosed hypercholesterolemic patients before and after initiation of statin therapy (not shown). The benefits of fenofibrate therapy on top of statin treatment are currently being investigated in the ACCORD (Action to Control Cardiovascular Risk in Diabetes) study (19–22). It would be of interest to investigate whether fenofibrate prevents the anticipated upregulation of PCSK9 mediated by statins in this clinical trial.

**References**


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