# Modulation of HDL metabolism : studies in APOE*3-Leiden.CETP mice

Haan, W. de

# Chapter 2

## FENOFIBRATE INCREASES HDL-CHOLESTEROL BY REDUCING CHOLESTERYL ESTER TRANSFER PROTEIN EXPRESSION

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**Journal of Lipid Research 2007; 48: 1763-1771**

**Abstract**

In addition to efficiently decreasing VLDL-triglycerides (TG), fenofibrate increases HDL-cholesterol levels in humans. We investigated whether the fenofibrate-induced increase in HDL-cholesterol is dependent on the expression of the cholesteryl ester transfer protein (CETP). To this end, *APOE*3-Leiden* (*E3L*) transgenic mice without and with the human CETP transgene, under control of its natural regulatory flanking regions, were fed a Western-type diet with or without fenofibrate. Fenofibrate (0.04% in the diet) decreased plasma TG in *E3L* and *E3L.CETP* mice (-59% and -60%; $P<0.001$), caused by a strong reduction in VLDL. Whereas fenofibrate did not affect HDL-cholesterol in *E3L* mice, fenofibrate dose-dependently increased HDL-cholesterol in *E3L.CETP* mice (up to +91%). Fenofibrate did not affect the turnover of HDL-cholesteryl esters (CE), indicating that fenofibrate causes a higher steady-state HDL-cholesterol level without altering the HDL-cholesterol flux through plasma. Analysis of the hepatic gene expression profile showed that fenofibrate did not differentially affect the main players in HDL metabolism in *E3L.CETP* mice compared with *E3L* mice. However, in *E3L.CETP* mice, fenofibrate reduced hepatic *CETP* mRNA (-72%; $P<0.01$) as well as the CE transfer activity in plasma (-73%; $P<0.01$). We conclude that fenofibrate increases HDL-cholesterol by reducing the CETP-dependent transfer of cholesterol from HDL to (V)LDL, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool.

## Introduction

High plasma TG levels are correlated with an increased risk for cardiovascular disease.[1] Fibrates are widely used to reduce hypertriglyceridemia, thereby generating a less atherogenic lipid phenotype. Fibrates perform their actions through activation of peroxisome proliferator-activated receptor alpha (PPARα).[2,3] Activated PPARα heterodimerizes with retinoid X receptor (RXR) and subsequently binds to specific peroxisome proliferator response elements (PPREs) in target genes to alter their transcription.[2,4] Fibrates thus decrease TG levels by inhibiting hepatic TG production through increased hepatic ß-oxidation and inhibition of *de novo* fatty acid synthesis, increasing LPL-mediated lipolysis, and providing a higher affinity of remnants for the LDL receptor (LDLr).[3]

A meta-analysis of 53 clinical studies using fibrates enrolling 16,802 subjects indicated that apart from a 36% reduction in plasma TG, fibrates increase HDL-cholesterol levels by ~10% in humans.[5] Studies *in vitro* and in (transgenic) mice showed that fibrates increase the hepatic transcription of human *APOA1*[6] and *APOA2*,[7] decrease hepatic receptor B type I (SR-BI) protein,[8] increase the SR-BI-mediated[9] and ABCA1-mediated[10] cholesterol efflux from human macrophages, and increase plasma phospholipid transfer protein (PLTP) activity.[11,12] All of these effects may thus potentially contribute to the increase in HDL-cholesterol as observed in humans.

In contrast to humans, fibrates decrease HDL-cholesterol levels in apolipoprotein E (apoE)-deficient mice[13] and do not affect HDL-cholesterol levels but increase the HDL particle size in wild-type mice and human APOA1 transgenic mice by downregulation of SR-BI[8] and/ or induction of PLTP.[11] The fact that fibrates do not increase the level of regularly sized HDLs in mice may be attributed to the fact that, in contrast to the human *APOA1* promoter, which contains a functional positive PPRE leading to increased *APOA1* transcription, the murine *apoa1* promoter contains a nonfunctional PPRE.[6] However, another major difference between both species is that, in contrast to humans,[14] mice do not express the cholesteryl ester transfer protein (CETP).[15] CETP is a hydrophobic plasma glycoprotein that is involved in the exchange of cholesteryl esters (CE) and TG between HDL and apoB-containing lipoproteins (*e.g.* VLDL and LDL), resulting in the net transfer of CE from HDL to apoB-containing lipoproteins.[16] CETP deficiency in humans is associated with increased HDL-cholesterol levels[17] and inhibition of CETP activity by small-molecule inhibitors increases HDL-cholesterol levels.[18-21] Interestingly, bezafibrate,[22,23] fenofibrate,[24] and ciprofibrate[25] increase HDL-cholesterol in subjects with hyperlipidemia with a concomitant reduction in plasma CETP activity. In the latter study, plasma apoAI levels were not affected, which indicates that fibrates may increase HDL-cholesterol levels via apoAI-independent mechanisms, including a potential effect of fibrates on CETP expression.

Therefore, our aim was to investigate whether the fibrate-induced increase in HDL-cholesterol depends on CETP expression. To this end, we used, *APOE*3-Leiden* (*E3L*) mice that express a natural mutation of the human APOE3 gene (i.e., tandem repeat of codons 120-126, yieling a protein of 306 amino acids) in addition to the human APOC1 gene. Introdution of these genes results in an attenuated clearance of apoB-containing lipoproteins via the LDL receptor pathway. Therefore these mice show modlerately increased cholesterol and TG levels on a chow diet and a human-like lipoprotein profile an a cholesterol rich diet.[26,27] E3L mice were crossbred with mice expressing human *CETP* under control of its natural flanking regions,[28] resulting in *E3L.CETP* mice.[29] *E3L.CETP* and *E3L* littermates were fed a cholesterol-rich (0.25%, w/w) diet with or without fenofibrate. After 2 weeks of administration, fenofibrate dose-dependently increased HDL-cholesterol in *E3L.CETP* mice, but did not affect HDL levels in *E3L* mice. In addition, in *E3L.CETP* mice fenofibrate reduced hepatic *CETP* mRNA expression, as well as CE transfer activity in plasma. From these studies, we conclude that fenofibrate increases HDL-cholesterol by reducing CETP-dependent transfer of CE from HDL to apoB-containing lipoproteins.

## Materials and Methods

*Animals*

Hemizygous human CETP transgenic (*CETP*) mice, expressing a human CETP minigene under the control of its natural flanking sequences[28] were purchased from the Jackson Laboratory (Bar Harbor, ME) and cross-bred with hemizygous *E3L* mice[30] at our Institutional Animal Facility to obtain *E3L* and *E3L.CETP* littermates.[29] In this study, male mice were used, housed under standard conditions in conventional cages with free access to food and water. At the age of 8 weeks, mice were fed a semi-synthetic cholesterol-rich diet, containing 0.25% (w/w) cholesterol and 15% (w/w) fat (Western-type diet) (Hope Farms, Woerden, The Netherlands) for 3 weeks. Upon randomization according to total plasma cholesterol (TC) levels, mice received Western-type diet without or with 0.004%, 0.012%, or 0.04% (w/w) fenofibrate (Sigma, St. Louis, MO). Experiments were performed after 4 h of fasting at 12:00 PM with food withdrawn at 8:00 AM, unless indicated otherwise. The institutional Ethical Committee on Animal Care and Experimentation approved all experiments.

*Plasma lipid and lipoprotein analysis*

Plasma was obtained via tail vein bleeding as described[31] and assayed for TC and TG, using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN), respectively. The distribution of lipids over plasma lipoproteins was determined by fast-

performance liquid chromatography using a Superose 6 column as described previously.[31]

*Cholesteryl ester transfer activity in plasma*
The transfer of newly synthesized CE in plasma was assayed by a radioisotope method as described previously.[32] In short, [$^3$H]cholesterol was complexed with BSA and incubated overnight at 4°C with mouse plasma to equilibrate with plasma free cholesterol. Subsequently, the plasma samples were incubated for 3 h at 37°C. VLDL and LDL were then precipitated by addition of sodium phophotungstate/$MgCl_2$. Lipids were extracted from the precipitate by methanol-hexane (1:2, v/v) and [$^3$H]CE was separated from [$^3$H]cholesterol on silica columns, followed by counting of radioactivity.

*Plasma apoAI concentration*
Plasma apoAI concentrations were determined using a sandwich ELISA. Rabbit anti-mouse apoAI polyclonal antibody (ab20453; Abcam plc, Cambridge, UK) was coated overnight onto Costar strips (Costar, Inc., New York, NY) (3 µg/ml) at 4°C and incubated with diluted mouse plasma (dilution 1:400,000) for 90 min at 37°C. Subsequently, goat anti-mouse apoAI antibody (600-101-196; Rockland Immunochemicals, Inc., Gilbertsville, PA; dilution 1:3000) was added and incubated for 90 min at 37°C. Finally, HRP-conjugated rabbit anti-goat IgG antibody (605-4313; Rockland Immunochemicals; dilution 1:15,000) was added and incubated for 90 min at 37°C. HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Purified mouse apoAI (A23100m; Biodesign International, Saco, ME) was used as a standard.

*Radiolabeling of autologous HDL*
One mouse from each experimental group was euthanized by cervical dislocation, and blood was drawn from the retro-orbital vein. Sera were collected and HDL was isolated after density ultracentrifugation in a SW 40 Ti rotor (Beckman Instruments, Geneva, Switzerland) (4°C; 40,000 rpm; overnight).[33] HDL (0.4 µmol HDL-cholesterol) was radiolabeled by incubation (37°C; 24 h) with [$^3$H]cholesteryl oleyl ether ([$^3$H]COEth)-labeled egg yolk phosphatidylcholine vesicles (40 µCi; 0.5 mg phospholipid) in the presence of lipoprotein deficient serum (1 ml) from *E3L.CETP* mice. Subsequently, HDL was reisolated after density ultracentrifugation (12°C; 40,000 rpm; 24 h).

*In vivo clearance of autologous HDL*
After 6 weeks of diet, mice were injected via the tail vein with a trace of autologous [$^3$H]COEth-labeled HDL ($0.2x10^6$ cpm in PBS) at 8:00 am. At the indicated time points after injection, blood was collected to determine the plasma decay of [$^3$H]COEth by scintillation counting (Packard Instruments,

Dowers Grove, IL). The total plasma volumes of the mice were calculated from the equation V (ml) = 0.04706 x body weight (g), as determined from previous $^{125}$I-BSA clearance studies.[34] The fractional catabolic rate (FCR) was calculated as pools of HDL-CE cleared per h from the plasma decay curves as described previously.[35] Briefly, curves were fitted using GraphPad Prism software, giving the best fit for one-phase exponential decay curves, described by the formula Y=span*exp(-k*x)+plateau. Subsequently the FCR was calculated as span/(area under the curve). Taking into account the fact that the plasma level of HDL was altered by the expression of CETP and fenofibrate treatment, the FCR was also calculated from these data as millimolar HDL-CE cleared per hour, based on the actual level of HDL-CE in the various mouse groups.

*Hepatic mRNA expression, SR-BI protein analysis, and lipid analysis*

Livers were isolated after cervical dislocation. Total RNA was isolated using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. RNA expression was determined in duplicate by real-time PCR on a MyiQ Single-Color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Primers for *CETP*[36] and *sr-b1*[37] have been described previously. Primers for *abca1*, *apoa1*, *cyp7A1,* and *pltp* are listed in Table 1. Expression levels were normalized using hypoxanthine-guanine phosphoribosyl transferase (HPRT) and cyclophilin as housekeeping genes.[37,38] Hepatic SR-BI protein was determined by immunoblot analysis as described previously.[39] Liver lipids were determined by homogenizing liver samples in water (~10% wet w/v) using a mini-bead beater (Biospec Products, Inc., Bartlesville, OK; 20 sec; 5,000 rpm), followed by lipid extraction as described by Bligh and Dyer.[40] Extracts were assayed for TC as described above. Protein was determined according to the method of Lowry et al.[41]

**Table 1.** Primers for quantitative real-time PCR analysis

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| *abca1* | CCCAGAGCAAAAAGCGACTC | GGTCATCATCACTTTGGTCCTTG |
| *apoa1* | GGAGCTGCAAGGGAGACTGT | TGCGCAGAGAGTCTACGTGTGT |
| *cyp7a1* | CAGGGAGATGCTCTGTGTTCA | AGGCATACATCCCTTCCGTGA |
| *pltp* | TCAGTCTGCGCTGGAGTCTCT | AAGGCATCACTCCGATTTGC |

*Abca1*, ATP-binding cassette transporter a1; *apoa1*, apolipoprotein a1; *cyp7a1*, cholesterol 7α-hydroxylase; *pltp*, phospholipid transfer protein.

*Statistical analysis*

All data are presented as means ± SD unless indicated otherwise. Data were analyzed using the unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

## Results

*Fenofibrate increases HDL-cholesterol in E3L.CETP mice*

To study the dose-dependent effect of fenofibrate on plasma lipid levels on a hyperlipidemic background, *E3L.CETP* mice were fed a cholesterol-rich diet with increasing doses of fenofibrate in the diet (0%, 0.004%, 0.012%, and 0.04%) for 2 weeks each (Fig. 1). Fenofibrate caused a dose-dependent decrease in plasma TG levels (up to -62% at the highest dose; $P<0.05$) (Fig. 1A), and only tended to reduce plasma cholesterol levels (up to -35%; NS) (Fig. 1B). However, fenofibrate had a great impact on the distribution of cholesterol over the various lipoproteins. Whereas on a cholesterol-rich diet, most cholesterol in *E3L.CETP* mice is carried in (V)LDL, fenofibrate resulted in a dose-dependent shift of cholesterol from (V)LDL to HDL (Fig. 1C).

Subsequently, we compared the effect of fenofibrate on plasma lipid levels in *E3L.CETP* mice with those in *E3L* mice by using the highest dose of fenofibrate (0.04%) (Fig. 2). In *E3L* mice, fenofibrate decreased plasma TG levels (-59%; $P<0.001$) (Fig. 2A) to a similar extent as in *E3L.CETP* mice (-60%; $P<0.01$)

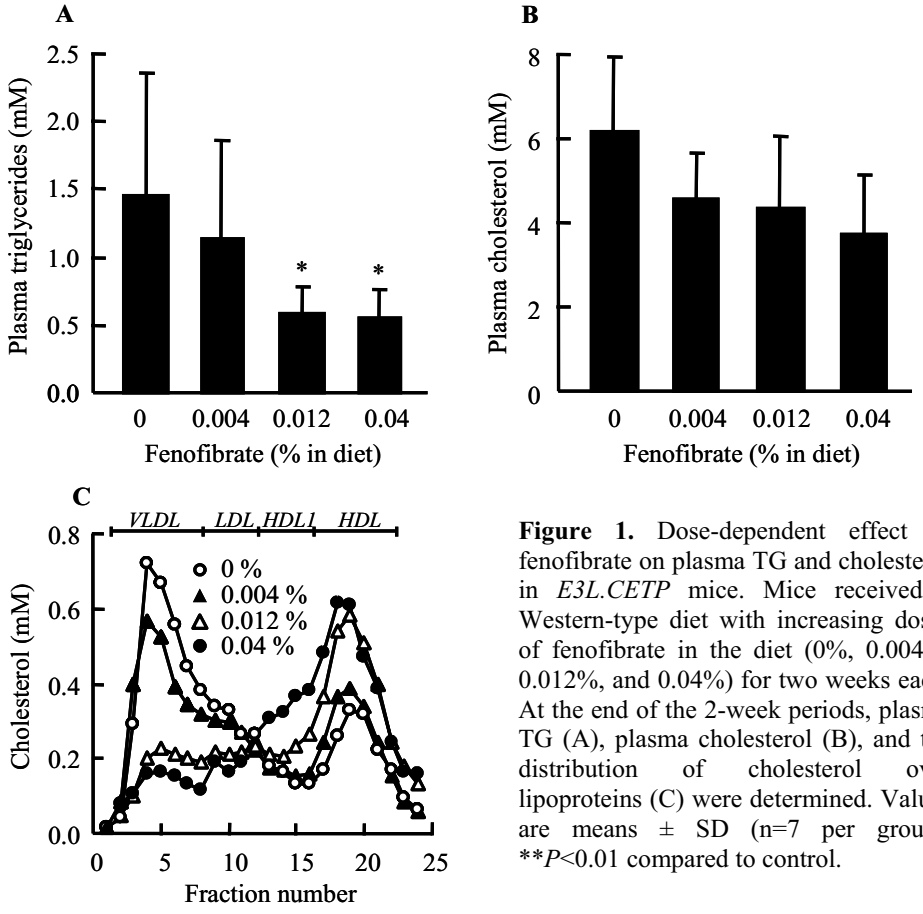


**Figure 1.** Dose-dependent effect of fenofibrate on plasma TG and cholesterol in *E3L.CETP* mice. Mice received a Western-type diet with increasing doses of fenofibrate in the diet (0%, 0.004%, 0.012%, and 0.04%) for two weeks each. At the end of the 2-week periods, plasma TG (A), plasma cholesterol (B), and the distribution of cholesterol over lipoproteins (C) were determined. Values are means ± SD (n=7 per group). **$P<0.01$ compared to control.

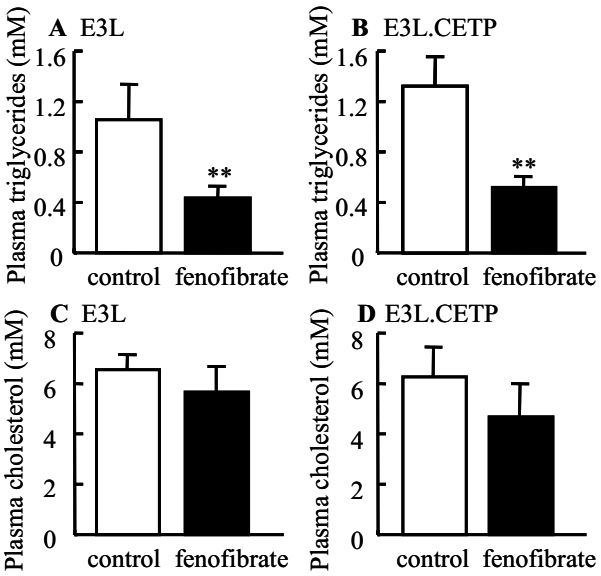


**Figure 2.** Effect of fenofibrate on plasma TG and cholesterol in *E3L* and *E3L.CETP* mice. *E3L* mice (A, C) and *E3L.CETP* mice (B, D) received a Western-type diet without (white bars) or with (black bars) fenofibrate for 2 weeks, and plasma TG (A, B) and cholesterol (C, D) were determined. Values are means ± SD (n=6 per group). ***P*<0.01 compared with controls.

(Fig. 2B). In both *E3L* mice and *E3L.CETP* mice, these effects of fenofibrate on plasma TG levels were reflected by a strong reduction in VLDL-TG (not shown). Fenofibrate also caused small trends towards lower plasma cholesterol levels in both *E3L* and *E3L.CETP* mice (Fig. 2C,D). Fenofibrate similarly decreased (V)LDL-cholesterol in both *E3L* mice (-91%) and *E3L.CETP* mice (-93%). However, whereas fenofibrate did not affect HDL-cholesterol levels in *E3L* mice, fenofibrate increased HDL-cholesterol in *E3L.CETP* mice (+91%) (Fig. 3), consistent with the dose-escalating study (Fig. 1).

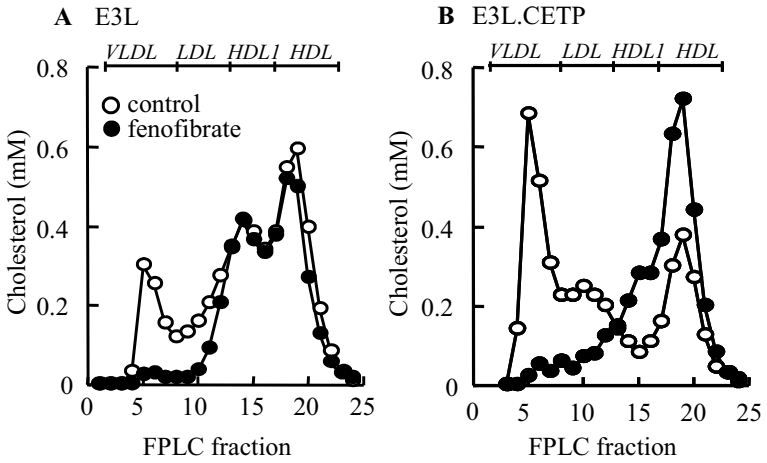


**Figure 3.** Effect of fenofibrate on the distribution of cholesterol over lipoproteins in *E3L* and *E3L.CETP* mice. *E3L* mice (A) and *E3L.CETP* mice (B) received a Western-type diet without (white circles) or with (black circles) fenofibrate. Plasmas of the various mouse groups were pooled (n=6 per group). Lipoproteins were separated by FPLC, and fractions were analyzed for cholesterol.

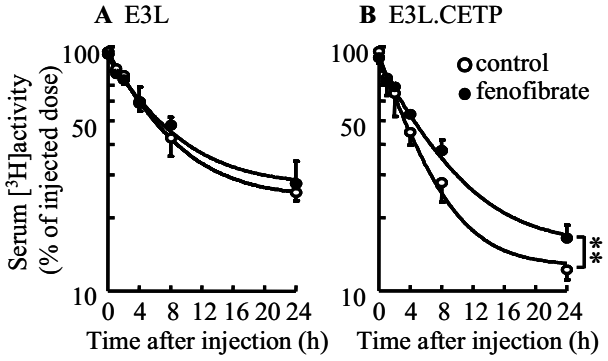


**Figure 4.** Effect of fenofibrate on the plasma clearance of HDL in *E3L* and *E3L.CETP* mice. *E3L* mice (A) and *E3L.CETP* mice (B) received a Western-type diet without (white circles) or with (black circles) fenofibrate. Mice were injected with autologous [$^3$H]COEth-labeled HDL and plasma $^3$H-activity was determined at the indicated time points. Values are means ± SD (n=5 per group). $^{**}P<0.01$ compared with controls.

*Fenofibrate increases the steady-state plasma HDL level without affecting net HDL-CE output in E3L.CETP mice*

To examine the mechanism underlying the fenofibrate-induced increased HDL-cholesterol in *E3L.CETP* mice, *E3L* and *E3L.CETP* mice were injected with autologous [$^3$H]COEth-labeled HDL and the plasma decay was determined (Fig. 4). The expression of CETP *per se* appeared to accelerate the plasma decay, reflected by an increased fractional catabolic rate (FCR) as calculated pools of HDL-CE cleared per hour (+65%; $P<0.01$; Table 2). In *E3L* mice, fenofibrate administration did not affect the clearance of HDL-CE (Fig. 4A; Table 1). In contrast, fenofibrate decreased the FCR of HDL in *E3L.CETP* mice (-27%; $P<0.01$). However, taking into account the fact that CETP expression and fenofibrate treatment influence plasma HDL levels (Fig. 3), the FCR was also calculated as millimolar HDL-CE cleared per hour. In fact, CETP expression in E3L mice, or fenofibrate feeding of either *E3L* or *E3L.CETP* mice, did not affect the amount (mM) of HDL-CE cleared per hour (Table 2). This indicates

**Table 2.** Effect of fenofibrate on the FCR of HDL-CE in E3L and E3L.CETP mice

| | Control | Fenofibrate |
|---|---|---|
| FCR (pools HDL-CE per h) | | |
| *E3L* | 0.067±0.003 | 0.057±0.004 |
| *E3L.CETP* | 0.111±0.006 | 0.081±0.003* |
| FCR (mM HDL-CE per h) | | |
| *E3L* | 0.166±0.008 | 0.162±0.011 |
| *E3L.CETP* | 0.142±0.008 | 0.162±0.007 |

*E3L* and *E3L.CETP* mice were fed a Western-type diet with or without fenofibrate, and mice were injected with autologous [$^3$H]COEth-labeled HDL. The data from Figure 4 were used to calculate the FCR as pools of HDL-CE cleared per hour or millimolar HDL-CE cleared per hour. Values are expressed as means ± SEM relative to control mice (n=5 mice per group). $^*P<0.01$ compared with controls.

that CETP expression and fenofibrate feeding alter the steady-state plasma HDL-cholesterol level without affecting the net HDL-cholesterol flux through plasma. These data indicate that the residual CETP activity in E3L.CETP mice on fenofibrate is sufficient to maintain net HDL-CE output.

*Fenofibrate does not differentially affect hepatic mRNA expression of genes involved in plasma HDL metabolism*

Because differences in genes encoding proteins that are crucially involved in HDL metabolism may account for the increase in HDL-cholesterol in *E3L.CETP* mice upon fenofibrate treatment, we examined the effect of fenofibrate on their hepatic expression (Fig. 5). The expression of these genes was not substantially different in E3L.CETP mice compared with E3L mice. Fenofibrate increased *Pltp* in *E3L* (3.5-fold; $P<0.01$) and *E3L.CETP* mice (2.7-fold; $P<0.05$), consistent with previously reported effects of fenofibrate.[11,12] The expression of *abca1*, which is involved in HDL formation, was similarly decreased in *E3L* (-50%; $P<0.05$) and *E3L.CETP* (-33%; $P<0.05$) mice. Likewise, s*r-b1* was decreased in *E3L* (-48%; $P<0.05$) and *E3L.CETP* (-42%; $P<0.05$) mice to a similar extent, as reflected by similar reductions in hepatic SR-BI protein levels (~-25%) for *E3L* ($P=0.06$) and *E3L.CETP* mice ($P<0.05$) (data not shown). *Apoa1* expression was decreased in *E3L* (-49%; $P<0.05$) and *E3L.CETP* (-41%; $P<0.05$) mice, without substantially affecting the plasma apoAI level (~80 mg/dL in all groups). The expression of *cyp7a1*, *pltp*, *abca1*, *sr-b1*, and *apoa1* is thus similarly affected by fenofibrate in *E3L* and *E3L.CETP* mice, and cannot explain the differentially raised HDL in *E3L.CETP* mice as compared to *E3L* mice.

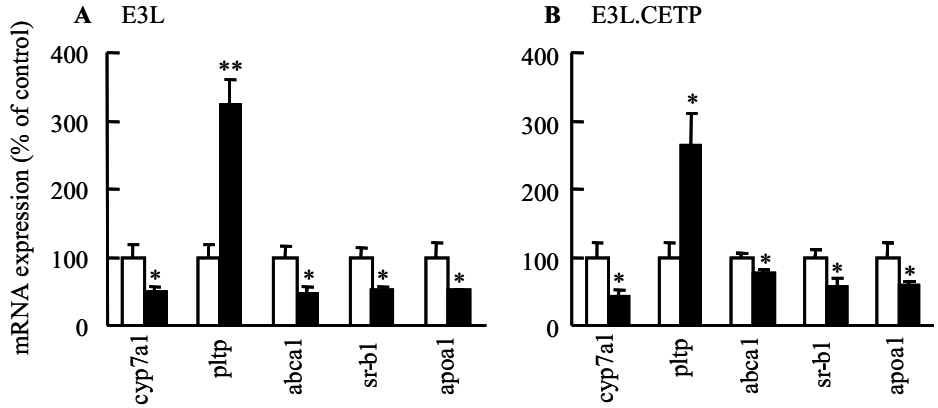


**Figure 5.** Effect of fenofibrate on hepatic mRNA expression in *E3L* and *E3L.CETP* mice. *E3L* mice (A) and *E3L.CETP* mice (B) were fed a Western-type diet with or without fenofibrate. Mice were euthanized, and livers were collected to determine mRNA expression. Values are expressed as means ± S.E. relative to control mice (n=4 per group). *$P<0.05$; **$P<0.01$ compared with controls.

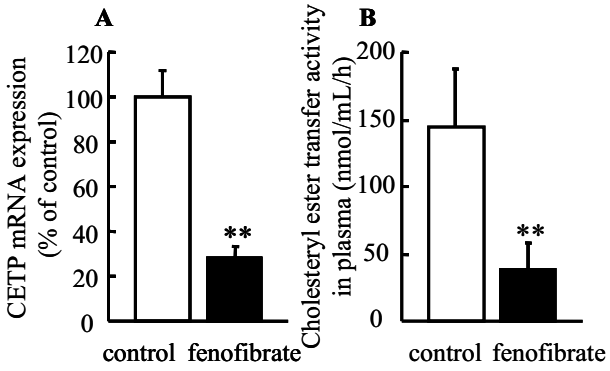


**Fig. 6.** Effect of fenofibrate on hepatic CETP mRNA expression and cholesteryl ester transfer activity in plasma of *E3L.CETP* mice. *E3L.CETP* mice received a Western-type diet with or without fenofibrate, and mice were euthanized. Livers were collected to determine CETP mRNA expression (A), and plasma was assayed for CE transfer activity (B). Values are means ± SD (n=4-6 per group). ***P*<0.01 compared with controls.

*Fenofibrate decreases hepatic CETP mRNA expression and CE transfer activity in plasma*

To investigate whether the effect of fenofibrate on increasing HDL-cholesterol in *E3L.CETP* mice is caused by reduction of CETP activity, we determined the hepatic *CETP* expression and CE transfer activity in plasma (Fig. 6). Indeed, fenofibrate markedly decreased *CETP* expression in *E3L.CETP* mice (-72%; *P*<0.01) (Fig. 6A). Because the liver X receptor (LXR) strongly regulates the expression of *CETP*,[42] we determined whether fenofibrate feeding would decrease the cholesterol content in the liver. Indeed, fenofibrate reduced the hepatic cholesterol content in *E3L* mice (4.9±2.6 vs 9.6±3.7 μg TC/mg protein) and *E3L.CETP* mice (3.6±1.0 vs 13.0±3.7 μg TC/mg protein; *P*<0.05). The fenofibrate-induced reduction in hepatic *CETP* expression was accompanied by a similar reduction in the CE transfer activity in plasma of *E3L.CETP* mice (-73%; *P*<0.01) (Fig. 6B). Therefore, the HDL-raising effect of fenofibrate in *E3L.CETP* mice is thus likely to be a direct consequence of lower CETP expression.

## Discussion

In this study, we investigated whether CETP might play a role in the fenofibrate-induced increase in HDL-cholesterol. Here we show that fenofibrate increases HDL-cholesterol in *E3L.CETP* mice, as paralleled by a reduction in hepatic *CETP* mRNA and plasma CE transfer activity, whereas fenofibrate does not increase HDL in *E3L* mice.

We showed previously that *E3L* mice are highly susceptible to dietary interventions with respect to modulating plasma lipid levels, and that these mice show a human-like response to drug interventions aimed at reducing plasma levels of apoB-containing lipoproteins, including statins (atorvastatin[43] and rosuvastatin)[44] and fibrates (gemfibrozil).[45] This is in sheer contrast with wild-

type mice[6,13] and more conventional hyperlipidemic mice such as apoE-deficient[13,46] or LDL receptor-deficient[47] mice, which show either an adverse or no response to such interventions. In particular, administration of fenofibrate to wild-type[13] and apoE-deficient[13,48] mice showed an unexpected increase in plasma TG and TC levels caused by elevated levels of lipoprotein remnants, with a concomitant reduction in HDL-cholesterol. Here we demonstrate that *E3L* mice also show a human-like response to fenofibrate with respect to decreasing TG and cholesterol in apoB-containing particles, although HDL-cholesterol was not increased after 2 weeks of intervention (Fig. 3A). We reasoned that introduction of human CETP in these *E3L* mice, which permits CE exchange between HDL and apoB-containing lipoproteins, would result in an excellent mouse model to study the effects of fenofibrate on HDL metabolism.

Indeed, we demonstrate that although *E3L.CETP* mice retain their ability to respond to fenofibrate with respect to a similar reduction of VLDL-TG and VLDL-cholesterol as compared with *E3L* mice, they also respond with an increase in HDL-cholesterol level. Apparently, the fact that mice normally do not express CETP prevents a human-like response to HDL-modulating drug interventions, such as fibrate treatment. In agreement with this hypothesis, we observed previously that treatment of *E3L* mice with statins also did not increase HDL-cholesterol even though VLDL reductions of as much as 60% were achieved.[43,45,49]

HDL-cholesterol levels can theoretically be modulated by several key proteins involved in HDL metabolism, including ABCA1,[10] SR-BI,[9] PLTP,[11,23] apoAI,[3,6,48,50,51] and CETP.[22,24,25] Therefore, we examined the potential contribution of each of these factors in the fenofibrate-induced increase of HDL-cholesterol in *E3L.CETP* mice. The HDL-cholesterol level in mice is largely determined by the hepatic expression of ABCA1, which plays an important role in HDL formation by mediating hepatic cholesterol efflux to apoAI.[52] In fact, it has been reported that treatment of chow-fed rats with ciprofibrate increased their hepatic *abca1* expression, concomitant with an increase in plasma HDL-cholesterol levels.[53] However, fenofibrate did not increase hepatic *abca1* expression in either *E3L* or *E3L.CETP* mice. On the contrary, fenofibrate decreased *abca1* mRNA in both genotypes and thus can not explain the selective increase of HDL-cholesterol in E3L.CETP mice.

Whereas bezafibrate did not increase the plasma PLTP mass and activity levels in humans,[23] fenofibrate has been shown to increase the hepatic *pltp* expression in mice, which was associated with increased plasma PLTP activity and HDL size, at least in human apoAI transgenic mice.[11] Accordingly, we found that fenofibrate induced the hepatic *pltp* expression both in *E3L* and *E3L.CETP* mice. However, the relative increase was even more pronounced in *E3L* mice as compared to *E3L.CETP* mice, whereas HDL-cholesterol was not affected in *E3L* mice. It is also of note that adenovirus-mediated hepatic expression of

PLTP results in a dose-dependent reduction of HDL-cholesterol levels, instead of increasing HDL-cholesterol, in both wild-type and human apoAI-transgenic mice.[54] It is thus unlikely that the induction of PLTP is the cause of the increase in HDL-cholesterol as observed in *E3L.CETP* mice.

In mice, hepatic SR-BI represents the most important pathway for the selective clearance of HDL-associated cholesteryl ester from plasma.[55] It has been shown that fenofibrate can downregulate hepatic SR-BI protein in wild-type mice, independent of *sr-b1* expression, via a posttranscriptional mechanism. This was correlated with a substantially increased HDL size, based on fast-performance liquid chromatography profiling.[8] We found that fenofibrate treatment did result in a similar reduction of *sr-b1* expression in *E3L* mice (-48%) and *E3L.CETP* mice (-42%), with a concomitant reduction in hepatic SR-BI protein levels (~-25%). Although fenofibrate did not increase in large HDL-1 in E3L mice after only 2 weeks of fenofibrate intervention (Fig 3A), cholesterol within large HDL-1 was indeed increased (+69%) after prolonged treatment of *E3L* mice (i.e., 6 weeks), as has also been shown for wild-type mice,[8] In *E3L.CETP* mice fenofibrate treatment for 2 weeks increased the levels of cholesterol in regularly sized HDLs but also increased the levels of HDL-1 to some extent (Figs. 1C, 3B). Therefore, the reduction in hepatic SR-BI levels may contribute to the appearance of HDL-1 in both *E3L* and *E3L.CETP* mice but does not explain the increase of regularly sized HDL in E3L.CETP mice.

In *APOA1* transgenic mice, human apoAI hepatic mRNA and plasma protein levels were increased after fenofibrate treatment,[6] probably by the binding of PPARα to a positive PPRE in the human apoAI gene promoter.[51] Given the tight relation between HDL-cholesterol and apoAI levels in humans, it could be expected that upregulation of apoAI expression would be the main causal factor for increasing HDL-cholesterol levels in humans. Fenofibrate treatment has an opposite effect on murine apoAI (i.e., reduction of expression and plasma levels),[6] which theoretically could easily explain why fenofibrate does not increase HDL-cholesterol in mice. However, although we do observe a reduction in hepatic *apoa1* expression upon fenofibrate treatment of *E3L* (-49%) and *E3L.CETP* (-41%) mice, HDL-cholesterol was nevertheless markedly increased in *E3L.CETP* mice. The fact that plasma apoAI was not affected by fenofibrate treatment may thus be explained by increased lipidation of apoAI, thereby preventing the clearance of apoAI.

Collectively, these data thus suggest that downregulation of CETP expression is the predominant cause of the fenofibrate-induced elevation of HDL-cholesterol. Expression of CETP in *E3L* mice decreased the HDL-cholesterol level (~-35%) but did not affect the HDL turnover, calculated as millimolar HDL-CE cleared per hour. Likewise CETP inhibition in rabbits, although increasing HDL-cholesterol, does not compromise the HDL-CE clearance from plasma.[56] Treatment of *E3L.CETP* mice with fenofibrate resulted in an increased HDL-cholesterol level, strongly decreased hepatic CETP expression levels, and

reduced cholesteryl ester transfer activity in plasma. Thus the increase in HDL-cholesterol may be caused by the combination of reduced hepatic *CETP* expression and reduced levels of apoB-containing lipoproteins as CE acceptors, thereby inhibiting the CETP-mediated transfer of CE from HDL to (V)LDL.

It is tempting to speculate about the mechanism(s) underlying the effect of fenofibrate on hepatic *CETP* expression. Dietary cholesterol has been shown to increase *CETP* mRNA expression in CETP transgenic mice,[28,29] presumably via an LXR responsive element in the CETP promoter.[42] Conversely, a decrease in hepatic *CETP* mRNA expression might thus be the consequence of a reduction in LXR signaling. Fenofibrate treatment indeed decreased hepatic cholesterol, which is likely to reduce the level of oxysterols, the natural ligands of LXRα. Down-regulation of LXRα is supported by a concomitant decrease in the expression of *cyp7a1*, another LXR-target gene.[57] This is in accordance with the observation that administration of ciprofibrate to wild-type mice caused a 65% reduction in hepatic *cyp7a1* mRNA.[58] Nevertheless it should be mentioned that *cyp7a1* is also regulated directly by fibrates via a negative PPRE in its promoter sequence.[59] A reduction in LXRα might also explain the reduction of *abca1* expression.[60] In addition to these mechanisms explaining reduced CETP expression by fenofibrate, a potential PPRE in the promoter region of CETP was recently identified,[61] which provides the possibility for direct regulation of CETP by PPARα agonists, although it is unclear whether this potential PPRE is functional.

Our finding that fenofibrate reduced CETP activity in E3L.CETP mice corroborates the outcome of two human studies. Although one study failed to detect an effect of fenofibrate on plasma CETP activity,[62] fenofibrate treatment did decrease CETP activity by 26% in subjects with combined hyperlipidemia[24] and by 18% in subjects with the metabolic syndrome.[63] Based on our experimental study, the fenofibrate-induced decrease in CETP activity in humans is likely also a causal factor for the generally observed increase in HDL-cholesterol.

Fibrate treatment has been associated with a reduction of cardiovascular disease.[5] The recent FIELD study, which assessed the effects of fenofibrate on cardiovascular risk in subjects with type 2 diabetes mellitus in a long-term, controlled trial, showed a reduction in total cardiovascular events, but did not reveal a reduced risk of the primary outcome of coronary events.[64] Nevertheless, the authors suggested that a more beneficial outcome might have been masked by a larger portion of statin treatment in the placebo group as compared with the fenofibrate group. Even though the benefit of an increase in HDL-cholesterol by CETP inhibition is still under debate,[65-68] and despite the recent failure of the CETP inhibitor torcetrapib in the ILLUMINATE study,[69] increasing HDL-cholesterol levels is still generally considered anti-atherogenic. Besides the ability of fibrates to potently reduce plasma TG, their concomitant effect on increasing HDL by reducing CETP expression may thus be an additional

advantageous anti-atherogenic property. We speculate that combination therapies of fibrates (i.e., reducing CETP expression) with small molecule CETP inhibitors (i.e., reducing plasma CETP activity) may help to further reduce cardiovascular risk.
Together, our data show that fenofibrate increases HDL-cholesterol by reducing CETP expression and plasma CE transfer activity in *E3L.CETP* mice. Therefore, we postulate that reduction of CETP expression also contributes to the increase in HDL that is found in human subjects treated with fibrates. Furthermore, we anticipate that the *E3L.CETP* mouse is a valuable model in which to test the effect of combination therapies (i.e., fibrates and CETP inhibitors) on plasma lipid metabolism and atherosclerosis.

**Acknowledgements**

This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO, and supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), the Netherlands Organization for Scientific Research (NWO grant 908-02-097 and NWO VIDI grant 917.36.351 to P.C.N.R.), the Netherlands Heart Foundation (NHS grant 2003B136 to P.C.N.R.) and the Center for Medical Systems Biology (project 115). J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (2001D032). We thank L.C. van der Zee-van Vark and E. Hoegee-de Nobel for excellent technical assistance.

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