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## Modulation of VLDL triglyceride metabolism

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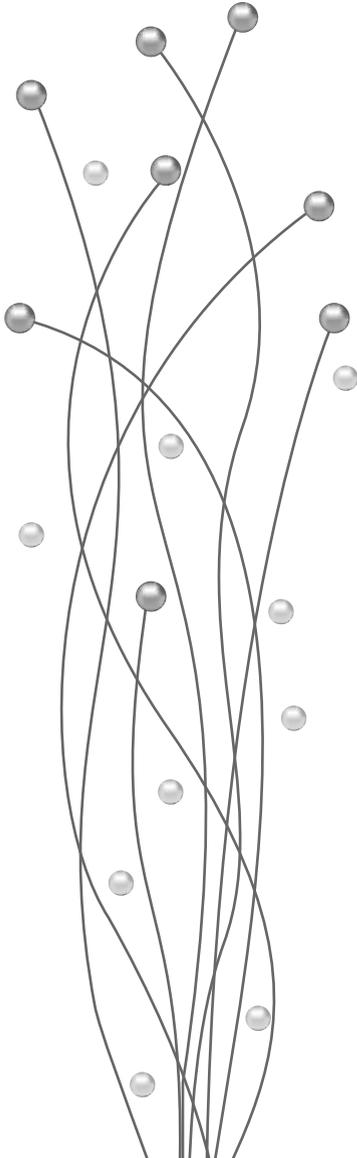
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# CHAPTER 3

## Fenofibrate increases VLDL-triglyceride production despite reducing plasma triglyceride levels in ApoE\*3-Leiden.CETP mice



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The PPAR $\alpha$  activator fenofibrate efficiently decreases plasma triglycerides (TG), which is generally attributed to enhanced VLDL-TG clearance and decreased VLDL-TG production. However, since data on the effect of fenofibrate on VLDL production are controversial, we aimed to investigate in (more) detail the mechanism underlying the TG-lowering effect by studying VLDL-TG production and clearance using ApoE\*3-Leiden.CETP mice, a unique mouse model for human-like lipoprotein metabolism. Male mice were fed a Western-type diet for 4 weeks, followed by the same diet without or with fenofibrate (30 mg/kg bodyweight/day) for 4 weeks. Fenofibrate strongly lowered plasma cholesterol (-38%; P<0.001) and TG (-60%; P<0.001) caused by reduction of VLDL. Fenofibrate markedly accelerated VLDL-TG clearance, as judged from a reduced plasma half-life of intravenously injected glycerol tri[ $^3$ H]oleate-labeled VLDL-like emulsion particles (-68%; P<0.01). This was associated with an increased post-heparin LPL activity (+110%; P<0.0001) and an increased uptake of VLDL-derived fatty acids by skeletal muscle, white adipose tissue and liver. Concomitantly, fenofibrate markedly increased the VLDL-TG production rate (+73%; P<0.0001) but not the VLDL-apoB production rate. Kinetic studies using [ $^3$ H]palmitic acid showed that fenofibrate increased VLDL-TG production by equally increasing incorporation of re-esterified plasma FA and liver TG into VLDL, which was supported by hepatic gene expression profiling data. We conclude that fenofibrate decreases plasma TG by enhancing LPL-mediated VLDL-TG clearance, which results in a compensatory increase in VLDL-TG production by the liver.

## Introduction

The lipid-lowering agent fenofibrate reduces plasma triglyceride (TG) levels and increases HDL-cholesterol (HDL-C) levels, which generates a less atherogenic lipid phenotype<sup>120, 121</sup>. Fenofibrate acts through activation of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) thereby altering the expression of genes involved in lipid metabolism<sup>145, 146, 147</sup>. Several mechanisms of action have been proposed through which fenofibrate lowers TG levels, including increased VLDL-TG clearance and decreased hepatic TG production<sup>147</sup>.

VLDL-TG clearance is governed by lipoprotein lipase (LPL), of which the expression is potently induced by PPAR $\alpha$ <sup>64</sup>. In addition, it has been shown that PPAR $\alpha$  agonists down-regulate the expression of the LPL inhibitor apoCIII, and up-regulate the expression of the LPL activator apoAV<sup>63</sup>. Altogether this results in an increase in LPL-mediated lipolysis and clearance of VLDL. Indeed, two human studies show that fenofibrate increases the fractional catabolic rate (FCR) of VLDL-apoB in patients with hypertriglyceridemia without or with type 2 diabetes<sup>148, 149</sup>, which is associated with increased LPL activity<sup>148</sup>.

Hepatic VLDL production is dependent on the availability of fatty acids (FA) which is determined by *de novo* FA synthesis, FA/TG uptake from the circulation and  $\beta$ -oxidation of FA in the liver. PPAR $\alpha$  has been shown to influence VLDL production in mice. PPAR $\alpha$  deficiency in mice increased hepatic VLDL-TG production<sup>150, 151</sup>, and the selective PPAR $\alpha$  agonist Wy14643 lowered VLDL-TG production, at least in severely hypertriglyceridemic Angptl4 transgenic mice<sup>152</sup>. Limited data exist on the specific effect of fenofibrate on hepatic VLDL production. Although *in vitro* experiments using cultured hepatocytes show that fenofibrate, among other fibrates, decreases the production of both VLDL-TG and apoB<sup>153, 154</sup>, in patients with the metabolic syndrome, fenofibrate treatment had no effect on the VLDL-apoB production rate<sup>149</sup>.

Our aim was to investigate in detail the mechanism underlying the VLDL-TG lowering effect of fenofibrate *in vivo*. We used ApoE\*3-Leiden.CETP (E3L.CETP) mice<sup>108, 116</sup> that express human CETP under control of its natural flanking regions<sup>129</sup>. These mice have an attenuated clearance of apoB-containing lipoproteins and, therefore, show a human-like lipoprotein profile on a cholesterol-rich Western-type diet<sup>114, 116</sup>. Our data show that treatment of

E3L.CETP mice on a Western-type diet with fenofibrate decreases plasma VLDL-TG as explained by increased VLDL-TG clearance resulting from enhanced LPL activity, but increases VLDL-TG production by increasing lipitation of apoB with TG that is equally derived from esterification of plasma FA and hepatic stores.

## Materials and Methods

### *Animals*

42 Hemizygous human CETP transgenic mice, expressing a human CETP minigene under the control of its natural flanking regions<sup>129</sup> were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossbred with hemizygous E3L mice<sup>108</sup> at our Institutional Animal Facility to obtain E3L.CETP mice<sup>116</sup>. In this study, male E3L.CETP and wild-type mice (both C57Bl/6 background) were used, housed under standard conditions in conventional cages with free access to food and water. At the age of 12 weeks, mice were fed a semi-synthetic cholesterol-rich diet, containing 0.25% (w/w) cholesterol, 1% (w/w) corn oil and 14% (w/w) bovine fat (Western-type diet) (Hope Farms, Woerden, The Netherlands) for four weeks. Upon randomization according to plasma total cholesterol (TC) and triglyceride (TG) levels, mice received Western-type diet without or with 30 mg/kg bodyweight/day (0.03%, w/w) fenofibrate (Sigma, St. Louis, MO, USA). This dose is relevant to the human situation, as it corresponds with 210 mg fenofibrate per day for a 70 kg-person taking into account a 10-fold higher (drug) metabolism in mice. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

### *Plasma parameters*

Plasma was obtained via tail vein bleeding as described<sup>130</sup> and assayed for TC and TG, using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively. The distribution of lipids over plasma lipoproteins was determined using fast protein liquid chromatography (FPLC). Plasma was pooled per group, and 50

$\mu\text{L}$  of each pool was injected onto a Superose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of  $50 \mu\text{L}/\text{min}$  in PBS, 1 mM EDTA, pH 7.4. Fractions of  $50 \mu\text{L}$  were collected and assayed for TC and TG as described above. HDL was isolated after precipitation of apoB-containing lipoproteins from  $20 \mu\text{L}$  EDTA plasma by adding  $10 \mu\text{L}$  heparin (LEO Pharma, The Netherlands;  $500 \text{ U}/\text{mL}$ ) and  $10 \mu\text{L}$   $0.2 \text{ M MnCl}_2$ . The mixtures were incubated for 20 min at room temperature and centrifuged for 15 min at  $13,000 \text{ rpm}$  at  $4^\circ\text{C}$ . HDL-C was measured in the supernatant using enzymatic kit 236691 (Roche Molecular Biochemicals, Indianapolis, IN, USA).

#### *In vivo clearance of VLDL-like emulsion particles*

Glycerol tri $^3\text{H}$ oleate (triolein, TO)- and  $[1\alpha,2\alpha(n)\text{-}^{14}\text{C}]$ cholesteryl oleate (CO)-double labeled VLDL-like emulsion particles (80 nm) were prepared as described by Rensen *et al.*<sup>135</sup>. In short, radiolabeled emulsions were obtained by adding  $100 \mu\text{Ci}$  of  $^3\text{H}$ TO and  $10 \mu\text{Ci}$  of  $^{14}\text{C}$ CO to  $100 \text{ mg}$  of emulsion lipids before sonication (isotopes obtained from GE Healthcare, Little Chalfont, U.K.). Mice were fasted for 4 h, sedated with  $6.25 \text{ mg}/\text{kg}$  acepromazine (Alfasan),  $6.25 \text{ mg}/\text{kg}$  midazolam (Roche), and  $0.3125 \text{ mg}/\text{kg}$  fentanyl (Janssen-Cilag) and injected with the radiolabeled emulsion particles ( $1.0 \text{ mg TG}$  in  $200 \mu\text{L}$  PBS) via the tail vein. At indicated time points after injection, blood was taken from the tail vein to determine the serum decay of  $^3\text{H}$ TO and  $^{14}\text{C}$ CO. At 30 min after injection, plasma was collected by orbital puncture and mice were sacrificed by cervical dislocation. Organs were harvested and saponified to determine  $^3\text{H}$ TO and  $^{14}\text{C}$ CO uptake.

#### *Hepatic lipase and lipoprotein lipase assay*

To liberate LPL from endothelium, 4 h fasted mice were injected intraperitoneally with heparin ( $0.5 \text{ U}/\text{g}$  bodyweight; Leo Pharmaceutical Products BV., Weesp, The Netherlands) and blood was collected after 20 min. Total hepatic lipase (HL) and LPL activity was analyzed as modified from Zechner<sup>155</sup>. In short,  $10 \mu\text{L}$  of post-heparin plasma was incubated with  $0.2 \text{ mL}$  of TG substrate mixture containing triolein ( $4.6 \text{ mg}/\text{mL}$ ) and  $^3\text{H}$ TO ( $2.5 \mu\text{Ci}/\text{mL}$ ) for 30 min at  $37^\circ\text{C}$  in the presence or absence of  $1 \text{ M NaCl}$ , which completely inhibits

LPL activity, to estimate both the HL and LPL activity. The LPL activity was calculated as the fraction of total triacylglycerol hydrolase activity that was inhibited by the presence of 1 M NaCl and is expressed as the amount of free FA released per hour per mL of plasma.

#### *Hepatic VLDL-TG and VLDL-apoB production*

Mice were fasted for 4 h prior to the start of the experiment. During the experiment, mice were sedated as described above. At t=0 min blood was taken via tail bleeding and mice were i.v. injected with 100  $\mu$ L PBS containing 100  $\mu$ Ci Trans<sup>35</sup>S label to measure *de novo* total apoB synthesis. After 30 min, the animals received 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG<sup>131</sup>. Additional blood samples were taken at t=15, 30, 60, and 90 min after tyloxapol injection and used for determination of plasma TG concentration. At 120 min, the animals were sacrificed and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation. <sup>35</sup>S-apoB was measured in the VLDL fraction after apoB-specific precipitation with isopropanol<sup>134, 133, 132</sup>.

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#### *Hepatic lipid analysis*

Livers were isolated and partly homogenized (30 sec at 5,000 rpm) in saline (approx. 10% wet w/v) using a mini-bead beater (Biospec Products, Inc., Bartlesville, OK, USA). Lipids were extracted as described<sup>156</sup> and separated by high performance thin layer chromatography (HPTLC). Lipid spots were stained with color reagent (5 g MnCl<sub>2</sub>·4H<sub>2</sub>O, 32 mL 95-97% H<sub>2</sub>SO<sub>4</sub> added to 960 mL of CH<sub>3</sub>OH:H<sub>2</sub>O 1:1 v/v) and quantified using TINA<sup>®</sup> version 2.09 software (Raytest, Straubenhardt, Germany).

#### *Hepatic gene expression analysis*

Total RNA was extracted from individual livers using RNA-Bee (Bio-Connect, Huissen, The Netherlands) and glass beads according to the manufacturer's instructions. The RNA was further purified using the nucleospin RNA II kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. The integrity of each RNA sample obtained was examined by

Agilent Lab-on-a-chip technology using a RNA 6000 Nano LabChip kit and a Bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). The Affymetrix 3' IVT-Express labeling Kit (#901229) and the protocols optimized by Affymetrix were used to synthesize Biotin-labeled cRNA (from 100 ng of total RNA) for microarray hybridization. For the hybridization 15  $\mu$ g cRNA was used for further fragmentation and finally 10  $\mu$ g for the hybridizations. The quality of intermediate products (that is, biotin-labeled cRNA and fragmented cRNA) was again checked.

Microarray analysis was carried out using an Affymetrix technology platform and Affymetrix GeneChip<sup>®</sup> mouse genome 430 2.0 arrays. Briefly, fragmented cRNA was mixed with spiked controls and hybridized with murine GeneChip<sup>®</sup> 430 2.0 arrays. The hybridization, probe array washing and staining, and washing procedures were executed as described in the Affymetrix protocols, and probe arrays were scanned with a Hewlett-Packard Gene Array Scanner (ServiceXS, Leiden, The Netherlands). Quality control of microarray data was performed using BioConductor packages (including *simpleaffy* and *affyplm*), through the NuGO pipeline that is available as a Genepattern procedure on <http://nbx2.nugo.org><sup>157</sup>. All samples passed the QC. Raw signal intensities (from CEL-files) were normalized using the GCRMA algorithm (*gc-rma slow*). For annotation of probes and summarization of signals from probes representing one gene the custom MNBI CDF-file was used (based on EntrezGene, version 11.0.2) (<http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/cdfreadme.htm>). This resulted in expression values for 16331 genes, represented by unique Entrez gene identifiers. Genes were filtered for expression above 5 in 3 or more samples, resulting in a set of 11587 genes that was used for further analysis. Gene expression data were log-transformed (base 2).

Statistical analysis on resulting data was performed using the moderated t-test (Limma: <http://bioinf.wehi.edu.au/limma/>) with correction for multiple testing<sup>158</sup>. Cut-off for statistically significant changes was set at corrected P-value (q-value) <0.05. In addition, T-profiler analysis<sup>159</sup> was performed using expression values corrected for mean expression in the control group. This analysis resulted in scores (t-scores) and significance values for functional gene sets and biological processes (based on gene ontology annotation). Gene sets

and biological processes with significant scores ( $>4$  or  $<-4$ ) in 5 or 6 animals per group were selected. A hierarchical clustering of these pathways and biological processes and their scores in all samples was generated in GenePattern (Broad Institute, MIT, USA)<sup>160</sup>.

#### *Analysis of pathways contributing to hepatic VLDL-TG secretion*

Mice were fasted for 2 h with food withdrawn at 8:00 a.m. prior to the start of the experiment. During the experiment, mice were sedated as described above. At  $t=-2$  h mice received a continuous i.v. infusion of  $^3\text{H}$ -labeled FA ([9,10(n)- $^3\text{H}$ ] palmitic acid in sterile saline with 2% BSA) at a rate of 100  $\mu\text{L}/\text{h}$  (1.6  $\mu\text{Ci}/\text{h}$ ). Blood samples were taken at  $t=-30$  and 0 min, and the mice received 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline. Additional blood samples were taken at  $t=15$ , 30, 60, and 90 min after tyloxapol injection. All blood samples were taken using chilled paraoxon-coated capillaries.

46 Plasma TG and FA were determined as described above. The amount of  $^3\text{H}$ -activity in the FA and TG fraction of all plasma samples was determined after lipid extraction from plasma according to Bligh and Dyer<sup>156</sup>. The lipid fraction was dried under nitrogen, dissolved into chloroform/methanol (5:1 [vol/vol]) and subjected to thin layer chromatography (TLC) on silica gel plates by using hexane/diethylether/acetic acid (83:16:1, vol/vol/vol) as resolving solution. Triolein and palmitic acid were used as internal standards. Plasma samples obtained at  $t=-30$  and 0 min were used to ensure steady state was reached for the specific activity (SA) of both [ $^3\text{H}$ ]FA as well as [ $^3\text{H}$ ]TG prior to the tyloxapol injection.

The production rate of [ $^3\text{H}$ ]TG and TG was calculated from the slope of the curve from  $t=0$  to  $t=90$  min and expressed as dpm/min and nmol/min, respectively. As the production rate of [ $^3\text{H}$ ]TG represents the rate of FA produced as TG, the production rate of FA within TG was calculated by multiplying the TG production rate by 3. This production rate represents the total rate of FA incorporation into VLDL-TG. The specific activity (SA) of the VLDL precursor pool (pTG) is given by the ratio of [ $^3\text{H}$ ]TG production and TG production (Equation 1; production rate is indicated with an accent  $\prime$ ). The relative contribution of plasma FA to the VLDL-TG precursor pool can

be estimated by dividing the SA of the pTG pool by the average SA of plasma FFA (Equation 2; average was taken over the time interval  $t=-30$  to  $+60$  min). Equation 2 gives the ratio of the rate of incorporation of FA in VLDL-TG, that is directly derived from plasma ( $V_1$ ), and the total rate of FA incorporation into VLDL-TG ( $V_1+V_2$ ; see also Fig. 6C); the contribution of liver TG to VLDL-TG is represented by  $V_2$ . By Equation 1 and 2 and the definition  $(TG)' = V_1+V_2$ , we obtain an expression for  $V_1$  (Equation 3).

Equation 1:  $SA\ pTG = ([^3H]TG)' / (TG)'$  [dpm/ $\mu$ mol TG]

Equation 2: Contribution of plasma FA to VLDL-TG production  
 $= (SA\ pTG / SA-FA_{t-30-+60}) * 100$  (%)

Equation 3:  $V_1 = ([^3H]TG)' / SA-FA_{t-30-+60}$  [nmol/min]

### Statistical analysis

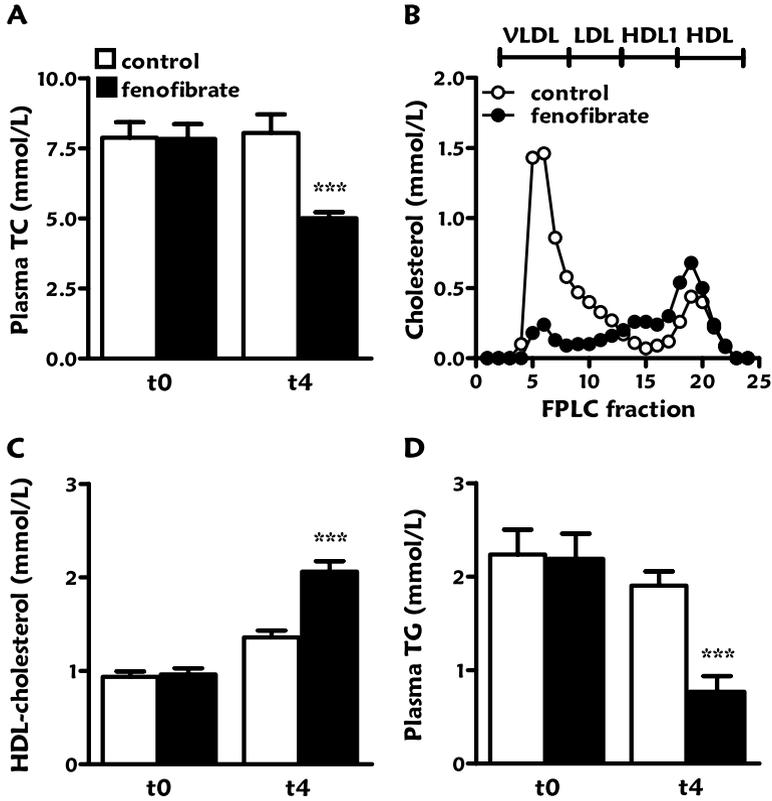
All data are presented as means  $\pm$  SEM unless indicated otherwise. Data were analyzed using the unpaired Student's t test. P-values less than 0.05 were considered statistically significant.

## Results

### *Fenofibrate decreases VLDL and increases HDL in E3L.CETP mice*

To confirm that fenofibrate decreases VLDL-TG in E3L.CETP mice, mice were fed a cholesterol-rich Western-type diet for 4 weeks ( $t_0$ ). Mice were randomized, and fed the same diet without (time-matched control group) or with fenofibrate (30 mg/kg bodyweight/day) for another 4 weeks ( $t_4$ ). At both  $t_0$  and  $t_4$ , plasma was assayed for lipids (Fig. 1). As compared to the time-matched control group, fenofibrate decreased plasma cholesterol (-38%;  $5.0 \pm 0.2$  versus  $8.0 \pm 0.7$  mmol/L;  $P < 0.001$ ) (Fig. 1A). Lipoprotein profiling showed that this was the net effect of a large reduction of VLDL-C (~85%) and an increase in HDL-C (~55%) (Fig. 1B). The increase in HDL-C was confirmed by directly measuring HDL-C in plasma after precipitation of apoB-containing lipoproteins (+52%;  $2.1 \pm 0.1$  versus  $1.4 \pm 0.1$  mmol/L;  $P < 0.001$ ) (Fig. 1C).

The decrease in VLDL-C was accompanied by a large reduction in plasma TG (-60%;  $0.7 \pm 0.2$  versus  $1.9 \pm 0.2$ ;  $P < 0.001$ ) (Fig. 1D), which was specific for VLDL (not shown).

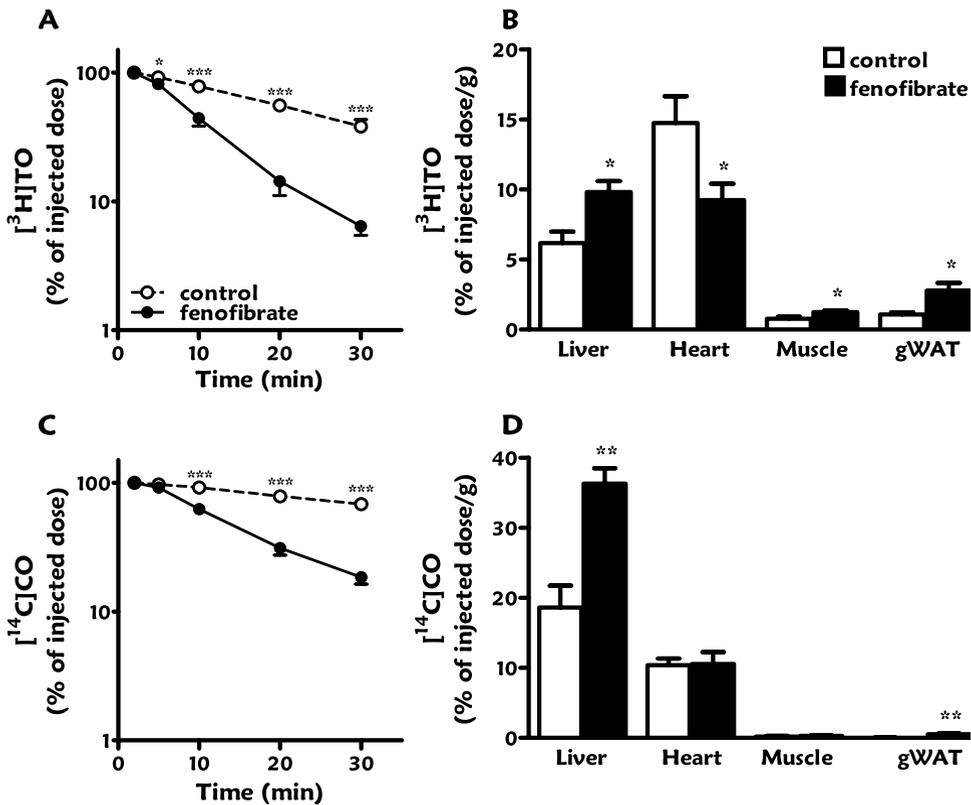


**Figure 1. Fenofibrate decreases VLDL-TG and VLDL-cholesterol in E3L.CETP mice.** Mice received a Western-type diet without and with fenofibrate (0.03% w/w). At baseline (t0) and after 4 weeks of intervention (t4), 4 h-fasted blood was taken and plasma was assayed for total cholesterol (A). After 4 weeks of intervention cholesterol distribution over lipoproteins was determined (B). At t0 and t4, HDL-cholesterol (C) and TG (D) were also assayed. Data are means  $\pm$  SEM (n=8). \*\*\* $P < 0.001$ .

*Fenofibrate increases VLDL-TG clearance by enhancing VLDL-TG hydrolysis and hepatic uptake of VLDL remnants*

Plasma VLDL-TG levels are determined by the balance between VLDL-TG production and VLDL-TG clearance. To evaluate whether an increased VLDL-TG clearance may contribute to the fenofibrate-mediated reduction in VLDL-TG levels, control and fenofibrate-treated E3L.CETP mice were injected with

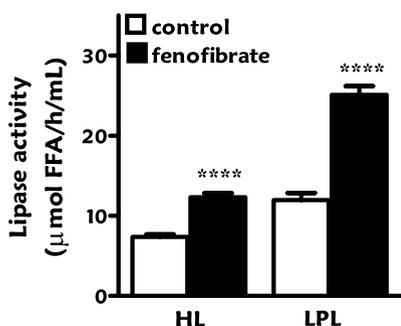
[<sup>3</sup>H]TO- and [<sup>14</sup>C]CO-labeled VLDL-like emulsion particles and the plasma clearance was determined (Fig. 2). Fenofibrate reduced the plasma half-life of [<sup>3</sup>H]TO as compared to time-matched control mice by -68% ( $t_{1/2} = 6.8 \pm 0.9$  min versus  $21.0 \pm 6.3$  min;  $P < 0.005$ ) (Fig. 2A) as reflected by an increase in uptake of [<sup>3</sup>H]TO-derived activity by liver, skeletal muscle and white adipose tissue (Fig. 2B). Likewise, fenofibrate reduced the plasma half-life of [<sup>14</sup>C]CO by -80% ( $t_{1/2} = 11.0 \pm 1.7$  min versus  $56.6 \pm 11.7$  min;  $P < 0.005$ ) (Fig 2C), as mainly reflected by an increased uptake of [<sup>14</sup>C]CO by the liver (Fig. 2D).



**Figure 2. Fenofibrate increases the clearance of VLDL-like emulsion particles in E3L.CETP mice.**

Mice received a Western-type diet without or with fenofibrate (0.03% w/w). After 4 h fasting, mice were injected with [<sup>3</sup>H]TO and [<sup>14</sup>C]CO-labeled VLDL-like emulsion particles (1 mg TG) and plasma samples were taken at indicated time points to determine the plasma clearance of [<sup>3</sup>H]TO (A) and [<sup>14</sup>C]CO (C). At 30 min after injection, the uptake of <sup>3</sup>H-activity (B) and <sup>14</sup>C-activity (D) was determined in liver, heart, skeletal muscle and gonadal white adipose tissue (gWAT). Data are means  $\pm$  SEM (n=5). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

To determine whether the accelerated clearance of VLDL-like emulsion particles was due to an increase in the lipolytic activity of plasma, HL and LPL activity was determined in plasma after heparin injection (Fig. 3). Fenofibrate increased postheparin plasma HL activity by +67% ( $12.3 \pm 1.4$  versus  $7.4 \pm 0.9$   $\mu\text{mol FFA/h/mL}$ ;  $P < 0.0001$ ) as well as postheparin plasma LPL activity by +110% ( $25.1 \pm 3.1$  versus  $12.0 \pm 2.5$   $\mu\text{mol FFA/h/mL}$ ;  $P < 0.0001$ ).



**Figure 3. Fenofibrate increases hepatic lipase and lipoprotein lipase activity in postheparin plasma of E3L.CETP mice.**

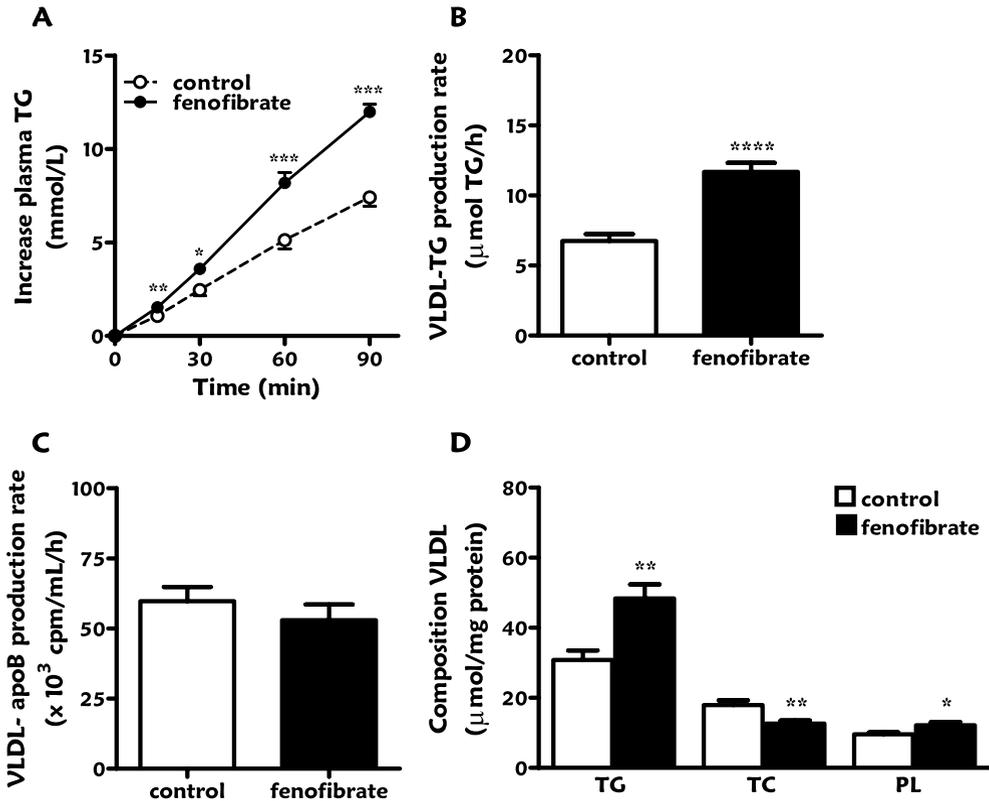
Mice received a Western-type diet without or with fenofibrate (0.03% w/w). After 4 h fasting, heparin was injected and postheparin plasma was collected. Plasma was incubated with a [ $^3\text{H}$ ] TO-containing substrate mixture in the absence or presence of 1 M NaCl, to estimate both the HL and LPL activity. Data are means  $\pm$  SEM ( $n=8$ ). \*\*\*\* $P < 0.0001$

*Fenofibrate increases VLDL-TG production but not VLDL-apoB production*

To determine the effect of fenofibrate on VLDL-TG production by the liver, control and fenofibrate-treated E3L.CETP mice were injected with Trans $^{35}\text{S}$  and tyloxapol and VLDL production was determined (Fig. 4). Surprisingly, fenofibrate caused an increase in the accumulation of plasma TG (Fig. 4A). The VLDL-TG production rate, as determined from the slope of the curve, was increased by +73% ( $11.8 \pm 0.6$  versus  $6.8 \pm 0.5$   $\mu\text{mol TG/h}$ ;  $P < 0.0001$ ) (Fig. 4B). The rate of VLDL-apoB production was not altered (Fig. 4C), indicating that fenofibrate increases the lipidation of VLDL particles in the liver rather than the number of VLDL particles secreted from the liver. Fenofibrate thus increased the amount of TG per mg of VLDL protein by +58% ( $48.3 \pm 4.0$  versus  $30.9 \pm 2.7$   $\mu\text{mol/mg}$ ;  $P < 0.01$ ) (Fig. 4D).

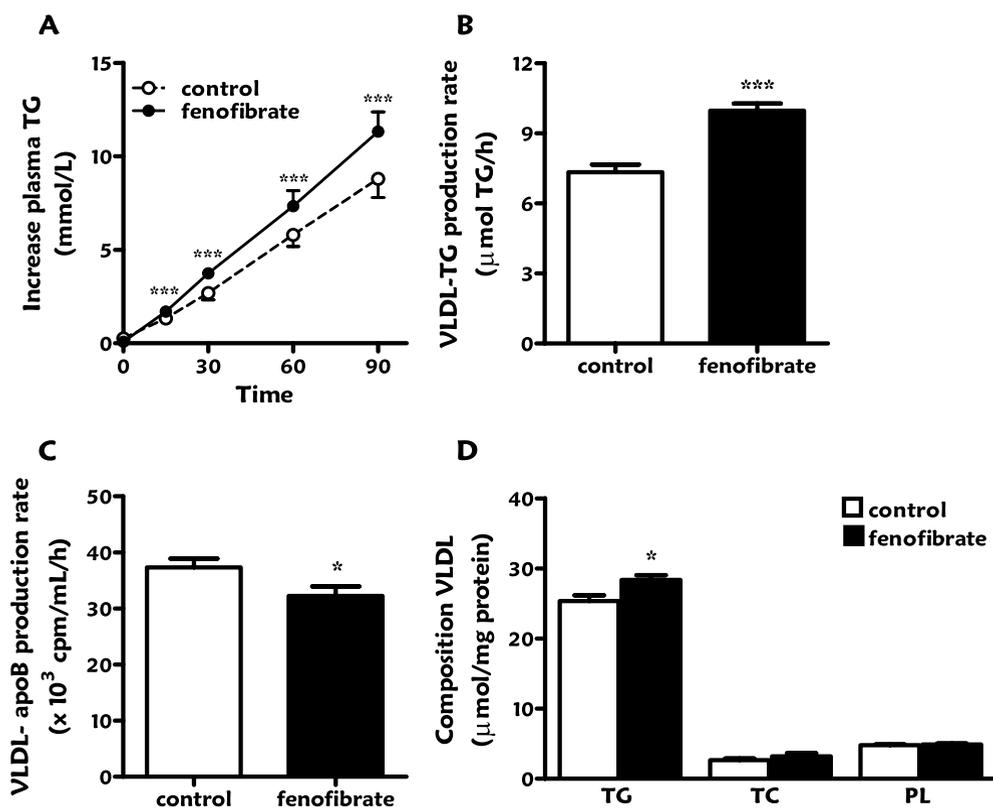
To exclude the possibility that the paradoxical increase VLDL-TG production was specific for E3L.CETP mice, we also determined the effect of fenofibrate on VLDL production in wild-type mice (Fig. 5). Like in E3L.CETP mice, fenofibrate increased the VLDL-TG production rate ( $10.0 \pm 0.3$  versus  $7.3 \pm 0.3$   $\mu\text{mol TG/h}$ ;  $P < 0.0001$ ) (Fig. 5A, B). Furthermore, fenofibrate slightly

decreased the VLDL-apoB production rate ( $32.2 \pm 1.7$  versus  $37.3 \pm 1.6$ ;  $P < 0.05$ ) (Fig. 5C) and increased the amount of TG per mg of VLDL protein ( $28.4 \pm 0.7$  versus  $25.4 \pm 0.8$   $\mu\text{mol}/\text{mg}$ ;  $P < 0.05$ ) (Fig. 5D).



**Figure 4. Fenofibrate increases hepatic VLDL-TG production in E3L.CETP mice.**

Mice received a Western-type diet without or with fenofibrate (0.03% w/w). After 4 h fasting, mice were consecutively injected with Trans- $^{35}\text{S}$  label ( $t = -30$  min) and tyloxapol ( $t = 0$  min) and blood samples were drawn up to 90 min after tyloxapol injection. Plasma TG concentrations were determined and plotted as the increase in plasma TG as compared to baseline (A). The rate of TG production was calculated from the slopes of the curves from the individual mice (B). After 120 min, the total VLDL fraction was isolated by ultracentrifugation and the rate of newly synthesized VLDL- $^{35}\text{S}$ -apoB (C) as well as the amount of triglycerides (TG), total cholesterol (TC) and phospholipids (PL) per mg VLDL protein (D) was measured. Data are means  $\pm$  SEM ( $n = 9$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$



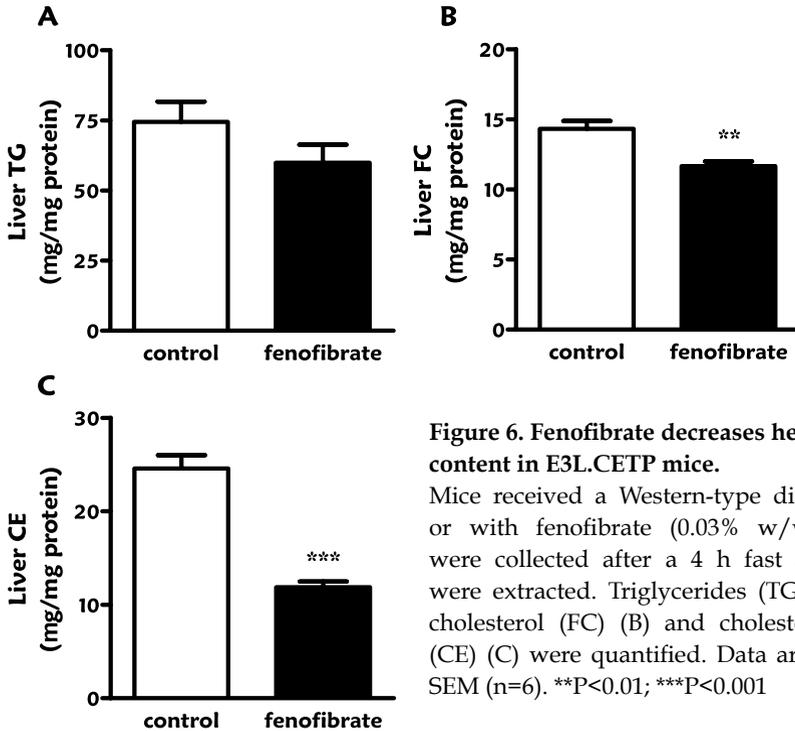
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**Figure 5. Fenofibrate increases hepatic VLDL-TG production in wild-type mice.** Mice received a Western-type diet without or with fenofibrate (0.03% w/w). After 4 h fasting, mice were consecutively injected with Trans<sup>35</sup>S label and tyloxapol and blood samples were drawn up to 90 min after tyloxapol injection. Plasma TG concentrations were determined and plotted as the increase in plasma TG (A). The rate of TG production was calculated from the slopes of the curves from the individual mice (B). After 120 min, the total VLDL fraction was isolated by ultracentrifugation and the rate of newly synthesized VLDL-apoB (C) as well as the amount of triglycerides (TG), total cholesterol (TC) and phospholipids (PL) per mg protein (D) was measured. Data are means  $\pm$  SD (n=8-10). \*P<0.05; \*\*\*P<0.001.

#### *Fenofibrate decreases hepatic lipid content*

Since an increase in VLDL production may result from an increased hepatic TG content<sup>161, 27</sup>, we determined the effect of fenofibrate on hepatic lipid levels (Fig. 6). However, fenofibrate in fact tended to reduce the hepatic TG content by -20% (59.9 $\pm$ 6.5 versus 74.5 $\pm$ 7.2 mg/mg protein; P=0.162) (Fig. 6A). In addition, fenofibrate decreased the hepatic free cholesterol content by -19%

( $11.7 \pm 0.3$  versus  $14.3 \pm 0.6$  mg/mg protein;  $P < 0.01$ ) (Fig. 6B) as well as the hepatic cholesteryl ester content by -51% ( $11.9 \pm 0.6$  versus  $24.6 \pm 1.4$ ;  $P < 0.0001$ ) (Fig. 6C).



**Figure 6. Fenofibrate decreases hepatic lipid content in E3L.CETP mice.**

Mice received a Western-type diet without or with fenofibrate (0.03% w/w). Livers were collected after a 4 h fast and lipids were extracted. Triglycerides (TG) (A), free cholesterol (FC) (B) and cholesteryl esters (CE) (C) were quantified. Data are means  $\pm$  SEM (n=6). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

*Fenofibrate increases expression of hepatic genes involved in FA uptake and transport*  
To further investigate the mechanism(s) by which fenofibrate affects VLDL-TG metabolism, we determined the hepatic expression profile of 16331 well characterized mouse genes. As compared to the control group, fenofibrate resulted in 2924 differentially expressed genes. Pathway analysis, based on gene ontology annotation, revealed that fenofibrate up-regulated gene sets related to PPAR activation and lipid/energy metabolism and down-regulated gene sets related to inflammation (Supplemental Fig. 1).

A selection of genes involved in VLDL metabolism is depicted in Table 1. As expected, fenofibrate upregulated genes involved in FA oxidation including *Cpt1a*, *Cpt1b*, *Acox1*, *Acaa1a*, *Acaa1b* and *Acaa2*. In line with our observation that fenofibrate increases VLDL-TG clearance and LPL activity in postheparin plasma, fenofibrate largely upregulated *Lpl* and slightly decreased *Apoc3*.

Fenofibrate did not largely affect FA/TG synthesis genes, apart from increasing *Dgat1*. Rather, fenofibrate upregulated genes involved in FA uptake (*Cd36*, *Ldlr*), FA transport (*Slc27a1*, *Slc27a2*, *Slc27a4* and *Slc27a5*), FA binding (*Fabp1*, *Fabp2*, *Fabp4* and *Fabp7*), FA activation (*Acsl1*, *Acsl3*, *Acsl4* and *Acsl5*) and VLDL assembly (*Mttp*). Taken together, these data suggest that fenofibrate increases hepatic VLDL-TG secretion through increased hepatic uptake, intracellular trafficking and secretion of excess FA derived from extrahepatic LPL-derived lipolysis, rather than by increased *de novo* FA synthesis.

**Table 1.** Effect of fenofibrate on hepatic expression of genes involved in VLDL metabolism

Protein	Gene	Fold change (vs control)	Corrected P-value
<b>FA oxidation</b>			
CPT1a	<i>Cpt1a</i>	<b>+1.28</b>	<b>0.010</b>
CPT1b	<i>Cpt1b</i>	<b>+6.08</b>	<b>&lt;0.001</b>
ACO	<i>Acox1</i>	<b>+1.51</b>	<b>&lt;0.001</b>
Thiolase	<i>Acaa1a</i>	<b>+1.89</b>	<b>&lt;0.001</b>
Thiolase	<i>Acaa1b</i>	<b>+1.29</b>	<b>&lt;0.001</b>
Thiolase	<i>Acaa2</i>	<b>+1.21</b>	<b>&lt;0.001</b>
<b>Lipolysis</b>			
LPL	<i>Lpl</i>	<b>+4.63</b>	<b>&lt;0.001</b>
ApoCI	<i>Apoc1</i>	-1.03	0.235
ApoCII	<i>Apoc2</i>	-1.04	0.402
ApoCIII	<i>Apoc3</i>	<b>-1.08</b>	<b>0.014</b>
ApoAV	<i>Apoa5</i>	-1.15	0.065
GPIHBP1	<i>Gpihbp1</i>	1.08	0.344
<b>FA/TG synthesis</b>			
SREBP1a/c	<i>Srebf1</i>	-1.11	0.350
FAS	<i>Fasn</i>	+1.90	0.068
DGAT1	<i>Dgat1</i>	<b>+1.48</b>	<b>0.002</b>
DGAT2	<i>Dgat2</i>	<b>-1.17</b>	<b>0.046</b>
SCD1	<i>Scd1</i>	+1.24	0.207
SCD2	<i>Scd2</i>	+1.09	0.247

Protein	Gene	Fold change (vs control)	Corrected P-value
<b>FA uptake and transport</b>			
FATPa1	<i>Slc27a1</i>	<b>+4.59</b>	<b>&lt;0.001</b>
FATPa2	<i>Slc27a2</i>	<b>+1.15</b>	<b>0.001</b>
FATPa4	<i>Slc27a4</i>	<b>+3.30</b>	<b>&lt;0.001</b>
FATPa5	<i>Slc27a5</i>	-1.07	0.181
CD36	<i>Cd36</i>	<b>+3.42</b>	<b>&lt;0.001</b>
LDLR	<i>Ldlr</i>	<b>+1.52</b>	<b>0.016</b>
PCSK9	<i>Pcsk9</i>	+1.64	0.110
<b>FA binding and activation</b>			
FABP1	<i>Fabp1</i>	<b>+1.10</b>	<b>0.012</b>
FABP2	<i>Fabp2</i>	<b>+1.36</b>	<b>0.006</b>
FABP4	<i>Fabp4</i>	<b>+2.19</b>	<b>&lt;0.001</b>
FABP6	<i>Fabp6</i>	+1.05	0.412
FABP7	<i>Fabp7</i>	<b>-3.48</b>	<b>&lt;0.001</b>
ACSL1	<i>Acsl1</i>	<b>+2.15</b>	<b>&lt;0.001</b>
ACSL3	<i>Acsl3</i>	<b>+3.07</b>	<b>&lt;0.001</b>
ACSL4	<i>Acsl4</i>	<b>+1.58</b>	<b>&lt;0.001</b>
ACSL5	<i>Acsl5</i>	<b>+1.49</b>	<b>0.001</b>
<b>VLDL assembly</b>			
ApoB	<i>Apob</i>	-1.02	0.374
ApoBEC	<i>Apohec1</i>	1.00	0.515
MTP	<i>Mttp</i>	<b>+1.42</b>	<b>&lt;0.001</b>

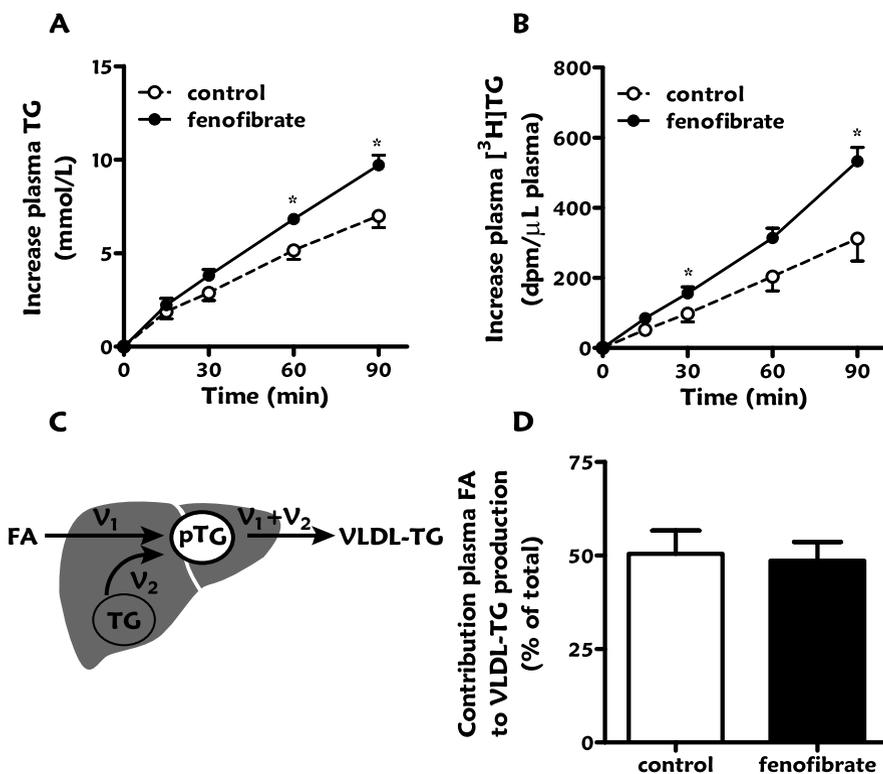
Mice received a Western-type diet without or with fenofibrate (0.03% w/w). Livers were collected after a 4 h fast, total RNA was extracted, and gene expression analysis was performed using Affymetrix GeneChip mouse genome 430 2.0 arrays. Data are expressed as mean fold change as compared to the control group (n=6 per group). Values in bold are considered significant (corrected P-value or q-value <0.05).

*Fenofibrate increases VLDL-TG production by equally increasing incorporation of re-esterified plasma FA and liver-derived TG into VLDL*

We next investigated whether the increase in VLDL-TG production is solely the result of an increased flux of plasma FA towards the liver, and subsequent incorporation into nascent VLDL after re-esterification into TG. Hereto, we continuously infused [<sup>3</sup>H]palmitic acid while measuring the linear accumulation of VLDL-derived TG and [<sup>3</sup>H]TG in plasma after injection of tyloxapol (Fig. 7).

Fenofibrate not only increased the production rate of both VLDL-TG (+31%;  $P < 0.05$ ) (Fig. 7A), which is consistent with Fig 4A, but also increased the production rate of VLDL- $^{[3]H}$ TG (+63%;  $P < 0.05$ ) (Fig. 7B). Fig. 7C schematically represents the model used to determine the contribution of plasma FA ( $V_1$ ) as compared to contribution of liver TG ( $V_2$ ) to the VLDL-TG production ( $V_1 + V_2$ ). Calculating the ratio of  $V_1$  over  $V_1 + V_2$  shows that the estimated contribution of re-esterification of plasma FA to the total VLDL production was

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**Figure 6. Fenofibrate equally increases the incorporation of both plasma FA and liver TG in VLDL-TG in E3L.CETP mice.**

Mice received a Western-type diet without or with fenofibrate (0.03% w/w). Mice were continuously infused with  $^{[3]H}$ palmitic acid after 2 h of fasting, and received tyloxapol after 4 h of fasting, and the increase in plasma TG (A) and  $^{[3]H}$ TG (B) was subsequently measured. According to the equations as schematically represented (C), the relative contribution of re-esterified plasma FA to VLDL-TG production was calculated (D). Data are means  $\pm$  SEM ( $n=5$ ). \* $P < 0.05$ .  $V_1$ , incorporation rate of plasma FA in VLDL-TG;  $V_2$ , incorporation rate of liver TG in VLDL-TG; pTG, VLDL precursor pool.

equal for both control and fenofibrate treated mice ( $46\pm 15\%$  vs  $49\pm 11\%$ ; n.s.) (Fig. 7D). These data imply that fenofibrate increases VLDL-TG production by equally increasing incorporation of re-esterified plasma FA and liver-derived TG. Indeed, fenofibrate increased VLDL-TG production by an increased incorporation of plasma FA (+38%) as well as liver TG (+25%).

## Discussion

We investigated the mechanisms underlying the effect of fenofibrate on VLDL metabolism. Our data show that fenofibrate decreases plasma TG levels in E3L.CETP mice, which is solely explained by an increased LPL-mediated TG clearance from plasma. Despite the generally accepted dogma that fenofibrate reduces VLDL-TG production thereby contributing to the plasma TG-lowering effect, fenofibrate in fact increases VLDL-TG production as caused in part by an increased flux of FA to the liver.

In this study, we have used E3L.CETP mice to study the effect of fenofibrate on VLDL metabolism, at a dose relevant to clinical practice in humans. We have previously observed that these mice show a human-like response to drug interventions aimed at reducing plasma levels of apoB-containing lipoproteins, including atorvastatin<sup>118</sup>, niacin<sup>117</sup> and fenofibrate<sup>114</sup>. In the present study, we confirmed that fenofibrate effectively reduces plasma TG levels (-60%) and cholesterol levels (-38%), which was the combined result of a decrease in VLDL-C (~85%) and an increase in HDL-C (~55%). Recently, we have shown that the HDL-raising effect of fenofibrate is explained by a reduction in hepatic cholesterol levels, which decreases hepatic CETP expression and consequently reduces plasma CETP levels<sup>114</sup>, an effect that has also been observed in humans<sup>162</sup>.

Since plasma VLDL-TG levels are determined by the balance between VLDL-TG production and VLDL-TG clearance, we evaluated the individual contribution of both pathways to the TG-lowering effect of fenofibrate. We observed that the TG-lowering effect of fenofibrate can be fully explained by an accelerated TG clearance from plasma. Fenofibrate appeared to increase the total triacylglycerol hydrolase capacity of plasma, as evidenced by increased HL activity (+67%) and LPL activity (+110%) in post-heparin plasma, which is

likely due to increased whole-body expression of the lipase genes as reflected by a large 4.6-fold increase in hepatic Lpl mRNA. It is likely that fenofibrate further increases TG clearance by indirectly enhancing LPL activity via reduced hepatic mRNA expression of the main LPL inhibitor apoCIII. These changes are consistent with previous observations on the effects of fibrates on LPL<sup>64</sup> and apoCIII<sup>63</sup> in rodents. Likewise, fibrates have been shown to increase LPL activity<sup>148, 163</sup> and decrease apoCIII synthesis<sup>63, 149, 164</sup> in humans.

In contrast to the generally accepted view that a reduction of the VLDL-TG production contributes to the TG-lowering effect of fenofibrate, we now clearly demonstrate that fenofibrate in fact increases the VLDL-TG production in E3L.CETP mice. To exclude that this effect would be genotype-specific, we confirmed that fenofibrate increases VLDL-TG production in wild-type mice. In both mouse types, fenofibrate did not increase the VLDL-apoB production rate, implying that fenofibrate increases the lipidation of VLDL particles rather than affecting the VLDL particle production rate.

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It is tempting to speculate about the mechanism(s) underlying the increasing effect of fenofibrate on VLDL-TG production. VLDL-TG production is determined by the balance between hepatic FA  $\beta$ -oxidation, FA/TG entry into the liver, hepatic FA/TG synthesis and hepatic TG stores. Fenofibrate increased hepatic genes involved in FA  $\beta$ -oxidation, which was expected from its PPAR $\alpha$  agonistic activity<sup>165, 166, 167</sup>. Affymetrix analysis indicated that hepatic genes involved in FA/TG synthesis were generally not affected. However, many genes involved in FA uptake and transport (*Slc27a*, *Cd36*, *Ldlr*), FA binding and activation (*Fabp*, *Acs1*) and FA esterification (*Dgat1*) were upregulated, in addition to *Mttp* that is crucial for VLDL assembly. In general, these data are in line with previous reports that PPAR $\alpha$  induces genes involved in TG synthesis and apoB lipidation<sup>168, 169</sup>. Collectively, these data suggest that fenofibrate may increase the uptake of FA by the liver, followed by the intrahepatic binding, activation, esterification, and ultimately secretion of FA as VLDL-TG. Indeed, by performing kinetic analyses using intravenously injected <sup>3</sup>H-FA, we demonstrated that fenofibrate increased the incorporation rate of plasma FA into nascent VLDL-TG. At the same time, fenofibrate also increased the incorporation rate of a hepatic pool of FA/TG into nascent VLDL-TG. The observed tendency towards a reduction in liver TG content can

thus be explained by a combination of an increased FA  $\beta$ -oxidation as well as an increased secretion of hepatic TG stores incorporated in VLDL.

Taken together, the effect of fenofibrate with respect to reduction of plasma TG levels is primarily caused by an enhanced TG hydrolysis in the capillary bed of LPL-expressing tissues such as skeletal muscle and white adipose tissue. Liberated FA are not only taken up by these tissues, but also redirected towards the liver as bound to albumin<sup>136</sup>. An increased FA flux towards the liver may then drive increased lipidation of nascent VLDL particles without influencing the VLDL particle production rate as judged from an unaltered VLDL-apoB production rate. Although the effect of fenofibrate on the VLDL-TG production rate has not been studied in humans, fenofibrate has been shown to reduce VLDL-apoB levels without affecting the VLDL-apoB production rate<sup>170, 149</sup>, which is agreement with our data.

So, how can we reconcile our data with the accepted dogma that fenofibrate would decrease VLDL-TG production? First, it has been generally assumed that, via increased FA  $\beta$ -oxidation, fenofibrate reduces the amount of FA available for VLDL-TG output<sup>147</sup>. However, we previously showed that specific inhibition of FA  $\beta$ -oxidation using methyl palmoxirate does not affect VLDL-TG production despite increasing hepatic TG content<sup>171</sup>. Moreover, this simplified assumption does not take into account that fenofibrate in fact increases the liver-directed flux of FA/TG. Second, a few models have suggested that fenofibrate reduces VLDL-TG production<sup>150, 151, 172, 173</sup>. For example, mice deficient for PPAR $\alpha$  have increased VLDL-TG production<sup>150, 151</sup>. However, this could easily be caused by compensatory changes resulting from complete deficiency of PPAR $\alpha$ . It would thus be more relevant to study PPAR $\alpha$  activation instead of complete PPAR $\alpha$  deficiency. Activation of PPAR $\alpha$  has been shown to inhibit VLDL-TG secretion by primary rat hepatocytes *in vitro*<sup>172</sup>. This can indeed be explained by an increase in FA  $\beta$ -oxidation, but it should be realized that such a study set-up obviously does not take into account an increased FA flux from peripheral tissues towards hepatocytes. A single *in vivo* study in mice, which were deficient for the LDL receptor (LDLr) and fed a high fat diet, showed that fenofibrate reduced VLDL-TG production<sup>173</sup>. It is likely that the absence of the LDLr may have prevented the efficient hepatic uptake of VLDL remnants in that study. We observed that fenofibrate increased both the plasma clearance

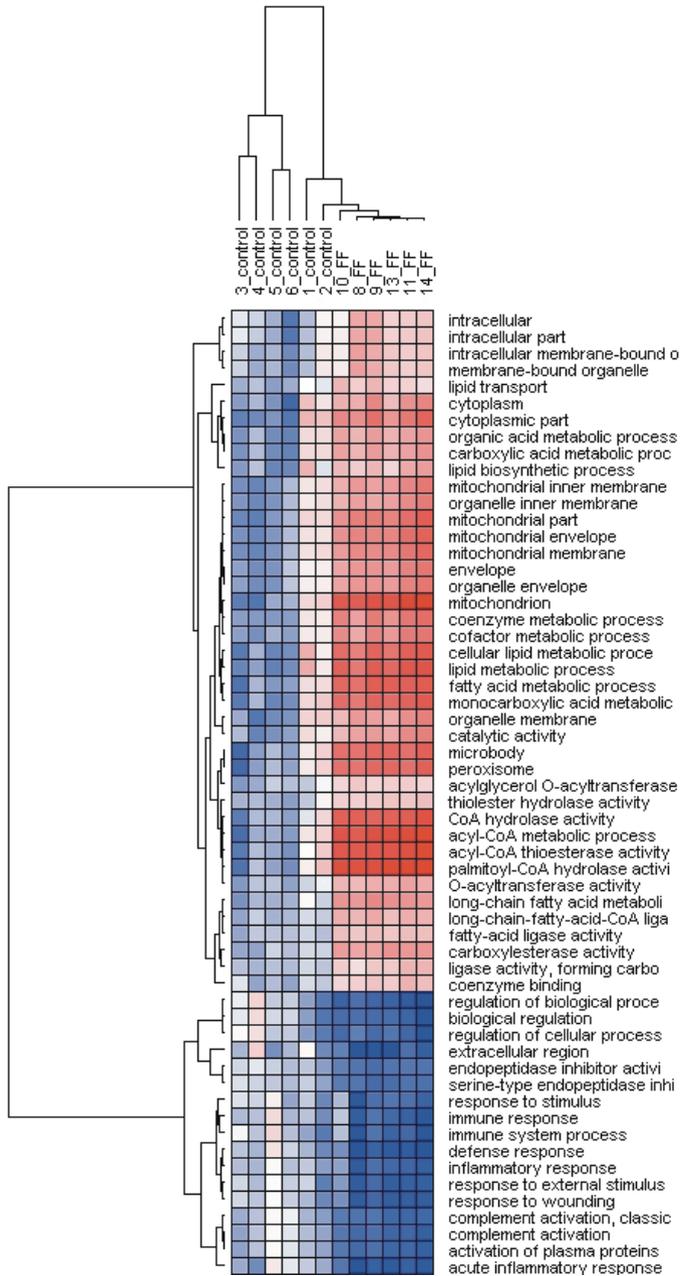
and liver uptake of VLDL core remnants. Furthermore, fenofibrate decreased the hepatic cholesterol content (which was also observed in the LDLr-deficient mice<sup>173</sup> and resulted in an expected increase in hepatic LDLr expression in our study. It is thus conceivable that the LDLr plays an important role in the influx of remnant-TG upon treatment with fenofibrate. In addition, an alternative uptake route of VLDL remnants, in case of LDLr-deficiency, may result in a different intracellular distribution of remnant-derived TG that is a less accessible source for VLDL-TG production.

In conclusion, our data demonstrate that fenofibrate decreases plasma TG by enhancing LPL-mediated VLDL-TG clearance. As a consequence, fenofibrate increases VLDL-TG production by the liver as caused by 1) enhanced hepatic FA/TG uptake resulting from strongly accelerated peripheral LPL-mediated VLDL-TG hydrolysis, and 2) increased incorporation of TG from a separate pool in the liver resulting from *de novo* FA/TG synthesis and/or lipoprotein uptake. Since the primary mechanism of action underlying the lipid-lowering effect of fibrates (i.e. increased LPL-mediated VLDL-TG clearance) is different as compared to that of statins (i.e. decreased hepatic VLDL-TG and VLDL-C output), future studies addressing the combined action of these two drug classes on lipid metabolism and their combined effect on atherosclerosis development are thus warranted.

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### Supplemental Figure 1. Hierarchical clustering of scores for biological processes.

T-profiler analysis was performed using expression values corrected for mean expression in the control group. Pathways and biological processes with significant scores ( $>4$  or  $<-4$ ) in 5 or 6 animals of the fenofibrate group were selected. A hierarchical clustering of these pathways and biological processes and their scores in all samples was generated in GenePattern (Broad Institute, MIT, USA). Red indicates positive score (majority of genes in set are up-regulated), blue indicate negative score (majority of genes in set are down-regulated).

