CETP does not affect triglyceride production or clearance in ApoE*3-Leiden mice

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The cholesteryl ester transfer protein (CETP) facilitates the bidirectional transfer of cholesteryl esters and triglycerides (TG) between HDL and (V)LDL. By shifting cholesterol in plasma from HDL to (V)LDL in exchange for VLDL-TG, CETP aggravates atherosclerosis in hyperlipidemic ApoE*3-Leiden (E3L) mice. The aim of this study was to investigate the role of CETP in TG metabolism and high fat diet-induced obesity by using E3L mice with and without the expression of human CETP gene. On chow, plasma lipid levels were comparable between both male and female E3L and E3L.CETP mice. Further mechanistic studies were performed using male mice. CETP expression increased the level of TG in HDL. CETP did not affect the postprandial plasma TG response, nor the hepatic VLDL-TG and VLDL-apoB production rate. Moreover, CETP did not affect the plasma TG clearance rate or organ-specific TG uptake after infusion of VLDL-like emulsion particles. In line with the absence of an effect of CETP on tissue-specific TG uptake, CETP also did not affect weight gain in response to a high fat diet. In conclusion, the CETP-induced increase of TG in the HDL fraction of E3L mice is not associated with changes in the production of TG or with tissue-specific clearance of TG from the plasma.
Introduction

Cardiovascular disease (CVD) is one of the major causes of mortality in the Western world, and dyslipidemia is an important risk factor in the development of CVD. Low levels of HDL-cholesterol (C), high levels of VLDL- and LDL-C and high levels of triglycerides (TG) are independent risk factors for CVD\textsuperscript{120, 121}. The ratio of (V)LDL-C to HDL-C is to a great extent affected by the cholesteryl ester transfer protein (CETP).

CETP mediates the bidirectional exchange of cholesteryl esters and TG between HDL and (V)LDL. CETP shifts cholesterol in plasma from HDL to (V)LDL and thereby aggravates atherosclerosis in the E3L mouse model which has a human-like lipoprotein metabolism\textsuperscript{116}. The HDL-C-lowering effect of CETP has prompted the development of pharmacological CETP inhibitors, such as torcetrapib, as adjuvant therapy to the widely prescribed LDL-lowering statins. Although the first CETP inhibitor torcetrapib, recently failed\textsuperscript{116, 122}, at least two novel CETP inhibitors (i.e. JTT-705 and anacetrapib) are currently in different phases of clinical trials\textsuperscript{123, 124}.

Despite the well described effects of CETP on plasma cholesterol metabolism, the role of CETP in TG metabolism has been studied less well. The torcetrapib and JTT-705 trials showed that inhibition of CETP in humans affects both the cholesterol and TG distribution over lipoproteins\textsuperscript{125, 126}. It was also reported that JTT-705 reduces plasma TG in patients with combined hyperlipidemia\textsuperscript{127}, which may reveal a beneficial effect of CETP inhibition on plasma TG levels. Furthermore, in rabbits, enrichment of HDL with TG by CETP increases the catabolism of HDL by hepatic lipase (HL)\textsuperscript{128}. Thus, CETP has the potential to affect TG metabolism, which may have effects on tissue-specific lipid accumulation.

VLDL-derived TG are lipolyzed in peripheral tissues by the enzyme lipoprotein lipase (LPL), whereas HDL-derived TG are presumably lipolyzed by HL and thus shunted to the liver. Therefore, we hypothesized that the CETP mediated net transfer of TG from (V)LDL to HDL modulates the tissue-specific uptake of plasma TG and, as a consequence, affects the development of high fat diet-induced obesity.
**Materials and Methods**

*Animals*
Human CETP transgenic mice which express CETP under control of its natural flanking regions (strain 5203)\textsuperscript{129} were obtained from Jackson Laboratories (Bar Harbor, MC) and crossbred with E3L mice\textsuperscript{108} in our local animal facility to obtain heterozygous E3L.CETP mice\textsuperscript{116}. Mice (12-16 weeks old) were housed in a temperature and humidity-controlled environment and were fed a standard chow diet with free access to water. Mice of 12 weeks of age were fed a high fat diet (60 energy\% derived from bovine fat; D 12492, Research Diet Services, Wijk bij Duurstede, The Netherlands) for 12 weeks to induce obesity. Body weight was measured during the intervention and the delta was calculated. All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center and The Netherlands Organization for Applied Scientific Research (TNO), Leiden, The Netherlands.

*Plasma parameters*
Plasma was obtained after overnight fasting (unless indicated otherwise) via tail vein bleeding in chilled paraoxon-coated capillary tubes to prevent *ex vivo* lipolysis, and assayed for TG and total cholesterol using commercially available kits 1488872 and 236691 from Roche Molecular Biochemicals (Indianapolis, IN, USA), respectively. Plasma CETP mass was analyzed using the CETP ELISA kit from ALPCO Diagnostics (Salem, NH, USA). FFA were measured using NEFA C kit from Wako Diagnostics (Instruchemie, Delfzijl, the Netherlands). HL activity in plasma was determined by measuring plasma triacylglycerol hydrolase activity as described earlier\textsuperscript{130}.

*Lipoprotein profiling*
To determine the lipid distribution over plasma lipoproteins, lipoproteins were separated using fast protein liquid chromatography (FPLC). Plasma was pooled per group, and 50 μL 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 μL/min in PBS, 1 mM EDTA,
pH 7.4. Fractions of 50 μL were collected and assayed for cholesterol and TG as described above.

Postprandial response
Mice were fasted overnight with food withdrawn at 6:00 p.m. the day before the experiment. Mice received an intragastric olive oil load (Carbonell, Cordoba, Spain) of 200 μL. Prior to the bolus and 1, 2, 3, 4, 6 and 10 h after the bolus, blood samples (30 μL) were drawn via tail bleeding for TG determination as described above. The circulating levels were corrected for the levels of TG prior to the bolus and the area under the curve (AUC) was calculated over the period of 0-10 h using GraphPad software.

Hepatic VLDL-TG and VLDL-apoB production
Mice were fasted for 4 h with food withdrawn at 5:00 a.m. prior to the start of the experiment. During the experiment, mice were sedated with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag). At t=0 min blood was taken via tail bleeding and mice were i.v. injected with 100 μL PBS containing 100 μCi Trans35S 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. 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. 35S-labeled total apoB content was measured in the VLDL fraction after precipitation with isopropanol.

In vivo clearance of VLDL-like emulsion particles
Glycerol tri[3H]oleate-labeled VLDL-like emulsion particles (80 nm) were prepared as described by Rensen et al. In short, radiolabeled emulsions were obtained by adding 200 μCi of glycerol tri[3H]oleate (triolein, TO) to 100 mg of emulsion lipids before sonication (isotope obtained from GE Healthcare, Little Chalfont, U.K.). Mice were fasted 4 h, sedated as described above and injected...
with the radiolabeled emulsion particles (1.0 mg TG in 200 μL PBS) in the tail vein at 9:00 a.m. At indicated time points after injection, blood was taken from the tail vein to determine the serum decay of [3H]TO. At 15 min after injection, plasma was collected by orbital puncture and mice were sacrificed by cervical dislocation. Organs were harvested and saponified to determine [3H]TO uptake.

Tissue-specific FFA uptake from plasma TG
Mice were fasted for 4 h with food withdrawn at 5:00 a.m. 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 received a continuous i.v. infusion of [3H]TO-labeled VLDL-like emulsion particles for 2 h (4.4 μCi [3H]TO and 1.2 μCi [14C]FA)\textsuperscript{136}. Blood samples were taken using chilled paraoxon-coated capillaries by tail bleeding at 90 and 120 min of infusion to ensure that steady-state conditions had been reached. Subsequently, mice were sacrificed and organs were quickly harvested and snap-frozen in liquid nitrogen. Analysis and calculations were performed as described\textsuperscript{136}.

Statistical analysis
Differences between groups were determined with the unpaired T-test for normally distributed data (GraphPad Prism 5 software, La Jolla, CA). A P-value of less than 0.05 was considered statistically significant. Data are presented as means ± SEM.

Results

Plasma lipids, lipoprotein profiles and hepatic lipase activity
To investigate the role of CETP in TG metabolism, male and female E3L and E3L.CETP mice were fasted overnight and plasma lipid levels were determined (Table 1). Expression of CETP had no effect on total plasma lipid levels. Since we did not detect a difference between males and females, we decided to use males only for all subsequent experiments. Plasma lipoprotein profiles were determined on pooled plasma. Expression of CETP resulted in a shift of cholesterol from HDL to VLDL (Fig. 1A), as seen previously\textsuperscript{116,119}. Furthermore,
a small amount of TG was detected in the HDL fraction upon expression of CETP (insert Fig. 1B). HL activity was did not differ between E3L and E3L.CETP mice (3.9 ± 1.5 vs 3.2 ± 1.3 μmol FFA/h/mL, respectively). This indicates that there is exchange of TG from the VLDL to the HDL fraction by CETP, which may be indicative of changes in TG metabolism.

Table 1. Plasma parameters after an overnight fast.

<table>
<thead>
<tr>
<th></th>
<th>males</th>
<th>females</th>
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<tr>
<td></td>
<td>E3L</td>
<td>E3L.CETP</td>
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<tr>
<td>triglycerides (mM)</td>
<td>2.39 ± 0.13</td>
<td>2.45 ± 0.26</td>
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<tr>
<td>total cholesterol (mM)</td>
<td>3.26 ± 0.11</td>
<td>2.91 ± 0.29</td>
</tr>
<tr>
<td>free fatty acids (mM)</td>
<td>1.09 ± 0.06</td>
<td>1.18 ± 0.07</td>
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<tr>
<td>CETP (μg/mL)</td>
<td>n.d.</td>
<td>3.78 ± 0.36</td>
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Plasma was obtained from overnight fasted male and female E3L and E3L.CETP mice on a chow diet (n=12 per group). Plasma triglycerides, total cholesterol, free fatty acids and CETP levels were measured, n.d.; not detected.

Figure 1. Plasma lipoprotein profiles.
12 h fasted mice were bled. Plasma was collected, pooled per group (n=12), and subjected to FPLC to separate lipoproteins Distribution of cholesterol (A) and triglycerides (B) over lipoproteins was determined.
Postprandial TG clearance
To examine whether CETP-mediated transfer of TG from VLDL to HDL influences plasma TG metabolism, we determined postprandial TG response. After an overnight fast, mice received an intragastric gavage of olive oil. Plasma TG concentrations were measured over a 10 h-period and the AUC was calculated. Expression of CETP in E3L mice did not affect the postprandial TG changes in plasma (Fig. 2).

Figure 2. Postprandial plasma TG response. Overnight fasted mice received an intragastric olive oil gavage and blood samples were drawn up to 10 h (n=6-9 per group). Plasma TG concentrations were determined and area under the curve (AUC) was calculated.

Figure 3. VLDL-TG and VLDL-apoB production. 4 h fasted mice were consecutively injected with Trans-35S label and tyloxapol and blood samples were drawn up to 90 min (n=4-6 per group). TG concentrations were determined in plasma and plotted as the increase in plasma TG (A). After 120 min, the total VLDL fraction was isolated by ultracentrifugation and the rate of newly synthesized VLDL-apoB (B) and the ratio of TG over apoB (C) was calculated.
VLDL-TG and VLDL-apoB production
To further examine the effect of CETP on TG metabolism we determined VLDL production. After 4 h of fasting, mice were injected with Trans[^35]S and tyloxapapol and the accumulation of endogenous VLDL-TG in plasma was measured over time. As is evident from Fig. 3A, the VLDL-TG production rate, as determined from the slope of the curve, was unchanged upon expression of CETP. Furthermore, the rate of VLDL-apoB production (Fig. 3B) as well as the ratio of TG over apoB (Fig. 3C), reflecting the amount of TG per VLDL particle, did not differ between E3L and E3L.CETP mice.

VLDL-like emulsion-TG clearance
Clearance of TG from circulation is also a major determinant of TG metabolism and therefore we examined the effect of CETP on TG clearance from VLDL-like emulsions, which have previously been shown to mimic the metabolic behaviour of TG-rich lipoproteins[^135, ^137]. After 4 h fasting, mice were injected with a bolus of [^3]H]-labeled VLDL-like emulsion particles. The decay of [^3]H]-TO in plasma was not affected by the expression of CETP (Fig. 4). The tissue-specific uptake of [^3]H]-TO was not different between E3L and E3L.CETP mice (data not shown).

![Figure 4. Plasma TG clearance.](image)

4 h fasted mice were injected with 1 mg TG as a constituent of VLDL-like [^3]H]-labeled emulsion particles (n=4-8 per group). Blood was collected at the indicated time points and radioactivity was measured in plasma.

To determine the body distribution of TG-derived FA in steady state, [^3]H]-labeled VLDL-like emulsion particles together with albumin-bound [^14]C]-FA were continuously infused for 2 h. No difference was observed in the serum half-life of [^3]H]-TO between E3L and E3L.CETP mice (Fig. 5A). Also, the uptake of [^3]H]-TO-derived radioactivity by liver, muscle, white adipose tissue (WAT)
and brown adipose tissue (BAT) was not altered due to the expression of CETP (Fig. 5B). The serum half-life and organ specific uptake of \[^{14}\text{C}]\text{FA}\) were also not changed upon expression of CETP (data not shown).

**Figure 5. Plasma derived TG distribution over tissues.**
4 h fasted mice were infused for 2 h with a trace of VLDL-like \[^{3}\text{H}]\text{TO}\)-labeled emulsion particles (n=7 per group). Blood and organs were collected. Radioactivity was measured in plasma lipid fractions after thin layer chromatography and the plasma half-life of \[^{3}\text{H}]\text{TO}\) was calculated (A). The specific \[^{3}\text{H}]\text{TG}\) activity in plasma was calculated based on the TG level, and the uptake of plasma TG by liver, skeletal muscle, white adipose tissue (WAT) and brown adipose tissue (BAT) was calculated (B).

**High fat diet-induced obesity**
We and others have previously shown that modulation of tissue-specific TG-derived FA delivery can have a major impact on the development of high fat diet-induced obesity (reviewed in\(^{138}\)). To exclude the possibility that CETP expression results in a minor change in tissue-specific TG-derived FA uptake that over a prolonged period would affect the development of obesity, E3L and E3L.CETP mice were fed a high fat diet (60% energy% in the form of fat) for 12 weeks, and body weight was measured over time. The high fat diet did not affect plasma CETP levels in E3L.CETP mice (3.8 ± 0.4 \(\mu\text{g/mL}\) on chow and 3.6 ± 0.3 \(\mu\text{g/mL}\) on high fat diet). Furthermore the high fat diet resulted in a similar decrease in plasma TG in both E3L and E3L.CETP mice (1.04 ± 0.11 and 0.92 ± 0.14 \(\text{mmol/L}\), respectively). CETP did not affect the high fat diet-induced body weight gain at any time point during the 12 weeks (Fig. 6).
Discussion

Novel drugs that inhibit CETP activity as therapy to increase HDL-C levels are in various stages of development. The rationale for development of these drugs is based on HDL-lowering effect of CETP due to the redistribution of cholesterol from HDL toward (V)LDL. Since CETP transfers both cholesterol and TG between lipoproteins, we here focused on the effect of CETP on TG metabolism. We studied the effect of CETP expression in E3L mice. The E3L mice display a human-like lipoprotein metabolism, and are an established model for hyperlipidemia and atherosclerosis (as reviewed in). We recently reported the HDL-lowering and pro-atherogenic properties of CETP expression on the E3L background. In this study, after 4 hour fasting, plasma cholesterol were somewhat higher in the E3L.CETP mice. In the current study, we find no changes in plasma total cholesterol and TG after overnight fasting. We do find a small increase in TG in the HDL fraction upon expression of CETP in E3L mice. Despite this relative increase in HDL-TG, CETP did not affect the postprandial TG response, hepatic VLDL-TG production, clearance of TG from VLDL-like emulsion particles and the development of high fat diet-induced obesity. These findings suggest that CETP-mediated transfer of TG from (V)LDL to HDL does not reflect a substantial effect on overall plasma TG metabolism in E3L mice.

There is some controversy on the effects of CETP on TG metabolism in various mouse models. Studies in mice, expressing simian CETP, show that on an atherogenic diet expression of CETP results in increased production...
and clearance of TG\textsuperscript{139}. Others have demonstrated that mice expressing human CETP when fed a regular chow diet show no alterations in VLDL-TG production\textsuperscript{140, 141}. However, Salerno \textit{et al}.\textsuperscript{140} showed that CETP-expressing mice have an increased postprandial TG response and decreased clearance of TG from the circulation. This was attributed to a decrease in LPL activity and LPL gene transcription. We did not find changes in the postprandial TG response or in the clearance of TG from the circulation in E3L.CETP mice versus E3L mice. We also did not find an effect of CETP in E3L mice on high fat diet-induced obesity. Since modification of tissue-specific FA delivery can significantly affect high-fat diet-induced obesity, this further confirms the absence of even a subtle effect of CETP on tissue-specific TG-derived FA uptake. It seems likely that the explanation for the discrepancy of our data with those of Salerno \textit{et al}.\textsuperscript{140} is associated with the more human-like lipoprotein metabolism on the E3L background as indicated by presence of a substantial amount of apoB-containing lipoproteins.

Enrichment of HDL with TG has a major impact on HDL metabolism. In humans, it has been demonstrated that cholesterol and apoAI within TG-rich HDL are cleared more rapidly as compared to those within TG-poor HDL\textsuperscript{142}. Similar observations have been made in various animal models\textsuperscript{128, 143, 144}. Thus, CETP-mediated TG enrichment of HDL has measurable effects on the kinetics of HDL-C and HDL-apoAI. Although these changes in HDL kinetics have the potential to have a substantial effect on TG metabolism, our results implicate that the CETP-mediated TG transfer does not alter the kinetics of TG clearance from the circulation.

This may be explained by the apparently small contribution of HDL-TG to the overall flux of TG. Especially in the postprandial state, the amount of TG in chylomicrons exceeds the amount of TG in HDL by far, even when CETP activity is high. Alternatively, HDL-TG may be readily lipolyzed by HL and the fate of the resulting FA may not be quantitatively different from FA derived from VLDL-TG. During lipolysis of VLDL-TG by LPL, a significant fraction of FA leaks to the circulation and is subsequently cleared by the liver\textsuperscript{136}. Since it has been postulated that HDL-TG-derived FA are also cleared by the liver\textsuperscript{128}, the fate of a substantial fraction of VLDL-TG derived FA and HDL-TG derived FA will thus be indistinguishable.
In conclusion, we show that expression of CETP does not affect overall TG metabolism and high fat diet-induced obesity in E3L mice. This implicates that, at least under relatively normolipidemic conditions, pharmacological CETP inhibition is unlikely to disturb TG metabolism.

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