Novel mechanistic insight in cholesteryl ester transfer protein production and pharmacological inhibition

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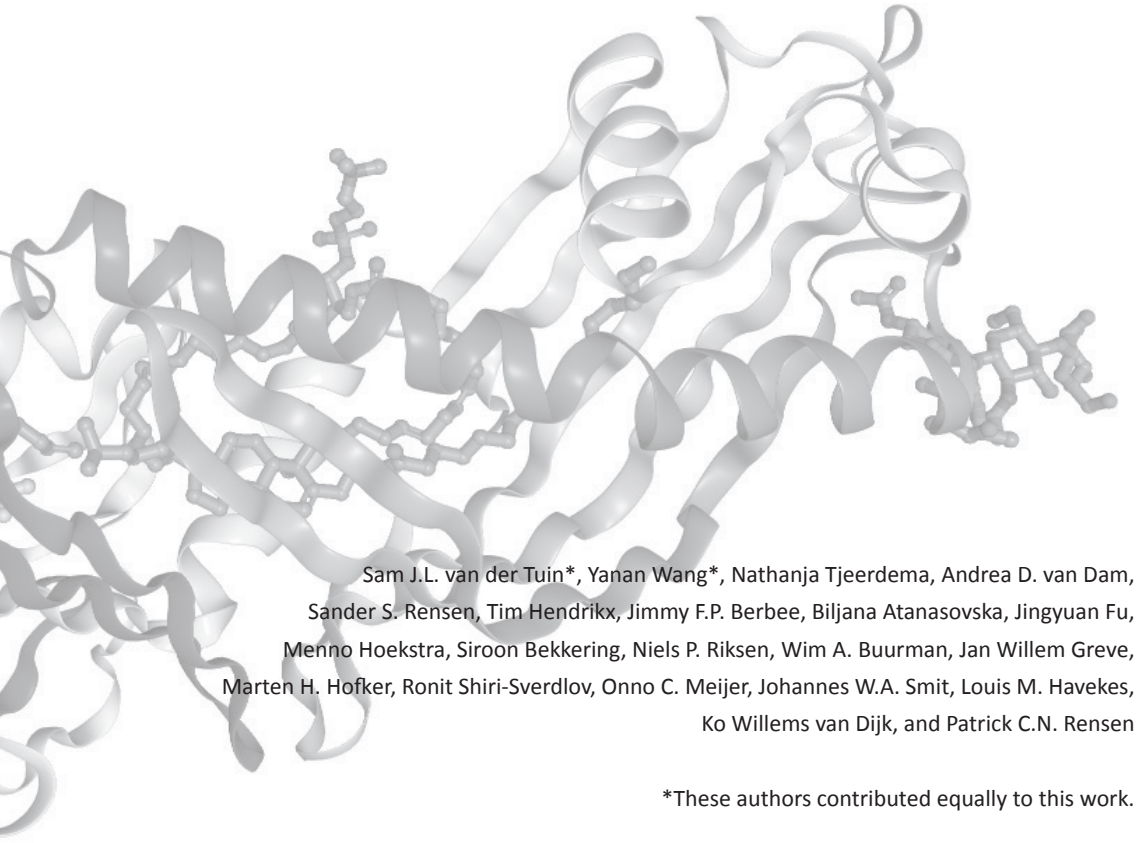
Chapter 2

Plasma cholesteryl ester transfer protein is predominantly derived from Kupffer cells



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ABSTRACT

The role of Kupffer cells (KCs) in the pathophysiology of the liver has been firmly established. Nevertheless, KCs have been underexplored as a target for diagnosis and treatment of liver diseases owing to the lack of noninvasive diagnostic tests. We addressed the hypothesis that cholesteryl ester transfer protein (CETP) is mainly derived from KCs and may predict KC content. Microarray analysis of liver and adipose tissue biopsies, obtained from 93 obese subjects who underwent elective bariatric surgery, showed that expression of *CETP* is markedly higher in liver than adipose tissue. Hepatic expression of *CETP* correlated strongly with that of KC markers, and CETP mRNA and protein colocalized specifically with KCs in human liver sections. Hepatic KC content as well as hepatic *CETP* expression correlated strongly with plasma CETP concentration. Mechanistic and intervention studies on the role of KCs in determining the plasma CETP concentration were performed in a transgenic (Tg) mouse model expressing human CETP. Selective elimination of KCs from the liver in CETP Tg mice virtually abolished hepatic *CETP* expression and largely reduced plasma CETP concentration, consequently improving the lipoprotein profile. Conversely, augmentation of KCs after vaccination largely increased hepatic *CETP* expression and plasma CETP. Also, lipid-lowering drugs fenofibrate and niacin reduced liver KC content, accompanied by reduced plasma CETP concentration. Conclusions: Plasma CETP is predominantly derived from KCs, and plasma CETP concentration predicts hepatic KC content in humans.

INTRODUCTION

Kupffer cells (KCs) have been identified as the resident macrophages in the liver more than a century ago. KCs have a firmly established function in host defense,[1, 2] bilirubin metabolism,[2] and liver regeneration.[3] In addition, KCs play an important role in the pathogenesis of various liver diseases, including liver failure and ischemia-reperfusion injury during liver resection or transplantation, alcohol-induced liver disease, and nonalcoholic fatty liver disease (NAFLD).[4] NAFLD is currently the leading cause of chronic liver diseases in the Western world and the estimated prevalence in the general population ranges between 20% and 30%, rising to as high as 90% in morbidly obese individuals.[5, 6] NAFLD embraces a spectrum of liver pathology, from simple steatosis to severe non-alcoholic steatohepatitis (NASH),[7] that is characterized by accumulation of lipid in the liver (steatosis) accompanied by hepatic inflammation (hepatitis). Treatment options for NAFLD are limited.[8]

Experimental studies in mice suggest a pivotal role of KCs in the development of NASH.[9-11] Preliminary data from clinical trials indicate that the number of hepatic KCs correlates with the severity of liver damage in patients with NASH.[12, 13] To distinguish NASH from simple steatosis and design more effective and specific treatments for liver injury and inflammation for patients with NASH, it is essential to diagnose KC content in clinical practice. However, liver biopsies are still the gold standard used for this purpose[14] owing to the fact that noninvasive modalities or plasma biomarkers are currently not available to predict KC content. Obviously, liver biopsies have severe limitations, such as sampling error, differences in histopathological interpretation, as well as patient stress and discomfort, risk of bleeding, and long hospitalization. Therefore, noninvasive biomarkers with a high sensitivity and specificity for hepatic KC content are eagerly awaited.

Cholesteryl ester transfer protein (CETP) is a plasma protein that is mainly bound to high-density lipoproteins (HDL) in plasma and plays a pivotal role in metabolism of HDL and very-low-density lipoprotein (VLDL). Currently, CETP inhibition is a target for the treatment of dyslipidemia to ultimately reduce cardiovascular disease risk.[15, 16] Previous studies have indicated that liver and adipose tissue are the two major sources of circulating CETP in humans.[17, 18] However, the relative contribution of liver and adipose tissue to total plasma CETP and the cell types involved in CETP synthesis remain to be unambiguously determined. Some studies suggested that hepatocytes may be responsible for hepatic expression and secretion of CETP,[17, 19] whereas another study suggested that nonparenchymal cells, including KCs, are the principal source of hepatic CETP.[20] Our previous studies indicated that pharmacological treatments that lead to a reduction in KCs are associated with a reduction in plasma CETP.[21] Therefore, in this study, we aimed to determine the cellular origin of CETP in humans. We hypothesized that hepatic CETP expression is confined to KCs, and plasma CETP is predominantly derived from KCs.

MATERIALS AND METHODS

Design of Human Studies

Two independent populations were selected. The first cohort was obtained from the general population in Rijswijk, The Netherlands, consisting of 1434 nondiabetic subjects between 40-70 years of age (654 males, 780 females). Exclusion criteria included diagnosed diabetes, known terminal disease, and a history of psychiatric disorder or substance abuse. Waist circumference was measured and venous blood samples were taken after overnight fasting for measurement of plasma CETP concentration. The Rijswijk study was approved by the review board of South West Holland and performed in accord with the Declaration of Helsinki.

The second study consisted of 93 severely obese subjects (body mass index [BMI]: 30-74) who underwent elective bariatric surgery from 2006 to 2009 at the Department of General Surgery, Maastricht University Medical Center (Maastricht, The Netherlands).[22] Subjects using anti-inflammatory drugs or having acute or chronic inflammatory diseases, degenerative diseases, and subjects reporting alcoholic intake >10 g/day were excluded. During surgery, biopsies from liver, subcutaneous adipose tissue, and visceral adipose tissue were taken for messenger RNA (mRNA) isolation and hybridization. Venous blood samples were obtained after overnight fasting on the morning of surgery for analysis of the plasma CETP concentration and lipid parameters. Six weeks after bariatric surgery, blood samples were collected for analysis of plasma CETP concentration. This study was approved by the Medical Ethics Board of Maastricht University Medical Center and was in line with the Declaration of Helsinki. All participants provided informed written consent.

Details of all parameters measured in both population cohorts are provided in the Supplemental data.

Design of Mouse Studies

Female APOE*3- Leiden.CETP transgenic (Tg) mice expressing the human CETP gene under the control of its natural flanking regions were used[23] and housed under standard conditions with a 12-hour light/dark cycle with free access to food and water, unless indicated otherwise. Mice were fed a chow diet or semisynthetic Western-type diet (WTD), containing 0.1% (w/w) cholesterol, 1% (w/w) corn oil, and 15% (w/w) cocoa butter (AB-Diets).

In a first experiment, mice were fed WTD for 4 weeks, randomized according to body weight and plasma lipid levels (total cholesterol [TC] and triglyceride [TG]), received two intraperitoneal injections of 4 mL/kg body weight liposomal clodronate (20 mg/kg body weight; purchased from Dr. N. van Rooijen, Amsterdam, The Netherlands) at a 3-day interval to deplete macrophages from the liver,[24] and were terminated 3 days after the second injection.

In a second experiment, mice were fed chow diet and randomized according to body weight and plasma lipid levels (TC and TG), received two intravenous injections of Bacille-Calmette-Guérin (BCG) vaccine from the State Serum Institute (SSI; 0.75 mg; 5 x 10^6 colony forming units in 100 mL of phosphate-buffered saline; SSI Denmark, Copenhagen, Denmark)[25] at the beginning of the study and after 2 weeks. Mice were terminated 4 weeks after the first injection.

In a third experiment, mice were fed WTD, without (control) and with 0.04% (w/w) fenofibrate or 1% (w/w) niacin (both from Sigma-Aldrich) for 4 additional weeks before sacrificing.

The institutional ethical committee on animal care and experimentation from Leiden University Medical Center (Leiden, The Netherlands) had approved all animal experiments. In all experiments, blood was obtained by tail vein bleeding into heparin-coated capillary tubes after 4 hours of fasting at 12:00 PM with food withdrawn at 8:00 AM. Tubes were placed on ice and centrifuged, and the obtained plasma was snap-frozen in liquid nitrogen and stored at -20°C until further analysis. Plasma was assayed for CETP and lipid concentrations and lipoprotein profiles (see the Supplemental data). After mice had been sacrificed, liver and gonadal adipose tissue samples were collected to measure expression of selected genes by quantitative real-time polymerase chain reaction and proteins by immunohistochemistry (see the Supplemental data).

Statistical Analysis

Categorical variables are presented as frequencies and percentages, and continuous variables as mean±SD, or medians and interquartile ranges for variables with skewed distributions. Pearson's correlation was used to estimate the association between waist circumference and plasma CETP in the Rijswijk study. In the bariatric surgery cohort, Spearman's correlation was used to determine the correlation between expression of *CETP* and macrophage receptor with collagenous structure (*MARCO*) in the liver, subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT); the association between CETP expression in liver, SAT, VAT, and plasma CETP concentration; as well as the association between CETP expression in liver, SAT, VAT, and plasma HDL-cholesterol (HDL-C) level, respectively. For correlation analyses, P values are provided in addition to q values that were calculated after correction for multiple testing. Colocalization between $CETP^+$ cells and $CD68^+$ cells in liver biopsies was determined by Pearson's correlation. For mouse studies, statistical differences between groups were assessed with the Student t test for two independent groups or two-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. All reported *P* values are two-tailed, and *P/q* values of less than 0.05 were considered statistically significant.

RESULTS

Waist circumference does not correlate with the plasma CETP concentration in humans

Previous studies in a small cohort of 13 men demonstrated a correlation between *CETP* expression in adipose tissue with plasma CETP concentration,[26] suggesting that adipose tissue may contribute to the plasma CETP pool. To investigate whether central adiposity correlates with plasma CETP concentration, we first assessed the correlation between waist circumference and plasma CETP concentration in a general population in Rijswijk. The characteristics of 1434 subjects (654 males and 780 females) are shown in Table S1.

Table S1: Characteristics of the non-diabetic population cohort in Rijswijk study.*

Characteristic	Male (N=654)	Female (N=780)
Age (yr)	54.0±8.2	53.7±8.3
Current smoking no. (%)	189 (28.9)	202 (25.9)
Metabolic Syndrome† no. (%)	293 (45)	245 (32)
Medication no. (%)		
Statin	82 (12.5)	61 (7.8)
Blood pressure lowering medication	128 (19.6)	132 (16.9)
Blood pressure (mmHg)		
Systolic blood pressure	136±19	127±20
Diastolic blood pressure	84±11	81±11
Waist circumference (cm)	99±11	89±12
Total cholesterol (mmol/L)		
Median	5.58	5.67
Interquartile range	4.84-6.27	4.99-6.38
HDL-cholesterol (mmol/L)		
Median	1.13	1.44
Interquartile range	0.96-1.33	1.23-1.70
Triglycerides (mmol/L)		
Median	1.32	1.05
Interquartile range	0.93-1.90	0.81-1.45
Glucose (mmol/L)	5.44±1.16	5.13±0.77
CETP (µg/mL)		
Median	2.31	2.44
Interquartile range	1.90-2.72	2.02-2.86

*Plus-minus values are mean±SD. To convert values for cholesterol from mmol/L to mg/dL, divide by 0.02586. To convert values for triglycerides from mmol/L to mg/dL, divide by 0.01129. HDL, high-density lipoprotein; CETP, cholesteryl ester transfer protein.

†The diagnosis of metabolic syndrome is based on the IDF criteria.[28] Therefore two or more of the following criteria in addition to an increased waist circumference (male ≥94 cm, female ≥80 cm) had to be present: triglycerides ≥1.7 mmol/L (≥150 mg/dL), HDL-cholesterol <1.03 mmol/L (<40 mg/dL) in male and <1.29 mmol/L (<50 mg/dL) in female, fasting glucose ≥5.6 mmol/L (150 mg/dL), blood pressure ≥130/85 mmHg or the use of blood pressure lowering medication.

Mean±SD waist circumference was 99±11 cm for males and 89±12 cm for females. Median value for plasma CETP concentration was significantly lower in males (2.31 µg/mL [1.90-2.72]), compared to females (2.44 µg/mL [2.02-2.86]; P<0.001). However, plasma CETP concentration did not differ between quintiles of waist circumference in either males (Fig. 1A) or females (Fig. 1B), and no correlation between waist circumference and plasma CETP concentration was observed in either males (Fig. 1C) or females (Fig. 1D). Plasma CETP concentration does not correlate with plasma HDL-C level in either males (r=-0.015) or females (r=0.011). Exclusion of subjects who received statins (82 males and 61 females), known to reduce plasma CETP concentration,[27] did not change the association between waist circumference and plasma CETP concentration for males (Fig. S1A) and females (Fig. S1B). Thus, these findings suggest that adipose tissue mass and plasma CETP are not correlated in humans.

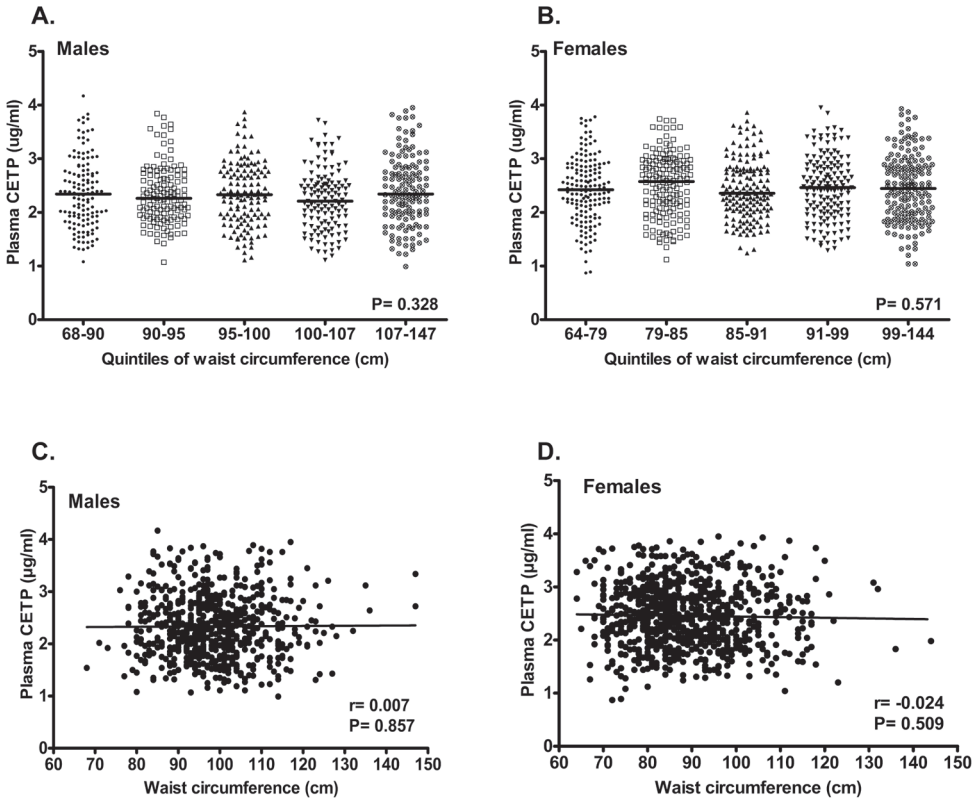

Figure 1: Waist circumference does not correlate with plasma CETP concentration in a general population

(A and B) A total of 1,434 subjects were enrolled in the Rijswijk study. Plasma CETP concentration over quintiles of waist circumference in 654 male (A) and 780 female (B) subjects were determined, and medians are indicated. (C and D) Correlations between waist circumference and plasma CETP concentration in male (C) and female (D) subjects.

Hepatic CETP expression is exclusive to KCs and correlates with the plasma CETP concentration in humans

We next compared expression of CETP in biopsies from livers, SAT, and VAT of 93 subjects who underwent elective bariatric surgery, on which microarray analyses have previously been performed.[22] The *CETP* mRNA transcript appeared to be much more abundant in liver than in SAT and VAT (Fig. 2A). Given that the liver contains multiple cell types, we set out to evaluate the cell type(s) responsible for hepatic *CETP* expression. First, we evaluated the correlation between hepatic expression of *CETP* with hepatic expression of the other genes determined by microarray. *CETP* showed the highest correlation with the macrophage-specific marker, *MARCO* (r=0.590; P=9.78 x 10^{9}; q=8.89 x 10^{9}; Fig. 2B), which is exclusively expressed in KCs.[29] *MARCO* expression in liver is higher than in SAT and VAT (Fig. 2A). Using a publicly available, considerably larger data set of subjects undergoing bariatric surgery (n=1008),[30] we were able to replicate the strong association between *CETP* expression and *MARCO* in liver (Spearman's correlation: r=0.624; p=1.74 x 10^{-71}; q=1.22 x 10^{-68}; Fig. S3). In addition to *MARCO*, expression of hepatic *CETP* also correlates with other macrophage/KC markers, for example, *CD68* (r=0.474, P=9.20 x 10^{-6}; q=0.03), *CD163* (r=0.430; P=6.62 x 10^{-5}; q=0.096), and *VSIG4* (r=0.408; P=1.62 x 10^{-4}; q=0.147). In contrast, the expression of hepatic *CETP* does not correlate with hepatocyte/parenchymal cell-specific markers, for example, *ALB* (r=0.042; P=0.709; Fig. 2C), *ASGR1* (r=-0.012; P=0.908), and *APOA1* (r=0.238; P=0.832).

To evaluate whether KCs are the primary site of *CETP* expression and synthesis, we next performed *in situ* hybridization and immunohistochemistry (IHC) on liver sections from a healthy donor and subjects of the bariatric surgery cohort. Indeed, *CETP* mRNA could only be detected in cells that also express *CD68* (Fig. 2E), and CETP protein colocalized with CD68 protein (Fig. 2F). The number of CETP-expressing cells was significantly correlated with the number of $CD68^{+}$ cells (r=0.624; P<0.001; Fig. 2D), with 39.0% colocalization (slides were available of 46 patients; two to four slides were examined per patient). A similar percentage of colocalization was observed in a healthy subject (38.5%). These data confirm that CETP is expressed and synthesized by KCs in the liver.

Importantly, hepatic *CETP* expression positively correlated with plasma CETP concentration (r=0.500; P<0.001; Fig. 3A) and tended to inversely correlate with the plasma HDL-C level (r=-0.222; P=0.054; Fig. 3B). These data suggest that hepatic *CETP* expression by KCs determines plasma CETP concentration as well as the CETP-induced effects on plasma HDL-C. A significant correlation was found between the number of $CD68^{+}$ KCs and plasma CETP concentration (r=0.393; P<0.01; Fig. 3C). This correlation is independent of other clinical parameters, such as gender, BMI, and age. In addition, 6 weeks after bariatric surgery, plasma CETP concentration was significantly decreased, as compared to baseline (P<0.001; Fig. 3D).

In addition to the liver, adipose tissue also contains a large population of resident macrophages. However, CETP expression did not correlate with MARCO in either SAT (Figs. S2A and S3) or VAT (Figs. S2B and S3) in both cohorts of subjects who underwent bariatric surgery.

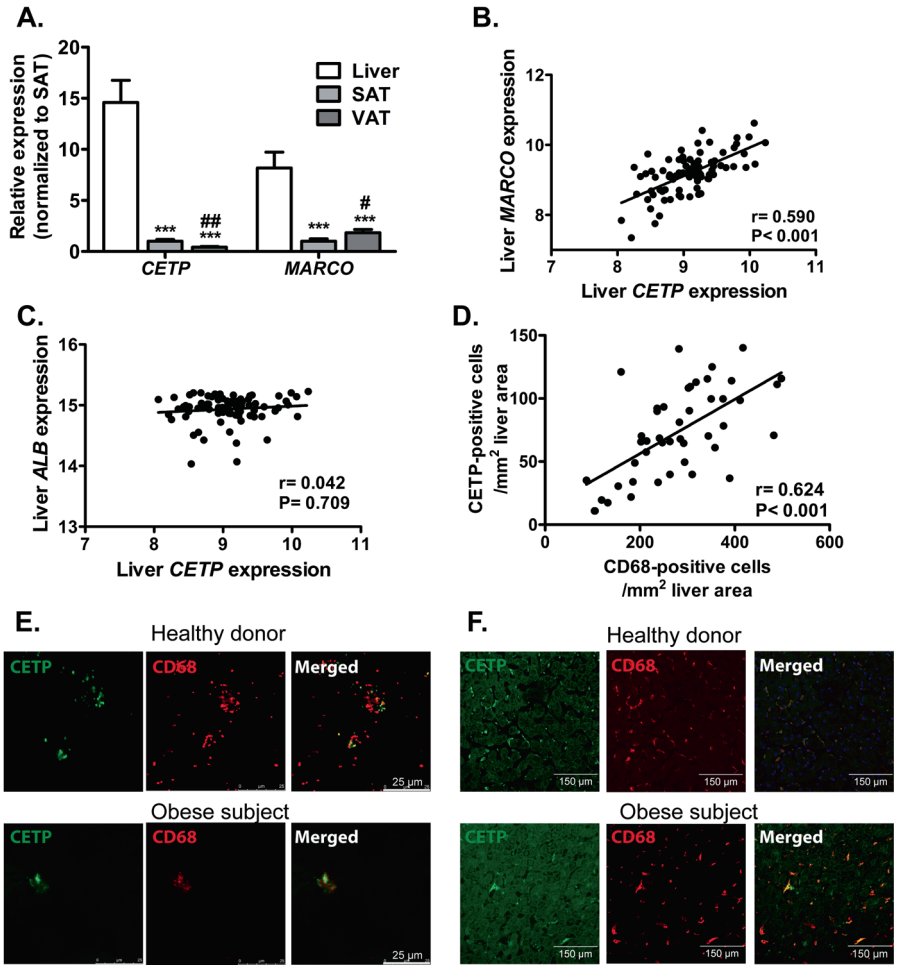

Figure 2: KCs are the principal source of CETP in the human liver
(A) Biopsies from liver, SAT, and VAT were taken during bariatric surgery from 93 patients and assayed for genome-wide gene expression profiles. *CETP* and *MARCO* expression in liver and VAT were normalized to expression in SAT, respectively. Data are represented as mean±SD. Two-way ANOVA, ***P<0.001, as compared to liver; #P<0.05, ##P<0.01, as compared to SAT. (B) Correlation of expression of *CETP* with *MARCO* in liver. (C) Correlation of expression of *CETP* with *ALB* in liver. (D) Correlation of number of CETP^{+} cells with CD68^{+} cells in liver. (E) Representative pictures of *in situ* hybridization of *CETP* mRNA (green), *CD68* mRNA (red), and merged in liver sections both from a healthy donor and an obese subject. (F) Representative pictures of immunofluorescent staining of CETP (green), CD68 (red), and merged in liver sections both from a healthy donor and an obese subject.

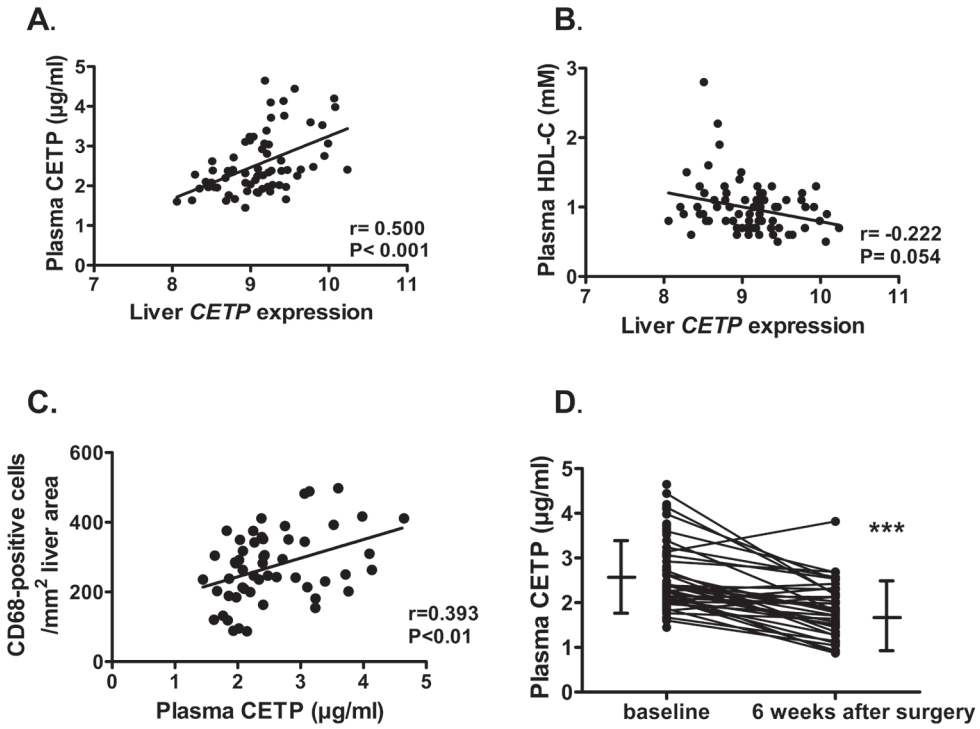

Figure 3: *CETP* expression in KCs correlates with plasma CETP and HDL-C in humans
Biopsies from liver, SAT, and VAT were taken during bariatric surgery from 93 patients and assayed for genome-wide gene expression profiles. (A) Correlation of hepatic *CETP* expression and plasma CETP concentration. (B) Correlation of hepatic *CETP* expression and plasma HDL-C level. (C) After IHC staining of CD68 in liver biopsies, $CD68^+$ KC number were quantified and correlated with plasma CETP concentration. (D) Before and 6 weeks after bariatric surgery, plasma CETP concentration was measured.
All data are represented as mean±SD; n=42-68; Student paired t test; ***P<0.001, as compared to baseline.

Elimination of KCs abolishes hepatic CETP expression and largely reduces plasma CETP concentration in human CETP Tg mice

To further investigate the contribution of CETP expression in KCs to the plasma CETP concentration and lipoprotein metabolism, detailed mechanistic studies were performed using APOE*3-Leiden.CETP Tg mice, a well-established mouse model for human-like lipoprotein metabolism that express the human *CETP* gene under control of its natural regulatory flanking regions.[31] *CETP* expression in liver was much higher than in adipose tissue, peritoneal macrophages, and bone marrow- derived macrophages (Fig. S4A). The higher ratio between *CETP* expression and *F4/80* expression in liver versus adipose tissue suggests that the capacity of KCs to produce CETP is much higher than that of macrophages in adipose tissue (Fig. S4B). Moreover, both *CETP* mRNA (Fig. S4B) and protein (Fig. S4C)

specifically colocalized with F4/80⁺ KCs in liver of mice. In APOE*3-Leiden.CETP mice fed a chow diet, 55.9±13.3% F4/80⁺ KCs expressed CETP, and the WTD did not affect colocalization percentage (57.4±12.4%). These data suggest that APOE*3-Leiden.CETP Tg mice have a similar CETP expression pattern as humans.

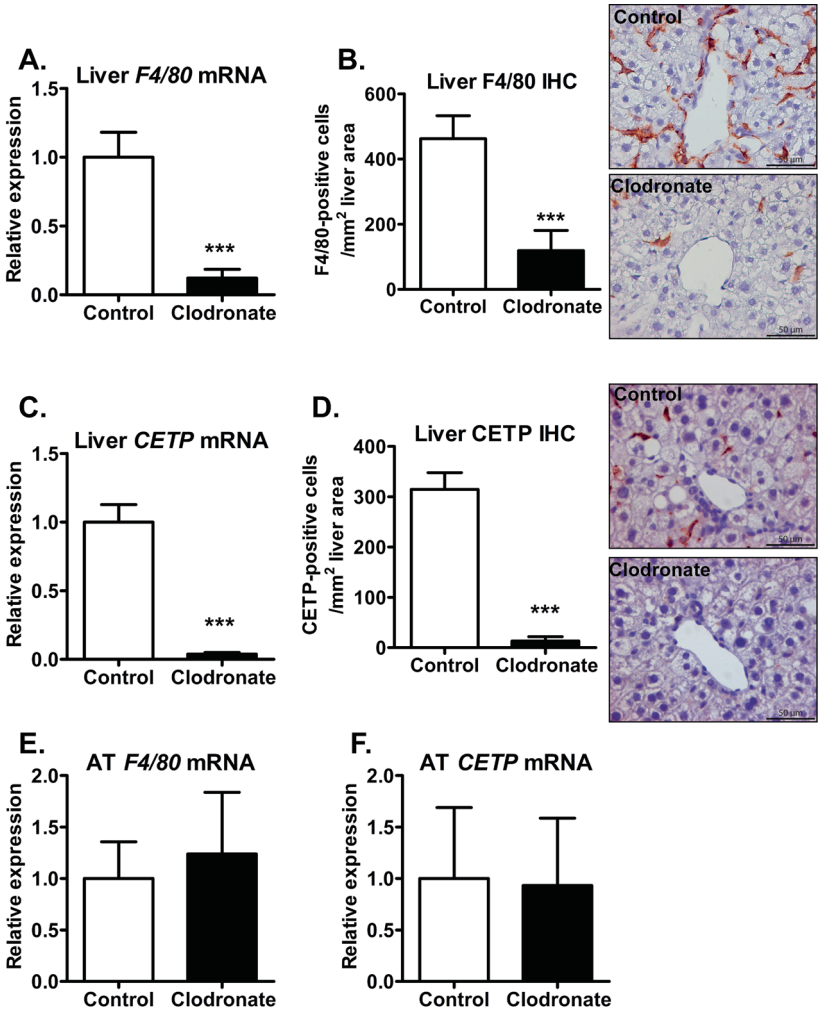

Figure 4: Elimination of KCs abolishes hepatic *CETP* expression in human CETP Tg mice
(A) APOE*3-Leiden.CETP mice fed a WTD were treated with or without liposomal clodronate and livers were assayed for *F4/80* mRNA expression. (B) KC content (F4/80⁺ cells) of the liver. Right panel shows representative pictures of IHC staining of F4/80 in liver sections of each group. (C) mRNA expression of *CETP* in liver. (D) Quantification of hepatic content of CETP⁺ cells. Right panel shows representative pictures of IHC staining of CETP in liver sections of each group. (E and F) Expression of *F4/80* mRNA (E) and *CETP* mRNA (F) in gonadal adipose tissue.
All data are represented as mean±SD; n=6; Student unpaired t test, ***P<0.001, as compared to control group.

Next, mice fed a WTD were injected with liposomal clodronate, a well-established method to deplete macrophages from liver in rodents.[24] Indeed, as compared to controls, liposomal clodronate markedly reduced hepatic *F4/80* expression (-88%; Fig. 4A) and the number of F4/80$^+$ KCs (-75%; Fig. 4B). Depletion of KCs almost completely abolished hepatic *CETP* expression (-96%; Fig. 4C) as well as the number of CETP$^+$ cells (-95%; Fig. 4D). Liposomal clodronate did not influence expression of other hepatic immune cell markers, including those of monocytes (CD11b), T cells (CD3, CD4, and CD8), and B cells (CD19 and CD20). In contrast to the liver, liposomal clodronate did not alter mRNA expression of *F4/80* (Fig. 4E) or *CETP* (Fig. 4F) in adipose tissue. Elimination of KCs largely reduced plasma CETP concentration (Fig. 5A) and improved dyslipidemia with respect to decreasing plasma TGs (Fig. 5B), and tended to decrease plasma total cholesterol (Fig. 5C). Lipoprotein profiling showed a reduction in VLDL-cholesterol (VLDL-C; Fig. 5D) and increase in HDL-C (Fig. 5D). Liposomal clodronate had essentially the same effects in mice fed a regular chow diet with respect to decreasing hepatic *CETP* mRNA, CETP-positive (CETP$^+$) cells in liver, and plasma CETP concentration, without affecting *F4/80* mRNA and *CETP* mRNA expression by adipose tissue (Fig. S5).

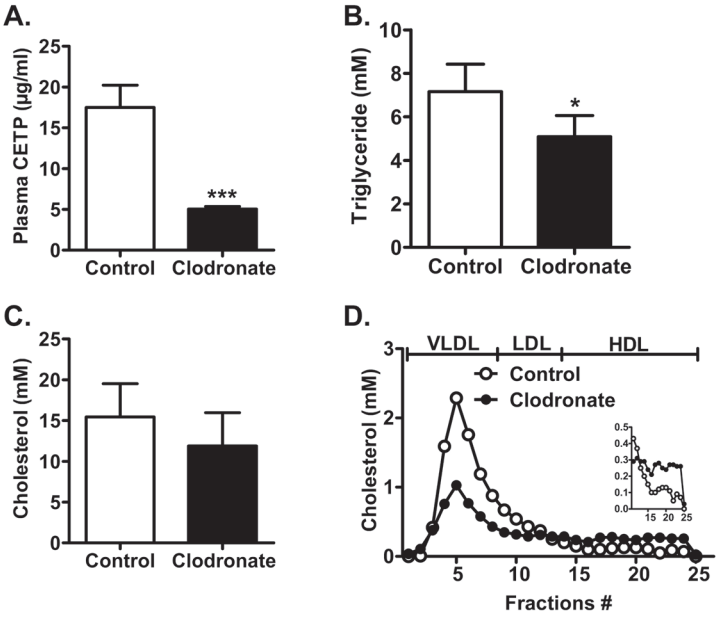

Figure 5: Elimination of KCs largely reduces plasma CETP concentration in human CETP Tg mice
APOE*3-Leiden.CETP mice fed a WTD were treated with or without liposomal clodronate. Plasma level of (A) CETP, (B) TGs, and (C) total cholesterol was determined. (D) Plasma cholesterol distribution over lipoproteins was assayed by FPLC, with HDL-C fraction as shown in the insert. Abbreviations: FPLC, fast protein liquid chromatography; LDL, low-density lipoprotein.
All data are represented as mean±SD; n=6; Student unpaired t test: *P<0.05, ***P<0.001, as compared to control group.

Augmentation of KCs largely increases hepatic *CETP* expression and plasma CETP concentration in human CETP Tg mice

To investigate whether an increase of KCs by a nondietary trigger increases plasma CETP concentration, APOE*3-Leiden.CETP mice fed a regular chow diet were treated with BCG vaccine. As compared to controls, BCG vaccination did not affect food intake or body weight, whereas it largely increased hepatic *F4/80* expression (5.6-fold; Fig. 6A), concomitant with increased hepatic *CETP* expression (2.9-fold; Fig. 6B) and increased plasma CETP concentration (3.3-fold; Fig. 6C). This was accompanied by increased plasma total cholesterol (Fig. 6E), specifically within VLDL (Fig. 6F).

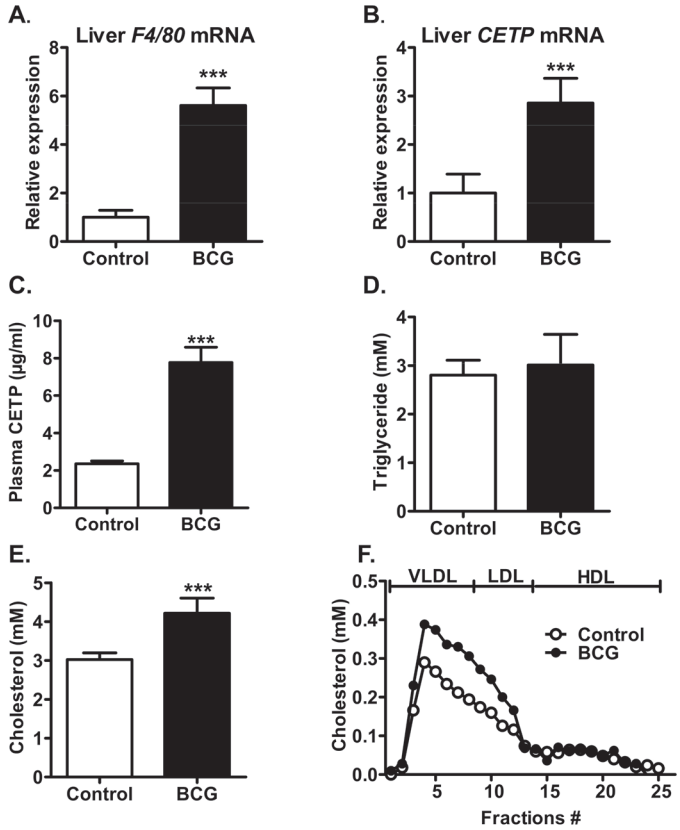

Figure 6: Augmentation of KCs increases hepatic CETP expression and plasma CETP concentration in human CETP Tg mice

APOE*3-Leiden.CETP mice fed a regular chow diet were treated with BCG vaccine (t=0 and 2 weeks) and terminated after 4 weeks. Hepatic mRNA expression of *F4/80* (A) and *CETP* (B) were determined. Plasma level of CETP (C), TGs (D), and total cholesterol (E) was determined. (F) Plasma cholesterol distribution over lipoproteins was assayed by fast protein liquid chromatography. Abbreviations: Con, control; Fen, fenofibrate; Nia, niacin.

All data are represented as mean±SD; n=6; Student unpaired t test: ***P<0.001, as compared to control group.

Collectively, we showed, by elimination or augmentation of hepatic KC content, that KCs are the principal source of the plasma CETP pool and that *CETP* expression in KCs determines plasma cholesterol distribution, which is in full accord with the data of our human studies.

Lipid-lowering agents reduce plasma CETP concentration accompanied by reduced KC content in human CETP Tg mice

Fibrates and niacin, classical lipid-lowering drugs used for the treatment of dyslipidemia,[32, 33] both increase plasma HDL-C in addition to decreasing plasma (V)LDL-C and TGs. This is, at least partly, explained by a reduction in plasma CETP concentration.[34, 35] Based on our present data that CETP is largely derived from KCs, we studied whether these lipid-lowering drugs lower hepatic KC content. In line with our previous findings,[34, 35] treatment of APOE*3-Leiden.CETP mice with fenofibrate or niacin for 3 weeks markedly decreased plasma CETP concentration (Fig. 7A) and hepatic expression of *CETP* (Fig. 7B). Both drugs also lowered plasma TGs (Fig. 7C) and cholesterol (Fig. 7D), explained by a reduction in VLDL-TGs (Fig. 7E) and VLDL-C (Fig. 7F), and an increase in HDL-C (Fig. 7F). Fenofibrate and niacin decreased liver KC content, reflected by a reduction in hepatic *F4/80* expression (Fig. 8A), as well as the number of F4/80$^+$ KCs (Fig. 8B, C). Moreover, plasma CETP concentration strongly correlates to liver F4/80$^+$ KC content ($r=0.633$; $P<0.001$; Fig. 8D). We cannot rule out that lipid-lowering agents reduce KC content as a consequence of improving lipoprotein profile. However, our data are consistent with the hypothesis that fenofibrate and niacin reduce liver KC content, thereby decreasing hepatic CETP production and plasma CETP concentration, concomitantly improving plasma lipoprotein cholesterol distribution.

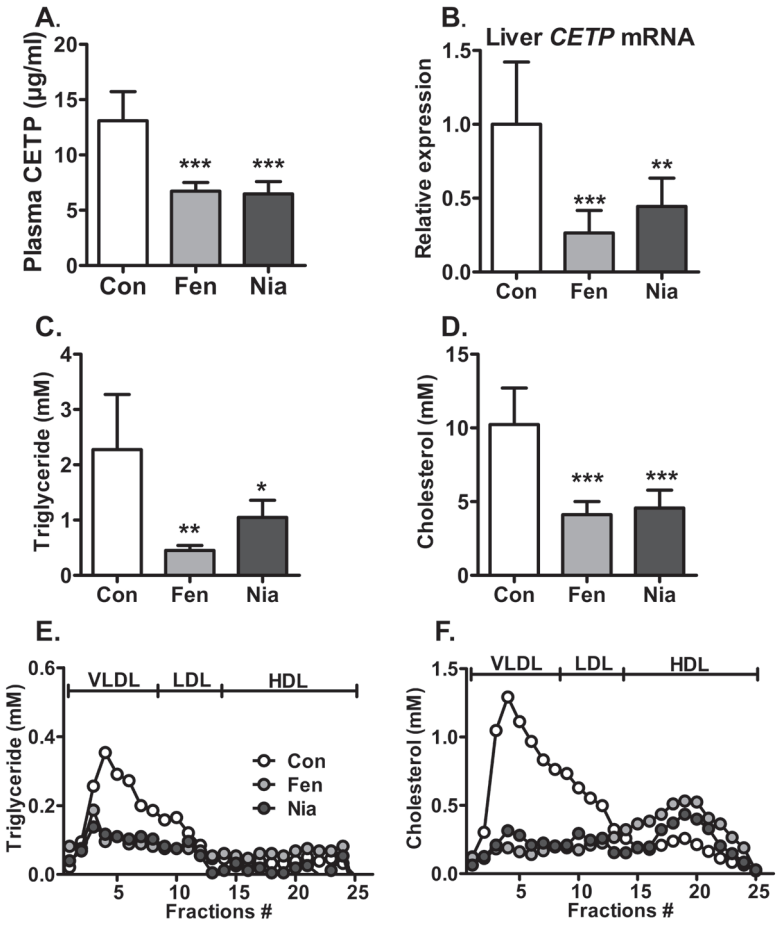

Figure 7: Lipid-lowering agents reduce plasma CETP concentration in human CETP Tg mice
APOE*3-Leiden.CETP mice fed a WTD were treated without (Con) or with fenofibrate (Fen) or niacin (Nia) for 4 weeks and plasma was assayed for CETP concentration (A), TG (C), and total cholesterol level (D), and hepatic mRNA expression of *CETP* (B) were determined. Distribution of triglyceride (E) and cholesterol (F) over plasma lipoproteins was assayed by fast protein liquid chromatography.
All data are represented as mean±SD; n=8; two-way ANOVA: *P<0.05, **P<0.01, ***P<0.001, as compared to control (Con) group.

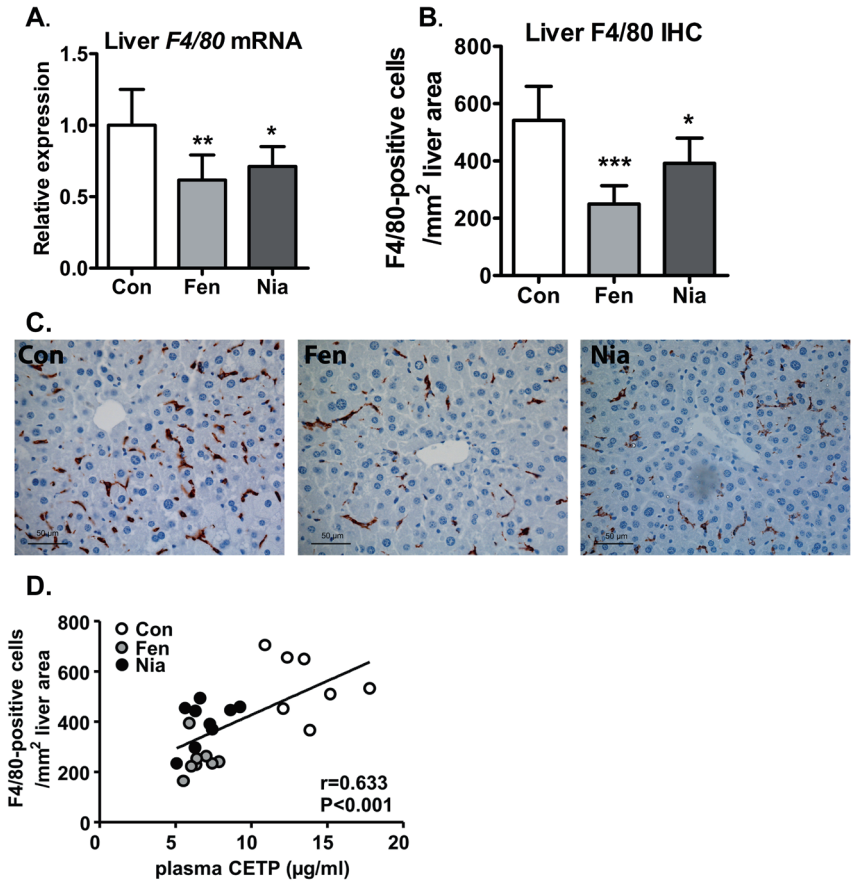

Figure 8: Lipid-lowering agents reduce the Kupffer cell content in human CETP Tg mice.
APOE*3- Leiden.CETP mice fed a WTD diet were treated without (Con) or with fenofibrate (Fen) or niacin (Nia) for 4 weeks. Hepatic mRNA expression of *F4/80* (A) and KC content ($F4/80^+$ cells; B) were determined. (C) Representative pictures of IHC staining of F4/80 in liver sections of each group. (D) Correlation of plasma CETP concentration with KC content ($F4/80^+$ cells).
All data are represented as mean±SD; n=8; two-way ANOVA: *P<0.05, **P<0.01, ***P<0.001, as compared to control (Con) group.

DISCUSSION

In this study, we show that the liver is the main source of plasma CETP, and that KCs are responsible for hepatic expression of CETP in humans. Previous studies have shown that *CETP* mRNA is not only expressed in the liver, but also in adipose tissue in several mammalian species.[18] A small human cohort study also found a correlation between adipose tissue *CETP* expression and plasma CETP concentration.[26] We now show that CETP is expressed more

prominently in the liver, as compared to adipose tissue. In addition, we found no association between waist circumference and plasma CETP concentration in a large cohort study. Furthermore, by analyzing liver biopsies from patients undergoing bariatric surgery, we found that plasma CETP concentration strongly correlates with *CETP* expression in the liver, but not in adipose tissue, indicating that *CETP* expression in liver contributes largely to the total plasma CETP pool in humans. Previous studies have shown that lowering adiposity induced by body-weight reduction reduced *CETP* expression and improved lipoprotein metabolism, implying that a reduction in adipose tissue reduces plasma CETP.[36, 37] However, in addition to reducing adiposity, body-weight reduction also significantly attenuates hepatosteatosis.[38, 39] Because we recently demonstrated that a decrease in hepatic lipid content is accompanied by a decrease in plasma CETP concentration,[39] it is thus tempting to speculate that body-weight reduction by attenuation of hepatosteatosis reduces production of CETP by the liver. Indeed, we observed that plasma CETP concentration was largely decreased after bariatric surgery. Thus, reduction in plasma CETP concentration is fully in line with the reduction in liver steatosis and inflammation after bariatric surgery.

Owing to the fact that the liver consists of multiple cell types, we set out to evaluate the cell type responsible for expression of *CETP*. In the present study, we found that expression of established macrophage markers strongly associates with expression of hepatic *CETP*, and that CETP is specifically colocalized with $CD68^+$ KCs in the liver. Hepatic $CD68^+$ cell number and *CETP* expression did not differ among the various liver lobular inflammation scores,[40] indicating no association between the inflammatory foci number and KC content or CETP expression. However, our results clearly indicated that plasma CETP and the number of $CD68^+$ KCs were correlated. Mechanistic studies in APOE*3-Leiden.CETP mice showed that depleting KCs from the liver by clodronate liposomes virtually abolished hepatic *CETP* expression and largely reduced plasma CETP concentration. On the other hand, BCG vaccination markedly increased KCs, as reflected by increased hepatic *F4/80* expression, accompanied by marked increased in hepatic *CETP* expression and increased plasma CETP concentration. These data fully corroborate our findings in humans that KCs, rather than hepatocytes, are the main cellular source of hepatic *CETP* expression and the plasma CETP pool. In contrast to KCs, we could hardly detect any *CETP* expression in extrahepatic macrophages, including peritoneal macrophages, bone marrow-derived macrophages, and macrophages in adipose tissue (Figs. S4A, B). It has been reported that *CETP* expression is regulated by the activation of liver X receptor α (LXRα),[41] which is highly expressed in multiple organs. Recently, Gautier *et al.*[42] demonstrated that, in addition to an LXRα-responsive element in the CETP promoter, the CETP gene contains an ER8 farnesoid X receptor (FXR) response element in the first intron. Therefore, bile acids that are the natural ligand for FXR and are produced by hepatocytes may be essential for maintaining high expression of *CETP* in hepatic versus extrahepatic macrophages. In fact, treatment of APOE*3-Leiden.CETP mice with the bile acid taurocholic acid, greatly increased the hepatic CETP transcript as well as plasma CETP concentration.[42]

Previously, hepatic expression of *CETP* in mice has been attributed to both KCs and hepatocytes, based on studies assessing hepatic *CETP* expression 8 weeks after transplantation of bone marrow from wild-type (WT) littermates into human CETP Tg mice, and vice versa, suggesting that KCs contribute approximately 50% to total hepatic *CETP* expression.[19] However, it should be realized that the replacement of liver KCs after bone marrow transplantation (BMT) occurs slowly. In the same study, it was found that only 50% of KCs were replaced by donor cells 8 weeks after BMT, accompanied by a 50% reduction in plasma CETP concentration as well as a 2-fold lower hepatic *CETP* expression in WT→CETP Tg mice, as compared to control transplanted CETP Tg→CETP Tg mice.[19] Interestingly, we found in a prolonged BMT study in which *CETP*-expressing bone marrow cells were replaced by cells from CETP-deficient mice, hepatic *CETP* expression was approximately -95% lower at 36 weeks after transplantation, compared to the CETP Tg→CETP Tg transplanted group (Fig. S6). Although we did not include a CETP Tg→WT transplantation group, liver KCs are likely the predominant source of CETP expression.

In addition, depletion of KCs by liposomal clodronate generates a less atherogenic lipid phenotype (e.g., decreasing TG and increasing HDL-C in APOE*3-Leiden.CETP mice). Our findings might shed new light on the development of new strategies for CETP inhibition and treatment of dyslipidemia. Strategies focusing on inhibiting CETP synthesis at its cellular origin may be a promising alternative, to avoid potentially adverse effects of the current CETP inhibitors on the function of HDL.[43, 44] We also observed that lipid-lowering drugs, including fenofibrate and niacin, reduce plasma CETP concentration accompanied by reducing the KC content in APOE*3-Leiden.CETP mice. In addition, fenofibrate and niacin improved the lipoprotein profile. The question of whether these pharmacological treatments reduce KCs in response to the improved lipoprotein profile or whether they primarily reduce KCs, which, as a consequence, results in the improved lipoprotein profile, is still obscure and needs to be determined by future research.

In this study, we showed that both a dietary trigger and BCG vaccination increase KC content of the liver, accompanied by increased hepatic *CETP* expression and plasma CETP concentration, which suggest that increased plasma CETP may be a marker for increased hepatic KC content. However, inflammatory stimuli, such as lipopolysaccharide (LPS), despite raising KC content, reduce *CETP* expression per se,[45, 46] thereby precluding a rise in plasma CETP concentration. Thus, although increased plasma CETP concentration indicates increased KCs, equal or lower plasma CETP concentration does not necessarily indicate a decrease in KCs in intervention studies modulating such inflammatory stimuli.

We conclude that CETP is mainly produced by KCs, and that plasma CETP concentration significantly correlates with the number of KCs in humans. Moreover, treatment of CETP Tg mice with niacin and fenofibrate reduces KC content accompanied with a reduction in plasma CETP concentration. Taken together, these data suggest that measurement of

plasma CETP concentration can be developed as a diagnostic and predictive test for liver KC content in clinical practice, which should be tested in large population cohorts. However, it should be noted that use of CETP as a marker for hepatic KC content may be limited under conditions of exposure to LPS or other inflammatory stimuli.

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SUPPLEMENTAL DATA

Assessment for Rijswijk study

A cross-sectional observational study was performed in a general population in Rijswijk, The Netherlands. All non-diabetic subjects between 40-70 years of age of four general practitioners were identified using computer assisted screening. In addition to known diabetes, persons with known terminal disease, a history of psychiatric disorder or substance abuse were excluded. Screening was carried out in 2079 of the 2942 considered eligible subjects (response rate 70.6%) after written informed consent. During the screening visit, medical history including use of medication was taken, if necessary additional information was retrieved from the medical record. Subsequently, waist circumference was measured in standing position, midway between the lower limit of the rib cage and the iliac crest using a Seca 200 circumference measuring tape (Seca Gmbh). Venous blood samples were taken after a 12 h overnight fast. Storage of samples for plasma CETP was performed after evaluation of the first 645 subjects. The Rijswijk study was approved by the review board of South West Holland and performed in accordance with the Declaration of Helsinki.

Statistical analysis was performed using SPSS for Windows (version 17.0; SPSS, Chicago, Illinois, USA). Data are expressed as mean±SD or median (interquartile range). To compare plasma CETP concentration between quintiles of waist circumference a Kruskal-Wallis test for non-parametric continues variables was used.

Assessment for bariatric surgery study

Tissue sampling, histology preparation, and mRNA isolation

Tissue sampling and mRNA isolation were performed as described earlier.[22] Wedge biopsies from liver, subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were taken during bariatric surgery. mRNA was isolated using the Qiagen Lipid Tissue Mini Kit (74804, Qiagen) and mRNA quality and concentration were assessed with an Agilent Bioanalyzer (5067-1521, Agilent Technologies). mRNA integrity numbers (RIN) of these samples ranged between 4.5 and 9.3 (average 7.5), 5.8 and 8.7 (average 7.5), and 6.2 and 9.4 (average 7.6) for liver, SAT, and VAT respectively.

mRNA pre-hybridization processing and hybridization

mRNA pre-hybridization processing and hybridization were performed as described earlier.[22] Starting with 200 ng of mRNA, the Ambion Illumina TotalPrep Amplification Kit was used for anti-sense RNA synthesis, amplification, and purification, according to the manufacturer's protocol (Applied Biosystems/Ambion). 750 ng of complementary RNA was hybridized to Illumina HumanHT12 BeadChips (Illumina\) and scanned on the Illumina BeadArray Reader. These micro arrays contain 48813 different probes targeting 37812 different genes; some genes are targeted by more than one probe.

Data normalization and quality control

Quantile-quantile normalization was applied to all genome-wide data from liver, VAT and SAT using LIMMA package (version 3.4.5) in R (version 2.11.1) (R foundation for statistical computing, Vienna, Austria). Only samples were included that passed quality control filtering, which was based on the median probe intensity, general behavior of known housekeeping genes, and principal component analysis over the samples. Available genome-wide genotype data were used to rule out sample mix-ups,[47] and qPCR was performed to estimate the technical quality of the micro array.[22] We obtained reliable RNA measures for 82 liver samples, 90 SAT samples and 84 VAT samples. All expression data has been made freely available by submission to GEO under GSE22070 (SAT data), and GSE22071 (VAT data). Liver expression data will be made available soon.

Calculations and data visualization

Two probes targeting CETP were present on the micro array. Values of these probes were very strongly correlated (r=0.82, 0.87, and 0.80 in liver, SAT and VAT respectively). We determined the expression of *CETP* as the average value of these two probes. Statistical analysis was performed using SPSS for Windows (version 17.0; SPSS). mRNA expression levels of *CETP* and *MARCO* in liver, SAT, and VAT were correlated to each other and to plasma CETP and HDL-C levels using Spearman correlation.

Plasma CETP concentration, lipid and lipoprotein profiles analysis

Plasma CETP concentration was measured using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi). Plasma cholesterol and triglycerides were assayed using the commercially available enzymatic kits 236691, 11488872 (Roche Molecular Biochemicals), respectively. The distribution of lipids over plasma lipoproteins was determined 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) 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 triglycerides as described above.

Assessment for mouse studies

RNA extraction, reverse transcription and qPCR

Total RNA was extracted from frozen liver and adipose tissue pieces using the Nucleospin RNAII kit (Macherey-Nagel) according to manufacturer's instructions. RNA quality was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad) and RNA concentration was determined by Nanodrop technology (Thermo Scientific). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (1708891, Bio-Rad), and the obtained cDNA was purified with Nucleospin Extract II kit (636973, Macherey-Nagel). qPCR

was performed on a CFX96™ (Bio-Rad), the reaction mixture consisting of iQ™ SYBR® Green Super mix (Bio-Rad), cDNA, primers (Biolegio; see Table S2 for primer sequences), and nuclease-free water in a total reaction volume of 10 µL. mRNA values of each gene were normalized to mRNA levels of ß-2 microglobulin and hypoxanthine ribosyltransferase. Data were expressed as relative expression normalized to control group.

Table S2: Primer sequences used for qPCR in mouse study

Gene	Forward primer	Reverse Primer
ß-2m	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATAG
Cd68	ATCCCCACCTGTCTCTCTCA	TTGCATTTCCACAGCAGAAG
CETP	CAGATCAGCCACTTGTCCAT	CAGCTGTGTGTTGATCTGGA
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG

ß-2m, ß-2 microglobulin; Cd68, cluster of differentiation 68; CETP, cholesteryl ester transfer protein; Hprt, hypoxanthine ribosyltransferase.

In situ hybridization

OCT-embedded frozen liver sections (5 µm) from a healthy donor, an obese subject and APOE*3-Leiden.CETP mice were prepared. mRNA of human *CD68* (Probe set: VA6-13284), human CETP (Probe set: VA1-15420), and mouse *F4/80* (*Emr1*, Probe set: VB6-12917) were detected in those sections by using the QuantiGene ViewRNA ISH Tissue Assay (Affymetrix Inc) according to manufacturer's instructions.

Immunohistochemistry

Paraffin-embedded human liver sections (5 µm) were stained for macrophage marker CD68 (M0814; 1/800, Dako, California, USA) and CETP (ab51771; 1/1000, Abcam). We were able to obtain liver sections of 46 subjects from the bariatric surgery cohort. Paraffin-embedded liver sections of APOE*3-Leiden.CETP mouse (5 µm) were stained for macrophage marker F4/80 (MCA497; 1/600, Serotec) as described previously,[48] and human CETP (ab51771; 1/1000, Abcam).

Bone marrow transplantation

APOE*3-Leiden.CETP mice were fed chow diet. At the age of 8 weeks, mice were randomized according to age and plasma lipid levels (TC and TG), and irradiated at 8 Gy using the Orthovolt X-ray machine (CAM/P10008, 081806, Varian Medical Systems) as described before.[49] After overnight recovery, mice received an intravenous tail injection with $1.2x10^6$ bone marrow cells from either wild-type mice or APOE*3-Leiden.CETP mice (both C57Bl/6 background). Mice were housed in sterile cages for 8 weeks until their hematopoietic system was fully recovered. The first 3 weeks following bone marrow transplantation mice were

administered with a mixture of Ciprofloxacin (0.13 mg/kg/day, Bayer b.v.), Amphotericin B (0.15 mg/kg/day, Bristol-Myers Squibb) and Polymixin B (0.105 mg/kg/day, Bupha) in the drinking water. Eight weeks after bone marrow transplantation, the diet of the mice was replaced by a Western-type diet and mice were sacrificed at 36 weeks after bone marrow transplantation. Livers were assayed for *CETP* and *F4/80* mRNA expression.

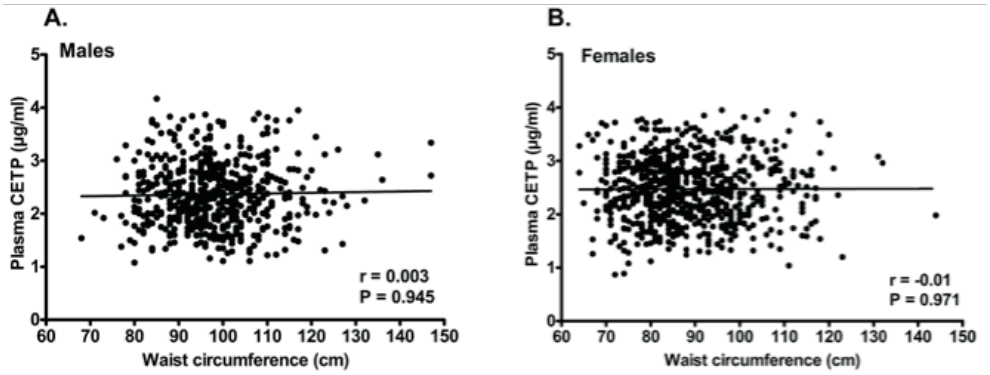

Figure S1: Waist circumference does not correlate with the plasma CETP concentration in a general population excluding subjects who received statins (related to Fig. 1)
(A and B) 82 males and 61 females who received statin treatment were excluded from the Rijswijk cohort. Correlations between waist circumference and the plasma CETP concentration were determined in 572 male (A) and 719 female (B) subjects.

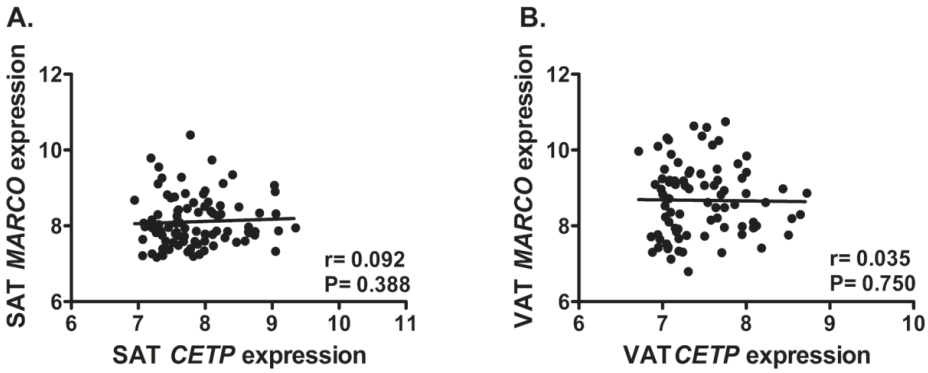

Figure S2: The expression of *CETP* does not correlate with *MARCO* in subcutaneous and visceral adipose tissue (related to Fig. 2)
(A and B) Biopsies from subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were taken during bariatric surgery from 97 patients, and assayed for genome-wide gene expression profiles. The correlation between the expression of *CETP* and *MARCO* in SAT (A) and VAT (B) were determined.

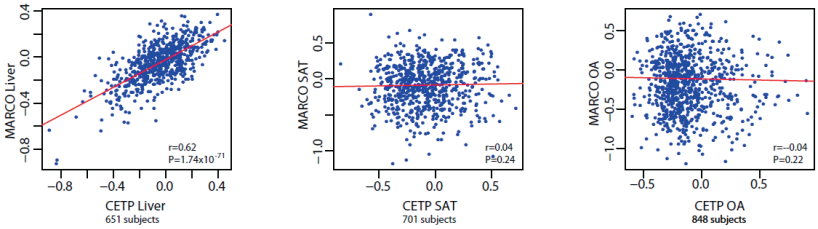

Figure S3: The expression of *CETP* correlates with *MARCO* in liver but not in adipose tissue
Scatter plots of the correlation between the expression of *CETP* and *MARCO* in liver, subcutaneous adipose tissue (SAT) and omental adipose (OA) were determined by using a publicly available dataset consisting of 1008 subjects.[30]

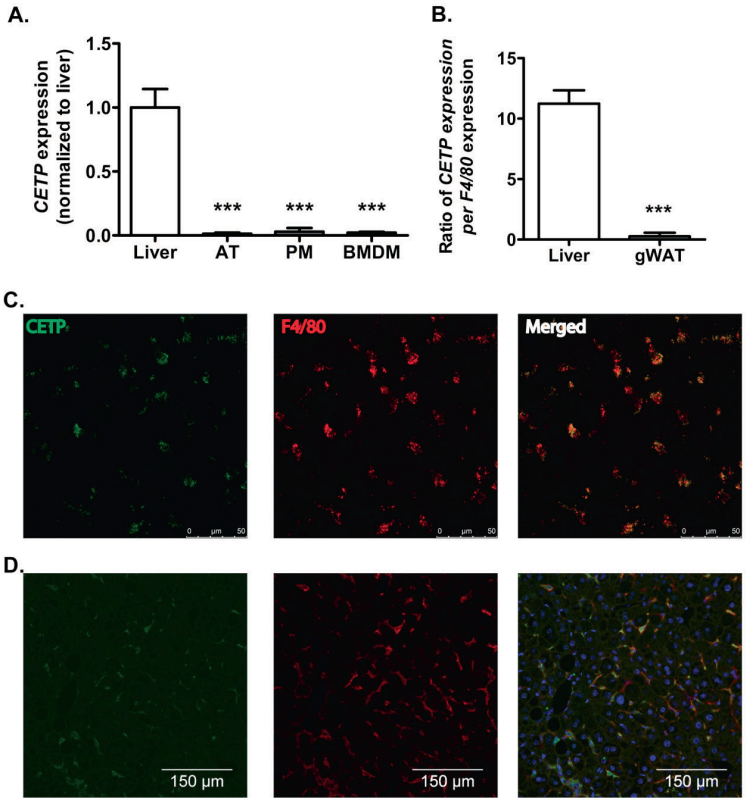

Figure S4: APOE*3-Leiden.CETP mice have a similar *CETP* expression pattern as humans, and KCs are the predominant source of CETP expression
(A) Liver, gonadal adipose tissue (AT), peritoneal macrophages (PM) and bone marrow-derived macrophages (BMDM) were isolated from APOE*3-Leiden.CETP transgenic mice, and qPCR of *CETP* and the reference gene *ß-2 microglobulin* (ß-2) were performed. *CETP* mRNA expression in extrahepatic tissue and cells was normalized to *CETP* mRNA expression in liver. (B) The ratio of *CETP* mRNA expression per *F4/80* mRNA expression was calculated. (C) Representative pictures of *in situ* hybridization of *CETP* mRNA (green), *F4/80* mRNA (red) and merged in liver sections. (D) Representative pictures of immunofluorescent staining of CETP (green), F4/80 (red), and merged in liver sections.
The data are represented as mean±SD (n=6). Student's unpaired t test, ***P<0.001, as compared to liver.

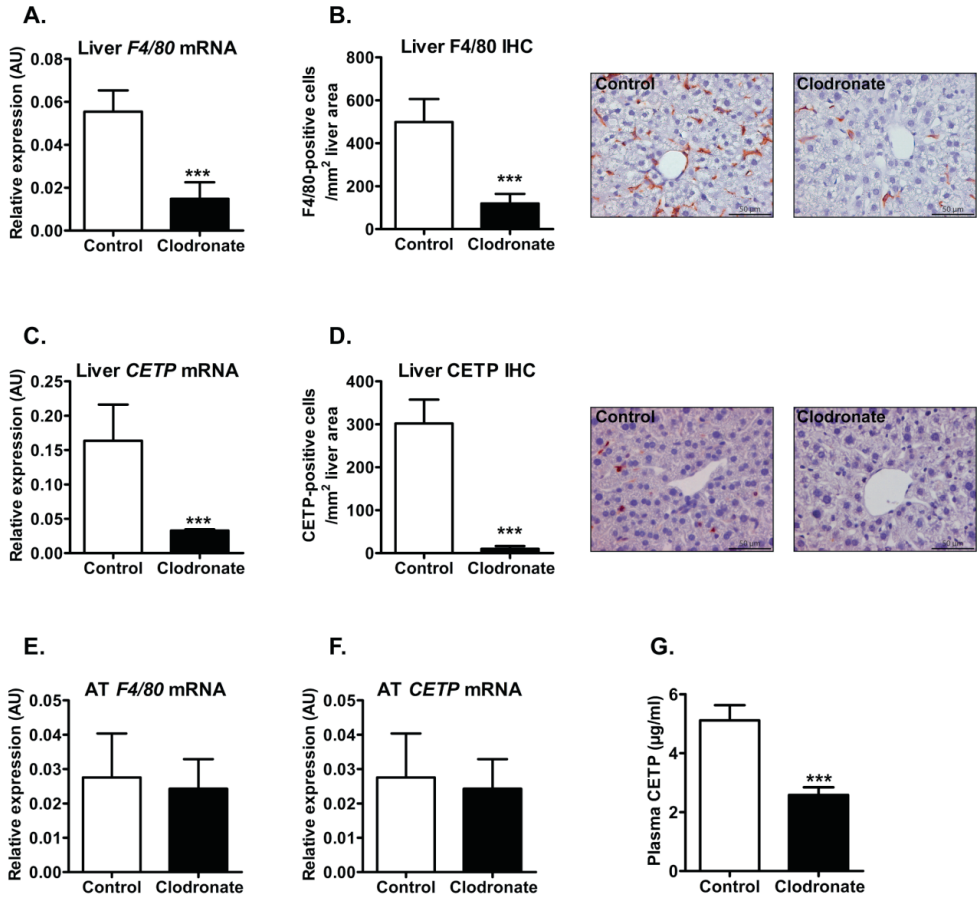

Figure S5: Elimination of Kupffer cells abolishes hepatic *CETP* expression and largely reduces plasma CETP concentration in APOE*3-Leiden.CETP mice fed a chow diet

(A) APOE*3-Leiden.CETP mice fed a chow diet (CD) were treated without or with liposomal clodronate and livers were assayed for *F4/80* mRNA expression. (B) Kupffer cell content (F4/80$^+$ cells) of the liver. The right panel shows representative pictures of IHC staining of F4/80 in liver sections of each group. (C) *CETP* mRNA expression in the liver. (D) Content of CETP$^+$ cells in the liver. The right panel shows representative pictures of IHC staining of CETP in liver sections of each group. (E and F) *F4/80* mRNA (E) and *CETP* mRNA (F) expression in gonadal adipose tissue. (G) Plasma CETP concentration.

All data are represented as mean±SD. Student's unpaired t test; ***P<0.001, as compared to control group.

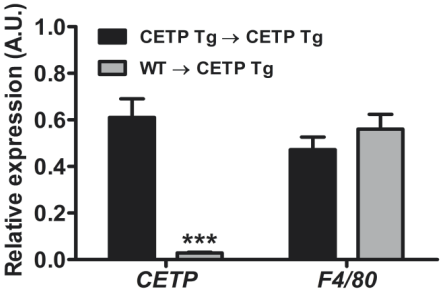

Figure S6: Bone marrow transplantation with CETP-deficient bone marrow abolishes hepatic *CETP* expression in APOE*3-Leiden.CETP mice

APOE*3-Leiden.CETP mice were transplanted with bone marrow from APOE*3-Leiden.CETP mice or wild-type (WT) mice. 36 weeks after bone marrow transplantation, mice were sacrificed and livers were assayed for *CETP* and *F4/80* mRNA expression.

All data are represented as mean±SD. Student's unpaired t test; ***P<0.001, as compared to CETP Tg→CETP Tg group.