6

PLASMA CHOLESTERYL ESTER TRANSFER PROTEIN: A BIOMARKER FOR HEPATIC MACROPHAGES

Yanan Wang, Sam J.L. van der Tuin, Nathanja Tjeerdema, Veerle Bieghs, Sander S. Rensen, Jingyuan Fu, Marcel G. Wolfs, Menno Hoekstra, Jan Willem Greve, Wim A. Buurman, Marten H. Hofker, Ronit Shiri-Sverdlov, Johannes W.A. Smit, Louis M. Havekes, Ko Willems van Dijk, Patrick C.N. Rensen

Submitted for publication

ABSTRACT

Cholesteryl ester transfer protein (CETP) reduces HDL-cholesterol and is a pharmaceutical target for treating dyslipidemia. However, the cellular origin of plasma CETP in humans is uncertain. We aimed to elucidate the relative contribution of adipose tissue and liver to plasma CETP pools and the cellular origin of plasma CETP. To assess the association between adipose tissue mass and CETP, waist circumference and plasma CETP were measured in 1434 subjects from a general population. To further characterize the source of CETP, biopsies of liver and adipose tissue from 94 subjects were analysed for gene and protein expression, and these were correlated with plasma CETP and lipid parameters. Detailed mechanistic studies on the cellular origin of CETP and mode of action of lipid-lowering drugs that lower plasma CETP were performed using *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice, a well-established model for human-like lipoprotein metabolism. Plasma CETP did not correlate with waist circumference, suggesting that central adipose tissue does not contribute to plasma CETP. Microarray analysis of liver and adipose tissue biopsies showed that CETP expression was highest in liver. Immunohistochemistry revealed that hepatic CETP is primarily expressed by macrophages. CETP expression in liver, but not adipose tissue, positively correlated with plasma CETP, and inversely correlated with plasma HDL-cholesterol. Selective elimination of macrophages from liver versus adipose tissue in *E3L.CETP* mice virtually abolished hepatic CETP expression, accompanied by largely reduced plasma CETP and increased plasma HDL-cholesterol. Lipid-lowering drugs that are known to reduce plasma CETP and to increase HDL in humans, reduced the hepatic macrophage content, simultaneously reducing plasma CETP and increasing HDL-cholesterol in *E3L.CETP* mice. In conclusion, plasma CETP is predominantly derived from hepatic macrophages, and may serve as a plasma biomarker for hepatic macrophage content in non-alcoholic steatohepatitis.

INTRODUCTION

The metabolic syndrome (MetS) is a complex disorder defined by interrelated risk factors for type 2 diabetes and cardiovascular disease, including central obesity, hyperglycemia, hypertension and dyslipidemia. In particular, many patients with the clinical diagnosis of MetS show a pro-atherogenic lipid profile with increased very low density lipoprotein (VLDL)-triglycerides (TG) and decreased high density lipoprotein (HDL)-cholesterol (C) [1]. Cholesteryl ester (CE) transfer protein (CETP) plays a pivotal role in the metabolism of VLDL and HDL by mediating the transfer of TG from VLDL to HDL in exchange for CE, resulting in increased (V)LDL-C and decreased HDL-C [2]. Genetic deficiency for CETP increases HDL-C and lipid-lowering compounds that reduce plasma CETP induce a beneficial lipoprotein profile including reduced VLDL-TG and raised HDL-C [3,4]. Therefore, CETP inhibition is a current target for the treatment of low HDL and the pro-atherogenic lipid profile associated with MetS to reduce cardiovascular disease risk [5].

Previous studies have demonstrated that liver and adipose tissue are the two major sources of circulating CETP in several mammalian species. Other tissues, such as spleen, heart, small intestine, adrenal gland and skeletal muscle express CETP mRNA to only a minor extent [6-10]. 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. Few studies have suggested that hepatocytes may be responsible for the expression and secretion of CETP from the liver [6]. However, other studies suggested that nonparenchymal cells including Kupffer cells are the principal source of CETP in the liver [6,11]. Although adipose tissue CETP expression was shown to be associated with plasma CETP in a small cohort of 13 men [12], an association between plasma CETP and adiposity has not been conducted in large general populations as yet.

To design novel CETP-target strategies and to understand their ability to reduce cardiovascular disease risk, it is crucial to understand the cellular origin of plasma CETP. Therefore, in this study we aimed to elucidate the cellular origin of CETP in the *APOE*3-Leiden.CETP* mouse model and in a translational setting using human cohorts. These mice express the human CETP gene under control of its natural regulatory flanking regions, ensuring a similar CETP expression pattern as in humans [13-16]. Our data show that the liver, rather than adipose tissue, predominantly contributes to plasma CETP, and that hepatic CETP is exclusively produced by macrophages. Moreover, specific lipid-lowering drugs decrease the hepatic macrophage content and thus increase HDL-C via reduction of hepatic CETP production. We therefore conclude that plasma CETP is a biomarker for the macrophage content of the liver.

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 non-diabetic 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 (approx. 12 h) for measurement of the plasma CETP concentration. The Rijswijk study was approved by the review board of South West Holland and performed in accordance with the Declaration of Helsinki.

The second study consisted of 93 severely obese subjects (BMI 30-74 kg/m^2) who underwent elective bariatric surgery from 2006 to 2009 at the Dept. of General Surgery, Maastricht University Medical Center, Maastricht, The Netherlands, as described [17]. 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 (SAT) and visceral adipose tissue (VAT) were taken for mRNA isolation and hybridization. Venous blood samples were obtained after overnight fasting (approx. 8 h) on the morning of surgery for analysis of the plasma CETP concentration and lipid parameters. This study was approved by the Medical Ethics Board of Maastricht University Medical Centre, 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 Methods section in the Supplementary Appendix.

Design of mouse studies

Female *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice expressing the human CETP gene under the control of its natural flanking regions [18] were used, and housed under standard conditions with a 12 h light/dark cycle with free access to food and water unless indicated otherwise. Mice were fed a semi-synthetic Western-type diet (WTD), containing 0.1% (w/w) cholesterol, 1% (w/w) corn oil and 15% (w/w) cocoa butter (Hope Farms, Woerden, The Netherlands).

In a first experiment, mice were fed WTD for 4 weeks, randomized according to body weight, plasma total cholesterol (TC) and triglyceride (TG) levels, and received two intraperitoneal injections of liposomal clodronate (4 ml/kg bodyweight; purchased from Dr. N. van Rooijen, VUmc, Amsterdam) at a 3-day interval to deplete macrophages from the liver [19, 20], and were terminated 3 days after the second injection. In a second

experiment, mice were fed WTD, without (control) and with 0.04% (w/w) fenofibrate or 1% (w/w) niacin (both from Sigma, St. Louis, MO, USA) for 4 additional weeks before sacrificing. 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 from the Leiden University Medical Center, Leiden, The Netherlands had approved all animal experiments.

In both experiments, blood was obtained via tail vein bleeding into heparin-coated capillary tubes. The 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 Methods section in the Supplementary Appendix). After mice had been sacrificed, liver and gonadal adipose tissue samples were collected to measure the expression of selected genes by quantitative real-time PCR and proteins by immunohistochemistry (see the Methods section in the Supplementary Appendix).

Statistical Analysis

Categorical variables are presented as frequencies and percentages, and continuous variables as means and standard deviations, or medians and interquartile ranges for variables with skewed distributions. Pearson correlation was used to estimate the association between waist circumference and plasma CETP in Rijswijk study. In the bariatric surgery cohort, Spearman correlation was used to determine the correlation between expression of *CETP* and *MARCO* in the liver, SAT, VAT; the association between *CETP* expression in liver, SAT, VAT and plasma CETP level; as well as the association between *CETP* expression in liver, SAT, VAT and plasma HDL-C level, respectively. Statistical differences between groups were assessed with the nonparametric Mann-Whitney U test for two independent groups or two-way ANOVA with Tukey's post-hoc test for multiple comparisons. All reported *P* values are two-tailed, and *P* values of less than 0.05 were considered statistically significant.

RESULTS

Waist circumference is not associated with plasma CETP concentration in humans

The characteristics of 1434 subjects (654 males and 780 females) from a non-diabetic population in Rijswijk are shown in Supplementary Table 1. Mean (±SD) waist circumference was 99 ± 11 cm for males (n=654) and 89 ± 12 cm for females (n=780). The

median value for plasma CETP concentration was significantly lower in males [2.31 (1.90-2.72) µg/ml] compared to females [2.44 (2.02-2.86) µg/ml] (P<0.001). However, plasma CETP did not differ between quintiles of waist circumference in either males (Fig. 1A; P=0.328) or females (Fig. 1B; P=0.571), and no correlation between waist circumference and plasma CETP concentration was observed in either males (Fig. 1C; r=0.007, P=0.857) or females (Fig. 1D; r=-0.024, P=0.509). Excluding subjects who received lipid-lowering medication (statins) (82 males and 61 females) did not change the results for males (r=0.023, P=0.583) and females (r=-0.003, P=0.930). Our findings suggest that central obesity measured as waist circumference does not correlate with the plasma CETP in a general population.

Supplementary Table 1 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/liter		
Median	5.58	5.67
Interquartile range	4.84-6.27	4.99-6.38
HDL-cholesterol — mmol /liter		
Median	1.13	1.44
Interquartile range	0.96-1.33	1.23-1.70
Triglycerides — mmol /liter		
Median	1.32	1.05
Interquartile range	0.93-1.90	0.81-1.45
Glucose – mmol/liter	5.44±1.16	5.13±0.77
CETP — µg/ milliliter		
Median	2.31	2.44
Interquartile range	1.90-2.72	2.02-2.86

* Plus-minus values are means±SD. To convert values for cholesterol to milligrams per deciliter, divide by 0.02586. To convert values for triglycerides to milligrams per deciliter, divide by 0.01129. HDL denotes high-density lipoprotein; CETP denotes cholesteryl ester transfer protein.

† The diagnosis of metabolic syndrome is based on the IDF criteria.[36] 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 (≥ 150mg/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.

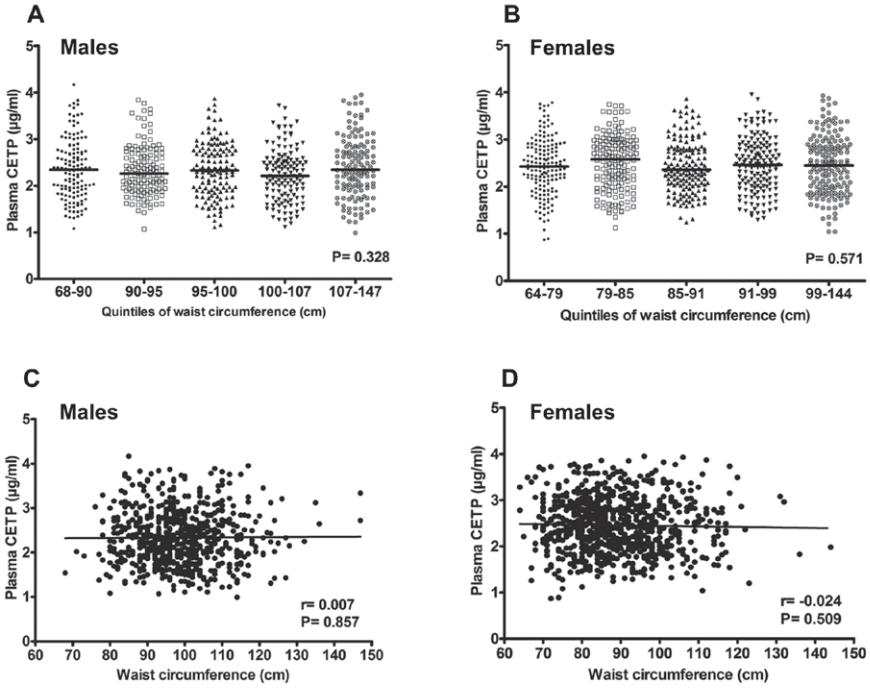


Figure 1. Waist circumference is not associated with plasma CETP concentration in a general population. In 1434 subjects that were enrolled in the Rijswijk study, median plasma CETP concentration over quintiles of waist circumference in male (A) and female (B) subjects, associations between plasma CETP concentration and waist circumference in male (C) and female (D) subjects were determined.

Hepatic CETP expression, specifically in macrophages, determines the plasma CETP concentration and HDL-cholesterol level in humans

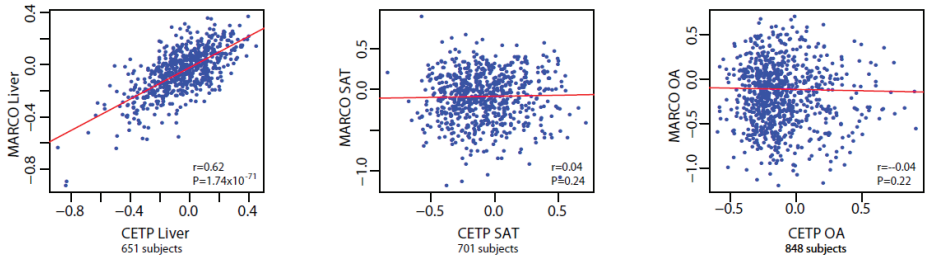
The characteristics of 93 severely obese patients who underwent elective bariatric surgery are shown in Table 1. Biopsies from livers and adipose tissue [i.e. subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT)] were taken during surgery and gene expression profiles were determined by micro-array analyses as described previously.[17] These profiles revealed that the CETP mRNA transcript is much more abundant in liver than in VAT and SAT (P=2.22x10^{-33} and P=1.9x10^{-27}, respectively). Next, the associations between the expression of *CETP* and other genes in VAT, SAT and liver was evaluated. Strikingly, the top genes that correlated highest with hepatic CETP expression are specific macrophage markers including *TIMD4* (r=0.631) and *MARCO* (r=0.590). In contrast, *CETP* did not correlate with macrophage-specific genes

in adipose tissue. For example, expression of *CETP* and *MARCO* were correlated in liver (r=0.590; P<0.0001; Fig. 2A) in liver but not in SAT (r=-0.092, P=0.388; Fig. 2B) or VAT (r=0.035, P=0.750; Fig. 2C). Using a publicly available considerably larger dataset undergoing bariatric surgery (1008 subjects) [21], we were able to replicate the strong association between *CETP* expression and *MARCO* in liver (r=0.624, P=1.7391e-71), but not in VAT (e.g. omental adipose tissue, r=-0.0423, P=0.2183) or SAT (r=0.043, P=0.2448) (Supplementary Fig. 1). Immunohistochemical stainings revealed that, in the liver, CETP specifically co-localized with $CD68^+$ macrophages (Fig. 2D).

Table 1 Characteristics of severely obese subjects underwent the bariatric surgery

Age — yr	44.2±9.7
Male sex — no. (%)	26 (28.0%)
Body mass index†	46.1±9.5
Glucose- mmol/liter	46.45±1.98
Cholesterol — mmol /liter	
Total	5.08±1.12
LDL	3.21±1.02
HDL	0.98±0.37
Triglycerides — mmol /liter	2.22±1.98
CETP — µg/ milliliter	2.70±1.02

Importantly, hepatic CETP expression positively correlated with plasma CETP (r=0.519, p<0.0001; Fig 2E), and inversely correlated with plasma HDL-C (r=-0.204, p=0.075; Fig 2H). In contrast, no significant correlation between expression of CETP in VAT and plasma CETP (p=0.140; Fig 2G) or HDL-C (p=0.250; Fig 2J) was noted. Although CETP expression in SAT correlated with plasma CETP (P<0.01; Fig 2F), it did not correlate with plasma HDL-C (p=0.123; Fig 2I). These data suggest that CETP expression in hepatic macrophages determines plasma CETP levels as well as the CETP-induced effects on HDL levels.



Supplementary Figure 1· Association of expression of CETP and MARCO in liver and adipose tissue
Scatter plots of the correlation between mRNA level of CETP and MARCO in liver (A), subcutaneous adipose tissue (SAT) (B) and omental adipose (OA) (C) obtained from 1008 subjects undergoing bariatric surgery.[21]

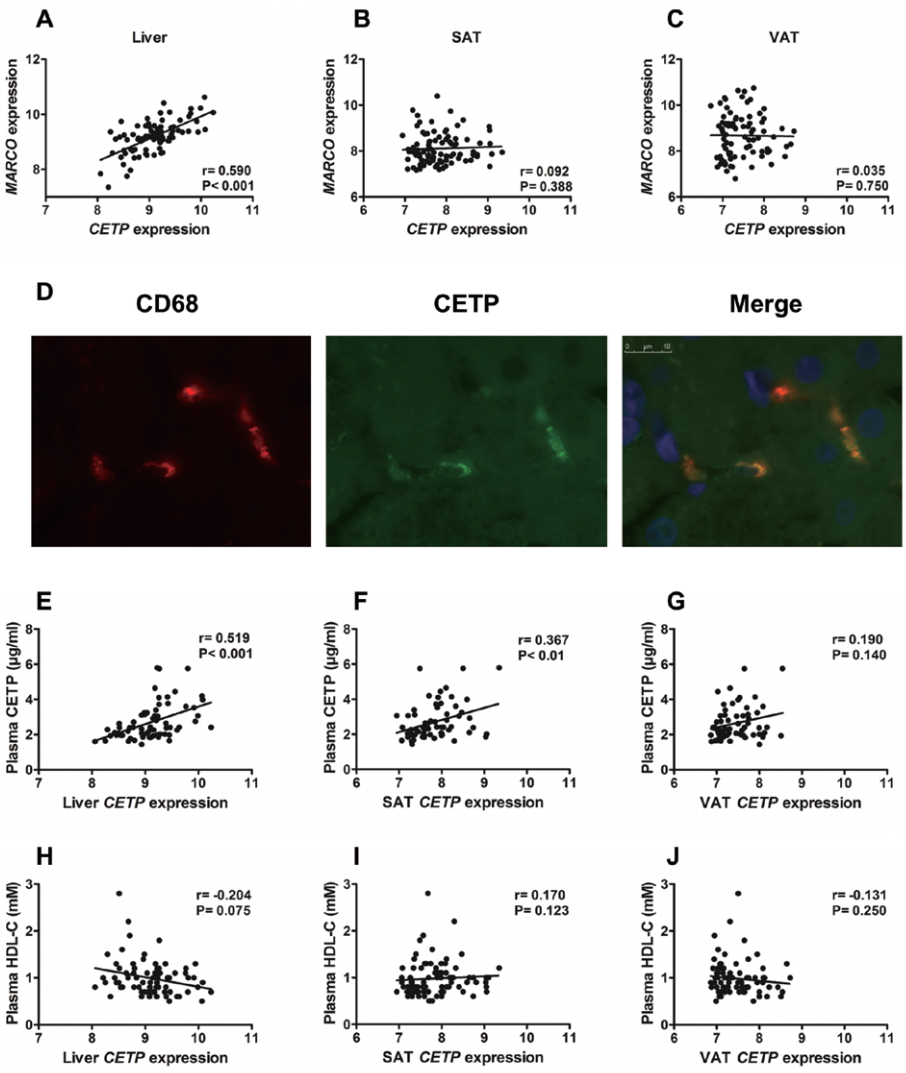


Figure 2. Hepatic CETP expression in macrophages is associated with plasma CETP concentration and HDL-cholesterol level in humans. Biopsies from visceral adipose tissues (VAT), subcutaneous adipose tissues (SAT) and livers and were taken during bariatric surgery from 97 severely obese patients, and assayed for genome-wide gene expression profiles. The expression of *CETP* was correlated with *MARCO* in liver (A), but not in SAT (B) and VAT (C). Panel D shows representative pictures of double stainings of CD68 (red) and CETP (green) in a liver section. Hepatic *CETP* expression positively correlated with plasma CETP level (E), and reversely correlated with plasma HDL-cholesterol level (H). The correlation between plasma CETP level and the expression of *CETP* in the SAT (F) and VAT (G), and the correlation between plasma HDL-C level and the expression of *CETP* in the SAT (I) and VAT (J) are shown.

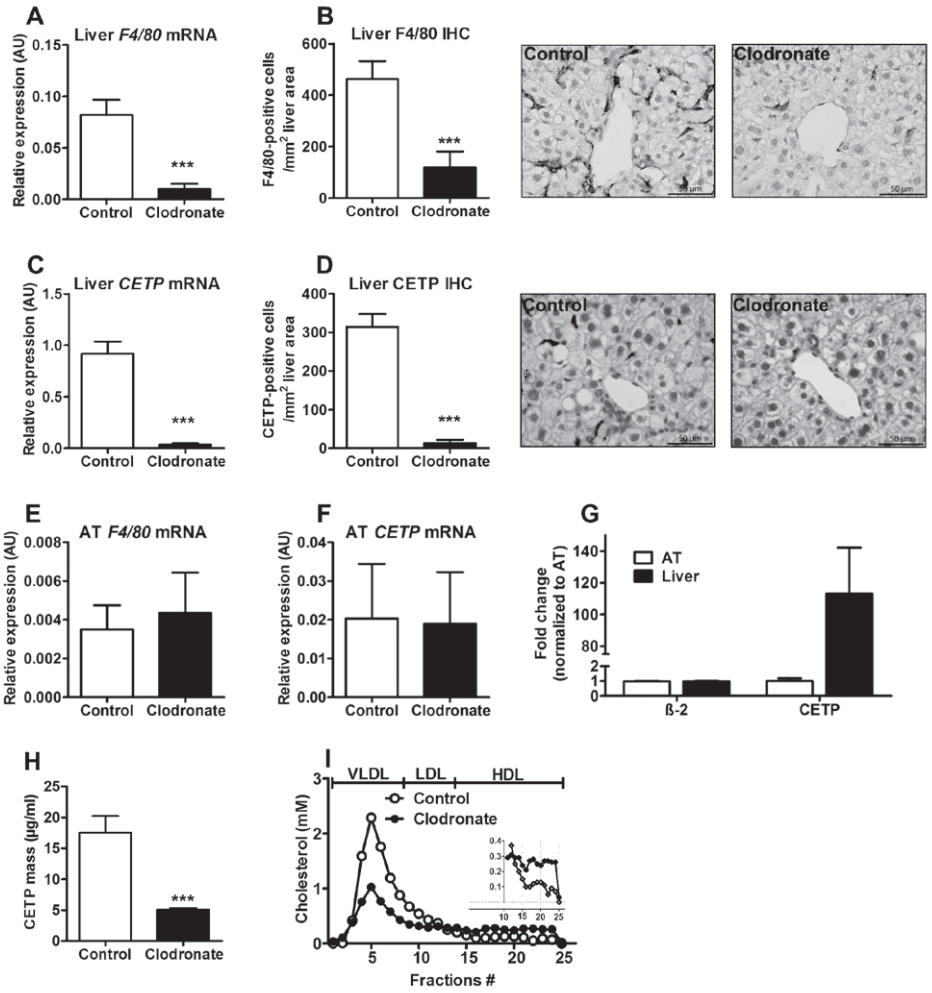


Figure 3. Elimination of hepatic macrophages abolishes hepatic CETP expression and largely reduces plasma CETP in *E3L.CETP* mice. *APOE*3-Leiden.CETP* (*E3L.CETP*) mice fed a western-type diet (WTD) were treated with or without liposomal clodronate. Livers were assayed for mRNA expression of *F4/80* (A) and *CETP* (C), as well as for F4/80 (B) and CETP (D)-positive cells. Gonadal adipose tissues were assayed for expression of *F4/80* (E) and *CETP* (F). The expression levels of *ß-2 microglobulin* (*ß-2*) and *CETP* were compared between livers and adipose tissue (G). Plasma was assayed for CETP concentration (H) and the cholesterol distribution over lipoproteins (I).

Elimination of hepatic macrophages abolishes hepatic CETP expression and largely reduces plasma CETP concentration in *E3L.CETP* mice

To further investigate the contribution of CETP expression in hepatic macrophages to plasma CETP as well as lipoprotein metabolism, *E3L.CETP* mice were injected with

liposomal clodronate, a well-established method to deplete macrophages from liver, but not adipose tissue in rodents [19, 20]. Indeed, as compared with controls, liposomal clodronate markedly reduced hepatic *F4/80* expression (-88%, $P<0.001$; Fig. 3A) and F4/80$^+$ cells (-74%, $P<0.001$; Fig. 3B). Concomitantly, depletion of hepatic macrophages almost completely abolished hepatic *CETP* expression (Fig. 3C, -96%, $P<0.001$) as well as CETP$^+$ macrophages (Fig. 3D, -96%, $P<0.001$). In contrast to the liver, liposomal clodronate did not alter the mRNA expression of *F4/80* (Fig. 3E) or *CETP* (Fig. 3F) in adipose tissue. Strikingly, *CETP* expression in the liver is 113-fold higher than in adipose tissue (Fig. 3G, $P<0.001$), despite a comparable expression of the reference gene *ß-2 microglobulin*. This corroborates the human studies suggesting that the liver, as opposed to adipose tissue, is the main source of CETP. In further support of this notion, elimination of hepatic macrophages largely reduced plasma CETP levels (-71%, $P<0.001$; Fig. 3H), accompanied by a decreased VLDL-C (-49%; Fig. 3I) and increased HDL-C level (+130%; Fig. 3I).

Lipid-lowering agents reduce plasma CETP concentration by reducing the hepatic macrophage content in *E3L.CETP* mice

Fibrates and niacin, which are used for the treatment of dyslipidemia in humans [22, 23], both decrease plasma CETP and increase HDL-cholesterol [15, 16]. Since our data show that CETP is largely derived from hepatic macrophages, we hypothesized that these lipid-lowering drugs could act by decreasing hepatic macrophages. In line with our previous findings [15, 16], treatment of *E3L.CETP* mice with fenofibrate and niacin for 3 weeks decreased plasma CETP level (-49% and -51%, both $P<0.001$; Fig. 4A) and hepatic expression of *CETP* (-74% and -56%, both $P<0.001$; Fig. 4B). It also lowered plasma TG (-80% and -54%, both $P<0.001$; Fig. 4C) and cholesterol level (-60% and -55%, both $P<0.001$; Fig. 4D), explained by a reduction in VLDL-TG (Fig. 4E) and VLDL-C (Fig. 4F), and an increase in HDL-C (Fig. 4F). Indeed, fenofibrate and niacin decreased hepatic macrophage content reflected by a reduction in hepatic F4/80 expression (-38% and -29%, both $P<0.001$; Fig. 4G), as well as F4/80$^+$ macrophages (-54% and -28%, both $P<0.001$; Fig. 4H and Fig. 4I). Taken together, these data indicate that fenofibrate and niacin reduce the hepatic macrophage content, thereby decreasing hepatic CETP production, resulting in a decreased plasma CETP concentration and increased HDL-cholesterol.

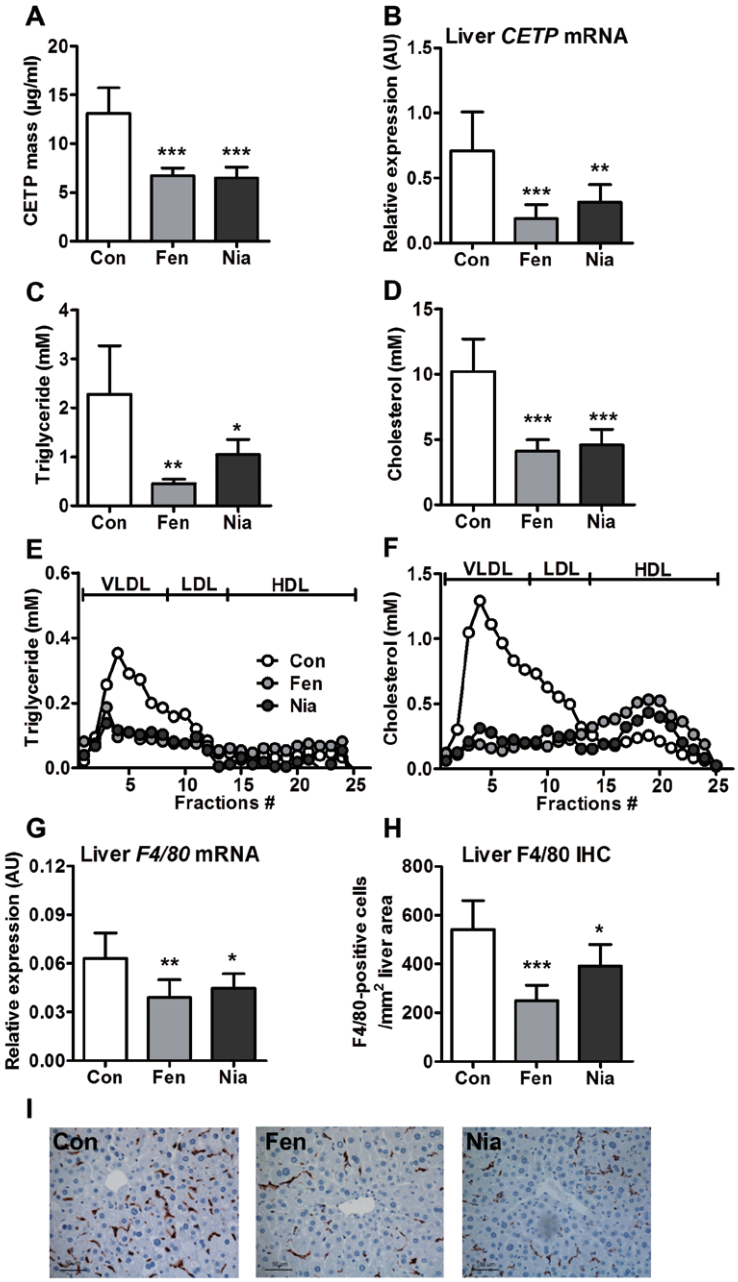


Figure 4. Lipid-lowering agents reduce plasma CETP concentration by reducing the hepatic macrophage content in *E3L.CETP* mice. *E3L.CETP* mice fed a WTD were treated without (Con) or with fenofibrate (Fen) or niacin (Nia) for 4 weeks. Plasma was assayed for CETP concentration (A), triglycerides (C) and cholesterol (D) as well as the distribution of triglycerides (E) and cholesterol (F) over lipoproteins. Livers were assayed for expression of *CETP* (B) and *F4/80* (G), and for F4/80-positive cells (H) with representative pictures shown (I).

DISCUSSION

In this study, we show that liver is the main source of plasma CETP, and that the cells responsible for expression of *CETP* are the hepatic macrophages. Adipose tissue does not appear to contribute significantly to the plasma CETP pool because central adiposity is not associated with the plasma CETP level in the general population.

Previous studies have shown that *CETP* mRNA is abundantly expressed in the liver and adipose tissue in several mammalian species [24]. A small human cohort study also found a correlation between adipose tissue *CETP* expression and plasma CETP concentration [12]. In contrast, our data show much more prominent *CETP* expression in the liver as compared to adipose tissue. In addition, we found no association between waist circumference and plasma CETP level in a large cohort study (n~1,500). Furthermore, by analyzing liver biopsies from obese patients undergoing bariatric surgery, we found that the plasma CETP was strongly correlated with the *CETP* expression in the liver but not in adipose tissue, indicating that liver is the main site of *CETP* expression and is a determinant of the total plasma CETP pool in humans. Some studies have suggested that changes in the degree of adiposity induced by body weight reduction reduced *CETP* expression and improved lipoprotein metabolism, implying that a reduction in adipose tissue reduces plasma CETP [25, 26]. However, in addition to reducing adiposity, body weight reduction significantly attenuates hepatosteatosis [27, 28]. Since we recently demonstrated that a decrease in hepatic lipid content is accompanied by a decrease in plasma CETP level [28], it is thus tempting to speculate that body weight reduction via attenuation of hepatosteatosis reduces the production of CETP by the liver.

Since the liver consists of multiple cell types including hepatocytes, endothelial cells and macrophages, also known as Kupffer cells, we set out to evaluate the cell type responsible for the expression of *CETP*. In the present study, we found that the expression of established macrophage markers strongly associated with the expression of hepatic *CETP*, and CETP was specifically co-localized with the $CD68^+$ macrophages in liver. Mechanistic studies in *E3L.CETP* mice showed that depleting the macrophages from liver by clodronate liposomes virtually abolished hepatic *CETP* expression and largely reduced plasma CETP, fully corroborating our findings in humans that hepatic macrophages, rather than hepatocytes, are the main cellular source of hepatic *CETP* expression and the plasma CETP pool. In contrast to Kupffer cells, we could hardly detect any *CETP* expression in extrahepatic macrophages, including peritoneal macrophages and macrophages in adipose tissue. It has been reported that *CETP* expression is regulated by the activation of liver X receptor α (LXRα) [29], which is highly expressed in multiple organs. Recently, Gautier *et al.* [30] demonstrated that in addition

to an LXRα responsive element in the CETP promotor, 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 *E3L.CETP* mice with the bile acid taurocholic acid greatly increased the hepatic CETP transcript as well as the plasma CETP level [30].

Previously, hepatic expression of *CETP* in mice has been attributed to both macrophages and hepatocytes, based on studies assessing hepatic *CETP* expression 8 weeks after transplantation of bone marrow from wild-type (WT) littermates into human CETP transgenic (Tg) mice and vice versa, suggesting that hepatic macrophages contribute ≈ 50% to the total hepatic *CETP* expression [31]. However, it should be realized that the replacement of liver macrophages after bone marrow transplantation occurs slowly. In the same study, it was found that only 50% of Kupffer cells were replaced by the donor cells 8 weeks after bone marrow transplantation, accompanied by a 50% reduction in plasma CETP 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 [31]. Interestingly, we found hepatic *CETP* expression decreased by approximately -90% 12 weeks after transplantation when more Kupffer cells were replaced (data not shown), confirming that hepatic macrophages are the predominant source of *CETP* expression.

So far, no plasma biomarkers exist for assessing hepatic macrophage content. Therefore, quantification of the accumulation of hepatic macrophages, for example in studies on non-alcoholic steatohepatitis (NASH), is performed by immunohistochemical analysis of liver biopsies. Our present data, showing that CETP is mainly produced by hepatic macrophages, and that hepatic *CETP* mRNA correlates with plasma CETP, imply that plasma CETP is a plasma biomarker for the hepatic macrophage content. It is therefore attractive to hypothesize that the extent of hepatosteatosis or the extent of inflammation are the main determinants of hepatic macrophage *CETP* expression.

Our findings that *CETP* expression in the hepatic macrophages determines the plasma CETP and affects lipoprotein metabolism provides new avenues for the development of new strategies of CETP inhibition. Although the precise mechanism by which CETP inhibitors (e.g. torcetrapib, dalcetrapib and anacetrapib) decrease CETP activity is not known, they all change the conformation of the plasma CETP protein and induce tight binding of CETP with HDL particles [32]. However, tight binding of CETP with HDL particles by torcetrapib for instance might compromise the function of HDL to generates large CE-rich HDL particles instead of small HDL particles and nascent discoidal HDL [33]. To avoid the potentially adverse effects of the current CETP inhibitors on HDL function, strategies focusing on inhibiting CETP synthesis at its cellular origin may be a promising

alternative. In fact, depletion of hepatic macrophages by liposomal clodronate or decreasing hepatic macrophage content by lipid-lowering drugs including fenofibrate and niacin reduce hepatic expression and plasma level of CETP, consequently generating a less atherogenic lipid phenotype, e.g. decreasing TG and increasing HDL-C.

In conclusion, we demonstrate that hepatic macrophages are the main cellular source of CETP in humans. Therefore, we propose that plasma CETP concentration measurement can be developed as a diagnostic and predictive test for hepatic macrophage content. Moreover, CETP expression in hepatic macrophages determines plasma CETP concentration and affects lipoprotein metabolism. We propose that elimination of hepatic macrophages resulting in decreasing hepatic expression and plasma level of CETP would be a promising strategy for the treatment of dyslipidemia and cardiovascular disease.

ACKNOWLEDGMENTS

We thank Froukje Verdam, Yanti Slaats, Jeroen Nijhuis, Charlotte de Jong (Dept. Surgery, Maastricht University Medical Center, Maastricht, The Netherlands) for excellent technical assistance. We also thank Tim Hendrikx (Dept. Molecular Genetics, Maastricht University, Maastricht, The Netherlands) for providing human liver slides.

This research was supported by the Netherlands Heart Foundation (NHS grant 2007B81 to PCNR), the Dutch Diabetes Foundation (DFN grant 2007.00.010 to PCNR), the Center for Translational Molecular Medicine (CTMM; www.ctmm.nl), project PREDICCt (grant 01C-104 to KWvD), the Center of Medical Systems Biology (CMSB), the Netherlands Consortium for Systems Biology (NCSB) established by The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NGI/NWO), and the Netherlands CardioVascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organisation for Health Research and Development and the Royal Netherlands Academy of Sciences for the GENIUS project "Generating the best evidence-based pharmaceutical targets for atherosclerosis" (CVON2011-19). P.C.N.R. is an Established Investigator of the Netherlands Heart Foundation (grant 2009T038).

REFERENCES

1 Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 2009;120:1640-5.

2 Le GW, Guerin M, Chapman MJ. Pharmacological modulation of cholesteryl ester transfer protein, a new therapeutic target in atherogenic dyslipidemia. Pharmacol Ther 2004;101:17-38.

3 Chapman MJ, Le GW, Guerin M, Kontush A. Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. Eur Heart J 2010;31:149-64.

4 Nagano M, Yamashita S, Hirano K, Takano M, Maruyama T, Ishihara M, Sagehashi Y, Kujiraoka T, Tanaka K, Hattori H, Sakai N, Nakajima N, Egashira T, Matsuzawa Y. Molecular mechanisms of cholesteryl ester transfer protein deficiency in Japanese. J Atheroscler Thromb 2004;11:110-21.

5 Karalis I, Rensen PC, Jukema JW. Journey through cholesteryl ester transfer protein inhibition: from bench to bedside. Circ Cardiovasc Qual Outcomes 2013;6:360-6.

6 Drayna D, Jarnagin AS, McLean J, Henzel W, Kohr W, Fielding C, Lawn R. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. Nature 1987;327:632-4.

7 Jiang XC, Moulin P, Quinet E, Goldberg IJ, Yacoub LK, Agellon LB, Compton D, Schnitzer-Polokoff R, Tall AR. Mammalian adipose tissue and muscle are major sources of lipid transfer protein mRNA. J Biol Chem 1991;266:4631-9.

8 Bruce C, Chouinard RA, Jr., Tall AR. Plasma lipid transfer proteins, high-density lipoproteins, and reverse cholesterol transport. Annu Rev Nutr 1998;18:297-330.

9 Pape ME, Rehberg EF, Marotti KR, Melchior GW. Molecular cloning, sequence, and expression of cynomolgus monkey cholesteryl ester transfer protein. Inverse correlation between hepatic cholesteryl ester transfer protein mRNA levels and plasma high density lipoprotein levels. Arterioscler Thromb 1991;11:1759-71.

10 Nagashima M, McLean JW, Lawn RM. Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. J Lipid Res 1988;29:1643-9.

11 Pape ME, Ulrich RG, Rea TJ, Marotti KR, Melchior GW. Evidence that the nonparenchymal cells of the liver are the principal source of cholesteryl ester transfer protein in primates. J Biol Chem 1991;266:12829-31.

12 Radeau T, Robb M, Lau P, Borthwick J, McPherson R. Relationship of adipose tissue cholesteryl ester transfer protein (CETP) mRNA to plasma concentrations of CETP in man. Atherosclerosis 1998;139:369-76.

13 Jiang XC, Agellon LB, Walsh A, Breslow JL, Tall A. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. J Clin Invest 1992;90:1290-5.

14 van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van VB, van der Boom H, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993;268:10540-5.

15 van der Hoogt CC, de HW, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM, Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. J Lipid Res 2007;48:1763-71.

16 van der Hoorn JW, de HW, Berbee JF, Havekes LM, Jukema JW, Rensen PC, Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. Arterioscler Thromb Vasc Biol 2008;28:2016-22.

17 Wolfs MG, Rensen SS, Bruin-Van Dijk EJ, Verdam FJ, Greve JW, Sanjabi B, Bruinenberg M, Wijmenga C, van Haeften TW, Buurman WA, Franke L, Hofker MH. Co-expressed immune and metabolic genes in visceral and subcutaneous adipose tissue from severely obese individuals are associated with plasma HDL and glucose levels: a microarray study. BMC Med Genomics 2010;3:34.

18 Westerterp M, van der Hoogt CC, de HW, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. Arterioscler Thromb Vasc Biol 2006;26:2552-9.

19 Geier A, Zollner G, Dietrich CG, Wagner M, Fickert P, Denk H, van RN, Matern S, Gartung C, Trauner M. Cytokine-independent repression of rodent Ntcp in obstructive cholestasis. Hepatology 2005;41:470-7.

20 Van HN, Lanthier N, Espanol SR, Abarca QJ, van RN, Leclercq I. Kupffer cells influence parenchymal invasion and phenotypic orientation, but not the proliferation, of liver progenitor cells in a murine model of liver injury. Am J Pathol 2011;179:1839-50.

21 Greenawalt DM, Dobrin R, Chudin E, Hatoum IJ, Suver C, Beaulaurier J, Zhang B, Castro V, Zhu J, Sieberts SK, Wang S, Molony C, Heymsfield SB, Kemp DM, Reitman ML, Lum PY, Schadt EE, Kaplan LM. A survey of the genetics of stomach, liver, and adipose gene expression from a morbidly obese cohort. Genome Res 2011;21:1008-16.

22 Birjmohun RS, Hutten BA, Kastelein JJ, Stroes ES. Efficacy and safety of high-density lipoprotein cholesterol-increasing compounds: a meta-analysis of randomized controlled trials. J Am Coll Cardiol 2005;45:185-97.

23 Bodor ET, Offermanns S. Nicotinic acid: an old drug with a promising future. Br J Pharmacol 2008;153 Suppl 1:S68-S75.

24 Tall AR. Plasma cholesteryl ester transfer protein. J Lipid Res 1993;34:1255-74.

25 Asztalos BF, Swarbrick MM, Schaefer EJ, Dallal GE, Horvath KV, Ai M, Stanhope KL, Austrheim-Smith I, Wolfe BM, Ali M, Havel PJ. Effects of weight loss, induced by gastric bypass surgery, on HDL remodeling in obese women. J Lipid Res 2010;51:2405-12.

26 Johansson LE, Danielsson AP, Parikh H, Klintenberg M, Norstrom F, Groop L, Ridderstrale M. Differential gene expression in adipose tissue from obese human subjects during weight loss and weight maintenance. Am J Clin Nutr 2012;96:196-207.

27 Lam B, Younossi ZM. Treatment options for nonalcoholic fatty liver disease. Therap Adv Gastroenterol 2010;3:121-37.

28 Wang Y, Snel M, Jonker JT, Hammer S, Lamb HJ, de RA, Meinders AE, Pijl H, Romijn JA, Smit JW, Jazet IM, Rensen PC. Prolonged caloric restriction in obese patients with type 2 diabetes mellitus decreases plasma CETP and increases apolipoprotein AI levels without improving the cholesterol efflux properties of HDL. Diabetes Care 2011;34:2576-80.

29 Luo Y, Tall AR. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. J Clin Invest 2000;105:513-20.

30 Gautier T, de HW, Grober J, Ye D, Bahr MJ, Claudel T, Nijstad N, Van Berkel TJ, Havekes LM, Manns MP, Willems SM, Hoogendoorn PC, Lagrost L, Kuipers F, Van EM, Rensen PC, Tietge UJ. Farnesoid X receptor activation increases cholesteryl ester transfer protein expression in humans and transgenic mice. J Lipid Res 2013.

31 Van EM, Ye D, Hildebrand RB, Kar KJ, de HW, Hoekstra M, Rensen PC, Ehnholm C, Jauhiainen M, Van Berkel TJ. Important role for bone marrow-derived cholesteryl ester transfer protein in lipoprotein cholesterol redistribution and atherosclerotic lesion development in LDL receptor knockout mice. Circ Res 2007;100:678-85.

32 Barter PJ, Rye KA. Cholesteryl ester transfer protein inhibition as a strategy to reduce cardiovascular risk. J Lipid Res 2012;53:1755-66.

33 Catalano G, Julia Z, Frisdal E, Vedie B, Fournier N, Le GW, Chapman MJ, Guerin M. Torcetrapib differentially modulates the biological activities of HDL2 and HDL3 particles in the reverse cholesterol transport pathway. Arterioscler Thromb Vasc Biol 2009;29:268-75.

34 Westra HJ, Jansen RC, Fehrmann RS, te Meerman GJ, van HD, Wijmenga C, Franke L. MixupMapper: correcting sample mix-ups in genome-wide datasets increases power to detect small genetic effects. Bioinformatics 2011;27:2104-11.

35 Lanthier N, Molendi-Coste O, Horsmans Y, van RN, Cani PD, Leclercq IA. Kupffer cell activation is a causal factor for hepatic insulin resistance. Am J Physiol Gastrointest Liver Physiol 2010;298:G107-G116.

36 Alberti KG, Zimmet P, Shaw J. The metabolic syndrome--a new worldwide definition. Lancet 2005;366:1059-62.

SUPPLEMENTARY APPENDIX

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, Hamburg, Germany). 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 ± standard deviation or median (interquartile range). To compare plasma CETP 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 [17]. 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 (Qiagen, Hilden, Germany, 74804) and mRNA quality and concentration were assessed with an Agilent Bioanalyzer (Agilent Technologies,Waldbronn, Germany, 5067-1521). 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 [17]. 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, Austin, TX, USA). 750 ng of complementary RNA was hybridized to Illumina HumanHT12 BeadChips (Illumina, San Diego, CA, USA) 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 [34], and qRT-PCR was performed to estimate the technical quality of the micro array [17]. 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.

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, Tokyo, Japan).

Plasma cholesterol and triglycerides were assayed using the commercially available enzymatic kits 236691, 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 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 triglycerides as described above.

RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was extracted from frozen liver and adipose tissue pieces using the Nucleospin RNAII kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad, Hercules, CA) and RNA concentration was determined by Nanodrop technology (Thermo Scientific, Wilmington, USA). 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, Bioké). Real-time PCR was performed on a CFX96 machine (Bio-Rad), the reaction mixture consisting of iQ™ SYBR® Green Super mix (Bio-Rad), cDNA, primers (Biolegio, Nijmegen, The Netherlands; see Supplementary Table 2 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 (Hprt). Data were expressed as relative expression using the dCt method (Pfaffl, 2001, Nucleic Acids Res).

Supplementary Table 2 primer sequences used for RT-qPCR

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.

Immunohistochemistry (IHC)

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, Cambridge, UK).

Paraffin-embedded sections of *E3L.CETP* mouse liver and adipose tissue (5 µm) were stained for macrophage marker F4/80 (MCA497; 1/600, Serotec, Oxford, UK) as described previously [35], and human CETP (ab51771; 1/1000, Abcam, Cambridge, UK).