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Genetic determinants of cholesterol and energy metabolism : implications for cardiometabolic health

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Metabolic liver inflammation in obesity does not robustly decrease hepatic and circulating CETP

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Abstract

Introduction

We recently showed that plasma cholesteryl ester transfer protein (CETP) is mainly derived from VSIG4-positive Kupffer cells, and that activation of these cells by the bacterial endotoxin lipopolysaccharide (LPS) strongly decreases CETP expression. As Kupffer cell activation plays a detrimental role in the progression of non-alcoholic fatty liver disease (NAFLD), we aimed to study if metabolic liver inflammation, as a component of NAFLD, is also associated with a decrease in hepatic expression and plasma concentrations of CETP in obese individuals.

Methods

We collected plasma and liver biopsy samples at various stages of NAFLD from 93 obese individuals who underwent bariatric surgery. Liver lobular inflammation was histologically determined, and liver sterols, liver triglycerides, liver *CETP* expression, CETP positive cells, circulating CETP concentrations, and liver VSIG4 expression were quantified.

Results

Body mass index ranged from 30.6 to 73.6 kg/m^2. Mean (SD) plasma CETP concentration was 2.68 (0.89) µg/mL. Compared to absence of liver pathology, in the presence of liver lobular inflammation, the age and sex-adjusted difference in hepatic *CETP* expression was -0.03 arbitrary units (95% CI -0.26, 0.20), the difference in the number of hepatic CETP positive cells (range 11 to 140 per mm^2) was -20.0 per mm^2 (95% CI -41.6, 1.9), and the difference in plasma CETP was -0.35 μg/mL (95% CI -0.80, 0.10). Hepatic *VSIG4* expression was not associated with liver inflammation (0.00; 95% CI -0.15, 0.15).

Conclusion

We found no strong evidence for a negative association between metabolic liver inflammation and CETP-related outcomes in obese individuals, although we observed consistent trends. Given that LPS strongly decreases *CETP* expression by Kupffer cells, our results suggest that metabolic and infection-induced liver inflammation have different effects on the expression and production of CETP by Kupffer cells. Further research into the role of CETP in liver inflammation and host defence is therefore warranted.

Introduction

Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl esters from high-density lipoproteins (HDL) towards (very) low-density lipoproteins ((V)LDL), coupled to a net flux of triglycerides from (V)LDL to HDL, thereby contributing to an atherogenic lipoprotein profile.[1] Recently, we showed in human studies that circulating levels of CETP are mainly determined by resident hepatic macrophages (Kupffer cells),[2] without a contribution of adipose tissue.[3] In fact, hepatic Kupffer cell content strongly correlated with both hepatic CETP expression and plasma CETP concentration.[2]

Besides its established role in lipid metabolism, accumulating evidence suggests that CETP is involved in immunity and inflammatory processes.[4] This is in line with the primary expression of *CETP* by Kupffer cells, which play a pivotal role in inflammation and host defence against e.g. Gram-negative bacterial infections. Kupffer cells can detect lipopolysaccharide (LPS), a potent endotoxin released from Gram-negative bacteria, and induce an antibacterial response via the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β).[5] Interestingly, LPS reduces liver CETP expression and circulating CETP concentrations in CETP-transgenic mice.[6] Similarly, LPS, TNF-α and IL-1β decrease *CETP* expression and CETP concentration in hamsters.[7] LPS administration also decreased plasma CETP concentrations in humans.[8] Collectively, inflammatory stimuli seem to downregulate CETP expression by Kupffer cells.

Recently, we showed in *CETP*-expressing mice that hepatic expression of CETP is confined to a specific subset of Kupffer cells that also express *Vsig4*, which is a marker of resting Kupffer cells.[8] Also in humans, hepatic *VSIG4* expression correlated with both liver *CETP* expression and plasma CETP concentration.[Li et al., resubmitted] In this same study, we showed that LPS inoculation of mice markedly reduced liver CETP expression and production, accompanied by a similar loss of the Kupffer cell marker *Vsig4*. Combined, these data indicate that liver CETP expression is exclusively confined to a resting Kupffer cell subset, which loses *CETP* expression when activated by LPS.

Although the mechanisms by which inflammatory stimuli reduce *CETP* expression are not yet fully understood, they may counteract liver X receptor (LXR)-induced *CETP* expression. LXRs regulate a variety of genes to control cholesterol and lipid homeostasis and protect cells from an overload of toxic sterol.[9] *CETP* gene transcription is induced via an LXRα response element in the *CETP* promotor region.[10,11] Natural ligands for LXRα are oxidized derivatives of cholesterol (i.e. oxysterols)[12,13] and the cholesterol precursor desmosterol.[14] Interestingly, *in vitro* exposure of murine macrophages to LPS, TNF-α or interferon γ (IFN-γ) supresses an LXRα-induced increase in *CETP* expression.[15] In ad-

dition, we recently showed that LPS also reduces CETP expression as induced by an LXR agonist in human macrophages.[8] Taken together, LXRα-induced expression of *CETP* by Kupffer cells may be counteracted by inflammatory stimuli.

Apart from their beneficial role in host defence,[5] Kupffer cells play a detrimental role in the progression of non-alcoholic fatty liver disease (NAFLD) from simple steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by liver inflammation.[16–21] With the growing prevalence of obesity worldwide, an increasing number of individuals suffer from NAFLD.[22] The central role of Kupffer cells in NAFLD is partly explained by excess free cholesterol that cannot be detoxified by esterification, leading to cholesterol crystallization not only within hepatocytes but also within Kupffer cells, which consequently activates inflammatory pathways.[17,23,24] The aim of the present study is to determine whether metabolic liver inflammation, as a component of NAFLD, is associated with a decrease in liver *CETP* expression and CETP production similar to LPS. To this end, we collected liver biopsy samples at various stages of NAFLD from a bariatric surgery cohort to histologically determine liver inflammation, and to quantify liver *CETP* and *VSIG4* expression, liver lipids, liver CETP positive cells and circulating CETP concentrations.

Materials and Methods

Study design and study population

The study population consisted of 93 severely obese men and women who underwent elective bariatric surgery between 2006 and 2009 at the Department of General Surgery, Maastricht University Medical Center (Maastricht, The Netherlands).[25] Subjects using anti-inflammatory drugs or with acute or chronic inflammatory diseases, degenerative diseases, and subjects reporting alcoholic intake >10 g/day were not included in this study. There were no specific dietary protocols that the participants had to follow before surgery. Venous blood samples were drawn on the morning of surgery after 8 hours of overnight fasting. During surgery, wedge biopsies of the liver were taken. The study was approved by the medical ethics board of Maastricht University Medical Centre, in line with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each participant.

Data collection

Because of the limited samples available, random samples from this study population were selected for data collection. Liver *CETP* and *VSIG4* mRNA expression were determined from microarrays of 82 samples, as described previously.[2,25] Biopsy specimens were formalin-fixed and paraffin-embedded, and subsequently immunohistochemistry was per-

formed. The number of CETP positive cells per mm^2 was obtained (n=44).[2] Plasma CETP concentrations were measured for 73 participants from whom plasma was available with ELISA kits (DAIICHI CETP ELISA, Alpco, Salem, USA), according to the manufacturer's instructions.

Histology

For histological scoring of the liver biopsies, Hematoxylin-eosin and Klatskin (Masson) trichrome stains were used to assess histopathology. Samples were scored for steatosis (n=93), lobular inflammation (n=89), and hepatocellular ballooning (n=89), by an experienced liver pathologist who was blinded for clinical and biochemical parameters, according to the criteria of the NAFLD activity score described by Kleiner et al.,[26] Steatosis was grouped into four categories based on the amount of surface area involved in steatosis (i.e. score 0: <5%, score 1: 5-33%, score 2: >33-66%, score 3: >66%). Lobular inflammation was grouped into four categories based on the number of inflammatory foci per 200X field (i.e. score 0: no foci, score 1: <2 foci, score 2: 2-4 foci, score 3: >4 foci). Ballooning was grouped into three categories based on the number of ballooning cells (i.e. score 0: none, score 1: few, score 2: many). To compose the NAFLD activity score, scores for steatosis, lobular inflammation, and hepatocellular ballooning were summed.[26]

Nuclear magnetic resonance (NMR) spectroscopy

Liver samples (approx. 1 mg) were stored at -80˚C in plastic Eppendorf tubes. After thawing at room temperature, 100 μL of liquid chromatography-mass spectrometry (LC-MS) grade water and 5 stainless steel beads were added and samples were placed in a bullet blender for 3 min. Ten μL of homogenised liver sample was taken and transferred to a fresh 1.5 mL Eppendorf tube for analysis of protein concentration (BCA, ThermoFisher Scientific, Waltham, USA), according to the manufacturer's instructions. Lipids were subsequently extracted from the remaining 90 μL homogenized liver sample, applying protein precipitation as follows. In short, 200 μL isopropanol (Sigma-Aldrich, LC-MS Chromasolv) was added to the sample, vortexed for 5 seconds and centrifuged for 5 min at 16,100×g. Two hundred microliters of the supernatant was transferred into a 2 mL glass vial (Agilent Technologies). The remaining pellet was again homogenised by placing in a bullet blender for 3 min with the addition of 150 μL isopropanol. After centrifugation (5 min at 16,100 ×g), 175 μL of the supernatant was collected and combined with organic extracts. The combined extract was dried under a gentle stream of nitrogen. After drying, the extract was reconstituted in 190 μL deuterated chloroform and transferred to a 3 mm NMR tube.

NMR was used to measure total cholesterol, free cholesterol, cholesteryl ester and triglyceride contents in 68 liver samples. Liver lipids were expressed as nmol per mg protein.

Proton NMR spectra were recorded on a Bruker Avance II 600 NMR spectrometer equipped with a 5 mm TCI cryogenic probe head and a z-gradient system. A Bruker SampleJet sample changer was used for sample insertion and removal. The NMR spectra were recorded using a simple one-pulse experiment. 256 scans of 65,536 points covering a sweep width of 12,336 Hz were acquired. After applying an exponential window function of 0.3 Hz, the time-domain signal was Fourier transformed yielding a spectrum of 32,768 complex data points. The spectrum was imported into a KNIME workflow[27] using a Python 2.7 script that utilizes the Nmrglue module.[28] Subsequently, automatic zeroth-order phase correction and fourth-degree polynomial baseline correction were applied. Relevant sections of the spectra were extracted and individually baseline corrected in order to remove background signals. Well-separated peaks, multiplets, or parts of multiplets were integrated, and divided by the associated number of protons. Peak areas were converted into concentrations using the peak area of the reference material (0.05% Tetramethylsilane). The integration intervals are given in Supplementary table 1.

Biochemical analyses

Plasma concentrations of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, alanine transaminase (ALT), and aspartate transaminase (AST) were measured, as described before.[25]

Statistical analyses

We assessed the associations of the following determinants with CETP-related outcomes: steatosis score, lobular inflammation score, hepatocellular ballooning score, presence of NASH, plasma ALT concentration, plasma AST concentration, liver total cholesterol content, liver free cholesterol content, liver cholesteryl ester content and liver triglyceride content. For steatosis, lobular inflammation, hepatocellular ballooning, and NASH separately, the presence of disease (score ≥1) was compared with absence of pathology (score 0), as defined by the criteria of the NAFLD activity score.[26] As CETP-related outcome variables we used liver *CETP* expression, CETP protein positive cells, and plasma CETP concentration. Linear regression analyses were used to determine all associations. Crude models (Model 1) were adjusted for age and sex (Model 2). Differences were expressed as beta coefficients with corresponding 95% CIs. Liver sterol and triglyceride contents and liver transaminases were non-normally distributed, and therefore we used their natural logarithms in the analyses. For interpretation purposes, beta coefficients were multiplied by ln(1.1), and differences with corresponding 95% CIs were expressed per 10% relative increase in the determinant. In addition, we assessed the correlations between NMR-determined liver sterol and triglyceride data and reported Pearson's correlation coefficient.

All analyses were performed using STATA Statistical Software (Statacorp, College Station, Texas, USA), version 12.0.

Results

Population characteristics

Table 3.1 shows the characteristics of the elective bariatric surgery cohort. Body mass index (BMI) ranged from 30.6 to 73.6 kg/m^2. Mean age was 44 years, and most participants were women. Mean (SD) CETP concentration was 2.68 (0.89) μg/mL in the total population, 2.56 (0.92) μg/mL for men and 2.72 (0.88) μg/mL for women. 15% of men and 18% of women had a NAFLD activity score ≥5, which is indicative of NASH.[26] Plasma ALT concentration was comparable between men and women, and plasma AST concentration was somewhat lower in women. Liver sterol and triglyceride contents were all higher in women than in men.

Lobular inflammation and hepatocellular ballooning tend to associate with less CETP positive cells in the liver and a lower plasma CETP concentration

Associations of the separate NAFLD activity score components, i.e. steatosis, lobular inflammation and ballooning score, with the CETP-related outcomes are presented in Figure 3.1 and Supplementary table 3.A.2. The age and sex-adjusted difference in *CETP* mRNA expression in the liver for an inflammation score ≥1 compared with a score of 0 was -0.03 arbitrary units (95% CI -0.26, 0.20). Comparable small, null associations were observed for steatosis and ballooning scores. The number of CETP positive cells per mm^2 liver ranged from 11 to 140. The presence of liver lobular inflammation was associated with a lower number of CETP positive cells of -20.0 per mm^2 (95% CI -41.6, 1.9). Steatosis and ballooning scores were also negatively associated with CETP positive cell number, although confidence intervals overlapped with zero. Plasma CETP concentration was within the range of 1.45 to 5.56 μg/mL. The presence of lobular inflammation was associated with a lower plasma CETP of -0.35 μg/mL (95% CI -0.80, 0.10), although confidence intervals overlapped with zero. Hepatocellular ballooning was associated with a -0.42 μg/mL (95% CI -0.84, -0.00) μg/mL lower plasma CETP concentration, which represents approx. 15% of the mean CETP concentration. Steatosis score also showed a negative association with CETP concentration, although the confidence intervals overlapped with zero. Steatosis, inflammation and ballooning scores were not negatively associated with *VSIG4* expression (Figure 3.1, Supplementary table 3.A.2).

Table 3.1: Characteristics of the elective bariatric surgery cohort aged 17-67 years (n=93), stratified by sex.

Characteristic	Men	Women
Number of participants (% of total)	26 (28%)	67 (72%)
Age (year)	46 (11)	43 (9)
BMI (kg/m2)	50 (10)	45 (9)
Fasting plasma concentrations		
CETP (μg/mL)	2.56 (0.92)	2.72 (0.88)
Total cholesterol (mmol/L)	4.66 (0.89)	5.26 (1.15)
HDL-cholesterol (mmol/L)	0.88 (0.27)	1.02 (0.40)
LDL-cholesterol (mmol/L)	2.90 (0.86)	3.34 (1.03)
Triglycerides (mmol/L)	1.95 (0.94)	2.33 (2.27)
ALT (IU/L)	26.6 (11.8)	26.5 (17.6)
AST (IU/L)	27.1 (10.4)	23.5 (13.1)
Liver biopsies		
Total cholesterol content (nmol/mg protein)[b]	12 (9)	37 (68)
Free cholesterol content (nmol/mg protein)[b]	8 (6)	25 (44)
Cholesteryl ester content (nmol/mg protein)[b]	4 (3)	11 (26)
Triglyceride content (nmol/mg protein)[b]	90.4 (71.1)	268 (618)
Number of patients with NAFLD activity score ≥5 (% of total men or women)[c]	4 (15%)	12 (18%)

Results are presented as mean (SD) or percentage.

[a] Missing data: n=1 for BMI, n=20 for plasma CETP concentration, n=6 for total cholesterol concentration, n=7 for HDL-cholesterol, LDL-cholesterol and triglyceride concentration, n=6 for ALT and AST concentration, n=4 for NAFLD activity score, n=25 for liver content of total cholesterol, free cholesterol, cholesteryl esters and triglycerides.

[b] Measured with NMR.

[c] A NAFLD activity score≥5 is indicative of NASH.[26]

ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; IU, international unit; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NMR, nuclear magnetic resonance.

CETP-related outcomes are not different in patients with a NAFLD activity score ≥5

A NAFLD activity score ≥5 was associated with a higher ALT concentration of 18.2 IU/L (95% CI 10.1, 26.3) and a higher AST concentration of 13.5 IU/L (95% CI 7.2, 19.8). However, no difference in CETP-related outcomes was observed between participants with a NAFLD activity score ≥5 compared with participants with a NAFLD activity score <5 (Figure 3.2, Supplementary table 3.A.3). Plasma ALT and AST concentrations were negatively associated with liver *CETP* expression, although associations were weak (Figure 3.3, Supplementary table 3.A.4). Age- and sex-adjusted differences in *CETP* expression were -0.020 arbitrary units (95% CI -0.040, 0.000) and -0.024 arbitrary units (95% CI -0.047, -0.001) per 10% relative increase in plasma ALT and AST concentration, respectively. Associations of both liver transaminases with CETP positive cells and CETP concentration were also negative, although effect sizes were small and confidence intervals overlapped with zero (Figure 3.3, Supplementary table 3.A.4).

Associations of liver sterol and triglyceride contents with CETP-related outcomes were around the null

NMR-determined liver sterol and triglyceride data clearly show that hepatic contents of total cholesterol and free cholesterol were highly correlated (r^2=0.97, P<0.0001). In addition, the correlations between the hepatic content of total cholesterol and cholesteryl esters (r^2=0.77, P<0.001), and between total cholesterol and triglycerides (r^2=0.71, P<0.001) were high. Strikingly, all of the associations of liver triglyceride and sterol contents with CETP-related outcomes were around the null (Figure 3.4, Supplementary table 3.A.5).

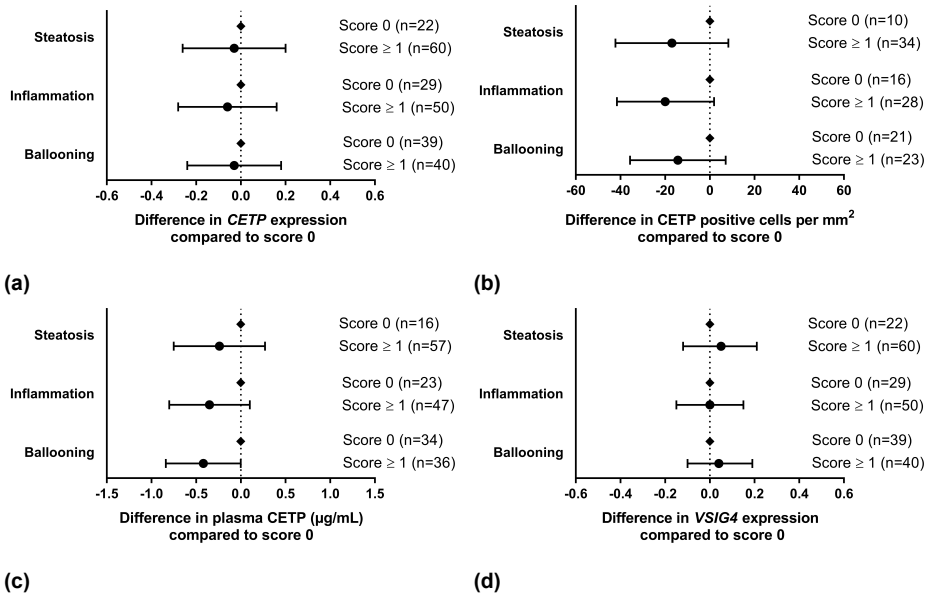

Figure 3.1: CETP-related outcomes, but not liver *VSIG4* expression, show a consistent trend for negative association with metabolic liver steatosis, inflammation and ballooning. Age and sex-adjusted differences (i.e. beta coefficients from linear regression) in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells, (**c**) plasma CETP concentration, and (**d**) liver VSIG4 mRNA expression between absence (score 0, reference category) and presence (score ≥1) of liver steatosis, lobular inflammation and ballooning. Error bars represent 95% confidence intervals.

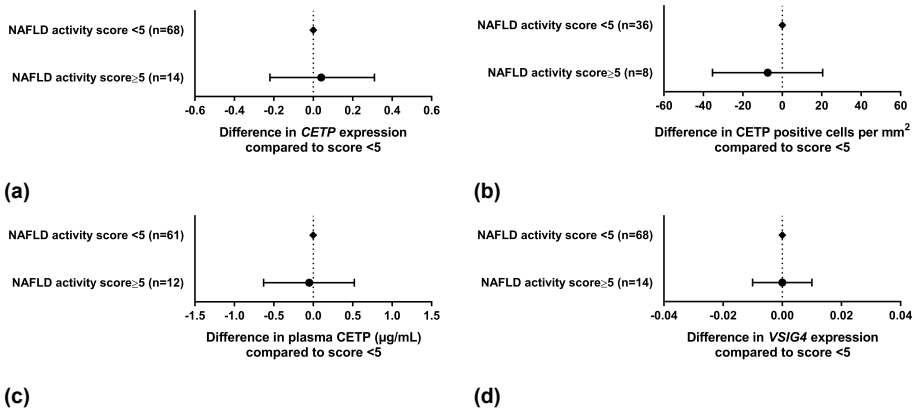

Figure 3.2: CETP-related outcomes and *VSIG4* expression are not different between participants with a non-alcoholic fatty liver disease (NAFLD) activity score <5 (reference category) and participants with a NAFLD activity score ≥5, which is indicative of non-alcoholic steatohepatitis (NASH). Age and sex-adjusted differences (i.e. beta coefficients from linear regression) in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells, (**c**) plasma CETP concentration, and (**d**) liver VSIG4 mRNA expression between participants with a NAFLD activity score <5 (reference category) and participants with a NAFLD activity score ≥5. Error bars represent 95% confidence interval.

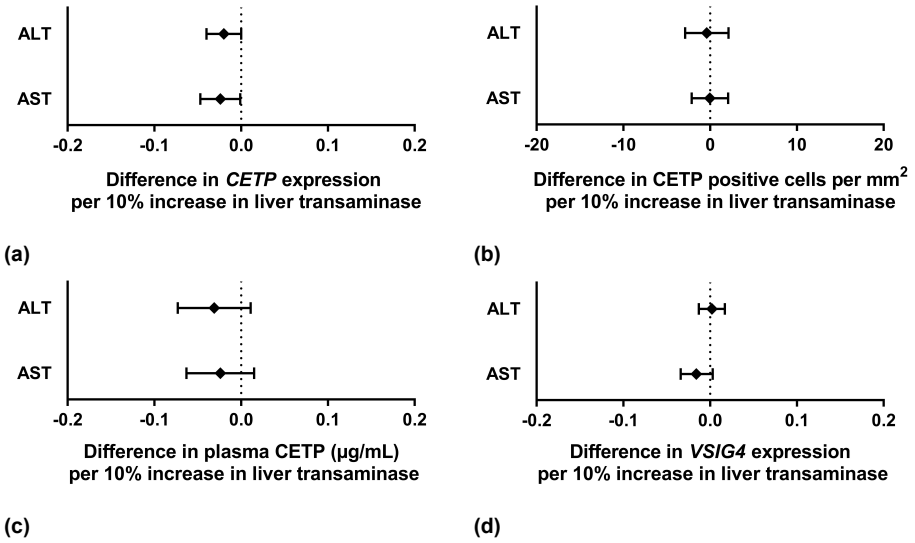

Figure 3.3: Both plasma alanine transaminase (ALT) and aspartate transaminase (AST) concentrations are negatively associated with CETP mRNA expression and plasma CETP concentration, although confidence intervals overlap with zero and effect sizes are small. Age and sex-adjusted differences[a] in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells, (**c**) plasma CETP concentration, and (**d**) liver VSIG4 mRNA expression, per 10% relative increase in the plasma concentrations of ALT and AST. Error bars represent 95% confidence interval.

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver lipid content.

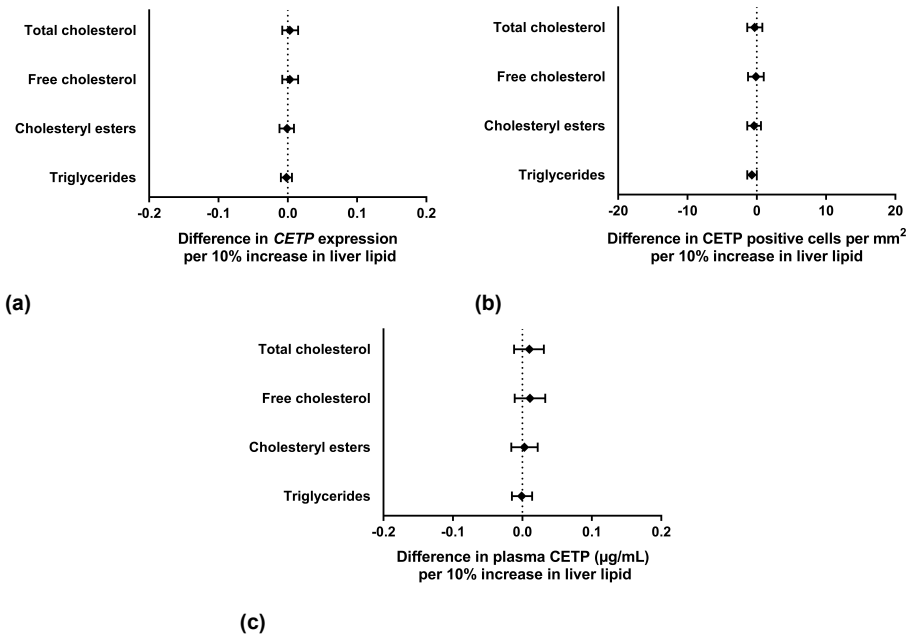

Figure 3.4: Both plasma alanine transaminase (ALT) and aspartate transaminase (AST) concentrations are negatively associated with CETP mRNA expression and plasma CETP concentration, although confidence intervals overlap with zero and effect sizes are small. Age and sex-adjusted differences[a] in (**a**) liver CETP mRNA expression, (**b**) liver CETP positive cells and (**c**) plasma CETP concentration, per 10% relative increase in the plasma concentrations of ALT and AST. Error bars represent 95% confidence interval.

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver sterol and triglyceride content.

Discussion

VSIG4-positive Kupffer cells have recently been identified as the main determinants of circulating CETP levels,[2,8] but the influence of the hepatic environment on CETP expression and CETP production is far from elucidated. Based on previous studies, showing that inflammatory stimuli downregulate CETP expression in mice and *in vitro*,[6,7,15,29,30] we hypothesized that hepatic *CETP* expression and circulating CETP concentrations are decreased in humans with metabolic liver inflammation. Therefore, we aimed to study the associations of histologically-determined liver inflammation, as a component of NAFLD, with liver *CETP* expression, CETP positive cells and circulating CETP concentrations, using liver biopsy data from an elective bariatric surgery cohort. We found no strong evidence for a negative association between liver lobular inflammation and CETP-related outcomes, as 95% confidence intervals overlapped with zero. However, we did observe consistent trends towards less CETP expression, a lower number of CETP positive cells, and a lower

circulating CETP concentration when liver inflammation was present. In line, we also observed trends for a negative association of ALT and AST concentrations, both markers for liver damage, with CETP-related outcomes.

It is interesting to speculate why only limited associations were observed between metabolic inflammation and CETP-related outcomes. Previous animal and *in vitro* studies showed a decrease in CETP expression specifically in response to infection-related inflammatory stimuli, such as LPS, IL-1β and IFN-γ.[6,7,15] In addition, we recently showed that LPS also reduces plasma CETP concentration in humans.[8] In the present study, all participants were morbidly obese, and individuals that used anti-inflammatory drugs or had acute or chronic inflammatory diseases were not able to participate. Thus, liver inflammation in the present study was assumed to be metabolically-induced. Interestingly, metabolic liver inflammation, which is a component of NAFLD, has been shown to differ from infection-induced liver inflammation.[31] In mice, LPS and IL-1β cause intrahepatic inflammation that is exclusively mediated by macrophages, while metabolic triggers (i.e. carbohydrate and cholesterol) of liver inflammation induce a NASH phenotype with mixed intrahepatic infiltrates including both macrophages and neutrophils.[31] Since CETP is primarily produced by hepatic macrophages, infection-related inflammatory stimuli may affect hepatic expression and production of CETP to a different extent than metabolic triggers. Notably, severe sepsis was previously shown in humans to largely decrease CETP concentration, i.e. by -25% after 3 days of sepsis,[32] while we only observed a trend towards a negative association between metabolic liver inflammation and CETP-related outcomes. This is in line with a previous study in CETP-transgenic mice, in which we observed that long term high-fat diet-induced obesity did not affect plasma CETP concentration.[33] The difference between metabolic versus infection-induced liver inflammation is further highlighted by our observation that *VSIG4* expression, which is a marker of resting Kupffer cells, was not lower in the presence of metabolic liver inflammation, while we previously showed that LPS injection largely reduced both *Vsig4* and *CETP* expression, at least in mice.[8] Taken together, we propose that acute and/or whole-body inflammatory responses to invading pathogens are required to induce a robust reduction in CETP production by Kupffer cells.

In the context of infection-induced *versus* metabolic liver inflammation, it is important to consider the biological relevance underlying the relationship between CETP and inflammation. CETP mediates the bidirectional exchange of triglycerides and cholesteryl esters between (V)LDL and HDL, thereby raising LDL-cholesterol and decreasing HDL-cholesterol, which is generally regarded as a biologically unwanted proatherogenic property of CETP. Since HDL is known for its inflammation-modulatory properties and beneficial role in host-defence,[34–37] we reason that the biological function of CETP in humans is modulation of

the immune response via HDL. In fact, the reduction in *CETP* expression by Kupffer cells in CETP-transgenic mice as induced by LPS is accompanied by an increase in HDL.[8] We thus postulate that inflammatory stimuli activate resting Kupffer cells to decrease CETP expression and thereby raise HDL to combat invading microorganisms. In line with this hypothesis, it seems plausible that metabolic liver inflammation will not largely reduce the expression and production of CETP.

Comparable with liver inflammation, liver steatosis score also tended to be negatively associated with the number of CETP positive cells. This negative effect direction can possibly be explained by a decrease in macrophage cell numbers per area rather than by an absolute decrease in Kupffer cell number, as the presence of steatosis results in occupation of a larger area of the microscopic field by lipid-filled hepatocytes. This is a plausible explanation, as associations between NMR-determined triglycerides content and CETP-related outcomes were all around zero. NMR triglyceride measurements were expressed per mg protein, and can therefore be interpreted as mean triglyceride content per hepatocyte. Therefore, in the light of the NMR findings, the negative association between histologically-determined steatosis and CETP-related outcomes is most likely explained by the measurement technique.

This study is one of the first studies to include NMR spectroscopy for the quantitative analysis of several lipid and sterol classes in human liver biopsies. By using NMR spectroscopy, we revealed a strong correlation between hepatic contents of total cholesterol and free cholesterol (r^2=0.97) as well as those of total cholesterol and triglycerides (r^2=0.71). The finding that none of the measured liver sterol components (i.e. total cholesterol, free cholesterol, nor cholesteryl esters) was associated with *CETP* expression in the present study, may seem counterintuitive given that *CETP* expression is under the control of LXRα.[10,11] Cholesterol derivatives activate LXRα,[12,13] which subsequently binds to an LXR-response element in the *CETP* promotor region to increase gene transcription.[10,11] It should be realized that we measured cholesterol rather than the actual LXR agonists, e.g. oxysterols and desmosterol. For future studies, mass spectrometric platforms might complement NMR analysis to measure oxysterols and desmosterol, as the commonly observed low concentrations are likely out of the measurement range of NMR spectroscopy.

Notably, compared with participants with a NAFLD activity score <5, CETP-related outcomes were not different in participants with a NAFLD activity score ≥5, which is indicative of NASH.[26] This score is composed of three summed, separate components (i.e. steatosis, lobular inflammation and ballooning scores).[26] We argue that this score may not be sufficiently specific to detect an association of specific aspects of NAFLD with CETP-related outcomes, which is the main reason that we studied the associations between the differ-

ent components of the NAFLD activity score and CETP-related outcomes separately. The same reasoning may also hold for plasma ALT and AST concentrations, which are relatively non-specific markers for liver function.[38] Associations of both liver transaminases with CETP-related outcomes were around the null.

The main strength of the present study is the availability of liver biopsies from a population of obese men and women, with a high prevalence of metabolic liver inflammation. Liver samples were extensively characterized with regard to CETP, sterol and triglyceride content, steatosis, inflammation and cell damage. Also, several limitations of the study design should be considered. Firstly, inherent to the observational cross-sectional design we cannot exclude residual confounding, or draw conclusions on causality. Secondly, this study possibly lacked power to identify statistically significant differences in CETP-related outcomes due to the relatively small study population size. Lastly, results may not be generalizable to other populations, as participants were selected from an obese cohort that underwent bariatric surgery.

In conclusion, we found no strong evidence for a negative association between metabolic liver inflammation and CETP-related outcomes, although we observed consistent trends. Given that infection-related inflammatory stimuli do significantly decrease *CETP* expression by Kupffer cells, our results suggest that metabolic liver inflammation affects the expression and production of CETP by Kupffer to a more modest extent than infection-induced liver inflammation. It is tempting to speculate that acute and/or whole-body inflammatory responses to invading pathogens are required to induce a robust reduction in CETP production by Kupffer cells. Further research into the effects of liver inflammation and host defence on hepatic CETP production is therefore eagerly warranted.

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Appendix

3.A. Supplementary tables

Table 3.A.1: Sections of the NMR spectra used to determine liver total cholesterol, free cholesterol, cholesteryl ester, and triglyceride content. In the case of free cholesterol and cholesteryl esters, due to partial overlap of the multiplet, two intervals were integrated separately, and the second interval was counted twice to account for the overlap on the other side of the multiplet.

Liver lipid	Integration interval		Integration interval 2		# of protons
	From/ppm	To/ppm	From/ppm	To/ppm	
Total cholesterol	0.659	0.696	-	-	3
Free cholesterol	3.502	3.548	3.548	3.581	1
Cholesteryl esters	4.589	4.635	4.575	4.589	1
Triglycerides	4.12	4.17	-	-	2

NMR, nuclear magnetic resonance.

Table 3.A.2: Crude (model 1) and age and sex-adjusted (model 2) differences[a] in liver *CETP* mRNA expression, liver CETP positive cells, plasma CETP concentration and liver *VSIG4* mRNA expression between absence (score 0, reference category) and presence (score ≥1) of liver steatosis, lobular inflammation and ballooning.

	Model	N	Difference in[a]	95% Confidence interval
Steatosis score				
			Liver *CETP* expression	
0	1	22	0 (reference)	-
	2	22	0 (reference)	-
≥1	1	60	-0.07	-0.30, 0.17
	2	60	-0.03	-0.26, 0.20
			CETP positive cells per mm^2	
0	1	10	0 (reference)	-
	2	10	0 (reference)	-
≥1	1	34	-15.8	-41.5, 9.8
	2	34	-17	-42.2, 8.2
			Plasma CETP concentration (μg/mL)	
0	1	16	0 (reference)	-
	2	16	0 (reference)	-
≥1	1	57	-0.23	-0.74, 0.27
	2	57	-0.24	-0.75, 0.27
			Liver *VSIG4* expression	
0	1	22	0 (reference)	-
	2	22	0 (reference)	-
≥1	1	60	0.04	-0.12, 0.20
	2	60	0.05	-0.12, 0.21
Inflammation score				
			Liver *CETP* expression	
0	1	29	0 (reference)	-
	2	29	0 (reference)	-
≥1	1	50	-0.11	-0.33, 0.11
	2	50	-0.06	-0.28, 0.16
			CETP positive cells per mm^2	
0	1	16	0 (reference)	-
	2	16	0 (reference)	-
≥1	1	28	-22.2	-43.8, -0.5
	2	28	-20	-41.6, 1.9
			Plasma CETP concentration (μg/mL)	
0	1	23	0 (reference)	-
	2	23	0 (reference)	-
≥1	1	47	-0.36	-0.80, 0.08
	2	47	-0.35	-0.80, 0.10
			Liver *VSIG4* expression	

0	1	29	0 (reference)	-
	2	29	0 (reference)	-
≥1	1	50	-0.02	-0.16, 0.13
	2	50	0	-0.15, 0.15
Ballooning score				
			Liver *CETP* expression	
0	1	39	0 (reference)	-
	2	39	0 (reference)	-
≥1	1	40	-0.07	-0.28, 0.14
	2	40	-0.03	-0.24, 0.18
			CETP positive cells per mm^2	
0	1	21	0 (reference)	-
	2	21	0 (reference)	-
≥1	1	23	-17	-38.3, 4.2
	2	23	-14.3	-35.7, 7.1
			Plasma CETP concentration (μg/mL)	
0	1	34	0 (reference)	-
	2	34	0 (reference)	-
≥1	1	36	-0.43	-0.84, -0.02
	2	36	-0.42	-0.84, -0.00
			Liver *VSIG4* expression	
0	1	39	0 (reference)	-
	2	39	0 (reference)	-
≥1	1	40	0.03	-0.11, 0.17
	2	40	0.04	-0.10, 0.19

[a] Beta coefficients from linear regression.

Table 3.A.3: Crude (model 1) and age and sex-adjusted (model 2) differences in liver *CETP* mRNA expression, liver CETP positive cells, plasma CETP concentration and liver *VSIG4* mRNA expression between participants with a non-alcoholic fatty liver disease (NAFLD) activity score <5 (reference category) and participants with a NAFLD activity score ≥5.

NAFLD activity score	Model	N	Difference in[a]	95% Confidence interval
			Liver *CETP* expression	
<5	1	68	0 (reference)	-
	2	68	0 (reference)	-
≥5	1	14	0.03	-0.25, 0.31
	2	14	0.04	-0.22, 0.31
			CETP positive cells per mm2	
<5	1	36	0 (reference)	-
	2	36	0 (reference)	-
≥5	1	8	-9.1	-37.3, 19.1
	2	8	-7.4	-35.4, 20.5
			Plasma CETP concentration (ćg/mL)	
<5	1	61	0 (reference)	-
	2	61	0 (reference)	-
≥5	1	12	-0.06	-0.63, 0.50
	2	12	-0.05	-0.63, 0.52
			Liver *VSIG4* expression	
<5	1	68	0 (reference)	-
	2	68	0 (reference)	-
≥5	1	14	0.17	-0.01, 0.36
	2	14	0	-0.01, 0.01

[a] Beta coefficients from linear regression.

Table 3.A.4: Crude (model 1) and age and sex-adjusted (model 2) differences in liver *CETP* mRNA expression, liver CETP positive cells, plasma CETP concentration and liver *VSIG4* mRNA expression, per 10% relative increase in the plasma concentrations of alanine transaminase (ALT) and aspartate transaminase (AST).

Liver transaminase	Model	N	Difference in[a]	95% Confidence interval
			Liver *CETP* expression	
ALT	1	79	-0.021	-0.041, -0.001
	2	79	-0.02	-0.040, 0.000
AST	1	79	-0.013	-0.033, 0.008
	2	79	-0.024	-0.047, -0.001
			CETP positive cells per mm^2	
ALT	1	42	-0.35	-2.89, 2.19
	2	42	-0.38	-2.88, 2.12
AST	1	42	0.28	-1.77, 2.33
	2	42	-0.03	-2.12, 2.07
			Plasma CETP concentration (μg/mL)	
ALT	1	69	-0.031	-0.073, 0.010
	2	69	-0.031	-0.073, 0.011
AST	1	69	-0.026	-0.063, 0.012
	2	69	-0.024	-0.063, 0.015
			Liver *VSIG4* expression	
ALT	1	79	0.001	-0.013, 0.015
	2	79	0.002	-0.013, 0.017
AST	1	79	0.002	-0.012, 0.015
	2	79	-0.016	-0.034, 0.003

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver sterol and triglyceride content.

Table 3.A.5: Crude (model 1) and age and sex-adjusted (model 2) differences in liver *CETP* mRNA expression, liver CETP positive cells and plasma CETP concentration, per 10% relative increase in liver total cholesterol, free cholesterol, cholesteryl ester, and triglyceride content.

Liver lipid	Model	N	Difference in[a]	95% Confidence interval
			Liver *CETP* expression	
Total cholesterol	1	63	0.003	-0.008, 0.015
	2	63	0.003	-0.008, 0.015
Free cholesterol	1	63	0.003	-0.008, 0.015
	2	63	0.003	-0.008, 0.015
Cholesteryl esters	1	63	-0.002	-0.012, 0.009
	2	63	-0.001	-0.012, 0.009
Triglycerides	1	63	-0.003	-0.010, 0.005
	2	63	-0.002	-0.010, 0.006
			CETP positive cells per mm^2	
Total cholesterol	1	41	-0.1	-1.16, 0.96
	2	41	-0.29	-1.37, 0.80
Free cholesterol	1	41	0.07	-1.04, 1.18
	2	41	-0.12	-1.26, 1.02
Cholesteryl esters	1	41	-0.25	-1.24, 0.74
	2	41	-0.39	-1.37, 0.60
Triglycerides	1	41	-0.62	-1.32, 0.08
	2	41	-0.69	-1.37, 0.00
			Plasma CETP concentration (μg/mL)	
Total cholesterol	1	64	0.012	-0.009, 0.032
	2	64	0.01	-0.012, 0.031
Free cholesterol	1	64	0.014	-0.007, 0.035
	2	64	0.011	-0.011, 0.033
Cholesteryl esters	1	64	0.005	-0.014, 0.023
	2	64	0.003	-0.016, 0.022
Triglycerides	1	64	0	-0.014, 0.014
	2	64	-0.001	-0.015, 0.014

[a] (Beta coefficients from linear regression)*ln(1.1), i.e. difference per 10% relative increase in liver lipid content.