The role of ApoCI, LPL and CETP in plasma lipoprotein metabolism - studies in mice

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6 Cholesteryl Ester Transfer Protein Decreases HDL and Severely Aggravates Atherosclerosis in *APOE*3-Leiden* Mice

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Objective - The role of cholesteryl ester transfer protein (CETP) in the development of atherosclerosis is still under debate. Therefore, we evaluated the effect of human CETP expression on atherosclerosis in *APOE*3-Leiden* (*E3L*) mice with a human-like lipoprotein profile.

Methods and Results - *E3L* mice were crossbred with human *CETP* transgenic mice. On a chow diet, CETP expression increased plasma total cholesterol (TC) (+43%; $P<0.05$). To evaluate the effects of CETP on the development of atherosclerosis, mice were fed a Western-type diet containing 0.25% cholesterol, leading to 4.3-fold elevated TC levels in both *E3L* and *CETP.E3L* mice ($P<0.01$). On both diets, CETP expression shifted the distribution of cholesterol from HDL towards VLDL/LDL. Moreover, plasma of *CETP.E3L* mice had reduced capacity (-39%; $P<0.05$) to induce SR-BI-mediated cholesterol efflux from Fu5AH cells than plasma of *E3L* mice. After 19 weeks on the Western-type diet, *CETP.E3L* mice showed a 7.0-fold increased atherosclerotic lesion area in the aortic root compared to *E3L* mice ($P<0.0001$).

Conclusion - CETP expression in *E3L* mice shifts the distribution of cholesterol from HDL to VLDL/LDL, reduces plasma-mediated SR-BI-dependent cholesterol efflux, and represents a clear pro-atherogenic factor in *E3L* mice. We anticipate that the *CETP.E3L* mouse will be a valuable model for the preclinical evaluation of HDL-raising interventions on atherosclerosis development.

Cardiovascular disease (CVD) is the first cause of death in the Western world and its prevalence is increasing in Eastern Europe and developing countries.[1] The main cause of CVD is atherosclerosis, characterized by the combination of chronic inflammation and/or hyperlipidemia.[1] Both low HDL-cholesterol plasma levels and high VLDL/LDL-cholesterol levels are independent risk factors for atherosclerosis development.[2] The ratio of VLDL/LDL to HDL is to a great extent affected by the cholesteryl ester transfer protein (CETP).[3]

CETP is a transfer factor that mediates the exchange of cholesteryl esters (CE) and triglycerides (TG) between the apoB-containing lipoproteins (*i.e.* chylomicrons, VLDL, and LDL) and HDL in plasma.[3] As such, CETP may be anti-atherogenic by facilitating reverse cholesterol transport (RCT) from peripheral tissues to the liver via the VLDL/LDL pathway. Another potential role of CETP in RCT has recently been supported by the observation that CETP mediates HDL-CE uptake by hepatocytes independently of SR-BI and the LDL receptor (LDLr) *in vitro*.[4] On the other hand, CETP may be pro-atherogenic by enhancing the levels of VLDL/LDL with concomitant reduction of anti-atherogenic HDL levels.

Many studies in humans have been performed regarding the association between CETP and lipoprotein levels and the subsequent development of CVD.[5-9] For example, CETP deficiency that was observed in a Japanese population, increased CVD despite increased HDL levels.[8,9] In contrast, high CETP concentrations are associated with a faster atherosclerosis progression in men with proven CVD.[6] This finding is corroborated by a correlation study in humans, which showed that the Taq1B polymorphism in CETP is associated with increased plasma CETP, decreased plasma HDL, and an increased progression of CVD.[7] However, this might be confined to hypertriglyceridemic subjects as it has been shown in the prospective EPIC-Norfolk study that CETP correlated positively with future CVD risk only in humans with high TG levels (>1.7 mM).[5]

As the studies in humans have been associative and the effects of CETP expression on lipid metabolism and atherosclerosis gave conflicting results, the role of CETP has been addressed in mice that are naturally deficient for CETP.[10] To evaluate the direct effect of CETP on atherosclerosis development, CETP transgenic mouse models have been generated[11] and crossbred on different genetic backgrounds. CETP expression was found to be anti-atherogenic in *APOC3* and lecithin:cholesterol acyltransferase (*LCAT*) transgenic mice.[12,13] However, these mouse models may not be the preferred models for atherosclerosis studies since *APOC3* and *LCAT* mice develop only very small atherosclerotic lesions.[12,13]

In contrast to *APOC3* and *LCAT* mice, CETP was shown to be pro-atherogenic in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice.[14] As those mice exhibit both nearly complete blockage of the clearance of VLDL/LDL particles by the liver, it has been hypothesized that the cholesterol-rich particles that are formed as a result of CETP expression, accumulate in the vessel wall of these mice.[14] However, the suitability of these particular mouse models for evaluating the effect of CETP on atherosclerosis development may be limited as the potential effect of CETP in the facilitation of RCT from peripheral tissues to the liver via the VLDL/LDL pathway is completely abrogated by apoE or LDLr deficiency. Furthermore, expression of CETP in *Apoe*$^{-/-}$ mice resulted in unnaturally elevated TG

levels.[14] Finally, the *CETP.APOB* mouse has lipoprotein profiles on a chow diet comparable to normolipidemic humans,[15] but does not develop atherosclerosis unless treated with a cholesterol-rich diet containing cholate,[16] that, next to facilitating cholesterol absorption, induces chronic inflammation.[17]

In the present study, we crossbred the human *CETP* transgenic mouse[11] with the *APOE*3-Leiden* (*E3L*) mouse.[18] The *E3L* mouse expresses a mutation of the human *APOE3* gene resulting in an attenuated clearance of apoB-containing particles via the LDL receptor (LDLr) pathway.[19] As a result, cholesterol and TG levels are moderately increased on a chow diet.[19] On a Western-type diet containing 0.25% cholesterol, these mice exhibit a human-like lipoprotein cholesterol distribution.[20] Its VLDL-cholesterol levels are highly susceptible to cholesterol levels in the diet, whereas VLDL-TG levels decline to a normotriglyceridemic human level.[20] In the present study, we thus aimed to investigate the effect of CETP on atherosclerosis development in this human-like mouse model.

Materials and Methods

Animals and Diet

Human *CETP* transgenic mice expressing the human *CETP* gene under control of its natural flanking regions (strain 5203, heterozygous expression of CETP),[11] were obtained from Jackson Laboratories (Bar Harbor, ME, USA), and were crossbred with *E3L* mice,[18] of which female mice were used for experiments. *CETP.E3L* and *E3L* mice were housed under standard conditions with a 12 h light cycle (7.00 am – 7.00 pm) and were fed *ad libitum* with regular chow. Blood samples were collected by tail vein bleeding 1 week before feeding the mice a Western-type diet (semi-synthetic cholesterol-rich diet, containing 15% (w/w) fat and 0.25% (w/w) cholesterol) (Diet W; Hope Farms, Woerden, The Netherlands) and every 4 weeks thereafter. Hereto, mice were fasted for 4 h with food withdrawal at 9.00 am as described previously.[21] The experiments were approved by the institutional Ethical Committee on Animal Care and Experimentation.

Lipid and Lipoprotein Analysis

Plasma TC and TG levels were determined using enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN), respectively. For determination of the lipid distribution over plasma lipoproteins by fast performance liquid chromatography (FPLC), 50 µl of pooled plasma from 15 mice per group was injected onto a Superose 6 HR 10/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 µl/min PBS, 1 mM EDTA (Sigma), pH 7.4. Fractions of 50 µl were collected and assayed for TC and TG using enzymatic assays (Roche Molecular Biochemicals). For the analysis of the apolipoprotein distribution, fractions of 50 µl were diluted 1:1 (v/v) in sample buffer (0.125 M Tris, pH 6.8; 4% (w/v) SDS, 20% (w/v) glycerol; 10% (v/v) ß-mercaptoethanol; 0.01% (w/v) bromophenolblue). Samples were then applied onto a 4-20% Tris Glycine precast polyacrylamide minigel (Gradipore Ltd., French Forest, Australia). Electrophoresis was performed ac-

cording to manufacturer's instructions. Protein bands were stained with Coomassie Brilliant Blue R250 (Sigma), and apparent molecular masses were identified.

CETP Activity and Protein Levels

CETP activity in plasma was measured as the transfer of [^{3}H]cholesteryl oleate ([^{3}H]CO) from exogenous LDL to HDL as described elsewhere.[22] Hereto, 2.5 µl of plasma of animals on chow, and 0.5 µl of plasma of animals on the Western-type diet was added as a CETP source, with and without a preceding precipitation of apoB-containing particles using sodium phosphotungstate in the presence of magnesiumchloride.[23] CETP activity was calculated as µmol CE transfer per ml plasma per h. Plasma CETP mass was analyzed as described previously.[24] In short, a two-antibody sandwich immunoassay with a combination of the monoclonal antibodies TP1 and TP2 as coating was used. TP20 labeled with digoxigenine was used as secondary antibody.

Murine ApoAI ELISA

Plasma apoAI concentrations were determined using a sandwich ELISA. Hereto, goat-anti-mouse apoAI polyclonal antibody (ab7614; Abcam plc, Cambridge, UK; dilution 1:1000) was coated overnight onto Costar strips (Costar, Inc., New York, NY) (1 µg/ml) at 4°C and incubated with diluted mouse plasma (dilution 1:40400) for 2 h at RT. Subsequently, rabbit-anti-mouse apoAI antibody (ab20453; Abcam; dilution 1:2000) was added and incubated for 1 h at RT. Finally, horse radish peroxidase (HRP)-conjugated swine-anti-rabbit IgG antibody (SWARPO; dilution 1:2000) was added and incubated for 1 h at RT. HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Purified mouse apoAI (A23100m; Biodesign International, Saco, Maine, USA) was used as a standard.

Cholesterol Efflux

The effect of macrophage CETP on lipid accumulation and cholesterol efflux was investigated using thioglycollate-elicited peritoneal macrophages from *E3L* and *CETP·E3L* mice. Macrophages were loaded with acetylated LDL (AcLDL, 50 µg/ml) and [^{3}H]cholesterol (2 µCi/ml) for 48 h and subsequently half of the cells was lysed to determine the [^{3}H]cholesterol association related to cell protein.[25] Cholesterol efflux for a period of 10 h was assessed in the remainder of those cells, with and without human HDL (50 µg/ml) as a cholesterol acceptor.

The capacity of the plasma from mice fed the Western-type diet to induce ABCA1 dependent cholesterol efflux was determined using J774 murine macrophage-like cells. To induce cholesterol loading, J774 cells were incubated with acetylated LDL (AcLDL, 50 µg/ml) and [^{3}H]cholesterol (2 µCi/mL) for 48 h. Subsequently, cells were incubated without and with 0.3 mM 8-(4-chlorophenyl thio)adenosine 3':5'-cyclic monophosphate (cAMP analogue, Sigma) for 16 h to induce ABCA1 expression. Then, J774 macrophages were incubated for 4 h in the absence or presence of 1% of a plasma pool of 10 mice each. Human apoAI (10 µg/ml) and HDL (50 µg/ml) served as positive controls. The cAMP dependent cholesterol efflux from J774 cells was considered the ABCA1 mediated efflux.[26]

The capacity of the plasma from mice fed the Western-type diet to induce SR-BI dependent cholesterol efflux was determined using Fu5AH rat hepatoma cells (generous gift from Dr N. Fournier, Chatenay-Malabry, France). First, cells were loaded with cholesterol (30 μg/ml) in the presence of [^{3}H]cholesterol (2 μCi/ml) for 24 h. Then, cholesterol laden Fu5AH cells were incubated for 4 h in the absence or presence of 1% of a plasma pool of 10 mice each. Human apoAI (10 μg/ml) and HDL (50 μg/ml) served as positive controls. Cholesterol efflux was interpreted as the SR-BI-mediated efflux.[27]

Atherosclerosis Study and Atherosclerotic Lesion Analysis

At 8 weeks of age, *CETP.E3L* and *E3L* littermates were fed the Western-type diet. Mice were sacrificed after 19 weeks of diet. Hearts were isolated and fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin, and were cross-sectioned (5 μm) throughout the entire aortic root area. Per mouse, 4 sections with 40 μm intervals were used for quantification of atherosclerotic lesion area and characterization of lesion severity. Sections were routinely stained with hematoxylin-phloxine-saffron (HPS). Lesion area was determined using Leica Qwin image analysis software (EIS, Asbury, NJ). Atherosclerotic lesions were also categorized for severity, according to the American Heart System for humans,[28] which we have adapted to categorize lesions in mice.[29] Three types of categories were discerned: (1) no lesions (type 0), (2) early lesions were fatty streaks containing only foam cells (type 1-3), (3) advanced lesions showing foam cells in the media and presence of fibrosis, cholesterol clefts, mineralization and/or necrosis (type 4-5). The number observed in each category is expressed as a percentage of the total number of lesions present within one group of mice (*CETP.E3L* or *E3L* control group).

Statistical Analysis

All data are presented as means ± SD. Statistical differences were assessed using the Mann-Whitney U test for all experiments, except for the typing of the atherosclerotic lesions, where statistical differences were determined using the chi-square test. *P*-values less than 0.05 were regarded as statistically significant.

Results

Effect of CETP Expression on Lipids and Lipoprotein Profiles on a Chow Diet and a Western Type Diet

The effect of CETP expression on plasma parameters in *E3L* mice on a chow and a Western type diet are summarized in Table 1. On a chow diet, CETP expression resulted in a CETP concentration of 6.2±3.3 μg/ml and activity of 0.25±0.05 μmol CE/ml/h as measured in whole plasma. Precipitation of apoB-containing lipoproteins before determination of CETP concentration and activity did not affect these values (results not shown), indicating that plasma CETP resided specifically on HDL. CETP expression increased plasma TC levels (+43%; $P<0.05$), and tended to increase plasma TG levels (+23%).

As compared to the chow diet, the Western-type diet increased TC levels 4.3-fold ($P<0.001$) whereas TG levels decreased approx. 60% ($P<0.01$), in both mouse groups (Table 1). In *CETP.E3L* mice, the Western-type diet increased the plasma CETP concentration 11.7-fold ($P<0.001$) (Table 1), with a concomitant increase in plasma CETP activity of 4.4-fold ($P<0.05$). This led to increased TC levels (+43%; $P<0.01$) and a tendency to increased TG levels (+26%) in *CETP·E3L* as compared to *E3L* mice (Table 1).

Lipoprotein fractionation showed that CETP increased cholesterol in VLDL 2-fold and decreased cholesterol in regularly sized HDL (fractions 17-22) by approximately

Table 1. Plasma parameters in *E3L* and *CETP.E3L* mice fed a chow diet and a Western-type diet.

Genotype	CETP protein (μg/ml)	CETP activity (μmol CE/ml/h)	TC (mM)	TG (mM)
Chow diet				
E3L	n.d.	n.d.	3.7±1.2	3.5±1.1
CETP.E3L	6.2±3.3	0.25±0.05	5.3±2.3*	4.3±0.6
Western type diet				
E3L	n.d.	n.d.	16±5	1.4±0.5
CETP.E3L	72.8±8.7	1.1±0.5	23±6**	1.9±1.1

Plasma was obtained from 7-weeks-old 4 h fasted *E3L* (n=10) and *CETP.E3L* (n=9) mice on a chow diet, or from 4 h fasted *E3L* (n=13) and *CETP.E3L* (n=15) mice fed a Western-type diet for 19 weeks. Plasma CETP protein, CETP activity, TC and TG levels were determined and are represented as means ± SD. Asterisks indicate significant differences as compared with *E3L* mice. *$P<0.05$, **$P<0.01$. n.d., not detectable.

25% (Fig. 1A). Likewise, the plasma apoAI content was reduced by 25% ($P<0.05$) (Fig. 1D). In addition, the lipoprotein particle eluting in fractions 14-16 in *E3L* mice almost disappeared upon CETP expression (Fig. 1A). This particle was rich in apoE and did not contain apoAI (Fig. 1B-C), and thus represented large apoE-rich HDL_1, consistent with previous observations.[19] Therefore, CETP expression reduced the cholesterol content in total HDL 2-fold.

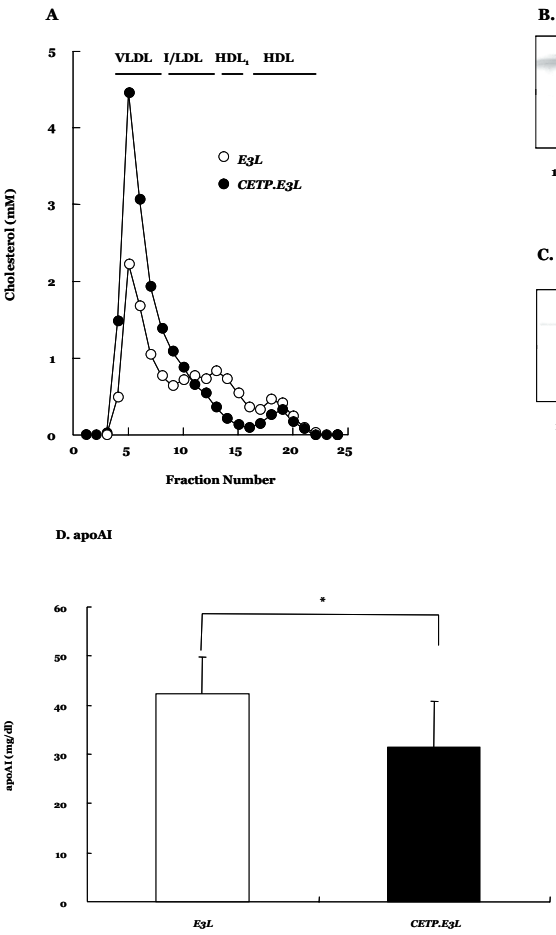

Figure 1. Effect of CETP on cholesterol distribution among lipoproteins in *E3L* mice fed a Western-type diet containing 0.25% cholesterol. Plasma from 4 h fasted *E3L* (white symbols) and *CETP.E3L* mice (black symbols) fed a Western-type diet containing 0.25% cholesterol for 19 weeks was pooled per genotype (n=11 per pool). Lipoproteins were size-fractionated by FPLC on a Superose 6 column, and the individual fractions were assayed for TC (A). Lipoprotein fractions 14-19 were assayed for protein and apparent molecular masses were identified representing apoAI and apoE in female *E3L* (B) and *CETP.E3L* (C) mice. Total apoAI levels were determined using a sandwich ELISA (D).

Effect of CETP Expression in E3L Macrophages on Cholesterol Uptake and Cholesterol Efflux

To investigate whether macrophage-associated CETP affects the uptake of cholesterol, peritoneal macrophages were isolated from *E3L* and *CETP.E3L* mice and incubated with AcLDL and [^{3}H]cholesterol. Macrophages from *CETP.E3L* mice showed no different cholesterol uptake as compared to those from *E3L* mice (Fig. 2A). Also, CETP expression did not affect cholesterol efflux from macrophages using HDL as a cholesterol acceptor (Fig. 2B). Taken together, CETP expression in macrophages did not affect either the uptake or efflux of cholesterol.

Effect of CETP Expression on the Cholesterol Accepting Capacity of Plasma
We determined the effect of plasma from *E3L* and *CETP.E3L* mice on cellular cholesterol efflux, either from cholesterol-laden cAMP analogue-treated J774 cells (representing ABCA1-mediated efflux)[26] or Fu5AH cells (representing SR-BI-mediated efflux).[27]

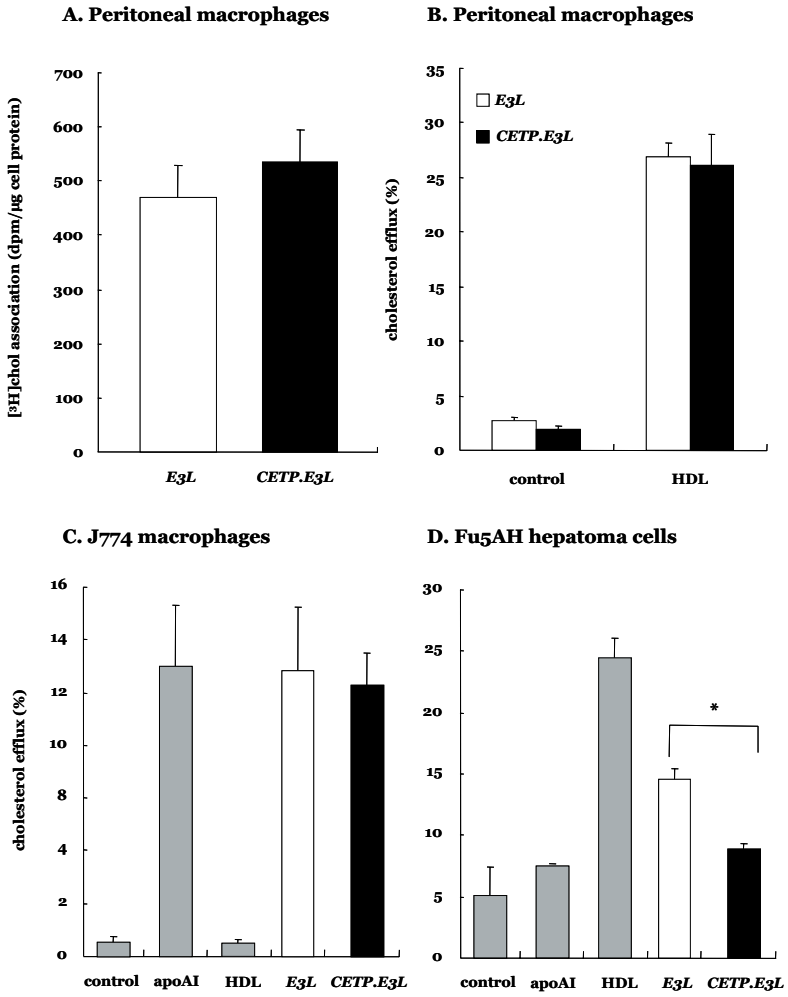

Figure 2. Effect of CETP expression on cholesterol association and cholesterol efflux. Peritoneal macrophages were isolated from *E3L* (white bars) and *CETP.E3L* (black bars). Macrophages were laden with AcLDL (48 h; 50 µg/ml) in the presence of [^{3}H]cholesterol (2 µCi/ml) and the accumulation of label was assessed (A). Subsequently, cholesterol efflux with and without HDL (50 µg/ml) was determined over a period of 10 h (B). After 4 h of incubation ABCA1-mediated cholesterol efflux from lipid-laden J774 macrophages (C) and SR-BI mediated cholesterol efflux from lipid-laden Fu5AH cells (D) was assessed in the absence (control) or presence of apoAI (10 µg/ml) or HDL (50 µg/ml) or a plasma-pool from 10 *CETP.E3L* (1%) or *E3L* (1%) mice fed the Western type diet for 19 weeks. *P<0.05

Cholesterol efflux from J774 cells was largely induced in the presence of apoAI, whereas HDL had no effect, which is consistent with ABCA1-mediated efflux (Fig. 2C). The ABCA1-dependent cholesterol accepting potencies of plasma of *E3L* and *CETP.E3L* mice were similar (approximately 12%). Cholesterol efflux from Fu5AH cells was hardly induced upon incubation with apoAI, yet largely induced upon incubation with HDL, consistent with SR-BI-mediated efflux (Fig. 2D). Plasma of *CETP.E3L* mice was 39% ($P<0.05$) less efficient in inducing SR-BI-mediated cholesterol efflux as compared to plasma of *E3L* mice (Fig. 3D). Taken together, CETP expression reduced the potency of plasma to mediate SR-BI-dependent cholesterol efflux, without compromising the ABCA1-mediated cholesterol efflux.

Effect of CETP Expression on Atherosclerosis Development

To investigate the effect of CETP on atherosclerosis development, mice were fed the Western-type diet from 8 weeks of age. In *E3L* mice, plasma cholesterol levels raised up to 16 mM and in *CETP.E3L* mice to 23 mM, which remained stable throughout the whole study. After 19 weeks of the Western-type diet, the development of atherosclerosis in *E3L* mice was still in the early phase as a lot of segments were either unaffected (type 0) or contained foam cell rich lesions (type 1-3) (Fig. 3A and B). In contrast, *CETP.E3L* mice developed much more advanced lesions that affected the integrity of the media, contained cholesterol clefts, and showed calcification (type 4-5) (Fig. 3A and B). The much more advanced atherosclerosis in *CETP.E3L* mice was reflected in a 7.0-fold increase in atherosclerotic lesion area (Fig. 3C). Collectively, CETP represents a clear pro-atherogenic factor in *E3L* mice.

Discussion

The role of CETP in atherosclerosis is still under debate.[5-9,12-14] In the present study, the effect of CETP expression on atherosclerosis development was evaluated in *E3L* mice, a mouse model with a human-like cholesterol distribution over lipoproteins. We found that CETP expression led to a net shift of cholesterol from HDL towards VLDL, resulting in 2-fold increased VLDL-cholesterol plasma levels and 2-fold decreased HDL-cholesterol levels. This led to a reduced capacity of the plasma to induce SR-BI-mediated cholesterol efflux, yet did not affect ABCA1-mediated cholesterol efflux. Furthermore, CETP expression resulted in much more advanced atherosclerotic lesions and a 7.0-fold increase in atherosclerotic lesion area in *E3L* mice.

CETP permits bidirectional transfer between apoB-containing lipoproteins and HDL, resulting in net flux of TG from VLDL and LDL to HDL, and net flux of cholesterol from HDL to VLDL and LDL.[30] Since *apoE*3-Leiden* has a reduced affinity for the hepatic LDLr as compared to wild-type apoE, *E3L* mice have increased VLDL-cholesterol.[19] CETP expression in *E3L* mice caused an additional increase in VLDL-cholesterol, probably by increasing the net cholesterol flux from HDL to VLDL,[30] thereby further impeding VLDL clearance. Furthermore, CETP expression in *E3L* mice reduced HDL levels. Next to a 25% decrease in HDL_2 and HDL_3, as reflected by a decrease in both apoAI and cholesterol in these HDL subpopulations, CETP expression resulted in disappear-

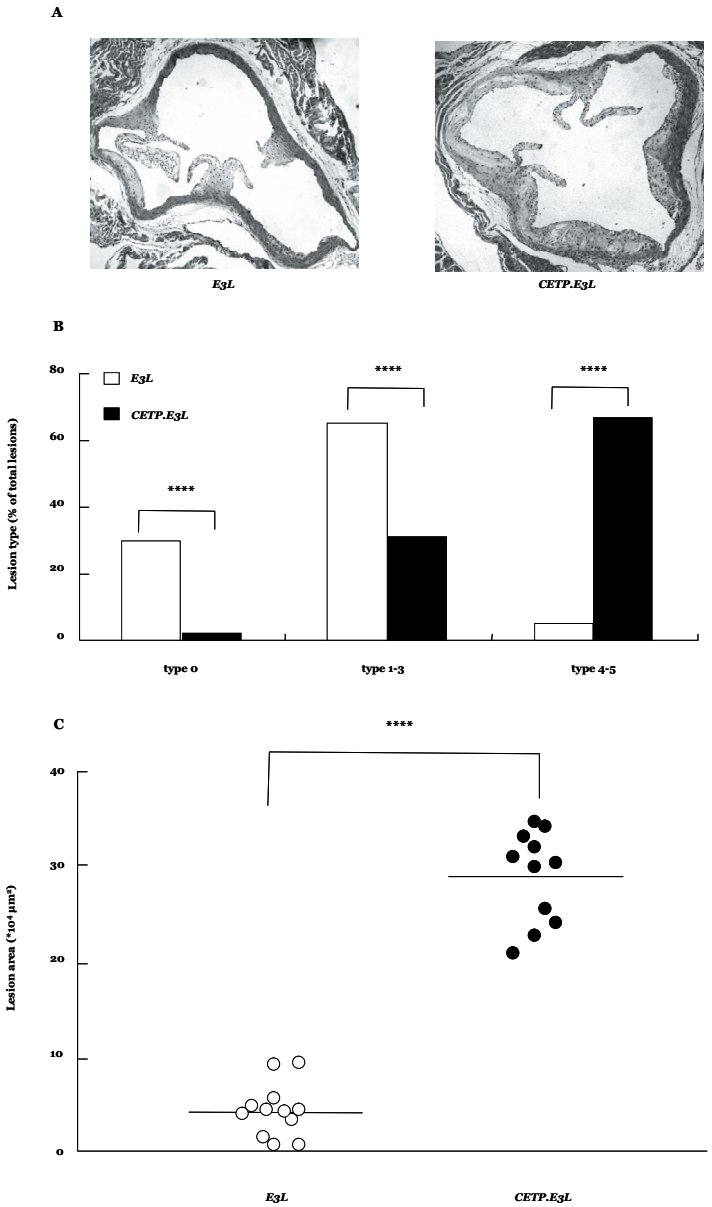

Figure 3. Effect of CETP on the development of atherosclerotic lesion severity and area in the aortic root. *E3L* (white symbols) (n=12) and *CETP.E3L* (black symbols) (n=11) mice were sacrificed after 19 weeks of Western type diet (containing 0.25% cholesterol) feeding, and hearts were isolated, fixed, dehydrated and embedded in paraffin. Hearts were cross-sectioned (5 µm) throughout the entire aortic root, and stained with hematoxylin-phloxine-saffron (HPS). Representative pictures are shown (A). Four sections per mouse with 40 µm intervals were typed and categorized according to lesion severity (B) and the extent of atherosclerosis was quantified (C). Each data point represents the mean lesion area per mouse (C). ****P<0.0001.

ance of apoAI-deficient and apoE-rich HDL_1, which is present in *E3L* mice.[19] Likewise, CETP expression has been shown to eliminate HDL_1 that accumulates in LCAT transgenic mice.[12] Apparently, HDL_1 is a preferential substrate for CETP. This hypothesis is corroborated by the finding that HDL_1 accumulates in CETP deficient humans.[8,9] The CETP-induced reduction in HDL may be explained by 1) reduced lipidation of HDL-apolipoproteins, resulting in enhanced renal clearance of lipid-poor apoAI, 2) enrichment of HDL in TG, resulting in a more efficient hepatic lipase-mediated HDL catabolism,[31] and/or 3) direct uptake of HDL-CE by liver-associated CETP, as has recently been proposed by Gauthier *et al*.[4]

On the Western-type diet, plasma CETP activity and mass were 4-fold and 12-fold increased, respectively, as compared to the chow diet. This indicates that inactive CETP accumulated on HDL on a Western-type via an as yet unidentified mechanism. The observation that a cholesterol-rich diet leads to CETP accumulation in plasma is consistent with previous observations in apoE-deficient and LDLr-deficient mice.[32] Also in humans, a correlation between plasma lipid levels and plasma CETP concentration was found.[33,34] Regulation of CETP expression involves an LXR-response element[35] that is present in the natural flanking regions of the CETP transgenic mouse strain that we used for cross-breeding with *E3L* mice.[11] Most likely, the cholesterol diet-induced hypercholesterolemia thus results in increased hepatic cholesterol as well as oxysterols, the natural ligands for the liver X receptor (LXR),[36] thereby increasing CETP expression, as reflected by increased plasma CETP levels.

In previous studies in *E3L* mice, VLDL-cholesterol has been found to correlate well with atherosclerotic lesion area, most probably by initiating atherosclerosis upon entry of VLDL into the vascular wall.[19] Specifically, feeding *E3L* mice a high cholesterol diet as compared to a low cholesterol diet resulted in 2-fold increased VLDL-cholesterol levels and a 2-fold increased atherosclerotic lesion area.[37] We now observed that a similar 2-fold increase in VLDL-cholesterol levels as induced by the introduction of CETP in *E3L* mice caused even a 7-fold increase in atherosclerotic lesion area. This can thus not simply be explained by a CETP-mediated increase in VLDL, and suggests that other mechanisms are involved in this process, which may include a local effect of CETP on lipid accumulation in macrophages and/or the observed reduction in HDL.

We found that the expression of CETP in macrophages did not affect AcLDL-induced foam cell formation or cholesterol efflux to human HDL. This is in contrast with findings in a monkey fibroblast cell line (COS-7), which showed that transfection with a CETP construct induces cholesterol efflux.[38] This seeming discrepancy may be caused by a difference in CETP expression. However, it is thus unlikely that CETP expression in macrophages contributed to the observed increased atherosclerosis development by affecting the cellular lipid homeostasis.

Alternatively, CETP may affect RCT by reducing plasma levels of HDL, which is crucially involved in RCT. The first step in this process is cholesterol efflux from the macrophage, as mediated by ABCA1 and SR-BI. The shift of plasma cholesterol from HDL to VLDL as induced by CETP expression did not affect ABCA1-mediated cholesterol efflux yet reduced SR-BI-mediated efflux. It has been shown that small lipid-poor HDL has the strongest association with ABCA1-mediated cholesterol efflux, even in

the presence of other HDL subpopulations.[39] Regarding the HDL cholesterol distribution, *CETP.E3L* mice mostly express small HDL, probably as a consequence of HDL remodeling by CETP. Apparently, the difference in levels of small HDL-particles between plasma from *CETP.E3L* and *E3L* mice is not sufficient to affect ABCA1-mediated cholesterol efflux. The observation that CETP expression does not compromise ABCA1-mediated cholesterol efflux to HDL is in agreement with data from a previous study in rabbits treated with a CETP-inhibitor.[40]

Whereas CETP expression did not affect ABCA1-mediated efflux, it decreased the SR-BI-mediated cholesterol efflux. As different HDL subpopulations contribute equally to SR-BI-mediated cholesterol efflux,[39] and total HDL levels were lower in the plasma of *CETP.E3L* mice (especially HDL_1), this can thus easily explain the reduced SR-BI efflux. Nevertheless, ABCA1 and SR-BI do not constitute all the pathways that mediate cholesterol efflux from macrophages.[41] ABCG1 also mediates cholesterol efflux, and has been shown to be highly functional in inducing cholesterol efflux to HDL from CETP-deficient subjects.[42] Since ABCG1 and SR-BI both use HDL as cholesterol acceptor,[39,42] an additional effect of the CETP-induced lipoprotein shift on ABCG1-mediated efflux can not be ruled out. Finally, it may be postulated that VLDL contributes to cholesterol efflux, similarly as has been documented for LDL.[43] However, even if VLDL contributes to cholesterol efflux, plasma from *CETP.E3L* mice showed a decreased SR-BI mediated cholesterol efflux despite higher VLDL levels.

It remains to be elucidated whether CETP-induced reduced HDL will be rate-limiting for integrated RCT *in vivo*, *i.e.* the transport of cholesterol from macrophages to the liver, leading to fecal secretion. A recent study has demonstrated that CETP inhibition in rabbits does not affect the clearance of HDL-cholesterol, and we have obtained initial data that CETP expression does not affect HDL-CE turnover in *E3L* mice (unpublished data). However, our observations that CETP expression in *E3L* mice reduced cholesterol efflux *in vitro*, and strongly increased atherosclerosis *in vivo*, suggest that CETP reduced RCT in *E3L* mice.

Collectively, we have now shown that CETP is a clear pro-atherogenic factor in *E3L* mice. Our data are in line with the pro-atherogenic effect of CETP in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, in which the clearance of VLDL-particles is also decreased.[14] The *E3L* mouse model has been proven very suitable for testing hypolipidemic drugs that affect VLDL/LDL-metabolism.[19,44] Atorvastatin,[44] rosuvastatin,[45] and gemfibrozil[46] reduced the levels of the VLDL/LDL in *E3L* mice comparable to humans. As the introduction of CETP results in the potential to modulate HDL-cholesterol levels in addition to VLDL-cholesterol levels, we anticipate that the *CETP.E3L* mouse will be suitable for the preclinical evaluation of HDL-increasing therapies (including CETP inhibitors), which constitute a novel target in the treatment of CVD.

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