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

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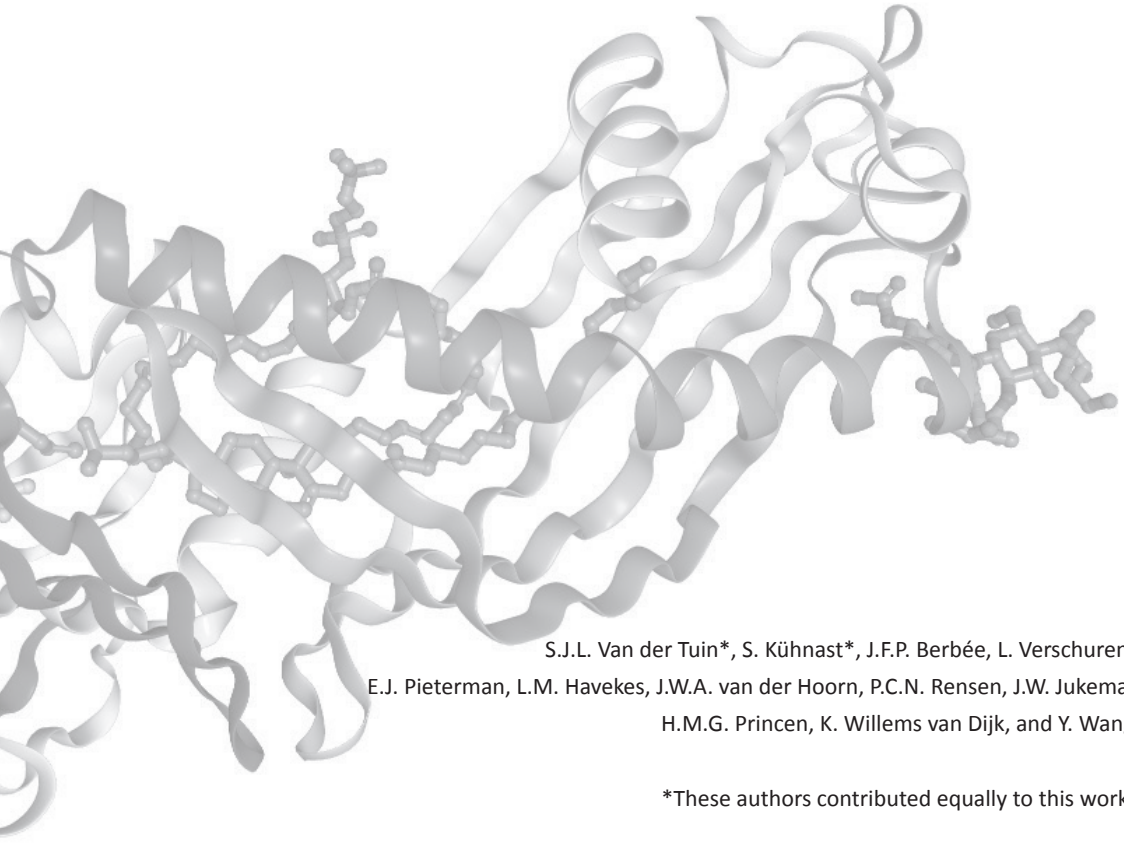
Chapter 5

Anacetrapib reduces (V)LDL cholesterol by inhibition of CETP activity and reduction of plasma Pcsk9



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ABSTRACT

Recently, we showed in APOE*3-Leiden.CETP mice that anacetrapib attenuated atherosclerosis development by reducing (V)LDL cholesterol [(V)LDL-C] rather than by raising HDL cholesterol. Here, we investigated the mechanism by which anacetrapib reduces (V)LDL-C and whether this effect was dependent on the inhibition of APOE*3-Leiden.CETP mice were fed a Western-type diet alone or supplemented with anacetrapib (30 mg/kg body weight per day). Microarray analyses of livers revealed downregulation of the cholesterol biosynthesis pathway (P<0.001) and predicted downregulation of pathways controlled by sterol regulatory element-binding proteins 1 and 2 (z-scores -2.56 and -2.90, respectively; both P<0.001). These data suggest increased supply of cholesterol to the liver. We found that hepatic proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) expression was decreased (-28%, P<0.01), accompanied by decreased plasma Pcsk9 levels (-47%, P<0.001) and increased hepatic LDL receptor (LDLr) content (+64%, P<0.01). Consistent with this, anacetrapib increased the clearance and hepatic uptake (+25%, P<0.001) of [^{14}C]cholesteryl oleate-labeled VLDL-mimicking particles. In APOE*3-Leiden mice that do not express CETP, anacetrapib still decreased (V)LDL-C and plasma Pcsk9 levels, indicating that these effects were independent of CETP inhibition. We conclude that anacetrapib reduces (V)LDL-C by two mechanisms: 1) inhibition of CETP activity, resulting in remodeled VLDL particles that are more susceptible to hepatic uptake; and 2) a CETP-independent reduction of plasma Pcsk9 levels that has the potential to increase LDLr-mediated hepatic remnant clearance.

INTRODUCTION

High plasma levels of (V)LDL cholesterol [(V)LDL-C] and TGs, as well as low levels of HDL cholesterol (HDL-C), are important risk factors for cardiovascular diseases. The standard treatment for the reduction of cardiovascular disease risk is statin therapy aiming to reduce plasma (V)LDL-C. However, a substantial residual risk remains despite statin treatment. This has prompted the search for secondary treatment targets.[1, 2] Prospective epidemiological studies indicate HDL-C as a potential target.[3] The ratio of plasma (V)LDL-C to HDL-C is to a great extent affected by cholesteryl ester transfer protein (CETP). CETP facilitates the transfer of cholesteryl esters from HDL to (V)LDL in exchange for TG.[4] In several mouse models, including C57Bl/6, Ldlr$^{-/-}$, and APOE*3-Leiden transgenic mice, CETP expression aggravates the development of atherosclerosis.[5-7] Although human studies have shown conflicting results with regard to the association between CETP deficiency and decreased cardiovascular disease risk,[8, 9] CETP inhibition is actively pursued as a potential strategy to reduce this risk. [10]This has led to the development of pharmacological CETP inhibitors, such as torcetrapib, dalcetrapib, anacetrapib, and evacetrapib.

In clinical trials, torcetrapib, anacetrapib, and evacetrapib have been shown to increase HDL-C [up to +72%,[11]+139%,[12] and +129%,[13] respectively] and to reduce LDL-C [down to -25%,[11] -40%,[12] and -36%,[13] respectively], whereas dalcetrapib only increased HDL-C [up to +40%[14]]. Although torcetrapib showed favourable effects on the lipoprotein profile, it failed in phase III clinical development due to increased risk of major cardiovascular events and mortality. These adverse effects were ascribed to an off-target effect[11] and proinflamatory lesions.[15] A large phase III clinical trial with dalcetrapib was prematurely terminated due to a lack of clinical benefit.[14] Nonetheless, the effects of anacetrapib and evacetrapib on cardiovascular outcomes are currently being evaluated in phase III clinical trials. [16]Neither compound shows increased blood pressure as observed with torcetrapib,[12, 13] and both compounds are more potent in increasing HDL-C and reducing LDL-C as compared with torcetrapib and dalcetrapib.

While regular C57Bl/6 mice have a very rapid clearance of ApoB-containing lipoproteins, APOE*3-Leiden mice have an attenuated clearance and increased TG level, thereby mimicking the slower clearance observed in humans, particularly in patients with familial dyslipoproteinemia.[17] Similarly as in familial dyslipoproteinemia patients, in APOE*3-Leiden and APOE*3-Leiden.CETP mice as a model for mixed dyslipoproteinemia, a major part of plasma cholesterol is contained in the VLDL (remnant) particles, leading to the formation of β-VLDL particles, which further increases after cholesterol feeding. The APOE*3-Leiden. CETP mouse model, unlike Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice, responds in a human-like way to the lipid-lowering effects of statins,[18] fibrates,[19] niacin,[20, 21] torcetrapib,[15] anacetrapib,[22] and anti-PCSK9mabs,[23] with respect to both direction and magnitude of the change. In conclusion,

APOE*3-Leiden.CETP mice have a more human-like lipoprotein metabolism when compared with C57Bl/6, Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice.

Recently, we have shown that anacetrapib treatment increased HDL-C, reduced (V)LDL-C and TG, and dose-dependently reduced atherosclerotic lesion size and severity in APOE*3-Leiden.CETP mice. ANCOVA showed that the effect on lesion size was mainly explained by a reduction in (V)LDL-C.[22] However, the mechanism by which anacetrapib reduces plasma (V)LDL-C and TG is not fully understood. To elucidate this, we performed microarrays on the livers from this latter study, identifying pathways affected by anacetrapib. To confirm physiological consequences of these identified pathways, we performed a VLDL production experiment and studied the clearance of VLDL-mimicking particles. By using APOE*3-Leiden mice with or without CETP expression,[7] we also determined whether these effects of anacetrapib were CETP dependent.

MATERIALS AND METHODS

RNA isolation, microarray, and qPCR validation

Liver pieces were obtained from a previous experiment performed by Kühnast *et al.*,[22] investigating the effects of anacetrapib on atherosclerosis in female APOE*3-Leiden.CETP mice.[22] In this study, mice were treated with a semisynthetic cholesterol-rich diet, containing 15% (w/w) cacao butter, 1% corn oil, and 0.1% cholesterol (Western-type diet; AB-Diets) with or without anacetrapib (30 mg/kg body weight per day) for 21 weeks. Total RNA was extracted from these liver pieces using the Nucleospin RNAII kit (Macherey-Nagel) according to the manufacturer's protocol. The microarray, including quality control, RNA labelling, hybridization, and data extraction, was performed by ServiceXS B.V. (Leiden, The Netherlands). To perform qPCR for validation, RNA quality was verified by the lab-on-a-chip method using Experion™ RNA StdSens analyses kit (Bio-Rad). Total RNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad), and qPCR was performed using a CFX96™ Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized to beta-2 microglobulin and hypoxanthine-guanine phosphoribosyltransferase. Relative expression was calculated as compared with the control group using Bio-Rad CFX Manager™ software 3.0 (Bio-Rad).

Microarray data analyses

The probe-level background subtracted expression values were used as input for lumi package[24] of the R/Bioconductor (http://www.bioconductor.org; http://www.r-project.org) to perform quality control and a quantile normalization. Unexpressed probes (P>0.01 in all experiments) were removed from further analyses. Differentially expressed probes were

identified using the limma package of R/Bioconductor.[25] The calculated P values <0.05 were used as a threshold for pathway analyses using Ingenuity Pathway Analysis suite (http://www.ingenuity.com). Upstream regulator analysis was performed using the Ingenuity Pathway Analysis software. This analysis determines the activation state of transcription factors based on the observed differential gene expression and results in an overlap P value and activation z-score for each transcription factor in the Ingenuity Pathway Analysis knowledgebase. The overlap P value indicates the significance of the overlap between the known target genes of a transcription factor and the differentially expressed genes measured in an experiment. The activation z-score indicates activation (positive z-score) or inhibition (negative z-score) of a particular transcription factor. An activation z-score >2 or <2 indicates significant activation or inhibition of a pathway or process.

Experimental setup

To investigate the effects of anacetrapib on VLDL production and clearance, female APOE*3-Leiden[26] and APOE*3-Leiden.CETP[7] transgenic mice, 8-10 weeks of age, were fed a Western-type diet for a run-in period of 3-4 weeks. They were then matched based on plasma total cholesterol (TC), HDL-C, TG, body weight, and age into two groups receiving either no treatment (control) or anacetrapib (30 mg/kg body weight per day) for 4 weeks, after which VLDL production (APOE*3-Leiden.CETP only) or clearance (APOE*3-Leiden and APOE*3-Leiden.CETP) was determined. After both experiments, the mice were euthanized by CO_2 asphyxiation. The mice were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water during the experiment. Body weight and food intake were monitored during the study. The Institutional Ethics Committee for Animal Procedures from the Leiden University Medical Center, Leiden, The Netherlands, approved the protocol.

Plasma lipid measurements

Blood was collected after a 4 h fasting period in heparin-coated capillaries via tail vein bleeding, and plasma was isolated. TC and TG were determined using enzymatic kits (Roche) according to the manufacturer's protocol. To measure HDL-C, apoB-containing particles were precipitated from plasma with 20% polyethylene glycol 6000 (Sigma Aldrich) in 200 mM glycine buffer (pH 10), and HDL-C was measured in the supernatant.[22] The distribution of cholesterol over plasma lipoproteins was determined by fast-performance liquid chromatography as previously described.[7]

Hepatic LDL receptor protein and plasma proprotein convertase subtilisin/kexin type 9 measurements

Snap-frozen mouse livers were lysed in ice-cold lysis buffer containing 50 mM HEPES (pH 7.6), 50 mM NaF, 50 mM KCl, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 mM

β-glycerophosphate, 1 mM sodium vanadate, 1% NP40, and protease inhibitors cocktail (Roche). Thereafter, protein level was determined using the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. Laemmli buffer (Sigma-Aldrich) was added to samples containing equal amounts of protein. Samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted to polyvinylidene difluoride. Blots were incubated with goat-anti-mouse LDL receptor (LDLr; 1:1000, AF2255, R&D Systems), rabbit-anti-mouse low density lipoprotein receptor-related protein 1 (LRP1; 1:20000, ab92544, Abcam), and mouse-anti-α-tubulin (1:1000, T5168, Sigma-Aldrich) and subsequently incubated with the appropriate secondary antibody. Bands were visualized by enhanced chemiluminescence with Pierce ECL 2 substrate following the manufacturer's protocol and quantified using Image J software as previously described.[27] Plasma proprotein convertase subtilisin/kexin type 9 (Pcsk9) was measured by using ELISA (MCP900, R and D Systems) according to the manufacturer's instructions.

Hepatic VLDL-TG and VLDL-apoB production analyses

Mice (n=8/9 per group) were anesthetized with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.31 mg/kg fentanyl (Janssen-Cilag) after a 4 h fast. A basal blood sample was taken from the tail, and the mice received an intravenous injection of 100 µl PBS containing 100 µCi Tran^{35}S label (MP Biomedicals) via the tail vein. After 30 min, animals received an intravenous injection of Tyloxapol (Triton WR-1339, Sigma-Aldrich; 500 mg/kg body weight), as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG.[28] At indicated time points up to 90 min after Tyloxapol injection, blood was taken and plasma TG concentration was determined. After 120 min, mice were euthanized, and blood was collected for isolation of the VLDL fraction by density gradient ultracentrifugation.[29] Tran^{35}S activity was measured in the VLDL fraction, and VLDL-ApoB production rate was calculated as dpm/h.[30]

Clearance analysis of VLDL-mimicking particles

Glycerol tri(9,10(n)[^{3}H])oleate ([^{3}H]TO) and [1α,2α(n)-^{14}C] cholesteryl oleate ([^{14}C]CO) double-radiolabelled VLDL-mimicking particles (mean diameter 80 nm) were prepared as previously described.[31] In short, [^{3}H]TO (100 µCi) and [^{14}C]CO (10 µCi) tracers were added to a mixture of TO (70 mg), egg yolk phosphatidylcholine (22.7 mg), lysophosphatidylcholine (2.3 mg), CO (3.0 mg), and cholesterol (2.0 mg). Particles were prepared from this mixture at 54°C using a Soniprep 150 (MSE Scientific Instruments) at 10 µm output. After two consecutive density gradient ultracentrifugation steps, VLDL-mimicking particles (average size of 80 nm) were isolated. The particles were stored at 4°C under argon and intravenously injected into mice within 5 days. These VLDL-mimicking particles acquire a wide range on interchangeable apolipoproteins from serum, among which apoE[31, 32] mediates the hepatic uptake of their remnants.[32]

After a 4 h fast, particles were injected via the tail vein in conscious mice (n=8/9 per group). At 2, 5, 10, and 15 min postinjection, blood was taken to determine the plasma decay of [^{3}H]TO and [^{14}C]CO. Plasma volumes were calculated as 0.04706 × body weight (g) as previously described.[33] Mice were euthanized after 15 min, perfused with ice-cold PBS with 0.1% heparin (v/v), and organs were harvested to determine tissue-specific [^{3}H]TO and [^{14}C]CO uptake. Subsequently, organs were dissolved overnight at 56°C in tissue solubilizer (Amersham Biosciences) and quantified for ^{3}H and ^{14}C activity. Uptake of [^{3}H]TO- and [^{14}C]CO-derived radioactivity by the organs was calculated as dose per organ after correction for organ weight.

Statistical analyses

Significance of differences between the groups was calculated nonparametrically using a Mann-Whitney *U*-test. All reported P values are two-tailed, and P values of >0.05 were considered statistically significant.

RESULTS

Pathway analyses predict downregulation of pathways controlled by sterol regulatory element-binding proteins 1 and 2 by anacetrapib

To determine the effects of anacetrapib treatment on hepatic gene expression in APOE*3-Leiden.CETP mice,[22] microarray analyses were performed. A total of 95 genes (false discovery rate P<0.05; Table S1) were differentially expressed between control and anacetrapib-treated female mice of which 46 genes were upregulated and 49 genes were downregulated. To gain insight into affected biological processes, a gene-set enrichment analysis was performed using the Ingenuity Pathway Analysis suite (as described in Material and Methods). This analysis showed that the cholesterol biosynthesis pathway was significantly affected (Table 1). In silico prediction of transcription factor activity (Table 2), based on the differentially expressed genes, predicted inhibition of genes regulated by SREBP-1 (P<0.001; z-score -2.90) and SREBP-2 (P<0.001; z-score -2.56), which are key regulators of cholesterol synthesis. SREBP-2 target genes mevalonate (diphospho) decarboxylase [*Mvd*[34]], 7-dehydrocholesterol reductase [*Dhcr7*[35]], and transmembrane 7 superfamily member 2 [*Tm7sf2*[36]] were validated using qPCR. All genes showed a downregulation after anacetrapib treatment (Fig. S2). Furthermore, anacetrapib activated genes regulated by NR1I2 (P<0.001; z-score +2.75) and NR1I3 (P<0.001; z-score +2.94). Both nuclear receptors function as sensors of endobiotic and xenobiotic substances. These data indicate that anacetrapib reduces cholesterol biosynthesis and activates a xenobiotic response.

Anacetrapib decreases hepatic and circulating Pcsk9 and increases hepatic LDLr protein in APOE*3-Leiden.CETP mice

In addition to effects on cholesterol biosynthesis and xenobiotic metabolism, microarray analyses showed a decrease in the expression of *Pcsk9* mRNA, a downstream target of the SREBP-2 pathway[37] (Fig. S1), in the liver of anacetrapib-treated APOE*3-Leiden.CETP mice (-78%, P<0.05; Table S1), which was confirmed by qPCR (-27%, P<0.01; Fig. 1A). In accordance, anacetrapib reduced plasma Pcsk9 levels (-47%, P<0.01; Fig. 1B). Because Pcsk9 plays an important role in the degradation of intracellular LDLr[38-40] and Lrp1,[41] hepatic mRNA expression and protein levels were measured. Anacetrapib did not affect the hepatic mRNA expression of *Ldlr* (Fig. 1C) or *Lrp1* (Fig. 1E) but did increase hepatic LDLr protein expression (+64%, P<0.05; Fig. 1D). No effect on Lrp1 (Fig. 1F) protein levels was observed. The decrease in plasma Pcsk9 levels and increase in LDLr suggest an increased capability of the liver to take up lipoprotein remnants.

Anacetrapib does not affect TG metabolism but increases lipoprotein remnant clearance by the liver in APOE*3-Leiden.CETP mice

To further investigate the effects of anacetrapib on lipoprotein metabolism, we performed a new experiment with female APOE*3-Leiden.CETP mice fed a Western-type diet with or without anacetrapib for 4 weeks. Plasma lipid and lipoprotein levels were decreased to the same extent as shown in figure 2A (lipoprotein profile in Fig. S3A). Anacetrapib treatment did not affect the VLDL-TG production rate (Fig. 2B), the VLDL-^{35}S-apoB production rate (Fig. 2C), or the ratio of VLDL-TG to VLDL-apoB production rate (Fig. 2D), indicating no changes in number or composition of newly synthesized VLDL particles.

The effect of anacetrapib on VLDL clearance was assessed by an intravenous injection of [^{3}H]TO and [^{14}C]CO double-radiolabelled VLDL-mimicking particles. At the indicated time points, blood was taken to determine clearance from plasma. After 15 min, mice were euthanized and organs were harvested to determine tissue-specific uptake of radioactivity derived from [^{3}H]TO and [^{14}C]CO. We observed no effects on the plasma clearance (Fig. 3A) or the tissue-specific uptake of [^{3}H]TO-derived activity (Fig. 3B). However, anacetrapib increased the plasma clearance of the [^{14}C]CO label of the VLDL-mimicking particles (Fig. 3C), decreased plasma half-life of [^{14}C]CO (-56%, P<0.001; Fig. 3C, inlay), and increased the uptake of [^{14}C]CO by the liver (+25%, P<0.001; Fig. 3D). Because these particles reflect the behaviour of VLDL,[32, 42] these results indicate that anacetrapib increases the uptake of lipoprotein remnants by the liver.

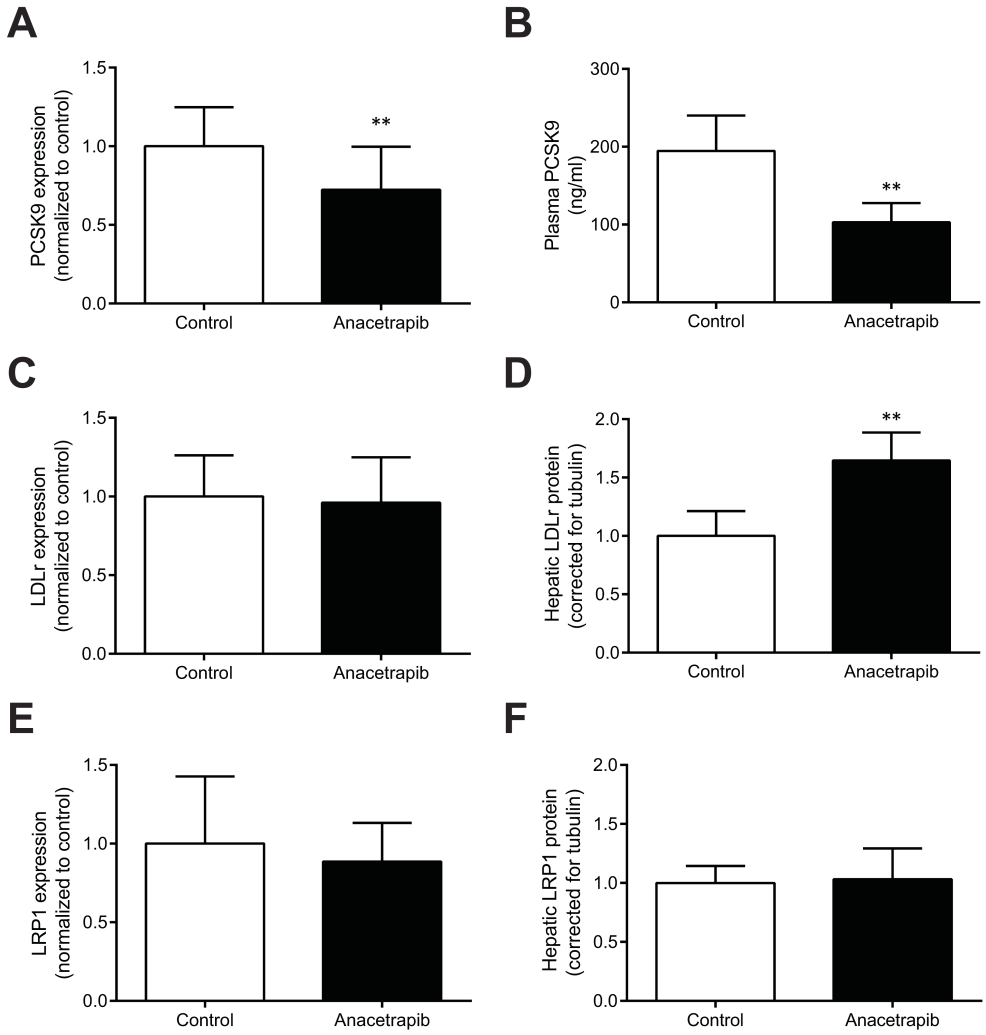

Figure 1: Anacetrapib decreases Pcsk9 mRNA expression and plasma levels and increases hepatic LDLr protein expression

Female APOE*3-Leiden.CETP mice were fed a Western-type diet with or without anacetrapib (30 mg/kg body weight per day) for 21 weeks; blood was collected for plasma PCSK9 levels and livers for mRNA expression. Hepatic Pcsk9 mRNA expression (A) and plasma levels (B). Hepatic LDLr mRNA (C) and protein (D) expression. Hepatic Lrp1 mRNA (E) and protein (F) expression.

Data are presented as mean±SD (n=14/15 per group). **P<0.01, ***P<0.001 when compared with control group.

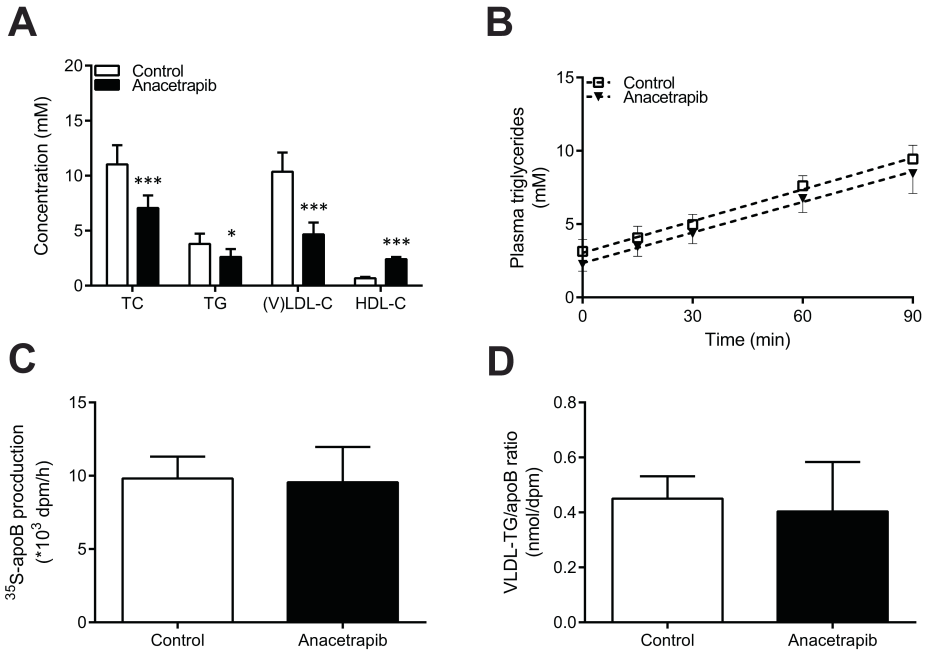

Figure 2: Anacetrapib does not affect hepatic VLDL-TG production in APOE*3-Leiden.CETP mice
Female APOE*3-Leiden.CETP mice were fed a Western-type diet with or without anacetrapib (30 mg/kg body weight per day) for 4 weeks, blood was collected by tail bleeding after 4 h fasting, and plasma TC, TG, non-HDL-C, and HDL-C were determined (A). After treatment, hepatic VLDL production was assessed (B). ^{35}S-apoB production was determined by scintillation counting of the isolated VLDL fraction (C), and the VLDL-TG production rate to VLDL-ApoB production rate ratio was calculated (D). Data are presented as mean±SD (n=8/9 per group). *P<0.05, ***P<0.001 when compared with control group.

Anacetrapib decreases (V)LDL-C and Pcsk9 levels in APOE*3-Leiden mice

To determine whether the effects of anacetrapib on (V)LDL metabolism were dependent on CETP inhibition, similar experiments were performed in female APOE*3-Leiden mice that do not express CETP. Notably, anacetrapib reduced TC (-17%, P<0.05) and (V)LDL-C (-20%, P<0.05; Fig. 4A; lipoprotein profile in Fig. S3B) levels in APOE*3-Leiden mice without CETP, concomitantly with a decrease in hepatic *Pcsk9* expression and plasma Pcsk9 levels (-37%, P<0.05; Fig. 4B). These data clearly show that anacetrapib has a CETP-independent lipid-lowering effect. Comparing APOE*3-Leiden with APOE*3-Leiden.CETP mice, anacetrapib increased the particle clearance in APOE*3-Leiden.CETP mice to the similar level as observed in APOE*3-Leiden mice without anacetrapib (Figs. 3C and 4C). Anacetrapib showed no additional effects on the plasma clearance of [^{3}H]TO and [^{14}C]CO labels of the VLDL-mimicking particles (Fig. 4C, E) or the tissue-specific uptake of [^{3}H]TO and [^{14}C]CO (Fig. 4D, F) in APOE*3-Leiden mice.

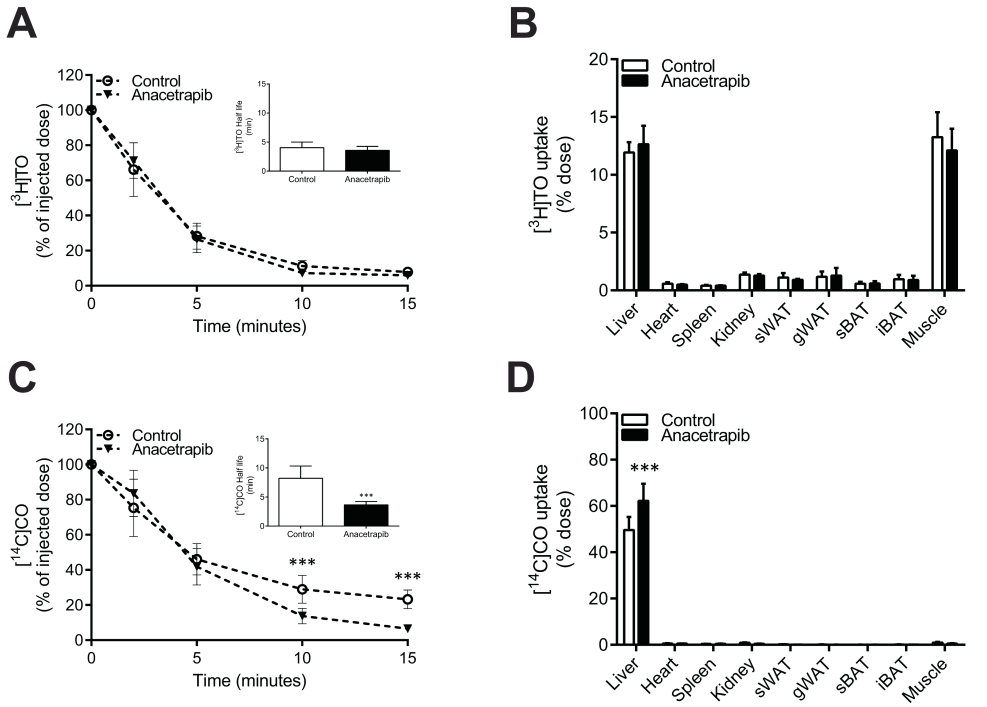

Figure 3: Anacetrapib increases lipoprotein remnant clearance by the liver in APOE*3-Leiden.CETP mice

Female APOE*3-Leiden.CETP mice were fed a Western-type diet with or without anacetrapib (30 mg/kg body weight per day) for 4 weeks. Mice received an injection with glycerol [^{3}H]TO and [^{14}C]CO double-labelled VLDL-mimicking particles. Blood was drawn at the indicated time points, and ^{3}H and ^{14}C plasma decay (A, C) and tissue-specific activity (B, D) were determined. The inlays in A and C show plasma half-life.

Data are presented as mean±SD (n=8/9 per group). ***P<0.001 when compared with control group.

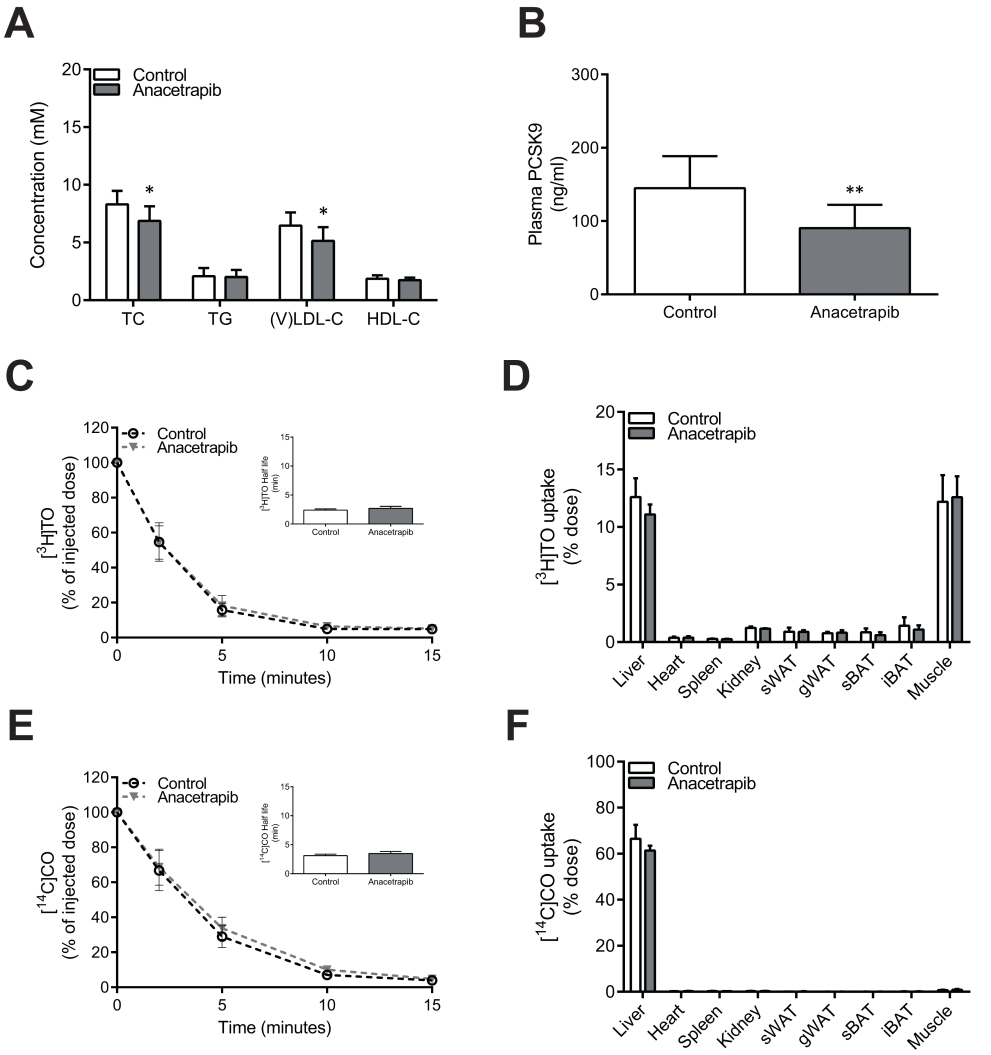

Figure 4: Anacetrapib decreases non-HDL-C and plasma Pcsk9 levels, without affecting lipoprotein remnant clearance in APOE*3-Leiden mice

Female APOE*3-Leiden mice were fed a Western-type diet with or without anacetrapib (30 mg/kg per day) for 4 weeks, blood was collected by tail bleeding after 4 h fasting, and plasma TC, TG, non-HDL-C, and HDL-C (A) and plasma Pcsk9 levels (B) were determined. After treatment mice received an injection with [^{3}H]TO and [^{14}C]CO double-labelled VLDL-mimicking particles. Blood was drawn at the indicated time points, and ^{3}H and ^{14}C plasma decay (C, E) and tissue-specific activity (D, F) were determined. The inlays in C and E show plasma half-life.

Data are presented as mean±SD (n=8/9 per group). *P<0.05 when compared with control group.

DISCUSSION

In this study, we investigated the mechanism by which anacetrapib reduces plasma (V)LDL-C and whether these effects are dependent on CETP. In APOE*3-Leiden.CETP mice, anacetrapib decreased gene expression of cholesterol biosynthesis pathways in the liver, most probably via inhibition of *Srebp-1* and/or *Srebp-2* signalling. In addition, we identified two important processes by which anacetrapib increases cholesterol clearance. First, anacetrapib increased cholesterol clearance by the liver, without affecting VLDL-TG production rate and clearance in APOE*3-Leiden.CETP mice. Second, in a CETP-independent manner, anacetrapib decreased hepatic *Pcsk9* expression and plasma Pcsk9 levels. In APOE*3-Leiden mice that do not express CETP, anacetrapib decreased (V)LDL-C and plasma Pcsk9 levels. However, no effects on cholesterol or VLDL-TG clearance were detected. These results indicate that CETP inhibition results in remodelled particles that are more susceptible for hepatic clearance.

The observed reduction in plasma Pcsk9 levels after anacetrapib treatment is in accordance with recent findings in rhesus macaques.[43] Here, we demonstrate that this effect is independent of CETP inhibition as this was also observed in APOE*3-Leiden mice without CETP. A recent study in C57Bl/6 mice also confirmed a CETP-independent decrease in plasma Pcsk9 levels by anacetrapib.[44] Pathway analyses of the gene expression data predicted that anacetrapib decreases liver cholesterol synthesis by reducing SREBP-2 regulated pathways. qPCR analysis of four SREBP-2 target genes (i.e., *Mvd*, *Dhcr7*, *Tm7sf2*, and *Pcsk9*) confirmed downregulation of genes in the cholesterol synthetic pathway. It is known that SREBP-2 is the principal nuclear transcription factor for the regulation of hepatic *Pcsk9* expression.[37] Therefore, the reduction of plasma Pcsk9 levels by anacetrapib may be attributed to the reduction of SREBP-2 pathway.

Accumulating evidence shows that inhibiting PCSK9 is an effective strategy to reduce LDL-C both in preclinical and clinical studies.[45, 46] This effect is attributed to a reduction of hepatic LDLr degradation and a subsequent increase of LDL remnant clearance. Our results showed an increase in hepatic LDLr protein levels in APOE*3-Leiden.CETP mice after 21 weeks of anacetrapib in parallel with a decrease in plasma Pcsk9 levels. Partially in contrast, we found after 4 weeks of anacetrapib treatment in APOE*3-Leiden mice with or without CETP expression, anacetrapib did not change hepatic LDLr protein levels (data not shown) despite a clear reduction of plasma Pcsk9 levels.

This discrepancy of anacetrapib affecting plasma Pcsk9 levels but not hepatic LDLr protein levels might be due to the duration of the treatment (i.e., 4 weeks vs. 21 weeks of treatment). Also, in C57BL/6 mice receiving anacetrapib for 1 week, anacetrapib reduced plasma Pcsk9 without affecting hepatic LDLr protein levels,[44] suggesting a time-dependent treatment effect of anacetrapib on Pcsk9 and hepatic LDLr protein. The mRNA expression of *Ldlr*, despite being a SREBP-2 target gene,[47] was not downregulated after anacetrapib treatment.

Apparently, SREBP-2 is not dominant for regulation of *Ldlr* mRNA expression under these conditions. In line with this contention, it is known that mouse liver cholesterol homeostasis is mainly dependent on endogenous cholesterol biosynthesis and to a lesser extent to on LDLr-mediated uptake of cholesterol.[48] Because PCSK9 has been reported to modulate LRP1 expression,[41] a backup receptor for (V)LDL clearance, we also measured LRP1 expression after anacetrapib treatment. No effect on either mRNA or protein expression of LRP1 was observed after 4 weeks or 21 weeks of anacetrapib treatment, suggesting that anacetrapib does not affect hepatic LRP1 level. Although the effects of anacetrapib in APOE*3-Leiden CETP mice closely resemble the effects in humans,[12, 22] the effects of anacetrapib on plasma PCSK9 and hepatic LDLr protein levels in humans remains to be determined.

The comparison of the clearance rates of VLDL remnants in APOE*3-Leiden mice with and without CETP indicates that the presence of CETP results in a decreased remnant particle clearance. This implies that inhibiting the activity of CETP alone is sufficient to increase lipoprotein remnant clearance in APOE*3-Leiden.CETP mice. The main activity of CETP is to transfer cholesteryl esters from HDL to (V)LDL in exchange for TG. Apparently, this lipoprotein remodelling activity of CETP renders the (V)LDL less susceptible to clearance. In APOE*3-Leiden.CETP mice, anacetrapib treatment results in an increase of plasma ApoE levels (+59%, $P<0.001$; data not shown), which is indicative for lipoprotein remodelling. Previously, we have shown the importance of a functional ApoE-LDLr pathway in the clearance of remnants from plasma and, subsequently, in the development of atherosclerosis.[23, 42] Interestingly, anacetrapib treatment in humans has also been shown to increase plasma ApoE levels.[12]

Although the direct effects of CETP inhibition in humans on lipoprotein remnant clearance have not been described, treatment with the CETP-inhibitor torcetrapib has been shown to increase the fractional catabolic rate of both VLDL-apoE[49] and VLDL-apoB100.[50] However, the potentially increased catabolism of (V)LDL and thus antiatherogenic properties were clearly not sufficient to offset or overrule the adverse side effects of torcetrapib.

Microarray analyses also revealed that anacetrapib activates genes regulated by NR1I2 and NR1I3. These transcription factors function as sensors of both toxic and xenobiotic exogenous compounds and toxic products derived from endogenous metabolism and activate pathways to eliminate these products.[51] In our study, activation of these receptors indicated that anacetrapib was recognized as a xenobiotic product by the liver. It is not yet known whether this has consequences beyond activation of elimination pathways and whether this will have clinical implications. Phase I/II clinical studies evaluating the effects of anacetrapib reported an acceptable side-effect profile.[12, 52-54] However, it should be noted that a nonsignificant increase in C-reactive protein was found after anacetrapib treatment.[52] We also found elevated serum amyloid A levels in anacetrapib-treated APOE*3-Leiden.CETP mice.[22]

In the present study, we present evidence that anacetrapib reduces (V)LDL-C by two mechanisms: 1) inhibition of CETP activity, resulting in remodelled lipoproteins that are more susceptible to clearance; and 2) a CETP-independent reduction of plasma Pcsk9 levels that has the potential to increase LDLr-mediated clearance. This reduction in (V)LDL-C is the crucial factor mediating the atheroprotective effects of anacetrapib in APOE*3-Leiden.CETP mice.[22] Whether the additional beneficial effects of anacetrapib on top of a statin translate into clinical benefit in humans will be elucidated in the current phase III REVEAL trial.[16]

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SUPPLEMENTAL DATA

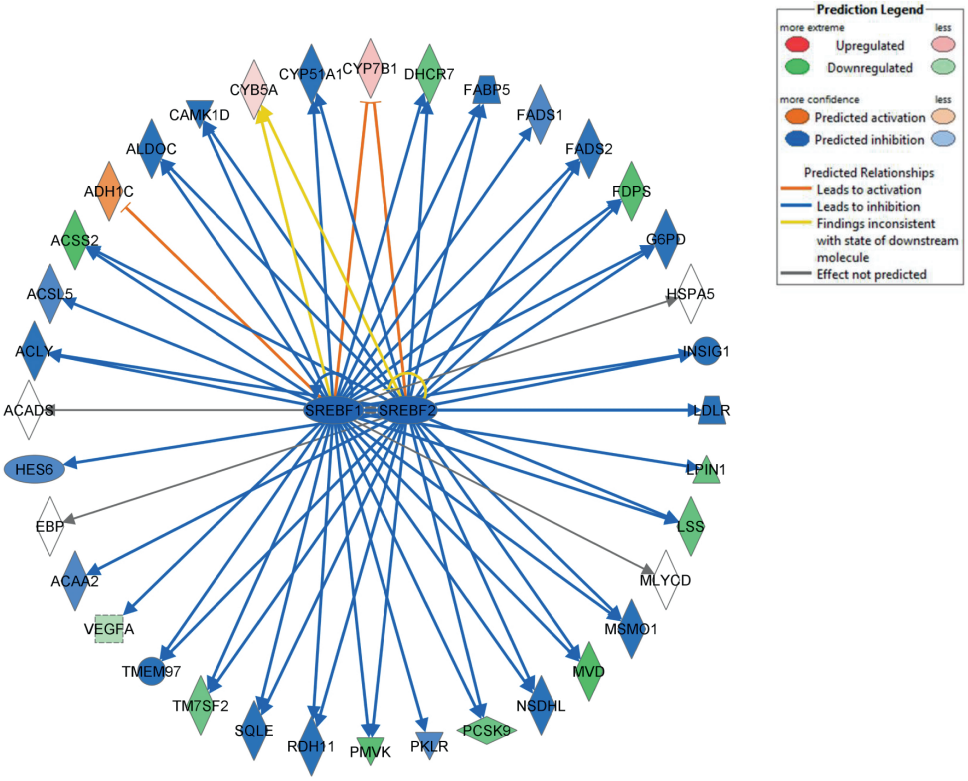

Figure S1: Regulation of sterol regulatory element-binding protein gene (*Srebf*) -1 and *Srebf*-2-related genes by anacetrapib

Molecular network showing *Srebf*-1 and -2-related gene regulation of anacetrapib. The molecular network consists of genes that are differentially expressed after anacetrapib treatment (cut off: P<0.01) and are coloured red if upregulated or green if downregulated (cut off: FDR<0.05). An in silico prediction based on the differentially expressed target genes was performed to indicate *Srebf*-1 and -2 activation state. Predicted regulation of genes is indicated by orange if they are predicted to be upregulated and blue if predicted to be downregulated.

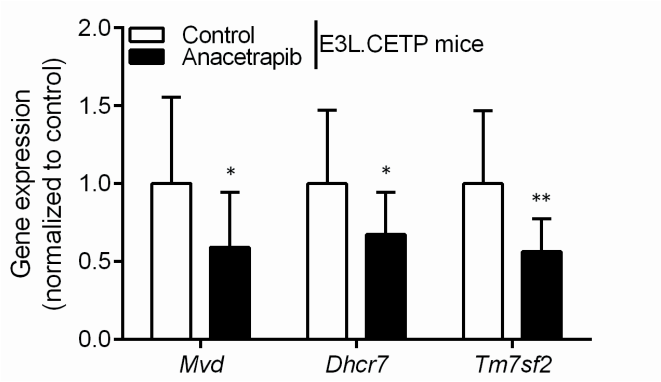

Figure S2: Hepatic expression of SREPB-2 target genes
Female E3L.CETP mice were fed a WTD with or without (control) anacetrapib (30 mg/kg body weight/d) for 21 weeks and livers were collected for mRNA isolation. Hepatic expression of the SREPB-2 target genes mevalonate (diphospho) decarboxylase (*Mvd*), 7-dehydrocholesterol reductase (*Dhcr7*) and transmembrane 7 superfamily member 2 (*Tm7sf2*) were determined in order to validate microarray results.
Data are presented as mean±SD (n=14/15 per group). *P<0.05, **P<0.01 when compared with control group.

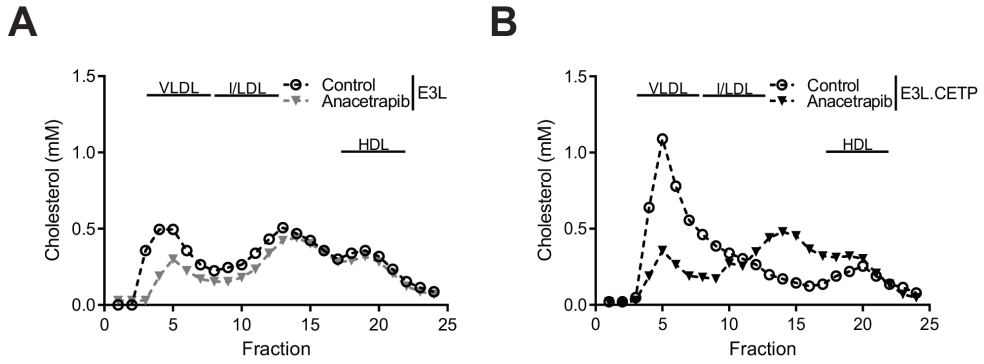

Figure S3: Lipoprotein profiles for cholesterol
Female E3L.CETP and E3L mice were fed a WTD with or without (control) anacetrapib (30 mg/kg body weight/d) for 4 weeks, and blood was collected by tail bleeding after 4h fasting to determine cholesterol lipoprotein profiles. The effects of 4 weeks of anacetrapib treatment on the distribution of cholesterol over lipoproteins in E3L.CETP mice (A) and E3L mice (B) were assessed by FPLC.

Table S1: Differentially expressed genes after 22 weeks of anacetrapib treatment.

Gene Name	Gene Symbol	Fold Change	Adjusted P-value
cytochrome P450, family 3, subfamily a, polypeptide 11	*Cyp3a11*	5.649	2.52E-08
cytochrome P450, family 2, subfamily b, polypeptide 10	*Cyp2b10*	103.604	2.09E-07
cytochrome P450, family 3, subfamily a, polypeptide 25	*Cyp3a25*	3.724	2.81E-05
cytochrome P450, family 2, subfamily c, polypeptide 29	*Cyp2c29*	10.133	2.93E-05
cytochrome P450, family 2. subfamily c, polypeptide 37	*Cyp2c37*	2.427	1.71E-04
sodium channel, nonvoltage-gated 1 alpha	*Scnn1a*	-3.941	2.43E-04
cytochrome P450, family 2, subfamily c, polypeptide 50	*Cyp2c50*	2.412	3.25E-04
aldehyde dehydrogenase family 1, subfamily A1	*Aldh1a1*	3.939	1.36E-03
cathepsin A	*Ctsa*	-4.641	1.36E-03
phospholipase A1 member A	*Pla1a*	-2.357	1.36E-03
leucine rich repeat containing 14B	*Lrrc14b*	5.494	1.80E-03
transmembrane protein 33	*Tmem33*	1.839	1.80E-03
breast cancer anti-estrogen resistance 1	*Bcar1*	2.217	2.60E-03
ectonucleoside triphosphate diphosphohydrolase 5	*Entpd5*	2.268	4.21E-03
glypican 1	*Gpc1*	5.006	5.04E-03
heme binding protein 1	*Hebp1*	-3.970	5.04E-03
transmembrane 7 superfamily member 2	*Tm7sf2*	-4.176	6.35E-03
7-dehydrocholesterol reductase	*Dhcr7*	-4.797	6.93E-03
cytochrome c oxidase subunit VIIa 1	*Cox7a1*	-4.018	7.26E-03
hosphomevalonate kinase	*Pmvk*	-6.690	7.84E-03
phosphate cytidylyltransferase 2, ethanolamine	*Pcyt2*	-2.142	8.66E-03
acetyl-Coenzyme A acetyltransferase 2	*Acat2*	-5.139	1.00E-02
heat shock protein 1	*Hspb1*	4.339	1.00E-02
serine dehydratase	*Sds*	-3.830	1.00E-02
cytochrome P450, family 2, subfamily d, polypeptide 9	*Cyp2d9*	9.285	1.06E-02
choline phosphotransferase 1	*Chpt1*	2.217	1.09E-02
RIKEN cDNA 9130409I23 gene	*9130409I23Rik*	7.743	1.11E-02
cytochrome P450, family 2, subfamily c, polypeptide 55	*Cyp2c55*	10.108	1.24E-02
cytochrome P450, family 7, subfamily b, polypeptide 1	*Cyp7b1*	2.965	1.24E-02
GM2 ganglioside activator protein	*Gm2a*	-1.804	1.24E-02
interleukin 11 receptor, alpha chain 1	*Il11ra1*	-2.312	1.31E-02
farnesyl diphosphate synthetase	*Fdps*	-7.399	1.35E-02
NFKB inhibitor interacting Ras-like protein 2	*Nkiras2*	-2.175	1.35E-02
PDZK1 interacting protein 1	*Pdzk1ip1*	-6.021	1.35E-02
low density lipoprotein receptor-related protein associated protein 1	*Lrpap1*	-2.775	1.55E-02
testis expressed gene 264	*Tex264*	1.913	1.55E-02
proprotein convertase subtilisin/kexin type 9	*Pcsk9*	-4.460	1.58E-02
C-type lectin domain family 4, member b1	*Clec4b1*	2.807	1.64E-02
nuclear receptor binding protein 2	*Nrbp2*	-2.046	1.73E-02
olfactory receptor 194	*Olfr194*	4.830	1.76E-02
predicted gene 5922	*Gm5922*	7.427	1.84E-02
RIKEN cDNA 0610012H03 gene	*0610012H03Rik*	2.458	1.94E-02
DEAQ RNA-dependent ATPase	*Dqx1*	-3.873	1.94E-02
cytochrome b-5	*Cyb5*	1.863	2.07E-02
replication factor C (activator 1) 5	*Rfc5*	-2.150	2.37E-02
vitelline membrane outer layer 1 homolog (chicken)	*Vmo1*	-1.908	2.67E-02
fasciculation and elongation protein zeta 2 (zygin II)	*Fez2*	1.651	2.73E-02
vomeronasal 1 receptor 63	*Vmn1r63*	4.944	2.73E-02

proteasome (prosome, macropain) 26S subunit, ATPase, 4	*Psmc4*	1.646	2.73E-02
proteasome (prosome, macropain) subunit, beta type 7	*Psmb7*	1.689	2.75E-02
cysteine conjugate-beta lyase 1	*Ccbl1*	-2.433	2.75E-02
ubiquitin-conjugating enzyme E2F (putative)	*Ube2f*	1.853	2.76E-02
RIKEN cDNA 1700034O15 gene	*1700034O15Rik*	3.533	3.02E-02
RIKEN cDNA 4931406C07 gene	*4931406C07Rik*	2.043	3.02E-02
glycine-N-acyltransferase	*Glyat*	1.651	3.02E-02
six transmembrane epithelial antigen of prostate 2	*Steap2*	-2.563	3.02E-02
TLC domain containing 1	*Tlcd1*	-2.980	3.02E-02
zinc finger, AN1-type domain 2A	*Zfand2a*	1.990	3.02E-02
cleft lip and palate associated transmembrane protein 1	*Clptm1*	-1.859	3.14E-02
interleukin 1 receptor accessory protein	*Il1rap*	-1.884	3.14E-02
annexin A6	*Anxa6*	-1.747	3.32E-02
cDNA sequence BC021614	*BC021614*	-2.597	3.32E-02
branched chain aminotransferase 2, mitochondrial	*Bcat2*	-2.513	3.32E-02
prune homolog (Drosophila)	*Prune*	1.950	3.47E-02
reversion-inducing-cysteine-rich protein with kazal motifs	*Reck*	-2.113	3.47E-02
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	*Abcc3*	3.802	3.50E-02
biliverdin reductase B (flavin reductase (NADPH))	*Blvrb*	1.968	3.50E-02
lipin 1	*Lpin1*	-5.103	3.50E-02
upstream transcription factor 2	*Usf2*	-2.389	3.50E-02
vascular endothelial growth factor A	*Vegfa*	-1.824	3.50E-02
cleavage and polyadenylation specific factor 1	*Cpsf1*	-2.048	3.56E-02
carboxylesterase 2A	*Ces2a*	4.824	3.59E-02
PHD finger protein 2	*Phf2*	-2.171	4.17E-02
argininosuccinate synthetase 1	*Ass1*	-2.753	4.23E-02
histone cluster 1, H2bk	*Hist1h2bk*	1.871	4.25E-02
aminolevulinic acid synthase 1	*Alas1*	3.070	4.64E-02
interferon, alpha-inducible protein 27 like 2B	*Ifi27l2b*	3.620	4.64E-02
lanosterol synthase	*Lss*	-5.130	4.64E-02
MACRO domain containing 1	*Macrod1*	-2.309	4.64E-02
mevalonate (diphospho) decarboxylase	*Mvd*	-9.687	4.64E-02
RAN GTPase activating protein 1	*Rangap1*	2.048	4.64E-02
epoxide hydrolase 1, microsomal	*Ephx1*	2.595	4.80E-02
EGF-like module containing, mucin-like, hormone receptor-like sequence 1	*Emr1*	3.535	4.91E-02
hypoxanthine guanine phosphoribosyl transferase	*Hprt*	2.247	4.93E-02
RIKEN cDNA 1600016N20 gene	*1600016N20Rik*	-2.797	4.95E-02
valosin containing protein	*Vcp*	1.573	4.95E-02
acid phosphatase, prostate	*Acpp*	-11.171	4.98E-02
acyl-CoA synthetase short-chain family member 2	*Acss2*	-9.253	4.98E-02
coagulation factor XII (Hageman factor)	*F12*	-1.529	4.98E-02
family with sequence similarity 213, member A	*Fam213a*	-1.987	4.98E-02
myotubularin related protein 11	*Mtmr11*	-3.126	4.98E-02
neurocalcin delta	*Ncald*	2.432	4.98E-02
phospholipid transfer protein	*Pltp*	-3.541	4.98E-02
pyrroline-5-carboxylate reductase-like	*Pycrl*	-1.688	4.98E-02
small G protein signaling modulator 1	*Sgsm1*	-3.306	4.98E-02

Female APOE*3-Leiden.CETP mice were fed a Western-type diet with or without anacetrapib (30 mg/kg bw/d) for 22 weeks. RNA was isolated from liver tissue and a microarray analysis was performed. Fold change is the change in expression for anacetrapib versus control treatment.

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