CHAPTER 5

Anacetrapib Reduces (V)LDL-Cholesterol by Inhibition of CETP Activity and Reduction of Plasma PCSK9

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Abstract

Objectives Recently, we showed in APOE*3-Leiden.CETP mice that anacetrapib attenuated atherosclerosis development by reducing (V)LDL-C rather than by raising HDL-C. Here, we investigated the mechanism by which anacetrapib reduces (V)LDL-C and whether this effect was dependent on the inhibition of CETP.

Approach and Results APOE*3-Leiden.CETP mice were fed a Western type diet alone or supplemented with anacetrapib (30 mg/kg bw/d). Microarray analyses of livers revealed down regulation of the cholesterol biosynthesis pathway (P<0.001) and predicted down regulation of sterol regulatory element-binding protein-1 and -2 controlled pathways (z-score -2.56 and z-score -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 protein 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 emulsion 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.

Conclusions Anacetrapib reduces (V)LDL-C by two mechanisms: 1) inhibition of CETP activity, resulting in remodelled 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 LDL receptor-mediated hepatic remnant clearance.

Keywords CETP, Cholesterol/Metabolism, Drug therapy/Hypolipidemic drugs, LDL/Metabolism, Lipids, Lipoproteins/Metabolism, PCSK9

Introduction

High plasma levels of (very) low-density lipoprotein [(V)LDL]-cholesterol (C) and triglycerides (TG), as well as low levels of high-density lipoprotein (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 of 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 (E3L) 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]; +129%,[13] respectively) and to reduce LDL-C (down to -25%[11]; -40%[12]; -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 pro-inflammatory 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 to torcetrapib and dalcetrapib.

Recently, we have shown that anacetrapib treatment increased HDL-C and reduced (V) LDL-C and TG, and dose-dependently reduced atherosclerotic lesion size and severity in APOE*3-Leiden.CETP (E3L.CETP) mice, a well-established mouse model for hyperlipidemia and atherosclerosis with a human-like lipoprotein metabolism.[17-19] Analysis of covariance showed that the effect on lesion size was mainly explained by a reduction in (V)LDL-C.[20] 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 emulsion particles. By using E3L mice with or without CETP expression,[7] we also determined whether these effects of anacetrapib were CETP-dependent.

Material and Methods

RNA isolation, microarray and qPCR validation

Liver pieces were obtained from a previous experiment performed by Kühnast and Van der Tuin *et al.*,[20] investigating the effects of anacetrapib on atherosclerosis in female E3L.CETP mice. In this study, mice were treated with a semi-synthetic cholesterol-rich diet, containing 15% (w/w) cacao butter, 1% corn oil and 0.1% cholesterol (Western-type diet; AB-Diets, Woerden, the Netherlands) with or without anacetrapib (30 mg/kg bw/d) for 21 weeks. Total RNA was extracted from these liver pieces using the Nucleospin RNAII kit (Macherey-Nagel) according to 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 real time quantitative PCR (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 to 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 package21 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.22 The calculated P-values <0.05 were used as a threshold for pathway analyses using Ingenuity Pathway Analysis suite (www.ingenuity.com, accessed 2013). 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 set-up

To investigate the effects of anacetrapib on VLDL production and clearance, female E3L[23] and E3L.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 bw/d) for four weeks after which VLDL production (E3L.CETP only) or clearance (E3L and E3L.CETP) was determined. After both experiments the mice were sacrificed by CO_2 asphyxiation. The mice were housed under standard conditions with a 12 hour 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 hour 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 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.[20]

Hepatic LDLr protein and plasma PCSK9 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 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 (1:1000, AF2255, R&D Systems) 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 manufacturer's protocol and quantified using Image J software as previously described.[24] Plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) was measured by using ELISA (MCP900, R&D Systems) according to 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-hour 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.[25] 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 sacrificed and blood was collected for isolation of the VLDL fraction by density gradient ultracentrifugation.[26] Tran^{35}S-activity was measured in the VLDL fraction and VLDL-apoB production rate was calculated as dpm/h.[27]

Clearance analysis of VLDL-mimicking emulsion particles

Glycerol tri(9,10(n)[^{3}H]oleate ([^{3}H]TO) and [1α,2α (n)-^{14}C]cholesteryl oleate ([^{14}C]CO) double-radiolabeled VLDL-mimicking emulsion particles (mean diameter 80 nm) were prepared as previously described.[28] After a 4 hour fast, particles were injected via the tail vein in conscious mice (n= 8/9 per group). At 2, 5, 10, and 15 min post-injection, blood was taken to determine the plasma decay of [^{3}H]TO and [^{14}C]CO. Plasma volumes were calculated as 0.04706 x body weight (g) as described.[29] Mice were sacrificed 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 analysis

Significance of differences between the groups was calculated non-parametrically using a Mann-Whitney U test. All reported p-values are two-tailed, and p-values of less than 0.05 were considered statistically significant.

Results

Pathway analyses predict down regulation of sterol regulatory element-binding protein-1 and -2 controlled pathways by anacetrapib.

To determine the effects of anacetrapib treatment on hepatic gene expression in E3L.CETP mice,[20] microarray analyses were performed. A total of 95 genes (FDR P<0.05; Supplemental Table I) were differentially expressed between control and anacetrapib-treated female mice of which 46 genes were up regulated and 49 genes were down regulated. 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 sterol regulatory element-binding protein (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. Furthermore, anacetrapib activated genes regulated by nuclear receptor subfamily 1, group I, member 2 (P<0.001; z-score +2.75) and member 3 (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.

Table 1: Significantly regulated pathways.

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Superpathway of Cholesterol Biosynthesis	10.50	0.24
PXR/RXR Activation	6.32	0.09
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	6.00	0.22
Bupropion Degradation	5.39	0.16
LPS/IL-1 Mediated Inhibition of RXR Function	5.28	0.04
Cholesterol Biosynthesis I	4.65	0.23
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	4.65	0.23
Cholesterol Biosynthesis III (via Desmosterol)	4.65	0.23
Isoleucine Degradation I	4.54	0.21
Mevalonate Pathway I	4.54	0.21

Female E3L.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. Selected differentially expressed genes (95 genes, see Table I) were used as input for pathway analysis through ingenuity pathway analysis suite.

Table 2: *In silico* prediction of transcription factor activity based on the expression changes of known target genes

Upstream Regulator	Molecule Type	Activation State	Z-score	p-value of overlap
Sterol regulatory element-binding protein 2 (SREBP2)	transcription regulator	Inhibited	-2.56	1.65E-13
Sterol regulatory element-binding protein 1 (SREBP1)	transcription regulator	Inhibited	-2.90	6.56E-12
Nuclear receptor subfamily 1, group I, member 1 (NR1I3)	ligand-dependent nuclear receptor	Activated	2.94	9.27E-12
Peroxisome proliferator-activated receptor alpha (PPARA)	ligand-dependent nuclear receptor	-	-0.24	6.74E-10
Nuclear receptor subfamily 1, group I, member 2 (NR1I2)	ligand-dependent nuclear receptor	Activated	2.75	6.01E-09
PXR ligand-PXR-Retinoic acid-RXRα	complex	Activated	2.42	1.34E-08
CAR ligand-CAR-Retinoic acid-RXRα	complex	Activated	2.22	1.71E-08
Ncoa-Nr1i3-Rxra	complex	Activated	2.00	6.83E-07
NAD-dependent deacetylase sirtuin-2 (SIRT2)	transcription regulator	Inhibited	-2.00	6.83E-07
Ncoa-Nr1i2-Rxra	complex	Activated	2.00	8.63E-07

To determine the activation status of transcription factors an upstream regulator analysis was performed. The z-score indicates activation (positive) or inhibition (negative).

Anacetrapib decreases hepatic and circulating PCSK9, and increases hepatic LDLr protein in E3L.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 (Supplemental Figure I),[30] in the liver of anacetrapib-treated E3L.CETP mice (-78%, P<0.05; Supplemental Table I), which was confirmed by qPCR (-27%, P<0.01; **Figure 1A**). In accordance, anacetrapib reduced plasma PCSK9 levels (-47%, P<0.01; **Figure 1B**). Since PCSK9 plays an important role in the degradation of intracellular LDL receptor (LDLr),[31-33] hepatic LDLr mRNA expression and protein levels were measured. Anacetrapib did not affect the hepatic *Ldlr* expression (**Figure 1C**), but did increase hepatic LDLr protein levels (+64%, P<0.05; **Figure 1D**). The decrease in plasma PCSK9 levels and increase in LDLr suggest an increased capability of the liver to take up lipoprotein remnants.

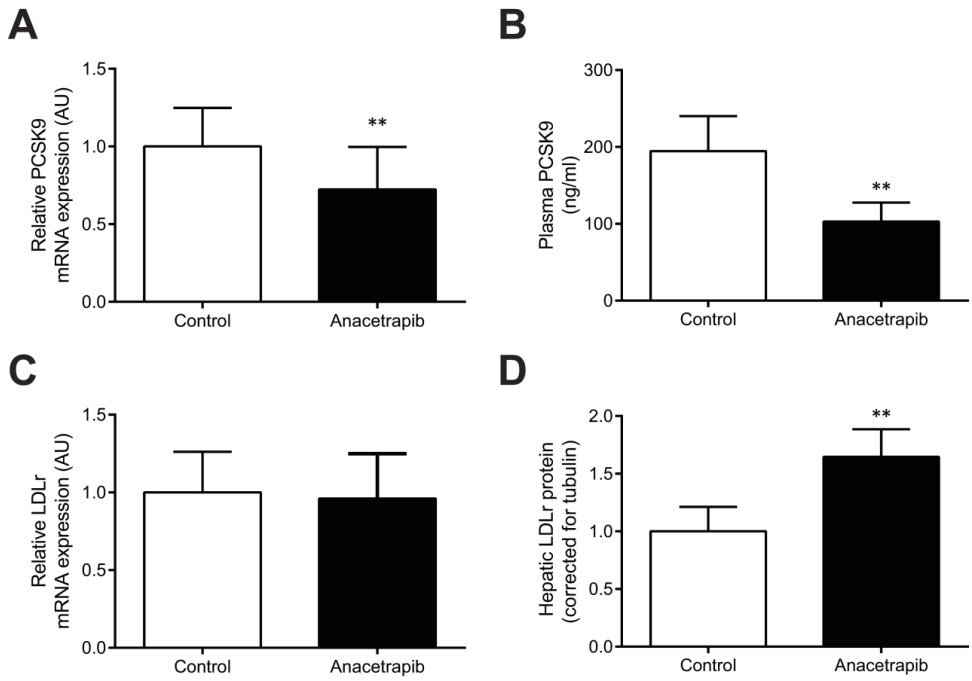

Figure 1. Anacetrapib decreases PCSK9 mRNA expression and plasma levels and increases hepatic LDLr protein levels in E3L.CETP mice.

Female E3L.CETP mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) 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) levels.

Data are presented as means ± SD (n= 14/15 per group). * P<0.05, ** P<0.01, ***P<0.001 when compared with control group.

Anacetrapib does not affect triglyceride metabolism, but increases lipoprotein remnant clearance by the liver in E3L.CETP mice

To further investigate the effects of anacetrapib on lipoprotein metabolism, we performed a new experiment with female E3L.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** (data not shown). Anacetrapib treatment did not affect the VLDL-TG production rate (**Figure 2B**), the VLDL-^{35}S-apoB production rate (**Figure 2C**) nor the ratio of VLDL-TG to VLDL-apoB production rate (**Figure 2D**), indicating no changes in number or composition of newly synthesized VLDL particles.

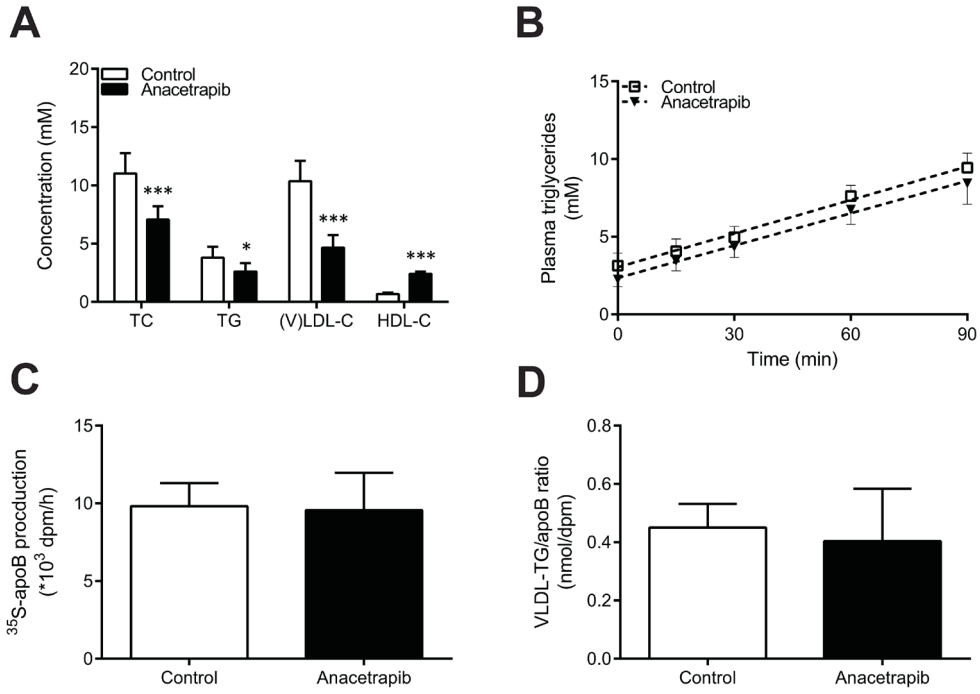

Figure 2. Anacetrapib does not affect hepatic VLDL-TG production in E3L.CETP mice.
Female E3L.CETP mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) for 4 weeks, blood was collected by tail bleeding after 4h fasting, and plasma TC, TG, (V)LDL-C and HDL-C were determined (A). After treatment, 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 means ± SD (n= 8/9 per group). * P<0.05, ***P<0.001 when compared with control group.

The effect of anacetrapib on VLDL clearance was assessed by an intravenous injection of glycerol tri(9,10(n)[^{3}H]oleate ([^{3}H]TO) and [1α,2α (n)-^{14}C]cholesteryl oleate ([^{14}C]CO) double-radiolabeled VLDL-mimicking emulsion particles. At indicated time points, blood was taken to determine clearance from plasma. After 15 min, mice were sacrificed 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 (**Figure 3A**) or the tissue specific uptake of [^{3}H]TO-derived activity (**Figure 3B**). However, anacetrapib increased the plasma clearance of the [^{14}C]CO label of the VLDL-mimicking emulsion particles (**Figure 3C**), decreased plasma half-life of [^{14}C]CO (-56%, P<0.001; **Figure 3C** inlay) and increased the uptake of [^{14}C]CO by the liver (+25%, P<0.001; **Figure 3D**). Since these particles reflect the behaviour of VLDL,[34] these results indicate that anacetrapib increases the uptake of lipoprotein remnants by the liver.

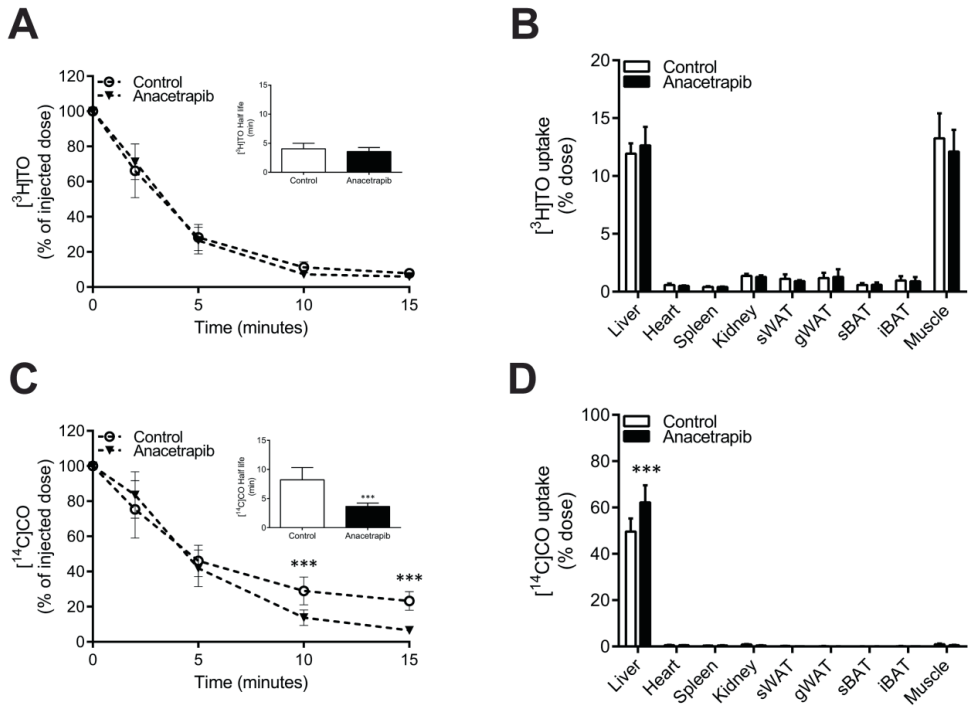

Figure 3. Anacetrapib increases lipoprotein remnant clearance by the liver in E3L.CETP mice.
Female E3L.CETP mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) for 4 weeks. Mice received an injection with glycerol tri[^{3}H]oleate- and [^{14}C]cholesteryl oleate-double labelled VLDL-mimicking emulsion particles. Blood was drawn at the indicated time points and ^{3}H and ^{14}C plasma decay (A and C) and tissue specific activity (B and D) were determined. The inlay in figure A and C show plasma half-life.
Data are presented as means ± SD (n= 8/9 per group). ***P<0.001 when compared with control group.

Anacetrapib decreases (V)LDL-C and PCSK9 levels in E3L mice

To determine whether the effects of anacetrapib on (V)LDL metabolism were dependent on CETP inhibition, similar experiments were performed in female E3L mice that do not express CETP. Notably, anacetrapib reduced TC (-17%, $P<0.05$) and (V)LDL-C (-20%, $P<0.05$; **Figure 4A**) levels in E3L mice without CETP, concomitantly with a decrease in hepatic *Pcsk9* expression and plasma PCSK9 levels (-37%, $P<0.05$; **Figure 4B**). These data clearly show that anacetrapib has a CETP-independent lipid-lowering effect. Comparing E3L with E3L.CETP mice, anacetrapib increased the particle clearance in E3L.CETP mice to the similar level as observed in E3L mice without anacetrapib (**Figure 3C and 4C**). Anacetrapib showed no additional effects on the plasma clearance of [^{3}H]TO and [^{14}C]CO (**Figure 4C and 4E**) labels of the VLDL-mimicking emulsion particles, nor the tissue specific uptake of [^{3}H]TO and [^{14}C]CO (**Figure 4D and 4F**) in E3L mice.

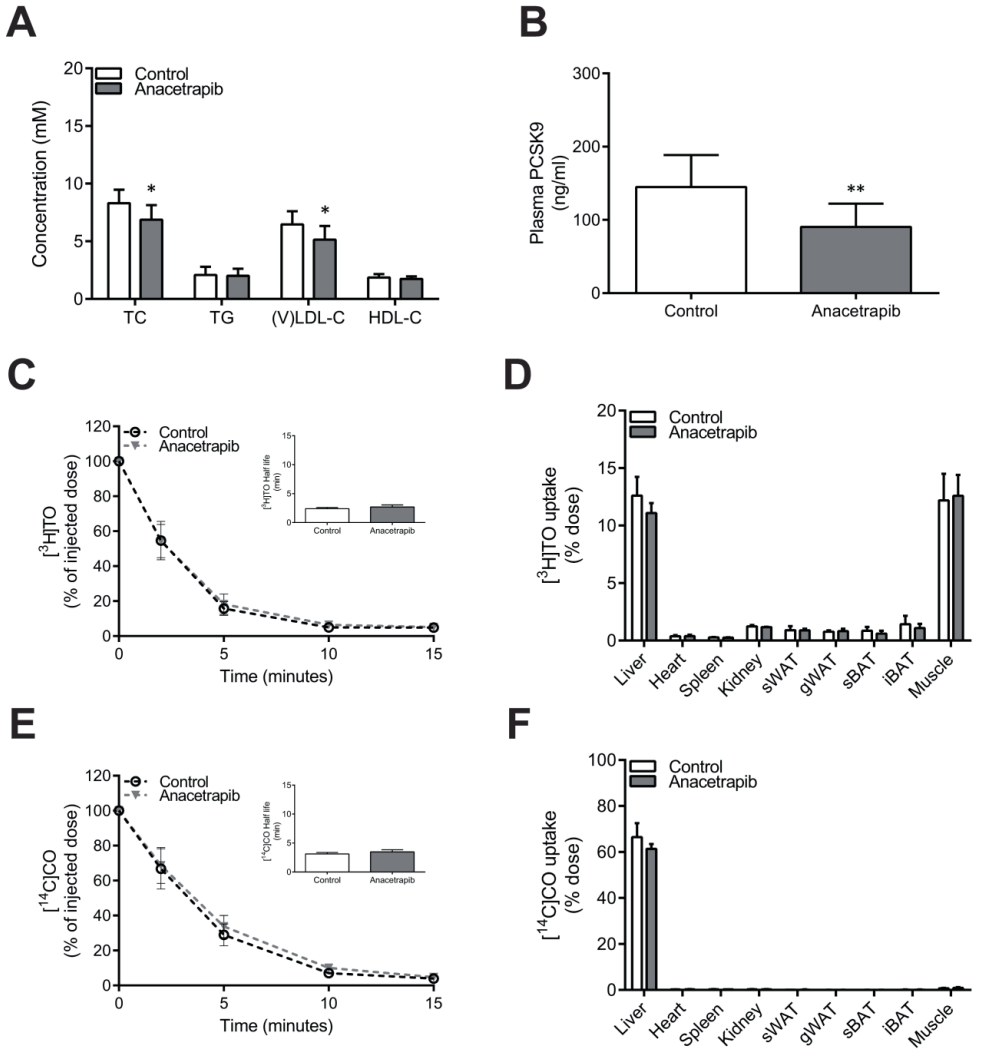

Figure 4. Anacetrapib decreases (V)LDL-C and plasma PCSK9 levels, without affecting lipoprotein remnant clearance in E3L mice.

Female E3L mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) for 4 weeks, blood was collected by tail bleeding after 4h fasting, and plasma TC, TG, (V)LDL-C, HDL-C (A) and plasma PCSK9 levels (B) were determined. After treatment mice received an injection with glycerol tri[^{3}H]oleate- and [^{14}C]cholesteryl oleate-double labelled VLDL-mimicking emulsion particles. Blood was drawn at the indicated time points and ^{3}H and ^{14}C plasma decay (C and E) and tissue specific activity (D and F) were determined. The inlay in figure A and E show plasma half-life.

Data are presented as means ± 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 E3L.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 E3L.CETP mice. Secondly, in a CETP-independent manner, anacetrapib decreased hepatic *Pcsk9* expression and plasma PCSK9 levels. In E3L 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.[35] Here, we demonstrate that this effect is independent of CETP inhibition as this was also observed in E3L mice without CETP. A recent study in C57Bl/6 mice also confirmed a CETP-independent decrease in plasma PCSK9 levels by anacetrapib.[36] Pathway analyses of the gene expression data predicted that anacetrapib decreases liver cholesterol synthesis by reducing SREBP-2 regulated pathways. It is known that SREBP-2 is the principal nuclear transcription factor for the regulation of hepatic *Pcsk9* expression.[30] 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.[37, 38] 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 E3L.CETP mice after 21 weeks of anacetrapib in parallel with a decrease in plasma PCSK9 levels. Partially in contrast, we found that after 4 weeks of anacetrapib treatment in E3L 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 (Figure 4B). 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 versus 21 weeks of treatment. Indeed, in the study in C57Bl/6 mice receiving anacetrapib for 1 week, anacetrapib reduced both plasma PCSK9 and hepatic LDLr protein levels,[36] suggesting a treatment time-dependent effect of anacetrapib on PCSK9 and LDLr. Although the effects of anacetrapib in E3L.CETP mice closely resemble the effects in humans,[12, 20] the effects of anacetrapib on plasma PCSK9 and hepatic LDLr protein levels in humans remain to be determined.

The comparison of the clearance rates of VLDL remnants in E3L mice with and without CETP (Figures 3C and 4C) 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 E3L.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 E3L.CETP mice, anacetrapib treatment results in an increase of plasma apolipoprotein E levels (+59%, P<0.001, data not shown), which is indicative for lipoprotein remodelling. Interestingly, anacetrapib treatment in humans has also been shown to increase plasma apolipoprotein E levels.[12]

Although the direct effects of CETP inhibition on lipoprotein remnant clearance in humans have not been described, treatment with the CETP-inhibitor torcetrapib has been shown to increase the fractional catabolic rate of both VLDL-apoE[39] and VLDL-apoB100.[40] However, the potentially increased catabolism of (V)LDL and thus anti-atherogenic 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 the nuclear receptor subfamily 1, group I (NR1I), members 2 and 3. 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.[41] 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, 42-44] However, it should be noted that a non-significant increase in C-reactive protein was found after anacetrapib treatment.[42] We also found elevated SAA levels in anacetrapib-treated E3L.CETP mice.[20]

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 E3L.CETP mice.[20] 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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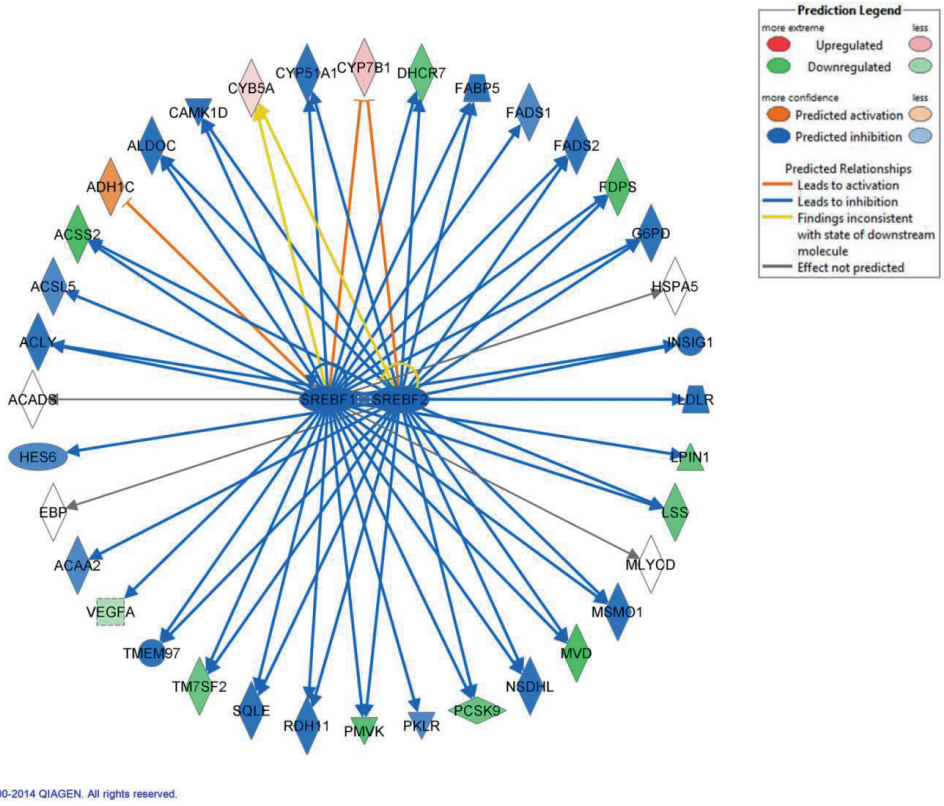
Supplemental Table I: Differentially expressed genes after 22 weeks of anacetrapib treatment.

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

Gene Name	Gene Symbol	Fold Change	Adjusted P-value
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 E3L.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.



Supplemental Figure I. 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.