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Anacetrapib Reduces Progression of Atherosclerosis, Mainly by Reducing non-HDL-cholesterol, Improves Lesion Stability and Adds to the Beneficial Effects of Atorvastatin

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Abstract

Objective The residual risk that remains after statin treatment supports the addition of other LDL-C-lowering agents and has stimulated the search for secondary treatment targets. Epidemiological studies propose HDL-C as a possible candidate. Cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from atheroprotective HDL to atherogenic (V) LDL. The CETP inhibitor anacetrapib decreases (V)LDL-C by ~15-40% and increases HDL-C by ~40-140% in clinical trials. We evaluated the effects of a broad dose range of anacetrapib on atherosclerosis and HDL function, and examined possible additive/synergistic effects of anacetrapib on top of atorvastatin in APOE*3Leiden.CETP mice.

Methods and Results Mice were fed a diet without or with ascending dosages of anacetrapib (0.03; 0.3; 3; 30 mg/kg/d), atorvastatin (2.4 mg/kg/d) alone or in combination with anacetrapib (0.3 mg/kg/d) for 21 weeks. Anacetrapib dose-dependently reduced CETP activity (-59% to -100%, $P<0.001$), thereby decreasing non-HDL-C (-24% to -45%, $P<0.001$) and increasing HDL-C (+30% to +86%, $P<0.001$). Anacetrapib dose-dependently reduced atherosclerotic lesion area (-41% to -92%, $P<0.01$) and severity, increased plaque stability index and added to the effects of atorvastatin by further decreasing lesion size (-95%, $P<0.001$) and severity. Analysis of covariance showed that both anacetrapib ($P<0.05$) and non-HDL-C ($P<0.001$), but not HDL-C ($P=0.76$), independently determined lesion size.

Conclusion Anacetrapib dose-dependently reduces atherosclerosis, and adds to the anti-atherogenic effects of atorvastatin, which is mainly ascribed to a reduction in non-HDL-C. In addition, anacetrapib improves lesion stability.

Keywords cholesteryl ester transfer protein, non-HDL-cholesterol, HDL-cholesterol, HDL function, atherosclerosis, anacetrapib, atorvastatin

Introduction

Intervention trials provide ample evidence that lowering of low-density lipoprotein-cholesterol (LDL-C) contributes to a reduction in cardiovascular (CV) risk.¹⁻³ However, the residual risk that remains after statin treatment, as well as failure for some patients to reach recommended LDL-C targets despite statin treatment, support the addition of other LDL-C-lowering agents and also stimulate the search for secondary treatment targets.^{3, 4} Prospective epidemiological studies propose high-density lipoprotein (HDL)-C as a potential target.⁵ Cholesteryl ester transfer protein (CETP) plays an important role in lipid metabolism by facilitating the transfer of cholesteryl esters from atheroprotective HDL to atherogenic (V)LDL in exchange for triglycerides (TG), and inhibition of CETP activity has been proposed as a therapeutic way to increase HDL-C levels.⁶⁻¹¹

In mouse models for atherosclerosis, CETP expression aggravated atherosclerosis development.^{12, 13} Most but not all studies in rabbits and mice showed that CETP inhibition reduced atherosclerosis development.¹⁴⁻¹⁹ However, torcetrapib failed to enhance the anti-atherogenic effects of atorvastatin and induced a pro-inflammatory, vulnerable plaque phenotype in APOE*3Leiden.CETP mice.¹⁹ In the large clinical outcome trial (ILLUMINATE), torcetrapib increased the risk of major CV events and mortality despite a 72% increase in HDL-C and a 25% reduction in LDL-C.²⁰ The unexpected detrimental effects were ascribed to either an off-target blood pressure effect or the possible generation of dysfunctional HDL particles.²⁰ The much less potent CETP inhibitor dalcetrapib increased HDL-C by 31% to 40% with a minimal reduction in LDL-C, but did not translate into clinical benefit and resulted in premature termination of the dal-OUTCOMES trial.²¹ Nonetheless, other CETP inhibitors are currently in clinical development. Amongst these, anacetrapib and evacetrapib have remarkable lipid-modulating abilities without the unwanted blood pressure effect as observed with torcetrapib.²² In phase II trials, anacetrapib (10 to 300 mg) decreased LDL-C by ~15% to 40% and increased HDL-C by ~40% to 140% and evacetrapib (30 to 500 mg) decreased LDL-C by ~15% to 35% and increased HDL-C by ~50% to 130%.^{23, 24}

To elucidate whether pharmacological CETP inhibition is anti-atherogenic and to what extent this is due to its LDL-C-lowering and HDL-C-raising abilities, we evaluated the effects of partial to full inhibition of CETP activity with a broad dose range of anacetrapib monotherapy on lipid modulation, atherosclerosis development and HDL functionality in APOE*3Leiden.CETP mice. Secondly, to mimic clinical intervention trials where dyslipidemic patients also receive statin treatment, we examined the possible additive/synergistic effects of anacetrapib on top of atorvastatin treatment in this well-established model for lipoprotein metabolism and atherosclerosis. These mice respond in a human-like manner to lipid-modulating interventions, including LDL-C-lowering^{19, 25} and HDL-C-raising drugs.^{19, 26, 27}

Methods

Animals and diet

Female APOE*3-Leiden.CETP transgenic mice¹³ (n=105) were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water. Body weight and food intake were monitored during the study. At the age of 9 to 16 weeks, mice were fed a semi-synthetic cholesterol-rich diet, containing 15% cacao butter, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch, 6.2% cellulose (Western-type diet, WTD; AB-Diets, Woerden, the Netherlands) and 0.1% cholesterol (SigmaAldrich, Zwijndrecht, the Netherlands) for a run-in period of 5 weeks. Animals were matched based on body weight, total cholesterol (TC), TG, HDL-C and age (n=15 per group) and received a control Western-type diet (WTD) without or with incremental dosages of anacetrapib (0.03; 0.3; 3 and 30 mg/kg/d; Dalton Chemical Laboratories Inc., Canada), atorvastatin (2.4 mg/kg/d) or a combination of atorvastatin (2.4 mg/kg/d) and anacetrapib (0.3 mg/kg/d) for a treatment period of 21 weeks. All animals were sacrificed by CO₂ inhalation and hearts were isolated to assess atherosclerosis development. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Research (TNO).

Plasma lipids, lipoprotein profile, endogenous cholesteryl ester transfer protein activity, cholesteryl ester transfer protein concentration and serum amyloid A

After 4 h fasting, blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated cups via tail vein bleeding and plasma was isolated every 2 to 4 weeks. To measure HDL-C, apoB-containing particles were precipitated from diluted plasma (15 μ L previously frozen plasma + 15 μ L PBS) by adding 5 μ L of 20% polyethylene glycol (PEG) in 200 mM glycine buffer (pH10). This mixture was incubated for 5 min at 25°C and centrifuged at 6000 rpm for 5 min at 25°C. Thirty μ L of supernatant was mixed with 20 μ L of 20% PEG in 200 mM glycine buffer and incubated for 5 min at 25°C and centrifuged at 6000 rpm for 20 min at 25°C. TC was measured in the supernatant to determine plasma HDL-C levels. Plasma TC and TG were determined individually using enzymatic kits (cat. no. 1458216 and cat. no. 1488872, Roche Diagnostics) according to manufacturer's protocol, and average plasma TC, TG, non-HDL-C and HDL-C levels were calculated. The distribution of cholesterol over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) as previously described.¹³

Plasma endogenous CETP activity and CETP concentration were determined as previously described.²⁸ Endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (Roar Biomedical, New York, USA), according to manufacturer's protocol. CETP activity was calculated as nmol

cholesteryl ester transfer/mL plasma/h. Plasma CETP concentration (ALPCO Diagnostics, Salem, USA) and serum amyloid A (SAA; Tridelta development Ltd, Maynooth, Ireland) were measured by ELISA according to manufacturer's instructions.

Atherosclerosis quantification

After sacrifice, hearts were isolated and fixed in formalin, embedded in paraffin and cross-sectioned (5 μ m) throughout the entire aortic root area. Cross sections were stained with hematoxylin-phloxine-saffron for histological analysis. Each cross section consisted of three segments separated by aortic valve leaflets and for each mouse four cross sections were used to assess atherosclerotic lesion area and severity. The lesions were classified into five categories according to the American Heart Association classification: I) Early fatty streaks: Up to ten foam cells in the intima with no other changes; II) Regular fatty streaks: Ten or more foam cells in the intima with no other changes; III) Mild plaque: A fibrotic cap and the presence of foam cells in the media; IV) Moderate plaque: More progressed lesions with an affected media, but without loss of architecture of the media; V) Severe plaque: The media is severely affected. Broken elastic fibers, cholesterol clefts, calcification and necrosis are frequently observed. Total lesion area, the number of lesions per cross section, as well as the percentage undiseased segments were determined as previously described.^{28, 29} To determine lesion severity, the type I-III lesions were classified as mild lesions and the type IV-V lesions were classified as severe lesions. Images were taken with the Olympus BX51 microscope and lesion areas were measured using Cell D imaging software (Olympus Soft Imaging Solutions).

The severe lesions (type IV-V) were further analyzed to assess lesion composition after immunostaining with mouse anti-human alpha actin (1:800; Monosan, Uden, The Netherlands) for smooth muscle cells (SMCs), and rat anti-mouse Mac-3 (1:50; BD Pharmingen, the Netherlands) for macrophages followed by sirius red staining for collagen. Necrotic area and cholesterol clefts, monocyte adhesion to the endothelium, and the calculation of plaque stability index (defined as the ratio of collagen and SMC area as stabilization factors to macrophage and necrotic area as destabilization factors) were determined as previously described.^{28, 29} All parameters of lesion composition were calculated per cross section and as a percentage of lesion area. Images of the lesions were taken with the Olympus BX40 microscope with Nuance 2 multispectral imaging system, and stained areas were quantified using Image J software. Evaluation of atherosclerosis development was performed under blinded conditions.

HDL functionality assays

Isolation of HDL

HDL from control and treated mice was isolated by sequential ultracentrifugation ($d = 1.063\text{--}1.21\text{ g/mL}$) according to the method of Havel *et al.*³⁰ using solid potassium bromide (Merck) for density adjustment as described previously.³¹

Vascular cell adhesion molecule detection by cell western

Human arterial endothelial cells (HAECs) (P7) were incubated with isolated HDL for 12 hours and treated with TNF-alpha (R&D Systems) for 4 hours. Cells were fixed in 3.7% formaldehyde, washed, blocked and incubated overnight with vascular cell adhesion molecule (VCAM-1) antibody (R&D Systems). Cells were washed and secondary antibody anti-goat (Odyssey Licor) with Draq-5 for normalization (680CW) was added. After incubation, cells were washed and fluorescence was measured.³²

Apoptotic cell death inhibition in a cellular system

HAECs (P7) were treated with an apoptosis-inducing agent in the presence and absence of isolated HDL. Cells were lysed and apoptosis was detected using a DNA fragmentation assay (Cell Death Detection ELISA_{PLUS}) according to the supplier's protocol (Roche Applied Science, 11 774 425 001). In short, supernatant was placed into a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase (POD) antibodies. Plates were washed to remove unbound components. The amount of nucleosome retained in the immunocomplex was quantitatively photometrically determined with ABTS as peroxidase substrate.

Statistical Analysis

Significance of differences between the groups was calculated non-parametrically using a Kruskal-Wallis test for independent samples, followed by a Mann-Whitney U-test for independent samples. An analysis of covariance (ANCOVA) was performed to test for group differences in lesion area with HDL-C and non-HDL-C exposure as covariates. To test whether collinearity was present between the explanatory variables, we calculated the variance inflation factor (VIF) and the condition index (CI). Values of $VIF > 5$ and values of $CI > 10$ were used as a cutoff for collinearity.^{33, 34}

SPSS 17.0 for Windows was used for statistical analysis. All groups were compared with the control group and the combination group was compared with the atorvastatin group. Bonferroni-Holm's method was used to determine the level of significance in the case of multiple comparisons. Values are presented as means \pm SD. P-values < 0.05 were considered statistically significant.

Results

Anacetrapib, atorvastatin and their combination decrease cholesteryl ester transfer protein activity despite an increase in cholesteryl ester transfer protein concentration

To assess the extent to which an ascending dose range of anacetrapib inhibits CETP, we measured CETP activity after 8 weeks of treatment and CETP concentration after 21 weeks of treatment (**Table 1**). Anacetrapib monotreatment (0.03; 0.3; 3 and 30 mg/kg/d) reduced CETP activity by -59% to -100% ($P < 0.001$) and increased plasma CETP concentration by +11% (NS) to +29% ($P < 0.001$). Both CETP activity and concentration were decreased by atorvastatin alone (-29% and -24%, $P < 0.001$) and in combination with 0.3 mg/kg/d anacetrapib (-84% and -23%, $P < 0.001$). Thus, adding anacetrapib to atorvastatin further reduced CETP activity (-78%, $P < 0.001$) without affecting CETP concentration when compared with atorvastatin.

Table 1 Effect of anacetrapib, atorvastatin and their combination on the cholesteryl ester transfer protein activity after 8 weeks of treatment and cholesteryl ester transfer protein concentration after 21 weeks of treatment

	Plasma CETP activity (nmol/mL/h)		Plasma CETP concentration (μ g/mL)	
Control	66.8 \pm 10.1		15.7 \pm 1.2	
0.03 mg/kg/d anacetrapib	27.1 \pm 7.1 ***	(-59%)	17.4 \pm 3.1	(+11%)
0.3 mg/kg/d anacetrapib	6.3 \pm 4.8 ***	(-91%)	19.9 \pm 2.4 ***	(+27%)
3 mg/kg/d anacetrapib	0.7 \pm 3.3 ***	(-99%)	20.2 \pm 3.1 ***	(+29%)
30 mg/kg/d anacetrapib	0.0 \pm 2.3 ***	(-100%)	18.5 \pm 4.1	(+18%)
Atorvastatin	47.5 \pm 8.2 ***	(-29%)	11.9 \pm 2.3 ***	(-24%)
Atorvastatin + 0.3 mg/kg/d anacetrapib	10.5 \pm 8.3 *** ###	(-84%)	12.1 \pm 2.1 ***	(-23%)

*** $P < 0.001$ when compared with control; ### $P < 0.001$ when compared with atorvastatin. Data are presented as means \pm SD (% inhibition or increase when compared with the control); $n = 15$ per group.

Anacetrapib alone and in combination with atorvastatin reduces plasma non-HDL-cholesterol and increases HDL-cholesterol

During the study, plasma lipids were measured every 2 to 4 weeks and average plasma TC (**Figure 1A**), TG (**Figure 1B**), non-HDL-C (**Figure 1C**) and HDL-C (**Figure 1D**) were calculated. In the control group, the Western-type diet resulted in an average plasma TC of 10.8 \pm 1.1 mmol/L, TG of 1.8 \pm 0.5 mmol/L, non-HDL-C of 9.5 \pm 1.1 mmol/L and HDL-C of 1.2 \pm 0.2 mmol/L. When compared with the control, anacetrapib monotreatment (0.03; 0.3; 3 and 30 mg/kg/d) decreased TC (-19%; -25%; -27% and -31%, $P < 0.001$ for all) mainly by decreasing non-HDL-C (-24%; -36%; -42% and -45%, $P < 0.001$ for all). In addition, anacetrapib monotreatment increased HDL-C (+30%; +60%; +86% and +86%, $P < 0.001$ for all) and decreased TG (-21%, $P = 0.07$; -22%, $P = 0.06$; -19%, NS and -27%, $P < 0.01$).

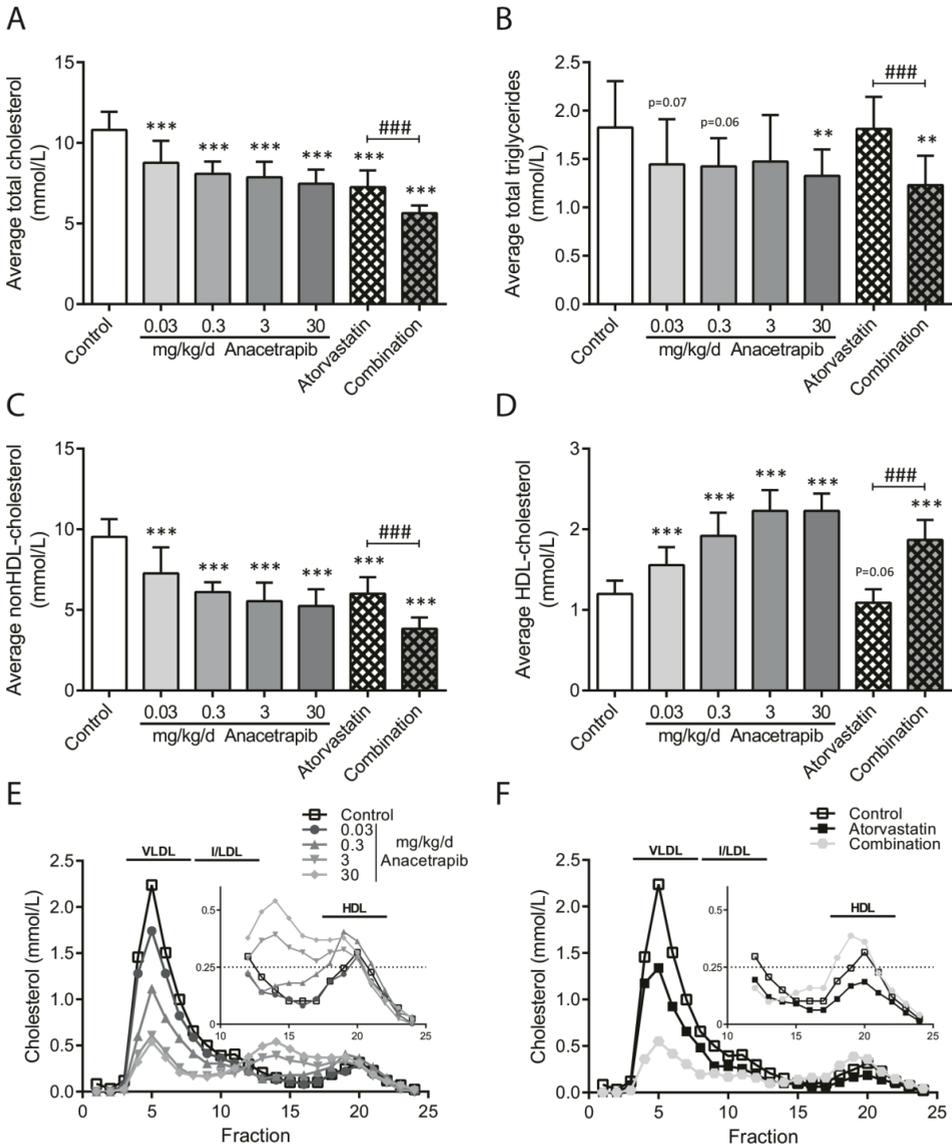


Figure 1. Effect of anacetrapib, atorvastatin and their combination on total cholesterol, triglycerides, non-HDL-cholesterol and HDL-cholesterol levels. Plasma total cholesterol (A), triglycerides (B) non-HDL-cholesterol (C) and HDL-cholesterol (D) were measured throughout the study and average levels were calculated. Lipoprotein profiles for cholesterol were assessed by FPLC lipoprotein separation to study effects of anacetrapib alone (E) and in combination with atorvastatin (F) after 18 weeks of treatment. **P<0.01, ***P<0.001 when compared with control; ###P<0.001 vln compared with atorvastatin. Data are presented as means ± SD (n=15 per group).

Atorvastatin decreased TC (-33%, $P < 0.001$) by decreasing non-HDL-C (-37%, $P < 0.001$) and with no effect on HDL-C and TG. The combination treatment decreased TC (-48%, $P < 0.001$), non-HDL-C (-60%, $P < 0.001$) and TG (-33%, $P < 0.01$) and increased HDL-C (+56%, $P < 0.001$). Anacetrapib enhanced the lipid-modifying effects of atorvastatin with greater reductions in TC (-22%, $P < 0.001$), non-HDL-C (-36%, $P < 0.001$) and TG (-32%, $P < 0.001$) and a greater increase in HDL-C (+72%, $P < 0.001$) when comparing the combination treatment with atorvastatin monotreatment. Lipoprotein profiles confirmed the lipid-modifying effects of anacetrapib and revealed the formation of larger HDL particles, as observed previously¹⁹ after treatment with higher dosages of anacetrapib (3 and 30 mg/kg/d; **Figure 1E and 1F**).

Atorvastatin in combination with anacetrapib reduces atherosclerosis progression to a greater extent than atorvastatin alone

After 21 weeks of treatment, the effects of anacetrapib, atorvastatin and their combination on the progression of atherosclerosis were assessed in the aortic root area as illustrated by representative images in **Figure 2**. The number of lesions (**Figure 3A**), lesion area (**Figure 3B**), undiseased segments (**Figure 3C**) and lesion severity (**Figure 3D**) were assessed as previously described.^{28, 29}

For the control group, 4.1 ± 0.6 lesions per cross section developed with a total lesion area of $169 \pm 51 \times 10^3 \mu\text{m}^2$. Approximately 71% of these lesions were severe lesions (type IV-V) and only 5% of the segments were undiseased. Anacetrapib monotreatment (0.03; 0.3; 3 and 30 mg/kg/d) dose-dependently reduced lesion area (-41%, $P < 0.01$; -72%; -86% and -92%, $P < 0.001$ for all) and the number of lesions and improved lesion severity as indicated by less severe lesions (down to 15%, $P < 0.001$) and more undiseased segments (up to 46%, $P < 0.001$). Atorvastatin monotreatment reduced total lesion area (-63%, $P < 0.001$) and improved lesion severity without affecting the number of lesions and undiseased segments. When compared with the control, the combination treatment further decreased total lesion area (-95%, $P < 0.001$), the number of lesions (-41%, $P < 0.01$), and lesion severity and increased the percentage undiseased segments. When compared with atorvastatin monotreatment, the combination treatment decreased total lesion area (-87%, $P < 0.001$), the number of lesions (-34%, $P = 0.06$) and severity and increased the percentage undiseased segments to a greater extent, indicative of an additional effect of anacetrapib on top of the statin.

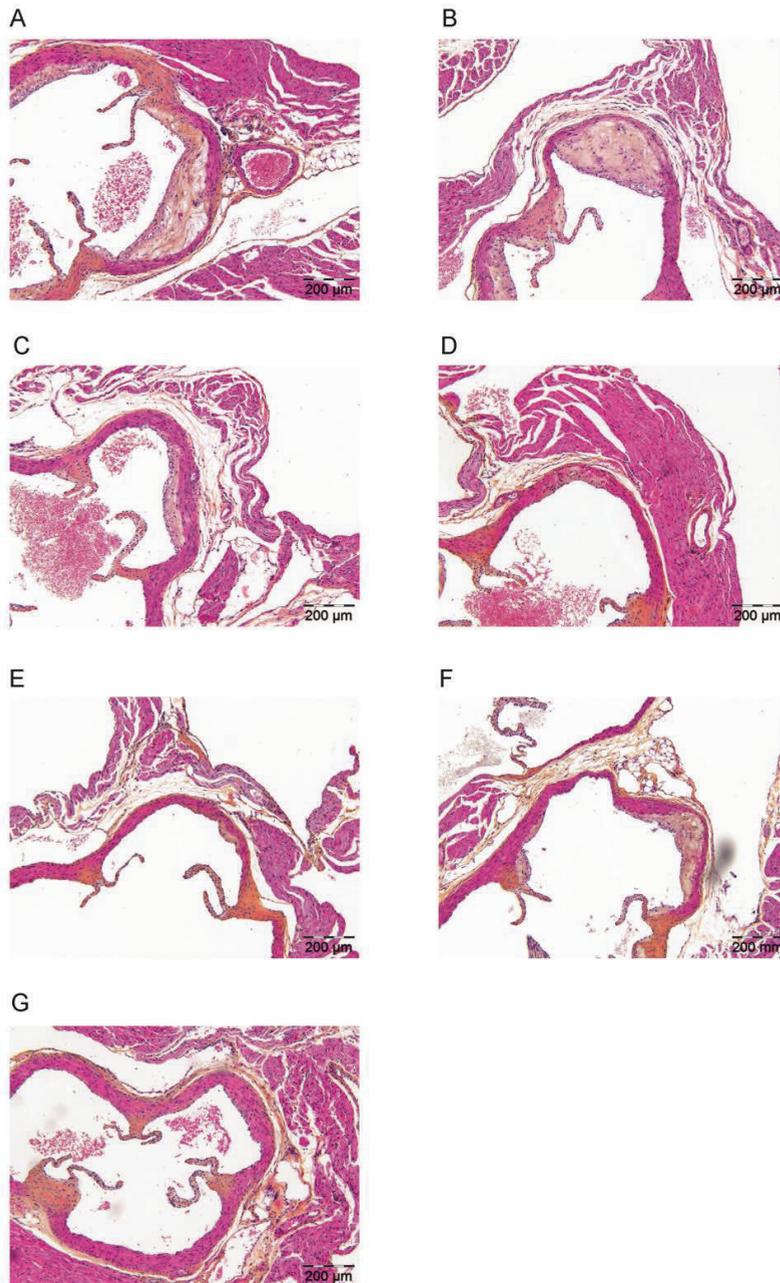


Figure 2. Effect of anacetrapib, atorvastatin and their combination on plaque morphology. Representative images of hematoxylin-phloxine-saffron-stained atherosclerotic lesions in a cross section of the aortic root area for the control group (A), 0.03 mg/kg/d anacetrapib (B), 0.3 mg/kg/d anacetrapib (C), 3 mg/kg/d anacetrapib (D), 30 mg/kg/d anacetrapib (E), atorvastatin group (F) and the combination group (G) after 21 weeks of treatment.

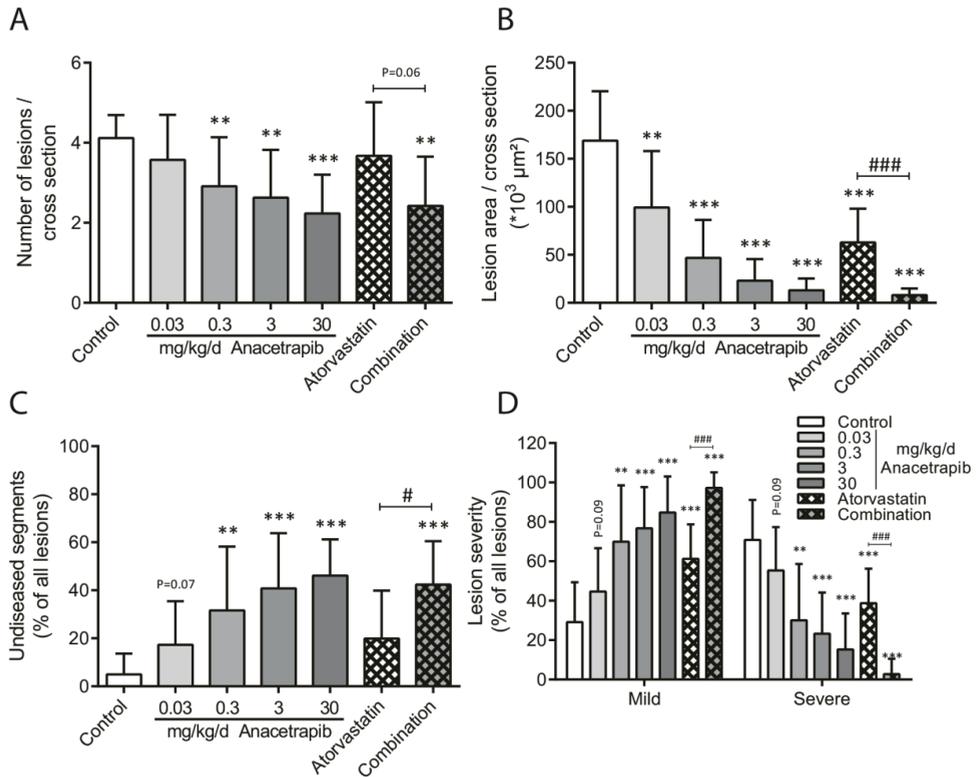


Figure 3. Effect of anacetrapib, atorvastatin and their combination on atherosclerosis development in the aortic root area. The number of lesions per cross section (A), total lesion area per cross section (B), the percentage undiseased segments (C) and lesion severity as a percentage of all lesions (D) were determined after 21 weeks of treatment. Lesion severity was classified as mild (type I-III) and severe (type IV-V) lesions.

** $P < 0.01$, *** $P < 0.001$ when compared with control; # $P < 0.05$, ### $P < 0.001$ when compared with atorvastatin. Data are presented as means \pm SD ($n = 15$ per group).

Anacetrapib, atorvastatin and their combination improve lesion stability

In addition to atherosclerotic lesion size and severity, we assessed the number of monocytes adhering to the endothelium as a functional marker for vascular inflammation (**Figure 4A**). Adhering monocytes per cross section in the control group (i.e. 4.1 ± 2.6) were reduced by the higher dosages of anacetrapib (-60%, $P < 0.01$ and -61%, $P < 0.01$), as well as by atorvastatin alone and in combination with anacetrapib (-48%, $P < 0.05$ and -78%, $P < 0.001$). When compared with atorvastatin, the combination treatment reduced the number of monocytes to a greater extent (-57%, $P < 0.01$). In addition, we analyzed the composition of the severe lesions (type IV-V), since these lesions are considered to be most vulnerable and prone to rupture. All parameters of lesion composition were calculated per cross section as absolute values and as a percentage of lesion area. To this end, collagen content (**Figure**

4B) and SMC content in the cap (**Figure 4C**) were considered as stabilization factors and macrophage content (**Figure 4D**) and necrotic content (**Figure 4E**) were considered as destabilization factors. The severe lesions in the control group consisted of approximately 54% collagen, 6% SMCs in the cap, 10% macrophages and 4% necrosis. The lesion stability index for the control group presented as the ratio of stabilization to destabilization factors was 4.9 ± 2.0 (**Figure 4F**).

When corrected for lesion area, the two higher dosages of anacetrapib (3 and 30 mg/kg/d) revealed a more stable plaque phenotype by increasing collagen content (+21%, $P < 0.001$ and +28%, $P < 0.001$) and SMC content in the cap (+120%, $P < 0.01$ and +119%, $P < 0.05$) and by decreasing macrophage (-53%, $P = 0.06$ and -60%, $P = 0.05$) and necrotic (-73%, $P < 0.001$ and -46%, $P < 0.05$) content. This is reflected by an increase in lesion stability index in these two treatment groups (+427%, $P < 0.001$ and +366%, $P < 0.01$). Atorvastatin in combination with anacetrapib tended to increase the SMC content in the cap (+194%, $P = 0.07$) and decreased necrotic content (-96%, $P < 0.05$) with no effect on lesion stability index. However, it should be noted that there were almost no lesions in the combination group and only two mice that received the combination treatment of anacetrapib and atorvastatin developed severe lesions.

Anacetrapib does not affect HDL function

To explore the contribution of the anacetrapib-induced increase in HDL-C to the reduction of atherosclerosis, we investigated the endothelial-vasoprotective properties of HDL, in particular with respect to anti-inflammatory and anti-apoptotic properties in cultured arterial endothelial cells. HDL isolated from anacetrapib-treated mice had no effect on pro-inflammatory cytokine-induced VCAM-1 expression (**Figure 5A**) or on apoptotic cell death (**Figure 5B**).

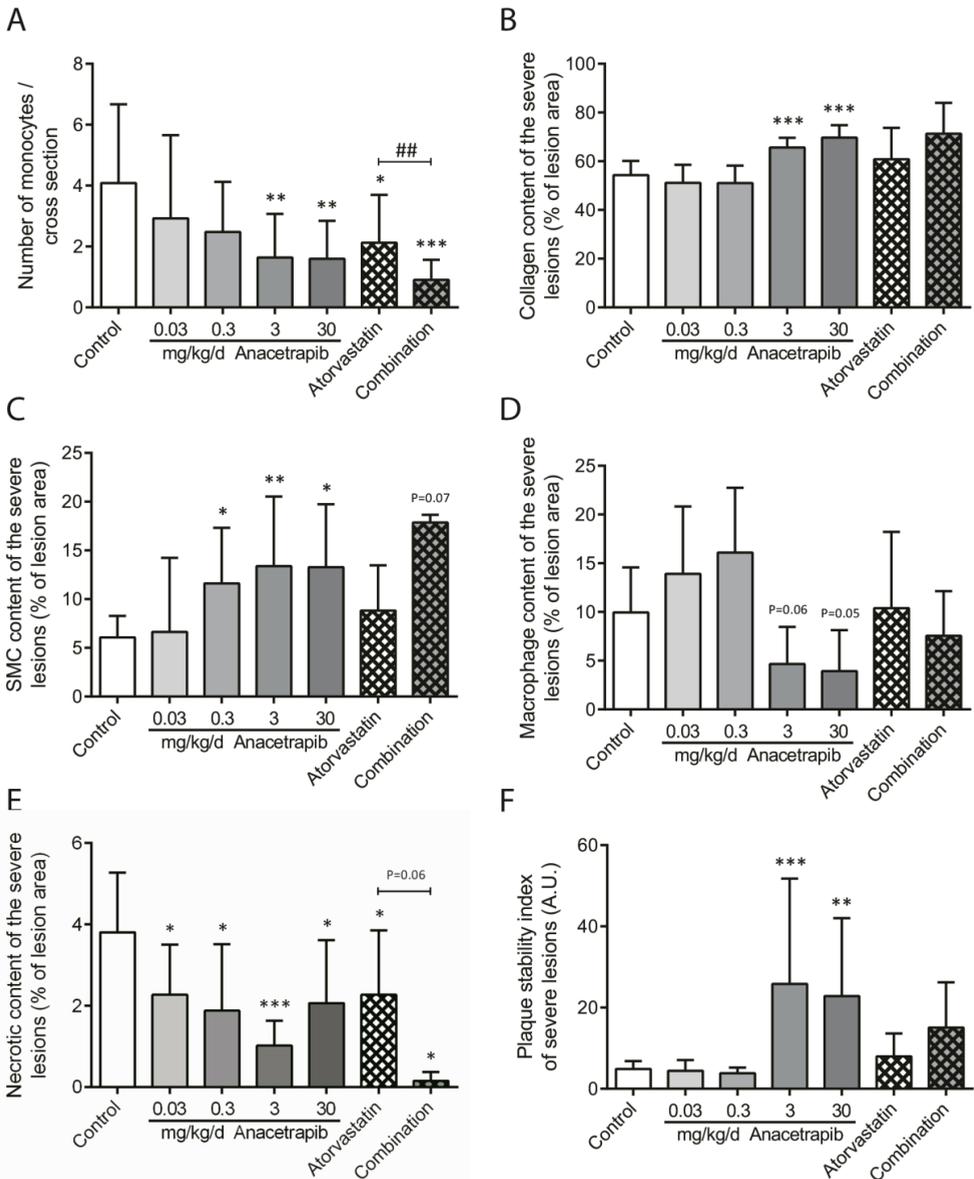


Figure 4. Effect of anacetrapib, atorvastatin and their combination on lesion composition. The number of monocytes adhering to the vascular endothelium per cross section (A) was calculated. In the severe lesions (type IV and V), collagen content (B) and SMC content in the cap (C) were determined as stabilization factors and macrophage content (D) and necrotic content (E) were determined as destabilization factors, all as a percentage of lesion area. The plaque stability index was calculated as the ratio of the stabilization factors to the destabilization factors (F).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with control; ## $P < 0.01$ when compared with atorvastatin. Data are presented as means \pm SD ($n = 15$ per group).

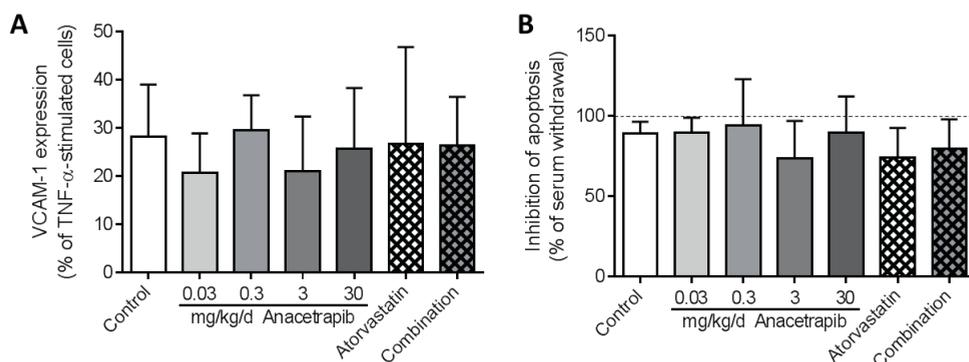


Figure 5. Effect of anacetrapib, atorvastatin and their combination on endothelial-vasoprotective properties of HDL, in particular pro-inflammatory cytokine-induced VCAM-1 expression (A) and apoptotic cell death (B).

Data are presented as means \pm SD.

Anacetrapib reduces atherosclerosis progression primarily by reducing non-HDL-cholesterol exposure

We evaluated whether the effects of anacetrapib and atorvastatin on atherosclerosis development could be explained by either an increase in HDL-C or a decrease in non-HDL-C or both. Lesion area was normalized by cubic root transformation (lesion area^(1/3)). Univariate regression analysis showed that lesion area was predicted by TC (**Figure 6A**), mainly non-HDL-C (**Figure 6B**) and to a lesser extent by HDL-C (**Figure 6C**). Analysis of covariance (ANCOVA) showed that both anacetrapib treatment, at the dosages of 3 and 30 mg/kg/d ($P < 0.05$) and non-HDL-C ($P < 0.001$), but not HDL-C ($P = 0.76$), independently determined lesion size. Importantly, the variance inflation factors of HDL-C and non-HDL-C (VIF = 4.42 and 3.18 respectively) and the condition index (CI = 4.43) did not exceed the threshold for collinearity between the explanatory variables. Collectively, these data are compatible with a mechanism that anacetrapib mainly decreases atherosclerotic lesion development via a reduction of non-HDL-C with an additional effect by the compound itself at the higher doses (**Figure 7**).

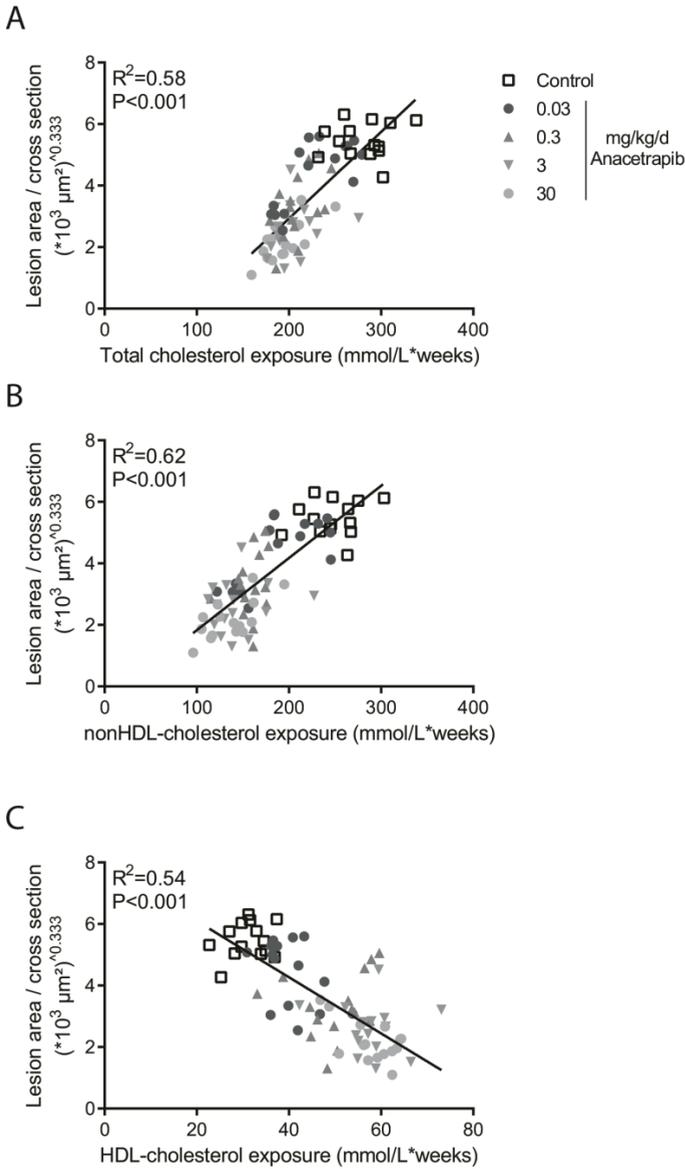


Figure 6. Correlation between plasma cholesterol exposure and lesion area. Linear regression analyses were performed on the cubic root of lesion area plotted against total cholesterol exposure (A), non-HDL-cholesterol exposure (B) and HDL-cholesterol exposure (C).

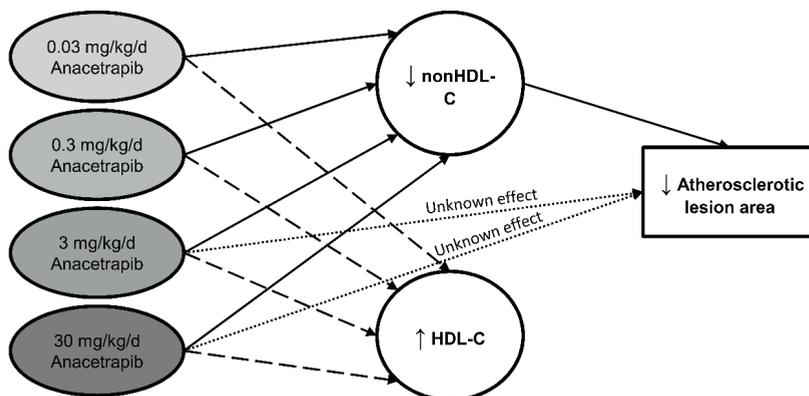


Figure 7. Hypothetical scheme of factors contributing to the effect of anacetrapib on atherosclerotic lesion area as suggested by statistical analyses. An analysis of covariance (ANCOVA) was performed to test for group differences in lesion area with HDL-C and non-HDL-C exposure as covariates. HDL-C was not an independent predictor of lesion area when non-HDL-C was included as covariate, suggesting that the effect of anacetrapib on atherosclerosis development was mainly mediated through the reduction of non-HDL-C. The higher dosages of anacetrapib (3 and 30 mg/kg/d) also revealed an effect on atherosclerosis that was independent of non-HDL-C, but this effect was not explained by the increase in HDL-C.

Anacetrapib slightly increased serum amyloid A as a marker of inflammation

To assess the effect of anacetrapib on general inflammatory status, we measured plasma SAA levels, a systemic inflammatory marker after 16 weeks of treatment (**Figure 8**). Plasma SAA levels in the control group were $1.6 \pm 0.5 \mu\text{g/mL}$. When compared with the control, 0.3 mg/kg/d anacetrapib tended to increase SAA levels (+37%, $P=0.07$). No effects on body weight (gain) and food intake were noted with any of the treatments (data not shown).

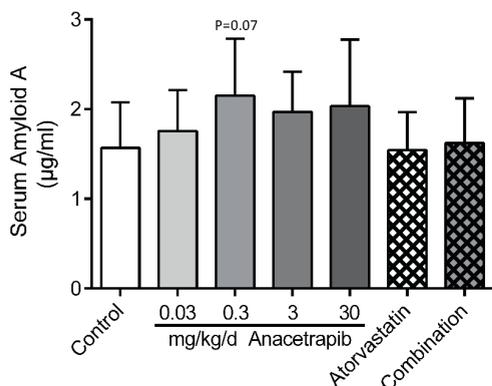


Figure 8. The effects of anacetrapib, atorvastatin and their combination on plasma SAA levels were measured after 16 weeks of treatment.

* $P < 0.05$ when compared with control. Data are presented as means \pm SD ($n=15$ per group)

Discussion

The present study is the first intervention study in a mouse model for atherosclerosis designed to investigate the effects of the CETP inhibitor, anacetrapib alone and in combination with atorvastatin on the progression of atherosclerosis, lesion stability and HDL function. In clinical trials, the effectiveness of novel treatment regimes in CVD is only being tested in patients on a statin background which makes this study unique in also evaluating the effects of anacetrapib monotreatment. In APOE*3Leiden.CETP mice, a broad dose range of anacetrapib dose-dependently reduced atherosclerosis development. This effect was mainly ascribed to the reduction in non-HDL-C despite a remarkable increase in HDL-C and without affecting HDL functionality. Anacetrapib improved lesion stability when given at a higher dose (3 and 30 mg/kg/d). In addition, a moderate dose of anacetrapib (0.3 mg/kg/d) added to the anti-atherogenic effects of atorvastatin.

In our study, incremental dosages of 0.03 to 30 mg/kg/d anacetrapib dose-dependently decreased CETP activity by > 60%, decreased non-HDL-C by 24% to 45% and increased HDL-C by 30% to 86%. These lipid-altering effects are comparable to findings from phase I, II and III clinical trials. In phase I trials, an anacetrapib-induced reduction in CETP activity of > 60% was accompanied by dose-dependent LDL-C-lowering and HDL-C-raising effects both in healthy subjects³⁵ and in patients with dyslipidemia.³⁶ In line with our results, these studies also report an increase in CETP concentration possibly due to the formation of an inactive complex between CETP and HDL.³⁷ In a phase II trial, 8 weeks of treatment with ascending dosages of anacetrapib monotreatment (10 to 300 mg/d) reduced LDL-C by 16% to 39% and increased HDL-C by 44% to 139%. Similar to our study, the addition of anacetrapib to atorvastatin produced incremental LDL-C reductions.²³

The present study in APOE*3Leiden.CETP mice demonstrates that total blockage of CETP does not reveal adverse effects on the clinical endpoint when compared with partial blockage: anacetrapib dose-dependently reduced the progression of atherosclerosis and increased plaque stability whereas the anti-atherogenic effects of atorvastatin were enhanced in combination with a moderate dose of anacetrapib.

Inconsistent data have been reported on the effect of other CETP inhibitors on atherosclerosis development in animals expressing CETP. In rabbits, dalcetrapib reduced atherosclerosis in one study with no effect in another study.^{15, 17} In contrast to the human situation,²¹ the reduction in atherosclerosis after dalcetrapib treatment was accompanied by a 40% to 50% decrease in non-HDL-C together with an increase in HDL-C.¹⁵ In mice, torcetrapib monotreatment decreased atherosclerosis.¹⁹ However, unlike the present study, these effects were not enhanced in combination with atorvastatin in the same mouse model.¹⁹ In rabbits, torcetrapib treatment decreased atherosclerosis where aortic lesion area correlated with TC/HDL-C ratio.¹⁸ This could suggest a possible anti-atherogenic role

of increased HDL-C or other pleiotropic effects of HDL. However, in the APOE*3Leiden.CETP mouse model, statistical analyses revealed that HDL-C was not an independent predictor of lesion area when non-HDL-C was included as covariate, suggesting that the effect of anacetrapib on atherosclerosis development was mainly mediated through the reduction of non-HDL-C. The higher dosages of anacetrapib (3 and 30 mg/kg/d) also revealed an effect on atherosclerosis that was independent of non-HDL-C, but this effect was not explained by the increase in HDL-C and could point to other hitherto unknown (off target) effects of anacetrapib.

Besides atherosclerotic lesion size, lesion composition should also be taken into consideration given that in the human situation, a vulnerable lesion consisting of more macrophages, a large necrotic core and a thin, collagen-poor, fibrous cap is more prone to rupture.³⁸ Previously, our group showed that torcetrapib produced a pro-inflammatory, unstable plaque phenotype as seen by increased monocyte adherence to the vascular endothelium and consequently increased macrophage content of the lesions.¹⁹ In the present study, anacetrapib decreased monocyte adherence and improved lesion composition as shown by an increase in stabilization factors (collagen and SMC content) and a decrease in destabilization factors (macrophage and necrotic content). The inconsistencies can be ascribed to the off-target activation of the renin-angiotensin-aldosterone system (RAAS) and blood pressure effect of torcetrapib.³⁹ Indeed, in our mouse model for atherosclerosis, torcetrapib also increased aldosterone levels in plasma.¹⁹

The large phase III DEFINE trial was designed to further assess the efficacy and tolerability of anacetrapib in statin-treated patients with or at risk for coronary heart disease.⁴⁰ Anacetrapib (100 mg/d) decreased LDL-C by 40% and increased HDL-C by 138% with an acceptable safety profile and no indication for an increase in CV events. In fact, *post hoc* analyses suggested a reduction in CV endpoints. These initial data provided a rationale for conducting a larger clinical endpoint trial of pharmacological CETP inhibition despite the conflicting outcomes of genetic CETP deficiency and the ILLUMINATE trial.²⁰ In view of the detrimental effects of torcetrapib in the ILLUMINATE trial, anacetrapib was thoroughly screened and revealed minimal side effects without any indication for an off-target pressure effect.^{23, 35, 36}

Despite the absence of reported side effects of anacetrapib, there are some concerns about target-related side effects due to formation of large buoyant cholesterol-rich HDL-2 particles after CETP inhibition,^{41, 42} which may be dysfunctional with regard to their endothelial-vasoprotective effects and consequently their atheroprotective properties^{10, 37, 43, 44} and that this may have contributed to the failure of torcetrapib.²⁰ In the present study, we investigated the effects of HDL isolated from control and anacetrapib-treated mice on parameters of vascular inflammation and function. HDL from anacetrapib-treated mice did not suppress cytokine-induced adhesion molecule expression or cell apoptosis in

endothelial cells. This is in line with results from recent studies where no differences were observed in the effect of HDL from control or anacetrapib-treated hamsters and humans on inflammatory markers (adhesion molecule expression, monocyte chemotactic protein-1 secretion, monocyte adhesion, NFkB activation and cytokine mRNAs) in endothelial cells⁴⁵ and macrophages.⁴⁶ Importantly, although anacetrapib treatment did not improve the anti-inflammatory and anti-apoptotic effects of HDL, it also did not adversely affect these functions of HDL. In addition, we found no effect of anacetrapib on serum paraoxonase 1 (PON-1) activity and the aortic content of reactive oxygen species (ROS) (data not shown). Formation of large cholesterol-rich HDL-2 particles in CETP-deficiency or after CETP inhibition has also been suggested to affect the cholesterol efflux capacity of these particles.^{41, 42} Although we did not address this in the present study, data from literature consistently indicate that the cholesterol efflux capacity is not impaired but improved. HDL from CETP-deficient patients displayed enhanced ability to promote cholesterol efflux from macrophages in an ABCG1-dependent manner.⁴¹ In humans, anacetrapib-treated HDL showed increased ABCA1- and ABCG1-mediated cholesterol efflux capacity.⁴⁶ Collectively, these data indicate that CETP inhibition does not result in formation of dysfunctional HDL with regard to its atheroprotective properties as assessed by *ex vivo* (cell) assays.

It should be noted that in the DEFINE trial, a non-significant 18% increase in C-reactive protein, a marker of inflammation, after anacetrapib treatment was found.⁴⁰ In the present study, the inflammatory marker, SAA was slightly elevated after anacetrapib treatment, but this effect was alleviated when anacetrapib was given in combination with atorvastatin.

The effects of two other CETP inhibitors, DRL-17822 and TA-8995 (DEZ-001), as well as a vaccine against CETP, ATH03, are being tested in phase I/II clinical development. In large phase III clinical trials, the effects of 100 mg anacetrapib (REVEAL) and 130 mg evacetrapib (ACCELERATE) in patients on standard statin treatment on CV outcomes are currently being investigated and results are expected in 2016/17.⁴⁴ The outcome of these trials will resolve the unanswered questions regarding possible beneficial effects of pharmacological CETP inhibition and may give additional insight into the HDL-hypothesis and the contribution of HDL and non-HDL to CV endpoints.

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