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Efficacy, safety and novel targets in cardiovascular disease : advanced applications in APOE*3-Leiden.CETP mice

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Triple treatment with alirocumab and
evinacumab on top of atorvastatin regresses
lesion size and improves plaque phenotype
in APOE*3-Leiden.CETP mice

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Submitted

Abstract

Objectives: Regression of atherosclerotic plaque is modest with the current standard therapy, therefore it is beneficial to evaluate new therapeutic options. We investigated the effect of aggressive lipid-lowering interventions using double and triple treatment with simple or combined inhibition of PCSK9 and ANGPTL3 using the monoclonal antibodies alirocumab and evinacumab, respectively, on top of atorvastatin on regression of pre-existent atherosclerosis in APOE*3-Leiden.CETP mice.

Methods and results: Mice were fed a Western-type diet (WTD) for 13 weeks and thereafter matched into a baseline group (sacrificed at t=13), and 5 groups that continued to receive WTD alone or with treatment for 25 weeks: regression control, atorvastatin, atorvastatin and alirocumab, atorvastatin and evinacumab or atorvastatin, alirocumab and evinacumab. All interventions decreased plasma total cholesterol (-37% with atorvastatin to -80% with triple treatment, all $p < 0.001$) by reduction of non-high-density lipoprotein cholesterol (non-HDL-C). Triple treatment decreased non-HDL-C levels at end-point from 10.7 mmol/L in control to 1.0 mmol/L (-91%, $p < 0.001$). Mono-treatment with atorvastatin reduced the progression of atherosclerosis (-28%, $p < 0.001$ vs control), double treatments completely blocked further progression and improved plaque stability, whereas triple treatment regressed lesion size in the thoracic aorta (-50%, $p < 0.05$ vs baseline) and in the aortic root (-36%, $p < 0.05$ vs baseline), diminished macrophage accumulation through reduced proliferation and further improved plaque stability.

Conclusions: This preclinical study using APOE*3-Leiden.CETP mice demonstrates that high-intensive cholesterol-lowering triple treatment with atorvastatin, alirocumab and evinacumab targeting all apoB-containing lipoproteins is a promising approach for regression of pre-existent atherosclerosis along with improvement in plaque phenotype.

Introduction

Atherosclerosis is the main cause of cardiovascular disease (CVD), and the annual number of deaths from CVD is predicted to rise from 17.5 million in 2012 to 22.2 million by 2030 (1). In addition to lifestyle changes (2), lipid-lowering has proven to be highly effective in reducing CVD, as every 1 mmol/L reduction in low-density-lipoprotein-cholesterol (LDL-C) is associated with a 23% CVD risk reduction (3). Since most patients at CVD risk are treated after development of atherosclerosis, therapies that regress pre-existent lesions are warranted.

Currently, statins are the 'golden standard' to lower LDL-C and to reduce CVD risk, but monotherapy with statins remains suboptimal as the achieved regression is modest, reflected by the small reductions in plaque volume (0.3-1.2% per year) (4,5). Furthermore, plaque regression is only seen in those patients with LDL-C reductions of >40% (6,7), or at plasma LDL-C levels below 78 mg/dL (2.0 mmol/L) (5,7), while a subgroup of patients still does not reach their LDL-C goals. Notably, the magnitude of regression is correlated with the percentage of LDL-C reduction (5,6), indicating the potential for further lipid-lowering. In this context, dual lipid-lowering therapies using ezetimibe or inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) on top of a statin further reduce plaque volume relative to monotherapy with statins (5). While currently available therapies aim mostly to decrease plasma LDL-C, remnant cholesterol and triglyceride (TG) levels are considered to be an important residual risk factor for CVD as well (8,9). Actually, the clinical benefit of lowering TG and LDL-C may be proportional to the absolute change in apoB, implicating that all apoB-containing lipoproteins have approximately the same effect on the risk of CVD per particle (10). Therefore, novel high-intensive lipid-lowering or combination therapies targeting all apoB-containing lipoproteins may provide additional benefit to regress atherosclerosis and further reduce clinical events.

Since the severity and progression of coronary atherosclerosis are associated with adverse cardiovascular outcomes (4,11), the modest reduction in plaque volume achieved by statins cannot fully explain the reduced CVD risk, suggesting an important role for improved lesion stability (5,12,13). Animal models represent an opportunity to study plaque composition during regression. However, many mouse models have limited translational capability due to lack of responsiveness to lipid-lowering treatment (13). In this study we utilized APOE*3-Leiden.CETP mice, a well-established model with a human-like lipoprotein metabolism and atherosclerosis development (14) that responds well to hypolipidemic drugs (15-17).

We tested alirocumab and/or evinacumab on top of atorvastatin as high-intensive lipid-lowering strategy to evaluate their effect on regression of pre-existent atherosclerosis in APOE*3-Leiden.CETP mice. In addition, we assessed the effects on plaque composition and stability, and further looked into the process of macrophage reduction during regression. Alirocumab is a fully human monoclonal antibody to PCSK9 that reduces the

risk of recurrent ischemic cardiovascular events in patients with acute coronary syndrome when administered on top of atorvastatin (18). Evinacumab (REGN1500) is a monoclonal antibody against angiotensin-like protein 3 (ANGPTL3) (19), a circulating protein that inhibits the hydrolysis of TG by lipoprotein lipase (LPL) in TG-rich lipoproteins. Loss-of-function mutations in the ANGPTL3 gene correlate with protection against CVD and treatment with evinacumab decreased plasma TG and LDL-C levels in human subjects (17,20).

Methods

Animals

Female APOE*3-Leiden.CETP transgenic mice on a C57BL/6 background (8-12 weeks of age) were obtained from the breeding facility of the Organization of Applied Scientific Research (TNO). The number of animals per group was calculated using a power of 0.80. Based on our experience from previous studies, we expected to have a variance of 23% in atherosclerosis, a minimal difference of 40% and a two-sided test with 95% confidence interval, which resulted in 16 animals per group. The mice entered the study in a staggered way of 5 weeks apart with two equal batches of each 8 mice per group to limit the difference in animal age. Groups that received the fully human monoclonal antibody evinacumab consisted of 32 (atorvastatin and evinacumab) or 48 (atorvastatin, alirocumab and evinacumab) mice as some mice develop mouse-anti-human auto-antibodies to evinacumab, leading to loss of efficacy. During the 38-week study with in total 144 mice, 4 mice were found dead in their cage and 4 mice were sacrificed based on human end-point criteria (atorvastatin: 3; atorvastatin and alirocumab: 2; atorvastatin and evinacumab: 1; atorvastatin, alirocumab and evinacumab: 2). In total, 48 mice developed auto-antibodies to evinacumab, as determined by Elisa (atorvastatin and evinacumab: 18; atorvastatin, alirocumab and evinacumab: 30) and were excluded from all analyses. The study was performed at the research facility of TNO-Metabolic Health Research, the Netherlands, and animal experiments were approved by the Animal Experiment Committee of The Netherlands Organization of Applied Scientific Research TNO under registration number 3682.

Diet and treatments

Mice were fed a Western-type diet (WTD) with 0.30% cholesterol and 15% saturated fat for 13 weeks to induce development of atherosclerosis. After 13 weeks mice were matched into 6 groups based on age, body weight, plasma total cholesterol (TC) and triglycerides (TG), and cholesterol exposure (mmol/L*weeks) measured at 12 weeks, and thereafter 16 mice were sacrificed as the baseline control group (see **Figure 1** for study design). The other 5 groups continued to receive WTD alone or with treatment for 25 weeks: regression

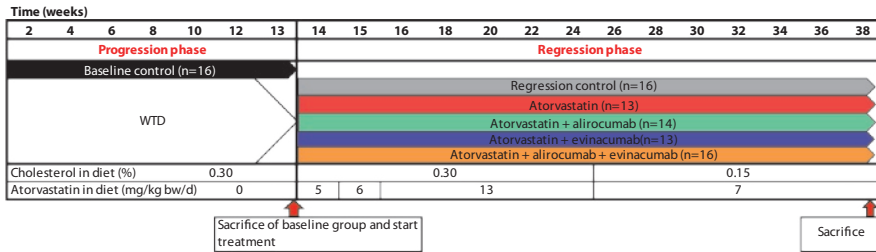


Figure 1 Study design. Female APOE*3-Leiden.CETP mice were fed a WTD diet for 13 weeks. Next, mice were matched in 6 groups based on age, body weight, plasma total cholesterol, triglycerides and cholesterol exposure (mmol/L*weeks). The baseline control group was sacrificed at t=13 weeks and the other 5 groups continued to receive a WTD alone or with treatment as indicated for 25 weeks until week 38. The number of mice used for the analyses are depicted, this number exclude the mice that died during the study (see Methods section) and mice that were excluded because of development of auto-antibodies to the human monoclonal antibody evinacumab. Abbreviations: WTD, Western type diet.

control, atorvastatin (5-13 mg/kg/d), atorvastatin and aliocumab (10 mg/kg/week), atorvastatin and evinacumab (25 mg/kg/week) or atorvastatin, aliocumab and evinacumab. Atorvastatin was mixed with the diet in a dose of 5 mg/kg/d (week 13-14), 6 mg/kg/d (week 15), 13 mg/kg/d (week 16-24) and 7 mg/kg/d (week 25-38). Aliocumab and evinacumab were administered by weekly subcutaneous injections. The cholesterol content in the diet was decreased from 0.30% to 0.15% in week 24 to reach plasma TC levels of an average 11-13 mmol/L to obtain more human-like levels, similarly as observed in untreated hyperlipidemic (FH) patients. Body weights, food intake per cage, and plasma parameters were measured throughout and the development of atherosclerosis was analyzed at t=13 weeks (baseline control group) and at t=38 weeks (control and treatment groups) in the aortic arch and aortic root. Lesion severity was determined in the aortic root. Plaque composition, monocyte adherence and macrophage proliferation were determined in the complex lesions of the aortic root.

Plasma lipids and lipoprotein analysis

Plasma TC and TG were determined at week 0, 4, 8, 12, 14, 15, 16, 20, 24, 28, 32, 36 and 38 using enzymatic colorimetric methods (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocols and total cholesterol exposure was calculated as mmol/L*weeks. HDL-C was measured at week 12, 18, 28 and 36 after precipitation of apoB-containing particles (21) and non-HDL-C was calculated by subtracting HDL-C from total cholesterol.

En face determination of atherosclerosis in the thoracic aorta

To determine the total plaque load in the aortic arch, perfusion-fixed aortas (from the aortic origin to the diaphragm) were cleaned of extravascular fat, opened longitudinally, pinned en face, and stained for lipids with oil-red O (Sigma-Aldrich Chemie BV) as described previously (22). Photographs of the aorta's were taken by an Olympus SZX10 microscope with an Olympus DP74 camera. Data were normalized for the analyzed surface area and expressed as percentage of the stained area.

Determination of lipid content in the thoracic aorta

The thoracic aortas were cleaned of extravascular fat, homogenized in phosphate-buffered saline, and the protein content was measured using a Lowry protein assay. Lipids were extracted as described previously (23), separated by high-performance thin-layer chromatography on silica gel plates, stained and analyzed with ChemiDoc Touch Imaging System (Bio-Rad). TG, cholesterol ester (CE) and free cholesterol (FC) content were quantified using Image-lab version 5.2.1 software (Bio-Rad) and expressed per mg protein.

Histological assessment of atherosclerosis in the aortic root

Atherosclerotic lesion area and severity were assessed in the aortic root area, as reported previously (24). Briefly, the aortic root was identified by the appearance of aortic valve leaflets, and serial cross-sections of the entire aortic root area (5 μm thick with intervals of 50 μm) were mounted on slides and stained with haematoxylin-phloxine-saffron (HPS). For each mouse, the lesion area was measured in 4 subsequent sections. Each section consisted of 3 segments (separated by the valves). The total lesion area and number of lesions were calculated per cross-section. Lesion severity was calculated as relative amount of early and complex lesions in which the lesion-free segments are included. The lesions were classified as early lesions (type I-III according to the American Heart Association (AHA)) and complex lesions, which include type IV-V lesions (according to the AHA (16,25)) and the so-called 'regression lesions'. Although the 'regression lesions' were generally smaller than type IV and V lesions, they could not be defined as early lesions/fatty streak since they did not consist of macrophages, but mainly of collagen and α -smooth muscle cells (SMCs). Slides were scanned by an Aperio AT2 slide scanner (Leica Biosystems) and atherosclerotic area was measured in Image Scope (version 12-12-2015).

Histological assessment of plaque composition

Lesion composition in complex lesions was assessed after double immunostaining with anti- α smooth muscle actin (1:400; PROGEN Biotechnik GmbH, Germany) for SMCs, and anti-mouse LAMP2 (M3/84) (1:500; BD Pharmingen, the Netherlands) for macrophages. Anti- α smooth muscle actin was labeled with Vina green (Biacare Medical, Pacheco, USA), and LAMP2 with DAB (Vector laboratories, Burlingame, USA). After slides were scanned and analyzed, cover slips were detached overnight in xylene and Sirius Red staining for

collagen was performed. Color intensity of Sirius Red staining was determined in ImageJ and the used threshold was confirmed by evaluation of the sections under polarized light. The necrotic area and cholesterol clefts were measured in the Sirius Red-stained slides. Lesion stability index, as the ratio of collagen and α SMC area (i.e. stabilization factors) to macrophage and necrotic area (i.e. destabilization factors) was calculated as described previously (24). In each segment used for lesion quantification, intracellular adhesion molecule (ICAM-1) expression and the number of monocytes adhering to the endothelium were counted after immunostaining with mouse monoclonal ICAM-1 antibody (1:400; Santa Cruz Biotechnology, Dallas, USA) and AIA 31240 antibody (1:500; Accurate Chemical and Scientific, New York, USA), respectively (25). The number of proliferating macrophages in the plaques was counted after triple staining with Ki67 (1:1600, Abcam, Cambridge, UK) for cellular proliferation labeled with DAB (black) (Vector laboratories, Burlingame, USA), anti-mouse LAMP2 (M3/84) (1:500; BD Pharmingen, the Netherlands) for macrophages labeled with DAB (brown) (Vector Laboratories, Burlingame, USA) and anti- α smooth muscle actin (1:400; PROGEN Biotechnik GmbH, Germany) labeled with vlna green (Biocare Medical, Pacheco, USA). Slides were scanned by an Aperio AT2 slide scanner (Leica Biosystems). Monocyte adherence, ICAM-1 expression and the number of Ki67 positive macrophages were assessed in Image Scope (version 12-12-2015), and plaque composition was measured in Fiji (version 30-5-2017).

Statistical analysis

Significance of differences between the groups was calculated using a one-way ANOVA, followed by Dunnett's 2-sided post-hoc test for comparisons against the control and baseline control group. The Bonferroni post-hoc test was used to correct for multiple comparisons between the different treatment groups. For the atherosclerosis measurements the non-parametric Kruskal-Wallis test was used to test for differences between groups, followed by a Mann-Whitney U test for comparisons against the baseline and control group and between the different treatment groups. Linear regression analyses were used to assess correlations between variables. IBM SPSS v24.0 was used for all analyses. p -values ≤ 0.05 were considered statistically significant.

Results

Double and triple treatment with alirocumab and evinacumab on top of atorvastatin gradually decrease total and non-HDL-cholesterol

Mice were fed WTD for 13 weeks which led to increased plasma TC levels of about 25 mmol/L. At that point, the mice were matched into groups and treatment started. All treatments decreased plasma cholesterol (**Figure 2A**) and cholesterol exposure (mmol/L*weeks) in comparison to control (**Figure 2B**) with a gradual decline in the

atorvastatin, double (alirocumab and atorvastatin, evinacumab and atorvastatin) and triple (alirocumab, evinacumab and atorvastatin) treatment groups. Triple treatment lowered plasma TC levels to 1.8 mmol/L at the end-point and reduced cholesterol exposure by 80% ($p<0.001$) relative to control, and by 68% ($p<0.001$), 45% ($p<0.001$) and 38% ($p=0.035$) when compared to atorvastatin or double treatment with alicocumab or evinacumab, respectively. All treatments, except monotreatment with atorvastatin, consistently decreased plasma TG levels (**Figure 2C**). Non-HDL-C levels were decreased by all treatments, with the largest reduction, down to 1.0 mmol/L, achieved by triple treatment at the end of the study (-91%, $p<0.001$), which was significantly lower when compared to double treatment with alicocumab (-74%, $p=0.010$) and evinacumab (-72%,

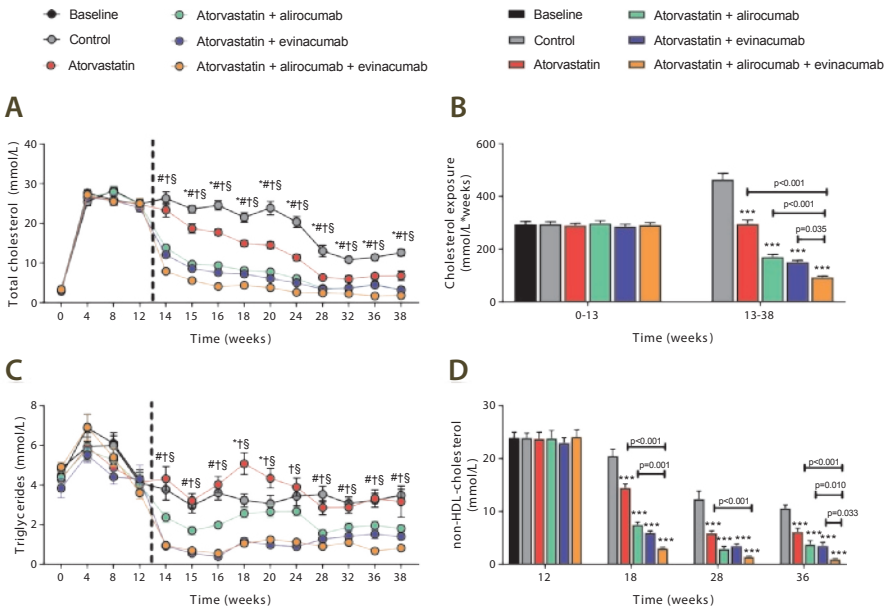


Figure 2 Double and triple treatment with alicocumab and evinacumab on top of atorvastatin gradually decrease triglycerides and total and non-HDL-cholesterol. APOE3-Leiden.CETP mice were fed a WTD for 13 weeks to induce atherosclerosis and remained on the diet without or with treatment until end-point. Plasma TC (A), total cholesterol exposure (mmol/L*weeks) (B), plasma TG (C). Non-HDL (D) was calculated by subtracting HDL-C from TC. The dotted line represents start of treatment and sacrifice of the baseline group. Data are presented as means \pm SEM ($n=13-16$ per group). Figure A and C: * $p<0.05$ atorvastatin vs control, # $p<0.05$ atorvastatin + alicocumab vs control, † $p<0.05$ atorvastatin + evinacumab vs control, § $p<0.05$ atorvastatin + alicocumab + evinacumab vs control. Figure B and D: *** $p<0.001$ compared to control. Abbreviations: WTD, western type diet; TC, total cholesterol; TG, triglycerides; HDL-C, high-density-lipoprotein-cholesterol.

$p=0.033$) (**Figure 2D**). The reduction in TC was confined to the apoB-containing lipoproteins (VLDL-LDL) (**Figure 3**). Altogether, these data demonstrate that evinacumab on top of atorvastatin and alirocumab has an additional cholesterol-lowering effect resulting in non-HDL-C levels of 1.0 mmol/L.

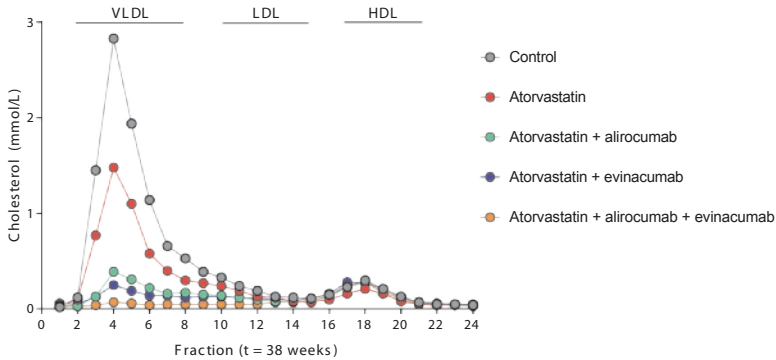


Figure 3 Lipoprotein profiles at end-point. APOE*3-Leiden.CETP mice were fed a WTD for 13 weeks to induce atherosclerosis and remained on the diet without or with treatment until end-point ($t=38$ weeks). Lipoprotein profiles were assessed by FPLC lipoprotein separation in group-wise pooled plasma ($n=13-16$ per group). Abbreviations: VLDL, very-low density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; WTD, Western type diet; FPLC, fast protein liquid chromatography.

Triple treatment with alirocumab and evinacumab on top of atorvastatin regresses pre-existent lesions and reduces lipid content in the thoracic aorta

We assessed the effect of intensive lipid-lowering on the progression and regression of pre-existing atherosclerosis at different sites along the aorta, in the thoracic aorta and the aortic root. After 13 weeks of WTD (at treatment baseline), 1.6% of the thoracic aorta was covered with oil-red-O positive lesions. WTD feeding for 25 more weeks led to further progression of atherosclerosis to 5.7% coverage in the control group. Treatment with alirocumab or evinacumab on top of atorvastatin fully blocked progression of atherosclerosis (**Figure 4A and C**). Double treatment with alirocumab and evinacumab decreased the amount of CE and double treatment with evinacumab the TG content beyond baseline (**Figure 4B**). Triple treatment did not only block the progression (-86%, $p<0.001$ vs control) but also resulted in regression of the pre-existent lesions by 50% ($p=0.045$) compared to baseline. Furthermore, triple treatment reduced CE and TG content beyond the baseline level in the thoracic aorta (-45%, $p=0.033$ and -83%, $p=0.001$, respectively).

The effect of triple and double treatments was stronger than atorvastatin monotreatment on all parameters.

In the aortic root, 208*1 000 μm^2 lesion area per cross-section was present at baseline, which further increased to 438*1 000 μm^2 in the control group. Atorvastatin modestly decreased lesion size (-28%, $p=0.001$ vs control), whereas double treatment with alirocumab or evinacumab on top of atorvastatin completely blocked the progression (-55%, $p<0.001$; -51%, $p<0.001$, respectively vs control). Triple treatment further decreased lesion size (-70%, $p<0.001$ vs control) and regressed the atherosclerotic lesion size (-36%, $p<0.001$ vs baseline) (**Figure 5A**). All treatments led to smaller lesions compared to control and triple treatment lesions were smaller than initial lesions size at baseline (**Figure 5B**). The area that consisted of complex lesions was decreased by triple treatment compared to control

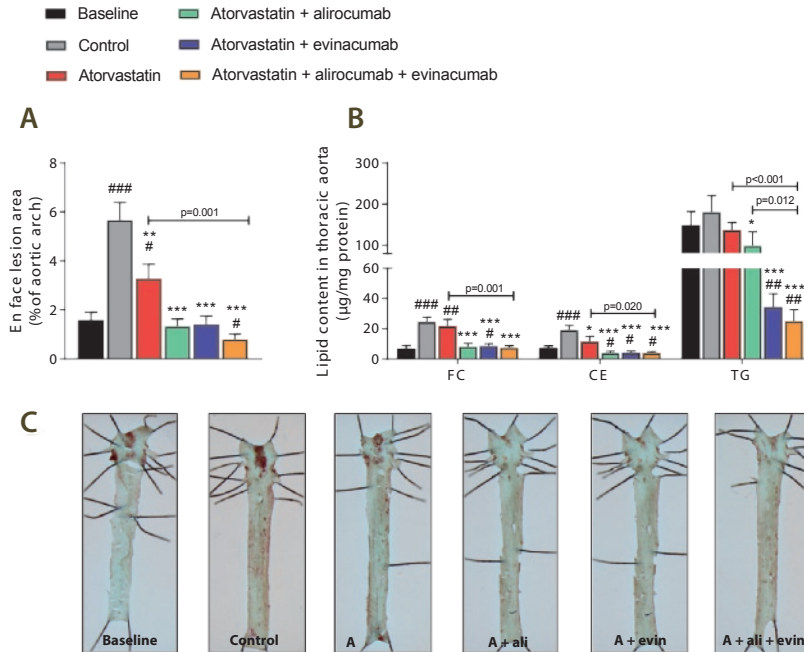


Figure 4 Triple treatment with alirocumab and evinacumab on top of atorvastatin regresses pre-existent lesions and reduces aortic lipid content in the thoracic aorta. En face analysis of atherosclerosis (A) and lipid content (B) in the thoracic aorta with representative images (C). Data are presented as means + SEM ($n=12$ per group). # $P<0.05$, ## $P<0.01$, ### $P<0.001$ when compared to baseline. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared to control. Abbreviations: A, atorvastatin; ali, alirocumab; evin, evinacumab; FC, free cholesterol; CE, cholesterol ester; TG, triglycerides.

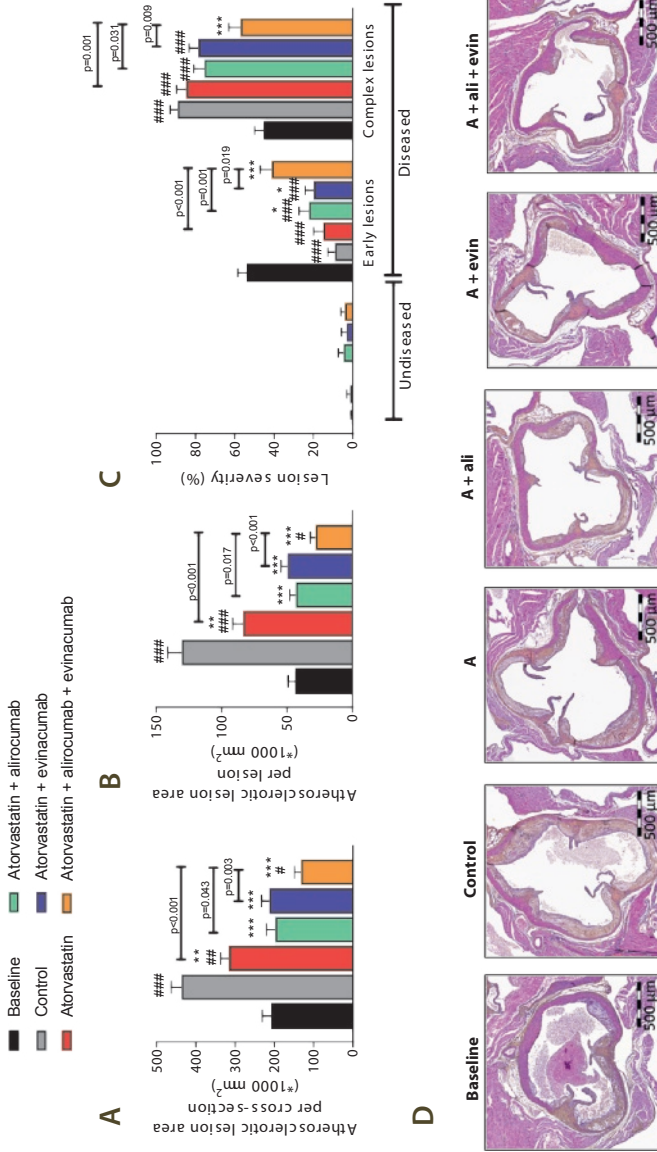


Figure 5 Double treatment with alirocumab or evinacumab on top of atorvastatin blocks the progression of atherosclerosis and triple treatment regresses pre-existing lesions in the aortic root. Lesion size (A) in aortic root after 13 weeks of WTD (baseline) and in control and treatment groups at end-point (week 38). Number of lesions per cross-section with lesion-free segment was assessed and the average size per lesion was calculated (B). Lesion severity as relative amount of early and complex lesions together with lesion-free segments (C). Representative images (D). Data are presented as means ± SEM (n=13-16 per group). #p<0.05, ##p<0.01, ###p<0.001 when compared to baseline. *p<0.05, **p<0.01, ***p<0.001 when compared to control. Abbreviations: A, atorvastatin; ali, alirocumab; evin, evinacumab.

(-36%, $p < 0.001$), with more early lesions present (**Figure 5C**). Additionally, triple treatment decreased lesion area and improved plaque phenotype as compared to mono- and double treatment. Representative images of the aortic root area are shown in **Figure 5D**. These data demonstrate that alirocumab and evinacumab on top of atorvastatin equally block the progression of atherosclerosis, but that regression of pre-existent, advanced atherosclerotic plaques is only achieved by aggressive lipid lowering using triple combination treatment.

The reduction in lesion size is correlated with the decrease in plasma cholesterol

We evaluated whether the reduction in lesion size could be explained by the reduction in plasma TC during treatment. The mean TC level at baseline was subtracted from the TC levels of each individual mouse at each time point and the cumulative decrease in cholesterol exposure was calculated as $\text{mmol/L} \cdot \text{weeks}$. These data were plotted against the lesion size at end-point minus the mean lesion size at baseline (**Figure 6**). A strong correlation between the difference in lesion area and the cumulative TC decrease during treatment was observed ($R = 0.85$, $p < 0.001$), indicating an important role of therapeutic cholesterol lowering in lesion regression.

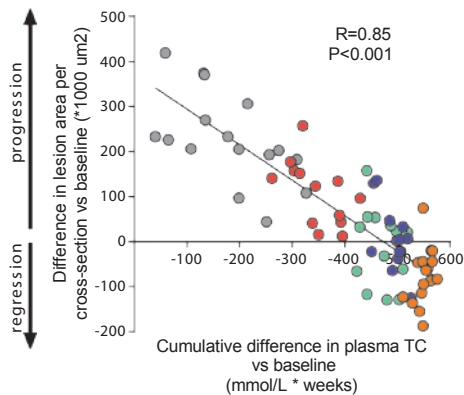


Figure 6 Correlation between the cumulative decrease in plasma cholesterol exposure and atherosclerotic lesion area. Mean TC at baseline was subtracted from TC levels of each individual mouse at each time point and the cumulative decrease in TC exposure during treatment was calculated as $\text{mmol/L} \cdot \text{weeks}$. Data were plotted against the difference in lesion size at end-point and mean lesion size at baseline. Linear regression analysis was performed ($n = 13-16$ per group). Abbreviations: TC, total cholesterol.

Double and triple treatment improve plaque composition

To evaluate whether plaque composition was affected by the treatments, the necrotic core, amount of macrophages, collagen and α SMC in the cap were quantified. Only triple treatment further decreased the macrophage content (-56%, $p=0.012$) compared to control, in parallel with increased α SMC (+38%, $p=0.015$) and collagen (+23%, $p<0.001$) content (**Figure 7A**). The plaque stability index improved by double (+66%, alirocumab and +64%, evinacumab, both $p<0.001$) and triple (+74%, $p<0.001$) treatment compared to control (**Figure 7B**). Representative images are shown (**Figure 7C**).

Triple treatment reduces monocyte adherence and macrophage proliferation

Vascular inflammation is recognized to play an important role in both the initiation and progression of atherosclerosis, whereas proliferation of macrophages further increases the plaque burden. Therefore, we measured endothelial ICAM-1 expression and adherence of monocytes to the activated endothelium as markers of vascular inflammation, and counted the number of currently proliferating macrophages after immunostaining for Ki67. All regimens except monotreatment with atorvastatin decreased ICAM-1 expression when compared to control, but only triple treatment decreased ICAM-1 expression when compared to baseline (-37%, $p=0.010$) (**Figure 8A and C**). In addition, triple treatment decreased the number of monocytes adhering to the endothelium when compared to baseline (-78%, $p<0.001$) and control (-69%, $p=0.003$), whereas mono- and double treatment only decreased monocyte adherence when compared to control (**Figure 8B**). The number of proliferating macrophages per plaque (**Figure 8D**) decreased over time by 91% ($p<0.001$; control vs baseline), which was further reduced by monotreatment with atorvastatin (-60%, $p=0.019$), double treatment with alirocumab or evinacumab on top of atorvastatin (-87%, $p=0.001$ and -58%, $p=0.012$ vs control) and triple treatment (-88%, $p<0.001$ vs control). Representative images are shown (**Figure 8E**).

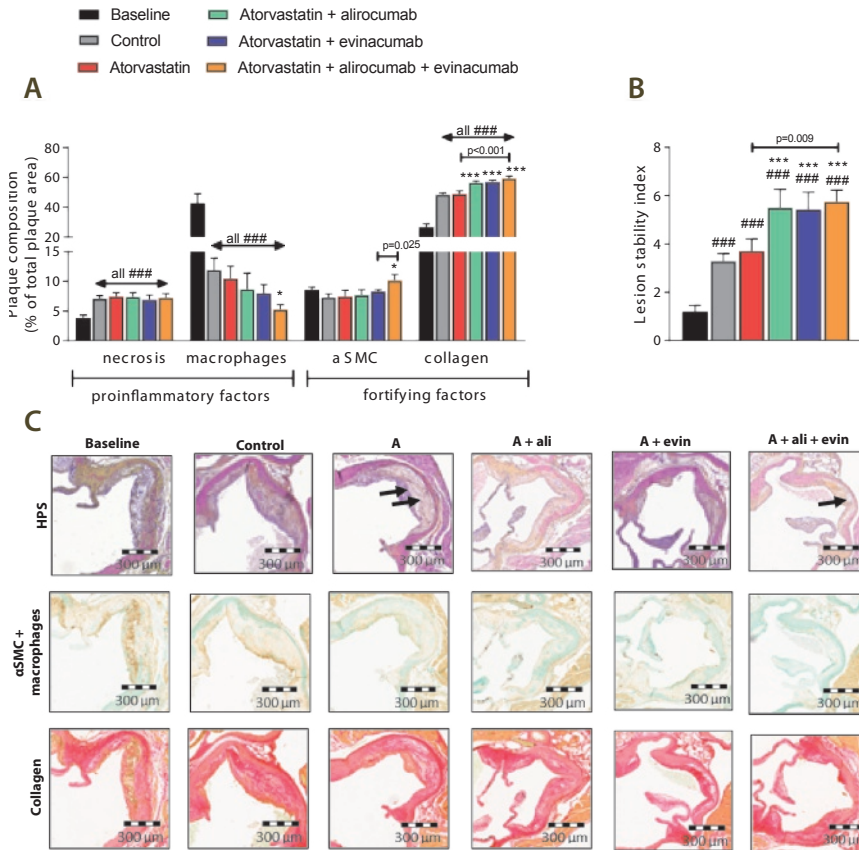


Figure 7 Double and triple treatment improve plaque phenotype. Necrotic and macrophage content as pro-inflammatory factors and αSMCs and collagen as fortifying factors were determined in the complex lesions in the aortic root and expressed as percentage of total plaque area (A). Lesion stability index, as the ratio of collagen and αSMC area (i.e. stabilization factors) to macrophage and necrotic area (i.e. destabilization factors) was calculated (B). Representative images of HPS staining, double-immunostaining with α-actin for SMCs (Vina green) and LAMP2 (M3/84) for macrophages (DAB, brown), and Sirius Red staining for collagen. The arrows depict necrotic areas, including cholesterol clefts (C). Data are presented as means ± SEM (n=13-16 per group). ###P<0.001 when compared to baseline. *p<0.05, ***p<0.001 when compared to control. Abbreviations: HPS, hematoxylin-phloxine-saffron; SMCs, smooth muscle cells; DAB, 3,3'-Diaminobenzidine; Abbreviations: A, atorvastatin; ali, aliocumab; evin, evinacumab.

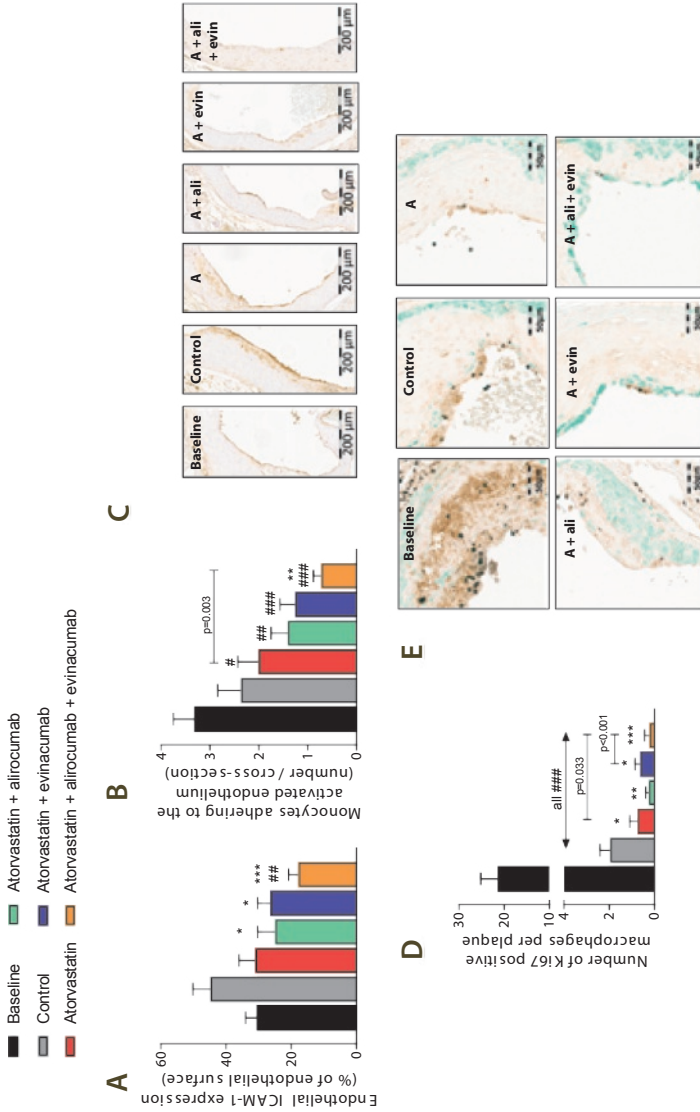


Figure 8 Monocyte adherence and the number of proliferative macrophages decrease in regression plaques. In each segment used for lesion quantification in the aortic root, endothelial ICAM-1 expression was determined as percentage of the luminal surface (A). Number of monocytes adhering to the activated endothelium per cross-section after staining with AIA 31240 (B). Representative images of ICAM-1 expression (C). Number of Ki67 positive macrophages as marker for proliferation in type IV and V plaques after triple-immunostaining with Ki67 (DAB, black), LAMP2 (M3/84) for macrophages (DAB, brown) and α -actin for α SMC (green). Number of proliferative macrophages was counted per plaque (D). Representative images (E). Four mice were excluded from figure D (2 mice in control, 2 mice in alirocumab + atorvastatin) due to extensive infiltration of the plaques by Ki67 positive inflammatory cells. Data are presented as means \pm SEM (n=11-16 per group), #p<0.05, ##p<0.01, ###p<0.001 when compared to baseline. *p<0.05, **p<0.01, ***p<0.001 when compared to control. Abbreviations: ICAM-1, intercellular adhesion molecule 1; SMCs, smooth muscle cells; DAB, 3,3'-Diaminobenzidine; A, atorvastatin; ali, alirocumab; evin, evinacumab.

Discussion

PCSK9 inhibition with alirocumab has been shown to strongly lower LDL-C and non-HDL-C alone and on top of a statin, and reduce the risk of recurrent ischemic cardiovascular events in patients with acute coronary syndrome (18). ANGPTL3 monoclonal antibody evinacumab was reported to reduce plasma TG and LDL-C levels in healthy subjects and homozygous hypercholesterolemia patients (17,20). Recent data suggest not only LDL-C but also remnant cholesterol, thus all apoB-containing lipoproteins are important predictors of cardiovascular outcome (9,10). The present study was designed to investigate the effect of gradual and aggressive reduction of cholesterol in both LDL and remnant lipoproteins by alirocumab and/or evinacumab on top of atorvastatin on regression of pre-existent atherosclerosis in hyperlipidemic mice. Our data revealed that alirocumab and evinacumab in combination with atorvastatin fully block further progression of atherosclerosis and triple treatment reduces lesion size beyond the treatment baseline level. In addition, double and triple treatments improve lesion morphology and composition in APOE*3-Leiden.CETP mice with pre-existent atherosclerosis. This is the first study in mice that shows real regression of lesion size using the combination of clinical hypolipidemic drugs.

Therapeutic interventions in mice have been hampered due to the lack of responsiveness to current lipid-lowering therapies in murine models of regression. Commonly used models are the aortic transplant model, the Reversa mouse (*Ldlr*^{-/-}*Apob*100/100Mx1-*Cre*^{+/+}), and *ApoE*^{-/-} and *LDLR*^{-/-} mice (reviewed in (13,26)). In these models, progression of atherosclerosis is induced by a WTD and regression is accomplished by a switch to chow, eventually together with genetic alterations or treatment strategies. Regression of atherosclerosis is generally defined by a decrease of macrophages or lipid content (13,26), though some studies reported a reduced total lesion size using experimental interventions, which was independent of plasma TC levels (reviewed in (13,26)). While these models are of great value to elucidate the molecular characteristics of the regressive plaque, they are less suitable for the evaluation of lipid-lowering interventions and their effect on atherosclerosis regression, as they poorly respond to registered lipid-lowering drugs (27,28). In the present study, we used the APOE*3-Leiden.CETP mouse, which possesses a delayed but intact apoE-LDLR-mediated clearance pathway and expresses CETP (14,28). These mice respond to all lipid-lowering drugs used in the clinic, including statins, alirocumab and evinacumab (15–17). Thus, treatments of APOE*3-Leiden.CETP mice on WTD with atorvastatin or alirocumab on top of the statin in our study decreased TC levels (-31% to -51% vs control and -32% to -52% vs atorvastatin, respectively) by a reduction of non-HDL-C, similarly as in humans (18,29). Evinacumab has an additive effect on treatment with atorvastatin and alirocumab by further reducing TC (-62% to -75% vs atorvastatin; -34% to -63% vs atorvastatin + alirocumab) and, in addition, TG levels (-62% to 80% vs atorvastatin; -42% to -65% vs atorvastatin + alirocumab).

We have previously shown that the lipid-modifying effects of PCSK9 and ANGPTL3 inhibition have an atheroprotective effect in a preventive design (16,17). However, to date,

the effect of pharmacological inhibition on regression of atherosclerosis, more closely mimicking the human situation, has not been investigated. Here, we show for the first time that double treatment with alirocumab or evinacumab on top of atorvastatin completely blocks progression of pre-existent atherosclerosis and that triple treatment regresses atherosclerosis in the aortic arch and the aortic root. The treatment effects on lesion area were mainly predicted by the gradual and aggressive reduction in plasma TC levels as illustrated by the strong association between the decreased cholesterol exposure and lesion size during treatment ($R=0.85$). All triple treated mice except one showed a lesion size below that at baseline, indicating regression. Reduction of non-HDL-C levels to about 1 mmol/L (38.7 mg/dL) was required to observe the regression. This finding is in accordance with studies in CVD patients that show exclusively reduction of plaque volume at LDL-C lowering of more than 40% or at a target level below 2.0 mmol/L (78 mg/dL) (5–7). Similar levels of 1 mmol/L were achieved in the recent outcome trials with PCSK9 inhibition which further reduced the risk of cardiovascular events as compared to statins and other hypolipidemic therapy (18,30).

Vulnerable plaques with high macrophage content, a large necrotic core and a thin, collagen-poor, fibrous cap are more prone to rupture (31). Thus, lesion composition, not only lesion size, is another important characteristic of the plaque. In the present study, the decline in plasma cholesterol reduced the lipid content of the aorta and resulted in smaller and less inflamed lesions. Double and triple treatment decreased endothelial expression of ICAM-1 and consequently reduced monocyte adhesion to the activated vascular endothelium, well-recognized processes in the initiation of atherosclerosis. In hypercholesterolemia, modified lipoproteins induce endothelium activation, thereby mediating the arrest and transmigration of circulating monocytes into the subendothelial space where they differentiate into macrophages (32). All treatments in the present study reduced the macrophage content, and double and triple treatment increased the amount of collagen in the lesions, resulting in a strongly improved plaque morphology. The large reduction in macrophage content in the present and other studies is a key feature of regression, and depends on the balance between recruitment of monocytes and their differentiation into macrophages, proliferation of macrophages, and on apoptosis and migratory egress from the plaques. However, whereas impaired monocyte transmigration during the initiation of atherosclerosis diminishes plaque volume (33), monocyte depletion per se does not affect further progression of plaque burden (34). Local proliferation of aortic macrophages has been reported to be a key event in the progression of atherosclerosis and to substantially contribute to lesional macrophage accumulation (34). Here we provide evidence that cholesterol lowering-induced regression decreases the number of Ki67-positive macrophages, a marker of currently proliferating macrophages. This finding suggests that diminished proliferation of macrophages is an important process in the reduction in macrophage content during regression of atherosclerosis.

In conclusion, we show that high-intensive lipid-lowering triple treatment with atorvastatin, alirocumab and evinacumab regresses atherosclerosis, improves plaque phenotype, and

reduces the proliferation of macrophages in the plaques. These data show that further reduction of plasma cholesterol together with TG-lowering to target all apoB-containing lipoproteins may be an effective approach to further reduce existing atherosclerosis in dyslipidemic patients at CV risk resulting in further decline of clinical events and increase of symptom-free years.

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Disclosures

Alirocumab (Praluent®) and evinacumab (REGN1500) are developed by Regeneron Pharmaceuticals and evinacumab is currently under trial. JG and VG are employees of Regeneron Pharmaceuticals and MGP, EJP, NW, NK, HMGP are employees of TNO during the conduct of this work.

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