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Efficacy of a novel PCSK9 inhibitory peptide alone and with evinacumab in a mouse model of atherosclerosis

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Abstract **Atherosclerosis is the major cause of cardiovascular disease. This study evaluated the effect of lipid lowering using a novel peptide inhibiting proprotein convertase subtilisin/kexin type 9 (PCSK9) and a monoclonal antibody against angiopoietin-like 3 (evinacumab), either alone or in combination in APOE*3-Leiden.CETP mice fed a Western diet. Effects on body weight, plasma lipids, atherosclerotic lesion size, severity, composition, and morphology were assessed. Treatment with PCSK9 inhibitory peptide significantly decreased both cholesterol and triglycerides (−69% and −68%, respectively). Similar reductions were seen in evinacumab-treated mice (−44% and −55%, respectively). The combination of evinacumab and PCSK9 inhibitory peptide lowered these levels to a larger extent than evinacumab alone (cholesterol: −74%; triglycerides: −81%). Reductions occurred in non-HDL-C without changes in HDL-C. Atherosclerotic lesion size was significantly reduced in all treatment groups compared to vehicle controls (evinacumab: −72%; PCSK9 inhibitory peptide: −97%; combination: −98%). Similarly, all interventions improved atherosclerotic lesion severity, with more undiseased segments and fewer severe lesions. Evaluation of the composition of severe atherosclerotic plaques revealed significant improvement in lesion stability in mice treated with both evinacumab and PCSK9 inhibitory peptide, attributable to decreased macrophage content and increased collagen content. Additionally, evaluation of lipid concentrations in cynomolgus monkeys revealed the beneficial effects of the PCSK9 inhibitory peptide on total cholesterol and LDL-C levels. Treatment with a novel PCSK9 inhibitory peptide alone or with evinacumab shows great potential to reduce and stabilize atherosclerotic lesions.**

Supplementary key words evinacumab • atherosclerosis • PCSK9 • ANGPTL3 • APOE*3-Leiden.CETP mice • combination therapy • lipid lowering • atherosclerotic plaque composition

Cardiovascular disease (CVD) is the leading cause of death worldwide, and its prevalence is predicted to rise further in the upcoming decades. While lifestyle changes remain the treatment of choice in patients at risk for developing CVD and atherosclerosis, pharmacological lipid-lowering therapy has proved an effective intervention for reducing cardiovascular burden as well. Statins are nowadays the golden standard for reducing cholesterol, yet large subgroups of patients do not reach their low-density lipoprotein cholesterol (LDL-C) targets or do not tolerate statins well. Most lipid-lowering strategies aim at reducing LDL-C because with each 1 mmol/L decrease in plasma LDL-C, there is a log-linear proportional reduction in cardiovascular events in patients at risk (1). Besides LDL-C, remnant cholesterol and triglyceride levels are considered important residual risk factors for CVD as well (2, 3), and non-high density lipoprotein cholesterol (non-HDL-C) has been reported to be an even more predictive risk factor than LDL-C (4). Essentially, the clinical benefit of lowering triglycerides and LDL-C is proportional to the absolute change in apolipoprotein B (apoB), implicating that all apoB-containing lipoproteins have approximately the same effect on the risk of CVD per particle (5). Reductions in LDL-C levels are necessary to stop progression of atherosclerosis, yet to induce the regression of atherosclerosis, more aggressive approaches are necessary, which may be achieved by combination therapies targeting all apoB-containing lipoproteins (6–8).

Proprotein convertase subtilisin/kexin type 9 (PCSK9) has been identified as an important target for cholesterol lowering since it binds to the LDL receptor, thereby targeting it for lysosomal degradation (9). Inhibition of this process results in LDL receptor recycling, which allows for additional removal of LDL-containing lipid particles from the circulation and consequently lowers plasma cholesterol concentrations. PCSK9 monoclonal

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antibodies have proven successful in reducing LDL-C and cardiovascular events in patients (10–13). Nevertheless, their relatively high costs make these monoclonal antibodies less appealing for the larger patient population requiring lipid lowering interventions. The PCSK9 inhibitory peptide used here is an analogue of the amino acid sequence of the epidermal growth factor-like domain A (EGF(A) domain) of the LDL receptor (amino acids 293–332) with improved binding capacity for PCSK9 (Ki about 1 nM) https://patents.google.com/patent/WO2017121850A1/en. To enhance intestinal absorption, to improve the stability of the peptide and to increase albumin affinity, Novo Nordisk's fatty acid acylation technology was used to obtain a long half-life and a long duration of action of the peptide (14). While the efficacy of PCSK9 inhibitors on top of statin intervention has been demonstrated before, we wanted to evaluate an approach more relevant for a patient-population that is intolerant to statins or that does not reach their LDL-C target levels with statin intervention. Since combination treatments are warranted to achieve profound apoB lowering, we used an approach where this novel PCSK9 inhibitory peptide was tested alone and in combination with the monoclonal anti-angiopoietin-like 3 (ANGPTL3) antibody evinacumab. The latter has been demonstrated to strongly improve plasma cholesterol and triglycerides in humans (15, 16) via a (different) mechanism that may act complementary to PCSK9 inhibition. By inhibiting ANGPTL3, triglyceride hydrolysis of triglyceride-rich lipoproteins by lipoprotein lipase is improved and consequently their removal from the circulation enhanced (17).

In the current study, we studied the effects of profound LDL-C lowering on the progression of atherosclerosis by treating apolipoprotein E*3-Leiden.CETP (APOE*3-Leiden.CETP) mice with evinacumab, the novel PCSK9 inhibitory peptide and their combination. This mouse model develops hyperlipidemia and associated cardiovascular complications in response to a Westernized diet. Importantly, these mice respond well to all lipid-lowering interventions that are being used in the clinic (18–20), including different ANGPTL3 (15, 21, 22) and PCSK9 (23–27) inhibiting modalities. Here, we evaluated how the lipid-lowering characteristics of the novel PCSK9 inhibitory peptide alone and in combination with evinacumab affected atherosclerotic lesion severity and composition in APOE*3-Leiden.CETP mice. Additionally, the effects of the PCSK9 inhibitory peptide on lipid concentrations were evaluated in a non-human primate model.

MATERIALS AND METHODS

APOE*3-Leiden.CETP mouse model

Experimental design. Experimental procedures were approved by The Netherlands Central Authority for Scientific Procedures on Animals (CCD; project license AVD5010020172064) and independent animal welfare body of The Netherlands Organization for Applied Scientific Research (IvD TNO; TNO-323). APOE*3-Leiden.CETP mice were bred and housed at the SPF animal facility at TNO (TNO Metabolic Health Research, Leiden, the Netherlands). For this study, female mice were selected because they are more responsive to dietary cholesterol than males and therefore develop more pronounced atherosclerosis (28, 29).

Mice (7–12 weeks old) were group-housed in a temperature-controlled room on a 12h light-dark cycle and at 50%–60% humidity. A total of 62 mice received a semi-synthetic modified diet containing 15% (w/w) cocoa butter with 0.15% (w/w) added cholesterol (Ssniff Spezialdiäten GmbH, Soest, Germany) and had free access to food and heat-sterilized tap water. Body weight, food intake at the cage level and clinical signs were monitored regularly. At t = 0, after a 3-weeks run-in period on the diet, mice were matched into four groups based on age, body weight, plasma cholesterol and triglycerides. Group sizes were calculated a priori by power analysis (GPower) (30) using a minimal effect size of 30% and two-sided test with 95% confidence interval, power of 90% and α of 0.05. For the two groups treated with evinacumab, two extra mice were added since this is the percentage of mice that may develop anti-drug antibodies (15, 21), which may undermine the effectiveness of evinacumab. A control group (n = 15) was treated daily with subcutaneous saline injections. The second group (n = 17) received weekly subcutaneous injections of evinacumab (25 mg/kg/week body weight) and received subcutaneous saline injections for the remaining six days of the week. The third group (n = 15) was treated daily with PCSK9 inhibitory peptide (NNC0385-0434; 0.6 mg/kg/day body weight) by subcutaneous injections. The last group (n = 17) was treated with both evinacumab and PCSK9 inhibitory peptide. After sixteen weeks, all mice were sacrificed non-fasted by gradual-fill CO_2 asphyxiation and terminal blood and hearts were collected for further analysis.

Biochemical analyses in plasma. Blood was drawn regularly throughout the study from the tail vein into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) either non-fasted for determination of post-prandial plasma parameters or after a 4h fasting period. Enzymatic colorimetric assays (Roche Diagnostics, Almere, the Netherlands) were used to determine plasma cholesterol and triglyceride concentrations. Cholesterol and triglyceride exposure were calculated as mmol/L*weeks. Cholesterol lipoprotein profile was determined in group pooled plasma samples after lipoprotein separation by fast protein liquid chromatography (FPLC) (31).

Histological assessment of liver steatosis. At sacrifice, livers were collected and tissue of the medial liver lobe was formalin-fixed and embedded in paraffin for histological analysis of hepatic steatosis. Cross-sections (3 μm) were stained with hematoxylin and eosin (H&E) and scored blindly for steatosis by a board-certified pathologist using an adapted grading system of human NASH (32, 33). Two cross-sections per mouse were analyzed for steatosis at 40× magnification and expressed as total percentage of steatosis relative to the total liver area.

Histological assessment of atherosclerosis. At sacrifice, hearts were collected, formalin-fixed and embedded in paraffin for histological analysis as previously described in detail (21, 34, 35). In short, cross-sections (5 μm) at 50 μm intervals were made perpendicular to the axis of the aorta. Sections were stained with hematoxylin-phloxine-saffron (HPS) and scanned with an

Aperio AT2 slide scanner (Leica Biosystems, Amsterdam, the Netherlands). Per mouse, four sections were assessed for atherosclerotic lesion severity in keeping with the classification by the American Heart Association. Accordingly, no lesions indicate undiseased segments, type I lesions indicates early fatty streak, II regular fatty streak, III mild plaque, IV moderate plaque and V severe plaque (21, 36).

All type IV and V lesions were further analyzed for atherosclerotic plaque composition as described previously in detail (21, 35). In short, double immunostaining with anti-α-smooth muscle actin (#61001; PROGEN Biotechnik GmbH) for smooth muscle cells (SMCs) and anti-LAMP2 (M3/84) (MA5-17861; Invitrogen) for macrophages was performed. Anti-α-smooth muscle actin was visualized with Vina Green (Biocare Medical) after incubation with a secondary HRP-conjugated goat anti-rat antibody (ab97057; Abcam). Anti-LAMP2 was visualized with 3,3′-diaminobenzidine (DAB; Vector Laboratories) after incubation with a secondary HRP-conjugated antibody (#P0260; Dako A/S, Glostrup). Slides were then scanned with an Aperio AT2 slide scanner and analyzed using customized macros in ImageJ, after which coverslips were detached overnight in xylene. Subsequently, Sirius Red staining was performed for visualization of collagen. These slides were used to evaluate collagen content and necrotic core content (defined as a pool of accumulated cellular debris and extracellular lipids and including cholesterol clefts) using customized macros in ImageJ (version 1.53; NIH). Plaque stability was calculated by dividing the sum of SMCs in the fibrotic cap and collagen content in the entire lesion as stabilizing factors by the sum of macrophage and necrotic core content, both in the entire lesion, as destabilizing factors. This ratio is derived from human pathology where vulnerable lesions present with increased macrophage content, large necrotic core and a thin fibrous cap (37).

To evaluate the number of monocytes adhering to the activated endothelium, sections of the aortic root area were immunostained with AIA antibody (#31240-1:1000; Accurate Chemical and Scientific, New York, NY, USA) and ICAM-1 antibody (sc-8439-1:400; Santa Cruz Biotechnology, Dallas, TX, USA). Segments that were used to evaluate atherosclerotic lesion severity were used to score monocyte adherence using ImageScope (version 12.3.2.8013) (21, 25). For cross-sections stained with AIA, monocytes adhering to the endothelium were counted and expressed as number per cross-section. For ICAM-1, the total length of the endothelial lining was determined as well as the area of this part of the endothelial lining that was ICAM-1 positive. ICAM-1 positive area is expressed as percentage of the total endothelial lining.

Expression of NLR family pyrin domain containing 3 (NLRP3) was evaluated in the severe (type IV-V) lesions after immunostaining with NLRP3 antibody (PA5-79740-1:70; ThermoFisher Scientific, Waltham, MA, USA). NLRP3 expression was quantified in ImageJ using customized macros and expressed as percentage of the total lesion area.

Statistical analysis. All data are presented as mean ± standard error of the mean (SEM). Differences between groups were determined non-parametrically by Kruskal-Wallis testing followed by Mann-Whitney U testing for independent samples. Correlations between total cholesterol exposure and atherosclerotic lesion area were determined by Spearman's rank-order correlation test after square root transformation of atherosclerotic lesion area. SPSS software (version 28; IBM Corp.; Armonk, NY, USA) was used for all statistical analyses. Two-tailed *P*-values are reported and a *P*-value < 0.05 was considered statistically significant.

PCSK9 inhibitory peptide efficacy in cynomolgus monkey model

Experimental design. Experimental procedures in cynomolgus monkeys were approved by the Protocol Peer Review Group in the Ethical Review and Approval Process (PPRG; Envigo) and the non-human primate FG at Novo Nordisk (Måløv). Vietnamese cynomolgus monkeys (*Macaca fascicularis*) were obtained from Envigo (Department of Primate Toxicology) non-naïve stock. Animals had a body weight of 3.67 ± 0.58 kg at the time of dosing and were housed in a temperature-controlled room (15–24°C) on a 12h light-dark cycle and at 40%–70% humidity. In total, 2 male and 2 female monkeys (average age 35 months) were used that received 100 g of a standard dry diet (Harlan Teklad 2050) supplemented with two biscuit supplements and fresh fruit per day. The animals received a bolus injection of PCSK9 peptide subcutaneously in the dorsal area (50 nmol/kg). The test compound (NNC0385-0434) was formulated in phosphate buffer (50 mM Na_2HPO_4, 70 mM NaCl, 0.05% Tween 80, pH 7.4). Dosing volumes were 0.2 ml/kg and calculated based on the most recently recorded body weight. Blood samples (0.8 ml) were collected from a suitable vein at predefined time points up to 20 days post-dosing time into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany). Upon finalization of the experimental procedures, animals were returned to stock at Envigo.

Biochemical analyses in plasma. Plasma concentration-time profiles were analyzed by a non-compartmental analysis using the calculation method Linear Trapezoidal Linear/Log Interpolation in Phoenix WinNonlin Professional 6.4 (Pharsight, Mountain View, CA, USA). Calculations were performed using individual concentration-time values from each animal using actual dose and actual time values. The actual time of sampling was used in the calculation. A deviation from target time point relative to dosing of 5% was allowed for the first 18 h and deviation of 1 h was accepted for samples taken after 24 h. Plasma lipid concentrations were determined using a Cobas 6000 c501 analyzer (Roche Diagnostics, Rotkreuz, Switzerland) in accordance with the manufacturer's protocols. Data are presented as percentage relative to baseline.

RESULTS

Safety aspects in APOE*3-Leiden.CETP mice

After three weeks of WTD feeding, APOE*3-Leiden.CETP mice were matched into four groups and continued on WTD feeding for an additional sixteen weeks. Mice were either left untreated or were treated with evinacumab, PCSK9 inhibitory peptide, or a combination of the latter two. Prolonged exposure to the Westernized diet gradually increased body weight in all groups (supplemental Fig. S1B) and food intake was similar across all groups as well (supplemental Fig. S1C). Neither of the compounds affected viability, body weight or food intake. Liver weight was slightly but significantly increased in mice treated with PCSK9

inhibitory peptide alone compared to vehicle controls (+8%, $P < 0.05$) (supplemental Fig. S1D). Further histological investigation of the liver revealed no effects of either compound on total steatosis (supplemental Fig. S1A, E).

Evinacumab, PCSK9 inhibitory peptide and their combination reduce plasma cholesterol and triglycerides in APOE*3-Leiden.CETP mice

Plasma cholesterol and triglyceride concentrations were determined postprandially at the study endpoint. Compared to vehicle controls, all interventions significantly reduced postprandial cholesterol levels (evinacumab: −33%, $P < 0.01$; PCSK9 inhibitory peptide: −71%, $P < 0.001$; combination: −77%, $P < 0.001$) and postprandial triglyceride levels (evinacumab: −66%; PCSK9 inhibitory peptide: −76%; combination: −84%, all $P < 0.001$) (**Fig. 1**A, B). Lipoprotein profile analysis of postprandial plasma samples showed that the decrease in cholesterol and triglycerides was confined to apoB-containing (V)LDL-sized particles in the evinacumab and combination treatment group (supplemental Fig. S2A, B). The effects of the PCSK9 inhibitory peptide on postprandial cholesterol were stronger than evinacumab alone (−57%, $P < 0.001$) and combination treatment lowered these levels even further (combination vs. evinacumab alone: −66%, $P < 0.001$; combination vs. PCSK9 inhibitory peptide alone: −20%, $P < 0.05$). Combination treatment resulted in significantly lower levels of postprandial triglycerides compared to PCSK9 inhibitory peptide alone as well (−36%, $P < 0.05$).

Additionally, 4h-fasted plasma cholesterol and triglyceride concentrations were determined throughout the study. Compared to vehicle controls, all interventions significantly lowered cholesterol concentrations at all timepoints (Fig. 1C). Mice treated with

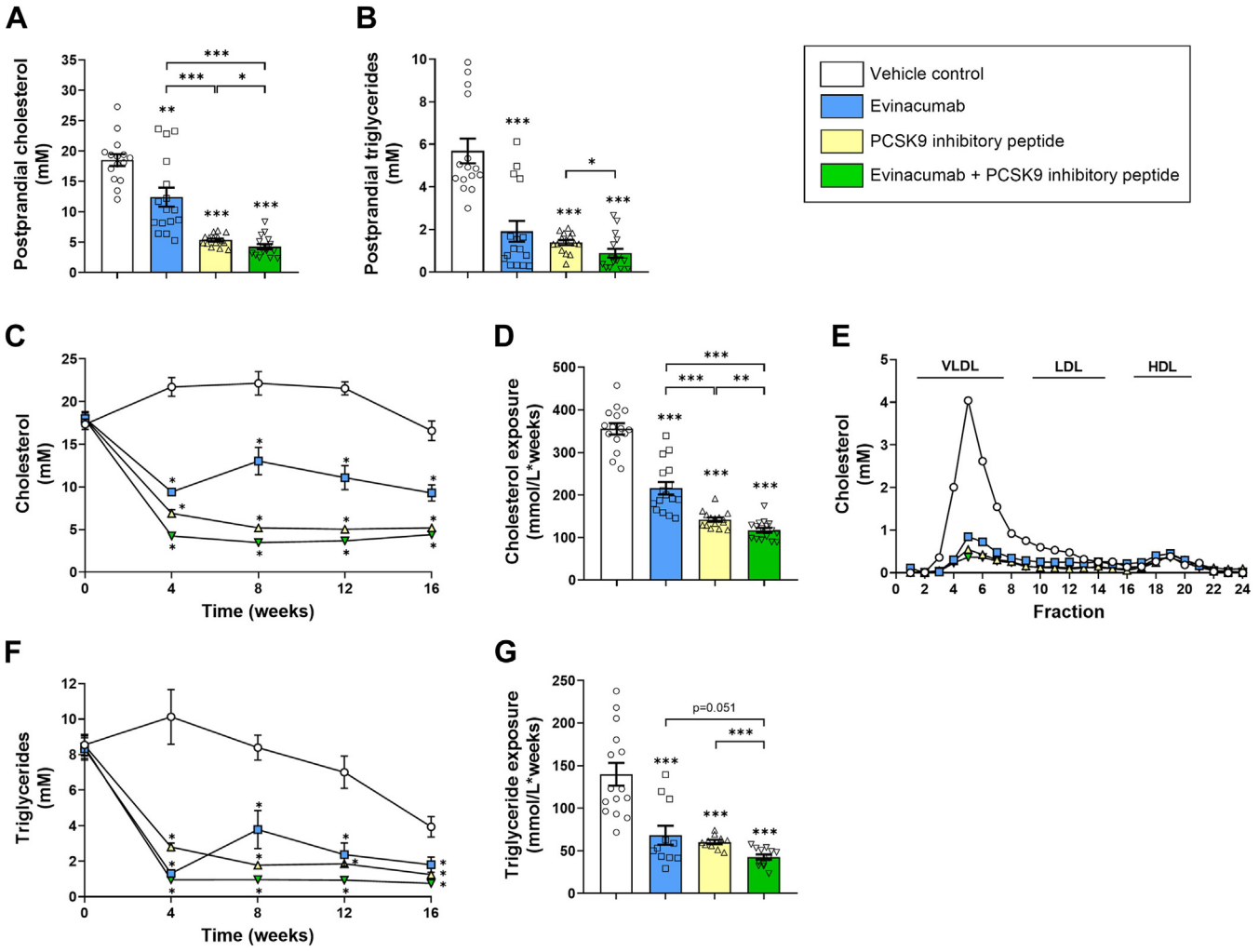

Fig. 1. Evinacumab, PCSK9 inhibitory peptide and their combination improve plasma cholesterol and triglycerides in APOE*3-Leiden.CETP mice. APOE*3-Leiden.CETP mice were fed a Westernized diet for 3 weeks, followed by 16 weeks on the same diet with or without treatment. Postprandial plasma cholesterol (A) and postprandial plasma triglycerides (B) were determined at the study endpoint. Four hour-fasted plasma cholesterol concentrations (C) were used to determine total cholesterol exposure (mmol/L*weeks) (D) and lipoprotein profiles for cholesterol were assessed in group-wise pooled plasma (E). Four hour-fasted plasma triglyceride concentrations (F) were used to determine total triglyceride exposure (mmol/L*weeks) (G). A, B, D, G: *$P < 0.05$, ***$P < 0.001$ versus vehicle control. C, F: *$P < 0.05$ versus vehicle control. Data are presented as mean ± SEM (n = 15–17 per group).

PCSK9 inhibitory peptide alone had lower plasma cholesterol levels compared to those treated with evinacumab alone. Moreover, at all timepoints, mice treated with the combination of evinacumab and PCSK9 inhibitory peptide had significantly lower plasma cholesterol levels relative to both monotreatments. Accordingly, total cholesterol exposure (concentration*weeks) was significantly reduced in all treatment groups (evinacumab: −39%; PCSK9 inhibitory peptide: −60%; combination: −67%, all $P < 0.001$ vs. vehicle controls) (Fig. 1D). Cholesterol exposure in mice that received PCSK9 inhibitory peptide monotreatment was 34% lower than mice that received evinacumab monotreatment ($P < 0.001$). Combination treatment was superior to both monotreatments in reducing total cholesterol exposure (combination vs. evinacumab alone: −46%, $P < 0.001$; combination vs. PCSK9 inhibitory peptide alone: −18%, $P < 0.01$). Lipoprotein profile analysis showed that the decrease was confined to the apoB-containing (V)LDL-sized (non-HDL-C) particles (Fig. 1E). Plasma triglycerides were significantly reduced in all treatment groups (Fig. 1F). Consequently, triglyceride exposure throughout the entire study was significantly lower in all intervention groups (evinacumab: −51%; PCSK9 inhibitory peptide: −57%; combination: −69%, all $P < 0.001$ vs. vehicle controls) (Fig. 1G). The ameliorative effects of the combination treatment on total triglyceride exposure were borderline significantly larger compared to evinacumab alone (−38%, $P = 0.051$) and significantly larger compared to PCSK9 inhibitory peptide alone (−29%, $P < 0.001$). Altogether, these data demonstrate the substantial cholesterol- and triglyceride-lowering capacity of evinacumab and PCSK9 inhibitory peptide and additional ameliorative effects on these levels of their combination.

Monotreatment with evinacumab or PCSK9 inhibitory peptide and combination treatment reduce the size, number and severity of atherosclerotic lesions

WTD feeding induced elevation of lipid levels and consequently induced development of pronounced atherosclerosis in the aortic root (**Fig. 2**A). Compared to vehicle controls, evinacumab monotreatment significantly reduced atherosclerotic lesion area and PCSK9 inhibitory peptide almost completely nullified atherosclerosis (evinacumab: −72%; PCSK9 inhibitory peptide: −97%; combination: −98%, all $P < 0.001$) (Fig. 2B). Total vessel area per cross-section did not differ between groups (supplemental Fig. S3A) and therefore lesion area per vessel area showed a similar pattern as atherosclerotic lesion area (supplemental Fig. S3B). Similar to PCSK9 inhibitory peptide monotreatment, the combination treatment almost completely abrogated atherosclerosis (combination vs. vehicle control: −97%; combination vs. evinacumab: −94%, both $P < 0.001$). The number of atherosclerotic lesions was significantly reduced with all interventions relative to vehicle controls (evinacumab: −27%, $P < 0.05$; PCSK9 inhibitory peptide: −81%, $P < 0.001$; combination: −80%, $P < 0.001$) (Fig. 2C). PCSK9 inhibitory peptide monotreatment and combination treatment improved the number of atherosclerotic lesions even further compared to evinacumab alone (both −73%, $P < 0.001$).

Further examination revealed a shift in lesion severity for all treatment groups compared to vehicle controls (Fig. 2D). In mice treated with evinacumab alone, there was a 3.0-fold increase in the percentage of undiseased segments ($P < 0.001$) and while the percentage of mild lesions was similar in this group compared to vehicle controls, there was a significant reduction in severe atherosclerosis lesions (−58%, $P < 0.001$). Likewise, monotreatment with PCSK9 inhibitory peptide improved lesion severity with significantly more undiseased segments (7.9-fold increase, $P < 0.001$), a borderline significant reduction in mild lesions (−42%, $P = 0.06$) and significantly less severe lesions (−91%, $P < 0.001$) relative to vehicle controls. The effect of the PCSK9 inhibitory peptide was significantly stronger than evinacumab monotreatment, with more undiseased segments (1.2-fold increase, $P < 0.001$) and fewer mild (−55%, $P < 0.01$) and severe lesions (−78%, $P < 0.01$). Compared to the vehicle control group, mice treated with both evinacumab and PCSK9 inhibitory peptide displayed mostly undiseased segments (8.6-fold increase, $P < 0.001$), owing to a reduction in mild lesions (−44%, $P < 0.05$) and almost complete absence of severe lesions (−96%, $P < 0.001$). Relative to mice that received evinacumab monotreatment, the combination group showed a significant increase in undiseased segments (1.4-fold increase, $P < 0.001$) and significant decrease in mild (−57%, $P < 0.01$) and severe (−91%, $P < 0.01$) lesions. The effect of combination treatment on atherosclerotic lesion severity was comparable to mice that received PCSK9 inhibitory peptide monotreatment.

Plasma cholesterol is a strong determinant for the progression of atherosclerosis and therefore, the correlation between total cholesterol exposure throughout the entire study and the square root of atherosclerotic lesion area at the study endpoint was determined. Atherosclerotic lesion area was strongly predicted by total cholesterol exposure ($R^2 = 0.83$, $P < 0.001$) (Fig. 2E). Together, these data demonstrate that cholesterol-lowering effects of evinacumab, PCSK9 inhibitory peptide and their combination result in robust improvements of atherosclerosis and atherosclerotic phenotype.

PCSK9 inhibitory peptide alone and in combination with evinacumab, but not evinacumab alone, improve atherosclerotic plaque composition

To more closely investigate the nature of the atherosclerotic plaques, necrotic core and macrophages

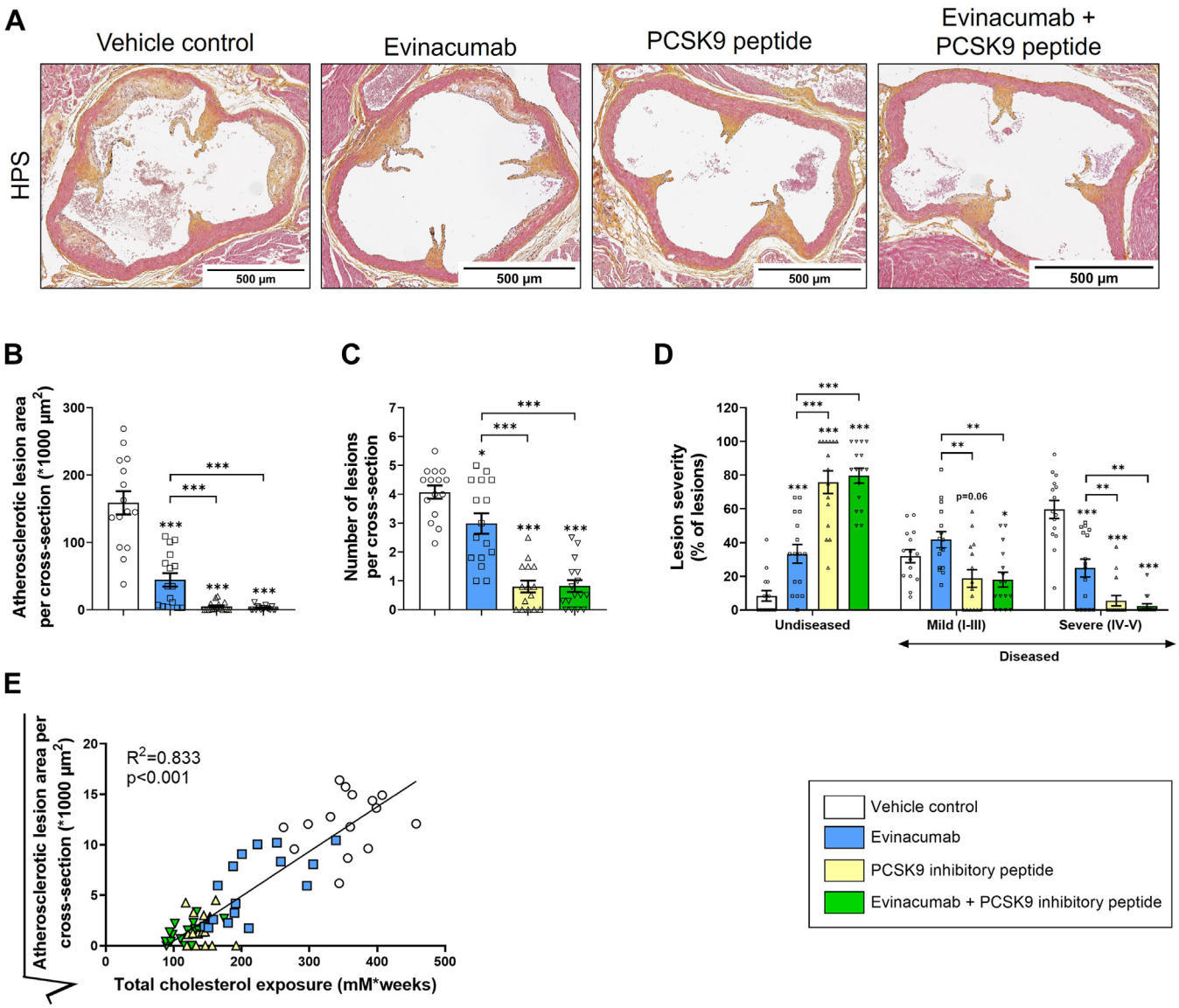

Fig. 2. Treatment with evinacumab, PCSK9 inhibitory peptide or both reduces atherosclerosis in the aortic root. Atherosclerotic lesion size and severity were determined in hematoxylin-phloxine-saffron (HPS)-stained cross-sections after 16 weeks of Western diet feeding with or without intervention. Representative HPS images (A), atherosclerotic lesion area per cross-section (B), number of atherosclerotic lesions per cross-section (C), and lesion severity as relative amount of mild (type I-III) and complex (type IV-V) lesions together with lesion-free (undiseased) segments (D). Correlation between the total cholesterol exposure throughout the study (mmol/L*weeks) and the square root of the total atherosclerotic lesion area (E). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle control. Data are presented as mean ± SEM (n = 15–17 per group).

as destabilizing factors and collagen and SMCs (in the fibrotic cap) as fortifying factors of severe type IV and V lesions were determined (**Fig. 3**A and supplemental Fig. S4). It should be noted that treatment with PCSK9 inhibitory peptide alone and in combination with evinacumab had profound ameliorative effects on the number of these severe type IV and V lesions. Therefore, only seven lesions could be analyzed in the PCSK9 monotreatment group and four lesions in the combination treatment group. Necrotic core content was similar in all groups, while macrophage content was significantly reduced in the combination treatment group relative to vehicle controls (−34%, $P < 0.01$) (Fig. 3B). With regard to the fortifying components of atherosclerotic plaques, SMC content was significantly reduced in the evinacumab monotreatment group (−35%, $P < 0.05$) and not affected in the other treatment groups relative to vehicle control (Fig. 3B). In contrast, collagen content was increased in plaques of mice that were treated with the combination of evinacumab and PCSK9 inhibitory peptide (+65%, $P < 0.05$) but not changed in both monotreatment groups (Fig. 3B). Plaques of mice treated with both evinacumab and PCSK9 inhibitory peptide consisted of almost twice the amount of collagen in the lesion area compared to mice treated with evinacumab alone (+98%, $P < 0.01$). Establishing

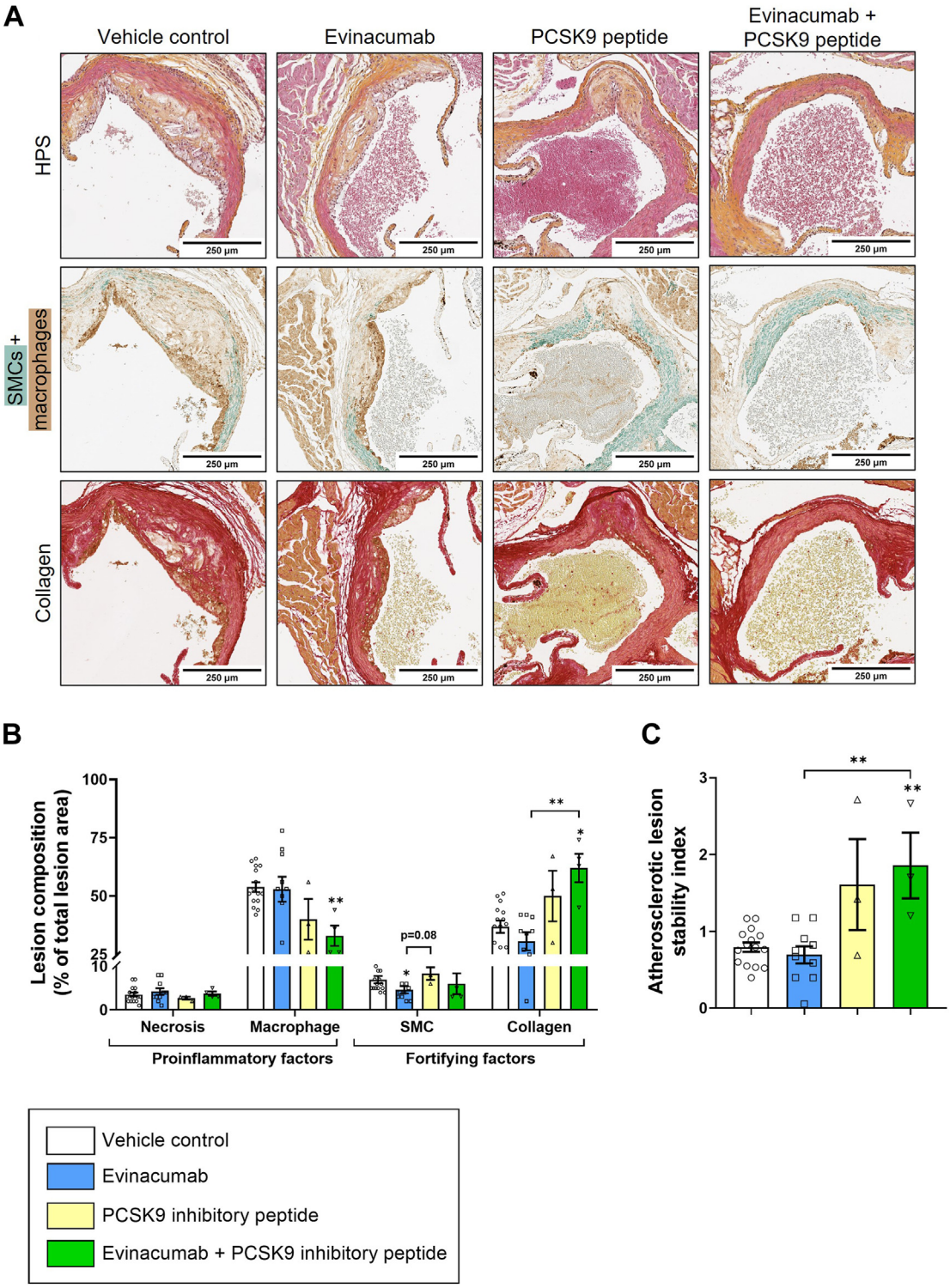

Fig. 3. Combination treatment with evinacumab and PCSK9 inhibitory peptide improve atherosclerotic plaque composition and activation of the endothelium. Representative images of hematoxylin-phloxine-saffron (HPS)-stained slides of the aortic root area, double-immunostaining with α-actin for smooth muscle cells (SMCs; Vina green), LAMP2 (M3/84) for macrophages (DAB, brown), and Sirius Red-stained slides for collagen (A). Complex (type IV-V) lesions in the aortic root area were analyzed for necrotic core and macrophage content as proinflammatory factors and for SMCs and collagen as plaque fortifying factors (B). Atherosclerotic plaque stability index was calculated by dividing collagen and SMC area (stabilizing factors) by necrotic core and macrophage area (destabilizing factors) (C). *$P < 0.05$, **$P < 0.01$ versus vehicle control. Data are presented as mean ± SEM (n = 15–17 per group).

the plaque stability index by calculating the ratio between stabilizing and destabilizing factors revealed a significant improvement in mice that received combination treatment both compared to the vehicle control group (2.1-fold increase, $P < 0.01$) and compared to evinacumab alone (2.2-fold increase, $P < 0.01$) (Fig. 3C).

Further analysis of inflammation in the aortic root area was performed by evaluating expression of endothelial intercellular adhesion molecule 1 (ICAM-1) and monocyte adherence to the endothelium surface (**Fig. 4**A). All interventions improved ICAM-1 expression compared to vehicle controls (evinacumab: −58%; PCSK9 inhibitory peptide: −94%; combination: −94%, all $P < 0.001$) (Fig. 4C). Combination treatment was significantly more effective in lowering endothelial ICAM-1 expression relative to evinacumab monotherapy (−85%, $P < 0.001$). Similarly, in all treatment groups, there was a significant reduction in the number of monocytes adhering the activated endothelium compared to vehicle controls (evinacumab: −51%; PCSK9 inhibitory peptide: −60%; combination: −74%, all $P < 0.001$) (Fig. 4B, D). The effects of combination treatment were significantly larger compared to evinacumab intervention alone (−47%, $P < 0.01$). Evaluation of NLRP3 expression as marker of macrophage activation and inflammation in the severe (type IV-V) lesions revealed a significant reduction in mice treated with the combination of evinacumab and PCSK9 inhibitory peptide (−74%, $P < 0.05$). Here as well, the effects of combination treatment were significantly larger than evinacumab monotreatment (−74%, $P < 0.05$).

Effects of PCSK9 inhibitory peptide on plasma lipids in cynomolgus monkeys

To further explore the effect of the PCSK9 inhibitory peptide, cynomolgus monkeys were administered the peptide via a single subcutaneous injection (50 nmol/kg). After two days, total cholesterol concentrations were lowered by 26% relative to baseline in monkeys that received the PCSK9 inhibitory peptide (**Fig. 5**A). LDL-C concentrations were lowered by approximately 60% in PCSK9 inhibitory peptide-treated monkeys two days after the treatment (Fig. 5B). Treatment with the PCSK9 inhibitory peptide did not affect plasma HDL cholesterol (supplemental Fig. S5A) or plasma triglycerides (supplemental Fig. S5B).

DISCUSSION

PCSK9 inhibition has shown to be an effective treatment strategy for lowering LDL-C and non-HDL-C and reduces atherosclerosis progression as well as clinical events (12, 13). Nevertheless, the relatively high cost of PCSK9 monoclonal antibodies makes them less attractive as therapeutic strategy for a larger patient population. To achieve profound apoB lowering and subsequently reduce clinical events, combination therapeutic strategies are often mandatory in accordance with ESC/EAS guidelines (38). Therefore, we evaluated the effects of a novel PCSK9 inhibitory peptide alone and in combination with the ANGPTL3 inhibitory antibody evinacumab on the development of atherosclerosis. Using a translational model of atherosclerosis, we confirm that evinacumab alone strongly improves plasma lipids and atherosclerosis (15). The effects on plasma and atherosclerosis parameters were more pronounced in mice treated with PCSK9 inhibitory peptide, reaching levels where combining PCSK9 inhibitory peptide with evinacumab did not yield additional improvements. Additionally, both monotreatments and combination treatment improved atherosclerotic lesion morphology and composition.

Statins are currently the first-line pharmacotherapy for dyslipidemia with the goal of lowering LDL-C and consequently CVD risk. However, despite the use of statins, more than half of the patients fail to meet their risk-based LDL-C goal (39, 40). Moreover, a subgroup of patients do not tolerate statin intervention well and develop adverse effects including muscle symptoms and liver dysfunction (41). While concurrently prescribing statins in combination with eg ezetimibe or conventional PCSK9 inhibitors is recommended in these patients, prescription rates of combination therapy in clinical practice remains low, even when a higher proportion of the patients could achieve lower LDL-C levels with this approach compared to statin monotherapy (40). Therefore, other approaches are warranted and the novel PCSK9 inhibitory peptide tested here may bridge this unmet need in pharmacotherapy. This peptide (NNC0385-0434) has recently been evaluated for oral administration as well, in a phase 2 clinical trial (NCT04992065) in patients with cardiovascular disease on maximally tolerated statin and stable lipid-lowering therapy (42). For this purpose, the PCSK9 inhibitory peptide was co-formulated with the oral absorption enhancer sodium N-[8-(2-hydroxybenzoyl) amino] caprylate to improve absorption in the gastrointestinal tract. Oral administration of the PCSK9 inhibitory peptide at the highest tested dose (100 mg) was shown to yield similar effects on LDL-C lowering (−61.8%) compared to subcutaneously administered (140 mg) evolocumab (−59.6%) (42). Secondary endpoints including total cholesterol, VLDL cholesterol, triglycerides, and apoB also showed similar reductions. Taking into consideration the relatively low production cost of the PCSK9 inhibitory peptide compared to established PCSK9 monoclonal antibodies, this peptide forms an interesting alternative in a patient population that does not reach their LDL-C goal with conventional statin intervention.

Prolonged exposure to high cholesterol concentrations is known to contribute to atherosclerosis development and progression (43). In line with clinical data using this peptide (42), we have demonstrated significant beneficial effects of the PCSK9 inhibitory peptide

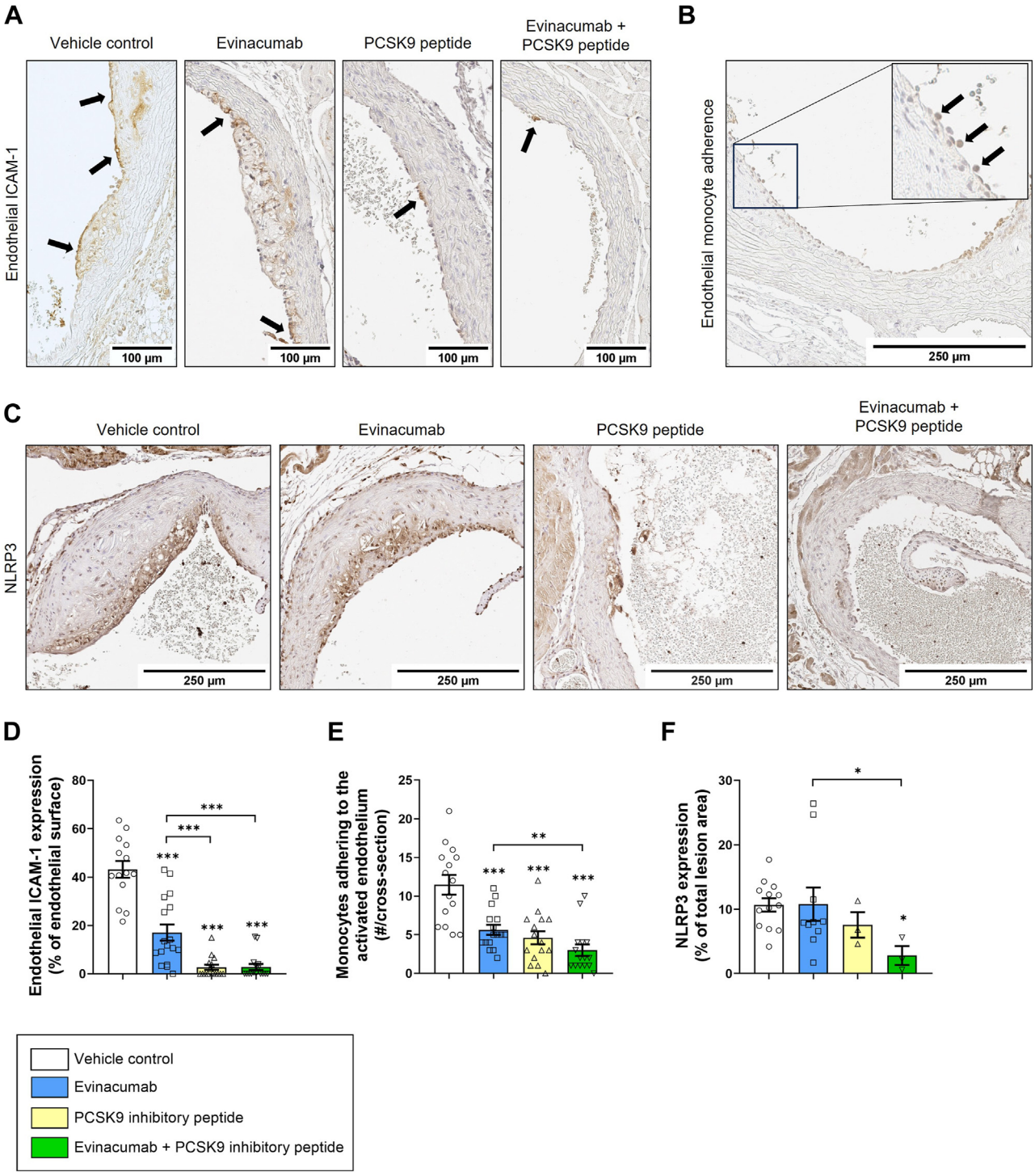

Fig. 4. Treatment with evinacumab, PCSK9 inhibitory peptide or both reduces endothelial activation. Endothelial ICAM-1 expression was determined in cross-sections of the aortic root area, with arrows indicating ICAM-1 positive areas (A). ICAM-1 expression is expressed as percentage of total endothelial surface area (D). The number of monocytes adhering to the activated endothelium was assessed after staining with AIA31240, with arrows indicating monocytes (B), and expressed as number per cross-section (E). NLRP3 expression was determined in severe type IV-V lesions (C) and expressed as percentage of total lesion area (F). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle control. Data are presented as mean ± SEM (n = 15–17 per group).

on plasma cholesterol and triglycerides. Our data are in line with a study in Golden Syrian hamsters on chow, where the PCSK9 inhibitory peptide (30–100 nmol/kg body weight) reduced LDL-C levels by up to 35% and increased LDL receptor protein expression by up to 1.8-fold https://patents.google.com/patent/WO2017121850A1/en. Here, evinacumab, PCSK9 inhibitory peptide and their combination strongly reduced apoB-containing (V)LDL-sized (non-HDL-C) particles, that play an important role in cardiovascular disease

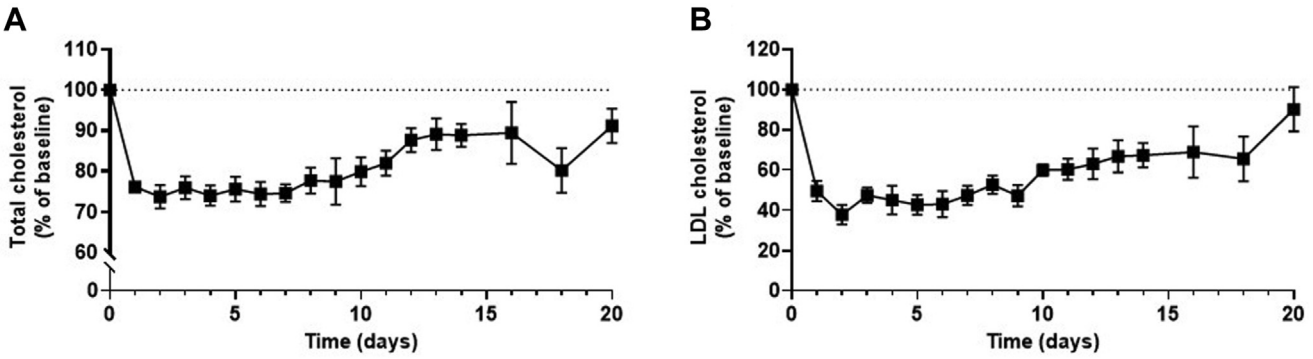

Fig. 5. Effects of the PCSK9 inhibitory peptide on plasma lipids in cynomolgus monkeys. Cynomolgus monkeys received a single subcutaneous injection of the PCSK9 inhibitory peptide (50 nmol/kg). Changes in plasma total cholesterol (A) and LDL cholesterol (B) were monitored throughout the 20 days after administration and data are presented as mean ± SEM (n = 4 per group).

progression (5). There was a strong and significant positive correlation between the total cholesterol exposure throughout the study and atherosclerotic lesion area (R^2 = 0.83). The reduced total cholesterol exposure in all treatment groups consequently contributed to substantial improvements in atherosclerosis with reduced atherosclerotic lesion size and number of lesions. Moreover, lesion severity was strongly improved, with both interventions resulting in more undiseased segments and fewer severe lesions. Compared to evinacumab monotreatment, combination treatment had additional ameliorative effects, with an even stronger reduction in lesion area, number of lesions and improved atherosclerotic plaque severity.

Atherosclerotic lesions with thin fibrous caps, high macrophage count, and large necrotic cores are unstable and therefore vulnerable to rupture (44, 45). In contrast, collagen-rich fibrous caps, lower macrophage count and lack of necrosis in the core of these plaques demonstrate a more stable phenotype. Assessment of these components that signify plaque stability revealed that in particular combination treatment with evinacumab and PCSK9 inhibitory peptide improved macrophage and collagen content in severe atherosclerotic plaques. It is important to emphasize that evaluation of lesion severity was confined to severe atherosclerotic lesions only. Considering the significant beneficial effects of the PCSK9 inhibitory peptide alone and in combination with evinacumab on lesion severity, only a limited number of lesions was available for analysis (PCSK9 inhibitory peptide: 7 lesions; combination: 4 lesions). Nevertheless, the limited number of severe plaques in these mice had a more stable phenotype compared to the severe plaques in mice treated with vehicle control or evinacumab monotreatment. The adhesion of monocytes to the activated endothelium is a key process in the initiation of atherosclerotic plaque development (46). Here, endothelial ICAM-1 expression was significantly reduced by evinacumab, PCSK9 inhibitory peptide and their combination. Subsequently, monocyte adhesion to the activated endothelium was reduced in all treatment groups, demonstrating the potential of both monotreatments and combination treatment in interfering in the early stages of atherosclerosis development. Furthermore, the PCSK9 inhibitory peptide in combination with evinacumab significantly reduced NLRP3 expression in severe (type IV-V) lesions, signifying reduced macrophage activation. In summary, these data demonstrate that besides significantly reducing the number of severe lesions, combination therapy with evinacumab and PCSK9 inhibitory peptide increases stability of the remaining severe lesions as well, making them less prone to rupture.

In cynomolgus monkeys, the PCSK9 inhibitory peptide was administered subcutaneously to study its effects on plasma lipid concentrations. As a consequence of the intervention, total cholesterol levels were lowered by 26% and LDL-C levels by 60% two days after administration. These findings illustrate that the PCSK9 inhibitory peptide has evident benefit for plasma lipid concentrations in a non-human primate model as well.

In conclusion, our findings demonstrate that treatment with a novel PCSK9 inhibitory peptide alone and in combination with evinacumab reduces atherogenic lipoproteins and subsequently reduces progression of atherosclerosis. The beneficial effects of the peptide on plasma lipid concentrations were confirmed in a non-human primate model. Recent clinical findings have shown that when administered orally, this PCSK9 inhibitory peptide has comparable efficacy to conventional PCSK9 monoclonal antibodies, which are underprescribed due to their relative costliness. The data presented in this study elaborate on these findings by showing the efficacy of the PCSK9 inhibitory peptide in diminishing atherosclerosis progression, characterized by fewer and more stable atherosclerotic lesions. Intervention with this PCSK9 inhibitory peptide therefore forms a promising approach for the large patient population with atherosclerotic CVD that fails to meet their LDL-C targets with conventional statin intervention or for statin-intolerant patients. The

combination of this novel PCSK9 inhibitory peptide with evinacumab has potential to reduce and stabilize atherosclerotic lesions even further.

Data availability

All data are contained within the manuscript.

Supplemental data

This article contains supplemental data.

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J. A. I., A. v. S. N., H. M. G. P., A. M. v. d. H., E. M. S., C. K. M., J. W. J., and B. R. writing–review & editing; J. A. I., H. M. G. P., and A. M. v. d. H. writing–original draft; J. A. I. visualization; J. A. I. and A. M. v. d. H. validation; J. A. I., H. M. G. P., A. M. v. d. H., and J. W. J. supervision; J. A. I., A. v. S. N., and E. M. S. investigation; J. A. I. and A. M. v. d. H. formal analysis; J. A. I., H. M. G. P., A. M. v. d. H., and B. R. conceptualization; A. M. v. d. H. project administration; B. R. funding acquisition.

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Conflict of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interest: Authors Bidda Rolin, Ellen Marie Staarup and Christina K. Morgensen are employees of Novo Nordisk A/S, Måløv, Denmark. The in-life phase of the non-human primate study was performed at Envigo and blood samples and raw data were analyzed by Novo Nordisk A/S. The funders had no role in data collection and raw data analysis of the mouse study.

Abbreviations

ANGPTL3, angiopoietin-like 3; Apo, apolipoprotein; CETP, cholesteryl ester transfer protein; CVD, cardiovascular disease; DAB, 3,3′-diaminobenzidine; FPLC, fast protein liquid chromatography; HDL, high density lipoprotein; HPS, hematoxylin-phloxine-saffron; ICAM1, intracellular adhesion molecule 1; NLRP3, NLR family pyrin domain containing 3; PCSK9, proprotein convertase subtilisin/kexin type 9; SMCs, smooth muscle cells; (V)LDL-C, (very) low-density lipoprotein cholesterol; WTD, Western-type diet.

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