Abstract  LDL cholesterol (LDL-C) contributes to coronary heart disease. Proprotein convertase subtilisin/kexin type 9 (PCSK9) increases LDL-C by inhibiting LDL-C clearance. The therapeutic potential for PCSK9 inhibitors is highlighted by the fact that PCSK9 loss-of-function carriers exhibit 15–30% lower circulating LDL-C and a disproportionately lower risk (47–88%) of experiencing a cardiovascular event. Here, we utilized psck9^-/- mice and an anti-PCSK9 antibody to study the role of the LDL receptor (LDLR) and ApoE in PCSK9-mediated regulation of plasma cholesterol and atherosclerotic lesion development. We found that circulating cholesterol and atherosclerotic lesions were minimally modified in psck9^-/- mice on either an LDLR- or ApoE-deficient background. Acute administration of an anti-PCSK9 antibody did not reduce circulating cholesterol in an ApoE-deficient background, but did reduce circulating cholesterol (-45%) and TGs (-36%) in APOE*3Leiden.cholesterol ester transfer protein (CETP) mice, which contain mouse ApoE, human mutant APOE3*Leiden, and a functional LDLR. Chronic anti-PCSK9 antibody treatment in APOE*3Leiden.CETP mice resulted in a significant reduction in atherosclerotic lesion area (~91%) and reduced lesion complexity. Taken together, these results indicate that both LDLR and ApoE are required for PCSK9 inhibitor-mediated reductions in atherosclerosis, as both are needed to increase hepatic LDLR expression. —Ason, B., J. W. A. van der Hoorn, J. Chan, E. Lee, E. J. Pieterman, K. K. Nguyen, M. Di, S. Shetterly, J. Tang, W-C. Yeh, M. Schwarz, J. W. Jukema, R. Scott, S. M. Wasserman, H. M. G. Princen, and S. Jackson. PCSK9 inhibition fails to alter hepatic LDLR, circulating cholesterol, and atherosclerosis in the absence of ApoE. J. Lipid Res. 2014. 55: 2370–2379.

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High levels of circulating LDL cholesterol (LDL-C) play a key role in the initiation and development of atherosclerosis. This contributes to the development of CVD and places patients at increased risk of experiencing an adverse cardiovascular event (1, 2).

Circulating LDL-C levels are dictated by the balance between dietary cholesterol absorption, hepatic cholesterol synthesis, storage, and clearance from the blood stream (3, 4). The LDL receptor (LDLR) plays a critical role in regulating the clearance of LDL-C (5–9). It has been shown that proprotein convertase subtilisin/kexin type 9 (PCSK9) promotes LDLR degradation, thereby reducing the number of LDLRs available to sequester LDL-C from circulation (10–16). PCSK9 is a member of the subtilisin family of serine proteases and is expressed primarily by the liver where it is secreted into circulation (17). Self-cleavage by PCSK9 enables secretion from hepatocytes and subsequent binding to the LDLR at the liver cell surface (13, 16, 18, 19). The LDLR:PCSK9 complex enters the cell and is transported to the lysosome compartment and degraded. This leads to a reduction in hepatic LDLR levels (12). Thus, higher circulating PCSK9 levels increase circulating LDL-C by preventing LDLR-mediated LDL-C clearance, whereas lower circulating PCSK9 levels decrease circulating LDL-C by increasing LDLR-mediated LDL-C clearance. The impact of PCSK9-mediated regulation of LDL-C is evident in studies of individuals with gain-of-function PCSK9 mutations. These individuals possess higher circulating LDL-C.

Abbreviations: CETP, cholesteryl ester transfer protein; CmAb1, chimeric mAb1; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; LDLR, LDL receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; TC, total cholesterol; TICE, transintestinal cholesterol excretion; VVG, Verhoeff-Van Gieson; WTD, Western-type diet.

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5 The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of nine figures.
and an increased risk of experiencing a cardiovascular event (20–22). Additionally, PCSK9 loss-of-function carriers have 15–30% lower circulating LDL-C and a disproportionately lower risk (47–88%) of experiencing a cardiovascular event (23). This disproportionate reduction in risk is in contrast to statins, where 5 year treatment reduced cardiovascular events by 40% even when LDL-C was reduced to 80 mg/dl (24). Whether this disproportionate reduction in risk is due to PCSK9 having a direct negative effect at the atherosclerotic lesion or if the additional benefit is driven by a modest lifelong reduction in serum cholesterol is unclear. These observations have led to the development of PCSK9 inhibitors as a means to therapeutically reduce LDL-C and the associated CVD risk (25–29). Inhibition of PCSK9 by monoclonal antibodies, adnectins, or siRNAs reduces LDL-C levels in patients, and clinical trials designed to assess the effect of anti-PCSK9 therapies on cardiovascular outcomes are underway (30–42).

ApoE, like ApoB, is present in lipoproteins and functions as a ligand of the LDLR and is important for the clearance of TG-rich lipoproteins. The decrease in HDL cholesterol (HDL-C) in pcsk9<sup>−/−</sup> mice has been attributed to the binding of ApoE containing HDL to the upregulated LDLR (11). Even with a functional LDLR and ApoB, mutations in APOE in humans can lead to hypercholesterolemia (43–46). To date the role of ApoE in the lipid lowering and atheroprotection of ApoE containing HDL to the upregulated LDLR has been reported to be proatherogenic, while PCSK9 deletion in apoE-deficient mice leads to a reduction in the amount of cholesterol ester found within the aorta, even though the plaque size and total plasma cholesterol levels remain unchanged (47). The contribution of cholesterol ester content to atherosclerotic lesion development in the absence of changes in lesion area are unknown, but these data hint that a functional ApoE-LDLR pathway is essential for PCSK9-mediated changes in atherosclerosis that are driven by decreases in plasma cholesterol. To investigate this, we utilized both genetically engineered knockout mice (pcsk9<sup>−/−</sup>) and an anti-PCSK9 antibody to examine the effect of PCSK9 inhibition on plasma lipoproteins and atherosclerotic lesion development in mice lacking the LDLR or ApoE, as well as in APOE*3Leiden.cholesteryl ester transfer protein (CETP) mice (47), which have mouse ApoE and LDLR but hampered clearance of the ApoB-containing lipoproteins due to the expression of human mutant APOE*3Leiden (48). The APOE*3Leiden.CETP mice are a well-established mouse model for familial dysbetalipoproteinemia with human-like lipoprotein metabolism and atherosclerosis development, which respond in a human-like manner to both lipid lowering as well as HDL-raising drugs (like statins, fibrates, niacin, etc.) used in the treatment of CVD (49–52).

**MATERIALS AND METHODS**

**Antibody generation and purification**

The fully human PCSK9-targeting antibody, mAb1, was generated as described previously (25). Briefly, mice engineered to express human IgG antibodies were immunized with human PCSK9. Determination of binding affinity, screening for cross-reactivity to mouse PCSK9, and activity in a cell-based LDL uptake assay led to mAb1 selection.

cDNA sequences encoding the variable domains of heavy and light chains of mAb1 were fused to constant domains of mouse IgG1 and lambda light chain. The resulting cDNA sequences encoding the chimeric mAb1 (CmAb1) heavy chains and light chains were inserted into pTT5 expression plasmid separately. CmAb1 mouse IgG1 was expressed by cotransfecting 293 E<sup>E</sup> cells with pTT5 plasmids containing light chain and heavy chain sequences. Expressed chimeric antibody was purified by capturing on a MabSelect SuRe column and polished on a SP-Sepharose column as previously described (25).

Binding of mAb1 and CmAb1 to mouse PCSK9 was measured in a kinetic binding assay by BLACore. Mouse anti-His antibody (Qiagen, Valencia, CA) was immobilized on all four flow cells of a CM5 chip using amine coupling reagents (GE Healthcare, Piscataway, NJ) with an approximate density of 5,000–6,000 RU. His-tagged PCSK9 was captured on the second and fourth flow cells at an approximate density of 130 RU for mouse PCSK9. Flow cells one and three were used as background controls. Anti-PCSK9 antibody at 100 nM was diluted in PBS plus 0.1 mg/ml BSA, 0.005% P20, and injected over the captured PCSK9 surface with a 50 μl/min flow rate (5 min association and 5 min dissociation). CmAb1 showed very similar binding activity compared with mAb1 (25).

Control mouse IgG1 was raised against a PeptiBody peptide AGP-3. The resulting antibody was produced in stably transfected Chinese hamster ovary cells and purified using the same method as CmAb1.

**In vivo**

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at Amgen for work performed at Amgen and by the Institutional Animal Care and Use Committee of the Netherlands Organization for Applied Research for work performed at TNO Metabolic Health Research. All mice were housed and maintained under standard environmental conditions with a 12 h light-dark cycle and had free access to food and water. All mice were in a C57Bl/6 background.

<sup>Ldlr</sup><sup>−/−</sup> and <sup>apoE</sup><sup>−/−</sup> mice were obtained from Jackson Laboratories. Each strain was crossed with <sup>pcsk9</sup><sup>−/−</sup> mice (Ozgene Pty Ltd, Bentley, Australia) to generate <sup>ldlr</sup><sup>−/−</sup> <sup>/pcsk9</sup><sup>−/−</sup> and <sup>apoE</sup><sup>−/−</sup> <sup>/pcsk9</sup><sup>−/−</sup> colonies. These <sup>pcsk9</sup><sup>−/−</sup> colonies were crossed again to generate the male double knockouts (<sup>ldlr</sup><sup>−/−</sup> <sup>/pcsk9</sup><sup>−/−</sup> and <sup>apoE</sup><sup>−/−</sup> <sup>/pcsk9</sup><sup>−/−</sup>) and the respective littermate controls (<sup>ldlr</sup><sup>−/−</sup> <sup>/pcsk9</sup><sup>−/−</sup> and <sup>apoE</sup><sup>−/−</sup> <sup>/pcsk9</sup><sup>−/−</sup>) used for these experiments. Mice (male) on the <sup>ldlr</sup><sup>−/−</sup> background were fed an atherogenic diet (Research Diets D12108C) containing 40% kcal from fat and 1.25% cholesterol. Mice (male) on the <sup>apoE</sup><sup>−/−</sup> background were fed chow diet (Harlan 2020X).

Female APOE*3Leiden.CETP transgenic mice (11–13 weeks of age) (53), expressing human CETP under control of its natural flanking regions, were used. APOE*3Leiden.CETP transgenic mice were fed a semi-synthetic cholesterol-rich diet containing 15% (w/w) cacao butter and 0.15% cholesterol (Western-type diet (WTD); Hope Farms, Woerden, The Netherlands) for a run-in period of 3–4 weeks to increase plasma total cholesterol (TC) levels to approximately 650 mg/dl. Mice were matched based on body weight, TG, TGs, and age.

In pharmacologic inhibitory studies, antibodies were administered by sc injection (10 mg/kg) every 10 days for 14 weeks, to examine effects on atherosclerotic plaque development.

Whole blood was collected by tail nick, vena cava, or cardiac puncture. At study termination, animals were euthanized either
by CO₂ asphyxiation or by exsanguination under anesthesia (100 mg/kg ketamine, 5 mg/kg diazepam).

For liver collection, sections of the right medial or left lobe were excised, flash frozen, and stored until further use. For heart and aorta isolation, hearts were either isolated and placed directly in formalin or animals were perfused by gravity flow under anesthesia. Perfusion was performed by inserting a 25 gauge needle into the apex of the left ventricle and nicking the right atrium. Animals were perfused with saline for 10 min followed by 4% paraformaldehyde for 10 min for fixation. Hearts and aortas were removed, immersed in 4% paraformaldehyde, and stored at 4°C.

Cholesterol, TG analysis, PCSK9 ELISA

Mouse serum or EDTA plasma was obtained from whole blood collected via centrifugation. Serum or plasma cholesterol and TGs were analyzed using either a Cobas Integra 400 chemistry analyzer or enzymatic kits according to the manufacturer’s instructions (catalog numbers 1458216 and 1488872, respectively; Roche/Hitachi). In some instances, pooled serum from mice treated with either control or anti-PCSK9 monoclonal antibodies was fractionated by fast protein liquid chromatography (Superose 6 10/300 GL column). Cholesterol content of each fraction was measured using the HDL-C E kit omitting the phosphotungstate-magnesium salt precipitation step (Wako Pure Chemical Industries, Osaka, Japan). Mouse PCSK9 serum protein levels were measured by sandwich ELISA (R&D Systems; MPC900) according to the manufacturer’s instructions.

Hepatic LDLR mRNA and protein expression

Total RNA was extracted from liver tissue samples using RNA-Bee (Amsbio, Oxon, UK) according to the manufacturer’s instructions. Random primers were used to convert RNA to single stranded cDNA by reverse transcription (Promega, Fitchburg, WI) according to the manufacturer’s protocol. Levels of cDNA were measured by real-time PCR using the 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Assay-on-demand primers and probes were obtained from Applied Biosystems. The mRNA levels were normalized to mRNA levels of three housekeeping genes (i.e., cyclophilin, HPRT, and GAPDH). The level of mRNA expression for each gene of interest was calculated according to the manufacturer’s instructions (Applied Biosystems).

For protein expression, liver tissues were homogenized in lysis buffer (Santa Cruz Biotechnology, Inc.) containing complete protease inhibitors (Roche Diagnostics) and incubated on ice for 30 min. The lysis buffer consisted of 50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40 (Igepal), 1 mM EDTA, protease inhibitor cocktail (complete, Roche), 1 mM magnesium salt precipitation step (Wako Pure Chemical Industries, Osaka, Japan). Aortas were mounted and photographed under a stereo microscope. Aortic plaque area was quantified by Image-Pro.

Statistical analysis

Significance between groups was calculated by two-way ANOVA, Sidak posttest, for longitudinal studies, by a two-tailed t-test for single end points containing two groups, and by a one-way ANOVA, Tukey posttest, using Prism (GraphPad, Inc). In the figures: *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

RESULTS

LDLR is the predominant means for PCSK9-mediated regulation of circulating cholesterol and is required for PCSK9 inhibitor-mediated regulation of atherosclerosis

To investigate whether LDLR influences circulating PCSK9 levels, we measured plasma PCSK9 levels in ldlr⁻/⁻ and WT mice and found a significant elevation in plasma PCSK9 in ldlr⁻/⁻ mice (2,083 ± 1,529 ng/ml and 98 ± 98 ng/ml) providing further confirmation that PCSK9 and LDLR influence the clearance of one another (Fig. 1A) (57, 58).

Psck9⁻/⁻ mice exhibit increased hepatic LDLR levels leading to lower circulating cholesterol levels (26). However, it is unclear if PCSK9 also influences the levels of circulating cholesterol independent of the LDLR (47). We investigated this possibility by comparing the levels of circulating cholesterol in 12-week-old ldlr⁻/⁻/psck9⁻/⁻ mice relative to ldlr⁻/⁻ littermate controls. Mice were fed a WTD (40% kcal from fat and 1.25% cholesterol) for 12 weeks, and circulating cholesterol and TG levels were measured at 5 and 12 weeks from the initiation of WTD feeding. The ldlr⁻/⁻/psck9⁻/⁻ mice exhibited a slight but significant (18-19%) decrease in both TC and LDL-C at 5 and 12 weeks and a decrease in HDL-C at 12 weeks (Fig. 1B, supplementary Fig. I) while circulating TG levels were not significantly different between groups at either time point (Fig. 1C). To determine whether these reductions in TC levels translated into reduced atherosclerosis development, we measured the amount of atherosclerotic plaque
within the aorta and the aortic root after 12 weeks. Atherosclerotic lesions covered 11 ± 5% and 10 ± 3% of the aortic area in the \( \text{ldlr}^{-/-} \) and \( \text{ldlr}^{-/-} / \text{pcsk9}^{-/-} \) mice, respectively (supplementary Fig. II). Similarly, no significant difference was observed in lesion area (0.26 ± 0.10 mm\(^2\) and 0.28 ± 0.11 mm\(^2\)) or macrophage content (0.04 ± 0.02 mm\(^2\) and 0.05 ± 0.02 mm\(^2\)) in the aortic sinus of \( \text{ldlr}^{-/-} \) and \( \text{ldlr}^{-/-} / \text{pcsk9}^{-/-} \) mice, respectively (Fig. 1D–F). Thus, deletion of PCSK9 in the absence of the LDLR reduces circulating cholesterol levels, suggesting that other receptors or mechanisms are involved in the PCSK9-mediated cholesterol clearance.

**PCSK9 deletion in \( \text{apoe}^{-/-} \) mice does not affect circulating cholesterol and atherosclerosis**

Another key player and essential protein for normal particle uptake by the liver via the LDLR gene family is ApoE, which is present on chylomicrons, VLDLs, IDLs, and LDLs, and also on large HDL particles. Consequently, \( \text{apoe}^{-/-} \) mice exhibit elevated circulating cholesterol levels leading to accelerated atherosclerotic plaque accumulation on a chow diet (59, 60). In contrast to \( \text{ldlr}^{-/-} \) mice, \( \text{apoe}^{-/-} \) mice exhibit comparable PCSK9 plasma and hepatic LDLR protein levels relative to WT mice (67 ± 23 ng/ml and 98 ± 98 ng/ml, respectively; Fig. 2A, supplementary Fig. III). Comparing \( \text{apoe}^{-/-} / \text{pcsk9}^{-/-} \) and \( \text{apoe}^{-/-} \) littermate controls revealed that there was no difference in circulating TC, HDLs, LDLs, or TGs, and there was no significant difference in hepatic LDLR protein levels (Fig. 2B, C; supplementary Figs. IV, V). In addition, there was no difference in either atherosclerotic plaque accumulation (0.18 ± 0.08 mm\(^2\) and 0.14 ± 0.07 mm\(^2\)) or macrophage content (0.006 ± 0.005 mm\(^2\) and 0.006 ± 0.003 mm\(^2\)) within the aortic root between \( \text{apoe}^{-/-} / \text{pcsk9}^{-/-} \) and \( \text{apoe}^{-/-} \) littermate controls at 32 weeks of age, respectively (Fig. 2D–F). Together these data demonstrate that in the absence of ApoE, expected lipid lowering and atheroprotective effects caused by the deletion of PCSK9 are not apparent.

**PCSK9 inhibition is effective in APOE*3Leiden.CETP mice but not in \( \text{apoe}^{-/-} \) mice**

Recently, several PCSK9 monoclonal antibodies have been developed as a therapy to reduce plasma lipids. To determine whether anti-PCSK9 antibody treatment can
In contrast, in APOE*3Leiden.CETP mice the single dose sc injection of anti-PCSK9 antibody significantly reduced both cholesterol (up to 69%) and TGs (up to 70%) during 14 days posttreatment (Fig. 3C, D) compared with control antibody. This corresponded to a significant increase in hepatic LDLR mRNA and protein expression (supplementary Fig. IX). We next assessed the effect of anti-PCSK9 antibody (10 mg/kg, sc, every 10 days) on atherosclerosis in APOE*3Leiden.CETP mice on a WTD. As compared with a chow diet, the WTD, containing 0.15% cholesterol, increased PCSK9 levels by 51% (from 135.4 ± 14.2 ng/ml to 205.2 ± 41.9 ng/ml, \( P < 0.05 \); Fig. 4A). Treatment with anti-PCSK9 antibody further increased the circulating PCSK9 levels by another 166% (to 545.8 ± 399.7 ng/ml, \( P < 0.01 \); Fig. 4A), demonstrating circulating complexes of antibody bound to PCSK9. During the 14 week treatment, consistent and significant reductions in TC and TG levels were observed as measured 3 and 10 days after the first (week 1) and ninth (week 12) injection (Fig. 4B, C). On average, TC was reduced by 67% (\( P < 0.001 \)), which was driven by a decrease in nonHDL-C (Fig. 4D), and TGs were reduced by 61% (\( P < 0.001 \)), as compared with control. After 14 weeks of treatment, atherosclerosis development was reduced by 91% (\( P < 0.001 \)) in the mice treated lower circulating lipids in the absence of ApoE, we administered a single dose (10 mg/kg, sc) of either an anti-PCSK9 antibody (CmAb1) or control antibody to either \( \text{apo}^e/^- \) or APOE*3Leiden.CETP mice, which express both mouse ApoE and the human mutant APOE3*Leiden, as well as a functional LDLR.

Consistent with our observations utilizing \( \text{pcsk}^9^-/^- \) mice, a single dose of anti-PCSK9 antibody did not significantly lower circulating cholesterol levels at up to 14 days posttreatment or affect hepatic LDLR protein levels in \( \text{apo}^e/^- \) mice (Fig. 3A, supplementary Fig. VI). This is in contrast to C57BL/6 mice, where a significant increase in hepatic LDLR was observed following anti-PCSK9 antibody treatment (supplementary Fig. VII), which is consistent with our previous findings for PCSK9 inhibition in WT mice (25). Circulating TGs were not significantly different at day 3, 10, or 14, but did reach significance at the day 5 time point (\( P < 0.05 \), Fig. 3B). Additionally, chronic administration of anti-PCSK9 antibody (10 mg/kg, sc, every 10 days) failed to reduce circulating lipid levels or atherosclerosis in \( \text{apo}^e/^- \) mice (supplementary Fig. VIII). Together, these data suggest that ApoE is required for cholesterol and TG lowering, and atherosclerosis reduction, by anti-PCSK9 antibody.
PCSK9 inhibition requires LDLR and ApoE for atheroprotection

LDLR and ApoE are required for the atheroprotective effects of PCSK9 inhibition. Moreover, we clearly demonstrate that an anti-PCSK9 antibody is highly efficacious in reducing lipid levels and atherosclerosis development in diet-induced hyperlipidemic APOE*3Leiden.CETP mice (a translational model for dysbetalipoproteinemia), which have an intact ApoE-LDLR clearance pathway.

Discussion

Classic work, such as that by Ishibashi et al. (61), has set the foundation of understanding of ApoE and LDLR in lipoprotein homeostasis. To study the role of LDLR and ApoE on PCSK9-mediated regulation of plasma cholesterol and atherosclerosis lesion development, we utilized ldlr−/−, apoe−/−, and APOE*3Leiden.CETP mice. We demonstrate that circulating cholesterol and atherosclerotic lesions are minimally modified in pse9k−/− mice on either an ldlr−/− or an apoe−/− background, strongly suggesting requirement of both proteins for robust atheroprotection mediated by PCSK9 inhibition. It is likely that the minor effects on plasma cholesterol lowering are the major reason for the lack of lesion reduction, as the key role of lipids in driving atherosclerotic lesion development in rodent models has been well-defined (62). We also demonstrate the ability of anti-PCSK9 monoclonal antibody to robustly reduce atherosclerosis in a mouse model with a functional ApoE-LDLR pathway, but with no effect when ApoE is absent.

We observed small but significant reductions in serum cholesterol levels after deletion of pse9k in ldlr−/− mice. These data are in contrast with previous studies showing no effect of pse9k deletion or PCSK9 inhibition by mAbs (25, 47). However, these studies used low-cholesterol diets (=0.2% w/w cholesterol) in contrast to the current study (1.25% w/w), which might be the reason for the discrepancy in effect. The small but significant reduction in serum cholesterol levels after deletion of pse9k in ldlr−/− mice might relate to potential effects of PCSK9 in enabling ApoB secretion in nascent VLDL, or perhaps in upregulation...

Fig. 3. Anti-PCSK9 antibody treatment reduces TC and TG levels in APOE*3Leiden.CETP mice but not apoe−/− mice. No significant reduction in TC (A) and only a slight but significant reduction in TGs 5 days posttreatment (B) are observed for anti-PCSK9 antibody-treated apoe−/− mice relative to control antibody-treated apoe−/− mice [10 mg/kg (sc) day 0, n = 5 per group]. This contrasts results with APOE*3Leiden.CETP mice, where anti-PCSK9 antibody treatment resulted in a significant decrease in TC (C) and TGs (D) (10 mg/kg (sc) day 0, n = 8 per group). Data represented as the means (bars) ± SD, *P < 0.05, **P < 0.01, ****P < 0.0001, as compared with control, two-way ANOVA, Sidak posttest.

with anti-PCSK9 antibody as compared with control (Fig. 4E-G). Lesion severity was also reduced, with 8-fold more lesion-free segments in the animals treated with anti-PCSK9 antibody, as compared with control (7.8 ± 9.2% in control and 62.5 ± 31.0% in anti-PCSK9 antibody; P < 0.001), and a strong significant reduction in the percentage of severe lesions (46.2 ± 23.9% in control and 7.8 ± 15.1% in anti-PCSK9 antibody; P < 0.001; Fig. 4H). All together these data suggest that LDLR and ApoE are required for the atheroprotective effects of PCSK9 inhibition. Moreover we clearly demonstrate that an anti-PCSK9 antibody is highly efficacious in reducing lipid levels and atherosclerosis development in diet-induced hyperlipidemic APOE*3Leiden.CETP mice (a translational model for dysbetalipoproteinemia), which have an intact ApoE-LDLR clearance pathway.
explains the absence of lipid lowering effects. This phenomenon, however, was not previously explained.

We hypothesize that in the absence of ApoE there is no uptake and intracellular trafficking of LDLR (bound to the lipoprotein), and consequently there is no shuttling of the LDLR into the lysosomal degradation pathway. In this situation when the LDLR is not degraded, PCSK9 inhibition, rescuing the LDLR from degradation, is not effective. Supportive data was provided by Mortimer et al. (70), showing that under normal circumstances, chylomicron remnants are rapidly internalized by the LDLR and catabolized in hepatocytes, with a critical requirement for ApoE. Ishibashi et al. (71) showed similar remnant clearance in apoe⁻/⁻/ldlr⁻/⁻ mice and apoe⁻/⁻/ldlr⁻/⁻/apoe⁻/⁻ mice, strongly suggesting a minor function of the LDLR in apoe⁻/⁻ mice.

In both ldlr⁻/⁻ and apoe⁻/⁻ strains, knockout of PCSK9 did not affect lesion formation, which is consistent with the results reported by Denis et al. (47), although the authors concluded a protection from atherosclerosis based on aortic cholesterol ester levels. They found that aortic cholesterol levels were reduced, without observing effects on lesion area in the aortic root or thoracic aorta.

Fig. 4. Anti-PCSK9 antibody treatment reduces atherosclerosis in APOE*3Leiden.CETP mice. Plasma PCSK9 levels (A) in APOE*3Leiden.CETP mice were determined on chow diet and WTD, as well as two weeks after a single injection with anti-PCSK9 antibody (10 mg/kg, sc) in mice fed WTD. Data represented as the means (bars) ± SD (n = 8 per group). *P < 0.01 versus chow; **P < 0.05 versus WD, one-way ANOVA, Tukey posttest. To assess the effect on atherosclerosis, control or anti-PCSK9 antibody was injected sc every 10 days for 14 weeks in APOE*3Leiden.CETP mice. Plasma TC (B) and TGs (C) were measured at 3 and 10 days postinjection in the first and twelfth week of treatment. Data represented as means (bars) ± SD (n = 15 per group). ***P < 0.001 versus control antibody. D: Fast protein liquid chromatography fractionation of pooled plasma samples are shown from week 8. Atherosclerosis development was determined in the aortic sinus of APOE*3Leiden.CETP mice. E–F: Representative pictures of control antibody- and anti-PCSK9 antibody-treated mice are shown. The total lesion area per cross-section (G) was measured and lesion severity (H) was determined. Data represented as means (bars) ± SD (n = 15 per group). ***P < 0.001 versus control antibody.
in apoe$^{-/-}$/pcsk9$^{-/-}$, as compared with their respective apoe$^{-/-}$ controls. We did not measure aortic cholesterol ester levels in our studies.

Here we provide further evidence that ApoE is necessary for the atheroprotective effects of PCSK9 inhibition, as treating APOE*3Leiden.CETP mice with anti-PCSK9 antibodies resulted in significant and sustained reductions in TC and TG levels, which translated to reduced atherosclerosis development in the aortic root. While normal WT mice have a very rapid clearance of apoB-containing lipoproteins, APOE*3Leiden mice have an impaired clearance and increased TG levels, and are thereby mimicking the slow clearance observed in humans, particularly in patients with familial dysbetalipoproteinemia (45). Upon feeding saturated fat and cholesterol, hyperlipidemia and atherosclerosis will develop. These animals also respond in a human-like manner to drugs used in the treatment of CVD (like statins, fibrates, antihypertensives, etc.) (49, 54, 72–74). However, APOE*3Leiden mice (like WT mice) do not possess a CETP gene, and therefore these mice do not respond to HDL-modulating interventions. By cross-breeding the APOE*3Leiden mice to mice expressing the CETP gene (75), APOE*3Leiden.CETP mice were obtained that respond to both lipid-lowering as well as HDL-raising interventions (50–53, 76). In the current study, we found significant lowering effects of anti-PCSK9 antibodies on TC and TG levels in APOE*3Leiden.CETP mice, but HDL-C was not affected (data not shown).

Other than the plasma cholesterol modulating effect, other potential atherosclerosis-related effects of PCSK9 have been described or suggested. Previously, Ferri et al. (77) reported that PCSK9 is expressed in human vessel walls and produced locally by vessel smooth muscle cells causing a local effect, and it was suggested that PCSK9 could enter the subendothelial space from the circulation either by itself or in association with LDL. In addition, it has been hypothesized that PCSK9 could impact the expression of LDLR on lesion monocytes and macrophages modulating foam cell formation and/or promoting apoptosis (78). Although our analysis was quite limited, we conclude that PCSK9-mediated local effects at the lesion, reflected by lesion area and macrophage number, is not significant in the models utilized here. However, we cannot rule out the possibility that certain biochemical changes may have occurred in the lesion due to the absence or inhibition of PCSK9.

The difficulty in examining the effect of human and humanized biologics in animal models, particularly when chronic dosing is required, is the potential appearance of neutralizing anti-drug antibodies reducing the efficacy of the therapeutic. In the study described here, there was clear evidence that after 12 weeks of treatment (9 injections) this was the case for approximately 30% of the mice, as demonstrated by a reduced efficacy in lipid lowering in those mice. Regardless, even with all study animals included in the analysis, the effect on atherosclerotic lesions was highly significant.

The ability of anti-PCSK9 therapies to lower LDL-C in human subjects is evident from numerous late stage clinical trials. The lipid lowering effect of statins has been shown to reduce the risk of cardiovascular events and death in several outcome trials (30–41, 79). Determining whether anti-PCSK9 antibody therapies will be efficacious in reducing the risk of cardiovascular events and death, as suggested by the current study using APOE*3Leiden.CETP mice, will be defined in the current outcome trials.

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