PCSK9 inhibition fails to alter hepatic LDLR, circulating cholesterol and atherosclerosis in the absence of ApoE

Short title: PCSK9 inhibition requires LDLR and ApoE for atheroprotection

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ABSTRACT

Low density lipoprotein 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 *pcsk9*$^{-/-}$ mice and an anti-PCSK9 antibody to study the role of the LDL-receptor (LDLR) and apolipoprotein E (ApoE) in PCSK9-mediated regulation of plasma cholesterol and atherosclerotic lesion development. We found that circulating cholesterol and atherosclerotic lesions were minimally modified in *pcsk9-/-* 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 triglycerides (-36%) in APOE*3Leiden.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.

SUPPLEMENTARY KEY WORDS: Apolipoprotein E, anti-PCSK9 antibody, atherosclerosis

ABBREVIATIONS: ApoB, Apolipoprotein B; ApoE, apolipoprotein E; apoe$^{-/-}$, apolipoprotein e knockout; CETP, cholesteryl ester transfer protein; CVD, cardiovascular disease; FD, familial dysbetalipoproteinemia; FPLC, fast protein liquid chromatography; HDL-C, high density lipoprotein cholesterol; HRP, horse radish peroxidase; IDL, intermediate density lipoprotein; LDL-C, low density lipoprotein cholesterol; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; sc, subcutaneously; TC, total cholesterol; TICE, trans-intestinal cholesterol excretion; VLDL, very low density lipoprotein; WTD, Western type diet

INTRODUCTION

High levels of circulating low density lipoprotein cholesterol (LDL-C) play a key role in the initiation and development of atherosclerosis. This contributes to the development of cardiovascular disease (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 low density lipoprotein 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 increases circulating LDL-C by preventing LDLR mediated LDL-C clearance, whereas lower circulating PCSK9 levels decreases 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 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).

Apolipoprotein E (ApoE), like Apolipoprotein B (ApoB), is present in lipoproteins and functions as a ligand of the LDLR and is important for the clearance of triglyceride rich lipoproteins. The decrease in high density lipoprotein cholesterol (HDL-C) in *pcsk9*$^{-/-}$ 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 athero-protective effects of PCSK9 inhibition is unclear. PCSK9 overexpression in an APOE deficient background 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*$^{-/-}$) 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.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 (FD) 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 cardiovascular disease (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. Hybridomas were evaluated for binding PCSK9 and inhibiting the interaction of PCSK9 with LDLR. 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 (H) and light (L) chains of mAb1 were fused to constant domains of mouse IgG1 heavy chain and mouse lambda light chain. The resulting cDNA sequences encoding the chimeric mAb1 HC and LC were inserted into pTT5 expression plasmid separately. Chimeric mAb1 (CmAb1) mouse IgG1 was expressed by co-transfecting 293 6E cells with pTT5 plasmids containing LC and HC 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 BIAcore. 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 5000-6000 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 ul/min flow rate (5 min association and 5 min dissociation). CmAb1 showed very similar binding activity compared to mAb1(25).

Control mouse IgG1 was raised against a PeptiBody peptide AGP-3. The resulting antibody was produced in stably transfected CHO 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 (IACUC) at Amgen for work performed at Amgen and by the IACUC 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-hour light-dark cycle and had free access to food and water. All mice were in a C57Bl/6 background.

Ldlr$^{-/-}$ and *apoE*$^{-/-}$ mice were obtained from Jackson Laboratories. Each strain was crossed with *pcks9*$^{-/-}$ mice (Ozgene Pty Ltd (Bentley, Australia)) to generate *ldlr*$^{+/-}$*/pcsk9*$^{+/-}$ and *apoe*$^{+/-}$*/pcsk9*$^{+/-}$ colonies. These *pcsk9*$^{+/-}$ colonies were crossed again to generate the male double knockouts (*ldlr*$^{-/-}$*/pcsk9*$^{-/-}$ *and apoe*$^{-/-}$*/pcsk9*$^{-/-}$) and the respective littermate controls (*ldlr*$^{-/-}$*/pcsk9*$^{+/+}$ *and apoe*$^{-/-}$*/pcsk9*$^{+/+}$) used for these experiments. Mice (male) on the *ldlr*$^{-/-}$ background were fed an atherogenic diet (Research Diets D12108C) containing 40% kcal from fat and 1.25% cholesterol. Mice (male) on the *apoe*$^{-/-}$ background were fed chow diet (Harlan 2020X).

Female APOE*3Leiden.CETP transgenic mice (11 to 13 weeks of age) (53), expressing human cholesteryl ester transfer protein (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 BW, TC, TG and age.

In pharmacologic inhibitory studies, antibodies were administered by subcutaneous 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 CO_2 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 minutes followed by 4% paraformaldehyde for 10 minutes for fixation. Hearts and aortas were removed, immersed in 4% paraformaldehyde and stored at 4 °C.

Cholesterol, triglyceride analysis, PCSK9 ELISA

Mouse serum or EDTA plasma was obtained from whole blood collected via centrifugation. Serum or plasma cholesterol and triglycerides were analyzed using either a Cobas Integra 400 chemistry analyzer or enzymatic kits according to the manufacturer's instruction (cat. no. 1458216 and cat. no. 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-Cholesterol E kit omiting 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 instruction.

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 instruction. Random primers were used to convert RNA to single stranded cDNA by reverse transcription (Promega, Fitchburg, USA) according to the manufacturer's protocol. Levels of cDNA were measured by real time polymerase chain reaction (real time-PCR) using the 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, USA), 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 minutes. The lysis buffer consisted of 50 mM Tris-HCL [pH=7.4], 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40 [Igepal], 1mM EDTA, protease inhibitor cocktail [complete, Roche], 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4. Samples were then centrifuged at 13,000 x g at 4˚C for 20 minutes. Protein concentration in cell lysates was determined by BioRad protein assay reagents (BioRad) according to manufacturer's instructions. 50 µg of protein lysates were separated by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (BioRad). Blots were subjected to goat anti-mouse LDLR from R&D Systems and rabbit anti-goat horseradish peroxidase (HRP) from Santa Cruz. Mouse anti-alpha-actin from Cell Signaling Technologies was used to confirm equal loading in conjunction with horse anti-mouse HRP from Santa Cruz Biotechnology Inc, according to the manufacturer's instructions. Blots were developed with Bio-Rad Clarity western ECL (BioRad) and subjected to ChemiDoc™ XRS+ imaging system. Intensities of protein bands were quantified using Image Lab™ Software.

Atherosclerosis measurements

Hearts embedded in paraffin were cross-sectioned (5 µm each) through the entire aortic root area. Sections were stained with either Verhoeff-Van Gieson (VVG) or Hematoxylin-Phloxin-Saffron to measure lesion area. In some studies, histological analysis was performed by Charles River Discovery Research Services and sections were stained with Mac-2 to monitor macrophage content. For each mouse, three or four sections at intervals of 40 to 50 µm were used for quantitative and qualitative assessment of the atherosclerotic lesions (54,55). To qualify lesion severity, the lesions were classified into one of five categories according to the American Heart Association classification: I) early fatty streak, II) regular fatty streak, III) mild plaque, IV) moderate plaque, and V) severe plaque, as previously described (56). To assess lesion severity as a percentage of all lesions, type I-III lesions were classified as mild lesions and type IV-V lesions were classified as severe lesions. Images were acquired with an Olympus BX51 microscope. Atherosclerosis development was quantified by measuring lesion areas using Cell D imaging software (Olympus Soft Imaging Solutions).

For *en face* analysis, aortas were soaked in PBS followed by 70% ethanol (5 minutes each). Aortas were subsequently soaked with Sudan IV stain for 6 minutes with occasional agitation. Aortas were then rinsed, twice, with 80% ethanol followed by PBS (3 minutes each). 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 post-test, for longitudinal studies, by a two-tailed t-test for single end points containing two groups, and by a one-way ANOVA, Tukey post-test, using Prism (GraphPad, Inc). In figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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 (2083 ± 1529 ng/ml and 98 ± 98 ng/ml) providing further confirmation that PCSK9 and LDLR influence the clearance of one another (**Fig. 1A**) (57,58).

Pcsk9$^{-/-}$ 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*$^{-/-}$*/pcsk9*$^{-/-}$ mice relative to *ldlr*$^{-/-}$ littermate controls. Mice were fed a Western Diet (40% kcal from fat and 1.25% cholesterol) for 12 weeks, and circulating cholesterol and triglyceride levels were measured at 5 and 12 weeks from the initiation of Western Diet feeding. *ldlr*$^{-/-}$*/pcsk9*$^{-/-}$ mice exhibited a slight but significant (18-19%) decrease in both total and LDL cholesterol at 5 and 12 weeks and a decrease in HDL cholesterol at 12 weeks (**Fig. 1B**, **Supplemental Fig. 1**) while circulating triglyceride 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 aorta area in the ldlr$^{-/-}$ and *ldlr*$^{-/-}$*/pcsk9*$^{-/-}$ mice, respectively (**Supplemental Fig. 2**). 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 *ldlr*$^{-/-}$ and *ldlr*$^{-/-}$*/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 *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, VLDL, IDL and LDL and also on large HDL particles (61). Consequently, *apoe*$^{-/-}$ mice exhibit elevated circulating cholesterol levels leading to accelerated atherosclerotic plaque accumulation on a chow diet (59,60). In contrast to *ldlr*$^{-/-}$ mice, *apoe*$^{-/-}$ mice exhibit comparable PCSK9 plasma and hepatic LDLR protein levels relative to *WT* mice (67 $\pm$ 23 ng/mL and 98 $\pm$ 98 ng/mL, respectively; **Fig. 2A, Supplemental Fig. 3**). Comparing *apoe*$^{-/-}$/*pcsk9*$^{-/-}$ and *apoe*$^{-/-}$ littermate controls revealed that there is no difference in circulating total cholesterol, HDL, LDL, or triglycerides, and there was no significant difference in hepatic LDLR protein levels (**Fig. 2B-C**, **Supplemental Fig. 4-5**). In addition, there was no difference in either atherosclerotic plaque accumulation (0.18 $\pm$ 0.08 mm^2 and 0.14 $\pm$ 0.07 mm^2) or macrophage content (0.006 $\pm$ 0.005 mm^2 and 0.006 $\pm$ 0.003 mm^2) within the aortic root between *apoe*$^{-/-}$/*pcsk9*$^{-/-}$ and *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 *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 lower circulating lipids in the absence of ApoE, we administered a single dose (10 mg/kg, subcutaneously [sc]) of either an anti-PCSK9 antibody (CmAb1) or control antibody to either *apoe*$^{-/-}$ 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 *pcsk9*$^{-/-}$ mice, a single dose of anti-PCSK9 antibody did not significantly lower circulating cholesterol levels at up to 14 days post-treatment or affect hepatic LDLR protein levels in *apoe*$^{-/-}$ mice (**Fig. 3A, Supplemental Fig. 6**). This is in contrast to C57Bl/6 mice, where a significant increase in hepatic LDLR was observed following anti-PCSK9 antibody treatment (**Supplemental Fig. 7**), which is consistent with our previous findings for PCSK9 inhibition in wild-type mice (25). Circulating triglycerides 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 *apoe*$^{-/-}$ mice (**Supplemental Fig. 8**). Together, these data suggest that APOE is required for cholesterol and triglyceride lowering, and atherosclerosis reduction, by anti-PCSK9 antibody.

In contrast, in APOE*3Leiden.CETP mice the single dose sc injection of anti-PCSK9 antibody significantly reduced both cholesterol (up to 69%) and triglycerides (up to 70%) during 14 days post-treatment (**Fig 3C-D**) compared to control antibody. This corresponded to a significant increase in hepatic LDLr mRNA and protein expression (**Supplemental Fig. 9**). 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 Western type diet. As compared to a chow diet, the Western type diet, containing 0.15% cholesterol, increased PCSK9 levels by 51% (from 135.4 ± 14.2 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 non-HDL-C (**Fig. 4D**) and TG was reduced by 61% ($p<0.001$) as compared to control. After 14 weeks of treatment atherosclerosis development was reduced by 91% ($p<0.001$) in the mice treated with anti-PCSK9 antibody as compared to 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 to 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.

DISCUSSION

Classic work, such as that by Ishibashi *et al.* has set the foundation of understanding of ApoE and LDLR in lipoprotein homeostasis (61). 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 *pcsk9*$^{-/-}$ mice on either an *ldlr*$^{-/-}$ or *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 is 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 *pcsk9* in *ldlr*$^{-/-}$ mice. These data are in contrast with previous studies showing no effect of *pcsk9* 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 *pcsk9* in *ldlr*$^{-/-}$ mice might relate to potential effects of PCSK9 in enabling ApoB secretion in nascent VLDL, or perhaps in upregulation of other LDLR family members, such as LRP1, that could also bind and clear lipoproteins (63-68). Alternatively, Le May *et al* demonstrated that transintestinal cholesterol excretion (TICE) is upregulated in *pcsk9*$^{-/-}$ mice driven by increased intestinal LDLR expression (69). Although TICE was not examined in this study, if upregulated in *pcsk9*$^{-/-}$/*ldlr*$^{-/-}$mice, this could point to an unidentified LDLR independent mechanism of TICE.

After deleting or inhibiting PCSK9 in *apoe*$^{-/-}$ mice we anticipated decreases in apoB containing lipoproteins (and hence some plasma cholesterol reduction) by the expected increase in liver LDLR expression, even though lipoproteins normally containing ApoE would be unaffected. However, our experiments clearly show that in absence of ApoE PCSK9 deletion or inhibition does not reduce lipids, whereas in the presence of ApoE, using the APOE*3Leiden.CETP mice, PCSK9 inhibition is effective. Further, we show that in the absence of ApoE PCSK9 inhibition does not lead to LDLR upregulation, which was also unexpected. Of course the lack of effect on LDLR expression after PCSK9 inhibition in *apoe*$^{-/-}$ mice most likely 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.*, showing that under normal circumstances, chylomicron remnants are rapidly internalized by the LDLR and catabolized in hepatocytes, with a critical requirement for ApoE (70). Ishibashi *et al*. showed similar remnant clearance in *apoe*$^{-/-}$ mice and *ldlr*$^{-/-}$/*apoe*$^{-/-}$ mice, strongly suggesting a minor function of the LDLR in *apoe*$^{-/-}$ mice (71).

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 aorta cholesterol ester levels. They found that aorta cholesterol levels were reduced, without observing effects on lesion area in the aortic root or thoracic aorta in *apoe*$^{-/-}$/*pcsk9*$^{-/-}$ as compared to their respective *apoe*$^{-/-}$ controls. We did not measure aorta 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 wild type mice have a very rapid clearance of apoB-containing lipoproteins, APOE*3Leiden mice have an impaired clearance and increased triglyceride levels and are thereby mimicking the slow clearance observed in humans, particularly in patients with Familial Dysbetalipoproteinemia (FD) (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 cardiovascular disease (like statins, fibrates, antihypertensives, etc.) (49,54,72-74). However APOE*3Leiden mice (like wild-type 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 human 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 total cholesterol and triglyceride 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 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 (77). 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 of 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 if 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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Figure 1. Minimal effect of deleting PCSK9 on circulating lipids or atherosclerosis in LDL receptor deficient mice. Plasma PCSK9 levels are higher in *ldlr*$^{-/-}$ mice compared to C57Bl/6 mice (**A**) consistent with the LDLR being a key clearance mechanism for PCSK9 (N = 9 *ldlr*$^{-/-}$, N = 12 C57Bl/6). *ldlr*$^{-/-}$, *pcsk9*$^{-/-}$ mice exhibit a slight decrease in total cholesterol (**B**) but not triglycercides (**C**) relative to *ldlr*$^{-/-}$ mice when fed a western type diet (N = 41 *ldlr*$^{-/-}$, N = 43 *pcsk9*$^{-/-}$, *ldlr*$^{-/-}$). The aortic sinus was sectioned and stained with Verhoeff-Van Gieson (VVG) to measure lesion area (blue) and Mac-2 to monitor macrophage content (red). No difference in atherosclerosis development or macrophage accumulation (**D-F**) was observed in the aortic sinus for *ldlr*$^{-/-}$ mice relative to *pcsk9*$^{-/-}$, *ldlr*$^{-/-}$ mice (N = 25 / group). Data represented as the means (bars) +/- S.D. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as compared to *ldlr*$^{-/-}$, two-tailed t-test, unpaired).

Figure 2. No effect of deleting PCSK9 on circulating lipids or atherosclerosis in APOE deficient mice. Comparable levels of PCSK9 are observed in *apoe*$^{-/-}$ mice compared to C57Bl/6 mice (**A**) (N = 12 *apoe*$^{-/-}$, N = 12 C57Bl/6). No significant reduction in circulating total cholesterol (**B**) and triglyceride (**C**) levels were observed in *pcsk9*$^{-/-}$, *apoe*$^{-/-}$ mice relative to *apoe*$^{-/-}$ mice (N = 9 (8 weeks), 21 (24 weeks) for *pcsk9*$^{-/-}$, *apoe*$^{-/-}$; N = 11 (8 weeks), N = 15 (24 weeks) for *apoe*$^{-/-}$). The aortic sinus was sectioned and stained with Verhoeff-Van Gieson (VVG) to measure lesion area (blue) and Mac-2 to monitor macrophage content (red), and consistent with these observations, no difference in atherosclerosis development or macrophage accumulation (**D-F**) was observed in the aortic sinus for *apoe*$^{-/-}$ mice relative to *pcsk9*$^{-/-}$, *apoe*$^{-/-}$ mice (N = 18 *pcsk9*$^{-/-}$, *apoe*$^{-/-}$; N = 20 *apoe*$^{-/-}$). Data represented as the means (bars) +/- S.D., two-tailed t-test, unpaired, as compared to *apoe*$^{-/-}$.

Figure 3. Anti-PCSK9 antibody treatment reduces total cholesterol and triglyceride levels in APOE*3Leiden.CETP mice but not *apoe*$^{-/-}$ mice. No significant reduction in total cholesterol (**A**) and only a slight but significant reduction in triglycerides 5 days post-treatment (**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 / group). This contrasts results with APOE*3Leiden.CETP mice, where anti-PCSK9 antibody

treatment resulted in a significant decrease in total cholesterol (**C**) and triglycerides (**D**) (10 mg/kg (sc) day 0, N = 8 / group). Data represented as the means (bars) +/- S.D., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, as compared to control, two-way ANOVA, Sidak post-test.

Figure 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 western diet (WD) as well as two weeks after a single injection with anti-PSCK9 antibody (10 mg/kg, sc) in mice fed WD. Data represented as the means (bars) +/- S.D. (n=8/group) ** P<0.01 vs chow; # P<0.05 vs WD, one-way ANOVA, Tukey post-test. To assess the effect on atherosclerosis, control or anti-PCSK9 antibody were injected sc every 10 days for 14 weeks in APOE*3Leiden.CETPmice. Plasma total cholesterol (**B**) and triglycerides (**C**) were measured at 3 and 10 days post-injection in the first and twelfth week of treatment. Data represented as means (bars) +/- S.D. (n=15/group), ***P<0.001 vs control antibody. (**D**) FPLC fractionation of pooled plasma samples are shown from week 8. Atherosclerosis development was determined in the aortic sinus of APOE*3Leiden.CETP mice. Representative pictures of control antibody and anti-PCSK9 antibody treated mice are shown (**E-F**). The total lesion area per cross section (**G**) was measured and lesion severity (**H**) was determined. Data represented as means (bars) +/- S.D. (n=15 /group), ***P<0.001 vs control antibody.

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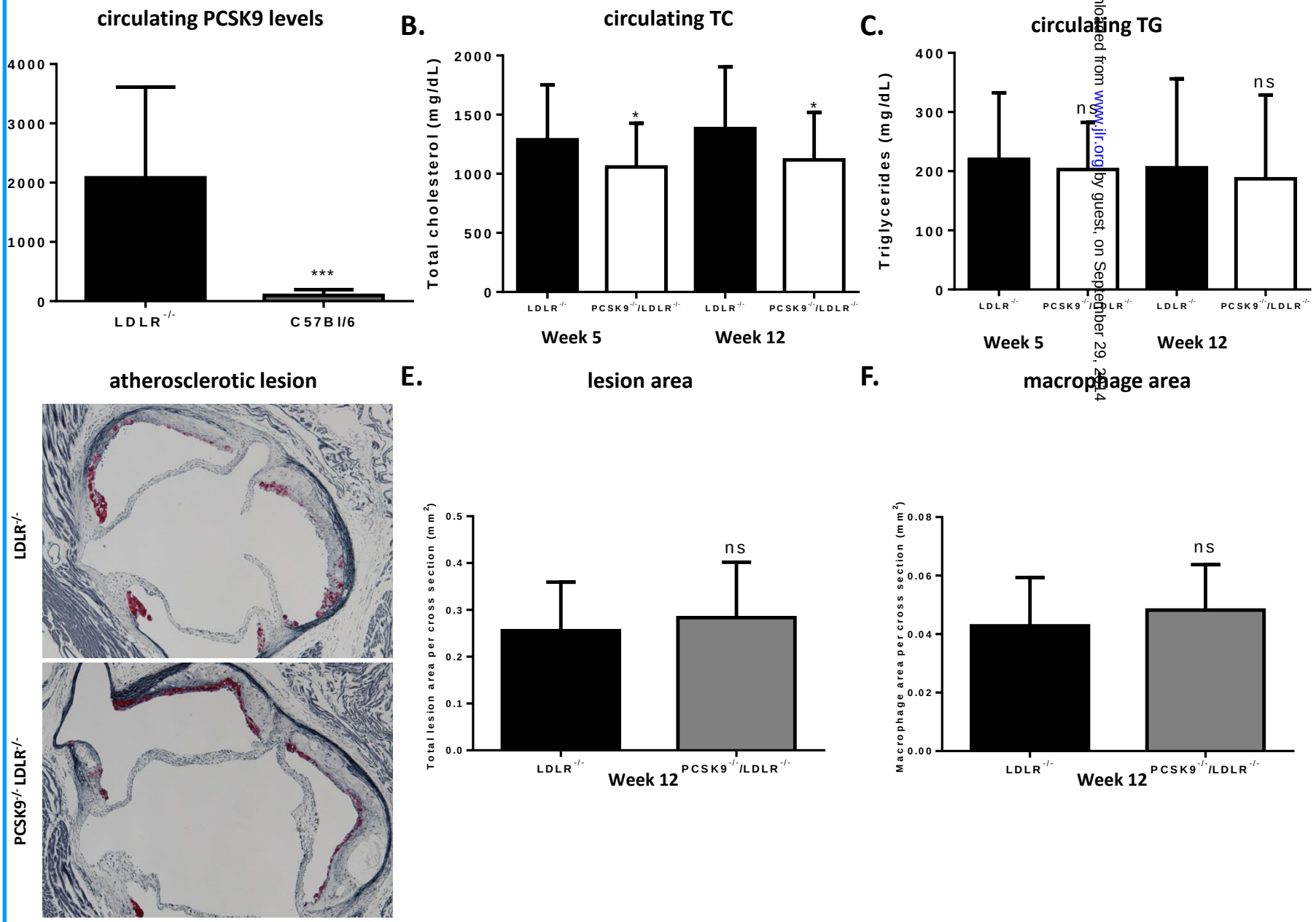

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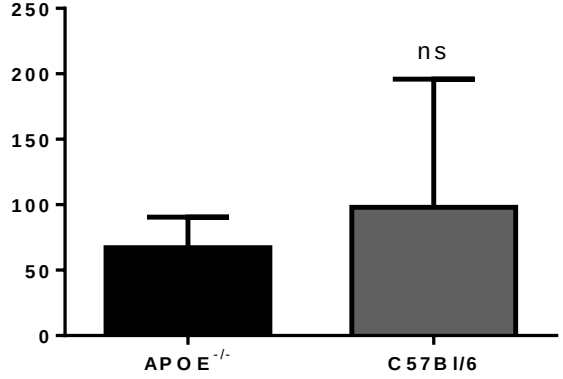

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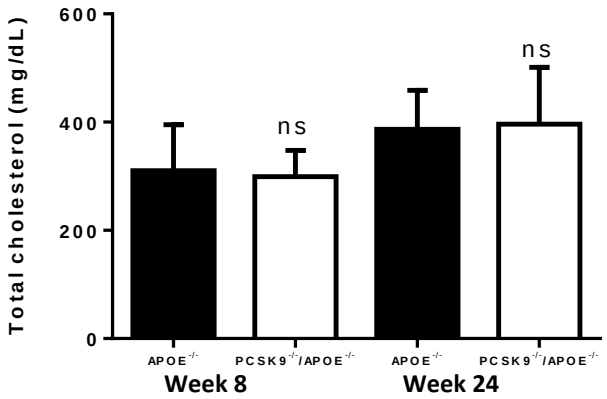

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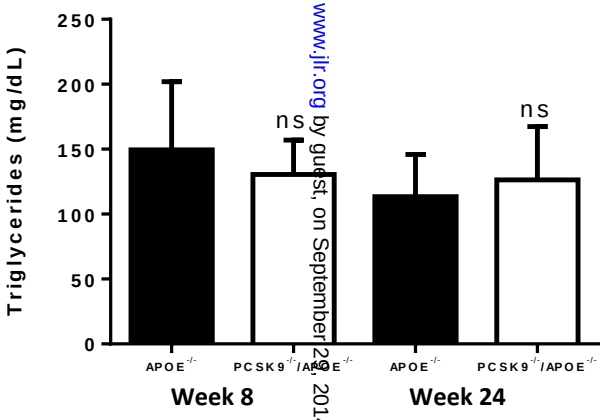

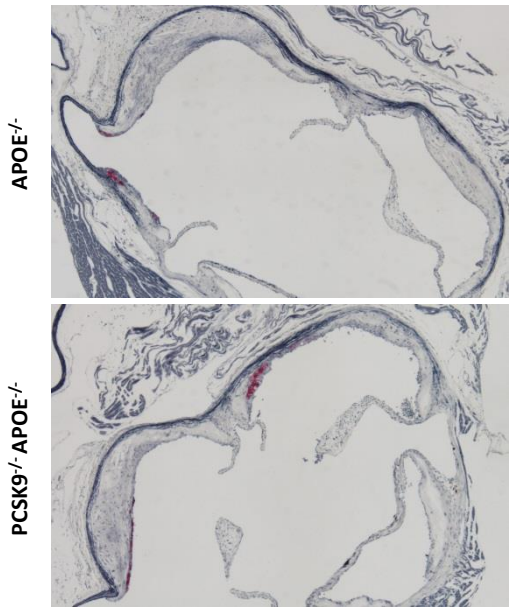

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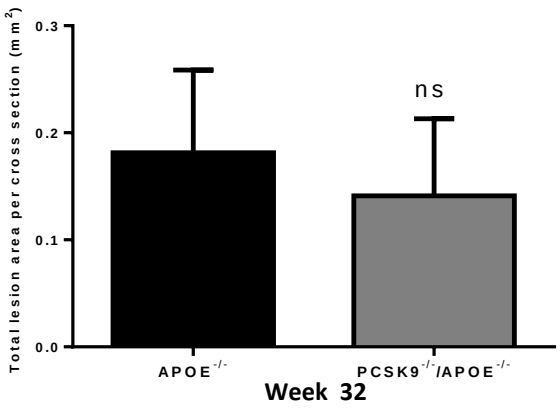

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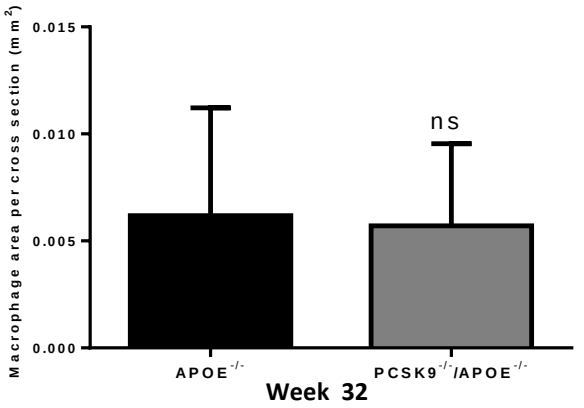

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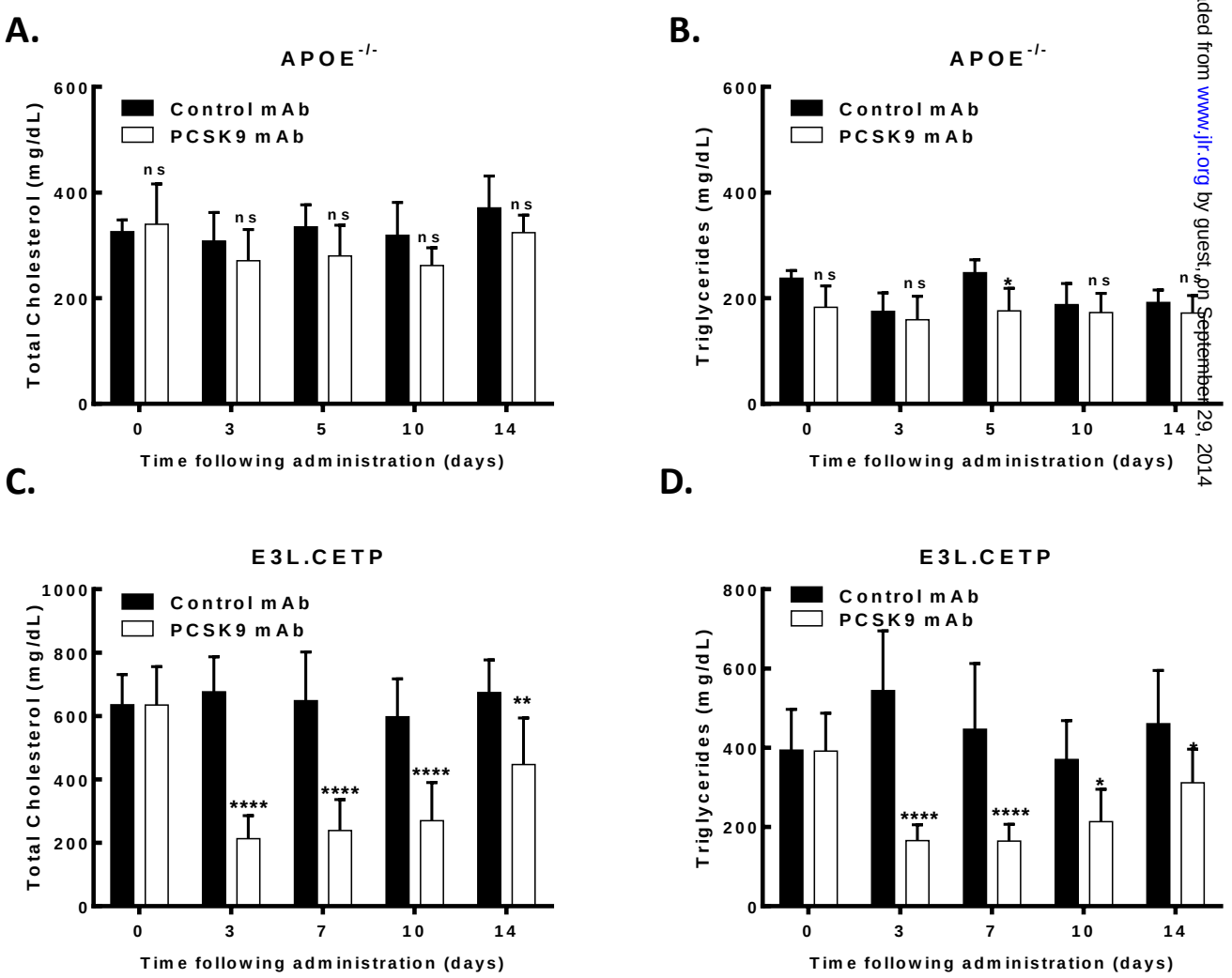

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