Inhibitory antibodies against PCSK9 reduce surface CD36 and mitigate diet-induced renal lipotoxicity

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*PCSK9 protects against diet-induced renal injury in both cultured cells and in mice by enhancing the degradation of renal CD36.

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Abstract:
Background: PCSK9 modulates the uptake of circulating lipids through a range of receptors, including the low-density lipoprotein receptor (LDLR) and CD36. In the kidney, CD36 is known to contribute to renal injury through pro-inflammatory and -fibrotic pathways. In this study, we sought to investigate the role of PCSK9 in modulating renal lipid accumulation and injury through CD36 using a high fat diet (HFD)-induced mouse model. Methods: The effect of PCSK9 on the expression of CD36 and intracellular accumulation of lipid was examined in cultured renal cells and in the kidneys of male C57BL/6J mice. The impact of these findings were subsequently explored in a model of HFD-induced renal injury in Pcsk9-/- and Pcsk9+/+ littermate control mice on a C57BL/6J background. Results: In the absence of PCSK9, we observed heightened CD36 expression levels, which increased free fatty acid (FFA) uptake in cultured renal tubular cells. As a result, PCSK9 deficiency was associated with an increase in long-chain saturated FFA-induced ER stress. Consistent with these observations, Pcsk9-/- mice fed HFD displayed elevated ER stress, inflammation, fibrosis, and renal injury relative to HFD-fed control mice. In contrast to Pcsk9-/- mice, pre-treatment of WT C57BL/6J mice with evolocumab, an anti-PCSK9 monoclonal antibody (mAbs) that binds to and inhibits the function of circulating PCSK9, protected against HFD-induced renal injury in association with reducing cell-surface CD36 expression on renal epithelia. Conclusions: We report that circulating PCSK9 modulates renal lipid uptake in a manner dependent on renal CD36. In the context of increased dietary fat consumption, the absence of circulating PCSK9 may promote renal lipid accumulation and subsequent renal injury. However, while the administration of evolocumab blocks the interaction of PCSK9 with the LDLR, this evolocumab/PCSK9 complex can still bind CD36, thereby protecting against HFD-induced renal lipotoxicity.

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Abbreviations: ATF6, activating transcription factor 6; BSA, bovine serum albumin; CD36, cluster of differentiation 36; CKD, chronic kidney disease; CVD, cardiovascular disease; ER, endoplasmic reticulum; FAS, fatty acid synthase; FFA, free fatty acid; FH, familial hypercholesterolemic; GFR, glomerular filtration rate; GOF, gain-of-function; GRP78, glucose-regulated protein of 78 kDa; GRP94, glucose-regulated protein 94 kDa; HFD, high-fat diet; HRP, horse radish peroxidase; IHC, immunohistochemical; IRE1, inositol-requiring enzyme 1; LDLc, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LOF, loss-of-function; mAb, monoclonal antibody; n-ATF6, nuclear ATF6; NCD, normal control diet; OA, oleic acid; ORO, oil-red-o; PERK, ox-LDL, oxidized-LDL; PA, palmitate; PoA, palmitoleic acid; PPAR, peroxisome proliferator-activated receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; p-PERK, phosphorylated-PERK; PERK, protein kinase RNA-like ER kinase; qRT-PCR, quantitative real-time PCR, ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum Ca2+-ATPase; siCD36, small interfering CD36; siScrambled, small interfering scrambled; SREBP-1, sterol regulatory element binding protein-1; TBS, tris-buffered saline; UPR, unfolded protein response
Key Points

- PCSK9 protects against diet-induced renal injury in both cultured cells and in mice by enhancing the degradation of renal CD36.
- PCSK9 mAbs (Evolocumab) do not block the ability of PCSK9 to bind to surface CD36, unlike its effects on degrading hepatic LDLR.
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Abstract

Background: PCSK9 modulates the uptake of circulating lipids through a range of receptors, including the low-density lipoprotein receptor (LDLR) and CD36. In the kidney, CD36 is known to contribute to renal injury through pro-inflammatory and -fibrotic pathways. In this study, we sought to investigate the role of PCSK9 in modulating renal lipid accumulation and injury through CD36 using a high fat diet (HFD)-induced mouse model.

Methods: The effect of PCSK9 on the expression of CD36 and intracellular accumulation of lipid was examined in cultured renal cells and in the kidneys of male C57BL/6J mice. The impact of these findings were subsequently explored in a model of HFD-induced renal injury in Pcsk9−/− and Pcsk9+/+ littermate control mice on a C57BL/6J background.

Results: In the absence of PCSK9, we observed heightened CD36 expression levels, which increased free fatty acid (FFA) uptake in cultured renal tubular cells. As a result, PCSK9 deficiency was associated with an increase in long-chain saturated FFA-induced ER stress. Consistent with these observations, Pcsk9−/− mice fed HFD displayed elevated ER stress, inflammation, fibrosis, and renal injury relative to HFD-fed control mice. In contrast to Pcsk9−/− mice, pre-treatment of WT C57BL/6J mice with evolocumab, an anti-PCSK9 monoclonal antibody (mAbs) that binds to and inhibits the function of circulating PCSK9, protected against HFD-induced renal injury in association with reducing cell-surface CD36 expression on renal epithelia.

Conclusions: We report that circulating PCSK9 modulates renal lipid uptake in a manner dependent on renal CD36. In the context of increased dietary fat consumption, the absence of circulating PCSK9 may promote renal lipid accumulation and subsequent renal injury. However, while the administration of evolocumab blocks the interaction of PCSK9 with the LDLR, this evolocumab/PCSK9 complex can still bind CD36, thereby protecting against HFD-induced renal lipotoxicity.
Introduction

The global prevalence of chronic kidney disease (CKD) is increasing at an alarming rate and is associated with a substantial burden on patients and the health care system. The progression of CKD in affected individuals commonly develops as a result of interstitial fibrosis, proteinuria, and tubular atrophy, which compromises the overall filtration capacity of the kidney. Dyslipidemia and obesity are considered prominent risk factors in CKD. Increased free fatty acid (FFA) uptake due to excess consumption of diets rich in fats promotes intra-renal lipid accumulation in several animal models and patients at various stages of CKD. As a result, excess renal uptake of FFAs damages podocytes, mesangial cells, and proximal tubular epithelial cells through various mechanisms, including increased reactive oxygen species (ROS) production and lipid peroxidation. This in turn promotes mitochondrial dysfunction and tissue inflammation, resulting in the formation of glomerular and tubular lesions.

Among the underlying mechanisms of FFA-induced CKD progression includes the induction of endoplasmic reticulum (ER) stress. Changes in the lipid composition of the ER membrane antagonizes the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump, leading to the depletion of ER Ca\(^{2+}\) levels. In support of this evidence, intracellular lipid accumulation is associated with ER stress. Because the chaperones expressed in the ER lumen that function to properly fold de novo proteins are dependent on ER Ca\(^{2+}\) as an essential co-factor, this state of Ca\(^{2+}\) depletion commonly leads to the accumulation and subsequent aggregation of misfolded proteins in the ER.

The ER is responsible for the synthesis and proper folding of secretory, transmembrane, and ER luminal proteins and addresses the accumulation of misfolded protein via a highly conserved signaling cascade known as the unfolded protein response (UPR). Activation of the UPR is dependent on the dissociation of ER chaperones such as the glucose-regulated protein of 78 kDa (GRP78) from three transmembrane sensors in the ER lumen; namely the activating transcription factor 6 (ATF6), the protein kinase RNA (PKR)-like ER kinase (PERK), and the inositol-requiring enzyme I (IRE1). Chronic activation of these transmembrane proteins and their downstream signaling pathways result in cellular apoptosis, inflammation, and fibrosis in the context of CKD progression.

The scavenger receptor known as the cluster of differentiation 36 (CD36) promotes intra-renal lipid accumulation, inflammatory signaling, cellular apoptosis and pro-fibrotic signaling pathways. CD36 is a multifunctional receptor that mediates the cellular uptake of long-chain fatty acids and oxidized lipoproteins and is abundantly expressed in proximal and distal tubular epithelium, as well as podocytes, mesangial cells, and interstitial macrophages. Previous studies have reported that CD36-dependent pathways can modulate the development of kidney fibrosis. In mouse MCT proximal tubular epithelial cells, overexpression of tubular CD36 increases intracellular lipid accumulation and induces the expression of pro-fibrotic genes and markers of UPR activation. In podocytes, CD36-dependent uptake of palmitic acid dose-dependently increases ER stress, mitochondrial reactive oxygen species (ROS) production, ATP depletion, and apoptosis. Thus, CD36 stands as a key driver of renal damage and its potential use as a therapeutic target for the management of renal disease has yet to be fully elucidated.
Patients with renal disease also are at a substantially higher risk for atherosclerotic cardiovascular diseases (CVD). In the general population, as well as in CKD patients, circulating low-density lipoprotein cholesterol (LDLc) is a well-known driver of atherosclerotic lesion development and CVD progression. It is well established that circulating PCSK9 binds to and degrades the LDLR, thereby increasing LDLc levels and the risk of CVD. PCSK9 also degrades several other receptors that promote extracellular lipid uptake into tissue such as the LDLR-related protein-1, the very low-density lipoprotein receptor, and CD36. Given the abundant expression of CD36 in renal tubular epithelium, deficiency of circulating PCSK9 may affect the expression of this receptor, thereby contributing to renal lipid accumulation and injury via the enhancement of FFA and lipoprotein uptake. In this study, we examined the role of circulating PCSK9 on the expression of renal CD36 and its ability to increase lipid uptake. We also explore the effect of evolocumab, an inhibitory antibody targeting PCSK9, on renal CD36 expression and its impact on diet-induced renal lipotoxicity.

Materials and Methods

Cell Culture, Transfections and Free Fatty Acid (FA) Treatments:
Human immortalized proximal tubule epithelial (HK-2), hepatocyte (HuH-7), and embryonic kidney (HEK293) cell lines, as well as Sprague-Dawley primary rat mesangial cells were used (passages 6 through 15). All cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 50 IU/ml of penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) and maintained in 5% CO₂ at 37°C. Cells were plated at a confluence of 60% for transfection experiments. Transfection cocktail consisted of the following ratio: 1 µg DNA:3 µl X-tremeGENE HP DNA reagent: 100 µl Opti-MEM. The cDNA of human WT PCSK9 and the PCSK9Q152H loss-of-function ER retention variant were cloned into pIRES-EGFP (Clontech) that contains a C-terminal V5 epitope, allowing protein detection with a V5 mAb (Invitrogen), as described.46 Cells were treated with either LDL (Lee Biosolutions), oxLDL (Alfa Aesar), oleate (Alfa Aesar) or palmitate (Sigma-Aldrich) that were conjugated to FA-free bovine serum albumin (BSA). All treatments were carried out overnight for 24 h unless specified otherwise.

Animal Studies
6-week old male Pcsk9⁻/⁻ mice on a C57BL/6J background and age-matched WT C57BL/6J controls (Jackson Laboratories) were placed on either a normal control diet (NCD) (n=10) or HFD (60% fat/Kcal; ENVIGO #TD06414; n=10) for 12 weeks with ad libitum access to food and water. Animals were fasted overnight prior to sacrifice. Pcsk9⁻/⁻ mice were a generous gift from Dr. Nabil G. Seidah and were generated as described. All animal procedures were approved by the McMaster University Animal Research Ethics Board.

PCSK9 mAbs Administration
10-week old male WT mice on a C57BL/6J background were administered the PCSK9 mAb, evolocumab (n=10) or saline (n=10) at 30 mg/kg via orbital injection and sacrificed 10 days later. All animal procedures were approved by the McMaster University Animal Research Ethics Board.

Immunoblotting
Total protein lysates were electrophoretically resolved on 7-10% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were then blocked using 5% w/v skimmed milk in tris-buffered saline (TBS) containing Tween-20 for 1 h and incubated with primary antibody overnight for 18 h at 4°C. To detect bound primary antibodies, horse radish peroxidase (HRP) conjugated antibodies were used (goat anti-rabbit, Bio-Rad; donkey anti-goat, Santa Cruz, goat anti-mouse, Bio-Rad) and developed with a chemiluminescent reagent (FroggaBio). Quantification of immunoblots was assessed after normalization to β-actin (Sigma-Aldrich). Please refer to Table 1 for a list of the antibodies used for this study.

**Real-time PCR**

Total RNA from tissues and culture cells was isolated using RNA purification kit according to the manufacturer’s instructions (ThermoFisher Scientific). A total of 2 μg of RNA was reverse-transcribed to cDNA using SuperScript Vilo IV cDNA synthesis kit (ThermoFisher Scientific). Quantitative real-time PCR (qRT-PCR) assessment of different mRNA species was used in conjunction with FAST SYBR Green (ThermoFisher Scientific). All primer sequences are listed in Table 2.

**Immunofluorescent Staining**

Cells were fixed using 4% paraformaldehyde and either non-permeabilized or permeabilized with 0.025% Triton-X in PBS, and blocked with 1% BSA for 30 min. Cells were then stained with anti-LDLR (catalog no. AF2148, R&D Systems), anti-CD36 (catalog no. NB400-144, Novus Biologicals) or anti-ATF6 (catalog no. NBP1-40256, Novus Biologicals) for 1h. Following primary antibody incubation, cells were fluorescently labelled with either Alexa 647 (catalog no. 21245, Thermo Fisher Scientific), Alexa 594 (catalog no. A11058, Thermo Fisher Scientific), or Alexa 488 (catalog no. A10468) as well as DAPI. Slides were mounted using permafluor and visualized using the EVOS FL color imaging system.

**Oil-Red-O (ORO) Staining**

Cells were fixed using 4% paraformaldehyde and stained with ORO (Sigma-Aldrich) for 5 min. After washing, cells were counterstained with Gills hematoxylin (no.5, Sigma Aldrich) and mounted on glass slides. For quantification purposes, ORO stain was extracted from cells using isopropanol for 20 min on an orbital shaker at 37°C. Optical density of ORO-exposed isopropanol extracts was quantified using a spectrophotometer (Molecular Devices) at a wavelength of 520 nm. Ten μm thick sections of mouse OCT-embedded kidneys were washed with propylene glycol and stained with ORO for 10 min. Following staining, tissues were repeatedly washed with propylene glycol and counterstained using alum hematoxylin.

**Dil-LDL/oxLDL uptake assay**

Approximately 3.0 x 10^4 cells were seeded per well in black clear-bottom 96-well plates for 24 h prior to treatment at the respective timepoints. During the last 5 h of treatment, cells were exposed to Dil-LDL/oxLDL (10 μg/ml) and then washed with pre-warmed (37 °C) HBSS containing 20 mM HEPES prior to analysis. The fluorescence intensity of Dil was subsequently quantified using the SpectraMax GeminiEM fluorescent spectrophotometer (Molecular Devices; ex 554/em 571).

**Thioflavin-T/S Staining**
Thioflavin staining is commonly used for the visualization of misfolded protein aggregates as a result of ER stress. Cells were fixed using 4% paraformaldehyde and stained with Thioflavin-T (Sigma-Aldrich) for 15 min at 37°C. For tissue, 10 µm thick sections of mouse OCT-embedded kidneys were stained with Thioflavin-S staining for 15 min. All images and quantifications were collected using a fluorescent microscope (EVOS FL).

**Triglyceride Quantification**
Renal cortical tissue was assessed for triglyceride and cholesterol content using a triglyceride assay kit (Abcam) and colorimetric cholesterol assay kit (Wako Diagnostics) respectively, according to manufacturer’s instructions. All data were normalized to tissue weight.

**PCSK9 ELISA**
Quantification of PCSK9 in media and throughout in vivo studies was determined using the R&D human and mouse Quantikine PCSK9 ELISA kits, respectively.

**Cystatin C ELISA**
Quantification of serum mouse Cystatin C was determined using the Abcam mouse Cystatin C ELISA kit as per manufacturer’s instructions.

**Immunohistochemical (IHC) Staining**
Four µm thick sections were deparaffinized, blocked in 5% v/v serum and subsequently incubated with primary antibodies for 18 h at 4°C. Sections were incubated with either biotin-labeled secondary antibodies (Vector Laboratories) or Alexa Fluor secondaries (Invitrogen) for fluorescent imaging. Streptavidin-labeled HRP solution (Vector Laboratories) and NovaRed developing solution (Vector Laboratories) were used to visualize the staining. Slides were examined using a Nikon light microscope (Model no. DS-Ri2). Relative staining intensity was quantified using ImageJ software (n=10). For picro Sirius red (PSR) staining, kidney sections were exposed to saturated picric acid solution and stained with Sirius red F3B (Colour Index 35782). Please refer to Table 1 for a list of the antibodies used for this study.

**Statistical Analysis**
Statistical analysis for two-group comparisons was conducted using unpaired Student's t-test and for multiple group comparison, one-way ANOVA was used. Statistically significant differences were considered at \( p<0.05 \). All error bars are represented as standard deviation of the mean.

**Results**

*Secreseed PCSK9 regulates surface CD36 and LDLR in HK-2 cells*
Renal proximal tubular cells express a variety of receptors targeted by PCSK9. Given that PCSK9 is secreted into the blood almost exclusively by the liver, we developed an in vitro model to mimic the effect of liver-secreted PCSK9 on HK-2 cells. In these experiments, cultured HuH7 cells were transfected with either WT PCSK9 or the PCSK9\(^{Q152H}\) LOF variant that fails to be secreted from cells. PCSK9-containing medium from PCSK9\(^{WT}\)-transfected cells or control medium from PCSK9\(^{Q152H}\)-transfected cells (devoid of secreted PCSK9) was harvested and used to culture HK-2 cells. Western blot and immunofluorescence analysis demonstrated that HK-2
cells exposed to PCSK9-containing medium express reduced levels of LDLR and CD36 (Fig. 1A-C). To confirm these observations, immunofluorescent staining intensity was quantified using ImageJ software (Fig. 1D; *, \(p<0.05\)). Secreted PCSK9 was also measured in the medium from HuH7 cells, along with non-transfected HuH7 and HK-2 cells. Using ELISAs, the majority of secreted PCSK9 originated from hepatocytes (Fig. 1E-F; *, \(p<0.05\)). To show that these findings were the direct result of PCSK9, western blot analysis revealed that the reduction of LDLR and CD36 in HK-2 cells treated with recombinant human PCSK9 (rhPCSK9) were comparable to the media harvested from HuH7 cells overexpressing WT-PCSK9 at equal concentrations of PCSK9 (Supp Fig. 1C; *, \(p<0.05\)). qRT-PCR analysis revealed that relative mRNA transcripts of CD36, SREBP 2, LDLR and PCSK9 in HK-2 cells were not significantly affected by the media swap compared to empty vector (EV) controls (Fig. 1G; NS, non-significant). Collectively, these data demonstrate that PCSK9 secreted from hepatocytes can downregulate CD36 expression on the surface of renal-derived cells.

**PCSK9 blocks OA- and LDL-induced lipid droplet accumulation in renal cells**

We next examined whether exogenous PCSK9 could regulate lipid uptake in a variety of renal-derived cell lines. HK-2 cells were exposed to medium harvested from PCSK9-transfected HuH7 cells, treated with oleic acid (OA), and stained with the ORO lipid stain. HK-2 cells exposed to medium from WT-PCSK9-transfected HuH7 cells exhibited reduced lipid accumulation relative to cells exposed to control medium from the PCSK9Q152H-transfected HuH7 cells (Fig. 2A). Consistent with visual observations, densitometric quantification of ORO staining via densitometry of isopropanol extract at 520 nm suggests that PCSK9 blocks OA-induced lipid accumulation in HK-2 cells (Fig. 2B, *, \(p<0.05\)). To further confirm this observation, both HK-2 and primary mesangial cells exposed to PCSK9-containing medium were treated with fluorescently-labelled DiI-LDL in order to visualize LDL uptake. A reduction in uptake of DiI-labelled LDL was observed in HK-2 cells exposed to PCSK9-containing medium (Fig. 2C); a finding confirmed using quantitative spectrophotometry (Fig. 2D-E, *, \(p<0.05\)). To examine whether the observed difference in lipid accumulation occurred due to exogenous V5-labelled PCSK9, anti-V5 antibodies were added to the media to block the binding of V5-labelled PCSK9 to CD36. Compared to IgG controls, the addition of anti-V5 antibody located on the C-terminus of PCSK9 reversed the attenuating effects of lipid accumulation by exogenous V5-PCSK9 in the media (Supp. Fig. 1A, *, \(p<0.05\)), which was also quantified (Supp. Fig. 1B, *, \(p<0.05\)). Altogether, these observations demonstrate that secreted PCSK9 modulates lipid uptake in renal-derived cell lines.

**PCSK9 attenuates lipid accumulation in renal-derived cells in a manner dependent on CD36**

Given that PCSK9 regulates CD36 expression, and that CD36 is an established scavenger receptor for FFA in renal cells, we examined whether PCSK9 regulates FFA uptake and subsequent accumulation in a manner dependent on CD36. First, ORO staining and quantification was carried out in HK-2 cells treated with SSO, a well-established blocker of CD36 (Fig. 3A-B, *, \(p<0.05\)). Then, HK-2 cells were treated with OA in the presence of either scrambled small interfering (si)RNA (siScrambled) or siRNA targeted against CD36 (siCD36), pre-treated for 18 h prior. Treatment with siCD36 was able to attenuate OA-induced lipid accumulation (Fig. 3C-D, *, \(p<0.05\)). The effectiveness of siRNA-mediated knockdown of CD36 was assessed via immunoblotting (Fig. 3E, *, \(p<0.05\)) and qRT-PCR (Fig. 3I, *, \(p<0.05\)). Next, primary rat mesangial cells were treated with Dil-oxLDL for 5 h. Visualization and
fluorescent staining quantification revealed that exogenous PCSK9 was able to attenuate DiI-oxLDL uptake (Fig. 3F-G, *, p<0.05). Lastly, as ORO staining measures for both esterified cholesterol and triglyceride, intracellular triglyceride content was measured in HK-2 cells following CD36 knockdown and OA treatment. Results revealed that knockdown of CD36 expression using siCD36 was able to attenuate relative triglyceride content in HK-2 cells compared to the scrambled siRNA control (Fig. 3H, *, p<0.05). In addition to regulating CD36 expression, these data demonstrate that PCSK9 also regulates the uptake and accumulation of FFA in cultured renal cells.

**PCSK9 attenuates palmitate- and oxidized LDL-induced ER stress in vitro**

CD36 contributes to ER stress in proximal tubular cells in a manner dependent on excess uptake of ER stress-inducing lipoproteins and FFAs, such as oxidized-LDL (ox-LDL) and palmitate (PA), respectively. Therefore, we next examined whether PCSK9 could protect against ER stress in the presence of ox-LDL and PA resulting from the attenuation of CD36 expression. Immunofluorescent staining of the UPR marker nuclear ATF6 (n-ATF6) demonstrated that PCSK9-containing medium reduces ox-LDL-induced ER stress (Fig. 4A), which was also quantified (Fig. 4B, *, p<0.05). Next, qRT-PCR assessment revealed that PCSK9-containing medium attenuated relative UPR activation induced by PA (Fig. 4C-F, *, p<0.05). Interestingly, palmitoleic acid (PoA) had no effect on UPR activation either in the presence or absence of HuH7-secreted circulating PCSK9 (Supp. Fig. 3C, NS, Non-Significant). To further correlate the lipotoxic effects of PA on the observed UPR activation, relative triglyceride content was measured in HK-2 cells treated with PA in the presence or absence of exogenously added PCSK9. The presence of PCSK9 led to a significant reduction in triglyceride uptake in HK-2 cells with PA (Fig. 4G, p<0.05). Lastly, protein aggregate accumulation, a hallmark characteristic of ER stress, was assessed via quantification of live staining in HK-2 cells with Thioflavin-T. Reduced fluorescence in Thioflavin-T staining was observed in HK-2 cells exposed to exogenous PCSK9 treated with PA, indicative of a reduction in misfolded protein aggregates (Fig. 4H, *, p<0.05; NS, non-significant). Together, these observations suggest that PCSK9 is able to protect against PA and oxLDL-induced ER stress by reducing extracellular FFA uptake.

**Pcsk9−/− mice exhibit increased renal lipid accumulation**

To gain insight into the ability of PCSK9 to modulate renal lipid uptake/accumulation in vivo, male Pcsk9−/− mice and age-matched WT C57BL/6J Pcsk9+/+ controls fed NCD were sacrificed at 12 weeks of age. Immunohistochemical analysis of the renal cortex revealed that Pcsk9−/− mice exhibited increased tubular lipid accumulation assessed via ORO staining relative to the controls (Fig. 5A). This observation was consistent with increased immunostaining for CD36 and the lipid droplet marker, perilipin, staining in Pcsk9−/− mice. Immunoblot analysis also demonstrated that Pcsk9−/− mice had increased protein expression of CD36 and LDLR, receptors known to promote lipid uptake in a variety of tissues including the kidney (Fig. 5B, *, p<0.05). Interestingly, as a compensatory response, Pcsk9−/− mice also exhibited increased β-oxidation through increased expression and cleavage (cPPARα) of the peroxisome proliferator-activated receptor (PPAR)α (c PPARα). Simultaneously, the nuclear form of sterol regulatory element binding protein 1 (nSREBP1) and fatty acid synthase (FAS) expression were reduced, which are well-established markers of lipogenesis (Fig. 5B). Interestingly, there were no significant differences in relative SREBP2 mRNA levels between the two groups (Supp. Fig. 1D, NS, non-
significant). In line with these observations, qRT-PCR analysis also showed a decrease in lipogenesis accompanied by marked increase in FA oxidation and lipolysis (Fig. 5C-F, *, \( p<0.05 \); NS, non-significant). Renal triglyceride content in the cortex was also significantly higher in \( \text{Pcsk9}^{-/-} \) mice relative to controls (Fig. 5G, *, \( p<0.05 \)). As expected, \( \text{Pcsk9}^{-/-} \) mice exhibited undetectable circulating PCSK9 levels compared to their respective controls (Fig. 5H, *, \( p<0.05 \)). Similar to our in vitro findings, these data suggest that PCSK9 deficiency increases renal CD36 expression and drives renal lipid levels in vivo.

**Pcsk9\(^{-/-}\) mice exhibit increased renal ER stress, fibrosis, and inflammation on a HFD**

To further investigate the role of PCSK9 on renal lipid accumulation in vivo, 6-week old male \( \text{Pcsk9}^{-/-} \) mice and age-matched C57BL/6J controls were fed either NCD or HFD for 12 weeks prior to sacrifice. IHC analysis revealed an increase in the expression of the ER stress marker phosphorylated-PERK (p-PERK) in the kidneys of HFD-fed \( \text{Pcsk9}^{-/-} \) mice compared to HFD-fed controls (Fig. 6A-B, *, \( p<0.05 \)). CD36 and perilipin expression were induced by HFD and elevated in the \( \text{Pcsk9}^{-/-} \) mice fed both NCD and HFD (Fig. 6A, C-D, *, \( p<0.05 \)). As well, we observed an increase in the expression of pro-fibrotic marker, \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), (Fig. 6A, E, *, \( p<0.05 \)) in \( \text{Pcsk9}^{-/-} \) mice on HFD relative to its controls. Along with these observations, qRT-PCR also revealed an increase in mRNA abundance of ER stress, inflammatory, apoptotic, and fibrotic markers in \( \text{Pcsk9}^{-/-} \) compared to controls (Fig 6F, *, \( p<0.05 \)). In support of these findings, renal triglyceride content was significantly higher in \( \text{Pcsk9}^{-/-} \) mice on both NCD and HFD (Fig 6G, *, \( p<0.05 \)). To assess renal function in these mice, we observed that \( \text{Pcsk9}^{-/-} \) mice on HFD exhibited significantly increased serum Cystatin C levels relative to WT controls (Fig. 6H, *, \( p<0.05 \)). Lastly, Thioflavin-S\(^{35} \) fluorescence was increased in the renal cortex of HFD-fed \( \text{Pcsk9}^{-/-} \) mice relative to HFD-fed controls (Supp. Fig 1E). Altogether, these data suggest that PCSK9 is able to attenuate HFD-induced ER stress by modulating lipid uptake within the cortex of the kidney.

**Evolocumab increases surface LDLR, but reduces surface CD36 in both cultured cells and mice**

Evolocumab is a commercially-available fully human mAb against PCSK9 that prevents the interaction between circulating PCSK9 and the LDLR\(^{60} \). Unlike genetic ablation of PCSK9, these anti-PCSK9 antibodies do not impact tissue expression or secretion of PCSK9. Importantly, it is not known whether binding of these anti-PCSK9 antibodies would also block PCSK9 from binding to CD36 since the epitope in PCSK9 that interacts with CD36 is not fully elucidated. To explore the outcome of this important question, cultured HK-2 cells were treated with evolocumab media swapped with Huh7-incubated media. Immunofluorescence microscopy and subsequent fluorescence quantification revealed that evolocumab was able to significantly reduce surface CD36 fluorescence, while upregulating surface LDLR levels (Figure 7A-C, *, \( p<0.05 \)). Consistent with previous reports, ELISA data demonstrate that evolocumab significantly increased circulating PCSK9 levels in mice, since circulating PCSK9 is no longer cleared by the LDLR (Fig. 7D, *, \( p<0.05 \)). Consequently, immunofluorescent microscopy of evolocumab-treated mice displayed reduced basolateral surface CD36 with a concurrent increased fluorescence localized in the tubules, compared to vehicle-treated mice (Fig. 7E), suggesting an internalization of CD36. In contrast, there was no discernable change in the fluorescence staining of total surface receptor expression of ATP1A1 (Supp. Figure 3D). Additionally, the administration of evolocumab was confirmed and detected in the renal cortex using a
fluorescently-labelled anti-human antibody (Supp. Figure 3E). Lastly, to confirm the established effects of evolocumab administration, we observed that evolocumab (30 mg/kg) upregulated surface hepatic LDLR (Supp. Fig 3A-B, *, p<0.05). Altogether, these findings suggest that the circulating evolocumab/PCSK9 complex increases circulating levels of PCSK9 (likely bound to the antibody) and does not alter the ability of PCSK9 to bind to cell surface CD36, unlike its neutralizing effect against the LDLR.

Evolocumab protects against HFD-induced renal stress by modulating surface expression of CD36 on renal epithelia

To assess the effects of evolocumab on diet-induced renal injury via CD36, male C57BL/6J mice (n=10) were placed on HFD and treated with evolocumab (30 mg/kg) once weekly. IHC analysis demonstrated a significant reduction in HFD-induced surface and total staining of CD36 on renal epithelia (Fig. 8A-B, *, p<0.05). Interestingly, administration of evolocumab reduced renal triglyceride levels, but not cholesterol (Fig. 8C-D, *, p<0.05; NS, non-significant). To confirm these findings, evolocumab treatment also reduced HFD-induced CD36 protein levels, while upregulating surface LDLR levels (Fig. 8E, *, p<0.05). At the mRNA level, qRT-PCR demonstrated that markers of ER stress, inflammation, apoptosis, and fibrosis were significantly reduced following the administration of evolocumab compared to non-treated controls placed on HFD (Fig. 8F, *, p<0.05). Consistent with these observations, qRT-PCR analysis demonstrated that evolocumab failed to reduce similar markers of ER stress, inflammation, and fibrosis induced by palmitate in HK-2 cells deficient in CD36 via siRNA (Supp. Fig. 2A-C, *, p<0.05; NS, non-significant). However, the administration of evolocumab failed to reduce serum Cystatin C levels of mice on HFD (Fig. 8G, *, NS, non-significant). Lastly, a visual summary has been supplemented, which compares the difference in mechanism of HFD-induced renal lipotoxicity in both Pcsk9⁻/⁻ mice and WT mice treated with evolocumab (Supp. Fig. 4A-C). Altogether, unlike the HFD studies using Pcsk9⁻/⁻ mice, our findings suggest that evolocumab protects against HFD-induced renal injury by enhancing the degradation of cell surface CD36 while increasing circulating PCSK9 levels independent of its binding to PCSK9.

Discussion:

The lipid nephrotoxicity hypothesis, first postulated by Moorhead and colleagues in 1982, suggests that dyslipidemia promotes CKD progression by inducing oxidative, inflammatory, and ER stress. Since this report, several studies have bridged the gap between renal lipid accumulation and kidney disease using a variety of in vitro and in vivo models, as well as clinical observations. Herein, we report that Pcsk9⁻/⁻ mice exhibit increased expression of renal tubular CD36 and LDLR, which was associated with increased renal lipid accumulation. Consistent with our observations, PCSK9 is known to interact with surface receptors expressed on renal epithelia, although not to the same extent as the liver. Previous studies have highlighted the pathological role of CD36 in promoting intra-renal oxidative and ER stress by driving the uptake of modified lipoproteins and FFAs. Given that HFD is reported to induce renal ER stress, inflammation, and fibrosis, we sought to investigate the effect of a HFD challenge in the context of elevated renal CD36 expression occurring as a result of PCSK9 deficiency. Throughout this study, we have observed that PCSK9 may have a protective effect against HFD-induced renal stress, inflammation, fibrosis, and apoptosis by modulating the levels of receptors known to promote lipid uptake from the circulation.
PCSK9 is primarily expressed in the liver, the small intestine and the kidney.\textsuperscript{46} Despite its presence in the kidney, little is currently known about its potential role in kidney function and renal disease. Preliminary studies demonstrate that circulating PCSK9 is significantly elevated in multiple \textit{in vivo} models of renal pathology.\textsuperscript{47,48} Recent studies have also shown a connection between circulating PCSK9 and its role in nephrotic syndrome.\textsuperscript{37} The primary pathology associated with nephrotic syndrome is damage to the glomeruli and podocytes. Patients with this disease are also at an increased risk of atherosclerosis and thromboembolism due to a well-established dyslipidemia observed in most patients with CKD.\textsuperscript{49} Haas et al. demonstrated that podocyte damage-induced nephrotic syndrome increases plasma PCSK9 levels and leads to dyslipidemia that was markedly attenuated in PCSK9 knockout mice.\textsuperscript{37} The up-regulation of circulating PCSK9 in multiple pathophysiological conditions is also consistent with our previous findings that diet-induced hepatic steatosis increases plasma PCSK9 concentrations.\textsuperscript{48} In other studies, Zhang et al. further strengthened the connection between PCSK9 and lipid-induced renal injury, reporting that inflammation-induced lipid deposition in the kidney was induced by primarily downregulating renal PCSK9 levels.\textsuperscript{50} However, as PCSK9 binds to circulating LDLc, the causal association of increased PCSK9 and dyslipidemia in renal disease remains unclear.

Accumulating evidence suggest that inhibition of PCSK9 has a protective effect against dyslipidemia in patients with renal disease.\textsuperscript{51–53} As circulating PCSK9 concentrations positively correlate with plasma cholesterol levels, these important findings highlight the role of PCSK9 as a driver of dyslipidemia in patients with CKD.\textsuperscript{54} In our studies, we demonstrate for the first time that the genetic ablation of PCSK9 in mice promotes renal lipid deposition, likely occurring as a result of increased expression in receptors known to endocytose lipids from the circulation, such as CD36 and the LDLR. Accordingly, PCSK9 deficiency further exacerbated HFD-induced renal ER stress, inflammation, and fibrosis. As well, we have previously reported that these \textit{Pcsk9}\textsuperscript{-/-} mice also display increased insulin resistance and circulating glucose levels in HFD conditions.\textsuperscript{44} These observations, therefore, suggest that PCSK9 may play an important role in regulating cholesterol and triglyceride levels in circulation and in target tissues via CD36. As such, it is crucial that future studies further investigate the influence of circulating PCSK9 on diet-induced renal injury by placing \textit{CD36}\textsuperscript{-/-} mice on HFD and assessing their relative renal function. Additionally, it is worth considering in later studies whether the paracrine or autocrine effects of secreted PCSK9 by renal cells may contribute to this homeostatic effect. Although our findings are consistent with several others regarding the pathogenic role of renal CD36\textsuperscript{11–13, 24-26}, Garbacz et al. reported that hepatic overexpression of CD36 was able to surprisingly attenuate HFD-induced hepatic steatosis and insulin resistance.\textsuperscript{55} These findings warrant further investigation, as the expression of major genes involved in lipogenesis and fatty acid oxidation in their study was not significantly different between HFD-fed transgenic CD36 mice compared to WT controls.

Clinically, FDA-approved mAbs against PCSK9, such as alirocumab and evolocumab, bind to the \textit{EGF}\textalpha-binding domain of PCSK9 in order to prevent the interaction of PCSK9 with the LDLR.\textsuperscript{36} Consequently, these mAbs increase circulating PCSK9 levels 7-fold as a result of the reduced rate of LDLR-mediated clearance of PCSK9 from the circulation.\textsuperscript{56} Additionally, familial hypercholesterolemic (FH) patients with LOF mutations in the LDLR also exhibit elevated levels of circulating PCSK9\textsuperscript{61}. As such, our findings suggest that using \textit{Pcsk9}\textsuperscript{-/-} mice, which are devoid of circulating PCSK9 levels, lower LDLc via a fundamentally different
mechanism than evolocumab. As a result, we investigated whether the compensatory increase in circulating PCSK9 by treating mice with evolocumab could enhance the degradation of renal CD36 and protect against lipid-induced renal injury. Additionally, the epitope binding domain in which PCSK9 interacts with CD36 currently remains unclear and may potentially differ from the LDLR. As such, we now report for the first time that evolocumab is able to reduce surface expression of CD36 on the renal epithelia. These data strongly suggest that (1) despite the ability of this circulating evolocumab/PCSK9 complex to mitigate the interaction of PCSK9 with the LDLR, it does not affect its binding to cell surface CD36, and (2) the binding domains in which PCSK9 uses to interact and degrade both the LDLR and CD36 are distinct. Thus, a reduction in surface CD36 expression on the basolateral epithelia, as a result of exposure to the circulating evolocumab-PCSK9 complex, protects against diet-induced renal ER stress, inflammation, fibrosis, and apoptosis. Hence, the pathophysiological changes that we observed in \( Pcsk9^{-/-} \) mice challenged on a HFD do not accurately represent the additional clinical benefit when compared to PCSK9 mAb therapy. We have supplemented a visual diagram comparing both HFD models to clarify our findings reported from this study.

Since the successful introduction of PCSK9 mAb therapy to patients, there has been a rapid development focused on generating new approaches at reducing circulating PCSK9 levels. One such approach is inclisiran, an siRNA targeted against PCSK9, which has recently undergone Phase III clinical trials. Although the siRNA approach has demonstrated great efficacy in reducing circulating LDLc, its potential effects on increasing surface CD36 levels on hepatocytes has not been investigated. This is akin to our findings when comparing the \( Pcsk9^{-/-} \) mice and evolocumab-treated mice on HFD; although both approaches may significantly lower LDLc levels, the specific mechanism in which it lowers LDLc may display significantly different off-target effects due to the ability of PCSK9 to modulate a wide range of receptors that play a role beyond LDLc regulation. Herein, our data provides a novel, yet crucial insight suggesting that the administration of PCSK9 mAbs may have additional clinical benefits in alleviating diet-induced renal stress and injury.

Overall, we report for the first time that PCSK9 has a direct effect on renal function beyond hepatic lipid homeostasis by modulating the LDLR and CD36 on renal epithelia. Given this effect, we observed that in turn, PCSK9 is able to attenuate CD36-driven ER stress, inflammation, and fibrosis in the context of diet-induced renal disease. Data demonstrating the extent to which the observed renal injury in \( Pcsk9^{-/-} \) occurs as a result of excess diet-induced lipid uptake represents a limitation of this study. Recently, we have identified a mechanism in which the loss-of-function PCSK9\(^{Q152H} \) variant acts as a co-chaperone within liver hepatocytes, as well as in kidney tubules (Byun and Austin, unpublished data), by stabilizing the protein levels of several critical ER chaperones, including GRP78 and GRP94. This raises the question as to whether the observed increased ER stress in HFD-fed \( Pcsk9^{-/-} \) may have occurred as a result of (1) increased lipid uptake, and (2) increased sensitivity to ER stress due to a lack of ER stress-response chaperones expressed in the kidney. As well, our HFD mouse model may portray significantly different lipid metabolic processes in comparison to other models such as in \( CD36^{-/-} \), \( Lpl^{-/-} \), and \( MTTP^{-/-} \) mice. To further clarify these findings, future studies will focus on determining whether intracellular or extracellular expression of PCSK9 act to mitigate ER stress in CKD, and to what extent the clinically-approved mAbs against PCSK9 protects against renal stress through cell surface CD36 degradation.
Disclosures
R. Austin reports the following: Scientific Advisor or Membership: Journal of Biological Chemistry. The remaining authors have nothing to disclose.

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Author Contributions
Jae Hyun Byun: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Supervision; Validation; Writing - original draft; Writing - review and editing. Paul Lebeau: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing - review and editing. Khrystyna Platko: Conceptualization; Data curation; Formal analysis; Investigation; Project administration; Writing - review and editing. Rachel Carlisle: Data curation; Formal analysis; Writing - review and editing. Mahi Faiyaz: Data curation; Formal analysis; Writing - review and editing. Jack Chen: Data curation; Writing - review and editing. Melissa MacDonald: Data curation; Formal analysis; Writing - review and editing. Yumna Makda: Formal analysis; Writing - review and editing. Tamana Yousof: Validation; Writing - review and editing. Edward Lynn: Formal analysis; Supervision; Writing - review and editing. Jeffrey Dickhout: Conceptualization; Funding acquisition; Investigation; Supervision; Writing - review and editing. Joan Krepinsky: Formal analysis; Investigation; Supervision; Writing - review and editing. Fiona Weaver: Data curation; Formal analysis. Suleiman Igdoura: Data curation; Formal analysis; Investigation; Writing - review and editing. Nabil Seidah: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Supervision; Writing - review and editing. Richard Austin: Conceptualization; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Supervision; Writing - original draft; Writing - review and editing.

Supplemental Materials
Supplementary Fig. 1
Supplementary Fig. 2
Supplementary Fig. 3
Supplementary Fig. 4
Supplementary Table 1
Supplementary Table 2
References


15. Inagi R, Ishimoto Y, Nangaku M: Proteostasis in endoplasmic reticulum—new


44. Lebeau PF, Byun JH, Platko K, Al-Hashimi AA, Lhoták Š, MacDonald ME, Mejia-


increases ER chaperones GRP78 and GRP94 and protects against liver injury. *J Clin Invest* **131**: 2021


Figure Legends

**Fig. 1** Secreted PCSK9 regulates the LDLR and CD36 in cultured renal cells
(A) Whole cell lysates and media immunoblotted for PCSK9 from HuH7 cells transfected with either PCSK9<sup>WT</sup> or PCSK9<sup>Q152H</sup> expression plasmids. (B) Relative protein expression of CD36 and LDLR in whole cell lysates from HK-2 cells exposed to media harvested from HuH7 cells. (C,D) Immunofluorescence microscopy staining for surface CD36 and LDLR in HK-2 cells post-media swap (*, p<0.05). (E) Relative secreted PCSK9 measured in HuH7 cells transfected with either PCSK9<sup>WT</sup> or PCSK9<sup>Q152H</sup> after 24 h (*, p<0.05). (F) Secreted PCSK9 was also measured in HuH7 and HK-2 cells to confirm that renal cells secrete less PCSK9 relative to hepatocytes in vitro (*, p<0.05; NS, non-significant). (G) mRNA transcript levels of PCSK9, SREBP2, LDLR, and CD36 in HK-2 cells exposed to harvested media from HuH7-transfected cells confirming that modulation of surface receptors on HK-2 cells were through exogenously added PCSK9 from hepatocytes (NS, non-significant). Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using t-tests or ANOVAs. Scale bars, 200 µm.

**Fig. 2** PCSK9 blocks OA- and LDL-induced lipid droplet accumulation in renal cells
(A,B) ORO staining of HK-2 cells exposed to media harvested from HuH7 cells and treated with OA (200 µM) for 18 h in the presence or absence of PCSK9 from media (*, p<0.05). (C,D,E) HK-2 and rat mesangial cells were treated with fluorescently-labelled DiI-LDL cholesterol and quantified (*, p<0.05). Data are represented as the mean and errors are represented as standard deviation. Differences between groups were determined using t-tests or ANOVAs. Scale bars, (A) 10 µm. (C) 200 µm.

**Fig. 3** PCSK9 regulates lipid accumulation in renal-derived cells in a manner dependent on CD36
(A,B) ORO staining of HK-2 cells treated with OA and or SSO (10 µM), a well-established blocker of CD36 activity for 18 h (*, p<0.05). (C,D) The effect of an siRNA targeted against CD36 was also assessed via ORO staining with the treatment of OA in HK-2 cells (*, p<0.05). (E) Immunoblot of CD36 confirming knockdown of its expression using siRNA targeted against CD36 (*, p<0.05). (F,G) Uptake of fluorescently-labeled oxLDL was also measured in HK-2 cells and quantified using a spectrophotometer (*, p<0.05). (H) Relative intracellular triglyceride levels measured in HK-2 cells treated with OA with the modulation of CD36 expression (*, p<0.05). (I) Knockdown of CD36 via siRNA was also confirmed using qRT-PCR (*, p<0.05). Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using t-tests or ANOVAs. Scale bars, 200 µm.

**Fig. 4** PCSK9 protects from palmitate and oxidized LDL-induced ER stress in vitro
(A,B) Immunofluorescence microscopy of HK-2 cells stained for n-ATF6 treated with ox-LDL either in the presence or absence of PCSK9 (*, p<0.05). (C,D,E,F) qRT-PCR analysis of UPR activation markers in HK-2 cells (*, p<0.05). (G) Relative intracellular triglyceride levels measured in HK-2 cells treated with PA in the presence or absence of PCSK9 (*, p<0.05). (H) Quantification of Thioflavin-T fluorescent staining in HK-2 cells treated with PA in media harvested from transfected HuH7 cells (*, p<0.05; NS, non-significant). Data are represented as
the mean and errors as the standard deviation. Differences between groups were determined using *t*-tests or ANOVAs. Scale bars, 200 µm.

**Fig. 5** *Pcsk9−/−* mice exhibit increased renal lipid accumulation on NCD
(A) Sections from the renal cortex in *Pcsk9−/−* mice were staining for ORO via cryosections and immunostained for CD36 and perilipin via paraffin-embedded sections and used to measure relative lipid accumulation in the renal cortex *in vivo*. (B) Immunoblot of lysates from the renal cortex in *Pcsk9−/−* mice and its relative controls for different markers of lipid metabolism (*, *p*<0.05). (C,D,E,F) qRT-PCR analysis of different genes involved in lipid droplet formation, lipogenesis, fatty acid oxidation, and lipolysis (*, *p*<0.05; NS, non-significant). (G) Relative intra-renal triglyceride content assessed in *Pcsk9−/−* mice relative to controls (*, *p*<0.05). (H) Serum PCSK9 levels were measured via ELISA in *Pcsk9−/−* mice compared to controls (*, *p*<0.05). Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using *t*-tests or ANOVAs. Scale bars, 100 µm.

**Fig. 6** *Pcsk9−/−* mice exhibit increased renal ER stress on a high-fat diet
(A-E) IHC analysis and quantification of the renal cortex of *Pcsk9−/−* mice and relevant controls on HFD for p-PERK, CD36, perilipin, and α-SMA (*, *p*<0.05). (F) qRT-PCR analysis of ER stress, inflammatory, apoptotic, and fibrotic markers in the renal cortex of the mice (*, *p*<0.05). (G) Relative intra-renal triglyceride content assessed in *Pcsk9−/−* mice fed either NCD or HFD relative to controls (*, *p*<0.05). (H) Serum Cystatin C levels were measured and assessed in *Pcsk9−/−* mice fed either NCD or HFD relative to controls (*, *p*<0.05). Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using *t*-tests or ANOVAs. Scale bars, 100 µm.

**Fig. 7** Evolocumab increases surface LDLR, but reduces surface CD36 in both cultured cells and in mice
(A-C) Immunofluorescence microscopy and quantifications of HK-2 cells co-stained for LDLR and CD36 treated with evolocumab exposed to media harvested from HuH7 cells (*, *p*<0.05). (D) Relative secreted PCSK9 measured in WT mice treated with either vehicle or evolocumab (*, *p*<0.05). (E) Immunofluorescence microscopy of mice treated with evolocumab stained for CD36 compared to controls. Differences between groups were determined using *t*-tests or ANOVAs. Scale bars, 100 µm (A) and 20 µm (E).

**Fig. 8** Evolocumab protects against HFD-induced renal stress by modulating surface expression of CD36 on renal epithelia
(A,B) IHC analysis and quantification of CD36 in the renal cortex of HFD mice treated with evolocumab, compared with relevant controls (*, *p*<0.05). (C,D) Total renal cholesterol and triglyceride content was measured in the renal cortex of these mice (*, *p*<0.05; NS, non-significant). (E) Immunoblot of CD36 and LDLR protein expression in renal cortex with quantifications (*, *p*<0.05). (F) qRT-PCR analysis of ER stress, fibrotic, inflammatory, and apoptotic markers in renal cortex (*, *p*<0.05). (G) Serum Cystatin C levels measured and assessed in evolocumab-treated mice on HFD relative to NCD controls (NS, non-significant). Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using *t*-tests or ANOVAs. Scale bars, 100 µm.
Figure 1.

Byun & Lebeau et al., 2022

A. HuH7 Transfection

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HuH7 Lysate

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Media Swap

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C. Media Swap from PCSK9-transfected HuH7

PCSK9<sup>WT</sup> vs PCSK9<sup>Q152H</sup>

PCSK9<sup>WT</sup> vs PCSK9<sup>Q152H</sup>

Cell-surface LDLR Staining

Fluorescence Intensity (490/525)

PCSK9<sup>WT</sup> vs PCSK9<sup>Q152H</sup>

Cell-surface CD36 Staining

Fluorescence Intensity (490/525)

PCSK9<sup>WT</sup> vs PCSK9<sup>Q152H</sup>

D. Cell-surface LDLR Staining

Cell-surface CD36 Staining

G. HK-2

E. Relative Secreted PCSK9 (Normalized to Protein)

F. Relative Secreted PCSK9 (Normalized to Protein)

Fold induction of PCSK9 mRNA (Relative to 18s)

Fold induction of LDLR mRNA (Relative to 18s)

Fold induction of CD36 mRNA (Relative to 18s)

Fold induction of SREBP2 mRNA (Relative to 18s)
Figure 2. Byun & Lebeau et al., 2022

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B

Optical Density of ORO Extract (normalized to protein)

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D

HK-2

dii-LDL Fluorescence Units (554/571)

E

Rat Mesangial

dii-LDL Fluorescence Units (554/571)
Figure 3.

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![Graph showing Optical Density of ORO Extract](image)

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D

![Graph showing Optical Density of ORO Extract](image)

E

![Western Blot showing CD36 and β-actin](image)

F

![Images showing DAPI, Dil-oxLDL, and MERGE](image)

G

![Graph showing diI-oxLDL Fluorescence Units](image)

H

![Graph showing Relative Triglyceride Content](image)

I

![Graph showing Fold Induction of CD36 mRNA](image)
Figure 4. Byun & Lebeau et al., 2022

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B

Nuclear-Positive ATF6 Fluorescence

C

Fold Induction of GRP78 mRNA (Relative to 18s)

D

Fold Induction of GRP94 mRNA (Relative to 18s)

G

Relative Triglyceride Content (Normalized to Protein)

E

Fold Induction of IRE1α mRNA (Relative to 18s)

F

Fold Induction of PERK mRNA (Relative to 18s)

H

Thioflavin-T Staining Intensity (% Area)

PCSK9^WT  PCSK9^Q152H
Vehicle  PA  Vehicle  PA  Vehicle  PA  Vehicle  PA

* NS
Figure 5.

A. ORO

B. Western Blot:

<table>
<thead>
<tr>
<th>Protein</th>
<th>PCSK9+/+</th>
<th>PCSK9-/-</th>
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<td>LDLR</td>
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<tr>
<td>β-Actin</td>
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C. Lipid Droplets

D. Lipogenesis

E. Fatty Acid Oxidation

F. Lipolysis

G. Renal Triglyceride Content (mg/g total protein)

H. Relative Secreted Mouse PCSK9
Figure 6.

Byun & Lebeau et al., 2022

<table>
<thead>
<tr>
<th>Control</th>
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<tbody>
<tr>
<td>PCSK9+/+</td>
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<td>P-PERK Staining Intensity</td>
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<td>CD36</td>
<td>CD36</td>
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<td>α-SMA</td>
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**ER Stress**

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<td>PCSK9/-</td>
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<th>E</th>
<th>F</th>
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<td>α-SMA Staining intensity</td>
<td>ER Stress</td>
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**Inflammation**

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<td>Renal Triglyceride Content (mg/g total protein)</td>
<td>Serum Cystatin C Concentration (mg/L)</td>
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**Apoptosis**

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Figure 7.

(A) HK-2

DAPI  LDLR  CD36  MERGE

Media Swap from PCSK9- transfected HuH7

Vehicle  Evolocumab

(B) LDLR Fluorescence Intensity (490/525)

Vehicle  Evolocumab

(C) CD36 Fluorescence Intensity (588/525)

Vehicle  Evolocumab

(D) Circulating PCSK9 Concentration (ng/mL)

Vehicle  Evolocumab

p=7.47E-8  5.73 Fold

(E) CD36

Vehicle  Evolocumab

Byun & Lebeau et al., 2022
Figure 8. Byun & Lebeau et al., 2022

A

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B

CD36 Staining Intensity (%Area)

C

Renal Cholesterol Content (mg/g total protein)

D

Renal Triglyceride Content (mg/g total protein)

E

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F

ER Stress

- Fold Induction of ATF6 mRNA (Relative to 18s)
- Fold Induction of IRE1α mRNA (Relative to 18s)
- Fold Induction of PERK mRNA (Relative to 18s)

Inflammation

- Fold Induction of TGFβ mRNA (Relative to 18s)
- Fold Induction of TNFα mRNA (Relative to 18s)
- Fold Induction of IL1β mRNA (Relative to 18s)
- Fold Induction of CASP1 mRNA (Relative to 18s)

Apoptosis

- Fold Induction of BAX mRNA (Relative to 18s)
- Fold Induction of BCL2 mRNA (Relative to 18s)
- Fold Induction of BCL2L1 mRNA (Relative to 18s)

G

Serum Cystatin C Concentration (mg/L)

ER Stress

- ![Graph](image10.png)
- ![Graph](image11.png)
- ![Graph](image12.png)

Inflammation

- ![Graph](image13.png)
- ![Graph](image14.png)
- ![Graph](image15.png)

Apoptosis

- ![Graph](image16.png)
- ![Graph](image17.png)
- ![Graph](image18.png)

Fibrosis

- ![Graph](image19.png)
- ![Graph](image20.png)
- ![Graph](image21.png)
Supplemental Figure Legends

Supplementary Fig. 1
(A,B) ORO staining and quantification of HK-2 cells exposed to media harvested from HuH7 cells and treated with OA (200 µM) for 18 hours pre-treated with either vehicle or anti-V5 antibodies (*, \( p<0.05 \)). Data are represented as the mean and errors as the standard deviation. (C) Immunoblot of LDLR and CD36 in HK-2 cells treated with either media from WT-PCSK9 overexpressed HuH7 cells or rhPCSK9 (*, \( p<0.05 \)). (D) Transcript levels of SREBP2 in Pcsk9\(^{-/-}\) mice compared to Pcsk9\(^{+/+}\) controls (NS, non-significant). (E) Thioflavin-S staining in the renal cortex of Pcsk9\(^{-/-}\) mice on HFD compared to relevant controls. Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using \( t \)-tests or ANOVAs. Scale bars, 100 µm.

Supplementary Fig. 2
(A,B,C) qRT-PCR analysis of ER stress, inflammatory, and fibrotic markers in HK-2 cells treated with PA either in the presence or partial knockdown of CD36 via siRNA (*, \( p<0.05 \); NS, non-significant). Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using \( t \)-tests or ANOVAs.

Supplementary Fig. 3
(A,B) IHC analysis and quantification of LDLR in the livers of mice treated with evolocumab, compared with vehicle controls (*, \( p<0.05 \)). (C) qRT-PCR analysis of ER stress markers in HK-2 cells treated with palmitoleic acid (PoA) for 18h overnight (NS, non-significant). (D) Immunofluorescence microscopy of ATP1A1 in mice treated with evolocumab compared to controls. (E) Immunofluorescence microscopy co-stained for CD36 and anti-human antibody in mice treated with evolocumab compared to controls. Data are represented as the mean and errors as the standard deviation. Differences between groups were determined using \( t \)-tests or ANOVAs. Scale bars, 100 µm (A) and 200 µm (D-E).

Supplementary Fig. 4
Visual summary outlining the underlying mechanisms of HFD-induced renal lipotoxicity reported within this study. (A) PCSK9 mAbs successfully block the interaction of PCSK9 to the LDLR, but fail to do so with CD36. (B) Pcsk9\(^{-/-}\) mice exhibit increased levels of basolateral surface CD36, thereby enhancing HFD-induced renal lipid accumulation and lipotoxicity. (C) PCSK9 mAbs fail to block the interaction of PCSK9 to CD36, thereby enhancing its degradation and reducing FFA uptake to protect against HFD-induced lipotoxicity. Created with BioRender.com.
Supplementary Figure 1.

A

<table>
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<th>IgG</th>
<th>Anti-V5 ab</th>
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<tr>
<td>HuH7 media-swap - PCSK9&lt;sup&gt;O152H&lt;/sup&gt;</td>
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<tr>
<td>Vehicle</td>
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<tr>
<td>OA</td>
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</tbody>
</table>

B

Optical Density of ORO Extract (normalized to protein)

- IgG + + + + -
- OA - + + + +
- V5-PCSK9 - - + + +
- anti-V5 ab - - - + +

C

Vehicle WT-PCSK9 M.S.

<table>
<thead>
<tr>
<th></th>
<th>LDLR</th>
<th>CD36</th>
<th>β-Actin</th>
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<td>180-</td>
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<tr>
<td>48-</td>
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<td>-</td>
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</table>

D

Fold Induction of SREBP2 mRNA (Relative to 18s)

- PC<sup>+/+</sup> PCSK<sup>-/-</sup>

E

Thioflavin-S

- NCD
- HFD

Byun & Lebeau et al., 2022
A  ER Stress

Folds Induction of GRP78 mRNA
(Relative to 18s)

Vehicle  PA  PA+Evo  Vehicle  PA+Evo

siScrambled  siCD36

B  Inflammation

Folds Induction of IL6 mRNA
(Relative to 18s)

Vehicle  PA  PA+Evo  Vehicle  PA+Evo

siScrambled  siCD36

C  Fibrosis

Folds Induction of FBN mRNA
(Relative to 18s)

Vehicle  PA  PA+Evo  Vehicle  PA+Evo

siScrambled  siCD36

**  *  NS

Byun & Lebeau et al., 2022

Supplementary Figure 2.
Supplementary Figure 4.

Byun & Lebeau et al., 2022
Table 1: Antibodies used for immunoblotting and immunohistochemical analysis

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<th>Application</th>
<th>Antibody Dilution</th>
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<td>Sigma-Aldrich</td>
<td>Immunoblot</td>
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<tr>
<td>PCSK9</td>
<td>NB300-959, Novus Biologics</td>
<td>Immunoblot</td>
<td>1:500</td>
</tr>
<tr>
<td>CD36</td>
<td>NB400-144, Novus Biologics</td>
<td>IHC, Immunoblot</td>
<td>1:500, no retrieval/1:1000</td>
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<td>V5</td>
<td>Sc-271944, Santa Cruz Biotechnology</td>
<td>Immunoblot</td>
<td>1:1000</td>
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<td>70B1413.1, Novus Biologics</td>
<td>Immunoblot</td>
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<td>LDLR</td>
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<td>Immunoblot</td>
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<td>PPARα</td>
<td>Sc-398394, Santa Cruz Biotechnology</td>
<td>Immunoblot</td>
<td>1:1000</td>
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<td>SREBP1</td>
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Table 2: Primers used for quantitative real time PCR

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