The Role of Proprotein Convertase Subtilisin/Kexin Type 9 in Nephrotic Syndrome-Associated Hypercholesterolemia

BACKGROUND: In nephrotic syndrome, damage to the podocytes of the kidney produces severe hypercholesterolemia for which novel treatments are urgently needed. PCSK9 (proprotein convertase subtilisin/kexin type 9) has emerged as an important regulator of plasma cholesterol levels and therapeutic target. Here, we tested the role of PCSK9 in mediating the hypercholesterolemia of nephrotic syndrome.

METHODS: PCSK9 and plasma lipids were studied in nephrotic syndrome patients before and after remission of disease, mice with genetic ablation of the podocyte (Podocyte Apoptosis Through Targeted Activation of Caspase-8, Pod-ATTAC mice) and mice treated with nephrotoxic serum (NTS), which triggers immune-mediated podocyte damage. In addition, mice with hepatic deletion of Pcsk9 were treated with NTS to determine the contribution of PCSK9 to the dyslipidemia of nephrotic syndrome.

RESULTS: Patients with nephrotic syndrome showed a decrease in plasma cholesterol and plasma PCSK9 on remission of their disease (P<0.05, n=47–50). Conversely, Pod-ATTAC mice and NTS-treated mice showed hypercholesterolemia and a 7- to 24-fold induction in plasma PCSK9. The induction of plasma PCSK9 appeared to be attributable to increased secretion of PCSK9 from the hepatocyte coupled with decreased clearance. Interestingly, knockout of Pcsk9 ameliorated the effects of NTS on plasma lipids. Thus, in the presence of NTS, mice lacking hepatic Pcsk9 showed a 40% to 50% decrease in plasma cholesterol and triglycerides. Moreover, the ability of NTS treatment to increase the percentage of low-density lipoprotein–associated cholesterol (from 9% in vehicle-treated Flox mice to 47% after NTS treatment), was lost in mice with hepatic deletion of Pcsk9 (5% in both the presence and absence of NTS).

CONCLUSIONS: Podocyte damage triggers marked inductions in plasma PCSK9, and knockout of Pcsk9 ameliorates dyslipidemia in a mouse model of nephrotic syndrome. These data suggest that PCSK9 inhibitors may be beneficial in patients with nephrotic syndrome–associated hypercholesterolemia.
Clinical Perspective

What Is New?

- Nephrotic syndrome increases plasma PCSK9 (proprotein convertase subtilisin/kexin type 9) up to 24-fold in mice.
- Pcsk9 ablation reduces plasma triglycerides and cholesterol by 40% to 60% in mice with nephrotic syndrome.
- Pcsk9 ablation abolishes the dramatic increase in the ratio of low-density lipoprotein to high-density lipoprotein cholesterol observed with the induction of nephrotic syndrome.

What Are the Clinical Implications?

- These studies suggest that PCSK9 inhibitors may be useful in treating nephrotic syndrome–associated hypercholesterolemia.

The association between podocyte dysfunction and hypercholesterolemia can be traced to 1830, when it was recognized that the plasma of nephrotic patients was so hyperlipidemic that it appeared milky.1 However, even today, definitive treatments for nephrotic syndrome–associated hypercholesterolemia are lacking and nephrotic patients experience a 6-fold increased risk of cardiovascular disease.3

The hyperlipidemia of nephrotic syndrome is complex, involving multiple mechanisms and tissues, and evolves over the course of the disease. The increase in cholesterol is caused by both the increased production and decreased clearance of lipoprotein particles, particularly the ApoB-containing particles (very low-density lipoprotein [VLDL], intermediate-density lipoprotein [IDL], and low-density lipoprotein [LDL]).4–9 Hepatic LDL receptor, which removes ApoB-containing particles from the plasma, is reduced at the protein, but not the mRNA, level.10–12

Proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged over the past decade as a posttranscriptional regulator of the LDL receptor, and several studies have suggested an association between PCSK9 and renal function.12–17 Circulating PCSK9 binds LDL receptors on the surface of the hepatocyte, causing the receptor to be internalized and degraded in the lysosome. Accordingly, patients with gain-of-function mutations in PCSK9 have increased LDL cholesterol, patients with loss-of-function mutations have decreased LDL cholesterol,18 and PCSK9 inhibitors are effective in reducing LDL cholesterol.19 The goal of this study was to determine the contribution of PCSK9 to nephrotic syndrome–associated hypercholesterolemia. We found that mice in the acute phase of nephrotic syndrome showed an increase in both plasma PCSK9 and plasma cholesterol, in particular, ApoB-associated cholesterol; moreover, knockout of Pcsk9 lowered plasma cholesterol and produced a more benign lipoprotein profile.

METHODS

Additional information can be found in the Methods in the online-only Data Supplement.

Human Samples

We identified 50 patients who were enrolled in NEPTUNE (Nephrotic Syndrome Study Network)20 as of October 2013 and had at least 1 plasma sample from the initial study visit during active disease (defined as urine protein/creatinine ratio ≥1) as well as from the first follow-up study visit during which remission was achieved (defined as urine protein/creatinine ratio <0.5). Thirty-eight percent of the patients were on immunosuppression therapy at baseline. In addition, 24% of patients were on medications potentially affecting plasma PCSK9 levels, such as statin drugs, at some point in the study; however, exclusion of these patients did not alter our results (see Methods in the online-only Data Supplement for additional information). Plasma PCSK9 concentrations were measured via enzyme-linked immunosorbent assay (CycLex CY-8079).

Mice

C57BL/6J mice were obtained from the Jackson Laboratory. Pcsk9lox/lox mice on a C57BL/6N background, which contain loxP sites in the introns between exons 1 and 2, and exons 3 and 4, were crossed to albumin-Cre on a C57BL/6N background to generate mice with liver-specific knockout of Pcsk9. Podocyte Apoptosis Through Targeted Activation of Caspase-8 (Pod-ATTAC) mice have been previously described.21 Pcsk9+/− mice and their approximate B6129SF2/J controls were also purchased from the Jackson Laboratory and intercrossed for at least 4 generations.

Nephrotic Models

Nephrotoxic serum was used as previously described.22 Five-to 8-week-old male mice were preimmunized with 100 μL of 1:1 sheep IgG:Complete Freund’s Adjuvant subcutaneously, then injected with either 50 μL of nephrotoxic serum (NTS) or normal sheep serum (vehicle) retro-orbitally daily for 3 days. Mice were euthanized in the nonfasted state 3 to 4 days after the first NTS or vehicle injection. Pod-ATTAC mice and their controls were injected with 0.5 μg/g body weight of AP20187 (MedChemExpress or Clontech), and euthanized 7 days after injection in the nonfasted state.

Plasma Lipid Analyses

Total plasma triglycerides and cholesterol were measured via colorimetric assays (Infinity Triglycerides and Cholesterol, Thermo). Size exclusion liquid chromatography was performed by the Lipid, Lipoprotein and Atherosclerosis Analysis Core Laboratory at Wake Forest University. In brief, equal volumes of plasma that were collected at the time of euthanization from 4 to 9 mice per group were pooled. The cholesterol content of the pooled plasma was measured and =15 μg of total cholesterol was diluted to a final volume of 120 μL by using phosphate-buffered saline solution containing 0.01% EDTA and 0.01% sodium azide, and loaded
onto a Superose 6 10/300 GL column powered by a LaChrom Elite HPLC system (Hitachi High Technologies) with online mixing of the column effluent and cholesterol enzymatic reagent (Cholesterol Liquid Reagent Set, Pointe Scientific, Inc). The relative cholesterol concentration, measured as the OD_{420nm}, and converted to an electric signal (response, measured in millivolts), was continuously monitored; raw traces of the response are shown in the online-only Data Supplement. The area corresponding to each lipoprotein (VLDL, LDL, high-density lipoprotein [HDL], and little HDL) was measured and used to calculate the percentage of the total cholesterol contained in each lipoprotein fraction. In the main text, the data are presented as pie charts, with HDL representing the sum of HDL and little HDL. Little HDL made up 3%, 1%, 2%, and 4% of cholesterol in plasma from vehicle-treated B6 mice, vehicle-treated wild-type (WT) mice, vehicle-treated PCSK9-KO mice, and vehicle-treated Flos mice, respectively, and was not present in other samples.

**Urine Analysis**

Spot urine was collected the morning of euthanization, and 2 μL was subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis and Coomassie staining.

**PCSK9 Clearance Assay**

Pod-ATTAC and control mice were injected retro-orbitally with 0.4 μg pf human recombinant PCSK9 7 days after AP20187 injection. Plasma samples were collected 1 to 121 minutes after PCSK9 injection. For each mouse, plasma concentrations of human recombinant PCSK9 were expressed as percentage of the t=1 minute concentration, and were plotted against time. The data from each mouse were fitted to a 1-phase exponential decay curve using GraphPad Prism, such that N(t)=N₀ – Plateaue^{-λt} + Plateau, and the half-life (t₁/₂) was calculated by the formula t₁/₂ = ln(2)/λ.

**Gene Expression**

Gene expression was measured by real-time polymerase chain reaction and normalized to Tbp.

**Protein Analysis**

Protein levels in liver and plasma were measured by western blotting or enzyme-linked immunosorbent assay. The Sec24a antibody used for Western blotting in this study was from Dr David Ginsburg23; all other antibodies were purchased from commercial sources. Mouse (CycLex CY-8078) and recombinant human PCSK9 (R&D Systems DPC900) concentrations in plasma were measured via enzyme-linked immunosorbent assay.

**Statistical Analysis**

**Human Studies**

Paired 2-tailed t tests and Wilcoxon signed rank tests were used to assess changes from baseline to remission. Spearman correlations were used to assess correlations at baseline and correlations of changes from baseline to remission. All tests were performed at 2-sided α-level of 0.05. IBM SPSS (version 21.0) software was used for all analyses. Data in the text are presented as mean±standard deviation.

**Mouse Studies**

Differences between groups were assessed by a 2-tailed Mann-Whitney test at a significance level of P<0.05; similar results were obtained by using a 2-tailed unequal variance Student t test at a significance level of P<0.05. No adjustment was made for multiple comparisons. These tests were performed using either Microsoft Excel 2010 or GraphPad Prism 6. Data are presented as mean and standard error of the mean in text and graphs (bars and error bars, respectively).

**Study Approval**

The human studies protocol was approved by the institutional review board at each patient recruiting site and at Boston Children’s Hospital; all subjects gave informed consent. All mouse studies were approved by the Institutional Animal Care and Research Advisory Committee at Boston Children’s Hospital.

**RESULTS**

We measured plasma PCSK9 concentrations in 50 patients (30% membranous nephropathy, 28% minimal change disease, 10% focal segmental glomerulosclerosis, and 11% other, online-only Data Supplement Table I) enrolled in the Nephrotic Syndrome Study Network (NEPTUNE) at 2 time points: during active disease (defined as urine protein/creatinine ratio >1 mg/mg) and during remission (urine protein/creatinine ratio <0.5 mg/mg). At baseline, patients were proteinuric, hypertriglyceridemic, and hypercholesterolemic. The urine protein/creatinine ratio was significantly correlated with total cholesterol (r=0.496), LDL cholesterol (r=0.398), and HDL cholesterol (r=0.321, Tables 1 and 2).

On remission, hyperlipidemia resolved (Table 1). In parallel, plasma PCSK9 decreased significantly from 348.0±139.5 ng/mL at baseline to 300.5±130.3 ng/mL at remission (P=0.04, Figure 1). The change in PCSK9 was correlated with the changes in total, LDL, and HDL cholesterol, although the correlation with LDL cholesterol did not quite reach significance (P=0.051, Table 2). Subgroup analysis revealed that minimal change

**Table 1. Clinical Variables at Baseline and Remission**

<table>
<thead>
<tr>
<th>Clinical Variables</th>
<th>Baseline</th>
<th>Remission</th>
<th>P Value ⤪</th>
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<tbody>
<tr>
<td>Urine protein/creatinine ratio, mg/mg</td>
<td>5.2 (4.9)</td>
<td>0.14 (0.10)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>298.0 (107.8)</td>
<td>197.5 (60.2)</td>
<td>&lt;0.0001</td>
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<td>LDL cholesterol, mg/dL</td>
<td>178.2 (91.1)</td>
<td>101.0 (44.7)</td>
<td>&lt;0.0001</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
<td>81.6 (28.1)</td>
<td>69.2 (25.2)</td>
<td>0.002</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>191.1 (109.1)</td>
<td>136.2 (73.3)</td>
<td>0.001</td>
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n=47 to 50. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and PCSK9, proprotein convertase subtilisin/kexin type 9.

*Data represent means (standard deviation).

†Paired t tests.
Podocyte injury can be induced in mice by treatment with nephrotoxic serum (NTS), prepared from sheep immunized with rodent glomeruli. Injection of NTS into preimmunized mice produces a complex immunologic attack on the mouse glomerulus. During the acute phase, NTS antibodies bind to the podocyte cell membrane, resulting in podocyte injury and severe proteinuria that persist as the lesion progresses to glomerular inflammation and crescent formation after 2 to 3 weeks. Changes in cholesterol metabolism also evolve during the course of nephrotic disease; in this model, maximal hypercholesterolemia was observed 4 days after NTS injection (online-only Data Supplement Figure IA).

We therefore injected wild-type C57BL/6J mice (hereafter referred to as B6 mice) with NTS or vehicle and euthanized them 4 days after the initial NTS injection. At this time, B6 mice were markedly proteinuric and dyslipidemic (Figure 2A through 2D). Plasma triglycerides were increased 5-fold and plasma cholesterol levels were increased 4-fold. Moreover, fractionation of the plasma by size exclusion chromatography revealed marked changes in the distribution of cholesterol among the different lipoprotein fractions. That is, although the absolute amount of VLDL, LDL, and HDL cholesterol all increased, there was a disproportionate increase in LDL cholesterol, from 12% to 53% of total plasma cholesterol (Figure 2D; online-only Data Supplement Figure IB). Consequently, the proportion of cholesterol associated with the ApoB-containing lipoproteins increased from 14% to 55%.

Consistent with previous studies, hyperlipidemia in NTS-treated B6 mice was associated with a reduction in LDL receptor protein (Figure 2E). The degradation of the LDL receptor is regulated by both the E3 ubiquitin ligase Idol (inducible degrader of the LDL receptor) and PCSK9. Interestingly, both Ldr and Idol mRNA levels were decreased in the livers of NTS-treated mice (Figure 2F).

PCSK9, on the other hand, was increased 16-fold in the plasma of NTS-treated mice (Figure 2G). Plasma PCSK9 is derived primarily from the liver. Although hepatic Pcsk9 mRNA levels were increased (Figure 2H), the increase was modest, ≈50%, suggesting that increased Pcsk9 transcription was unlikely to be the sole cause of elevated plasma PCSK9 levels. We therefore measured PCSK9 clearance in NTS-treated B6 mice by measuring the half-life of recombinant human PCSK9. The half-life of PCSK9 was increased by NTS treatment, but only 2-fold (P=0.009, online-only Data Supplement Figure IC). The modest changes in Pcsk9 mRNA and PCSK9 clearance in comparison with the large change in plasma PCSK9 suggested that PCSK9 secretion might also be increased and that posttranscriptional regulation of Pcsk9 could be involved. Consistent with this, clpA1, which is required for PCSK9 processing and secretion, and Sec24a, which facilitates packaging of PCSK9 into COPII vesicles for secretion, were increased (Figure 2I). Sortilin, another protein that appears to enhance PCSK9 secretion, however, was not changed (Figure 2I).

To specifically dissect the role of the podocyte in the regulation of plasma PCSK9, we also studied Podocyte Apoptosis Through Targeted Activation of Caspase-8 (Pod-ATTAC) mice. These mice carry the ATTAC transgene, which encodes a fusion protein that includes human caspase-8 and a mutant FK506 binding protein that binds the synthetic compound AP20187. AP20187 promotes...
dimerization of the fusion protein, activation of caspase-8, and apoptosis.\(^3\) In these mice, the transgene is driven by the podocin (\(Nphs2\)) promoter, which is specific to podocytes;\(^2\) therefore, injection of these mice with AP20187 results in selective ablation of the podocyte.

We injected AP20187 into mice heterozygous for the \(\text{Pod-ATTAC}\) transgene (\(\text{Pod-ATTAC}\) mice), and their wild-type littermates (controls), and studied mice 7 days later, at the point of maximal plasma cholesterol levels (data not shown). The phenotype of the \(\text{Pod-ATTAC}\) mice was remarkably similar to that of the NTS-treated mice (Figure 3). Both showed proteinuria, hypertriglyceridemia, and hypercholesterolemia, with a marked increase in the proportion of ApoB-associated cholesterol, from 14% in the controls to 48% in Pod-ATTAC mice, again primarily because of an increase in LDL-associated cholesterol (Figure 3A through 3D, online-only Data Supplement Figure II). Both models also showed a decrease in hepatic LDL receptor protein, although Pod-ATTAC livers showed no changes in \(\text{Ldlr}\), \(\text{Idol}\), or \(\text{Pcsk9}\) mRNA (Figure 3E, 3F, and 3H). Plasma PCSK9 was increased 7-fold (Figure 3G) and PCSK9 clearance was decreased, as the half-life of injected human recombinant PCSK9 increased from 5.4±0.3 minutes in control mice to 11.3±1.4 minutes in Pod-ATTAC mice (\(p=0.029\), Figure 3I). Finally, \(\text{cIAP1}\) and Sortilin were increased in Pod-ATTAC livers, although \(\text{Sec24a}\) was not (Figure 3J).

Taken together, these data showed that injury to the podocyte, either via NTS treatment or genetic ablation of the podocyte, could produce striking changes in plasma PCSK9 that were correlated with reduced levels of LDL receptor protein and an increased proportion of ApoB-associated cholesterol. To dissect the role of PCSK9 in nephrotic syndrome--associated hypercholesterolemia, we administered NTS to mice with global or liver-specific deletion of \(\text{Pcsk9}\).

**Figure 2. Nephrotic serum (NTS) increases plasma PCSK9.**

Six- to 8-week-old male B6 mice were injected with NTS or normal sheep serum (vehicle) and were euthanized 4 days after the initial injection. Two microliters of spot urine that was collected the morning of euthanization was subjected to SDS-PAGE and Coomassie blue staining (A). Plasma taken at the time of euthanization was used to measure triglycerides (B), total cholesterol (C), and PCSK9 (G), or subjected to size exclusion chromatography for lipoprotein analysis (D, brackets indicate the percentage of total cholesterol found in ApoB-containing lipoproteins [VLDL+LDL]). Hepatic protein was measured by Western blotting liver whole-cell lysates (E and I). Hepatic gene expression was measured by real-time PCR (F and H). \(n=3\) to 8. For lipoprotein analysis, equal amounts of plasma from 4 to 6 mice were pooled from each group. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Veh, vehicle; and VLDL, very low-density lipoprotein.
Mice with global deletion of Pcsk9 (hereafter referred to as PCSK9-KO mice) were compared to their wild-type controls (hereafter referred to as WT mice) on the same mixed genetic background. NTS treatment of WT mice led to marked proteinuria, with a 7-fold increase in plasma triglycerides, a 5-fold increase in plasma cholesterol, and an 8-fold increase in plasma PCSK9, with no change in liver Pcsk9 mRNA (online-only Data Supplement Figure IIIA and Figure 4A through 4D). The proportion of ApoB-associated cholesterol increased, but in this strain the effect was more modest, increasing from 11% in vehicle-treated WT mice to 21% in NTS-treated WT mice. Moreover, this increase was primarily attributable to an increase in VLDL, rather than LDL, cholesterol (Figure 4E and online-only Data Supplement Figure IIIB). Nonetheless, plasma levels of ApoB100 and ApoB48 increased markedly on NTS treatment in WT mice (Figure 4F). In parallel, LDL receptor protein was decreased (Figure 4G).

Knockout of Pcsk9 in mice with global deletion of Pcsk9 had no effect on renal histology or urine protein levels, either in the presence or absence of

Figure 3. Podocyte apoptosis increases plasma PCSK9.
Five- to 11-week-old mice with (Pod-ATTAC) or without (control) the Pod-ATTAC transgene were injected with dimerizer AP20187 and euthanized 7 days after injection. Two microliters of spot urine that was collected the morning of euthanization was subjected to SDS-PAGE and Coomassie blue staining (A). Plasma taken at the time of euthanization was used to measure triglycerides (B), total cholesterol (C), and PCSK9 (G), or subjected to size exclusion chromatography for lipoprotein analysis (D, brackets indicate the percentage of total cholesterol found in ApoB-containing lipoproteins [VLDL+LDL]). Hepatic protein was measured by Western blotting liver whole-cell lysates (E and J). Hepatic gene expression was measured by real-time PCR (F and H). PCSK9 clearance was measured as described in Methods (I). n=8 to 10. For lipoprotein analysis, equal amounts of plasma from 8 to 9 mice were pooled from each group. Con indicates control; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; Pod-ATTAC, Podocyte Apoptosis Through Targeted Activation of Caspase-8; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; and VLDL, very low-density lipoprotein.
NTS treatment (online-only Data Supplement Figure IIIA and data not shown). However, PCSK9-KO mice showed profound changes in lipoprotein metabolism, with reductions in total cholesterol, ApoB100, and ApoE (Figure 4B), consistent with previous studies.33 The effects of NTS treatment on plasma lipids were reduced, but not abolished, in PCSK9-KO mice. Thus, plasma triglycerides and total cholesterol were still significantly increased by NTS in PCSK9-KO mice (Figure 4A and 4B). However, the ability of the NTS to increase the proportion of cholesterol in ApoB-containing lipoproteins and plasma ApoB100/ApoB48 protein levels, as well as decrease LDL receptor protein levels was blunted (Figure 4E through 4G, online-only Data Supplement Figure IV).
Ldlr mRNA and Idol mRNA were not significantly changed by NTS treatment in either WT or PCSK9-KO mice (Figure 4H).

In parallel, we examined mice with liver-specific knockout of Pcsk9 (Cre+/–Pcsk9<sup>flox/flox</sup>, hereafter referred to as PCSK9 L-KO mice) on the C57BL/6 background, generated using Cre recombinase under the control of the albumin promoter. These mice were compared with their Cre–/–Pcsk9<sup>flox/flox</sup> littermates (hereafter referred to as Flox mice).

NTS treatment of Flox mice produced a 2-fold increase in Pcsk9 mRNA levels that did not reach significance, a 24-fold increase in plasma PCSK9, a 7-fold increase in plasma triglycerides, and a 4-fold increase in plasma cholesterol (Figure 5A through 5D). Here, as in wild-type B6 mice, NTS treatment produced a profound redistribution of plasma cholesterol, with LDL-associated cholesterol increased from 9% to 47% (Figure 5E, online-only Data Supplement Figure IVA). LDL receptor protein was decreased by NTS treatment, but Ldr and Idol mRNA levels were not (online-only Data Supplement Figure IVB and IVC).

In PCSK9 L-KO mice, plasma PCSK9 was barely detectable in vehicle-treated mice, consistent with previous reports that plasma PCSK9 is derived primarily from the liver.29 Interestingly, NTS treatment of PCSK9 L-KO mice nonetheless produced a 3-fold increase in plasma PCSK9 (P=0.004) (Figure 5B, inset). To explore this further, we profiled Pcsk9 mRNA in the other tissues known to express Pcsk9: brain, intestine, and kidney. We found that NTS treatment of B6 mice had no effect in the brain or intestine, but produced a 3-fold increase in renal Pcsk9. Interestingly, in situ hybridization revealed that Pcsk9 was induced not in podocytes or other intraglomerular cells, but rather in...

Figure 5. Liver-specific knockout of Pcsk9 prevents increased LDL cholesterol after nephrotoxic serum (NTS) treatment.

Five- to 8-week-old male Pcsk9<sup>flox/flox</sup> (Flox) and liver-specific knockout (L-KO) mice were injected with NTS or normal sheep serum (vehicle) and euthanized 4 days after the initial NTS injection. Hepatic gene expression was measured by real-time PCR (A), Plasma that was collected at the time of euthanization was used to measure PCSK9 (B), triglycerides (C), and cholesterol (D), or subjected to size exclusion chromatography for lipoprotein analysis (E, brackets indicate the percentage of total cholesterol found in ApoB-containing lipoproteins [VLDL+LDL]). n=4 to 8; *P<0.05. For lipoprotein analysis, equal amounts of plasma from 4 to 8 mice were pooled from each group. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; PCR, polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; and VLDL, very low-density lipoprotein.
a subset of tubule cells (online-only Data Supplement Figure VA and VB).

Despite this, the effects of liver-specific and global Pcsk9 knockout were quite similar, and both showed a significant 50% to 60% reduction in plasma cholesterol in the presence of NTS (Figures 4B and 5D). In addition, Flox mice showed a dramatic increase in ApoB-associated cholesterol, from 12% to 56% on NTS treatment, whereas PCSK9 L-KO mice showed only a modest increase in ApoB-associated cholesterol, from 11% to 16% on NTS treatment (Figure 5E and online-only Data Supplement Figure IVA). In parallel, the reduction in LDL receptor protein observed in Flox mice was blunted in Pcsk9 L-KO mice (online-only Data Supplement Figure IVB).

**DISCUSSION**

Our data reveal a novel podocyte/hepatic axis that contributes to the regulation of plasma lipid levels via PCSK9. NTS-treated mice and Pod-ATTAC mice showed increased levels of PCSK9, whereas patients with nephrotic syndrome showed decreased levels of PCSK9 on remission. Moreover, knockout of Pcsk9 in NTS-treated mice reduced plasma triglycerides and cholesterol, in particular ApoB-associated cholesterol.

Our studies in mice showed plasma PCSK9 to be increased up to 24-fold in nephrotic syndrome models. Previous cross-sectional studies in humans showed plasma PCSK9 to be increased 50% to 60% in proteinuric/nephrotic patients in comparison with control subjects.13,14 Similarly, our longitudinal human studies showed a 14% decrease in plasma PCSK9 with remission of nephrotic syndrome. These data demonstrate a consistent association between nephrotic syndrome and PCSK9 in humans. However, the changes in PCSK9 observed in humans with nephrotic syndrome were much more modest than those observed in mouse models. In our study, this may have been, in part, because one third of the patients were already undergoing immunosuppressive therapy to treat nephrotic syndrome at baseline. It is also possible that PCSK9 levels vary during the course of nephrotic disease and that our mouse studies, performed during the acute phase of the disease, reflected a time point with greater changes in PCSK9.

There appeared to be multiple mechanisms by which podocyte injury induced PCSK9, and these varied somewhat between the models we studied. In NTS-treated B6 mice, hepatic Pcsk9 mRNA was slightly increased, suggesting an increase in Pcsk9 transcription. The drivers of Pcsk9 transcription include sterol regulatory element binding protein 2 (SREBP2) and hepatocyte nuclear factor 1α (HNF1α).34–36 Gene expression analysis in NTS-treated B6 livers revealed an increase in Srebp2 and hepatocyte nuclear factor 1α (HNF1α). Thus, both SREBP2 and HNF1α may have contributed to the induction in Pcsk9 mRNA in NTS-treated B6 livers. However, hepatic Pcsk9 mRNA was not significantly changed in the other models studied.

PCSK9 clearance was also decreased in both NTS-treated and Pod-ATTAC mice. Curiously, the LDL receptor, a target of PCSK9, participates in the clearance of PCSK9 from the plasma.37 Moreover, LDL seems to impair PCSK9 binding to and uptake by the LDL receptor.38 Thus, the decrease in PCSK9 clearance could be secondary to the decrease in LDL receptor and increase in LDL cholesterol.

The facts that PCSK9 clearance was reduced only 2-fold after podocyte injury and that Pcsk9 mRNA was not usually increased imply the existence of a posttranscriptional mechanism by which podocyte injury increases PCSK9 secretion. This mechanism may involve cIAP1, because both NTS-treated and Pod-ATTAC mice showed an increase in hepatic cIAP1, which promotes PCSK9 secretion.39 How exactly the injured podocyte signals to the liver to increase cIAP1 has yet to be determined. One possible mediator is tumor necrosis factor-α, which can be secreted by the podocyte,39 is increased in nephrotic syndrome plasma,40,41 and is known to induce cIAP1.42 It is also possible that the increase in plasma PCSK9 observed in nephrotic syndrome is secondary to one of the systemic effects of podocyte injury, such as protein loss into the urine. Indeed, protein loss in the context of peritoneal dialysis is also associated with elevated levels of plasma PCSK9.14

Our studies in mice with both global and liver-specific knockout of Pcsk9 showed that Pcsk9 ablation has beneficial effects in NTS-treated mice. Although Pcsk9-KO and Pcsk9 L-KO mice were on different genetic backgrounds that varied somewhat in their response to NTS, it was clear that either global or liver-specific knockout of Pcsk9 could reduce plasma triglycerides and cholesterol by 40% to 60%. Moreover, both global and liver-specific knockout of Pcsk9 decreased the proportion of ApoB-associated cholesterol and increased the proportion of HDL-associated cholesterol.

Of course, knockout of Pcsk9 did not entirely prevent NTS-induced hyperlipidemia. This is consistent with the multiple mechanisms at play in nephrotic syndrome–associated dyslipidemia, which include increased cholesterol synthesis, increased synthesis and secretion of ApoB-containing lipoproteins, defective HDL maturation, and impaired triglyceride clearance.11,43–45 For example, acetyl-coenzyme A acetyltransferase-2, which promotes cholesterol ester formation, is increased in nephrotic syndrome and required for the development of hypercholesterolemia.11,46,47 Similarly, scavenger receptor class B, member 1, a component of the reverse cholesterol transport system that resides on the surface of the hepatocyte and removes HDL cholesterol from the plasma, is reduced in nephrotic syndrome.48,49 The fact that hypercholesterolemia and hypertriglyceridemia still occur in the absence of PCSK9 indicates that many of these processes may be PCSK9 independent. Thus, the correlation we observed between the change in PCSK9
and the change in HDL on remission in our patients with nephrotic syndrome was likely because the resolution of nephrotic syndrome was associated with the normalization of HDL metabolism, as well as a reduction in PCSK9.

In summary, our data show that PCSK9, although not solely responsible for nephrotic syndrome–associated dyslipidemia, is nonetheless an important participant. Plasma PCSK9 is increased in nephrotic syndrome, and Pcsk9 ablation not only reduces the magnitude of the hypercholesterolemia associated with podocyte injury, but also produces a more atheroprotective lipoprotein profile. These data raise the possibility that PCSK9 inhibitors, which were recently approved by the US Food and Drug Administration for the treatment of hypercholesterolemia, may be the long-sought treatment for the dyslipidemia of nephrotic syndrome.

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DISCLOSURES
Dr. Biddinger has received consulting fees from Novo Nordisk.

AFFILIATIONS
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FOOTNOTES
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Mary E. Haas, Amy E. Levenson, Xiaowei Sun, Wan-Hui Liao, Joseph M. Rutkowski, Sarah D. de Ferranti, Valerie A. Schumacher, Philipp E. Scherer, David J. Salant and Sudha B. Biddinger

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**SUPPLEMENTAL MATERIAL**

**Supplemental Methods**

**Human Studies**

**Patients**

The Nephrotic Syndrome Study Network (NEPTUNE) is a multicenter, prospective, observational cohort study of patients with nephrotic syndrome. Enrollment of patients at 21 clinical sites began in August 2010, with a target enrollment of 450 patients with minimal change disease, focal segmental glomerulosclerosis, and membranous nephropathy. Inclusion criteria were ≥500 mg/day of proteinuria from either a 24-h or spot urine collection, and a clinically indicated renal biopsy. Exclusion criteria included evidence of other renal disease (e.g., lupus, diabetic nephropathy), prior solid organ transplant, and life expectancy of <6 months. Participants whose biopsy was not consistent with minimal change disease, focal segmental glomerulosclerosis, or membranous nephropathy were retained in the study. Detailed information regarding socio-demographics, medical history, and medication exposure was collected by subject interview and chart review, as previously described.

In this ancillary study, 38% of patients were on immunosuppression therapy (steroids, mycophenolate mofetil, cytoxan, calcineurin inhibitors or rituximab) at baseline, and 44% were on immunosuppression therapy at baseline, remission or both. 24% were on medications which could potentially affect PCSK9: atorvastatin, rosuvastatin, lovastatin, simvastatin, oral contraceptives, testosterone, medroxyprogesterone, gliclazide, cyproterone acetate/ethinyl estradiol, ezetimide, fenofibrate, gemfibrozil, glipizide, gliclazide, insulin, hydrocortisone, sitagliptin, levo-norgestrel, levothyroxine, pitavastatin, norethindrone estradiol, omega-3, metformin, methylprednisone, methylprednisolone, niacin, ethinyl estradiol norgestimate, pravastatin, estrogen, progesterone, cholestyramine, thyroxine, colesevelam. When patients on these medications were included, plasma PCSK9 decreased by 14% from baseline to remission (348.0 ± 139.5 vs 300.5 ± 130.3 ng/mL, p = 0.04), as shown in Figure 1. When patients on these medications were excluded, PCSK9 decreased 18% from baseline to remission (343.8 ± 150.3 vs 283.6 ± 135.5 ng/mL, p = 0.03).

**Assays**

Plasma PCSK9 levels were measured via a quantitative sandwich enzyme immunoassay ELISA (CircuLex CY-8079, CycLex Co., Ltd., Japan; distributed by MBL International), according to manufacturer’s instructions. All samples were assayed in duplicate. Plasma lipid profiles and urine protein/creatinine ratio were measured at a single, central laboratory.

**Statistical Analysis**

Descriptive statistics are reported as mean (±SD). Paired, two tailed t-tests were used to assess pre-post changes from baseline to remission. Changes in UPCR and total cholesterol were non-normally distributed, and did not achieve normality after logarithmic transformation. Changes in UPCR, total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides were therefore also assessed using non-parametric tests (Wilcoxon signed-rank tests), which yielded similar results as paired t-tests. Associations between change in PCSK9 and changes in continuous variables, as well as UPCR at baseline and plasma parameters at baseline, were assessed using Spearman correlation and testing for zero correlation; assessments using Pearson correlation yielded similar results.
Mouse Studies

Mice

To generate Pcsk9\textsuperscript{flx/flx} mice, a targeting construct containing a loxP site between exons one and two, and a neomycin resistance cassette flanked by Frt sites followed by a second loxP site between exons three and four was electroporated into B6-3 (C57BL/6J) embryonic stem cells. G418-resistant colonies were screened by 5’ PCR using forward primer TTGCTCAAAGGTATGGGTGCCA and reverse primer CTGTGCTCGACGTTGTCACTG. Four potential clones were confirmed with southern blot analysis using 5’ probe located before exon one, 3’ probe between exons five and six, and a Neomycin resistance cassette probe. Two of these clones were electroporated with Flp and screened for G418 sensitivity. Subsequent clones were examined via PCR to confirm deletion of the neomycin resistance cassette and presence of the 5’ loxP site. Resulting Pcsk9\textsuperscript{flx/flx} (Flox) mice were backcrossed onto a C57BL/6N background and crossed to albumin-Cre mice on a C57BL/6N background to generate PCSK9 L-KO (C\textsuperscript{re}+/Pcsk9\textsuperscript{flx/flx}) mice, which were maintained on the C57BL/6N background.

Pcsk9\textsuperscript{−/−} mice and their approximate controls B6129SF2/J were purchased from Jackson Laboratory and intercrossed for four or more generations, to minimize differences in genetic background. Mice were subsequently mated Pcsk9\textsuperscript{−/−} x Pcsk9\textsuperscript{−/−} (WT x WT) and Pcsk9\textsuperscript{−/−} x Pcsk9\textsuperscript{−/−} (PCSK9-KO x PCSK9-KO) to generate sufficient numbers of experimental mice. For Pod-ATTAC, Pcsk9\textsuperscript{−/−} L-KO and C57BL/6J mice, littermates were used as controls.

Animals were housed in a twelve-hour light/dark cycle (0700–1900 hours). Mice were given standard chow and water ad libitum. On the day of sacrifice, spot urine was collected in the morning; mice were sacrificed in the non-fasted state at 1400 hours.

Pod-ATTAC mice (FVB background, aged five to eleven weeks) were injected i.p. with 0.5 μg/g body weight dimerizer AP20187 dissolved in 4% ethanol, 10% PEG-400, 1.75% Tween-20 in H\textsubscript{2}O. Wildtype littermates injected with either dimerizer or vehicle served as controls. Mice were sacrificed seven days after dimerizer injection. Two cohorts of males (using wildtype mice injected with dimerizer as controls) and one cohort of females (using wildtype mice injected with vehicle as controls) were examined. Pod-ATTAC mice that were injected with dimerizer but did not exhibit proteinuria on the day of sacrifice were excluded from subsequent analysis.

PCSK9 clearance assay in NTS-treated mice

NTS- and vehicle-treated C57BL/6J mice were injected retro-orbitally with 1 μg recombinant human PCSK9 (Biovision) four days after initial NTS or vehicle injection. Plasma samples were collected 1-121 min after PCSK9 injection, and were analyzed as described in the main text for PCSK9 clearance in Pod-ATTAC mice.

Urine protein analysis

For precise quantification, proteinuria (albumin: creatinine ratio) in WT and PCSK9-KO mice was measured by Microalbumin/Creatinine tests on a DCA 2000 Vantage Analyzer (Siemens).

Gene expression analysis

Gene expression was measured using real-time PCR. Total RNA was isolated by RNaseasy kit (Qiagen). cDNA was synthesized by a reverse transcription kit (Applied Biosystems). The resulting cDNA was diluted ten-fold and used for real-time PCR analysis with SYBR green
reagents (Thermo Scientific) in Applied Biosystems 7900 HT or 7000 instruments. Data are presented in arbitrary units (A.U.) relative to the control group set to 1. Primer sequences are listed at the end of the Supplemental Methods.

**Western blotting**

Liver lysates were prepared by homogenizing 50-100 mg of liver in 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.3% SDS, and protease inhibitor (Roche), heating for 10 min at 95°C and centrifuging at 13,000 x g for 10 min at room temperature. Protein was measured by the BCA assay kit (Pierce). 60-100 μg of lysates, 0.3 μL plasma, or 50 μL of plasma size exclusion chromatography fractions pooled from 3 minutes of column elution was loaded onto sodium dodecyl sulfate–PAGE (SDS–PAGE) gels and transferred onto a PVDF membrane (Thermo Scientific). After blocking in SuperBlock buffer (Thermo Scientific), blots were incubated overnight with a primary antibody (1:500 to 1:5000 dilution). Secondary antibody conjugated with horseradish peroxidase (Thermo Scientific) and chemiluminescent ECL reagents (Thermo Scientific) were used to develop blots. The Sec24a antibody was a kind gift from Dr. David Ginsburg. Other commercial antibodies used in this study were: Actin, Santa Cruz sc-47778; ApoA1, Meridian Life Sciences K23001R; ApoB, Meridian Life Sciences K23300R; ApoE, Meridian Life Sciences K23100R; cIAP1, Enzo life sciences ALX 803-335-C100; GAPDH, Santa Cruz sc-25778; LDLR, Abcam ab30532; Sortilin Abcam ab16640.

**In situ hybridization**

A previously described mouse *Pcsk9* sequence⁹ (nucleotides 1197-2090, see table at end of Supplemental Methods for primer sequences) was cloned into the pGEM T-Easy vector, sequenced, and transcribed using DIG RNA Labeling Mix (Roche 11277073910) and T7 and SP6 RNA polymerases to make *Pcsk9* antisense and sense probes.

Kidneys were isolated from mice, incubated overnight at 4°C in 4% paraformaldehyde (PFA) in PBS, rinsed with PBS, equilibrated in 30% sucrose in PBS, and frozen in OCT. 10 μm sections were fixed in 4% PFA in PBS, digested in 15 μg/mL Proteinase K in PBS, fixed in 4% PFA in PBS, and acetylated in 1.33% triethanolamine, 0.175% 12M hydrochloric acid, 0.375% acetic anhydride. Slides were pre-hybridized in hybridization buffer (50% formamide, 1.3x SSC pH 4.5, 5 mM EDTA (pH 8.0), 50 mg/ml yeast tRNA, 0.2% Tween-20, 0.5% CHAPS, 100 mg/ml heparin) and incubated with 125 ng sense or anti-sense probe overnight at 80°C. Slides were then washed in 0.2x SSC at 72°C, and blocked in 5% heat-inactivated sheep serum, 2% blocking reagent (Roche 1096176) in NTT (0.15M sodium chloride, 0.1 M Tris (pH 7.5), 0.1% Tween-20). Slides were incubated overnight at 4°C with 1:500 Anti-Digoxigenin-AP (Roche 11093274910) in 1% heat-inactivated sheep serum in NTT with embryo powder. Finally, slides were washed in NTT, rinsed in 0.15 M sodium chloride, 0.1 M Tris (pH 7.5), 0.1% Tween-20, 50 mM magnesium chloride, 2 mM levamisole, and developed using BM Purple (Roche 11442074001). Sections from five vehicle-treated C57BL/6J mice (two mice from one cohort and three mice from a second cohort, and five NTS-treated C57BL/6J mice (three mice from one cohort and two mice in a second cohort) were examined.
### Primers used in this study:

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FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; MN, membranous nephropathy. Data represent means and standard deviations.
Supplemental Figure 1. *Multiple mechanisms contribute to increased PCSK9 in nephrotic syndrome.* (A) Five- to eight-week-old male control mice were injected with nephrotoxic serum (NTS) or normal sheep serum (Vehicle). Plasma cholesterol levels were measured in non-fasted samples taken over the next two months. n=4-7, *p < 0.05 compared to vehicle-treated mice. (B-D) Five- to eight-week-old male B6 mice were injected with nephrotoxic serum (NTS) or normal sheep serum (Vehicle) and sacrificed four days after the initial NTS injection. Approximately 15 μg of cholesterol from non-fasted, pooled plasma was subjected to plasma lipoprotein fractionation as described in Methods; raw traces depict the relative distribution of cholesterol among the different fractions (B). The distribution of ApoB100 was assessed by western blotting (B, inset). PCSK9 clearance was measured as described in the supplemental methods (C). Liver gene expression was measured by real-time PCR (D). n=4-12; *p < 0.05. For plasma lipoprotein analysis, equal amounts of plasma from 4-6 mice were pooled.
Supplemental Figure 2. LDL cholesterol is increased in Pod-ATTAC mice. Five- to eleven-week-old mice with (Pod-ATTAC) or without (Control) the Pod-ATTAC transgene were injected with dimerizer AP20187 and sacrificed seven days after injection. Approximately 15 μg of cholesterol from non-fasted, pooled plasma was subjected to plasma lipoprotein fractionation as described in Methods; raw traces depict the relative distribution of cholesterol among the different fractions. For each group, equal amounts of plasma from 8-9 mice were pooled.
Supplemental Figure 3. Global knockout of PCSK9 blunts the NTS-induced increase in ApoB-associated cholesterol. Five- to eight-week-old PCSK9-wild type (WT) or PCSK9 total body knockout (PCSK9-KO) mice were injected with nephrotoxic serum (NTS) or normal sheep serum (Vehicle) and were sacrificed three to four days after the initial injection. Proteinuria (albumin: creatinine ratio) was determined in spot urine collected the morning of sacrifice (A). Approximately 15 μg of cholesterol from non-fasted, pooled plasma was subjected to plasma lipoprotein fractionation as described in Methods; raw traces depict the relative distribution of cholesterol among the different fractions (B). For each group, equal amounts of plasma from 4-7 mice were pooled.
Supplemental Figure 4. Liver-specific knockout of PCSK9 blunts the NTS-induced increase in ApoB-associated cholesterol. Five- to eight-week-old male PCSK9^flox/flox (Flox) and liver-specific knockout (L-KO) mice were injected with nephrotoxic serum (NTS or N) or normal sheep serum (Vehicle or V) and sacrificed four days after the initial NTS injection. Approximately 15 μg of cholesterol from non-fasted, pooled plasma was subjected to plasma lipoprotein fractionation as described in Methods; raw traces depict the relative distribution of cholesterol among the different fractions (A). Hepatic protein levels were measured by western blotting whole-cell lysates (B); since LDLR protein levels were markedly higher in L-KO mice, Flox and L-KO samples were run on separate gels to allow comparisons between vehicle and NTS treatment. Hepatic gene expression was measured by real-time PCR (C). n= 4-8. For plasma lipoprotein analysis, equal amounts of plasma from 4-8 mice were pooled.
Supplemental Figure 5. *NTS treatment increases Pcsk9 expression in the kidney.* Five- to eight-week-old wild type male B6 mice (n= 4-12) were injected with nephrotoxic serum (NTS) or normal sheep serum (Vehicle) and sacrificed four days after the initial NTS injection. Gene expression was measured in the tissues indicated by real-time PCR, with average cycle threshold values of 26, 22, 24, 27 and 28 in kidney, liver (shown in Figure 2H), ileum, jejunum and brain, respectively (A). Pcsk9 expression in the kidney was determined via in situ hybridization of Pcsk9 antisense and sense probes (B). Pcsk9 was undetectable in vehicle-treated mice, but present in a limited subset of tubules in NTS-treated mice (upper panels, black arrow indicates glomerulus). No signal was detected in either vehicle- or NTS-treated kidneys using the sense Pcsk9 probe, confirming the specificity of the Pcsk9 signal. Expression was also occasionally noted on the outer edge of the glomerulus (lower panel, red arrows), which likely represents the proximal tubule as it connects to the glomerulus. *p < 0.05.
References


Nephrotic Syndrome Study Network Consortium (NEPTUNE) Sites and Investigators

Nelson, S. Hingorani, J. Hernandez, K. Tuttle, K. Klepach, J.D. Sandhu, C. Weber, C. Alpers;
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