Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) in Secondary Hyperlipidemias

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th>Citation</th>
<th>Haas, Mary Elizabeth. 2016. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) in Secondary Hyperlipidemias. Doctoral dissertation, Harvard University, Graduate School of Arts &amp; Sciences.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:33493577">http://nrs.harvard.edu/urn-3:HUL.InstRepos:33493577</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) in Secondary Hyperlipidemias

A dissertation presented

by

Mary Elizabeth Haas

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Human Biology and Translational Medicine

Harvard University

Cambridge, Massachusetts

April 2016
Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) has emerged over the past decade as an important regulator of plasma cholesterol and cardiovascular disease risk. PCSK9 promotes degradation of low density lipoprotein (LDL) receptors, thereby decreasing LDL clearance. Accordingly, patients with gain-of-function mutations in PCSK9 have increased LDL cholesterol and increased risk of cardiovascular disease. Conversely, PCSK9 inhibitors recently approved by the FDA are effective in reducing LDL cholesterol.

While the contribution of PCSK9 to familial hypercholesterolemia is well-established, less is understood about the role of PCSK9 in diseases in which hyperlipidemia results secondary to an initial disease insult. Hormonal regulation of PCSK9 is also incompletely understood. Understanding the regulators of PCSK9 and specific diseases in which it contributes to hypercholesterolemia is important for identifying additional mechanisms via which PCSK9 can be manipulated, and for choosing patient populations in which PCSK9 inhibitors will be effective.

Here, we investigate PCSK9 in two diseases of secondary hyperlipidemia. In nephrotic syndrome, damage to kidney podocytes causes extreme proteinuria and hypercholesterolemia of unclear etiology. We show that plasma cholesterol and PCSK9 are dramatically elevated in mice made nephrotic by nephrotoxic serum treatment or podocyte apoptosis. Moreover, knockout of Pcsk9 protects mice from the effects of nephrotic syndrome on plasma lipids, particularly increased LDL cholesterol. Similarly, nephrotic patients show decreased plasma PCSK9 and cholesterol upon disease remission. Second, loss of adipose tissue in lipodystrophy results in low levels of the hormone leptin. Treatment of lipodystrophic patients with leptin reduces LDL cholesterol through unknown mechanisms. We used this background of hypoleptinemia to investigate the effects of leptin on PCSK9. We found that in female
lipodystrophic patients, leptin treatment reduced plasma PCSK9, correlating with decreased LDL cholesterol. Similarly, in male leptin-deficient ob/ob mice, leptin also decreased plasma PCSK9 but did not affect plasma lipids.

Our data show that PCSK9 is a novel regulator of hypercholesterolemia in nephrotic syndrome, suggesting that PCSK9 inhibitors may be an important therapy for this patient population with ambiguous treatment options. They also show that leptin can suppress PCSK9 expression, which may explain the observed decreases in LDL cholesterol upon leptin treatment of lipodystrophic patients.
# Table of Contents

Abstract........................................................................................................................................ iii
Table of Contents ......................................................................................................................... v
List of Figures ................................................................................................................................. vii
List of Tables ................................................................................................................................ viii
Glossary.......................................................................................................................................... ix
Acknowledgements ...................................................................................................................... xii
Chapter 1 - Introduction .............................................................................................................. 1
  Lipoproteins and Cardiovascular Disease .................................................................................. 2
    Lipoprotein Structure and Function ......................................................................................... 2
    Lipoproteins and Cardiovascular Disease ............................................................................. 5
  PCSK9 Modulates LDL Metabolism and Cardiovascular Disease Risk ................................. 6
    PCSK9 Function ....................................................................................................................... 6
    Regulation of PCSK9 Expression and Activity ...................................................................... 9
  Nephrotic Syndrome-Associated Hyperlipidemia ................................................................. 13
    Podocyte Damage in Nephrotic Syndrome ........................................................................... 13
    Hyperlipidemia in Nephrotic Syndrome .............................................................................. 16
  Hyperlipidemia in Lipodystrophy ............................................................................................. 19
    Clinical Characteristics of Lipodystrophy ........................................................................... 19
    Functions of Adipose Tissue .................................................................................................. 20
    Leptin Treatment in Lipodystrophy and Leptin Deficiency ................................................ 22
    Leptin Function ....................................................................................................................... 22
  Overview of the Dissertation .................................................................................................... 24
Chapter 2 - The Role of Proprotein Convertase Subtilisin/Kexin Type 9 in Nephrotic Syndrome-Associated Hypercholesterolemia .............................................................................. 25
  Abstract ....................................................................................................................................... 26
  Introduction ................................................................................................................................. 26
  Methods ..................................................................................................................................... 28
  Results ....................................................................................................................................... 31
  Discussion .................................................................................................................................. 41
  Acknowledgements .................................................................................................................. 44
  Attributions ............................................................................................................................... 44
Chapter 3 - Effect of Leptin Replacement on PCSK9 in ob/ob Mice and Female Lipodystrophic Patients ................................................................................................................................. 46
List of Figures

Figure 1.1. Structure of lipoprotein particles ................................................................. 2
Figure 1.2. Progression of atherosclerosis from endothelial dysfunction to thrombosis. .... 5
Figure 1.3. PCSK9 stimulates degradation of the LDL receptor ..................................... 7
Figure 1.4. Structure and processing of PCSK9 ............................................................ 11
Figure 1.5. The glomerular filtration barrier ............................................................... 15
Figure 2.1. Plasma PCSK9 in nephrotic patients ......................................................... 33
Figure 2.2. Nephrotoxic serum increases plasma PCSK9 .............................................. 34
Figure 2.3. Podocyte apoptosis increases plasma PCSK9 .............................................. 36
Figure 2.4. Knockout of PCSK9 reduces plasma cholesterol in the presence and absence of NTS .................................................................................................................... 38
Figure 2.5. Liver-specific knockout of PCSK9 prevents increased LDL cholesterol during NTS 40
Figure 3.1. Leptin treatment suppressed PCSK9 expression in male ob/ob mice .............. 53
Figure 3.2. Leptin treatment reduced Pcsk9 mRNA and SREBP target genes in male ob/ob mice ........................................................................................................................................ 55
Figure 3.3. Leptin did not alter PCSK9 expression in female ob/ob mice ....................... 56
Figure 3.4. Comparison of plasma PCSK9 concentrations in lipodystrophic patients at baseline and after 4-6 months of leptin treatment .................................................................................................................. 58
Figure S1.1. Multiple mechanisms contribute to increased PCSK9 in nephrotic syndrome. 106
Figure S1.2. LDL cholesterol is increased in Pod-ATTAC mice ..................................... 107
Figure S1.3. Global knockout of PCSK9 blunts the NTS-induced increase in ApoB-associated cholesterol ................................................................................................................ 108
Figure S1.4. Liver-specific knockout of PCSK9 blunts the NTS-induced increase in ApoB-associated cholesterol ................................................................................................. 109
Figure S1.5. NTS treatment increases Pcsk9 expression in the kidney ......................... 110
Figure S2.1. Effects of leptin on the distribution of cholesterol among the different lipoprotein fractions in male ob/ob mice .............................................................................................................. 117
Figure S2.2. Effects of leptin on PolII occupancy of the Pcsk9 promoter in male ob/ob mice ................................................................. 117
Figure S2.3. Effects of leptin on mRNA levels of Hnf1α and its targets in male ob/ob mice .... 118
Figure S2.4. Effects of leptin on Pcsk9 mRNA and SREBP target genes in female ob/ob mice. ........................................................................................................................................... 118
List of Tables

Table 1.1. Characteristics of human lipoproteins .............................................................. 3
Table 2.1. Plasma PCSK9 in nephrotic syndrome .............................................................. 32
Table 3.1. Patient demographics, markers of glucose homeostasis, and lipid parameters at baseline and post-leptin treatment ................................................................. 57
Table S1.1. Characteristics of nephrotic syndrome subgroups at baseline ........................ 103
Table S1.2. Primers used in Chapter 2 ............................................................................. 104
Table S2.1. Primers used in Chapter 3 ............................................................................. 112
Table S2.2. Information on antibodies used in Chapter 3 ............................................... 114
Table S2.3. Laboratory reference ranges for measured patient parameters, as per the NIH Clinical Center laboratory .............................................................. 115
Table S2.4. Correlation between change in PCSK9 and metabolic parameters upon leptin treatment ........................................................................................................ 116
Glossary

18s: 18s ribosomal RNA
ACAT: Acetyl-CoA acetyltransferase
ACC: Acetyl-CoA carboxylase
AGL: Acquired generalized lipodystrophy
AgRP: Agouti-related peptide
APL: Acquired partial lipodystrophy
ApoA1: Apolipoprotein A1
ApoB: Apolipoprotein B
ApoE: Apolipoprotein E
B6: C57BL/6J
BACE1: \(\beta\)-site amyloid precursor protein cleaving enzyme 1
BMI: Body mass index
CGL: Congenital generalized lipodystrophy
ChIP: Chromatin immunoprecipitation
CHRD: Cysteine- and histidine-rich C-terminal domain
CPT-1a: Carnitine palmitoyl transferase 1a
CVD: Cardiovascular disease
EGF: Epidermal growth factor
ENaC: Epithelial sodium channel
FPL: Familial partial lipodystrophy
FSGS: Focal segmental glomerulosclerosis
FASN: Fatty acid synthase
FDFT1: Farnesyl-diphosphate farnesyltransferase 1
FDPS: Farnesyl diphosphate synthase
FGB: Fibrinogen beta chain
Flox: $Pcsk9^{\text{flox/flox}}, \text{Albumin-Cre}^{-/-}$
HDL: High density lipoprotein
HIV: Human immunodeficiency virus
HMGCR: 3-hydroxy-3-methylglutaryl CoA reductase
HNF: Hepatocyte nuclear factor
IDL: Intermediate density lipoprotein
IDOL: Inducible degrader of the LDLR
L-KO: $Pcsk9^{\text{flox/flox}}, \text{Albumin-Cre}^{+/-}$
LDL: Low density lipoprotein
LDLR: Low density lipoprotein receptor
LPL: Lipoprotein lipase
LRP1: LDL receptor-related protein 1
MCD: Minimal change disease
MN: Membranous nephropathy
NARC-1: Neural apoptosis-regulated convertase-1
NEPTUNE: Nephrotic Syndrome Study Network
NPY: Neuropeptide Y
NTS: Nephrotoxic serum
OGIS: Oral glucose insulin sensitivity index
OGTT: Oral glucose tolerance test
PBS: Phosphate-buffered saline
Pod-ATTAC: Podocyte apoptosis through targeted activation of caspase-8
POMC: Pro-opiomelanocortin
PPAR: Peroxisome proliferator-activated receptor
PCSK9: Proprotein convertase subtilisin/kexin type 9
PCSK9-KO: PCSK9 total body knockout
SCD1: Stearoyl-CoA desaturase-1
SR-BI: Scavenger receptor class B, member 1
SRE: Sterol responsive element
SREBP: Sterol responsive element binding protein
SS: Signal sequence
TBP: TATA-box binding protein
TNFα: Tumor necrosis factor alpha
UPCR: Urine protein/creatinine ratio
VLDL: Very low density lipoprotein
WT: PCSK9-wildtype
Acknowledgements

This dissertation would not have been possible without the help and support of so many people, which I am sincerely grateful for.

First, I would like to thank Sudha Biddinger, who has been a tremendous advisor for the past five years. I have learned so much from her, not only about the subject matter of lipid metabolism and diabetes, but also about the process of doing science and scientific research as a career. She has pushed me to become a better scientist, to think deeply about the question at hand and how to make the most impactful biological insights in any area of research. My approach to science has become so much more targeted and efficient, and for this I am very thankful. At the same time, she has also encouraged me to think outside of conventional dogma of the field, and has always supported exploring questions tangential to the lab’s main focus. Trying out our crazy ideas and seeing where they lead has been one of the most enjoyable parts of being in the lab. Finally, I’ve appreciated her balanced approach to science, and her continued demonstration that working on even very complex biological problems should always be fun. I could not have had a better mentor for my Ph.D. experience, and I am very grateful to have been able to learn so much from her.

I would also like to thank the other members of the Biddinger Lab for making my time in the lab so enjoyable. Thanks to Alisha Ling and Mary Gearing for fellow graduate student comradery and for indulging my neurotic tendency to talk through every problem I encounter. Ji Miao, former postdoc and now faculty member extraordinaire, has answered probably close to a million of my experimental questions by now with never-ending patience, for which I am very thankful. I am also grateful to Amy Levenson, who has been my clinical counterpart on many projects – she has provided crucial clinical insights into the biological questions we investigate in mice, and I have had so much fun working together with her.
There are many other faculty and staff at Harvard who have ensured that my experience here has been happy and enjoyable: David Cohen has been there every step of the way, from originally convincing me to come to Harvard to letting me rotate in his lab and for serving as the chair of my DAC ever since. He, Jordan Kreidberg and David Salant have provided tremendous guidance and encouragement on my project these past four years. Kate, Danny, Maria and Tucker of the BBS office have helped me navigate not only the bureaucracy of Harvard, but also the ups and downs inherent in any Ph.D. Finally, Thomas Michel and Connie Cepko, heads of the Leder Human Biology program, not only gave me a fantastic translational context with which to approach my research, but also have created a great community of like-minded students.

I am also deeply grateful for my friends. From those I have known from elementary school – Sophie Chowdhury, Lindsey Kline – to the deep friendships I made at Williams College – Ruby Dale-Brown, Joanna Palmer, Anna Hernandez-French – you continue to encourage me through tough times and push me to think critically about the role of science in my life. I would also like to thank the friends I have made here in BBS: Meg Emori, Laura Smith, Stéphane Ricoul, Niroshi Senaratne, Kaitlin Samocha, Ben Vincent, Kal Tsanov, Kostadin Petrov, and so many more I would need an appendix to list them exhaustively: thank you so much for your support through the highs and lows of grad school, for your insightful advice, and for all of the amazing adventures we’ve had outside of lab. It’s been a wonderful six years.

Finally, and most importantly, thank you to my family. You have always been so supportive of my dreams – even when they take me across the country from you for 11 years and counting. Thank you so much for instilling in me a passion for science from an early age, and providing with me with so many opportunities growing up to follow this interest. Your love, encouragement and support mean more to me than I can ever express in words. I couldn’t have done this without you.
Chapter 1 - Introduction
Lipoproteins and Cardiovascular Disease

Lipoprotein Structure and Function

In multicellular organisms, transport of lipids between different tissues within the body is crucial for balancing energy storage and nutrient availability. Tissues hydrolyze triglycerides into fatty acids for fuel, whereas additional cholesterol is required if cellular synthetic pathways are insufficient for current demand. Lipoproteins are the predominant means of transporting cholesterol and triglycerides through the circulation. Lipoprotein particles are comprised of a phospholipid monolayer which surrounds hydrophobic triglyceride and cholesterol ester molecules, enabling them to be transported through an aqueous, hydrophilic environment (Figure 1.1). Also found on the surface of lipoproteins are amphipathic apolipoproteins and unesterified free cholesterol. Lipoproteins can be classified according to their lipid and protein content, which also gives them distinct size and density and determines their biological function (Table 1.1).

Figure 1.1. Structure of lipoprotein particles.
Lipoproteins containing the protein Apolipoprotein B (ApoB) predominantly function to deliver triglycerides to peripheral tissues. Chylomicrons are synthesized in the enterocytes of the intestine, where ingested fatty acids are packaged into triglycerides and added to a single nascently-transcribed ApoB-48, which is 48% of the full-length ApoB protein due to the introduction of a premature stop codon into APOB mRNA by the editing enzyme APOBEC). The resulting chylomicrons contain the highest amount of triglycerides, and are therefore both largest and least dense lipoprotein particles. Upon secretion into the circulation, chylomicrons acquire several accessory apolipoproteins, including apolipoprotein E (ApoE) from other lipoprotein particles. In the circulation, triglycerides in the chylomicron are hydrolyzed into fatty acids by lipoprotein lipase (LPL) and delivered to peripheral tissues for use as energy or stored in adipose tissue, causing the chylomicron to shrink into a chylomicron remnant particle.

Very low density lipoprotein (VLDL) particles are synthesized in the hepatocytes of the liver. Fatty acids, both taken up from the circulation as well as synthesized by the hepatocyte, are added to a single nascent-translated ApoB particle to form a VLDL particle which is secreted into the circulation. In humans, VLDL particles contain exclusively ApoB-100 due to intestinal-

Table 1.1. Characteristics of human lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/mL)</td>
<td>&lt;0.95</td>
<td>0.95-1.006</td>
<td>1.006-1.019</td>
<td>1.019-1.063</td>
<td>1.063-1.210</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>75-1,200</td>
<td>30-80</td>
<td>25-35</td>
<td>18-25</td>
<td>5-12</td>
</tr>
<tr>
<td>Composition (%)a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>2</td>
<td>10</td>
<td>18</td>
<td>25</td>
<td>33</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>83</td>
<td>50</td>
<td>31</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Cholesterol b</td>
<td>8</td>
<td>22</td>
<td>29</td>
<td>45</td>
<td>30</td>
</tr>
</tbody>
</table>

Apolipoproteins B48, A1, C1, C2, C3 B100, E, C1, C2, C3 B100, E, C1, C2, C3 B100 A1, A2, C1, C2, C3, E

Adapted from 1.

a Percent of dry weight
b Cholesterol ester and free cholesterol
specific expression of APOBEC1. In mice, Apobec1 is expressed in both the liver and the intestine, and VLDL particles therefore contain either ApoB-48 or ApoB-100. Like chylomicrons, VLDL particles are predominantly comprised of triglycerides, acquire accessory apolipoproteins like ApoE in the circulation, and are hydrolyzed by LPL to deliver fatty acids to peripheral tissues. Consequently, the VLDL particle decreases in size, becomes denser and is categorized as an intermediate density lipoprotein (IDL) and then a low density lipoprotein (LDL). As IDL is converted to LDL by hepatic lipase, the ApoE protein dissociates from the lipoprotein particle, such that LDL contains ApoB as its only protein component. LDL particles are also notable for being cholesterol-enriched.

These ApoB-containing lipoproteins can be removed from the circulation via several receptors. The LDL receptor (LDLR) can bind both ApoB-100 and ApoE, although it has much greater affinity for ApoE; ApoB-48, on the other hand, lacks the ApoB domain necessary for binding and therefore cannot bind the LDL receptor. The LDL receptor-related protein 1 (LRP1) also binds ApoE. Finally, heparin sulfate proteoglycans also mediate uptake of ApoB-containing lipoproteins, by facilitating binding to the LDL receptor and LRP1 and/or by removing them directly. ApoB-48 containing lipoproteins are removed from circulation much faster than ApoB-100 containing lipoproteins, likely because they contain more ApoE to act as a receptor substrate. This may also explain why less VLDL containing ApoB-48 is converted to IDL and LDL in rodents. Finally, LDL particles are notable for their comparatively long half-life of 2-3 days in the circulation. LDL can be taken up by tissues which require exogenous cholesterol, but most LDL particles are removed from the circulation by the liver.

High density lipoprotein (HDL) particles, on the other hand, are characterized by their main protein component, Apolipoprotein A1 (ApoA1), as well as their small size, high density, and cholesterol enrichment. Synthesized and secreted from the liver, these particles can remove
excess cholesterol from peripheral tissues such as macrophages, and return it to the liver for further catabolism and excretion.\textsuperscript{11}

**Lipoproteins and Cardiovascular Disease**

Cardiovascular disease is the leading cause of mortality in the United States, responsible for 30\% of deaths annually.\textsuperscript{12} Atherosclerosis, the hardening of artery walls due to the accumulation of lipids and macrophages in the intimal space between endothelial and smooth muscle cells, is a leading cause of heart disease, as up to 75\% of examined acute myocardial infarctions have been due to coronary thrombosis.\textsuperscript{13, 14} The development of atherosclerosis is depicted in Figure 1.2.

Briefly, changes in the endothelial cells lining arteries allow the entry of both LDL particles and monocytes into the intimal space. These monocytes differentiate into macrophages, which engulf the LDL particles, becoming foam cells. Smooth muscle cells then migrate into the nascent plaque, where they proliferate and produce extracellular matrix proteins that form a fibrous cap covering the plaque. As the plaque grows, some of the macrophages and smooth muscle cells apoptose, resulting in the accumulation of cell debris and a necrotic plaque core.

![Figure 1.2. Progression of atherosclerosis from endothelial dysfunction to thrombosis. See text for details. Modified from 13.](image)

---

\textsuperscript{11}  Further catabolism and excretion.

\textsuperscript{12}  Cardiovascular disease.

\textsuperscript{13}  Atherosclerosis.

\textsuperscript{14}  Coronary thrombosis.
Finally, thrombosis occurs when the fibrous cap of the plaque ruptures, releasing the necrotic contents into the circulation which stimulates clot formation.\textsuperscript{16} Clots formed in the coronary arteries or which travel to the brain result in acute myocardial infarction or stroke, respectively.

The normal mechanisms of cholesterol homeostasis are vastly altered in atherogenic states: decreased concentrations of HDL cholesterol and increased LDL cholesterol are both well-established risk factors for atherosclerosis.\textsuperscript{16-18} Increased LDL cholesterol has been correlated with increased LDL particle accumulation in plaque-prone arteries.\textsuperscript{19} LDL particles can also be oxidized, and this oxidized LDL is much more rapidly engulfed by macrophage and thus stimulates foam cell formation.\textsuperscript{20} HDL, on the other hand, is generally atheroprotective. This is attributed to its ability to promote cholesterol efflux from macrophages to HDL, thus inhibiting foam cell formation. Independent of cholesterol transport, HDL also been shown to inhibit endothelial dysfunction and LDL oxidation.\textsuperscript{11, 17}

**PCSK9 Modulates LDL Metabolism and Cardiovascular Disease Risk**

**PCSK9 Function**

Familial hypercholesterolemia is a rare genetic disease which is characterized by severely increased plasma total cholesterol and LDL cholesterol and greatly accelerated atherosclerosis and heart disease. Mutations in the \textit{LDLR}, \textit{ApoB}, and \textit{LDLRAP1} genes were identified beginning in the 1970s as causal defects which result in familial hypercholesterolemia.\textsuperscript{21} In 2003, mutations in a novel gene, \textit{PCSK9}, were proposed to constitute a fourth cause of familial hypercholesterolemia.\textsuperscript{22} Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9), also known as Neural Apoptosis-Regulated Convertase 1 (NARC-1), is expressed predominantly in the liver, and to a smaller extent the intestine, kidney and brain,\textsuperscript{23} and efficiently secreted into the circulation.
Extensive work in the past decade has shown that PCSK9 stimulates post-translational degradation of the LDL receptor. In the absence of PCSK9, LDL binding to the LDL receptor at the N-terminal ligand binding domain results in endocytosis of the LDL-LDL receptor complex via clathrin-coated pits (Figure 1.3). A decrease in the pH of endosomes causes the LDL receptor to dissociate from its LDL cargo, and it is trafficked back to the plasma membrane with other receptors. LDL, on the other hand, remains in the endosome until it fuses with the lysosome, where ApoB is degraded to amino acids and the remaining cholesterol enters the cytoplasm to modulate cellular cholesterol homeostasis via several mechanisms.\textsuperscript{10}

**Figure 1.3. PCSK9 stimulates degradation of the LDL receptor.**

PCSK9 and LDLR are translated in the ER (1) and transported via the Golgi (2) to the plasma membrane (3). LDL binding to LDLR triggers internalization of the complex (4). Acidification of the endosomal compartment causes dissociation of LDLR and its trafficking back to the plasma membrane (5) whereas LDL is degraded in the lysosome. PCSK9 can also bind the LDLR on a domain distinct from that of LDL. PCSK9 binding also triggers internalization of the LDLR-PCSK9 complex (6). Acidification of the endosomal compartment enhances the binding of PCSK9 and LDLR, resulting in degradation of both proteins in the lysosome (7). From \textsuperscript{24}.
When PCSK9 is present in the circulation, it can bind the first epidermal growth factor-like repeat (EGF)-A repeat domain of the LDL receptor via its catalytic domain.\textsuperscript{25-27} Unlike binding to LDL, binding of the LDL receptor to PCSK9 is strengthened at lower pH; therefore the LDL receptor stays bound to PCSK9 through the endo-lysosomal pathway, and both proteins are eventually degraded in the lysosome.\textsuperscript{25-28} LDL receptors in the most tissues (kidney, lung, ileum) can be degraded by PCSK9, with the liver being the most susceptible; the LDL receptors in the adrenal gland appear, on the other hand, to be protected from PCSK9-mediated degradation.\textsuperscript{29-31} Inside the cell, the LDL receptor can also trafficked to the lysosome via binding to PCSK9,\textsuperscript{32} as well as be ubiquitinated and degraded by Inducible Degrader of the LDL Receptor (Idol).\textsuperscript{33}

Additional research on PCSK9 function has revealed a number of additional targets, although the biological significance of these interactions is not as clear as is with the LDL receptor. For example, PCSK9 can bind and enhance the degradation of other members of the LDL receptor family, including the VLDL receptor,\textsuperscript{34,35} ApoER2,\textsuperscript{34} and LRP1.\textsuperscript{36} It also appears to target the fatty acid scavenger receptor CD36 for degradation.\textsuperscript{37} As the VLDL receptor, LRP1, and CD36 are all involved in the removal of triglycerides and/or triglyceride-rich lipoproteins, this suggests an emerging role for PCSK9 in triglyceride metabolism.\textsuperscript{38} PCSK9 also stimulates degradation of CD81 as well as the LDL receptor.\textsuperscript{39} These receptors are important for Hepatitis C viral entry into the hepatocyte. PCSK9 expression reduced viral infection of cells, suggesting that an antiviral function in addition to its role in regulating plasma cholesterol.

The clinical significance of PCSK9 in regulating LDL cholesterol levels has been established by the discovery of additional loss-of-mutation and gain-of-mutations in the \textit{PCSK9} gene. For example, patients with gain-of-function mutations have increased risk of coronary heart disease,\textsuperscript{40} whereas the nonsense mutations Y142X and C679X, which are found in 1-2\% of African Americans populations studied, decrease plasma LDL cholesterol by 28\% and also reduce the risk of coronary heart disease by 88\%.\textsuperscript{41} Furthermore, a recently-identified patient
with no detectable circulating PCSK9 resulting from compound heterozygosity had extremely low LDL cholesterol but was otherwise healthy, suggesting that complete loss of PCSK9 function is not deleterious in humans. These latter studies suggested that PCSK9 inhibition could be an effective treatment for hypercholesterolemia; indeed, monoclonal antibodies against PCSK9 reduce LDL cholesterol by 60%43, 44 and were approved by United States Food and Drug Administration for the treatment of familial hypercholesterolemia and clinical atherosclerotic cardiovascular disease in 2015.

**Regulation of PCSK9 Expression and Activity**

Regulation of PCSK9 expression is perhaps best characterized at the transcriptional level, where multiple transcriptional factors have been shown to have profound effects on expression of PCSK9 mRNA. Regulation of PCSK9 by the Sterol Response Element Binding Protein (SREBP)s has important clinical implications. The SREBPs are membrane-bound transcription factors which basally reside in the ER membrane, but upon depletion of cholesterol and other sterols from the ER, can be processed into active proteins which induce transcription of genes whose promoters contain Sterol Response Elements (SREs). There are three isoforms of SREBP: SREBP1a and SREBP1c, which are transcribed from the same gene, and SREBP2. The SREBPs stimulate transcription of genes involved in fatty acid synthesis and cholesterol synthesis preferentially by SREBP1c (the predominant SREBP1 isoform in the liver) and SREBP2, respectively, although there is overlap between the two isoforms.

The PCSK9 promoter contains a SRE and therefore its expression is increased in states of increased SREBP activity. Thus, PCSK9 expression is increased upon refeeding, when insulin, which stimulates SREBP-1c, is high. Furthermore, statins, which decrease cellular cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coA reductase (HMGCR, the rate-limiting enzyme in cholesterol synthesis) and therefore activate SREBP2 processing, also increase Pcsk9 transcription and plasma PCSK9 in both mice & humans. Statins are also commonly
prescribed to lower LDL cholesterol via increased synthesis of the LDL receptor, which is also an SREBP target gene. The fact that statins increase transcription of both the LDL receptor and PCSK9, which degrades the LDL receptor, suggests that the maximal efficacy of statin therapy may be blunted by the presence of PCSK9; indeed, patients treated with a PCSK9 inhibitor in addition to statins show significantly reduced LDL cholesterol compared to statin treatment alone.

Several other transcription factors in addition to the SREBPs regulate expression of PCSK9. Hepatocyte Nuclear Factor (HNF)1α, which has also been implicated in the regulation of the cholesterol metabolites bile acids, stimulates PCSK9 transcription via a HNF binding site in the PCSK9 promoter, and knockdown of Hnf1α in mice reduces Pcsk9 mRNA, serum PCSK9, and increases LDL cholesterol. Pharmacological activation of the Liver X Receptor also increases Pcsk9 mRNA by activating SREBP1c. Finally, the Peroxisome Proliferator-activated Receptors (PPAR)s also appear to modulate PCSK9 expression: agonist activation of PPARα and PPARγ increases and decreases, respectively, PCSK9 mRNA.

PCSK9 is a member of the proprotein convertase family, a group of nine serine proteases originally named for their similarity to the yeast kexin and bacterial subtilisin enzymes, and which cleave secreted proteins as they are trafficked out of the cell. Human PCSK9 is a 692 amino acid protein comprised of a signal sequence, prodomain, catalytic domain and cysteine- and histidine-rich C-terminal domain (CHRD, Figure 1.4). In an autocatalytic reaction in the ER, the catalytic domain of PCSK9 removes the prodomain, which associates with the remaining mature protein now comprised of the catalytic and CHRD domains, inhibiting catalytic activity. This processing seems to be enhanced through an unknown mechanism by the E3-ubiquitin ligase cIAP1.

In other proprotein convertases, the prodomain is thought to prevent premature catalytic activity by associating with the catalytic domain until the complex arrives to the proper subcellular
compartment and the prodomain dissociates from the catalytic domain. In the case of PCSK9, however, the prodomain remains non-covalently associated with catalytic domain during its secretion out of the cell and in the circulation. Consistent with this, catalytic activity of PCSK9 is not required for its ability to stimulate degradation of the LDL receptor. To date, searches for additional PCSK9 enzymatic substrates have not been particularly fruitful. *In vitro* evidence using overexpression of PCSK9 has suggested that PCSK9 can cleave the epithelial sodium channel (ENaC) and β-site amyloid precursor protein cleaving enzyme 1 (BACE1); however, PCSK9 has either no or contradictory effects on these proteins in the kidney and brain.

Figure 1.4. Structure and processing of PCSK9.
PCSK9 is a 692 amino acid protein composed of a signal sequence (SS), prodomain, catalytic domain and cysteine and histidine rich C-terminal domain (CHR). The triad of amino acids required for catalytic activity are shown in green (top). Autocatalytic cleavage by PCSK9 in the ER removes the prodomain, which remains non-covalently associated with PCSK9 (middle). In the circulation and at plasma membrane, PCSK9 can be cleaved by the proprotein convertase furin, which removes amino acids 152-218 (bottom). This appears to decrease PCSK9 activity. Figure created using data from 64, 67, 68.
Regulation of mature, processed PCSK9 transportation from the ER through the trans-Golgi network and secretion out of the cell remains poorly understood. Transport of PCSK9 from the ER to the Golgi is dependent on the inside coat protein Sec24a. Sec24a is required for PCSK9 sorting into COPII transport vesicles, as mice deficient in Sec24a have increased intracellular accumulation of PCSK9 and decreased plasma PCSK9 levels. Sortilin is a Vps10p domain-containing sorting receptor which is involved in trafficking between the Golgi, plasma membrane and lysosomes. Sortilin appears to bind PCSK9 in the trans-Golgi network and facilitate its secretion; however, whether this has physiological consequences on degradation of the LDLR is unclear. Finally, agonism of sirtuin 1, through an unknown mechanism, seems to inhibit PCSK9 secretion and leads to an accumulation of intracellular PCSK9.

Activity of PCSK9 can also be modified via multiple mechanisms during secretion as well as in the circulation. Mature PCSK9 can be further processed by other members of the PC family, furin and to a lesser extent PC5/6, in the late secretory pathway and on the cell membrane. This processing removes aa153-218 of the catalytic domain on the N-terminal of the mature protein. The furin-processed form accounts for ~30% of total plasma PCSK9 in both humans and mice. Importantly, furin-cleaved PCSK9 appears to have impaired ability to degrade the LDL receptor and decrease LDL uptake in most, but not all studies, and is therefore considered to represent an inactive form of circulating PCSK9. Similarly, the phospholipid binding protein Annexin A2 can inactivate PCSK9 by binding its CHRD and inhibiting its ability to degrade the LDLR; high expression of Annexin A2 in organs such as the adrenal gland may provide an explanation as to why PCSK9 is not effective in degrading the LDL receptor in these tissues.

Circulating PCSK9 can also bind to LDL, but not other lipoproteins, with similar affinity as its binding to the LDL receptor. LDL binding to PCSK9 inhibits its ability to degrade the LDL receptor. In human plasma, ~30%-40% of PCSK9 is bound to LDL. In the circulation,
PCSK9 can also multimerize into higher molecular weight complexes; these multimers appear to have increased ability to degrade the LDL receptor. Finally, it is important to note that all of these modifications interact with each other, such that the distribution of PCSK9 in human plasma is quite complex: mature PCSK9 seems to preferentially associate with LDL particles, whereas the majority of furin-cleaved PCSK9 and PCSK9 multimers are found in the protein fractions of plasma. The net effects of these modification combinations on PCSK9 activity remain to be determined.

Finally, PCSK9 must be eventually cleared from the circulation. The turnover of PCSK9 is rapid, with a half-life of ~5 min. This appears to be primarily mediated by the hepatic LDL receptor, as injected PCSK9 accumulates primarily in the liver, and clearance of injected PCSK9 was increased ten-fold in mice lacking the LDL receptor. However, there is nonetheless some disappearance of injected PCSK9 from the plasma of these Ldlr-deficient mice, suggesting the existence of a LDL receptor-independent mode of PCSK9 clearance.

Nephrotic Syndrome-Associated Hyperlipidemia

Podocyte Damage in Nephrotic Syndrome

One of the primary functions of the kidney is to filter unwanted small molecules out of the bloodstream to be excreted in the urine. This filtration process occurs in the nephron, the basic unit of the kidney. Blood enters the nephron in the glomerulus, where small solutes are filtered from the circulation into the tubules of the nephron. As this filtrate travels through the proximal tubule, loop of Henle and then distal tubule, desired solutes are reabsorbed by the epithelial cells that comprise these segments of the nephron. Unwanted solutes remain in the filtrate, which is concentrated into urine in the collecting duct, and eventually excreted from the body.

Within the glomerulus, the initial filtration of solutes is a complex process involving multiple specialized cells. The glomerular filtration barrier is composed of three parts: a fenestrated
endothelium, an intermediary glomerular basement membrane, and specialized epithelial cells known as podocytes, which wrap around the glomerular capillary. Podocytes are so named for their extensive foot processes, elaborate cytoskeleton protrusions which interdigitate with foot processes from other podocytes to create a small filtration slit between the cells. This filtration slit is covered by a slit diaphragm, made up of extracellular proteins on the podocyte cell surface (Figure 1.5). It is this slit diaphragm which appears to create a physical barrier preventing the passage of the protein albumin and other similar plasma macromolecules into the urinary filtrate.86

Damage to podocytes, either by genetic or environmental causes, can lead to disruptions in the slit diaphragm and allow plasma proteins to enter the filtrate. In sufficient quantities, these proteins are unable to be reabsorbed by the rest of the nephron and are subsequently excreted in the urine, a condition known as proteinuria. High-grade proteinuria (excretion of 3 g protein/24 hr), hypoalbuminemia (<2.5 mg/dL, due to loss of albumin in the urine), and edema are diagnostic of the disease nephrotic syndrome. Nephrotic syndrome is a relatively rare kidney disease, with ~3 new cases per 100,000 adults per year and 1-7 new cases per 100,000 children per year.87, 88 Nephrotic syndrome can be classified into different disease subtypes, such as minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS) and membranous nephropathy (MN), based on the glomerular damage observed on renal biopsy. Primary nephrotic syndrome is often caused by genetic mutations in podocyte-specific proteins, underscoring the central role of the podocyte in nephrotic syndrome. A variety of systemic diseases, such as diabetes, Lupus erythematosus and viral infections, can result in secondary nephrotic syndrome.88
Figure 1.5. The glomerular filtration barrier.
Plasma filtration in the kidney occurs predominantly in the renal cortex (A). Blood enters the glomerulus, the site of filtration, via afferent arterioles (B). In the glomerular capillary (C), the filtration barrier is composed of fenestrated endothelial cells, covered by a basement membrane which supports podocytes, specialized epithelial cells. Foot processes of adjacent podocytes interdigitate to form a filtration slit. This filtration slit is covered by a slit diaphragm composed of podocyte proteins, which creates a physical barrier normally excluding proteins from passing into the filtrate (D). From 89.
**Hyperlipidemia in Nephrotic Syndrome**

Patients with nephrotic syndrome often have an associated secondary hyperlipidemia. This is generally characterized by increased plasma cholesterol, LDL cholesterol, small dense LDL, and plasma triglycerides.\(^90\)\(^{,}\)\(^91\) Absolute HDL cholesterol is usually unchanged, but the ratio of HDL to total cholesterol is often decreased, and HDL maturation can also be impaired.\(^92\) Subsequently, nephrotic patients are at increased risk of developing cardiovascular disease, and six times more likely to have a myocardial infarction and three times more likely to die from coronary heart disease.\(^93\)

Importantly, there is an urgent need for effective lipid-lowering therapies for patients with nephrotic syndrome. Statins are widely effective in lowering LDL cholesterol in other patient populations; while they show some efficacy in limited trials of small numbers of nephrotic patients,\(^94\)\(^{-}\)\(^96\) they fail to show improvements in LDL cholesterol in rigorous meta-analysis.\(^97\) Additionally, no other lipid-lowering therapy, such fibrates, has shown benefit via randomized clinical trial in this patient population.\(^97\)\(^,\)\(^98\)

**Mechanisms of Hyperlipidemia in Nephrotic Syndrome**

The molecular mechanisms underlying and their relative contributions to the hyperlipidemia in nephrotic syndrome remain unclear. Previous studies in humans and animal models of nephrotic syndrome suggest that increased production and/or decreased clearance of ApoB-containing lipoproteins are involved. These data are often contradictory, which could be due to several factors which make studying the hyperlipidemia of nephrotic syndrome challenging. First, different mechanisms may be involved depending on the specific hyperlipidemic phenotype and/or severity of nephrotic syndrome studied. For example, one study comparing nephrotic patients with only hypercholesterolemia with nephrotic patients who were both hypercholesterolemic and hypertriglyceridemic found that LDL clearance was decreased in hypercholesterolemic patients, while patients with combined hypercholesterolemia and
hypertriglyceridemia showed increased production of LDL. Second, although podocyte injury underlies all nephrotic syndrome, the specific damage, resulting phenotype and effective treatment all vary between the different subtypes of nephrotic syndrome (MCD, FSGS, MN, etc.); such differences could also affect plasma lipid metabolism. Finally, studies in animal models of nephrotic syndrome show that the regulation of lipid metabolism varies over the time course of the disease, suggesting that disease duration may also influence the relative importance or presence and absence of specific mechanisms contributing to nephrotic syndrome hyperlipidemia.

The lipoprotein overproduction hypothesis is perhaps the widest-known theory for nephrotic syndrome hyperlipidemia. It was originally proposed in 1960 based on data that rat liver slices from nephrotic rats showed increased secretion of ApoB-containing lipoproteins compared to control rats. The additional observation that ApoB secretion from cultured hepatocytes is stimulated by decreasing concentrations of albumin and other molecules which affect media viscosity led to the theory that the liver senses either hypoalbuminemia, decreased oncotic pressure or decreased viscosity in the plasma and responds by increasing secretion of albumin and other liver-specific proteins, including ApoB-containing lipoproteins. This theory is supported by the evidence that infusion of either albumin or other macromolecules in vivo to increase plasma albumin or oncotic pressure results in decreased plasma VLDL and LDL in both nephrotic patients and rats, as well as that plasma cholesterol is significantly inversely correlated with both plasma albumin and plasma oncotic pressure in nephrotic patients. Data from some recent kinetics studies of lipoprotein metabolism in patients with nephrotic syndrome using stable isotopes or radiolabeled tracers also partially supports this idea. Increased production of VLDL or LDL has been found in several studies. Increased production of LDL could result from either direct secretion of low density ApoB-containing particles, or from conversion of VLDL particles to LDL; in most of these studies, the relative
contribution of these two processes, and thus whether ApoB secretion is increased, is unclear. Furthermore, in several studies the rate of albumin secretion was not correlated with the rate of VLDL or LDL secretion in nephrotic patients, suggesting that hypoalbuminemia may not drive ApoB secretion in nephrotic syndrome in humans.

Kinetic studies of nephrotic patients have also documented decreases in clearance of VLDL and IDL particles. Analogous to increased production of lipoproteins, decreased clearance of VLDL and IDL could be due to either decreased conversion of VLDL to IDL and IDL to LDL, respectively, by lipases, or by decreased receptor-mediated removal of these particles from the circulation. In studies where these two possibilities can be distinguished, delayed VLDL clearance is due to decreased lipolysis, whereas conflicting results have been found for clearance of IDL. Lipolysis of VLDL and IDL particles is primarily mediated by LPL and hepatic lipase, respectively. Decreased expression and/or activity of both enzymes have been documented in animal models of nephrotic syndrome. Patients with nephrotic syndrome have also been shown to have decreased LPL and/or hepatic lipase activity, although this has not been found in all human studies.

Finally, several studies in humans have shown that LDL clearance can be decreased in nephrotic patients, and that this defect is due to decreased receptor-mediated clearance of LDL. This is consistent with several models of nephrotic syndrome in rats. Imai rats, which develop spontaneous focal glomerulosclerosis and renal insufficiency, have increased plasma cholesterol and LDL cholesterol compared to controls. Consistent with this, hepatic LDL receptor protein levels are also decreased 2.5-fold in these rats. Hepatic cholesterol metabolism has been best studied in the puromycin aminonucleoside model of nephrotic syndrome. Puromycin aminonucleoside causes podocyte injury via DNA damage induced by reactive oxygen species, resulting in podocyte effacement or glomerulosclerosis resembling MCD or FSGS, respectively, depending on the dose used. Rats made nephrotic by puromycin
aminonucleoside treatment show sustained increases in plasma total cholesterol and LDL cholesterol. During the initial increase in plasma cholesterol, hepatic LDL receptor protein is unchanged, but is later significantly decreased. Furthermore, LDL receptor protein was significantly correlated with plasma LDL cholesterol at this timepoint. Importantly, hepatic Ldlr mRNA and Ldlr transcription were not changed at this time, thus implicating post-translational degradation of LDL receptor protein as an important regulator of LDL cholesterol in this model of nephrotic syndrome. The mechanisms responsible for such post-translational degradation, however, were not investigated. LDL receptor protein is not changed in hypercholesterolemic Nagase rats with hereditary analbuminemia, suggesting that podocyte injury and/or its sequelae other than hypoalbuminemia is responsible for the decreased LDL receptor protein observed in experimental nephrosis.

Hyperlipidemia in Lipodystrophy

Clinical Characteristics of Lipodystrophy

Patients with lipodystrophy often exhibit hyperlipidemia secondary to the primary defect of selective loss of adipose tissue (lipoatrophy). There are multiple underlying causes of lipodystrophies, which can be categorized according to whether the adipose loss is generalized and most adipose depots in the body are affected, or partial, wherein adipose is mostly lost from limbs with varying concomitant lipo hypertrophy in the trunk, face, neck and legs. Lipodystrophies are further grouped according to whether they are congenital, arising from genetic mutations and generally presenting very early in life, or acquired, which manifest later in life.

Lipodystrophies are in general extremely rare. The most common form is associated with patients with human immunodeficiency virus (HIV) treated with protease inhibitors as part of highly active antiretroviral therapy, and affects ~100,000 patients in the United States. Other lipodystrophies, which the rest of this dissertation will focus on, are much rarer, with
approximately 300 cases of congenital generalized lipodystrophy (CGL), 350 cases of familial partial lipodystrophy (FPL), 100 cases of acquired generalized lipodystrophy (AGL) and 250 cases of acquired partial lipodystrophy (APL) reported in the literature to date.124, 125

Lipodystrophy results in a constellation of secondary metabolic abnormalities which vary somewhat depending on the underlying disorder and are generally correlated in severity with the extent of lipoatrophy.125 Patients with lipodystrophy tend to have severe insulin resistance, diabetes, and hepatic steatosis which can progress to cirrhosis.122, 126-129

Hyperlipidemia is a key additional complication of lipodystrophy. Most lipodystrophic patients have moderate to severe hypertriglyceridemia and those with particularly severe hypertriglyceridemia can develop pancreatitis.126-131 HDL cholesterol is commonly decreased.129, 130, 132 Total cholesterol and LDL cholesterol are usually normal, but can be elevated in some cases.131, 133-136 Moreover, LDL clearance can be decreased in lipodystrophic patients.137 Consequently, patients with lipodystrophy have increased incidence of cardiovascular disease.130, 132

**Functions of Adipose Tissue**

Many of the phenotypes seen in lipodystrophy can be traced back to loss of adipose tissue function. The canonical functions of adipocytes are energy storage and mechanical support. Energy in the adipocyte is efficiently stored as triglycerides which are extremely energy dense. These triglycerides can be cleaved into free fatty acids and released into the circulation for use as energy by many organs in the body. Mechanically, adipose tissue also functions to protect fragile organs and shield parts of the body which subjected to high levels of mechanical stress.138, 139 In recent decades, several novel and increasingly appreciated functions of adipose tissue have been discovered. In addition to adipocytes, adipose tissue contains a high number of immune cells such as macrophages, B and T lymphocytes, which can have important systemic effects. In settings of excess caloric intake, macrophages secrete substantial amounts
of Tumor Necrosis Factor alpha (TNFα) and other pro-inflammatory cytokines, which decrease insulin sensitivity in other metabolic organs in the body.\textsuperscript{139-141}

The adipose tissue is increasingly appreciated as an endocrine organ that secretes hormones or cytokines referred to as “adipokines” which stimulate changes in other metabolic organs in the body. Many important adipokines have been identified to date, including adiponectin, resistin, and RBP4, but the archetypal adipokine is leptin. Leptin was originally identified as the gene mutated in the profoundly obese \textit{ob/ob} mouse and expressed in adipose tissue;\textsuperscript{142} it was soon after shown to be a secreted protein which was specifically expressed in the adipocyte of adipose tissue.\textsuperscript{143} Although other tissues express the 16 kDa protein to some extent, the adipocyte is functionally the most important source of circulating leptin.\textsuperscript{139} Additionally, systemic leptin concentrations are proportional to the amount of white adipose tissue present in the body,\textsuperscript{144} and lipodystrophic patients are generally hypoleptinemic, having decreased plasma leptin concentrations.\textsuperscript{145}

The importance of leptin in mediating the pathophysiology of lipodystrophy was elegantly demonstrated in the A-ZIP/F1 mouse model of lipodystrophy. In these mice, adipocyte-specific expression of the A-ZIP/F1 protein blocks the activity of transcription factors necessary for adipogenesis, resulting in a mouse lacking all white adipose tissue.\textsuperscript{146} Similar to lipodystrophic humans, these mice are hyperphagic, diabetic and show increases in plasma and hepatic triglycerides and decreases in leptin.\textsuperscript{146} Transplantation of normal white adipose tissue ameliorates these phenotypes, as does leptin infusion.\textsuperscript{147,148} In contrast, transplantation of white adipose tissue from \textit{ob/ob} mice does not improve any of these metabolic sequelae, and there is no additional benefit of \textit{ob/ob} white adipose tissue transplantation in addition to leptin infusion as compared to leptin infusion alone.\textsuperscript{147} Taken together, these data suggest that the predominant defect in lipodystrophy is hypoleptinemia.
Leptin Treatment in Lipodystrophy and Leptin Deficiency

The importance of leptin in the pathology of human lipodystrophy has been established by the use of recombinant leptin as a treatment for the disease. Leptin treatment has many beneficial effects in this patient population. Concomitant with increased plasma leptin concentrations, leptin treatment can reduce appetite, decrease energy expenditure, decrease blood glucose and improve glycemic control, and reduce hepatic steatosis in lipodystrophic patients.

Leptin treatment also improves dyslipidemia in these patients. Dramatic decreases in hypertriglyceridemia are commonly observed. Hypercholesterolemia is also often improved. HDL cholesterol is generally not affected by leptin treatment. LDL cholesterol, on the other hand, is often decreased. Understanding the endogenous effects of leptin is important for determining the molecular mechanisms underlying these improvements in metabolic abnormalities.

Leptin Function

Many of the physiological functions of leptin have been elucidated by studying the ob/ob mouse and the complementary db/db mouse, which lacks functioning leptin receptors, either in comparison to control mice or as a null background on which the effects of exogenous leptin can be examined. Both ob/ob and db/db mice are severely hyperphagic, which led to investigations showing that leptin is a potent suppressor of feeding activity. Leptin can cross the blood-brain barrier, where it binds leptin receptors in the arcuate nucleus, stimulating anorexigenic pro-opimelanocortin (POMC) neurons and suppressing orexigenic agouti-related peptide (AgRP) and neuropeptide (NPY) neurons and eventually inhibiting feeding behavior. Leptin can also regulate energy homeostasis by increasing energy expenditure by stimulating thermogenesis in brown adipose tissue. Thus, treatment of ob/ob mice with leptin leads to a rapid reduction in food intake, increased energy expenditure, and decreased body weight.
Leptin also has profound effects on other peripheral metabolic tissues that can be independent of its effects on food intake and body weight. In the muscle, leptin stimulates fatty acid oxidation and promotes glucose uptake. In white adipose tissue, leptin decreases lipogenic and increases lipolytic gene expression. Leptin also inhibits secretion of insulin and glucagon from the pancreas.

Finally, leptin also has profound effects on glucose and lipid metabolism in the liver. Leptin signaling in the brain suppresses hepatic gluconeogenesis both dependent and independent of insulin, but independent of changes in body weight or food intake. Leptin also decreases hepatic triglyceride content, decreasing expression of lipogenic genes, such as fatty acid synthase (Fasn) and stearoyl-CoA desaturase-1 (Scd1), and increasing expression of fatty acid oxidation genes, such as carnitine palmitoyl transferase (Cpt1a) in the liver. SCD1 in particular appears to be a key mediator of leptin’s suppression of hepatic triglyceride levels, as Scd1 mutations in leptin-deficient or lipodystrophic mice protect against the increases in hepatic triglycerides seen in these models. Importantly, the effects of leptin on hepatic triglycerides are mediated via the central nervous system, and can be independent of leptin’s effects on food intake and body weight. On the other hand, leptin treatment does not appear to affect hepatic cholesterol levels.

Finally, leptin can also affect plasma lipid levels. Leptin treatment reduces plasma triglycerides via the central nervous system but independent of effects on food intake, likely through several mechanisms including decreased hepatic lipogenesis which decreases secretion of triglyceride-rich lipoproteins and increased clearance of plasma triglycerides via lipolysis. The mechanisms via which leptin treatment decreases plasma cholesterol metabolism, particularly that of ApoB-containing lipoproteins, have been less studied. Leptin treatment of leptin-deficient ob/ob mice decreases plasma and HDL cholesterol by normalizing the observed decreased clearance of HDL. However, leptin regulation
of LDL cholesterol and clearance of LDL particles is unclear. Chronic leptin deficiency as in
\textit{ob/ob} mice results in conflicting reports on both LDLR\textsuperscript{170-173} and PCSK9\textsuperscript{170, 172} expression. Moreover, chronic decreases in leptin result in a myriad of compensatory effects on hormonal regulation, including several known to affect PCSK9 and LDLR.\textsuperscript{172} Understanding how regulators of LDL cholesterol such as PCSK9 are affected by acute leptin treatment is therefore an important step to understanding the beneficial effects this hormone has on LDL cholesterol in lipodystrophic humans.

\section*{Overview of the Dissertation}

PCSK9 has emerged over the past decade as an important regulator of plasma cholesterol. The recently-approved PCSK9 inhibitors represent an exciting treatment option for normalizing hypercholesterolemia and reducing the risk of cardiovascular disease. Inhibition of PCSK9 in humans, however, necessitates further understanding of the regulation of PCSK9, and its contribution to the multitude of diseases in which hyperlipidemia occurs secondary to the underlying disease pathology. This knowledge will help ensure that PCSK9 inhibition in patients is both safe and effective.

This dissertation addresses this important clinical problem by research into two different diseases associated with hyperlipidemia. In Chapter 2, the role of PCSK9 in nephrotic syndrome-associated hypercholesterolemia and the regulation of PCSK9 by podocyte injury are investigated. The effects of the hormone leptin on PCSK9 and implications for leptin treatment of lipodystrophic patients are evaluated in Chapter 3. In both chapters, mouse models are used for mechanistic determination of PCSK9 regulation in combination with studies in humans to investigate the potential relevance of such findings. The data provided in this dissertation not only shed important light on the role and regulation of PCSK9 in different disease contexts, but also reveal novel mechanisms of inter-organ communication in the control of metabolic homeostasis.
Chapter 2 - The Role of Proprotein Convertase Subtilisin/Kexin Type 9 in Nephrotic Syndrome-Associated Hypercholesterolemia
Abstract

Background
In nephrotic syndrome, damage to the podocytes of the kidney produces severe hypercholesterolemia for which novel treatments are urgently needed. Proprotein convertase subtilisin/kexin type 9 (PCSK9) 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 and Results
Both nephrotoxic serum (NTS) treatment, which induces immune-mediated damage of the podocyte, and genetic ablation of the podocyte produced hypercholesterolemia and a 7- to 20-fold induction in plasma PCSK9 in mice. Conversely, patients with nephrotic syndrome showed a decrease in plasma PCSK9 and plasma cholesterol upon remission of their disease (p=0.04, n=47-50). The induction of plasma PCSK9 in nephrotic syndrome appeared to be due 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% decrease in plasma cholesterol and triglycerides. Moreover, the ability of NTS treatment to increase the proportion of LDL-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 reduces plasma lipids in a mouse model of nephrotic syndrome. These data suggest that PCSK9 inhibitors may be beneficial in patients with nephrotic syndrome-associated hypercholesterolemia.
Introduction

The association between podocyte dysfunction and hypercholesterolemia can be traced back to 1830, when it was recognized that the plasma of nephrotic patients was so hyperlipidemic as to appear milky.\textsuperscript{174} However, even today, definitive treatments for nephrotic syndrome-associated hypercholesterolemia are lacking\textsuperscript{97} and nephrotic patients suffer a six-fold increased risk of cardiovascular disease.\textsuperscript{93}

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 due to both the increased production and decreased clearance of lipoprotein particles, particularly the ApoB-containing particles (VLDL, IDL and LDL).\textsuperscript{99, 107-109, 112, 118} Hepatic LDL receptor (LDLR), which removes ApoB-containing particles from the plasma, is reduced at the protein, but not mRNA, level.\textsuperscript{100, 119, 175}

Proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged over the past decade as a post-transcriptional regulator of the LDL receptor, and several studies have suggested an association between PCSK9 and renal function.\textsuperscript{175-180} 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,\textsuperscript{181} and PCSK9 inhibitors are effective in reducing LDL cholesterol.\textsuperscript{43} 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, particularly ApoB-associated cholesterol; moreover, knockout of PCSK9 lowered plasma cholesterol and produced a more benign lipoprotein profile.
Methods

Further information can be found in Supplement 1, Supplemental Methods.

Human samples
We identified 50 patients who were enrolled in the Nephrotic Syndrome Study Network (NEPTUNE)$^{182}$ as of October 2013 and had at least one plasma sample from the initial study visit during active disease (defined as urine protein/creatinine ratio $\geq 1$) as well as from first follow-up study visit during which remission was achieved (defined as urine protein/creatinine ratio $< 0.5$). 38% of the subjects were on immunosuppression therapy at baseline. Additionally, 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 Supplement 1, Supplemental Methods for additional information). Plasma PCSK9 concentrations were measured via ELISA (CycLex CY-8079).

Mice
C57BL/6J mice were obtained from the Jackson Laboratory. $Pcsk9^{\text{flox/flox}}$ mice on a C57BL/6N background, which contain loxP sites in the introns between exons one & two, and exons three & four, were a generous gift from Merck & Co., and were crossed to Albumin-Cre mice on a C57BL/6N background to generate PCSK9 L-KO mice. Pod-ATTAC mice have been previously described.$^{183}$ PCSK9-KO mice and their approximate B6129SF2/J controls were also purchased from the Jackson Laboratory and intercrossed for at least four generations.

Nephrotic models
Nephrotoxic serum was used as previously described.$^{184}$ Five- to eight-week-old male mice were preimmunized with 100 μL sheep IgG:Complete Freund’s Adjuvant s.c., then injected with either 50 μL nephrotoxic serum (NTS) or normal sheep serum (Vehicle) daily for three days. Mice were sacrificed in the non-fasted state three to four days after the first NTS or Vehicle injection. Pod-ATTAC mice and their controls were injected with 0.5 μg/g BW of AP20187
(MedChemExpress or Clontech), and sacrificed seven days after injection in the non-fasted 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. Briefly, equal volumes of plasma collected at the time of sacrifice from 4-9 mice per group were pooled. The cholesterol content of the pooled plasma was measured and approximately 15 μg of total cholesterol was diluted to a final volume of 120 μL using phosphate-buffered saline 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 OD_{500nm}, which was proportional to the cholesterol concentration, was converted to an electrical signal (Response, measured in millivolts) and continuously monitored. Raw traces of Response as a function of time therefore show the relative amount of cholesterol in each fraction, and are shown in Figure S1.1-S1.4.

For Figures 2.2-2.5, the percentage of the total area under the cholesterol curve corresponding to each lipoprotein fraction detected (VLDL, LDL, HDL and little HDL) was calculated. For presentation in the pie charts, HDL represents HDL + little HDL. Little HDL made up 3%, 1%, 2%, and 4% of cholesterol in plasma from vehicle-treated B6 mice, vehicle-treated WT mice, vehicle-treated PCSK9-KO mice, and vehicle-treated Flox mice, respectively, and was not present in other samples.

**Urine analysis**
Spot urine was collected the morning of sacrifice, and 2 μL was subjected to SDS-PAGE and Coomassie staining.
**PCSK9 clearance assay**
Pod-ATTAC and Control mice were injected retro-orbitally with 0.4 μg human recombinant PCSK9 seven days after AP20187 injection. Plasma samples were collected 1 to 121 minutes after PCSK9 injection. For each mouse, the plasma concentration of human recombinant PCSK9 at t = 1 minute was set to 100%. Human recombinant PCSK9 concentrations were plotted against time, and fitted to a one-phase exponential decay curve using GraphPad Prism, such that \( N(t) = (N_0 - \text{Plateau})e^{-\lambda t} + \text{Plateau} \). The half-life \((t_{1/2})\) was calculated by the formula \( t_{1/2} = \frac{\ln(2)}{\lambda} \).

**Gene expression**
Gene expression was measured by real-time PCR and normalized to Tbp.

**Protein analysis**
Protein levels in liver and plasma were measured by western blotting or ELISA. The Sec24a antibody used for western blotting in this study was a kind gift from Dr. David Ginsberg, 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 ELISA.

**Statistical Analysis**
Human studies: Paired two-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 two-sided alpha-level of 0.05. IBM SPSS (version 21.0) software was used for all analyses. Data in the text are presented as mean ± St.Dev.

Mouse studies: Differences between groups were assessed by a two-tailed Mann-Whitney test at a significance level of \( p < 0.05 \); similar results were obtained using a two-tailed unequal variance Student’s 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 S.E.M. 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 subjects (30% membranous nephropathy, 28% minimal change disease, 10% focal segmental glomerulosclerosis or 11% other, Table S1.1 found in Supplement 1) enrolled in the Nephrotic Syndrome Study Network (NEPTUNE) at two time points: during active disease (defined as urine protein/creatinine ratio (UPCR) > 1 mg/mg) and upon remission (UPCR < 0.5 mg/mg). At baseline, patients were proteinuric, hypertriglyceridemic and hypercholesterolemic. The UPCR was significantly correlated with total cholesterol ($r_s = 0.496$), LDL cholesterol ($r_s = 0.398$) and HDL cholesterol ($r_s = 0.321$, Table 2.1).

Upon remission, hyperlipidemia resolved (Table 2.1). 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 2.1). The change in PCSK9 was correlated with the changes in total, LDL, and HDL cholesterol, though the correlation with LDL cholesterol did not quite reach significance ($p=0.051$, Table 2.1). Subgroup analysis revealed that minimal change disease was associated with the largest decreases in total cholesterol and plasma PCSK9 upon remission (data not shown).

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 immunological attack on the mouse glomerulus. During the acute phase, NTS
antibodies bind to the podocyte cell membrane, resulting in podocyte injury and severe proteinuria\textsuperscript{185, 186} that persist as the lesion progresses to glomerular inflammation and crescent

<table>
<thead>
<tr>
<th>Clinical Variables</th>
<th>Baseline*</th>
<th>Remission*</th>
<th>P-value‡</th>
</tr>
</thead>
<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>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>298.0 (107.8)</td>
<td>197.5 (60.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>178.2 (91.1)</td>
<td>101.0 (44.7)</td>
<td>&lt;0.0001</td>
</tr>
<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>
</tr>
</tbody>
</table>

**Correlations between UPCR and lipid parameters at baseline**

<table>
<thead>
<tr>
<th>Lipid parameter</th>
<th>Correlation Coefficient‡</th>
<th>P-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>0.496</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>0.398</td>
<td>0.006</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>0.321</td>
<td>0.028</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>0.189</td>
<td>0.203</td>
</tr>
</tbody>
</table>

**Correlations between change in PCSK9 and change in lipid parameters**

<table>
<thead>
<tr>
<th>Lipid parameter</th>
<th>Correlation Coefficient‡</th>
<th>P-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>0.384</td>
<td>0.006</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>0.277</td>
<td>0.051</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>0.462</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>0.076</td>
<td>0.602</td>
</tr>
</tbody>
</table>

n = 47-50
*Data represent means and SD
†Paired T tests
‡Spearman correlations on nontransformed data
formation after two to three weeks. Changes in cholesterol metabolism also evolve during the course of nephrotic disease; in this model, maximal hypercholesterolemia was observed four days after NTS injection (Figure S1.1A).

We therefore injected wildtype C57BL/6J (B6) mice with NTS or vehicle and sacrificed them four days after the initial NTS injection. At this time, B6 mice were markedly proteinuric and dyslipidemic (Figure 2.2A-D). Plasma triglycerides were increased four-fold and plasma cholesterol levels were increased five-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 2.2D; Figure S1.1B). Consequently, the proportion of cholesterol associated with the ApoB-containing lipoproteins increased from 14% to 55%.

Consistent with prior studies, hyperlipidemia in NTS-treated B6 mice was associated with a reduction in LDL receptor protein (Figure 2.2E). The degradation of the LDL receptor is regulated by both the E3 ubiquitin ligase Idol (Inducible degrader of the LDL receptor) and
Interestingly, both *Ldlr* and *Idol* mRNA levels were decreased in the livers of NTS-treated mice (Figure 2.2F).

PCSK9, on the other hand, was increased sixteen-fold in the plasma of NTS-treated mice (Figure 2.2G). Plasma PCSK9 is derived primarily from the liver. Though hepatic *Pcsk9* mRNA levels were increased (Figure 2.2H), the increase was modest, approximately 50%, suggesting
that increased \textit{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 two-fold (Figure S1.1C). The modest changes in \textit{Pcsk9} mRNA and PCSK9 clearance compared to the large change in plasma PCSK9 suggested that PCSK9 secretion might also be increased, and that post-transcriptional regulation of PCSK9 could be involved. Consistent with this, cIAP1, which is required for PCSK9 processing and secretion\textsuperscript{65} and Sec24a, which facilitates packaging of PCSK9 into COPII vesicles for secretion\textsuperscript{73} were increased (Figure 2.2I). Sortilin, another protein which appears to enhance PCSK9 secretion\textsuperscript{74} on the other hand, was not changed (Figure 2.2I).

To specifically dissect the role of the podocyte in the regulation of plasma PCSK9, we also studied Pod-ATTAC (Podocyte Apoptosis Through Targeted Activation of Caspase-8) 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\textsuperscript{188}. In these mice, the transgene is driven by the podocin (\textit{Nphs2}) promoter, which is specific to podocytes\textsuperscript{183}; therefore, injection of these mice with AP20187 results in selective ablation of the podocyte.

We injected AP20187 into mice heterozygous for the Pod-ATTAC transgene (Pod-ATTAC mice), as well as their wildtype littermates (Controls), and studied mice seven days later, at the point of maximal plasma cholesterol levels (data not shown). The phenotype of the Pod-ATTAC mice was remarkably similar to that of the NTS-treated mice (Figure 2.3). 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 due primarily to an increase in LDL-associated cholesterol (Figure 2.3A-D, Figure
Figure 2.3. Podocyte apoptosis increases plasma PCSK9.

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. 2 μL of spot urine collected the morning of sacrifice was subjected to SDS-PAGE and Coomassie Blue staining (A). Plasma taken at the time of sacrifice was used to measure triglycerides (B), total cholesterol (C) and PCSK9 (G), or subjected to size exclusion chromatography for lipoprotein analysis. Brackets indicate the percentage of total cholesterol found in ApoB-containing lipoproteins (VLDL + LDL) (D). Hepatic protein was measured by western blotting liver whole-cell lysates (E, J). Hepatic gene expression was measured by real-time PCR (F, H). PCSK9 clearance was measured as described in Methods (I). n=8-10. For lipoprotein analysis, equal amounts of plasma from 4-6 mice were pooled from each group.
Both models also showed a decrease in hepatic LDL receptor protein, though Pod-ATTAC livers showed no changes in Ldlr, Idol, or Pcsk9 mRNA (Figure 2.3E, F, H). Plasma PCSK9 was increased seven-fold (Figure 2.3G) 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 (Figure 2.3I). Finally, cIAP1 and Sortilin were increased in Pod-ATTAC livers, though Sec24a was not (Figure 2.3J).

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 treated mice with global knockout (PCSK9-KO) or liver-specific knockout of PCSK9 with NTS.

PCSK9-KO mice and their wildtype (WT) controls were maintained on the same mixed genetic background. NTS treatment of WT mice led to marked proteinuria, with a seven-fold increase in plasma triglycerides, a five-fold increase in plasma cholesterol, and an eight-fold increase in plasma PCSK9, with no change in liver Pcsk9 mRNA (Figure S1.3A, Figure 2.4A-D). 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 due primarily to an increase in VLDL, rather than LDL, cholesterol (Figure 2.4E, Figure S1.3B). Nonetheless, plasma levels of ApoB100 and ApoB48 increased markedly upon NTS treatment in WT mice (Figure 2.4F). In parallel, LDL receptor protein was decreased (Figure 2.4G).

Knockout of PCSK9 had no effect on renal histology or urine protein levels, either in the presence or absence of NTS treatment (Figure S1.3A and data not shown). On the other hand,
Figure 2.4. Knockout of PCSK9 reduces plasma cholesterol in the presence and absence of NTS.

Five- to eight-week-old wild type (WT) or PCSK9 global 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. Plasma taken at the time of sacrifice was used to measure triglycerides (A), cholesterol (B) and PCSK9 (C) or subjected to size exclusion chromatography for lipoprotein analysis. Brackets indicate the percentage of total cholesterol found in ApoB-containing lipoproteins (VLDL + LDL) (E). Hepatic gene expression was measured by real-time PCR (D, H). Protein levels were measured by western blotting plasma (F) or liver whole-cell lysates (G). n=4-7; * p < 0.05; N.M. not measured. For lipoprotein analysis, equal amounts of plasma from 4-6 mice were pooled from each group.
PCSK9-KO mice showed profound changes in lipoprotein metabolism, with reductions in total cholesterol, ApoB100, and ApoE (Figure 2.4B, F), consistent with prior studies.189

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 2.4A, B). 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 2.4E-G, Figure S1.3B). Ldlr mRNA and Idol mRNA were not significantly changed by NTS treatment in either WT or PCSK9-KO mice (Figure 2.4H).

In parallel, we examined mice with liver-specific knockout of PCSK9 (Cre+/−Pcsk9floxflox, PCSK9 L-KO). These mice were generated using Cre−/−Pcsk9floxflox (Flox) mice and mice expressing Cre recombinase under the control of the albumin promoter. The PCSK9 L-KO mice were compared to their littermate Flox controls, both on the C57BL/6 background.

NTS treatment of Flox mice produced a two-fold increase in Pcsk9 mRNA levels that did not reach significance, a 24-fold increase in plasma PCSK9, a seven-fold increase in plasma triglycerides, and a four-fold increase in plasma cholesterol (Figure 2.5A-D). Here, as in wildtype B6 mice, NTS treatment produced a profound redistribution of plasma cholesterol, with LDL-associated cholesterol increased from 9% to 47% (Figure 2.5E, Figure S1.4A). LDL receptor protein was decreased by NTS treatment, but Ldlr and Idol mRNA levels were not (Figure S1.4B,C).

In PCSK9 L-KO mice, plasma PCSK9 was barely detectable in vehicle-treated mice, consistent with prior reports that plasma PCSK9 is derived primarily from the liver.23 Interestingly, NTS treatment of PCSK9 L-KO mice nonetheless produced a three-fold increase in plasma PCSK9 (p=0.004) (Figure 2.5B insert). To explore this further, we profiled Pcsk9 mRNA in the other
Abstract

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 three-fold increase in renal Pcsk9. Interestingly, in situ hybridization revealed that Pcsk9 was induced not in podocytes or other intra-glomerular cells, but rather in a subset of tubule cells (Figure S1.5A, B).

Despite this, the effects of liver-specific and global PCSK9 knockout were quite similar, and both showed a significant 50-60% reduction in plasma cholesterol in the presence of NTS (Figure 2.5).
Additionally, Flox mice showed a dramatic increase in ApoB-associated cholesterol from 12% to 56% upon NTS treatment, whereas PCSK9 L-KO mice had only a modest increase in ApoB-associated cholesterol, from 11% to 16%, upon NTS treatment (Figure 2.5E, Figure S1.4A). In parallel, the reduction in LDL receptor protein observed in Flox mice was blunted in PCSK9 L-KO mice (Figure S1.4B).

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, while patients with nephrotic syndrome showed decreased levels of PCSK9 upon remission. Moreover, knockout of PCSK9 in NTS-treated mice reduced plasma triglycerides and cholesterol, particularly ApoB-associated cholesterol.

Our studies in mice showed plasma PCSK9 to be increased up to 24-fold in nephrotic syndrome models. Prior cross-sectional studies in humans showed plasma PCSK9 to be increased 50-60% in proteinuric/nephrotic patients compared to control subjects. Similarly, our longitudinal human studies showed a 14% decrease in plasma PCSK9 upon 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 due in part to the fact that 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 change 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, *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). Gene expression analysis in NTS-treated B6 livers revealed an increase in *Srebp2* and in some, but not all, of its targets, as well as an increase in the HNF1α targets *Alb*, *Fgb*, and *Serpina1*, though not in *Hnf1a* itself (Figure S1.1D). 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. Moreover, LDL seems to impair PCSK9 binding to and uptake by the LDL receptor. Thus, the decrease in plasma PCSK9 could be secondary to the decrease in the LDL receptor and increase in LDL cholesterol.

The facts that PCSK9 clearance was reduced only two-fold after podocyte injury, and that *Pcsk9* mRNA was not usually increased, imply the existence of a post-transcriptional mechanism by which podocyte injury increases PCSK9 secretion. This mechanism may involve cIAP1, as both NTS-treated and Pod-ATTAC mice showed an increase in hepatic cIAP1, which promotes PCSK9 secretion. How exactly the injured podocyte signals to the liver to increase cIAP1 has yet to be determined. One possible mediator is TNFα, which can be secreted by the podocyte, is increased in nephrotic syndrome plasma, and is known to induce cIAP1. 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 PCSK9.177

Our studies in mice with both global and liver-specific knockout of Pcsk9 showed that Pcsk9 ablation has beneficial effects in NTS-treated mice. While 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 40-60%. 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.92, 119, 195, 196 For example, acetyl-Coenzyme A acetyltransferase (ACAT)-2, which promotes cholesterol ester formation, is increased in nephrotic syndrome and required for the development of hypercholesterolemia.119, 197, 198 Similarly, scavenger receptor class B, member 1 (SR-B1), a component of the reverse cholesterol transport system which resides on the surface of the hepatocyte and removes HDL cholesterol from the plasma, is reduced in nephrotic syndrome.199, 200 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 in our nephrotic syndrome patients was likely due to the fact that 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, though not solely responsible for nephrotic syndrome-associated dyslipidemia, is nonetheless an important participant. Plasma PCSK9 is increased in
nephrotic syndrome, and \( \text{Pcsk9} \) 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 FDA for the treatment of hypercholesterolemia, may be the long-sought treatment for the dyslipidemia of nephrotic syndrome.

Acknowledgements

We thank Dr. Henry Feldman for excellent statistical support and Merck and Co. for PCSK9\(^{\text{flox/flox}}\) mice. Nephrotic patient samples were provided by the Nephrotic Syndrome Study Network Consortium (NEPTUNE).

Funding Sources

This work was funded by NIH grants R01HL109650 (S.B.B. & M.E.H.), 5K12DK094721-04 (A.E.L.), R01DK090029 (D.J.S.), American Heart Association grant 12SDG12050287 (J.M.R.), and by a Department of Defense National Defense Science & Engineering Graduate (NDSEG) fellowship to M.E.H. The Nephrotic Syndrome Study Network Consortium (NEPTUNE) was funded by NIH grant U-54-DK083912.

Attributions

This chapter has been accepted and will be published in Circulation by Wolters Kluwer Health, Lippincott Williams & Wilkins © as:


Other contributors to this work include Amy E. Levenson, Xiaowei Sun, Jordan Grant, Mansi Shah, Jie Zhang, Wan-Hui Liao, Joseph M. Rutkowski, Nephrotic Syndrome Study Network
(NEPTUNE), Sarah D. de Ferranti, Valerie A. Schumacher, Philipp E. Scherer, David J. Salant, Henry Feldman and Sudha B. Biddinger.

Human studies: Amy E. Levenson conducted research on nephrotic patient samples, including study design, plasma analysis and interpretation of results. NEPTUNE provided human samples; S.D.d.F and S.B.B. supervised study design and analysis. H.F. provided statistical support.

Mouse studies: Mary E. Haas performed all experiments with assistance from X.S., J.G., M.S. and J.Z. V.A.S., J.M.R., P.E.S. and D.J.S. provided mice or reagents and technique advice.

M.E.H., W-H.L. and S.B.B. conceived of the study. M.E.H. and S.B.B. designed the experiments, analyzed the data and wrote the manuscript.
Chapter 3 - Effect of Leptin Replacement on PCSK9 in ob/ob Mice and Female Lipodystrophic Patients
Abstract
Leptin treatment has beneficial effects on plasma lipids in patients with lipodystrophy, but the underlying mechanism is unknown. Proprotein convertase subtilisin/kexin type 9 (PCSK9) decreases LDL clearance, promotes hypercholesterolemia, and has recently emerged as a novel therapeutic target. To determine the effect of leptin on PCSK9, we treated male and female ob/ob mice with leptin for four days via subcutaneous osmotic pumps (~24 µg/day). Leptin reduced body weight and food intake in all mice, but the effects of leptin on plasma PCSK9 and lipids differed markedly between the sexes. In male mice, leptin suppressed PCSK9, but had no effect on plasma triglycerides or cholesterol. In female mice, leptin suppressed plasma triglycerides and cholesterol, but had no effect on plasma PCSK9. In parallel, we treated female lipodystrophic patients (8 females, ages 5-23 years) with subcutaneous metreleptin injections (~4.4 mg/day) for four to six months. In this case, leptin reduced plasma PCSK9 by 26% (298 ± 109 vs 221 ± 102 ng/mL; n=8; p=0.008), and the change in PCSK9 was correlated with a decrease in LDL cholesterol (r²=0.564, p=0.03). In summary, in leptin-deficient ob/ob mice, the effects of leptin on PCSK9 and plasma lipids appeared to be independent of one another and strongly modified by sex. On the other hand, in lipodystrophic females, leptin treatment reduced plasma PCSK9 in parallel with LDL cholesterol.

Introduction
Leptin plays a critical role in metabolic regulation: it decreases food intake, increases energy expenditure, enhances insulin sensitivity, and inhibits hepatic lipogenesis and fatty acid uptake. Consequently, patients with defects in leptin action, due to either a mutation in the leptin gene or a deficiency in circulating leptin, such as in lipodystrophy, manifest severe hyperphagia, insulin resistance, and hepatic steatosis. In addition, these patients exhibit dyslipidemia with hypertriglyceridemia, elevated total and low density lipoprotein (LDL) cholesterol, and reduced high density lipoprotein (HDL) cholesterol levels. In clinical studies,
leptin treatment decreases triglycerides and has also been shown to reduce LDL and total cholesterol.\textsuperscript{135, 150, 151, 201} The effect of leptin on plasma triglycerides has been ascribed to improved insulin sensitivity.\textsuperscript{127, 163} However, the mechanisms underlying the reduction of LDL are unclear. Given the central role of LDL in increasing the risk of cardiovascular disease (CVD),\textsuperscript{202} it is important to understand how leptin regulates LDL cholesterol.

One potential mediator of leptin’s effect on LDL is proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 promotes degradation of the LDL receptor (LDLR), resulting in reduced LDL clearance.\textsuperscript{22, 203, 204} Thus, gain-of-function PCSK9 variants lead to elevated levels of LDL cholesterol, whereas loss-of-function PCSK9 mutations are associated with reductions in both LDL cholesterol and CVD risk.\textsuperscript{41} Therapies targeting PCSK9 are now available and show great promise.\textsuperscript{205} Defining the endogenous regulators of PCSK9 is an important step towards understanding the biology of this important molecule.

Here, we studied the effects of leptin treatment on plasma PCSK9 in leptin-deficient ob/ob male mice, leptin-deficient ob/ob female mice, and lipodystrophic female patients. We found that the effects of leptin on PCSK9 and plasma lipids in ob/ob mice were sex-dependent, with no correlation between plasma PCSK9 and plasma cholesterol; in contrast, leptin suppressed PCSK9 in parallel with LDL cholesterol in female lipodystrophic patients.

Materials and methods

Animal Studies

Leptin Treatment of ob/ob Mice
Male and female ob/ob mice were purchased from Jackson Laboratories and acclimated for two weeks before the study. They were maintained in a twelve-hour light/dark cycle (7 a.m. to 7 p.m.) with standard chow and water ad libitum. Mice were implanted with osmotic pumps (1007D; Alzet, Cupertino, CA) containing either phosphate buffered saline (PBS) or recombinant
mouse leptin (24 μg/day; National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) for four days. Two days after pump implantation, 4-hour fasting glucose was obtained. Four days after pump implantation, mice were sacrificed in the non-fasted state at 2 p.m. All animal experimentation described above was conducted in accord with accepted standards of humane animal care, and all procedures were approved by the Institutional Animal Care and Research Advisory Committee at Boston Children’s Hospital.

**Plasma PCSK9 and Plasma Lipid Analysis**
Mouse plasma PCSK9 levels were measured by ELISA (MLB International), and plasma total cholesterol and triglyceride levels were measured by colorimetric assays (Thermo Scientific) according to the manufacturer’s instructions. Size-exclusion liquid chromatography analysis of plasma cholesterol lipoprotein distribution was performed by the Lipid, Lipoprotein and Atherosclerosis Analysis Core Laboratory at Wake Forest University.

**RNA Isolation and Gene Expression Analysis**
Total RNA from liver tissues was extracted and purified using an RNeasy kit (Qiagen). Reverse transcription was performed according to the manufacturer’s protocol (Applied Biosystems). The resulting cDNA was diluted ten-fold and used for real-time PCR analysis with SYBR green (Thermo Scientific) in an Applied Biosystems 7900 HT or 7000 instrument. Results were normalized to TATA-box binding protein (Tbp) gene or to 18s ribosomal RNA (18s). The value of the control group was set to 1. Primer sequences are listed in Table S2.1.

**Western Blotting**
Liver lysates were prepared by homogenizing 50 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, 10 mM NaF, 10 mM Na3VO4, 10 mM sodium β-glycerophosphate, and protease inhibitor (Roche) and centrifuging at 13,000 g for 10 min at 4°C. Protein was measured by a BCA assay kit (Pierce). The sample (100 μg) was subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon). After 1 hour in SuperBlock blocking buffer (Pierce), blots were incubated overnight
with a primary antibody (1:1,000 dilution). Primary antibodies used included the following (Table S2.2): SCD1 (Santa Cruz Biotechnology, sc-14720), HMGCR (Santa Cruz Biotechnology, sc-27578), FASN (Abcam, ab38844), FPDS (Abcam, ab22759), ACC (EMD Milipore, 04-322), LDLR (Abcam, ab30532, and a gift from Dr. Alan Attie), \(^{206}\) beta actin (Santa Cruz Biotechnology, sc-47778) and alpha tubulin (Cell Signaling Technology, 2125). Secondary antibodies conjugated with horseradish peroxidase (Pierce 31430 and 31460, Santa Cruz Biotechnology sc-2768) and chemiluminescent ECL reagents (Thermo Scientific) were used to develop blots. Bands were quantified by ImageJ software. All values were normalized to actin or tubulin, and the control (vehicle) ratio was set to 1.

Chromatin Immunoprecipitation (ChIP) Assays in Mouse Liver
ChIP assays were performed as previously described. \(^{207, 208}\) Briefly, mouse livers were finely minced and incubated in phosphate-buffered saline solution containing 1% formaldehyde at room temperature for 15 mins. The reaction was stopped by adding glycine to 125 mM for 5 min. Cells were resuspended in hypotonic buffer (10 mM KOH-Hepes (pH 7.9), 1.5 mM MgCl\(_2\), 10 mM KCl, 0.2% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose) and lysed by homogenization with a Dounce homogenizer. The cell lysate was layered on a sucrose cushion buffer (10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose). Nuclei were pelleted by centrifugation and resuspended in sonication buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.75% SDS). The samples were sonicated to reduce DNA length to between 200 and 1,000 bp. After centrifugation at 13,000 \( \times g \) for 10 min, 2 volumes of dilution buffer (2 mM EDTA, 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate) were added. The chromatin sample was pre-cleared followed by incubation with 2 \( \mu \)g of antisera against normal rabbit IgG (sc-2027), or polymerase II (sc-9001X) at 4 °C overnight. The immune complex was collected by centrifugation after incubation with protein A/G agarose beads (sc-2003). The beads were washed, the bound chromatin was eluted, and the genomic DNA was
purified and quantified by qPCR using primers specific to \textit{Pcsk9} and \textit{Fasn} promoters. The DNA in the immunoprecipitate was normalized to the input, with the value of the vehicle treated livers immunoprecipitated with IgG set to 1.

**Human Studies**

**Subjects**

Subjects were enrolled in an open-label, prospective study examining the effects of recombinant human methionyl leptin (metreleptin; Bristol Myers Squibb and Astra Zeneca) replacement on metabolic and hormonal derangements in patients with congenital or acquired lipodystrophy (NCT00025883). The dose of self-administered metreleptin (via subcutaneous injection) was adjusted over time for each patient to optimize triglycerides and hemoglobin A1c, while minimizing excessive weight loss. The average daily dose of metreleptin at the end of four to six months was ~4.4 mg/day. The study was approved by the National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board.

In this ancillary study, we included only patients who had available plasma samples, both pre- and post-leptin treatment, and who were not taking lipid-lowering drugs. The latter was done to avoid the confounding effects of these medications on PCSK9.

**Study Procedures**

Oral glucose tolerance tests (OGTTs) were performed at baseline and after four to six months of leptin administration. During each visit, after an overnight fast, patients underwent an OGTT with plasma samples obtained at baseline and 30, 60, 90, 120, and 180 min after glucose ingestion for the measurement of glucose, insulin, and C-peptide concentrations. Insulin sensitivity was calculated from the OGTT as the oral glucose insulin sensitivity index (OGIS), using the 2-h OGIS equation. This method provides a surrogate measure of insulin sensitivity that is an estimate of the glucose clearance during a euglycemic-hyperglycemic clamp, expressed in mL/min/m$^2$ body surface area.
Serum leptin levels were determined by immunoassay with the use of a commercial kit (Linco Research, St. Charles, MO). Human plasma PCSK9 levels were measured by ELISA (MLB International). All other measurements were done in the NIH Clinical Center laboratory using standard methodology.

Statistics

Human studies
Descriptive statistics are reported as mean ± SD. One-sample t-tests were used to assess changes from baseline to post-leptin therapy. Non-normally distributed variables were logarithmically transformed and non-parametric tests were used if normality was not attained. Associations between change in PCSK9 and change in continuous variables were assessed using Pearson correlation and testing for zero correlation. All tests were performed at two-sided alpha-level of 0.05. IBM SPSS (version 21.0) software was used for all analyses.

Mouse studies
Differences between groups were assessed by a two-tailed, unequal variance, Student’s t-test. Data are presented as the means ± SEM unless otherwise indicated. All studies were validated in a second, independent cohort.

Results

Leptin treatment of male mice suppresses PCSK9
Twelve-week old male ob/ob mice were administered leptin (24 μg/day recombinant leptin) or vehicle (PBS) subcutaneously, through an osmotic pump, for four days. As expected, leptin treatment decreased body weight and food intake, and also tended to lower blood glucose and liver weight (Figure 3.1A-D). In parallel, leptin treatment reduced plasma PCSK9 protein by 90% (Figure 3.1E). Surprisingly, however, the decrease in PCSK9 was not associated with an increase in LDLR protein: LDLR protein was decreased by 47%, Ldlr mRNA was decreased by 80%, and Inducible Degrader of the LDLR (Idol), another gene that promotes degradation of the
Figure 3.1. Leptin treatment suppressed PCSK9 expression in male ob/ob mice.

Twelve-week-old male, ob/ob mice were implanted with subcutaneous pumps containing either vehicle or leptin (24 μg/day). Body weight (A), food intake (B), and liver weights (D) were measured at time of sacrifice, four days after pump implantation. Fasting blood glucose (C) was measured after a four hour fast, two days after implantation. Plasma PCSK9 (E), triglycerides (I), and total cholesterol (J) were measured in nonfasted plasma taken at the time of sacrifice. Hepatic LDLR protein expression was measured by immunoblotting (F, left); band intensities were quantified by densitometry, normalized to tubulin, and expressed relative to vehicle-treated mice (F, right). Hepatic gene expression of Ldlr and Idol was measured by real time PCR, normalized to Tbp, and expressed relative to vehicle-treated mice (G, H). Data are presented as mean ± SEM, and are representative of two independent experiments. *P value ≤ 0.05 compared to vehicle treatment, Student’s t-test; n=3-5/group.
LDLR,\textsuperscript{33} was not altered (Figure 3.1F-H). Moreover, neither plasma triglycerides, cholesterol, nor the proportion of cholesterol present in the LDL fraction, were reduced with leptin treatment (Figure 3.1I-J, Figure S2.1).

The reduction in plasma PCSK9 was associated with a 93% reduction in hepatic \textit{Pcsk9} mRNA (Figure 3.2A). In parallel, PolII promoter occupancy of the \textit{Pcsk9} promoter was reduced by 60%, consistent with a decrease in \textit{Pcsk9} transcription (Figure S2.2). Several transcription factors have been implicated in the regulation of \textit{Pcsk9}, including Sterol Regulatory Element-Binding Protein (SREBP)-1c, SREBP-2, and Hepatocyte Nuclear Factor-1α (HNF1α).\textsuperscript{48, 211-213}

Leptin treatment decreased \textit{Srebp-1c} and its lipogenic targets, acetyl-CoA carboxylase (\textit{Acc}), fatty acid synthase (\textit{Fasn}), and stearoyl CoA desaturase 1 (\textit{Scd1}) by 50-95% (Figure 3.2B). Leptin also suppressed the cholesterologenic genes, 3-hydroxy-3-methylglutaryl-CoA reductase (\textit{Hmgcr}), farnesyl diphosphate synthase (\textit{Fdps}) and farnesyl diphosphate farnesyl transferase 1 (\textit{Fdft1}), which are targets of SREBP-2 (Figure 3.2C). \textit{Srebp-2} mRNA levels, however, were not changed with leptin treatment. Protein levels of \textit{ACC}, \textit{FASN}, \textit{SCD1}, \textit{HMGCR}, and \textit{FDPS} were also reduced in leptin-treated male \textit{ob/ob} mice (Figure 3.2D-E). In contrast, \textit{Hnf1α} and its targets were generally unchanged at the mRNA level (Figure S2.3).

\textbf{Leptin treatment of female mice fails to suppress PCSK9}

Female \textit{ob/ob} mice were similarly treated with leptin or vehicle (leptin or PBS, 24 μg/day, subcutaneously). As expected, leptin-treated female \textit{ob/ob} mice showed a 17% decrease in body weight, a significant reduction in food intake, a trend towards lower fasting blood glucose levels, and decreased liver weight (Figure 3.3A-D). Surprisingly, leptin treatment did not alter plasma PCSK9 (Figure 3.3E). LDLR protein was reduced by 63%, \textit{Ldlr} mRNA was reduced by 50%, and \textit{Idol} mRNA was unchanged (Figure 3.3F-H). Yet, leptin treatment reduced plasma triglyceride and cholesterol levels by 24-53% (Figure 3.3I-J). Furthermore, hepatic \textit{Pcsk9} mRNA
expression was not significantly changed with leptin treatment, even though the lipogenic and cholesterologenic genes were suppressed in females as in males (Figure S2.4).
Figure 3.3. Leptin did not alter PCSK9 expression in female ob/ob mice.

Twelve-week-old female ob/ob mice were implanted with subcutaneous pumps containing either vehicle or leptin (24 μg/day). Body weight (A), food intake (B), and liver weights (D) were measured at time of sacrifice, four days after pump implantation. Fasting blood glucose (C) was measured after a four hour fast, two days after implantation. Plasma PCSK9 (E), triglycerides (I), and total cholesterol (J) were measured in nonfasted plasma taken at the time of sacrifice. Hepatic LDLR protein expression was measured by immunoblotting (F, left); band intensities were quantified by densitometry, normalized to actin, and expressed relative to vehicle-treated mice (F, right). Hepatic gene expression of Ldlr and Idol was measured by real time PCR, normalized to 18s, and expressed relative to vehicle-treated mice (G, H). Data are presented as mean ± SEM, and are representative of two independent experiments. *P value ≤ 0.05 compared to vehicle treatment, Student’s t-test; n=3-6/group.
Leptin replacement decreased PCSK9 and improved lipid parameters in female lipodystrophic subjects

We further studied the effects of leptin on lipodystrophic patients. Our cohort, which was exclusively female, included patients with multiple forms of lipodystrophy (congenital generalized lipodystrophy, n=4; acquired generalized lipodystrophy, n=2; familial partial lipodystrophy, n=2), had a mean baseline age of 14 years (range 5-23 years), and a normal mean body mass index (BMI). Table 3.1 shows the characteristics of the study subjects, before and after four to six months of treatment with metreleptin (~4.4 mg/day via subcutaneous injection).

Table 3.1. Patient demographics, markers of glucose homeostasis, and lipid parameters at baseline and post-leptin treatment.

<table>
<thead>
<tr>
<th>Clinical Variables</th>
<th>Baseline</th>
<th>Post-leptin Treatment</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>18 ± 5.5</td>
<td>47.9 ± 21.2</td>
<td>0.07</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>50.9 ± 23.3</td>
<td>47.9 ± 21.2</td>
<td>0.07</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>20.8 ± 3.2</td>
<td>19.6 ± 3.4</td>
<td>0.05</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>6.4 ± 11.1</td>
<td>53.8 ± 51.2</td>
<td>0.09</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mg/dL)</td>
<td>144 ± 79</td>
<td>111 ± 41</td>
<td>0.34</td>
</tr>
<tr>
<td>Fasting Plasma Insulin (µU/mL)</td>
<td>119.4 ± 87.4</td>
<td>41.6 ± 42.5</td>
<td>0.03</td>
</tr>
<tr>
<td>2-hr OGTT Plasma Glucose (mg/dL)</td>
<td>180 ± 75</td>
<td>156 ± 71</td>
<td>0.35</td>
</tr>
<tr>
<td>Hemoglobin A1C (%)</td>
<td>7.9 ± 1.6</td>
<td>6.0 ± 1.8</td>
<td>0.04</td>
</tr>
<tr>
<td>OGIS (ml/min/m²)</td>
<td>360 ± 200</td>
<td>343 ± 155</td>
<td>0.89</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>195 ± 64</td>
<td>146 ± 38</td>
<td>0.08</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dL)</td>
<td>82 ± 46</td>
<td>68 ± 19</td>
<td>0.43</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)</td>
<td>28 ± 8</td>
<td>29 ± 6</td>
<td>0.89</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>402 ± 182</td>
<td>225 ± 89</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. aP values are shown for comparisons between the baseline and post-leptin treatment variables using a t-test. P values ≤ 0.05 are shown in bold.
Patients in this cohort showed elevated fasting plasma glucose (>100 mg/dL) and insulin levels (>15 μU/mL), as well as abnormal 2-hour plasma glucose on OGTT (>140 mg/dL) and lower OGIS measurements (<440 ml/min/m²), suggestive of insulin resistance. In addition, six out of eight patients had hemoglobin A1c levels in the diabetic range (≥ 6.5%). Lipid measurements at baseline were notable for elevated fasting triglycerides and low HDL levels, consistent with prior reports. LDL cholesterol was within the normal range, likely because patients with high LDL cholesterol would have been placed on lipid-lowering therapy, and therefore excluded from this study cohort. Normal reference ranges for laboratory measurements per the NIH Clinical Center laboratory are listed in Table S2.3.

Leptin treatment significantly decreased BMI, fasting plasma insulin levels, and hemoglobin A1c. Consistent with prior studies, leptin decreased triglycerides, total cholesterol, and LDL cholesterol by approximately 20-40%, although the results did not reach significance in this small cohort; HDL cholesterol levels were unchanged (Table 3.1). In parallel, plasma PCSK9 levels fell by more than 20% (P=0.008; Figure 3.4A). In addition, the change in

![Figure 3.4](image.png)

**Figure 3.4. Comparison of plasma PCSK9 concentrations in lipodystrophic patients at baseline and after 4-6 months of leptin treatment.**

Dashed lines connect baseline and post-leptin treatment values for individual subjects. The gray boxes denote the 75th and 25th percentiles; the horizontal lines within the boxes denote the median values; the whiskers represent the minimum and maximum values (n=8). One-sample t-tests were used to assess changes from baseline to post-leptin therapy. P=0.008. (B) Change in PCSK9 was correlated with change in LDL. Data shown are ln-transformed and analyzed using Pearson correlation.
PCSK9 was significantly correlated with the change in LDL cholesterol, but not with other variables (Figure 3.4B and Table S2.4).

Discussion

Leptin plays a central role in the regulation of energy balance, as well as in lipid metabolism. We show here that leptin suppresses plasma PCSK9 by 90% in male ob/ob mice, and by 20% in female lipodystrophic patients. In female lipodystrophic patients, the decrease in plasma PCSK9 is associated with a reduction in LDL cholesterol. However, leptin does not suppress plasma PCSK9 in female ob/ob mice.

In ob/ob males, leptin appears to suppress PCSK9 by reducing its transcription, as Pcsk9 mRNA is reduced by 90% in the liver, the predominant source of plasma PCSK9. In addition, the activity of the SREBPs, which can drive Pcsk9 transcription, appear to be reduced, as the lipogenic and cholesterologenic SREBP target genes are decreased 50-95%. Interestingly, in the livers of ob/ob females, while leptin suppresses the lipogenic and cholesterologenic targets of the SREBPs to a similar extent as in males, it does not suppress Pcsk9. These data suggest that sex hormones may modify the ability of the SREBPs to specifically activate the Pcsk9 promoter; alternatively, it is possible that the effects of leptin on Pcsk9 mRNA levels are not mediated by the SREBPs.

In any case, most of the changes in lipid metabolism produced by leptin in mice appear to be independent of PCSK9. First, leptin reduces hepatic LDLR protein in males, even as it suppresses PCSK9. We would note that the effects of leptin on LDLR appear to be very sensitive to the particular experimental paradigm used, as ob/ob mice are reported to have both increased and decreased amounts of LDLR relative to their lean controls. This is perhaps due to the fact that LDLR is under complex regulation at the transcriptional level, by miRNA binding sites and potentially other regulatory elements in the 3'UTR of the Ldlr, and,
of course, post-transcriptionally. Here, it appears that leptin may have reduced LDLR synthesis, as there was a 50-80% reduction in Ldlr mRNA in both sexes. Second, there was no correlation between PCSK9 and plasma lipid levels: male mice showed no change in plasma lipids despite a 90% reduction in plasma PCSK9, and female mice showed a 24-53% reduction in plasma lipids, but no change in plasma PCSK9. Finally, mice that lack both leptin and LDLR (ob/ob, Ldlr double knockout mice) show a five-fold increase in plasma triglycerides and total cholesterol compared to mice with knockout only of Ldlr, indicating that leptin is capable of suppressing plasma lipids entirely independently of the LDL receptor. Together, these data suggest that other effects of leptin on lipid metabolism, such as decreasing lipogenesis, increasing fatty acid oxidation and promoting HDL clearance, may be more important.

We also identified a dramatic role for sex in modulating the effects of acute leptin treatment on plasma lipids, as well as on PCSK9. Male and female ob/ob mice have similar lipid levels, and to our knowledge, this is the first report of sex-dependent effects on lipid metabolism in response to leptin treatment. However, this observation is perhaps not surprising given the extensive cross-talk between leptin and sex hormone signaling and the marked effects of estrogen on lipid metabolism. In addition, sex hormones also affect PCSK9 itself, as estrogen suppresses PCSK9 in vitro, in vivo, and in humans, and estrogen appears to mediate the effects of PCSK9 on the subcellular distribution of LDLR in the hepatocyte.

Our studies in lipodystrophic patients differed from those in ob/ob mice even beyond the obvious species differences. First, in contrast to ob/ob mice, which are entirely leptin-deficient and markedly obese, human lipodystrophies are characterized by selective loss of adipose tissue and varying degrees of hypoleptinemia. Second, given that both acquired and congenital lipodystrophy are extremely rare (~1 in 10 million) in the population and most of them are female, and considering our exclusion criteria of lipid-lowering medication use, our study was confined to a very small cohort of lipodystrophic patients that were all female. Interestingly,
human studies have not indicated any sex differences in lipid metabolism in response to metreleptin treatment.\textsuperscript{232} Third, the dose and duration of leptin treatment differed. Interestingly, in our patients, we noted a significant correlation between the change in PCSK9 and the change in LDL cholesterol upon leptin treatment. Thus, in this case, leptin may reduce LDL cholesterol by lowering PCSK9 levels. Consistent with the idea that leptin suppresses PCSK9 and increases LDLR protein in humans, leptin-deficient lipodystrophic patients show impaired LDL clearance.\textsuperscript{137}

In summary, our data show that leptin can suppress PCSK9 under some, but not all, conditions. In patients with lipodystrophy, leptin suppression of PCSK9 is associated with a reduction in LDL cholesterol, suggesting that the suppression of PCSK9 may contribute to the beneficial effects of leptin on their plasma lipid profiles. However, based on our studies in leptin-deficient mice, it is likely that leptin also acts via PCSK9-independent pathways to regulate plasma lipids.

Acknowledgements

We thank Dr. Henry Feldman for excellent statistical support, Dr. John Stafford for helpful discussion, and Mary Gearing and Abhiruchi Mehta for technical assistance.

Funding Sources

This work was funded by R01HL109650 (S.B.B.), 5K12DK094721-04 (A.E.L.), K99DK100539 (J.M.), and M.E.H. was supported by the Department of Defense through the National Defense Science & Engineering Graduate Fellowship (NDSEG) Program. Dr. de Ferranti was supported by the Farb Family Fund, and by the Kostin Family Innovation Fund. Human studies were funded by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases.
Attributions

This chapter was originally published as:

The work in this chapter was done in collaboration with Amy E. Levenson. Other contributors include Ji Miao, Rebecca J. Brown, Sarah D. de Ferranti, Ranganath Muniyappa, Henry Feldman and Sudha B. Biddinger.

Mouse studies: Leptin treatments of ob/ob mice in Figure 3.1-3.3, and the resulting data in Figure 3.1A-D and Figure 3.3A-D were performed by Mary E. Haas with assistance from A.E.L. M.E.H. and A.E.L. conducted PCSK9 ELISAs. A.E.L. performed real-time PCR and plasma lipid analyses. J.M. conducted all western blot and ChIP analysis, except for Figure 3.3F which was done by M.E.H.


Chapter 4 - Conclusions
PCSK9 and Cardiovascular Disease

Heart disease is the leading cause of death in the United States, accounting for ~25% of deaths annually.\textsuperscript{12} Increased concentrations of the ApoB-containing lipoproteins, particularly LDL and VLDL, are major risk factors for the development of atherosclerosis,\textsuperscript{202} a disease of the cardiovascular system which can result in myocardial infarction and stroke. Understanding the molecular regulators of these lipoproteins is therefore crucially important for developing both lifestyle modifications and therapies to decrease the incidence of these cardiovascular diseases.

Human genetics has for decades provided key insights into the regulatory pathways controlling ApoB lipoprotein metabolism. In the 1970s, the discovery of molecular defects in the LDL receptor as a cause of familial hypercholesterolemia by Brown and Goldstein\textsuperscript{233} clearly demonstrated the crucial importance of this receptor for removing LDL cholesterol from the circulation and therefore its key role in mediating atherosclerotic cardiovascular disease. Nearly 30 years later, PCSK9 was identified via patients with familial hypercholesterolemia lacking mutations in \textit{LDLR} as another important regulator of LDL cholesterol.\textsuperscript{22} Additional loss of function and gain of functions in \textit{PCSK9} were found to correlate with decreased LDL cholesterol and cardiovascular disease risk or increased LDL cholesterol and cardiovascular disease risk, respectively,\textsuperscript{40-42} leading to the development of PCSK9 inhibitors which reduce LDL cholesterol by 60%.\textsuperscript{43, 44}

These studies of PCSK9 in primary hypercholesterolemia clearly established the importance of PCSK9 in regulating LDL metabolism and cardiovascular disease risk. They also justify the need for research investigating regulation of PCSK9 itself. Understanding PCSK9 regulation is important for at least two reasons: first, knowledge of the endogenous regulators of PCSK9 will help identify patient populations in which the newly-approved PCSK9 inhibitors are likely to be effective, and second, it will help identify additional mechanisms via which PCSK9 can be manipulated and PCSK9 therapies can be improved.
PCSK9 in Nephrotic Syndrome

This dissertation investigates the regulation of PCSK9 using two very different disease contexts. The first is nephrotic syndrome, a disease in which damage to the podocytes in the kidney results in high-grade proteinuria as well as hypercholesterolemia of unclear etiology. We used two different mouse models of acute nephrotic syndrome, complemented with a longitudinal cohort of nephrotic patients, to investigate the contributions of PCSK9 to the hypercholesterolemia associated with this disease. In Chapter 2, we show that both nephrotoxic serum treatment and selective apoptosis of podocytes using the ATTAC transgenic system cause dramatic elevations in plasma PCSK9, hypercholesterolemia, and an increased proportion of LDL cholesterol. Moreover, both total-body and liver-specific knockout of Pcsk9 reduce this hypercholesterolemia and largely prevent the increases in ApoB-containing cholesterol. Finally, patients show significantly increased plasma PCSK9 during active nephrotic syndrome as compared to in remission.

These data demonstrate that PCSK9 is an important contributor to hypercholesterolemia, and suggest that PCSK9 inhibitors may be an attractive therapeutic, which is especially important given the ambiguous treatment options currently available for this patient population. To move towards trying PCSK9 inhibitors in nephrotic patients, several additional studies are likely to be informative. First, the mouse models used in Chapter 2 are acute models of nephrotic syndrome, in that both hypercholesterolemia and PCSK9 peak 4-7 days after disease induction (Figure S1.1A and data not shown). It would be helpful to investigate whether PCSK9 is also elevated in chronic rodent models of nephrotic syndrome. Second, the clinical symptoms of nephrotic syndrome result from a variety of damages to the podocyte and glomerulus, as evidence by the different pathologies observed upon kidney biopsy of nephrotic patients. Our data establishes that general podocyte damage or death is sufficient to induce PCSK9 in mice. However, the increases in PCSK9 in human nephrotic patients appeared to vary by disease
subtype, with minimal change disease showing the largest decrease upon remission. For clinical
development of PCSK9 inhibitors in nephrosis, it would be very informative to study PCSK9 in a
larger populations of nephrotic patients to determine if PCSK9 is elevated differentially in
different types of nephrotic syndrome, as this could indicate the populations in which PCSK9
inhibitors are mostly likely to be effective. Finally, there are a variety of secondary causes of
nephrotic syndrome, such as diabetes and HIV infection. Our studies examined whether
primary causes of nephrotic syndrome affect PCSK9, and it would be very interesting to see
whether PCSK9 is also increased in cases of secondary nephrotic syndrome.

In addition to the clinical implications of using PCSK9 inhibitors in nephrotic patients, this study
also resulted in important insights into the fundamental molecular biology of PCSK9. For
example, our data revealed a non-linear relationship between plasma PCSK9 and hepatic LDLR
protein levels. Thus, increasing PCSK9 from 0 ng/ml in PCSK9-KO mice to 71 ng/mL in WT
mice produces a ~60% decrease in LDLR protein, whereas a further increase to 550 ng/mL in
NTS-treated WT mice produces only a ~25% decrease in LDLR (Figure 2.4C,G). One possibility
is that there is a saturable dose response between PCSK9 and LDLR protein, such that
increasing PCSK9 over a certain level has only a modest effect. Another possibility is that
nephrotic syndrome may be associated with partial inactivation of PCSK9, for example by furin-
or proprotein convertase (PC)5/6-mediated cleavage, or by binding to Annexin A2 or LDL
particles. Finally, nephrotic syndrome could result in changes to the LDL receptor or
its trafficking that reduce its ability to be degraded as a result of PCSK9. Further studies on the
modifications of PCSK9 and/or the LDL receptor during nephrotic syndrome could help
elucidate the molecular mechanism underlying this observation. Additionally, this complex
relationship between PCSK9 and LDLR may help explain why variations in PCSK9
levels account for less than 8% of the population variation in LDL cholesterol, and why
PCSK9 levels do not predict cardiovascular disease risk.
Finally, this study revealed a novel axis of renal/hepatic communication, in which podocyte damage results in a dramatic accumulation of PCSK9 in the plasma. Indirect evidence suggests that the primary mechanism via which this occurs is through increased secretion of PCSK9 from the liver. Liver-specific deletion of Pcsk9 showed that the hepatocyte is predominant, although not exclusive, source of increased PCSK9 in nephrotic mice. Examination of hepatic Pcsk9 mRNA and PCSK9 clearance suggested that each of these processes contributes only minorly (i.e. two-fold or less) to elevated PCSK9. These data implicate increased synthesis or secretion of PCSK9 from the liver as the predominant mechanism underlying increased plasma PCSK9. In contrast to dysregulation of Pcsk9 transcription, these data are also, to our knowledge, the first indication of post-transcriptional dysregulation of PCSK9 which contributes to secondary hyperlipidemia

Our attempts to directly demonstrate increases in PCSK9 processing and secretion were unfortunately not successful. We were very interested in measuring PCSK9 secretion in vivo using stable isotopes. However, PCSK9 antibodies are in general rather nonspecific, and neither the commercial antibodies available nor the antibodies we generated were capable of immunoprecipitating PCSK9 with the necessary affinity and specificity required for such an experiment. We also spent significant effort and time towards developing an in vitro model with which to study the effects of nephrotic syndrome on PCSK9 secretion from hepatocytes. This included attempts to treat primary mouse hepatocytes with conditioned media from podocytes subjected to various stressors; studies of primary hepatocytes isolated from Pod-ATTAC and Control mice; and studies of primary rodent hepatocytes treated with plasma from Pod-ATTAC and Control mice. However, we were never able to achieve robust and reproducible results with these models (data not shown), either because the PCSK9 response is lost upon culturing the hepatocyte, or because the required factors for the induction of PCSK9 were missing.
The lack of success with these in vitro approaches suggests that it will be necessary to study the mechanisms responsible for PCSK9 upregulation in vivo. We have begun such studies by investigating the few proteins implicated to date in PCSK9 processing and secretion: cIAP1, sortilin and Sec24a. Of these proteins, only cIAP1 was consistently increased in the livers of both NTS-treated and Pod-ATTAC mice (Figure 2.2I, Figure 2.3J). cIAP1 is particularly intriguing both due to its regulation by TNFα, which can be increased in nephrotic syndrome, and its potential therapeutic application. cIAP1 has also been implicated in the pathogenesis of certain cancer types and Hepatitis B, prompting the development of SMAC mimetics which result in the degradation of cIAP1. Preclinical studies of these compounds show that they are effective in decreasing cIAP1 in murine livers. This suggests that they could be used to degrade cIAP1 in mouse models of nephrotic syndrome to investigate whether increases in cIAP1 are responsible for increased plasma PCSK9. An alternate strategy to elucidate the mechanism of increased PCSK9 secretion would be through the use of unbiased genomic approaches. For example, RNA-sequencing of NTS-treated and Pod-ATTAC livers compared to controls could reveal common biological pathways upregulated by podocyte injury that could modulate PCSK9 secretion. To eliminate pathways which are involved in response to podocyte injury but do not affect PCSK9 processing and secretion, this data could be compared to that of doxorubicin-treated mice, another common model of nephrotic syndrome which preliminary studies suggest does not result in increased plasma PCSK9 (data not shown). Alternatively, PCSK9 immunoprecipitation and mass spectrometry of control and nephrotic livers could also identify novel or dysregulated binding partners of PCSK9 during nephrosis. Such studies may ultimately provide more information about the dysregulation of hepatic metabolism and effects on PCSK9 in the disease state of nephrotic syndrome.
Regulation of PCSK9 by Leptin

In contrast to our attempt to understand the contribution of PCSK9 to the unknown etiology of hypercholesterolemia in nephrotic syndrome, our studies on leptin and PCSK9 were inspired by the clinical observation that leptin treatment in lipodystrophic, hypoleptinemic patients reduces plasma total and LDL cholesterol. The regulation of the LDL receptor by leptin has not been well-studied. In Chapter 3, we showed that in a small cohort of lipodystrophic patients, leptin treatment reduced plasma PCSK9, and this was correlated with decreases in LDL cholesterol. In order to investigate the mechanisms via which leptin reduces PCSK9, we treated male and female leptin-deficient \textit{ob/ob} mice with leptin. Here we uncovered a striking sex-dependent effect of leptin on PCSK9 levels. In male \textit{ob/ob} mice, leptin treatment strongly reduced \textit{Pcsk9} mRNA and plasma PCSK9, whereas in female mice no effect of leptin was observed on PCSK9, even though leptin had similar effects on food intake and body weight in both sexes. Moreover, in mice the effects of leptin on PCSK9 appear to be dissociated from plasma lipids, as leptin reduced plasma cholesterol and triglycerides in female but not male \textit{ob/ob} mice.

One important difference between our mouse and human studies in this area is the difference in underlying disease. Human lipodystrophic patients still possess some white adipose tissue, and depending on the severity of their disease, have varying levels of hypoleptinemia\textsuperscript{145}, whereas \textit{ob/ob} mice are entirely leptin deficient. It would be interesting to see if mouse models of lipodystrophy, such as A-ZIP/F-1 or aP2-nSREBP2 mice\textsuperscript{147}, \textsuperscript{162} show similar results to lipodystrophic humans in terms of the effect of leptin on PCSK9, and whether sex-dependent effects of leptin observed in \textit{ob/ob} mice are also present in these models.

These results also provide a basis on which additional experiments to determine the mechanism via which leptin suppresses PCSK9 can be based. In male \textit{ob/ob} mice, occupancy of the PCSK9 by RNA polymerase was reduced (Figure S2.2), suggesting that leptin decreases PCSK9 transcription. Examination of the transcription factors known to regulate PCSK9
revealed that the expression of target genes of both SREBP-1c and SREBP2 were decreased (Figure 3.2), whereas target genes of other transcription factors were unchanged (Figure S2.3 and data not shown), suggesting that the SREBPs may be responsible for the decrease in *Pcsk9* mRNA. To test this idea, it would be interesting to examine whether SREBP occupancy of the *Pcsk9* promoter is also reduced and if so, whether leptin is able to reduce *Pcsk9* expression in mice lacking one or both SREBPs.  

If the SREBPs do appear to mediate the decrease in *Pcsk9* transcription, it would also be interesting to determine whether this is dependent or independent of insulin. Insulin can stimulate expression of *Pcsk9* likely via SREBP1c, and leptin treatment can decrease plasma insulin in both *ob/ob* mice and lipodystrophic patients. Like PCSK9, SCD1 is a SREBP1c and insulin target gene whose expression is decreased by leptin treatment in *ob/ob* mice. However, the effect of leptin on SCD1 appears to be independent of insulin, as leptin was still able to decrease *Scd1* expression even when insulin was coadministered to counteract the inhibitory effect of leptin on insulin. A similar approach could be used to determine whether the regulation of *Pcsk9* by leptin is also dependent on insulin.

Finally, such mechanistic studies would have the additional benefit of examining the molecular basis for the sex-dependent effects of leptin on PCSK9. Expression of SREBP target genes is reduced by leptin to a similar extent in male and female *ob/ob* mice (Figure 3.2, Figure S2.4), suggesting that if the SREBPs mediate the decrease in *Pcsk9* mRNA by leptin, sex hormones may be modulating their activity specifically on the *Pcsk9* promoter. Thus, comparison of SREBP occupancy of the *Pcsk9* promoter in male and female mice, as well as the effects of leptin in male and female mice deficient in the SREBPs, for example, could reveal the point at which regulation of PCSK9 becomes sexually divergent and guide future studies determining how sex hormones modulate *Pcsk9* transcription. Similarly, differential responses of male and female *ob/ob* mice to leptin treatment may facilitate determining the mechanism via which leptin
suppresses PCSK9, as this comparison would allow separation of general effects of leptin on \textit{ob/ob} physiology, observed in both male and female mice, from the effects of leptin on processes which ultimately regulate PCSK9, which should only be observed in male mice.

In conclusion, this dissertation provides novel insights into the regulation of PCSK9 and its contribution to diseases where hyperlipidemia arises secondary to an initial insult. Our studies identified PCSK9 as a key contributor to the hypercholesterolemia associated with nephrotic syndrome, providing new ideas to help answer a century-old medical question and suggesting that PCSK9 inhibitors as a novel therapeutic option for this patient population. We also found new evidence to suggest that both the kidney, via podocyte injury, and the adipose tissue, via the hormone leptin, are important regulators of PCSK9 and therefore plasma cholesterol metabolism. This information can help inform selection of patient populations in which PCSK9 inhibitors will likely be effective, as well as further our understanding of inter-organ communication in metabolic homeostasis.
References


P, Brule LJ, Scott MM, Coppa R and Elmquist JK. Direct leptin action on POMC neurons

162. Shimomura I, Hammer RE, Ikemoto S, Brown MS and Goldstein JL. Leptin reverses

Kahn CR, Ntambi JM, Socci ND and Friedman JM. Site and mechanism of leptin action in a

R, Hudgins LC, Ntambi JM and Friedman JM. Role for stearoyl-CoA desaturase-1 in leptin-

165. Hyogo H, Roy S, Paigen B and Cohen DE. Leptin promotes biliary cholesterol
elimination during weight loss in ob/ob mice by regulating the enterohepatic circulation of bile

Y, Tamura Y, Amemiyako M, Yoshikawa T, Okazaki H, Ohashi K, Harada K, Matsuzaka T,
Sone H, Gotoda T, Nagai R, Ishibashi S and Yamada N. Severe hypercholesterolemia,
hypertriglyceridemia, and atherosclerosis in mice lacking both leptin and the low density

muscle lipoprotein lipase and postprandial lipid metabolism in mice. Metabolism: clinical and
experimental. 2011;60:438-43.

causes decreased recycling, degradation, and selective lipid uptake. The Journal of clinical

169. Silver DL, Jiang XC and Tall AR. Increased high density lipoprotein (HDL), defective
Possible role of leptin in stimulation of HDL turnover. The Journal of biological chemistry.

and Tall AR. Regulation of hepatic LDL receptors by mTORC1 and PCSK9 in mice. The Journal


221. Singh AB, Li H, Kan CFK, Dong B, Nicolls MR and Liu J. The critical role of mRNA destabilizing protein heterogeneous nuclear ribonucleoprotein d in 3' untranslated region-
mediated decay of low-density lipoprotein receptor mRNA in liver tissue. *Arteriosclerosis, thrombosis, and vascular biology*. 2014;34:8-16.


Supplement 1 - Material Related to Chapter 2
Supplemental Methods

Human Studies

Patients
The Nephrotic Syndrome Study Network (NEPTUNE) is a multicenter, prospective, observational cohort study of patients with nephrotic syndrome.\(^{182}\) 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 \(\geq 500\) 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.\(^{182}\) Blood and urine specimens were collected at baseline and at each follow-up visit.

In this ancillary study, 38% 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:\(^{60, 61, 229, 239-241}\) atorvastatin, rosuvastatin, lovastatin, simvastatin, oral contraceptives, testosterone, medroxyprogesterone, gliclazide, cyproterone acetate/ethinyl estradiol, estradiol, ezetimide, fenfibrate, gemfibrozil, glipizide, glimepiride, insulin, hydrocortisone, sitagliptin, levonorgestrel, levothyroxine, pitavastatin, norethindrone estradiol, omega-3, metformin, methylprednisone, methylprednisolone, niacin, ethinyl estradiol norgestimate, pravastatin, estrogen, progesterone, cholestyramine, thyroxine, colesvelam. When patients on these medications were included, plasma PCSK9 decreased by 14% from baseline to remission \((348.0 \pm 139.5 \text{ vs } 300.5 \pm 130.3\text{ ng/mL, } p = 0.04)\), as shown in Figure 2.1.
When patients on these medications were excluded, PCSK9 decreased 18% from baseline to remission (343.8 ± 150.3 vs 283.6 ± 135.5, 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-flox 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 TTGCTCCAAGGTATGGGTGCCA 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<sup>lox/lox</sup> mice were backcrossed onto a C57BL/6N background and crossed to Albumin-Cre mice on a C57BL/6N background to generate PCSK9 L-KO mice, which were maintained on the C57BL/6N background.

PCSK9-KO mice and their approximate controls B6129SF2/J were purchased from Jackson Laboratory and inbred for four or more generations, to minimize differences in genetic background. Mice were subsequently mated WT x WT and KO x KO to generate sufficient numbers of experimental mice. For Pod-ATTAC, PCSK9 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<sub>2</sub>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.
Abstract

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 RNeasy kit (Qiagen). cDNA was synthesized by a reverse transcription kit (Applied Biosystems). The resultant 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 in Table S1.2.

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 sequence\(^6^4\) (nucleotides 1197-2090, see Table S1.2 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.
## Supplemental Tables

### Table S1.1. Characteristics of nephrotic syndrome subgroups at baseline.

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Overall</th>
<th>FSGS</th>
<th>MCD</th>
<th>MN</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>50</td>
<td>10 (20)</td>
<td>14 (28)</td>
<td>15 (30)</td>
<td>11 (22)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37.6 (23.5)</td>
<td>41.5 (26.8)</td>
<td>20.7 (20.1)</td>
<td>51 (14.6)</td>
<td>37.4 (23.4)</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>48</td>
<td>50</td>
<td>43</td>
<td>53</td>
<td>45</td>
</tr>
<tr>
<td>Urine protein/creatinine ratio (mg/mg)</td>
<td>5.2 (4.9)</td>
<td>3.6 (4.3)</td>
<td>6.3 (4.1)</td>
<td>4.7 (3)</td>
<td>6.0 (7.7)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>298.0 (107.8)</td>
<td>261.6 (74.8)</td>
<td>365.9 (110.6)</td>
<td>259.7 (73.2)</td>
<td>296.8 (146.6)</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>178.2 (91.1)</td>
<td>151.2 (60.3)</td>
<td>232.5 (98.6)</td>
<td>142.9 (59.8)</td>
<td>183 (122.7)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>81.6 (28.1)</td>
<td>74.9 (20.9)</td>
<td>91.3 (34.7)</td>
<td>77.9 (22.7)</td>
<td>80.1 (32.6)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>191.1 (109.1)</td>
<td>177.5 (112.5)</td>
<td>210.4 (96.2)</td>
<td>194.5 (71.4)</td>
<td>168.1 (181.3)</td>
</tr>
<tr>
<td>PCSK9 (ng/mL)</td>
<td>348.0 (139.5)</td>
<td>315 (126.4)</td>
<td>362.5 (153.1)</td>
<td>378.3 (152.9)</td>
<td>318.1 (118.9)</td>
</tr>
</tbody>
</table>

FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; MN, membranous nephropathy. Data represent means and standard deviations.
### Table S1.2. Primers used in Chapter 2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse <em>Pcsk9</em>-F</td>
<td>CCCCATGTGGAGTACATTGA</td>
</tr>
<tr>
<td>Mouse <em>Pcsk9</em>-R</td>
<td>GTGGAAGCGTGTC CCCATC</td>
</tr>
<tr>
<td>Mouse <em>Ldlr</em>-F</td>
<td>GAGGAGCAGCCACATGGTAT</td>
</tr>
<tr>
<td>Mouse <em>Ldlr</em>-R</td>
<td>GCTCGTCCTCTGTGGTCTTTC</td>
</tr>
<tr>
<td>Mouse <em>Idol</em>-F</td>
<td>ACCGCCCAATACAGCTATGA</td>
</tr>
<tr>
<td>Mouse <em>Idol</em>-R</td>
<td>GTTTCCTCATCGCTGACACAA</td>
</tr>
<tr>
<td>Mouse <em>Srebp</em>1c*-F</td>
<td>GGCCCGGGAAGTC ACTGT</td>
</tr>
<tr>
<td>Mouse <em>Srebp</em>1c*-R</td>
<td>GGAGCCATGGATTGCACATT</td>
</tr>
<tr>
<td>Mouse <em>Fasn</em>-F</td>
<td>GCTGCGGAACTTCAGGAAAT</td>
</tr>
<tr>
<td>Mouse <em>Fasn</em>-R</td>
<td>AGAGACGTGTCACTCCTGGACTT</td>
</tr>
<tr>
<td>Mouse <em>Srebp</em>2-F</td>
<td>GCGTTCTGGAGACCATGGA</td>
</tr>
<tr>
<td>Mouse <em>Srebp</em>2-R</td>
<td>ACACAGTGCTCTGAAAAACAAATCA</td>
</tr>
<tr>
<td>Mouse <em>Fdps</em>-F</td>
<td>ATGGAGATGGGCGAGTTTCTT</td>
</tr>
<tr>
<td>Mouse <em>Fdps</em>-R</td>
<td>CCGACCTTTCCGCGTCA</td>
</tr>
<tr>
<td>Mouse <em>Hmgcr</em>-F</td>
<td>CTTGTGGAATGCGGCTTTGTGATTG</td>
</tr>
<tr>
<td>Mouse <em>Hmgcr</em>-R</td>
<td>AGCCGAAGCAGCACATGAT</td>
</tr>
<tr>
<td>Mouse <em>Cyp51</em>-F</td>
<td>AGCTGTACGCAGACCTGGAT</td>
</tr>
<tr>
<td>Mouse <em>Cyp51</em>-R</td>
<td>ACGCCCGTCTTTGTATGTAG</td>
</tr>
<tr>
<td>Mouse <em>Tbp</em>-F</td>
<td>ACCCTTCACCAATGACTCCTATG</td>
</tr>
<tr>
<td>Mouse <em>Tbp</em>-R</td>
<td>TGACTGCAGCAATC GCTTGG</td>
</tr>
<tr>
<td>Mouse <em>Albumin</em>-F</td>
<td>GCTGAGACCTTCACCTTCCA</td>
</tr>
<tr>
<td>Mouse <em>Albumin</em>-R</td>
<td>CTTGTGCTTCCAGCCTCAG</td>
</tr>
<tr>
<td>Mouse <em>Serpina1</em>-F</td>
<td>CAGGATAGGGGGCATAGACA</td>
</tr>
<tr>
<td>Mouse <em>Serpina1</em>-R</td>
<td>GACCTTCACCAATGACCTATG</td>
</tr>
<tr>
<td>Mouse <em>Fgb</em>-F</td>
<td>TCTTCACAGCGTGAGCACAGGA</td>
</tr>
<tr>
<td>Mouse <em>Fgb</em>-R</td>
<td>TGTAAGGCACCCCATGAG</td>
</tr>
<tr>
<td>Mouse <em>Hnf4α</em>-F</td>
<td>CGAGTG GGGCCAAGTACATCC</td>
</tr>
</tbody>
</table>

104
<table>
<thead>
<tr>
<th>Mouse</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hnf4α-R</td>
<td>GCAGCACGTCTTTAAACACC</td>
<td></td>
</tr>
<tr>
<td>Hnf1α-F</td>
<td>TTCTAAAGCTGAGCCAGCTGCAGACG</td>
<td></td>
</tr>
<tr>
<td>Hnf1α-R</td>
<td>GCTGAGGGTTCTCAGGTCTTTTCAGA</td>
<td></td>
</tr>
<tr>
<td>Lxra-F</td>
<td>ACTTCAGTTACACCGGAAG</td>
<td></td>
</tr>
<tr>
<td>Lxra-R</td>
<td>GAGCAAACTCAGCATCATTGAG</td>
<td></td>
</tr>
<tr>
<td>Lxrb-F</td>
<td>CAGAGAAGTTGTGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>Lxrb-R</td>
<td>AGGGCAACAGAGTCGGAGAC</td>
<td></td>
</tr>
<tr>
<td>Abcg5-F</td>
<td>TGCCCCATCCTTTAAAATCC</td>
<td></td>
</tr>
<tr>
<td>Abcg5-R</td>
<td>GATGAACTGGACCCCTTGG</td>
<td></td>
</tr>
<tr>
<td>Hnf1b-F</td>
<td>TCACCAGCATGTCTTTCCAGT</td>
<td></td>
</tr>
<tr>
<td>Hnf1b-R</td>
<td>CCAGAGACTGATGGTGTG</td>
<td></td>
</tr>
<tr>
<td>Ppara-F</td>
<td>ATTTCCCTGTTTGCTGCTGC</td>
<td></td>
</tr>
<tr>
<td>Ppara-R</td>
<td>ACAATCCCTCCTGCAACTT</td>
<td></td>
</tr>
<tr>
<td>Hmgcs2-F</td>
<td>ATACCACCAACGCCCTGTATGG</td>
<td></td>
</tr>
<tr>
<td>Hmgcs2-R</td>
<td>CAATGTACACAGACCACACCAG</td>
<td></td>
</tr>
<tr>
<td>Pparg-F</td>
<td>AGAGCTGACCAATGGTGC</td>
<td></td>
</tr>
<tr>
<td>Pparg-R</td>
<td>AAGGTTCTTTGATGAGCCTGT</td>
<td></td>
</tr>
<tr>
<td>Cd36-F</td>
<td>AAGCTATTGCGACATGATTAATGG</td>
<td></td>
</tr>
<tr>
<td>Cd36-R</td>
<td>TCTCAATGTCCGGAGACTTTTCAAC</td>
<td></td>
</tr>
<tr>
<td>Pcsk9 ISH Probe-F</td>
<td>AGGGCAGCTGAGC</td>
<td></td>
</tr>
<tr>
<td>Pcsk9 ISH Probe-R</td>
<td>CTTCTGGTGCGAGTGG</td>
<td></td>
</tr>
</tbody>
</table>
Abstract

Supplemental Figures

Figure S1.1. 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-fastened 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-fastened, 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.
Figure S1.2. 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.
Figure S1.3. 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.
Figure S1.4. Liver-specific knockout of PCSK9 blunts the NTS-induced increase in ApoB-associated cholesterol.

Five- to eight-week-old male PCSK9floxfloxflox (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.
Figure S1.5. 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 2.2H), 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.
Supplement 2 – Material Related to Chapter 3
### Table S2.1. Primers used in Chapter 3.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse <em>Pcsk9</em>-F</td>
<td>TTGCAGCAGCTGGGAACCTT</td>
</tr>
<tr>
<td>Mouse <em>Pcsk9</em>-R</td>
<td>CCGACTGTGATGACCTCTGGA</td>
</tr>
<tr>
<td>Mouse <em>Ldlr</em>-F</td>
<td>GAGGAGCAGCCACATGGTAT</td>
</tr>
<tr>
<td>Mouse <em>Ldlr</em>-R</td>
<td>GCTCGTCTCTGTGGTCTTC</td>
</tr>
<tr>
<td>Mouse <em>Idol</em>-F</td>
<td>ACAAAGTCTGCTCTGGACTT</td>
</tr>
<tr>
<td>Mouse <em>Idol</em>-R</td>
<td>ACAAAGTCTGCTCTGGACTT</td>
</tr>
<tr>
<td>Mouse <em>Srebp-1c</em>-F</td>
<td>GCCTCGTGGAGACCATGGA</td>
</tr>
<tr>
<td>Mouse <em>Srebp-1c</em>-R</td>
<td>AGAGGACGTGCTACTCTGGAGAAT</td>
</tr>
<tr>
<td>Mouse <em>Fasn</em>-F</td>
<td>GCTCGGAAACTGAGGAAAT</td>
</tr>
<tr>
<td>Mouse <em>Fasn</em>-R</td>
<td>GCTCGGAAACTGAGGAAAT</td>
</tr>
<tr>
<td>Mouse <em>Srebp-2</em>-F</td>
<td>GCCTCGTGGAGACCATGGA</td>
</tr>
<tr>
<td>Mouse <em>Srebp-2</em>-R</td>
<td>AGAGGACGTGCTACTCTGGAGAAT</td>
</tr>
<tr>
<td>Mouse <em>Hmgcr</em>-F</td>
<td>GCCGAAGCAGCAGCAGCAGCAGGAGAAT</td>
</tr>
<tr>
<td>Mouse <em>Hmgcr</em>-R</td>
<td>GCCGAAGCAGCAGCAGCAGGAGAAT</td>
</tr>
<tr>
<td>Mouse <em>Fdps</em>-F</td>
<td>ATGGAGATGGGCGAGTTTTTCTTC</td>
</tr>
<tr>
<td>Mouse <em>Fdps</em>-R</td>
<td>CCGAACCTTTCCCGTCACA</td>
</tr>
<tr>
<td>Mouse <em>Tbp</em>-F</td>
<td>ACCCTTCAATGACTCTCCTATG</td>
</tr>
<tr>
<td>Mouse <em>Tbp</em>-R</td>
<td>ACCCTTCAATGACTCTCCTATG</td>
</tr>
<tr>
<td>Mouse <em>18s</em>-F</td>
<td>GTAACCCGTTGAACCCCATTT</td>
</tr>
<tr>
<td>Mouse <em>18s</em>-R</td>
<td>GTAACCCGTTGAACCCCATTT</td>
</tr>
<tr>
<td>Mouse <em>Scd1</em>-F</td>
<td>CATCATTTCATATACCTGCTGTAGCG</td>
</tr>
<tr>
<td>Mouse <em>Scd1</em>-R</td>
<td>CATCATTTCATATACCTGCTGTAGCG</td>
</tr>
<tr>
<td>Mouse <em>Fgb</em>-F</td>
<td>TCTTCAGCAGTACGTACGACAGG</td>
</tr>
<tr>
<td>Mouse <em>Fgb</em>-R</td>
<td>TCTTCAGCAGTACGTACGACAGG</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Sequence</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Mouse <em>Fdf1</em></td>
<td>CCAACTCAATGGGTCTGTTCCT</td>
</tr>
<tr>
<td>Mouse <em>Hnf4α</em></td>
<td>GCATGGATATGGCCGACTAC</td>
</tr>
<tr>
<td>Mouse <em>Hnf1α</em></td>
<td>CTCGAGGCTCCGTAGTGTTT</td>
</tr>
<tr>
<td>Mouse <em>Apoa1</em></td>
<td>TATGTGGATGCGGTCAAAGA</td>
</tr>
<tr>
<td>Mouse <em>Acc</em></td>
<td>TGACAGACTGATCGCAGAGAAAG</td>
</tr>
<tr>
<td>Mouse <em>Serpina1</em></td>
<td>CAGATAGGGGGGACATAGACA</td>
</tr>
<tr>
<td>Mouse <em>Albumin</em></td>
<td>GCTGAGACCTCCACCTTCCA</td>
</tr>
<tr>
<td>Mouse <em>Pcsk9</em></td>
<td>CAGAAGGCTCCACCTTCCA</td>
</tr>
<tr>
<td>Mouse <em>Fasn</em></td>
<td>CCAAGCTGTCAGCCCATGT</td>
</tr>
<tr>
<td>Mouse <em>Fasn</em></td>
<td>CGTCTCTCTGGCTCCCTCTTA</td>
</tr>
<tr>
<td>Mouse <em>Pcsk9</em></td>
<td>CAGAAGGCTCCACCTTCCA</td>
</tr>
<tr>
<td>Mouse <em>Fasn-Chip</em></td>
<td>CCAAGCTGTCAGCCCATGT</td>
</tr>
<tr>
<td>Mouse <em>Pcsk9-Chip</em></td>
<td>GCTCAGTCCTCTAGCCTCAGAA</td>
</tr>
</tbody>
</table>
Table S2.2. Information on antibodies used in Chapter 3.

<table>
<thead>
<tr>
<th>Peptide/Protein Target</th>
<th>Antigen sequence (if known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog No., or Name of Source</th>
<th>Species Raised in, Monoclonal or Polyclonal</th>
<th>Dilution Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearoyl-CoA desaturase-1</td>
<td>Unknown</td>
<td>SCD1 Antibody</td>
<td>Santa Cruz Biotechnology, sc-14720</td>
<td>Goat Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A reductase</td>
<td>Unknown</td>
<td>HMGCR Antibody (C-18)</td>
<td>Santa Cruz Biotechnology, Sc-27578</td>
<td>Goat Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Fatty Acid Synthase</td>
<td>Unknown</td>
<td>Anti-Fatty Acid Synthase Antibody</td>
<td>Abcam, ab22759</td>
<td>Rabbit Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Farnesyl diphosphate synthase</td>
<td>Unknown</td>
<td>Anti-FDPS Antibody</td>
<td>Abcam, ab38854</td>
<td>Rabbit Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Acetyl CoA Carboxylase 1</td>
<td>Unknown</td>
<td>Anti-Acetyl CoA Carboxylase 1 Antibody</td>
<td>EMD Millipore, 04-322</td>
<td>Rabbit Monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>LDLR</td>
<td>Unknown</td>
<td>Anti-LDL Receptor Antibody</td>
<td>Abcam, ab30532</td>
<td>Rabbit Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Pol II Antibody</td>
<td>Unknown</td>
<td>Pol II Antibody (H-224)</td>
<td>Santa Cruz Biotechnology, sc-9001</td>
<td>Rabbit polyclonal</td>
<td>2 μg</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Unknown</td>
<td>Normal rabbit IgG</td>
<td>Santa Cruz Biotechnology, sc-2027</td>
<td>Rabbit</td>
<td>2 μg</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Unknown</td>
<td>β-Actin Antibody (C4)</td>
<td>Santa Cruz Biotechnology, Sc-47778</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>Unknown</td>
<td>α-Tubulin, (11H10) Antibody</td>
<td>Cell Signaling, 2125</td>
<td>Rabbit Monoclonal</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Table S2.3. Laboratory reference ranges for measured patient parameters, as per the NIH Clinical Center laboratory.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Mass Index (kg/m$^2$)</td>
<td>18.5 – 24.9</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>3.3-18.3 (BMI=22)</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mg/dL)</td>
<td>74 – 100</td>
</tr>
<tr>
<td>Fasting Plasma Insulin (µU/mL)</td>
<td>2.6 – 24.9</td>
</tr>
<tr>
<td>2-hr OGTT Plasma Glucose (mg/dL)</td>
<td>&lt; 140</td>
</tr>
<tr>
<td>Hemoglobin A1C (%)</td>
<td>4.0 – 6.0</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>&lt;200</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dL)</td>
<td>60-130</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)</td>
<td>40-60</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>&lt;150</td>
</tr>
</tbody>
</table>
Table S2.4. Correlation between change in PCSK9 and metabolic parameters upon leptin treatment.

<table>
<thead>
<tr>
<th>Change in Clinical Variable</th>
<th>Correlation with Change in ln(PCSK9)†</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Mass Index, kg/m²</td>
<td>0.59</td>
<td>0.12</td>
</tr>
<tr>
<td>Leptin, ng/mL</td>
<td>0.56</td>
<td>0.25</td>
</tr>
<tr>
<td>Fasting Plasma Glucose, mg/dL</td>
<td>-0.02</td>
<td>0.97</td>
</tr>
<tr>
<td>Fasting Plasma Insulin, µU/mL</td>
<td>0.52</td>
<td>0.23</td>
</tr>
<tr>
<td>2-hr OGTT Plasma Glucose, mg/dL</td>
<td>0.33</td>
<td>0.47</td>
</tr>
<tr>
<td>Hemoglobin A1C, %</td>
<td>-0.37</td>
<td>0.48</td>
</tr>
<tr>
<td>OGIS, ml/min/m²</td>
<td>-0.25</td>
<td>0.58</td>
</tr>
<tr>
<td>Total Cholesterol, mg/dL</td>
<td>0.41</td>
<td>0.31</td>
</tr>
<tr>
<td>LDL Cholesterol, mg/dL</td>
<td>0.75</td>
<td>0.03</td>
</tr>
<tr>
<td>HDL Cholesterol, mg/dL</td>
<td>0.58</td>
<td>0.17</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>-0.17</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Supplemental Figures

**Figure S2.1. Effects of leptin on the distribution of cholesterol among the different lipoprotein fractions in male ob/ob mice.**
Equal volumes of non-fasted plasma from leptin- or vehicle-treated mice were pooled (n=4-5/group) and were subjected to size-exclusion liquid chromatography. The proportion of cholesterol in each of the fractions was calculated. Similar results were obtained in a second cohort.

**Figure S2.2. Effects of leptin on PolII occupancy of the Pcsk9 promoter in male ob/ob mice.**
Male ob/ob mice were treated with leptin or vehicle as described in Fig. 1. Equal aliquots of liver from two mice per group were pooled and subjected to chromatin immunoprecipitation with antibodies against Pol II or control IgG, and the relative enrichment compared to input was calculated. Analysis of the Pcsk9 (A) and Fasn (B) promoters is shown. Data are presented as mean ± SEM of two independent experiments. * P value ≤ 0.05 compared to vehicle-treated mice, Student’s t-test.
Figure S2.3. Effects of leptin on mRNA levels of Hnf1α and its targets in male ob/ob mice.
Mice were treated as detailed in Figure 1. Gene expression was measured by real time PCR, normalized to Tbp, and expressed relative to vehicle-treated mice. Data represent mean ± SEM, and are representative of two independent experiments. *P value ≤ 0.05 compared to vehicle treatment, Student’s t-test; n=4-5/group.

Figure S2.4. Effects of leptin on Pcsk9 mRNA and SREBP target genes in female ob/ob mice.
Hepatic gene expression was measured by real time PCR, normalized to 18s, and expressed relative to vehicle-treated mice. Data are pooled from two independent experiments; mean and SEM are shown. *P value ≤ 0.05 versus vehicle-treated mice; n = 12/group.