Diet-induced hepatic steatosis abrogates cell-surface LDLR by inducing de novo PCSK9 expression in mice

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Running title: NAFLD affects the PCSK9-LDLR axis

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Key words: Cardiometabolic disease, CVD, ER stress, HFD, LDL, Liver, NAFLD, SREBP2

ABSTRACT

The worldwide prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing rapidly. Although this condition is generally benign, accumulating evidence now suggests that patients with NAFLD are also at increased risk of cardiovascular disease (CVD); the leading cause of death in developed nations. Despite the well-established role of the liver as a central regulator of circulating low-density lipoprotein (LDL) cholesterol levels, a known driver of CVD, the mechanism(s) by which hepatic steatosis contributes to CVD remains elusive. Interestingly, a recent study has shown that circulating proprotein convertase subtilisin/kexin type 9 (PCSK9) levels correlate positively with liver steatosis grade. Given that PCSK9 degrades the LDL receptor (LDLR) and prevents the removal of LDL from the blood into the liver, in the present study we examined the effect of hepatic steatosis on LDLR expression and circulating LDL cholesterol levels. We now report that in a manner consistent with findings in human patients, diet-induced steatosis increases circulating PCSK9 levels as a result of de novo expression in mice. We also report the novel finding that steatosis abrogates hepatic LDLR expression and increases circulating LDL levels in a PCSK9-dependent manner. These findings provide important mechanistic insights as to how hepatic steatosis modulates lipid regulatory genes like PCSK9 and the LDLR, and also highlights a novel mechanism by which liver disease may contribute to CVD.

INTRODUCTION

Liver fat accumulation due to reasons other than excessive alcohol consumption, or more often referred to as NAFLD, currently has an estimated occurrence of 30-46% in developed nations (1). Given the current trends in global consumption of unhealthy dietary fats and sugars, it is no surprise that NAFLD is increasing in prevalence as these factors play a central role in its development (2). Although NAFLD is characterized by liver fat accumulation with no sign of liver injury, this initial stage often progresses to non-alcoholic steatohepatitis (NASH); a state characterized histologically by necroinflammation and hepatocyte damage (3). NAFLD and its complications are estimated to be the primary cause of liver-related mortality and liver transplantation within the next 20 years (4).

Evidence that patients with NAFLD are at higher risk of developing CVD, which is among the leading causes of death worldwide (5), is now accumulating (6-9). Given that both
NAFLD and CVD share many comorbidities and frequently develop in patients at the same time, it has been a major challenge to discern the exact mechanism(s) by which one contributes to the other (10). Despite this challenge, several reports have demonstrated that NAFLD increases the expression or prevalence of factors known to contribute to CVD. These factors include circulating pro-inflammatory mediators, pro-thrombotic factors, hyperlipidemia and risk of type-2-diabetes. Recent studies have shown a link between the presence of NAFLD and increased intima-media thickness, impaired arterial vasodilation, plaque development as well as coronary artery calcium scores (10).

Interestingly, two independent clinical studies have also demonstrated that patients with NAFLD have increased levels of circulating PCSK9 (11,12). PCSK9 is an established driver of atherosclerotic lesion development and CVD due to its ability to enhance the degradation of cell-surface LDLR, thereby reducing the ability of the liver to clear pro-atherogenic LDL cholesterol from the circulation (13-15). Seminal studies in this field have also shown that gain-of-function mutations in PCSK9 correlate with increased risk of CVD in humans (16), and in a reciprocal manner, LOF mutations have the opposite outcome (17). Pre-clinical data also demonstrate that adenoviral-mediated overexpression of PCSK9 in mice, or hepatocyte-specific transgenic overexpression of PCSK9 leads to a similar phenotype as that of the well-established ldlr−/− mouse model used for the study of atherosclerosis (18, 19). In line with these data, human monoclonal antibodies targeted against PCSK9 were recently shown to reduce circulating LDL cholesterol levels by up to 60% in patients at high risk of CVD (20).

In the present study, we examined the effect of diet-induced hepatic steatosis on the expression and abundance of established drivers of CVD. Here, we show that the uptake and accumulation of the saturated fatty acid palmitate (PA), as well as high-fat diet (HFD), cause endoplasmic reticulum (ER) stress in cultured hepatocytes, and in the livers of mice, respectively. ER stress is a pathological cellular response that contributes to the development of liver disease and is also known to promote the activation of the sterol regulatory element-binding protein-2 (SREBP2); the major transcription factor responsible for the de novo synthesis of cholesterol regulatory proteins including PCSK9 and the LDLR. Accordingly, we also observed increased expression of PCSK9 in PA-treated hepatocytes, as well as in the livers and circulation of HFD-fed mice. Given the consistency of these data with studies done in patients with NAFLD, we next investigated the effect of this outcome on circulating LDL cholesterol and on the expression of its receptor in the liver. We also report the novel finding that HFD-induced hepatic steatosis caused a significant reduction of cell-surface LDLR expression and increased circulating LDL cholesterol levels in mice. Furthermore, because LDLR expression and serum LDL levels were unaffected by the HFD in Pcsk9−/− mice, we also identify that diet-induced hepatic steatosis affected these parameters in a PCSK9-dependent manner. Collectively, our data highlight a novel mechanism by which NAFLD may contribute to CVD by increasing PCSK9 expression to attenuate liver-mediated LDL cholesterol clearance.

RESULTS

**HFD increases circulating PCSK9 levels and attenuates hepatic cell-surface LDLR expression in mice** — Consistent with previous studies (12), we first confirmed that patients with liver fat accumulation exhibit increased plasma PCSK9 levels (Figure 1A; \( p=0.013; \) n=9), as well as circulating cholesterol (Figure 1B; \( p=0.0001; \) n=9) and triglyceride levels (Figure 1C; \( p=0.0020; \) n=9) (Figure 1A-C). Given that (a) PCSK9 contributes to CVD by degrading the LDLR and increasing plasma LDL levels (21) and (b) accumulating evidence suggests that hepatic steatosis contributes to CVD (10), we next examined the effect of steatosis on hepatic LDLR expression. Accordingly, male C57BL/6J mice were fed HFD or normal control diet (NCD) for a total of 12 weeks. Hepatic steatosis in these mice was confirmed via visualization of lipid droplets using H&E as well as oil-red-o (ORO) (Figure 2A). Immunohistochemical staining then revealed that HFD-fed mice had markedly reduced cell-surface LDLR expression.
compared to NCD-fed controls (Figure 2B). As expected, LDLR antibody staining specificity was confirmed by the absence of staining in the livers of Ldlr\(-/-\) mice. In contrast, only a modest reduction in LDLR expression was observed in the livers of HFD-fed mice via immunoblot (Figure 2C). These data are representative of whole-cell LDLR abundance, however, and not strictly that of the cell-surface LDLR population. Therefore, these data suggest a strong presence of an intracellular LDLR population in liver hepatocytes, that is not affected by the HFD. ORO and LDLR immunohistochemical staining intensities were also quantified using ImageJ software (Figure 2D). Consistent with previous studies, we observed that hepatic LDLR expression was inversely correlated with circulating PCSK9 levels in mice (Figure 2D) (14). The surrogate marker of circulating LDL cholesterol levels, apolipoprotein B (ApoB) (22), as well as total cholesterol and triglyceride levels were also examined and found to be increased in HFD-fed mice compared to NCD-fed controls (Figure 2E, F and G). These findings demonstrate a diet-induced hepatic steatosis effect on the PCSK9-LDLR axis, which could explain the observed increase in circulating lipid levels.

**Diet-induced hepatic steatosis causes hepatic ER stress and promotes de novo PCSK9 expression** — Our research group has previously demonstrated that ER stress causes the activation of SREBP2 (23,24) and expression of PCSK9 in cultured hepatocytes (25). As such, we next examined the livers of HFD-fed mice for markers of ER stress. Consistent with other reports (26,27), increased expression of ER stress and pro-apoptotic markers including the glucose-regulated proteins (GRP78, and GRP94), C/EBP homologous protein (CHOP), activating transcription factor 4 (ATF4), PKR-like endoplasmic reticulum kinase (PERK), as well as pro-fibrotic and apoptotic markers fibronectin 1 (FN1), Bcl-2-binding component 3 (BBC3), caspases (CASP1 and CASP3), inositol-requiring enzyme 1α (IRE1α) and spliced X-box-binding protein 1 (sXBP1) was observed in the livers of HFD-fed mice compared to controls via immunohistochemical staining, real time PCR and immunoblot (Figure 3A, B and C). Furthermore, similar to our previous studies done in cultured cells (25), we observed that hepatic ER stress was associated with increased mRNA expression of SREBP2, as well as PCSK9 and the LDLR (Figure 3C). Increased intracellular PCSK9 protein abundance was also observed in the livers of HFD-fed mice compared to NCD-fed controls (Figure 3D). Additional modulators of cholesterol and triglyceride homeostasis, including the SREBP2-regulated HMG-CoA reductase (HMGCR), SREBP1, fatty acid synthase (FAS) and ApoB were examined via real time PCR and found to be induced in the livers of HFD-fed mice (Figure 3F). Overall, because circulating PCSK9 originates almost exclusively from liver hepatocytes (20, 28), these findings suggest that diet-induced hepatic ER stress represents a significant contributor in the observed increase in circulating PCSK9 levels in the context of liver fat accumulation.

**Pharmacologic inhibition of ER stress, or lipid accumulation, blocks lipid-driven PCSK9 expression and restores LDLR function and expression in hepatocytes** — To further examine the effect of lipid-induced ER stress on PCSK9 expression, cultured hepatocytes were treated with the fatty acid, PA. PA represents one of many saturated fatty acids that is highly abundant in animal-derived dietary fats (29) and is also a well-established inducer of ER stress in a variety of cultured cell models (30-32). Similar to the livers of mice exposed to high levels of dietary fats from the HFD, cultured human HepG2 hepatocytes treated with bovine serum albumin (BSA)-conjugated PA yielded increased mRNA expression of SREBP2, PCSK9 and LDLR compared to those treated with the BSA vehicle control (Figure 4A). Because previous studies have shown that metformin (Met) and 4-phenylbutyrate (4PBA) attenuate hepatic lipid accumulation and protect against ER stress (33-35), two additional groups of cells were also pre-treated with these agents for 24 hours prior to PA treatment. Consistent with other studies, we observed that both agents reduced lipid accumulation (Figure 4B) (36,37) and attenuated PA-induced expression of ER stress markers, GRP78, GRP94 and IRE1α. Furthermore, Met and 4PBA also blocked the PA-induced expression of SREBP2, PCSK9 and LDLR. SREBP2 transcriptional activity was then
assessed in HuH7 cultured human hepatocytes transfected with a sterol-regulatory element (SRE)-driven GFP reporter construct (Figure 4C). Similar to mRNA transcript levels of SREBP2, PA increased GFP fluorescence intensity, which was in turn blocked by Met and 4PBA. Met and 4PBA also significantly reduced PA-induced secreted PCSK9 levels in the medium harvested from HepG2 and HuH7 cells (Figure 4D). To assess whether these treatments were affecting all secreted proteins, or if PCSK9 was affected with an acceptable level of specificity, medium harvested from these cells was electrophoretically resolved and stained using Coomassie Brilliant Blue protein stain. Given that the relative abundance of secreted proteins was not markedly affected by these treatments, these data suggest that PA, Met and 4PBA-induced changes in secreted PCSK9 levels are not the result of changes in global protein secretion.

Next, a quantitative assessment of fluorescently-labelled DiI-LDL uptake was carried out in HepG2 cells treated with vehicle, met or 4PBA in the presence or absence of PA (Figure 4E). Consistent with previous studies, elevated secreted PCSK9 levels were associated with a reduction of LDL uptake (38,39). Met and 4PBA also attenuated the PA-mediated inhibition of DiI-LDL uptake observed in these cells. PCSK9 mRNA expression and secretion in response to PA treatment was also examined in primary human hepatocytes, yielding findings that were consistent with those observed in HepG2 and HuH7 cells (Figure 4F).

Given our observation that 4PBA can attenuate PA-induced ER stress and PCSK9 expression in cultured cells, an additional cohort of mice was fed HFD in the presence or absence of 4PBA in the drinking water. Similar to PA-treated HepG2 cells, we observed that 4PBA attenuated the ability of the HFD to block LDLR expression (Figure 4G). A reduction in the expression of ER stress markers, GRP78 and GRP94, as well as the fibrosis marker, fibronectin, was also observed in the livers of HFD-fed mice exposed to 4PBA (Figure 4H).

Diet-induced hepatic steatosis attenuates hepatic LDLR expression in a PCSK9-dependent manner — Although LDLR expression is known to be affected by other proteins and conditions (40), our final aim was to identify the extent to which PCSK9 contributed to the observed reduction of cell-surface LDLR expression in HFD-fed mice. Accordingly, Pcsk9−/− mice on a C57BL/6J background were also fed a HFD for 12 weeks starting at 6 weeks of age. Similar to wild-type C57BL/6J mice, a significant increase in hepatic lipid content was observed in HFD-fed Pcsk9−/− mice compared to those fed the NCD (Figure 5A and B). Strikingly, immunohistochemical staining revealed that the HFD did not significantly reduce LDLR expression in these mice. ORO and LDLR staining intensities were also quantified using ImageJ Software (Figure 5C). Consistent with immunohistochemical staining of cell-surface LDLR, immunoblot data also demonstrate that HFD did not markedly reduce LDLR expression in the livers of Pcsk9−/− mice (Figure 5D). Furthermore, HFD also failed to increase circulating ApoB-containing LDL cholesterol in Pcsk9−/− mice (Figure 5E). PCSK9 knockout in these mice was confirmed using an ELISA for circulating PCSK9 (Figure 5F).

Collectively, these data suggest that intracellular lipid accumulation causes ER stress, which induces de novo PCSK9 expression and secretion from hepatocytes (Figure 6). In turn, heightened circulating PCSK9 levels enhance the degradation of hepatic cell-surface LDLR and increase the levels of circulating LDL cholesterol in the context of diet-induced hepatic steatosis.

DISCUSSION

Hepatocytes, like all secretory cells, are rich in ER and are thus susceptible to injury and damage as a result of conditions that lead to ER stress (41). It is well-established that lipid accumulation in hepatocytes can promote the activation of the unfolded protein response (UPR), a highly conserved signaling cascade that attempts to resolve ER stress (42). In a reciprocal manner, studies have also shown that ER stress can lead to intracellular lipid accumulation by inducing de novo lipid synthesis in a manner dependent on specific transcription factors, such as SREBP1 (42,43). Given that both processes appear to occur at the same time however, it has been challenging to discern which of the two factors in this
paradoxical relationship contributes most to the development of liver disease (10). Nonetheless, in a manner consistent with previous studies, we observed a significant increase in the expression of mediators of the UPR during conditions of hepatic steatosis in response to HFD (Figure 3A and B) (27). Although previous studies have demonstrated that HFD increases SREBP1 expression (44,45), we also report the finding that diet-induced hepatic steatosis increases the expression of SREBP2 (Figure 3C). Furthermore, we demonstrate that hepatic ER stress caused a significant increase in PCSK9 expression and secretion (Figure 3C and 2D, respectively); a process that we previously demonstrated to be dependent on SREBP2 (25). We also observed that PA, a saturated fatty acid known to cause ER stress in secretory cell types (46), increased SREBP2 activity and PCSK9 expression and secretion in cultured human hepatocytes (Figure 4). In response to elevated secreted PCSK9 levels, we observed that the livers of mice and cultured hepatocytes exhibited a significant reduction in LDLR expression and activity (Figure 2B and 4E, respectively). Given that this phenotype was not observed in HFD-fed Pcsk9−/− mice, we conclude that the effect of diet-induced hepatic steatosis on LDLR expression occurs in a manner dependent on de novo SREBP2-driven PCSK9 expression and secretion (Figure 6).

In a previous study, we observed that ER stress occurring as a result of ER Ca^{2+} depletion using thapsigargin, but not from the inhibition of N-glycosylation using tunicamycin, caused a significant increase in SREBP2 activation and PCSK9 expression in hepatocytes (25). These findings suggest that PCSK9 expression is affected only by certain ER stress-inducing stimuli. Despite increased PCSK9 protein abundance as a result of thapsigargin treatment, we observed that thapsigargin and tunicamycin blocked the exit of PCSK9 from hepatocytes. Interestingly, in the present study we demonstrate that lipid accumulation in hepatocytes increases SREBP2 activity and promotes PCSK9 expression, suggesting that this process could occur as a result of ER Ca^{2+} depletion. Consistent with this notion, previous studies have also demonstrated that fatty acid uptake and accumulation causes ER Ca^{2+}
depletion, ER stress and apoptosis in a variety of cell lines (30,47). In contrast to our previous study, however, diet-induced hepatic steatosis and ER stress not only increased the expression of PCSK9, but also increased its secretion from hepatocytes. Given the intricacies of the UPR and of the ER cargo receptors that are known to play a role in the secretion of PCSK9 and regulation of cholesterol (48,49), it is not surprising that different conditions of ER stress affect PCSK9 in different ways. Although ER stress can increase its expression, PCSK9 is also a lipid-responsive gene and therefore further studies are required to delineate the exact mechanism by which lipid accumulation influences PCSK9 secretion from hepatocytes.

Consistent with previous studies, we also observed that circulating PCSK9 levels were positively correlated with circulating LDL cholesterol levels, but inversely correlated with hepatic LDLR expression (Figure 2B, D and E) (38,50). Importantly, de novo synthesis of PCSK9 and the LDLR is regulated by the same transcription factor, and thus differences in the relative abundance of these proteins in the context of SREBP2 activation/inhibition can be attributed to differences in the stimuli being studied. Here, we observed that diet-induced hepatic steatosis increased de novo expression of the LDLR at the mRNA level (Figure 3C), but blocked its expression at the protein level (Figure 4A). Consistent with our observations, LDLR expression is inversely correlated with SREBP2 activation and in naringin-treated mice and in monkeys treated with siRNA targeted against the SREBP cleavage-activating protein (51,52); a protein known to interact with and stabilize the SREBPs (53). In contrast, it is also well-established that HMG-CoA reductase (HMGR) inhibitors activate SREBP2 and increase hepatic LDLR expression, as well as circulating PCSK9 levels (54,55). Interestingly, a recent study has demonstrated that HMGR inhibitors can increase circulating PCSK9 levels while blocking the occurrence of a gain-of-function phosphorylation at position 688 (56). Given that HMGR inhibitors are well-known to reduce CVD risk (57), the aforementioned findings rectify the long-lasting PCSK9-statin anomaly. Furthermore, with a half-life of only five minutes (58), PCSK9 protein expression is more
likely dependent on do novo synthesis than the LDLR, which has a half-life of 12 hours (59).

Although we are the first to demonstrate that diet-induced liver fat accumulation increases circulating PCSK9 levels in mice, similar reports in patients have been controversial. The large Dallas Heart study was the first to report a modest but significant positive correlation between hepatic steatosis and circulating PCSK9 levels (11). Ruscica and colleagues also reported a statistically significant positive correlation between steatosis grade and circulating PCSK9 levels (12). Although our findings also demonstrate that patients with hepatic steatosis exhibit increased plasma PCSK9 levels, the statistical power of the result in this study is limited by low patient number. In contrast to the aforementioned data, a recent study by Wargny and colleagues demonstrated that no significant correlation was observed between circulating PCSK9 levels and liver fat accumulation, plasma transaminase activity, NASH activity score or lobular/portal inflammation in three patient cohorts (60). Inconsistencies in these data sets, however, are likely attributable to differences in liver disease severity between patient cohorts (60).

The liver is a central regulator of lipid homeostasis and yet despite the established role of lipid in the development of atherosclerosis and other cardiovascular complications, the contribution of liver disease to CVD remains elusive. Although previous studies have also demonstrated that a HFD can increase circulating LDL cholesterol levels (61), we are the first to highlight a role of the liver in this process. Because recent studies have also demonstrated that PCSK9 itself has pro-inflammatory properties (62), it is also possible that steatosis-driven PCSK9 expression may contribute to CVD in a manner independent of LDL cholesterol. Collectively, the present study delineates a novel mechanism by which diet-induced liver fat accumulation can influence CVD by affecting the expression of central regulators in its development, PCSK9 and the LDLR.

EXPERIMENTAL PROCEDURES

**Patient cohort and ELISAs** — Plasma samples from healthy controls and patients with fatty liver disease were acquired from Discovery Life Sciences (Huntsville, Alabama, USA). All samples were acquired from Men over the age of 50. PCSK9 levels in human and mouse plasma samples, as well as mouse liver lysates, were examined using commercially available ELISAs (R&D Systems). Mouse ApoB levels were also assessed using ELISAs (Abcam).

**Cholesterol and triglyceride assays** — Circulating plasma total cholesterol and triglyceride levels were examined using commercially available colorimetric assays and were carried out as per manufacturer’s instructions (Wako Diagnostics).

**Immunohistochemical staining** — Formalin-fixed paraffin-embedded liver sections were deparaffinized and stained with primary antibodies for 18 hours following heat-induced epitope retrieval. Excess primary antibodies were removed via washing prior to exposure of sections to secondary antibodies conjugated to horseradish peroxidase. Staining was visualized using Nova Red (Vector Labs, USA) and quantified using ImageJ Software (NIH, USA). For quantification purposes, 20 representative images were taken from each treatment group at a magnification power of 20X. See Table 1 for antibodies used.

**Cell culture studies** — HepG2 cells were treated with BSA-conjugated PA (300 µM; Sigma-Aldrich) for 24 hours in the presence or absence of Met (1 mM; Sigma-Aldrich) or 4-PBA (1 mM; Sigma-Aldrich). Cells were fixed in 4% paraformaldehyde for the assessment of lipid droplet accumulation via Oil-Red-O (ORO; Sigma-Aldrich) staining. SREBP2 transcriptional activity was assessed in HepG2 cells transfected with a reporter plasmid encoding a sterol regulatory element-driven GFP [9] using X-tremeGENE HP transfection reagent (Sigma-Aldrich). PCSK9 mRNA expression and ELISAs were repeated in Hepatosure® 100-donor primary human hepatocytes purchased from Xenotech (Kansas, USA). For the quantitative assessment of LDL uptake, HepG2 cells were seeded in black clear-bottom 96-well plates and treated with the indicated interventions for 24 hours. 5 hours prior to quantification using a fluorescent spectrophotometer (Molecular Devices, Gemini EM; excitation 554/emission 571), cells were...
treated with DiI-fluorescently-labelled LDL (100 ng/ml; Alpha Aesar). Excess DiI-LDL in the medium was removed and the cell monolayer was vigorously washed with Hank’s buffered saline solution containing HEPES (20 mM).

**Animal Studies — Pcsk9<sup>−/−</sup>** (n=5) and age-matched *Pcsk9<sup>+/+</sup>* controls on a C57BL/6J background (n=10) were started on HFD (60% fat; Harland Teklad) *ad libitum* at 6 weeks of age and sacrificed at 18 weeks of age. A second cohort of C57BL/6J were started on the HFD at 10-11 weeks of age and provided with either normal drinking water (n=8) or water containing 4-PBA (n=9; 1g/kg/day) for 12 weeks. Mice were fasted for 12 hours prior to sacrifice. All animal studies were performed in accordance with the McMaster University animal care guidelines.

**Immunoblot Analysis** — Cells were lysed in 4X SDS-PAGE sample buffer and separated on 10% polyacrylamide gels in reducing conditions, as described previously (63), and transferred to nitrocellulose membranes using a Trans-Blot Semi-Dry transfer apparatus (Bio-Rad). Following transfer, membranes were blocked in 1X tris-buffered saline (TBS) and 5% BSA for 45 minutes. Membranes were then incubated with primary antibodies (diluted in TBS containing 1% BSA) for 18h at 4 °C. Following primary antibody incubation, membranes were incubated in secondary antibodies conjugated to horseradish peroxidase. EZ-ECL chemiluminescent reagent (FroggaBio) was used to visualize membranes on Amersham Biosciences Hyperfilm (GE Healthcare), which were developed using a Kodak X-Omat 1000A processor.

**RNA isolation and quantitative real time PCR** — Total RNA was isolated using RNeasy Mini Kit (Qiagen) and reverse-transcribed to cDNA using High-capacity cDNA Reverse Transcription kit (Applied Biosystems). Real time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems). Primer sequences used for real time PCR are listed in Table 2.

**Statistical analysis** — Values are expressed as the mean and error bars as standard deviation (SD). Comparisons between two groups were carried out using the unpaired Student’s *t*-test and those involving multiple groups using a one-way ANOVA. Differences between groups were considered significant at *p* < 0.05.

ACKNOWLEDGEMENTS: This work was supported in part by research grants to Richard C. Austin from the Heart and Stroke Foundation of Canada (G-13-0003064 and G-15-0009389), the Canadian Institutes of Health Research (74477), to Nabil G. Seidah from the Leducq Foundation (13 CVD 03), CIHR Foundation grant (148363) and Canada Research Chair (216684). Financial support from the Research Institute of St. Joe’s Hamilton is acknowledged. Richard C. Austin is a Career Investigator of the Heart and Stroke Foundation of Ontario and holds the Amgen Canada Research Chair in the Division of Nephrology at St. Joseph’s Healthcare and McMaster University.

CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS: PL and RCA wrote the manuscript. PL, JHB, KP, MEM and SP carried out all in vivo experiments. In vitro studies were conducted by PL, JHB and MF. The manuscript was revised by KP, JHB, MF and NGS.
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**Abbreviations:**

4PBA, 4-phenylbutyrate; ANOVA, analysis of variance; ApoB, apolipoprotein-B; ATF4, activating transcription factor 4; BBC3, Bel-2-binding component 3; CASP, caspase; CHOP, C/EBP homologous
protein; CVD, cardiovascular disease; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FN1, fibronectin 1; GFP, green fluorescent protein; GRP, glucose-regulated protein; H&E, hematoxylin and eosin; HFD, high-fat diet; HMGR, HMG-CoA reductase; IRE1α, inositol-requiring enzyme 1α; LDL, low-density lipoprotein; LDLR, LDL receptor; Met, metformin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NCD, normal control diet; ORO, Oil-Red-O; PA, palmitate; PCR, polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; PERK, PKR-like endoplasmic reticulum kinase; SD, standard deviation; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; TBS, tris-buffered saline; TG, thapsigargin; TM, tunicamycin; UPR, unfolded protein response; XBP1, X-box-binding protein.

Table 1. Antibodies used for immunoblotting and immunohistochemical staining.

| Antibody | Catalogue no. | Application | Dilution       |
|----------|---------------|-------------|----------------|
| GRP78    | SC-1050, Santa Cruz Biotechnology | IHC | 1:40, no retrieval |
| GRP94    | ADI-SPA-850, Enzo | IHC | 1:100, HIER |
| LDLR     | AF-2255, R and D systems | IHC | 1:100, HIER |
| Fibronectin | PA5-29578, ThermoFisher Scientific | IHC | 1:200, HIER |
| GRP78    | 610979, BD Bioscience | IB | 1:1000 |
| IRE1α    | 14C10, Cell Signaling Technologies | IB | 1:500 |
| XBP1     | Sc-8015, Santa Cruz Biotechnology | IB | 1:2000 |
| β-Actin  | MA5-15739, Thermofisher Scientific | IB | 1:5000 |
| LDLR     | AF2255, R and D Systems | IB | 1:1000 |

Table 2. Primers used for real time PCR.

| Gene | Species | Forward | Reverse |
|------|---------|---------|---------|
| ApoB | Mouse   | AAGCACCCTCCGAAAAGTAGTACGTG | TTCCAGCTCTACCTTACAGTTGA |
| ATF4 | Mouse   | ATGGCCGCGCTATGGATGAT | CGAAGTCAAACACTTTCCAGATCCATT |
| BBC3 | Mouse   | TGTTGAGGAGGAGAGTGAGG | TGCTGTTCAGCTTTTGGCTTCG |
| CASP3| Mouse   | CCTCAGAGAGACACTTCATGG | GCAGTAGTCGCCCTGAGAA |
| CHOP | Mouse   | CTTGCTTTTTCATGGAGAC | CGTTTTCTGAGGATGAGATA |
| FAS  | Mouse   | GCAGTAGAGAGCATGTGTTTAG | GGCCTAGGGGTCCATGTT |
| FN1  | Mouse   | CGAGGTTGACAGAGACCACAA | CTGGAGTCAAGGGCAACA |
| GRP78| Mouse   | GTCCTGCATCATCAGCAGAAG | GTGACCCACATAGTACACA |
| GRP94| Mouse   | GTTGGTCTGGCAACATGGGAG | CGCCTTGTGCTCCGTGTAAGA |
| HMGR | Mouse   | CTTGGAGGAAAGAGGCTGTCAC | CTAGTGGGAAGTGAATGGGCAATCATG |
| LDLR | Mouse   | GAGGAGCAGCCATGGATGT | GCTGTGCCTTGGTGCTTC |
| PCSK9| Mouse   | TCAGCAGTCTTAGGGAACTT | CCGAATGTCATCCTTGGGA |
| PERK | Mouse   | GTAGTCCTGCAATAGCCAAAG | CCTTTCCCGTGCCAACCTC |
| SREBP1| Mouse  | GGAGGCCAGGATTTGACATT | GGCGCAGGAGTACCTG |
| SREBP2| Mouse  | GCAGTTGCTGGAGACCATGGA | ACAAAGTGTGCTGAAAAACAAATCA |
Figure Legends

Figure 1. Circulating PCSK9 levels are increased in patients with hepatic steatosis. (A-C) Circulating PCSK9 levels, as well as total cholesterol and triglyceride levels, were assessed in patients with fatty liver (n=9) and compared to healthy volunteers (n=9). All samples were acquired from men over the age of 50.

Figure 2. Diet-induced hepatic steatosis increases circulating PCSK9 levels and blocks cell-surface LDLR expression in mice. C57BL/6J mice were fed either a normal control diet (NCD; n=10) or a high-fat diet (HFD; n=10) ad libitum starting at 6 weeks of age, for an additional 12 weeks. (A) Hepatic lipid droplet accumulation was confirmed using H&E as well as oil-red-o (ORO) staining. (B) Cell-surface LDLR protein expression was examined via immunohistochemical staining. LDLR antibody staining specificity was confirmed in the livers of Ldlr-/- mice. (C) Total hepatic LDLR expression was also examined via immunoblot analysis. (D) ORO and LDLR staining intensities were quantified using ImageJ Software. (E,F) Circulating PCSK9 and ApoB protein levels were examined using ELISAs (n=5). (G) Total cholesterol and triglyceride levels were also examined in the serum of NCD- and HFD-mice. *, p<0.05. Values are presented as the mean and error bars as SD.

Figure 3. HFD-fed mice exhibit hepatic ER stress and increased expression of SREBP2 and PCSK9. C57BL/6J mice were fed either a normal control diet (NCD; n=10) or a high-fat diet (HFD; n=10) ad libitum starting at 6 weeks of age, for an additional 12 weeks. (A) Immunohistochemical staining of ER stress markers, GRP78 and GRP94, as well as Masson’s Trichrome staining for fibrotic collagen deposition (blue) in the livers of HFD-fed mice. (B) Real time PCR analysis of hepatic ER stress marker expression (GRP78, GRP94, CHOP, ATF4, PERK, IRE1a) and apoptosis and fibrosis markers (CASP1, CASP3 and FN1). (C) Immunoblot used to examine LDLR expression in the livers of HFD-fed mice from figure 2C were re-probed for ER stress markers GRP78, IRE1a and XBP1. (D) Real time PCR analysis of hepatic PCSK9, LDLR and SREBP2 mRNA transcript levels. (E) Assessment of liver PCSK9 protein levels using an ELISA. (F) Analysis of mRNA transcript abundance of established cholesterol and triglyceride modulators HMGCR, SREBP1, FAS and APOB via real time PCR. *, p<0.05. Values are presented as the mean and error bars as SD.

Figure 4. Blocking ER stress and lipid accumulation attenuates PCSK9 expression and restores LDLR expression and function. (A) HepG2 cells were pre-treated with metformin (Met; 1 mM) or 4-phenylbutyrate (4-PBA; 1mM) for 24 hours and subsequently treated with bovine serum albumin (BSA)-conjugated palmitate (PA; 300 µM). SREBP2, PCSK9 and LDLR, as well as ER stress markers GRP78, GRP94 and IRE1a expression was assessed using real time PCR. (B) Lipid droplet accumulation in these cells was also assessed via Oil-Red-O staining. (C) HepG2 cells were transfected with a sterol-regulatory element (SRE) reporter plasmid encoding green fluorescent protein (GFP) and subsequently pre-treated with Met (1 mM) or 4-phenylbutyrate (4-PBA; 1 mM) in the presence or absence of BSA-conjugated palmitate (PA; 300 µM). SREBP2-mediated GFP expression was assessed using a fluorescent microscope. (D) Secreted PCSK9 levels from HepG2 and HuH7 cells grown in FBS-free medium were examined via ELISA. Coomassie staining of electrophoretically-resolved medium harvested from these cells demonstrates that treatments did not affect global protein secretion. (E) Fluorescently-labelled Dil-LDL uptake was examined in treated HepG2 cells. (F) Experiments were repeated in cultured primary human hepatocytes (PA, 300 µM, 24 hours). (G,H) Male C57BL/6J mice were fed HFD in the presence or absence of 4PBA in the drinking water. Hepatic LDLR expression, as well as GRP78, GRP94 and fibronectin expression was examined via immunohistochemical staining. *, p<0.05. Values are presented as the mean and error bars as SD.

Figure 5. HFD fails to affect hepatic cell-surface LDLR and plasma LDL in Pcsk9-/- mice. (A) Pcsk9-/- mice on the C57BL/6J background were fed either normal control diet (NCD; n=5) or high-fat
diet (HFD; n=5) ad libitum for 12 weeks starting at 6 weeks of age. (A,B) Hepatic lipid droplet accumulation was assessed via H&E and Oil-Red-O (ORO) and cell-surface LDLR expression via immunohistochemical staining. (C) Staining intensity was quantified using ImageJ Software (*, p<0.05; n=5). (D) Hepatic LDLR expression was also examined via immunoblot analysis. (E) Circulating ApoB levels were assessed using an ELSIA. (F) PCSK9 knockout was also confirmed via ELISA of circulating PCSK9. *, p<0.05. Values are presented as the mean and error bars as SD.

**Figure 6. Fatty liver increases de novo PCSK9 to block cell-surface LDLR expression.** The healthy liver regulates circulating LDL cholesterol levels by means of expressing high levels of cell-surface LDLR compared to most tissue types. Diet-induced hepatic steatosis however, causes ER stress, which leads to an increase in SREBP2 activation and expression of the natural inhibitor of the LDLR, PCSK9. In this pathologic milieu, PCSK9 prevails over the LDLR and contributes to dyslipidemia and risk of CVD.
Figure 1. Lebeau and Byun et al., 2019

A

Plasma PCSK9 Concentration (ng/ml)

Control Fatty Liver

p = 0.013

B

Total Plasma Cholesterol (mg/dl)

Control Fatty Liver

p = 0.0001

C

Total Plasma Triglycerides (mg/dl)

Control Fatty Liver

p = 0.0020
Figure 2. Lebeau and Byun et al., 2019

A

|       | H&E          | ORO          |
|-------|--------------|--------------|
| NCD   | ![H&E Image] | ![ORO Image] |
| HFD   | ![H&E Image] | ![ORO Image] |

B

|       | LDLR         |
|-------|--------------|
| NCD   | ![LDLR Image] |
| HFD   | ![LDLR Image] |

C

|        | LDLR | β-actin |
|--------|------|---------|
| NCD    | ![LDLR Image] | ![β-actin Image] |
| HFD    | ![LDLR Image] | ![β-actin Image] |

D

|        | ORO Staining Intensity (x Area) |
|--------|---------------------------------|
| NCD    | ![ORO Staining Image]           |
| HFD    | ![ORO Staining Image]           |

E

|        | LDLR Staining Intensity (x Area) |
|--------|---------------------------------|
| NCD    | ![LDLR Staining Image]          |
| HFD    | ![LDLR Staining Image]          |

F

|        | Serum ApoB Concentration (µg/ml) |
|--------|----------------------------------|
| NCD    | ![Serum ApoB Image]              |
| HFD    | ![Serum ApoB Image]              |

G

|        | Total Serum Cholesterol (mg/dl) |
|--------|---------------------------------|
| NCD    | ![Total Serum Cholesterol Image]|
| HFD    | ![Total Serum Cholesterol Image]|

|        | Total Serum Triglycerides (mg/dl) |
|--------|-----------------------------------|
| NCD    | ![Total Serum Triglycerides Image]|
| HFD    | ![Total Serum Triglycerides Image]|

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Figure 3. Lebeau and Byun et al., 2019

A

| NCD | GRP78 | GRP94 | Trichrome |
|-----|-------|-------|-----------|
|     | ![Image](GRP78_NCD.png) | ![Image](GRP94_NCD.png) | ![Image](Trichrome_NCD.png) |
| HFD | ![Image](GRP78_HFD.png)   | ![Image](GRP94_HFD.png)   | ![Image](Trichrome_HFD.png) |

200 µm

B

Fold Induction of mRNA (Normalized to 18S)

NCD | HFD
---|---
| ![Graph](NCD_HFD_graph.png) |

C

| NCD | HFD |
|-----|-----|
| IRE1α | ![Image](IRE1α_NCD.png) | ![Image](IRE1α_HFD.png) |
| GRP78 | ![Image](GRP78_NCD.png) | ![Image](GRP78_HFD.png) |
| XBP1  | ![Image](XBP1_NCD.png) | ![Image](XBP1_HFD.png) |
| sXBP1 | ![Image](sXBP1_NCD.png) | ![Image](sXBP1_HFD.png) |
| β-actin | ![Image](β-actin_NCD.png) | ![Image](β-actin_HFD.png) |

D

Fold Induction of PDCD3 mRNA

E

Fold Induction of ANKRD1 mRNA

F

Fold Induction of CASP8 RNA

Fold Induction of mRNA (Normalized to 18S)
Figure 5. Lebeau and Byun et al., 2019
Figure 6. Lebeau and Byun et al., 2019

Healthy Liver

Fatty Liver

Excess Dietary Fat Consumption

Hepatocyte

LDLR

ER

Clearance

Lipid Accumulation

ER Stress

SREBP2

LDLR Degradation

PCSK9

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