

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

Received for publication, February 19, 2019, and in revised form, April 9, 2019. Published, Papers in Press, April 19, 2019, DOI 10.1074/jbc.RA119.008094

Paul F. Lebeau<sup>†1</sup>, Jae Hyun Byun<sup>†1</sup>, Khrystyna Platko<sup>‡</sup>, Melissa E. MacDonald<sup>‡</sup>, Samantha V. Poon<sup>‡</sup>, Mahi Faiyaz<sup>‡</sup>, Nabil G. Seidah<sup>§</sup>, and Richard C. Austin<sup>†2</sup>

From the <sup>†</sup>Department of Medicine, McMaster University, The Research Institute of St. Joe's Hamilton, Hamilton, Ontario L8N 4A6 and the <sup>§</sup>Laboratory of Biochemical Neuroendocrinology, Montreal Clinical Research Institute, University of Montreal, Montreal, Quebec H2W 1R7, Canada

Edited by George N. DeMartino

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 patients, diet-induced steatosis increases circulating PCSK9 levels as a result of *de novo* expression in mice. We also report the 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, including PCSK9 and the LDLR, and also highlights a novel mechanism by which liver disease may contribute to CVD.

Liver fat accumulation due to reasons other than excessive alcohol consumption, or more often referred to as NAFLD,<sup>3</sup>

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 described as 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 proinflammatory mediators, prothrombotic 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, loss-of-function mutations have the opposite outcome (17). Pre-clinical data also demonstrate that

This work was supported by the Heart and Stroke Foundation of Canada Research Grants G-13-0003064 and G-15-0009389 and Canadian Institutes of Health Research Grant 74477 (to R. C. A.), Leducq Foundation Grant 13 CVD 03, CIHR Foundation Grant 148363, and Canada Research Chair Grant 216684 (to N. G. S.), and the Research Institute of St. Joe's Hamilton. The authors declare that they have no conflicts of interest with the contents of this article.

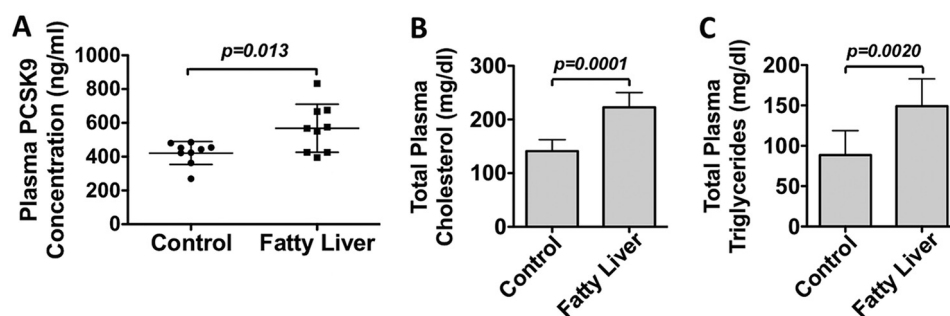
<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> A Career Investigator of the Heart and Stroke Foundation of Ontario and holds the Amgen Canada Research Chair in the Division of Nephrology at The Research Institute of St. Joe's Hamilton and McMaster University. To whom correspondence should be addressed: 50 Charlton Ave. East, Rm. T-3313, Hamilton, Ontario L8N 4A6, Canada. Tel.: 905-522-1155 (ext. 35175); Fax: 905-540-6589; E-mail: [austinr@taari.ca](mailto:austinr@taari.ca).

<sup>3</sup> The abbreviations used are: NAFLD, non-alcoholic fatty liver disease; 4PBA, 4-phenylbutyrate; ApoB, apolipoprotein-B; ATF4, activating transcription factor 4; CASP, caspase; CHOP, C/EBP homologous protein; CVD, cardiovascular disease; ER, endoplasmic reticulum; FN1, fibronectin 1; GFP, green fluorescent protein; GRP, glucose-regulated protein; H&E, hematoxylin

and eosin; HFD, high-fat diet; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IRE1 $\alpha$ , inositol-requiring enzyme 1 $\alpha$ ; LDL, low-density lipoprotein; LDLR, LDL receptor; Met, metformin; NASH, non-alcoholic steatohepatitis; NCD, normal control diet; ORO, Oil Red O; PA, palmitate; PCSK9, proprotein convertase subtilisin/kexin type 9; PERK, PKR-like endoplasmic reticulum kinase; SREBP, sterol regulatory element-binding protein; TBS, Tris-buffered saline; UPR, unfolded protein response; XBP1, X-box-binding protein.

This is an open access article under the CC BY license.



**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 with healthy volunteers ( $n = 9$ ). All samples were acquired from males over the age of 50.

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*<sup>−/−</sup> 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*<sup>−/−</sup> 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

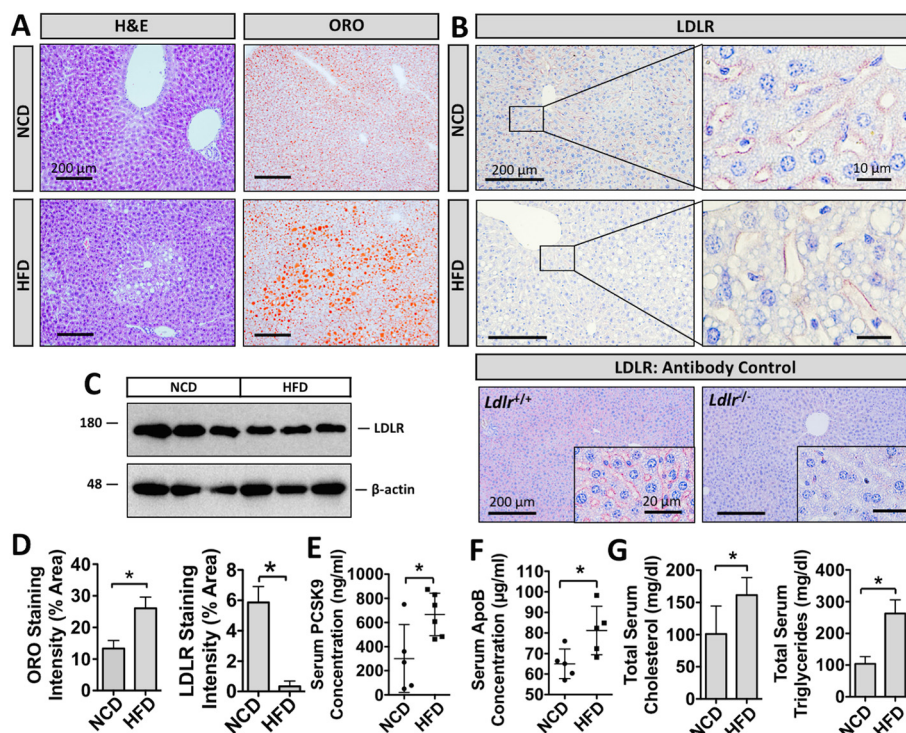
### HFD increases circulating PCSK9 levels and attenuates hepatic cell-surface LDLR expression in mice

Consistent with previous studies (12), we first confirmed that patients with liver fat accumulation exhibit increased plasma PCSK9 levels (Fig. 1A;  $p = 0.013$ ;  $n = 9$ ), as well as circulating cholesterol (Fig. 1B;  $p = 0.0001$ ;  $n = 9$ ) and triglyceride levels (Fig. 1C;  $p = 0.0020$ ;  $n = 9$ ). 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) (Fig. 2A). Strikingly, immunohistochemical staining revealed that HFD-fed mice had markedly reduced cell-surface LDLR expression compared with NCD-fed controls (Fig. 2B). As expected, LDLR antibody staining specificity was confirmed by the absence of staining in the livers of *Ldlr*<sup>−/−</sup> mice. In contrast, immunoblotting revealed a modest reduction in LDLR expression in the livers of HFD-fed mice (Fig. 2C). These data are representative of whole-cell LDLR abundance, however, and not strictly of the cell-surface LDLR population. Therefore, these data suggest a strong presence of an intracellular LDLR population in liver hepatocytes, which is not affected by the HFD. ORO and LDLR immunohistochemical staining intensities were also quantified using ImageJ software (Fig. 2D). Consistent with previous studies, we observed that hepatic LDLR expression was inversely correlated with circulating PCSK9 levels in mice (Fig. 2D) (14). The surrogate marker of circulating LDL cholesterol, 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 with NCD-fed controls (Fig. 2, E–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-



**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 NCD ( $n = 10$ ) or a 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 ORO staining. *B*, cell-surface LDLR protein expression was examined via immunohistochemical staining. LDLR antibody staining specificity was confirmed in the livers of *Ldlr*<sup>-/-</sup> mice. *C*, total hepatic LDLR expression was also examined via immunoblot analysis. *D*, ORO and LDLR staining intensities were quantified using ImageJ software. *E* and *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-fed mice. \*,  $p < 0.05$ . Error bars represent values presented as the mean  $\pm$  S.D.

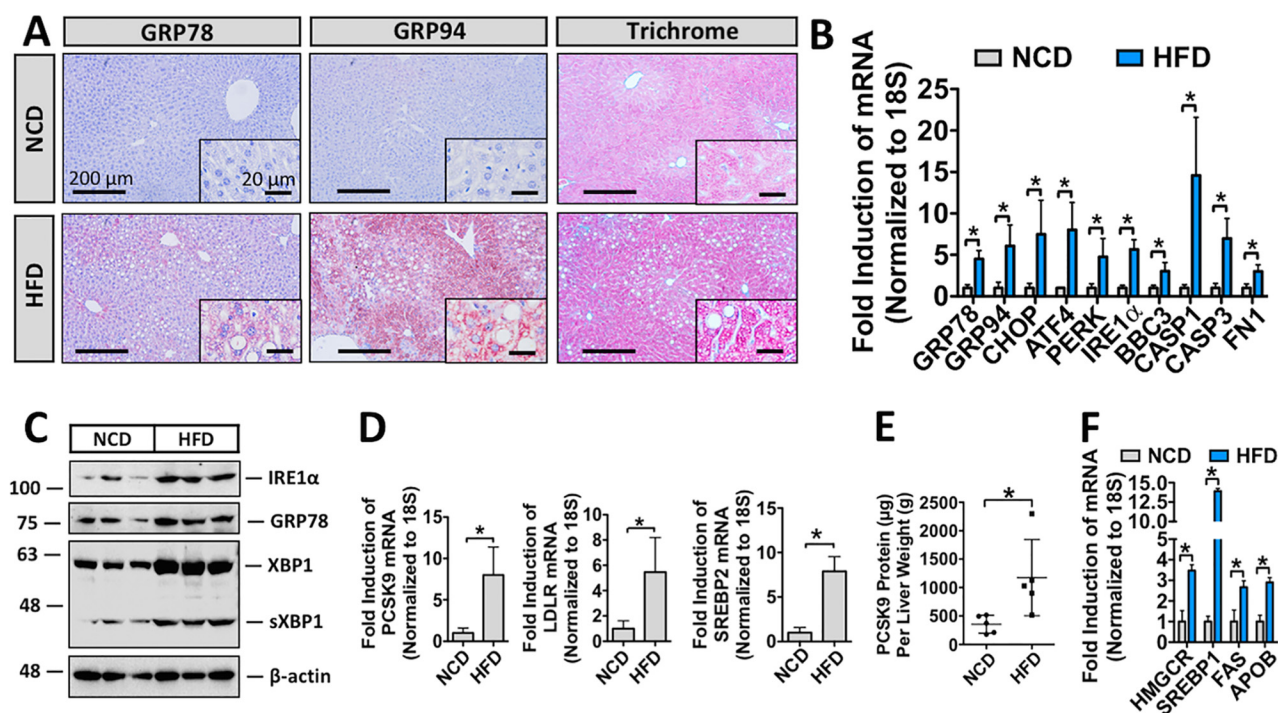
requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ), and spliced X-box-binding protein 1 (sXBP1) was observed in the livers of HFD-fed mice compared with controls via immunohistochemical staining, real-time PCR, and immunoblotting (Fig. 3, *A–C*). Furthermore, similar to our previous studies done in cultured cells (25), we observed that hepatic ER stress was associated with increased mRNA expression of SREBP2, as well as PCSK9 and the LDLR (Fig. 3*C*). Increased intracellular PCSK9 protein abundance was also observed in the livers of HFD-fed mice compared with NCD-fed controls (Fig. 3*D*). Additional modulators of cholesterol and triglyceride homeostasis, including the SREBP2-regulated HMG-CoA reductase, SREBP1, fatty acid synthase, and ApoB were examined via real-time PCR and found to be induced in the livers of HFD-fed mice (Fig. 3*F*). 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 are 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 with those treated with the BSA vehicle control (Fig. 4*A*). 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 h prior to PA treatment. Consistent with other studies, we observed that both agents reduced lipid accumulation (Fig. 4*B*) (36, 37) and attenuated PA-induced expression of the ER stress markers, GRP78, GRP94, and IRE1 $\alpha$ . 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 (Fig. 4*C*). 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 (Fig. 4*D*). 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





**Figure 3. HFD-fed mice exhibit hepatic ER stress and increased expression of SREBP2 and PCSK9.** C57BL/6J mice were fed either a NCD ( $n = 10$ ) or a HFD ( $n = 10$ ) *ad libitum* starting at 6 weeks of age, for an additional 12 weeks. **A**, immunohistochemical staining of the 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, and IRE1 $\alpha$ ), apoptosis, and fibrosis markers (CASP1, CASP3, and FN1). **C**, immunoblots used to examine LDLR expression in the livers of HFD-fed mice from Fig. 2C were re-probed for ER stress markers GRP78, IRE1 $\alpha$ , 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 HMG-CoA reductase (HMGCR), SREBP1, fatty acid synthase (FAS), and ApoB via real-time PCR. \*,  $p < 0.05$ . Error bars represent values presented as the mean  $\pm$  S.D.

changes in secreted PCSK9 levels are not the result of changes in global protein secretion.

Next, a quantitative assessment of fluorescently-labeled DiI-LDL uptake was carried out in HepG2 cells treated with vehicle, Met, or 4PBA in the presence or absence of PA (Fig. 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 (Fig. 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 (Fig. 4G). A reduction in the expression of the 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 (Fig. 4H).

#### Diet-induced hepatic steatosis attenuates hepatic LDLR expression in a PCSK9-dependent manner

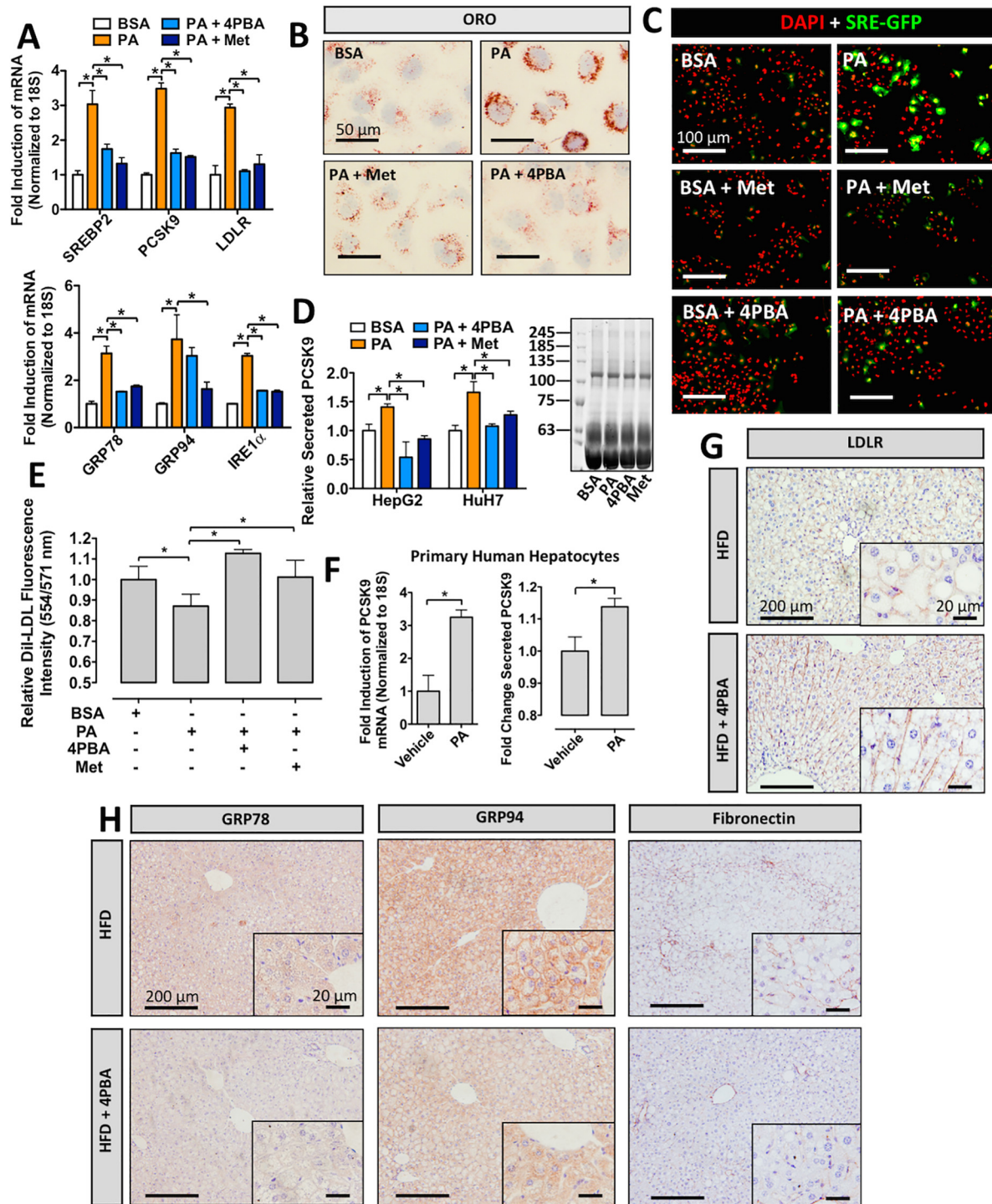
Because LDLR expression is known to be affected by other proteins and conditions (40), our final aim was to identify the extent to which PCSK9 contributed to the observed reduction of cell-surface LDLR expression in HFD-fed mice. Accordingly,

*Pcsk9*<sup>-/-</sup> 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*<sup>-/-</sup> mice compared with those fed the NCD (Fig. 5, A 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 (Fig. 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*<sup>-/-</sup> mice (Fig. 5D). Furthermore, HFD also failed to increase circulating ApoB-containing LDL cholesterol in *Pcsk9*<sup>-/-</sup> mice (Fig. 5E). PCSK9 knockout in these mice was confirmed using an ELISA for circulating PCSK9 (Fig. 5F).

Collectively, these data suggest that intracellular lipid accumulation causes ER stress, which induces *de novo* PCSK9 expression and secretion from hepatocytes (Fig. 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

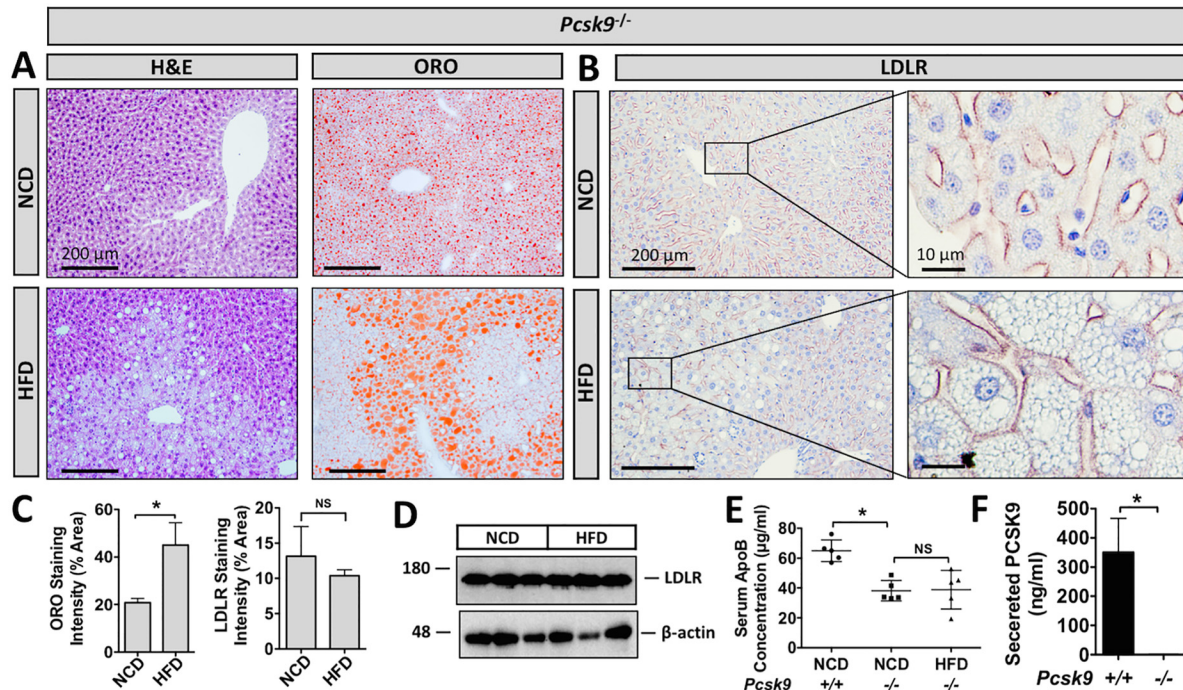


**Figure 4. Blocking ER stress and lipid accumulation attenuates PCSK9 expression and restores LDLR expression and function.** *A*, HepG2 cells were pre-treated with Met (1 mM) or 4PBA (1 mM) for 24 h and subsequently treated with BSA-conjugated PA (300  $\mu$ M). SREBP2, PCSK9, and LDLR, as well as the ER stress markers GRP78, GRP94, and IRE1 $\alpha$  expression was assessed using real-time PCR. *B*, lipid droplet accumulation in these cells was also assessed via ORO staining. *C*, HepG2 cells were transfected with a SRE reporter plasmid encoding GFP and subsequently pre-treated with Met (1 mM) or 4PBA (1 mM) in the presence or absence of BSA-conjugated PA (300  $\mu$ M). 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-labeled DiI-LDL uptake was examined in treated HepG2 cells. *F*, experiments were repeated in cultured primary human hepatocytes (PA, 300  $\mu$ M, 24 h). *G* and *H*, male C57BL/6J mice were fed a 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. \*,  $p < 0.05$ . Error bars represent values presented as the mean  $\pm$  S.D.

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





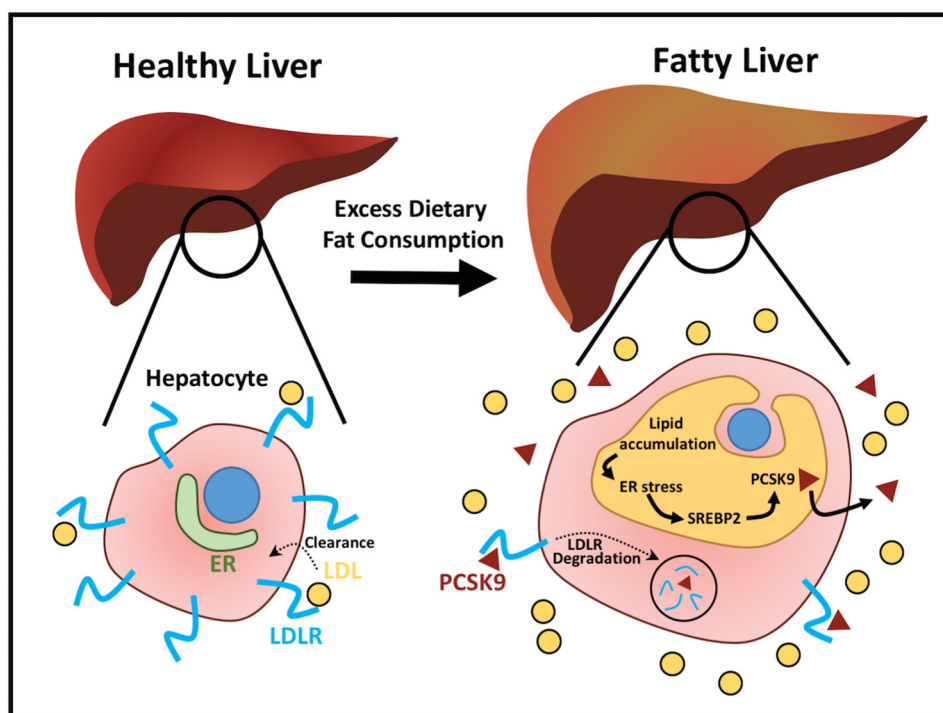
**Figure 5. HFD fails to affect hepatic cell-surface LDLR and plasma LDL in *Pcsk9*<sup>-/-</sup> mice.** A, *Pcsk9*<sup>-/-</sup> mice on a C57BL/6J background were fed either NCD (*n* = 5) or HFD (*n* = 5) *ad libitum* for 12 weeks starting at 6 weeks of age. A and B, hepatic lipid droplet accumulation was assessed via H&E and 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 ELISA. F, PCSK9 knockout was also confirmed via ELISA of circulating PCSK9 (\*, *p* < 0.05). Error bars represent values presented as the mean ± S.D.

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 (Fig. 3, A 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 (Fig. 3C). Furthermore, we demonstrate that hepatic ER stress caused a significant increase in PCSK9 expression and secretion (Figs. 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 (Fig. 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 (Figs. 2B and 4E, respectively). Given that this phenotype was not observed in HFD-fed *Pcsk9*<sup>-/-</sup> 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 (Fig. 6).

In a previous study, we observed that ER stress resulting from ER Ca<sup>2+</sup> depletion, but not from the inhibition of *N*-glycosylation, 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<sup>2+</sup> depletion. Consistent with this notion, previous studies have also demonstrated that fatty acid uptake and accumulation causes ER Ca<sup>2+</sup> 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 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 (Fig. 2, B, 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 (Fig. 3C), but blocked its expression at the protein level



**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 with 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.

(Fig. 4A). Consistent with our observations, LDLR expression is inversely correlated with SREBP2 activation 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 inhibitors activate SREBP2 and increase hepatic LDLR expression, as well as circulating PCSK9 levels (54, 55). Interestingly, a recent study has demonstrated that HMG-CoA reductase inhibitors can increase circulating PCSK9 levels while blocking the occurrence of a gain-of-function phosphorylation at position 688 (56). Given that HMG-CoA reductase 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 5 min (58), PCSK9 protein expression is more likely dependent on *de novo* synthesis than the LDLR, which has a half-life of 12 h (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 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 (12) also reported a statistically significant positive correlation between steatosis grade and circulating PCSK9 levels. 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 (60) 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. 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 proinflammatory 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, AL). All samples were acquired from males 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).

**Table 1**

Antibodies used for immunoblotting and immunohistochemical (IHC) staining

Antibody	Catalog No.	Application	Dilution
GRP78	SC-1050, Santa Cruz Biotechnology	IHC <sup>a</sup>	1:40, no retrieval
GRP94	ADI-SPA-850, Enzo Life Sciences	IHC	1:100, HIER <sup>c</sup>
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 <sup>b</sup>	1:1000
IRE1 $\alpha$	14C10, Cell Signaling Technologies	IB	1:500
XBP1	Sc-8015, Santa Cruz Biotechnology	IB	1:2000
$\beta$ -Actin	MA5-15739, ThermoFisher Scientific	IB	1:5000
LDLR	AF2255, R and D Systems	IB	1:1000

<sup>a</sup> IHC, immunohistochemistry.<sup>b</sup> IB, immunoblot.<sup>c</sup> HIER, heat-induced epitope retrieval.**Table 2**

Primers used for real-time PCR

Gene	Species	Forward	Reverse
ApoB	Mouse	AAGCACCTCCGAAAGTACGTG	CTCCAGCTCTACCTTACAGTTGA
ATF4	Mouse	ATGGCCGGCTATGGATGAT	CGAAGTCAAACCTTTTCAGATCCATT
BBC3	Mouse	TGTGGAGGAGGAGGAGTGG	TGCTGCTCTTCTGTCTCCG
CASP1	Mouse	TCCGCGGTGAATCCTTTTCAGA	ACCACAATTGTGTGTGTGCGCA
CASP3	Mouse	CCTCAGAGAGACATTATGG	GCAGTAGTCGGCTCTGAAGA
CHOP	Mouse	CTGCCTTTCACCTGGAGAC	CGTTTCCTGGGATGAGATA
FAS	Mouse	GCGATGAAGAGCATGGTTTAG	GGCTCAAGGGTTCATGTT
FN1	Mouse	CGAGGTGACAGAGACCACAA	CTGGAGTCAAGCCAGACACA
GRP78	Mouse	GTCTGCATCATCAGCGAAAG	GGTAGCCACATACTGAACACCA
GRP94	Mouse	GATGGTCTGGCAACATGGAG	CGCCTTGGTGTCTGGTAGAA
HMGCR	Mouse	CTTTCAGAAACGAAGTGTAGC	CTAGTGAAGATGAATGGACATGAT
LDLR	Mouse	GAGGAGCAGCCACATGGTAT	GCTCGTCTCTGTGGTCTTC
PCSK9	Mouse	TTGCAGCAGCTGGGAACCT	CCGACTGTGATGACCTCTGGA
PERK	Mouse	GATGACTGCAATTACGCTATCAAGA	CCTTCTCCCGTGCCAACTC
SREBP1	Mouse	GGAGCCATGGATTGCACATT	GGCCCCGGAAGTCACTGT
SREBP2	Mouse	GCGTCTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAACAAATCA

### Cholesterol and triglyceride assays

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

### Immunohistochemical staining

Formalin-fixed paraffin-embedded liver sections were deparaffinized and stained with primary antibodies for 18 h 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) and quantified using ImageJ. For quantification purposes, 20 representative images were taken from each treatment group at a magnification power of  $\times 20$ . See Table 1 for antibodies used.

### Cell culture studies

HepG2 cells were treated with BSA-conjugated PA (300  $\mu$ M; Sigma-Aldrich) for 24 h in the presence or absence of Met (1 mM; Sigma-Aldrich) or 4PBA (1 mM; Sigma-Aldrich). Cells were fixed in 4% paraformaldehyde for the assessment of lipid droplet accumulation via ORO (Sigma-Aldrich) staining. SREBP2 transcriptional activity was assessed in HepG2 cells transfected with a reporter plasmid encoding a SRE-driven GFP (9) using X-tremeGENE HP transfection reagent (Sigma-Aldrich). PCSK9 mRNA expression and ELISAs were repeated in Hepatosure<sup>®</sup> 100-donor primary human hepatocytes purchased from Xenotech (Kansas City, KS). 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 h. Five hours prior to quantification using a fluorescent spectrophotometer (Molecular Devices, Gemini EM; excitation 554/emission 571), cells were treated with DiI fluorescently-labeled 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 mice were started on the HFD at 10–11 weeks of age and provided with either normal drinking water ( $n = 8$ ) or water containing 4PBA ( $n = 9$ ; 1 g/kg/day) for 12 weeks. Mice were fasted for 12 h prior to sacrifice. All animal studies were performed in accordance with the McMaster University animal care guidelines.

### Immunoblot analysis

Cells were lysed in 4 $\times$  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 1 $\times$  Tris-buffered saline (TBS) and 5% BSA for 45 min. Membranes were then incubated with primary antibodies (diluted in TBS containing 1% BSA) for 18 h at 4  $^{\circ}$ C. Following primary antibody incubation, membranes were exposed to 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

Error bars represent values expressed as the mean  $\pm$  S.D. Comparisons between two groups were carried out using the unpaired Student's *t* test and those involving multiple groups using a one-way analysis of variance. Differences between groups were considered significant at  $p < 0.05$ .

**Author contributions**—P. F. L., J. H. B., K. P., N. G. S., and R. C. A. conceptualization; P. F. L., J. H. B., K. P., S. V. P., M. F., and R. C. A. data curation; P. F. L. software; P. F. L. formal analysis; P. F. L. supervision; P. F. L. validation; P. F. L., J. H. B., K. P., M. E. M., S. V. P., and R. C. A. investigation; P. F. L. visualization; P. F. L., J. H. B., K. P., M. E. M., S. V. P., and N. G. S. methodology; P. F. L. writing-original draft; P. F. L., J. H. B., and R. C. A. project administration; P. F. L., J. H. B., K. P., M. F., N. G. S., and R. C. A. writing-review and editing; R. C. A. resources; R. C. A. funding acquisition.

### References

- Vernon, G., Baranova, A., and Younossi, Z. M. (2011) Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment. Pharmacol. Ther.* **34**, 274–285 [CrossRef Medline](#)
- Sayiner, M., Koenig, A., Henry, L., and Younossi, Z. M. (2016) Epidemiology of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis in the United States and the rest of the world. *Clin. Liver Dis.* **20**, 205–214 [CrossRef Medline](#)
- Benedict, M., and Zhang, X. (2017) Non-alcoholic fatty liver disease: an expanded review. *World J. Hepatol.* **9**, 715–732 [CrossRef Medline](#)
- Calzadilla Bertot, L., and Adams, L. A. (2016) The natural course of non-alcoholic fatty liver disease. *Int. J. Mol. Sci.* **17**, e774 [Medline](#)
- World Health Organization (2016) *Hearts: technical package for cardiovascular disease management in primary health care*. World Health Organization, Switzerland
- Söderberg, C., Stål, P., Askling, J., Glaumann, H., Lindberg, G., Marmur, J., and Hultcrantz, R. (2010) Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* **51**, 595–602 [CrossRef Medline](#)
- Pickhardt, P. J., Hahn, L., Muñoz del Rio, A., Park, S. H., Reeder, S. B., and Said, A. (2014) Natural history of hepatic steatosis: observed outcomes for subsequent liver and cardiovascular complications. *Am. J. Roentgenol.* **202**, 752–758 [CrossRef Medline](#)
- Ekstedt, M., Hagström, H., Nasr, P., Fredrikson, M., Stål, P., Kechagias, S., and Hultcrantz, R. (2015) Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* **61**, 1547–1554 [CrossRef Medline](#)
- Patil, R., and Sood, G. K. (2017) Non-alcoholic fatty liver disease and cardiovascular risk. *World J. Gastrointest. Pathophysiol.* **8**, 51–58 [CrossRef Medline](#)
- Francque, S. M., van der Graaff, D., and Kwanten, W. J. (2016) Non-alcoholic fatty liver disease and cardiovascular risk: pathophysiological mechanisms and implications. *J. Hepatol.* **65**, 425–443 [CrossRef Medline](#)
- Lakoski, S. G., Lagace, T. A., Cohen, J. C., Horton, J. D., and Hobbs, H. H. (2009) Genetic and metabolic determinants of plasma PCSK9 levels. *J. Clin. Endocrinol. Metab.* **94**, 2537–2543 [CrossRef Medline](#)
- Ruscica, M., Ferri, N., Macchi, C., Meroni, M., Lanti, C., Ricci, C., Maggioni, M., Fracanzani, A. L., Badiali, S., Fargion, S., Magni, P., Valenti, L., and Dongiovanni, P. (2016) Liver fat accumulation is associated with circulating PCSK9. *Ann. Med.* **48**, 384–391 [CrossRef Medline](#)
- Benjannet, S., Rhainds, D., Essalmani, R., Mayne, J., Wickham, L., Jin, W., Asselin, M. C., Hamelin, J., Varret, M., Allard, D., Trillard, M., Abifadel, M., Tebon, A., Attie, A. D., Rader, D. J., et al. (2004) NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *J. Biol. Chem.* **279**, 48865–48875 [CrossRef Medline](#)
- Maxwell, K. N., and Breslow, J. L. (2004) Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 7100–7105 [CrossRef Medline](#)
- Seidah, N. G., Abifadel, M., Prost, S., Boileau, C., and Prat, A. (2017) The proprotein convertases in hypercholesterolemia and cardiovascular diseases: emphasis on proprotein convertase subtilisin/kexin 9. *Pharmacol. Rev.* **69**, 33–52 [Medline](#)
- Abifadel, M., Varret, M., Rabès, J. P., Allard, D., Ouguerram, K., Devillers, M., Cruaud, C., Benjannet, S., Wickham, L., Erlich, D., Derré, A., Villéger, L., Farnier, M., Beutler, L., Bruckert, E., et al. (2003) Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat. Genet.* **34**, 154–156 [CrossRef Medline](#)
- Mayne, J., Dewpura, T., Raymond, A., Bernier, L., Cousins, M., Ooi, T. C., Davignon, J., Seidah, N. G., Mbikay, M., and Chrétien, M. (2011) Novel loss-of-function PCSK9 variant is associated with low plasma LDL cholesterol in a French-Canadian family and with impaired processing and secretion in cell culture. *Clin. Chem.* **57**, 1415–1423 [CrossRef Medline](#)
- Kumar, S., Kang, D. W., Rezvan, A., and Jo, H. (2017) Accelerated atherosclerosis development in C57BL6 mice by overexpressing AAV-mediated PCSK9 and partial carotid ligation. *Lab. Invest.* **97**, 935–945 [CrossRef Medline](#)
- Denis, M., Marcinkiewicz, J., Zaid, A., Gauthier, D., Poirier, S., Lazure, C., Seidah, N. G., and Prat, A. (2012) Gene inactivation of PCSK9 reduces atherosclerosis in mice. *Circulation* **125**, 894–901 [CrossRef Medline](#)
- Sabatine, M. S., Giugliano, R. P., Keech, A. C., Honarpour, N., Wiviott, S. D., Murphy, S. A., Kuder, J. F., Wang, H., Liu, T., Wasserman, S. M., Sever, P. S., Pedersen, T. R., and FOURIER Steering Committee and Investigators. (2017) Evolocumab and clinical outcomes in patients with cardiovascular disease. *N. Engl. J. Med.* **376**, 1713–1722 [CrossRef Medline](#)
- Seidah, N. G. (2017) The PCSK9 revolution and the potential of PCSK9-based therapies to reduce LDL-cholesterol. *Global Cardiol. Sci. Pract.* **2017**, e201702 [Medline](#)
- Wadhwa, R. K., Steen, D. L., Khan, I., Giugliano, R. P., and Foody, J. M. (2016) A review of low-density lipoprotein cholesterol, treatment strategies, and its impact on cardiovascular disease morbidity and mortality. *J. Clin. Lipidol.* **10**, 472–489 [CrossRef Medline](#)
- Colgan, S. M., Tang, D., Werstuck, G. H., and Austin, R. C. (2007) Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2. *Int. J. Biochem. Cell Biol.* **39**, 1843–1851 [CrossRef Medline](#)
- Colgan, S. M., Hashimi, A. A., and Austin, R. C. (2011) Endoplasmic reticulum stress and lipid dysregulation. *Expert. Rev. Mol. Med.* **13**, e4 [CrossRef Medline](#)
- Lebeau, P., Al-Hashimi, A., Sood, S., Lhoták, Š., Yu, P., Gyulay, G., Paré, G., Chen, S. R., Trigatti, B., Prat, A., Seidah, N. G., and Austin, R. C. (2017) Endoplasmic reticulum stress and Ca<sup>2+</sup> depletion differentially modulate the sterol regulatory protein PCSK9 to control lipid metabolism. *J. Biol. Chem.* **292**, 1510–1523 [CrossRef Medline](#)
- Li, J., Huang, J., Li, J. S., Chen, H., Huang, K., and Zheng, L. (2012) Accumulation of endoplasmic reticulum stress and lipogenesis in the liver through generational effects of high fat diets. *J. Hepatol.* **56**, 900–907 [CrossRef Medline](#)

27. Lee, H. Y., Lee, G. H., Bhattarai, K. R., Park, B. H., Koo, S. H., Kim, H. R., and Chae, H. J. (2016) Bax inhibitor-1 regulates hepatic lipid accumulation via ApoB secretion. *Sci. Rep.* **6**, 27799 [CrossRef Medline](#)
28. Zaid, A., Roubtsova, A., Essalmani, R., Marcinkiewicz, J., Chamberland, A., Hamelin, J., Tremblay, M., Jacques, H., Jin, W., Davignon, J., Seidah, N. G., and Prat, A. (2008) Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. *Hepatology* **48**, 646–654 [CrossRef Medline](#)
29. Carta, G., Murru, E., Banni, S., and Manca, C. (2017) Palmitic acid: physiological role, metabolism and nutritional implications. *Front. Physiol.* **8**, 902 [CrossRef Medline](#)
30. Zhang, Y., Xue, R., Zhang, Z., Yang, X., and Shi, H. (2012) Palmitic and linoleic acids induce ER stress and apoptosis in hepatoma cells. *Lipids Health Dis.* **11**, 1 [CrossRef Medline](#)
31. Cui, W., Ma, J., Wang, X., Yang, W., Zhang, J., and Ji, Q. (2013) Free fatty acid induces endoplasmic reticulum stress and apoptosis of beta-cells by  $\text{Ca}^{2+}$ /calpain-2 pathways. *PLoS ONE* **8**, e59921 [CrossRef Medline](#)
32. Zhang, Y., Miao, L., Zhang, H., Wu, G., Zhang, Z., and Lv, J. (2018) Chlo-rogenic acid against palmitic acid in endoplasmic reticulum stress-mediated apoptosis resulting in protective effect of primary rat hepatocytes. *Lipids Health Dis.* **17**, 270 [CrossRef Medline](#)
33. Ben Mosbah, I., Alfany-Fernandez, I., Martel, C., Zaouali, M. A., Bintanel-Morcillo, M., Rimola, A., Rodes, J., Brenner, C., Rosello-Catafau, J., and Peralta, C. (2010) Endoplasmic reticulum stress inhibition protects steatotic and non-steatotic livers in partial hepatectomy under ischemia-reperfusion. *Cell Death Dis.* **1**, e52 [CrossRef Medline](#)
34. Ford, R. J., Fullerton, M. D., Pinkosky, S. L., Day, E. A., Scott, J. W., Oakhill, J. S., Bujak, A. L., Smith, B. K., Crane, J. D., Blüner, R. M., Marcinko, K., Kemp, B. E., Gerstein, H. C., and Steinberg, G. R. (2015) Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. *Biochem. J.* **468**, 125–132 [CrossRef Medline](#)
35. Zabielski, P., Hady, H. R., Chacinska, M., Roszczyc, K., Gorski, J., and Blachnio-Zabielska, A. U. (2018) The effect of high fat diet and metformin treatment on liver lipids accumulation and their impact on insulin action. *Sci. Rep.* **8**, 7249 [CrossRef Medline](#)
36. Woo, S. L., Xu, H., Li, H., Zhao, Y., Hu, X., Zhao, J., Guo, X., Guo, T., Botchlett, R., Qi, T., Pei, Y., Zheng, J., Xu, Y., An, X., Chen, L., et al. (2014) Metformin ameliorates hepatic steatosis and inflammation without altering adipose phenotype in diet-induced obesity. *PLoS ONE* **9**, e91111 [CrossRef Medline](#)
37. Nissar, A. U., Sharma, L., Mudasir, M. A., Nazir, L. A., Umar, S. A., Sharma, P. R., Vishwakarma, R. A., and Tasduq, S. A. (2017) Chemical chaperone 4-phenyl butyric acid (4-PBA) reduces hepatocellular lipid accumulation and lipotoxicity through induction of autophagy. *J. Lipid Res.* **58**, 1855–1868 [CrossRef Medline](#)
38. Khera, A. V., Qamar, A., Reilly, M. P., Dunbar, R. L., and Rader, D. J. (2015) Effects of niacin, statin, and fenofibrate on circulating proprotein convertase subtilisin/kexin type 9 levels in patients with dyslipidemia. *Am. J. Cardiol.* **115**, 178–182 [CrossRef Medline](#)
39. Gu, L., Wang, Y., Xu, Y., Tian, Q., Lei, G., Zhao, C., Gao, Z., Pan, Q., Zhao, W., Nong, L., and Tan, S. (2017) Lunasin functionally enhances LDL uptake via inhibiting PCSK9 and enhancing LDLR expression *in vitro* and *in vivo*. *Oncotarget* **8**, 80826–80840 [Medline](#)
40. Zelcer, N., Hong, C., Boyadjian, R., and Tontonoz, P. (2009) LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science* **325**, 100–104 [CrossRef Medline](#)
41. Dara, L., Ji, C., and Kaplowitz, N. (2011) The contribution of endoplasmic reticulum stress to liver diseases. *Hepatology* **53**, 1752–1763 [CrossRef Medline](#)
42. Basseri, S., Lhoták, S., Sharma, A. M., and Austin, R. C. (2009) The chemical chaperone 4-phenylbutyrate inhibits adipogenesis by modulating the unfolded protein response. *J. Lipid Res.* **50**, 2486–2501 [CrossRef Medline](#)
43. Ruiz, R., Jideonwo, V., Ahn, M., Surendran, S., Tagliabracchi, V. S., Hou, Y., Gamble, A., Kerner, J., Irimia-Dominguez, J. M., Puchowicz, M. A., DePaoli-Roach, A., Hoppel, C., Roach, P., and Morral, N. (2014) Sterol regulatory element-binding protein-1 (SREBP-1) is required to regulate glycogen synthesis and gluconeogenic gene expression in mouse liver. *J. Biol. Chem.* **289**, 5510–5517 [CrossRef Medline](#)
44. Shimomura, I., Bashmakov, Y., and Horton, J. D. (1999) Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J. Biol. Chem.* **274**, 30028–30032 [CrossRef Medline](#)
45. Jo, H. K., Kim, G. W., Jeong, K. J., Kim, D. Y., and Chung, S. H. (2014) Eugenol ameliorates hepatic steatosis and fibrosis by down-regulating SREBP1 gene expression via AMPK-mTOR-p70S6K signaling pathway. *Biol. Pharm. Bull.* **37**, 1341–1351 [Medline](#)
46. Cao, J., Dai, D. L., Yao, L., Yu, H. H., Ning, B., Zhang, Q., Chen, J., Cheng, W. H., Shen, W., and Yang, Z. X. (2012) Saturated fatty acid induction of endoplasmic reticulum stress and apoptosis in human liver cells via the PERK/ATF4/CHOP signaling pathway. *Mol. Cell. Biochem.* **364**, 115–129 [CrossRef Medline](#)
47. Xu, S., Nam, S. M., Kim, J. H., Das, R., Choi, S. K., Nguyen, T. T., Quan, X., Choi, S. J., Chung, C. H., Lee, E. Y., Lee, I. K., Wiederkehr, A., Wollheim, C. B., Cha, S. K., and Park, K. S. (2015) Palmitate induces ER calcium depletion and apoptosis in mouse podocytes subsequent to mitochondrial oxidative stress. *Cell Death Dis.* **6**, e1976 [CrossRef Medline](#)
48. Chen, X. W., Wang, H., Bajaj, K., Zhang, P., Meng, Z. X., Ma, D., Bai, Y., Liu, H. H., Adams, E., Baines, A., Yu, G., Sartor, M. A., Zhang, B., Yi, Z., Lin, J., et al. (2013) SEC24A deficiency lowers plasma cholesterol through reduced PCSK9 secretion. *eLife* **2**, e00444 [CrossRef Medline](#)
49. Emmer, B. T., Hesketh, G. G., Kotnik, E., Tang, V. T., Lascuna, P. J., Xiang, J., Gingras, A. C., Chen, X. W., and Ginsburg, D. (2018) The cargo receptor SURF4 promotes the efficient cellular secretion of PCSK9. *eLife* **7**, e38839 [CrossRef Medline](#)
50. Li, H., Dong, B., Park, S. W., Lee, H. S., Chen, W., and Liu, J. (2009) Hepatocyte nuclear factor 1 $\alpha$  plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine. *J. Biol. Chem.* **284**, 28885–28895 [CrossRef Medline](#)
51. Jensen, K. K., Tadin-Strapps, M., Wang, S. P., Hubert, J., Kan, Y., Ma, Y., McLaren, D. G., Previs, S. F., Herath, K. B., Mahsut, A., Liaw, A., Wang, S., Stout, S. J., Keohan, C., Forrest, G., et al. (2016) Dose-dependent effects of siRNA-mediated inhibition of SCAP on PCSK9, LDLR, and plasma lipids in mouse and rhesus monkey. *J. Lipid Res.* **57**, 2150–2162 [CrossRef Medline](#)
52. Sui, G. G., Xiao, H. B., Lu, X. Y., and Sun, Z. L. (2018) Naringin activates AMPK resulting in altered expression of SREBPs, PCSK9, and LDLR to reduce body weight in obese C57BL/6J mice. *J. Agric. Food Chem.* **66**, 8983–8990 [CrossRef Medline](#)
53. Shao, W., and Espenshade, P. J. (2014) Sterol regulatory element-binding protein (SREBP) cleavage regulates Golgi-to-endoplasmic reticulum recycling of SREBP cleavage-activating protein (SCAP). *J. Biol. Chem.* **289**, 7547–7557 [CrossRef Medline](#)
54. Mayne, J., Dewpura, T., Raymond, A., Cousins, M., Chaplin, A., Lahey, K. A., Lahaye, S. A., Mbikay, M., Ooi, T. C., and Chrétien, M. (2008) Plasma PCSK9 levels are significantly modified by statins and fibrates in humans. *Lipids Health Dis.* **7**, 22 [CrossRef Medline](#)
55. Welder, G., Zineh, I., Pacanowski, M. A., Trout, J. S., Cao, G., and Konrad, R. J. (2010) High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol. *J. Lipid Res.* **51**, 2714–2721 [CrossRef Medline](#)
56. Gauthier, M. S., Awan, Z., Bouchard, A., Champagne, J., Tessier, S., Faubert, D., Chabot, K., Garneau, P. Y., Rabasa-Lhoret, R., Seidah, N. G., Ridker, P. M., Genest, J., and Coulombe, B. (2018) Posttranslational modification of proprotein convertase subtilisin/kexin type 9 is differentially regulated in response to distinct cardiometabolic treatments as revealed by targeted proteomics. *J. Clin. Lipidol.* **12**, 1027–1038 [CrossRef Medline](#)
57. LaRosa, J. C., Grundy, S. M., Waters, D. D., Shear, C., Barter, P., Fruchart, J. C., Gotto, A. M., Greten, H., Kastelein, J. J., Shepherd, J., Wenger, N. K., and Treating to New Targets (TNT) Investigators (2005) Intensive lipid lowering with atorvastatin in patients with stable coronary disease. *N. Engl. J. Med.* **352**, 1425–1435 [CrossRef Medline](#)
58. Giunzioni, I., and Tavori, H. (2015) New developments in atherosclerosis: clinical potential of PCSK9 inhibition. *Vasc. Health Risk Manag.* **11**, 493–501 [Medline](#)



59. Wang, Y., Huang, Y., Hobbs, H. H., and Cohen, J. C. (2012) Molecular characterization of proprotein convertase subtilisin/kexin type 9-mediated degradation of the LDLR. *J. Lipid Res.* **53**, 1932–1943 [CrossRef Medline](#)
60. Wargny, M., Ducluzeau, P. H., Petit, J. M., Le May, C., Smati, S., Arnaud, L., Pichelin, M., Bouillet, B., Lannes, A., Blanchet, O., Lefebvre, P., Francque, S., Van Gaal, L., Staels, B., Vergès, B., Boursier, J., and Cariou, B. (2018) Circulating PCSK9 levels are not associated with the severity of hepatic steatosis and NASH in a high-risk population. *Atherosclerosis* **278**, 82–90 [CrossRef Medline](#)
61. Retterstøl, K., Svendsen, M., Narverud, I., and Holven, K. B. (2018) Effect of low carbohydrate high fat diet on LDL cholesterol and gene expression in normal-weight, young adults: a randomized controlled study. *Atherosclerosis* **279**, 52–61 [CrossRef Medline](#)
62. Ricci, C., Ruscica, M., Camera, M., Rossetti, L., Macchi, C., Colciago, A., Zanutti, I., Lupo, M. G., Adorni, M. P., Cicero, A. F. G., Fogacci, F., Corsini, A., and Ferri, N. (2018) PCSK9 induces a pro-inflammatory response in macrophages. *Sci. Rep.* **8**, 2267 [CrossRef Medline](#)
63. Lebeau, P., Platko, K., Al-Hashimi, A. A., Byun, J. H., Lhoták, Š., Holzapfel, N., Gyulay, G., Igldoura, S. A., Cool, D. R., Trigatti, B., Seidah, N. G., and Austin, R. C. (2018) Loss-of-function PCSK9 mutants evade the unfolded protein response sensor GRP78 and fail to induce endoplasmic reticulum stress when retained. *J. Biol. Chem.* **293**, 7329–7343 [CrossRef Medline](#)