PCSK7: a Novel Regulator of Apolipoprotein B and a Potential Target Against Non-Alcoholic Fatty Liver Disease

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**Keywords:** Proprotein Convertase Subtilisin Kexin 7 PCSK7, apoB, triglycerides, ER-stress, chaperone, escort, NAFLD, antisense oligonucleotide (ASO)

**Posted Date:** March 24th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-2724841/v1

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**Additional Declarations:** There is **NO** Competing Interest.
PCSK7: a Novel Regulator of Apolipoprotein B and a Potential Target Against Non-Alcoholic Fatty Liver Disease

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Short title: Hepatic function of PCSK7 and its relationship to apoB and NAFLD

Key Words: Proprotein Convertase Subtilisin Kexin 7 PCSK7, apoB, triglycerides, ER-stress, chaperone, escort, NAFLD, antisense oligonucleotide (ASO)

Abbreviations: ALT, alanine aminotransferase; Ab, antibody; apoB100, apolipoprotein B100; ASO, antisense oligonucleotide; apoA-V, apolipoprotein A-V; apoB, apolipoprotein B; CRISPR, clustered regularly interspaced short palindromic repeats; ER, endoplasmic reticulum; IHH, immortalized human primary hepatocytes; IRE1α, inositol-requiring enzyme-1α; HDL, high-density lipoprotein; HDLc, high density lipoprotein-cholesterol; HFFC, high fat/fructose/cholesterol; TfR1, transferrin receptor 1; hTfR1, human transferrin receptor 1; LD, lipid droplet; LDLc, low-density lipoprotein-cholesterol; LOF, loss-of-function; MTP, microsomal triglyceride transfer protein; NASH, nonalcoholic steatohepatitis; GOF, gain-of-function; NAFLD, nonalcoholic fatty liver disease; ORO, oil red O; PCSKs, proprotein convertases; PCSK7, proprotein convertase of the subtilisin/kexin type 7; PCSK9, proprotein convertase of the subtilisin/kexin type 9; sLDLR, shed LDLR; TG, triglycerides; SNP, single nucleotide polymorphism; sXBP1, spliced X-box binding protein 1; TfR1, transferrin receptor 1; TC, total cholesterol; VLDL, very low-density lipoproteins; UPR, unfolded protein response.
Abstract

Epidemiological evidence links the proprotein convertase subtilisin/kexin 7 (PCSK7) to triglyceride (TG) metabolism. PCSK7-SNPs gain-of-function and loss-of-function variants were associated with higher and lower levels of plasma apoB and TGs, respectively. Herein, we biochemically defined the in vivo role of PCSK7 in lipid metabolism using full-body Pcsk7−/− mice and hepatic cell lines. Non-enzymatically membrane-bound PCSK7 binds apoB100 in the endoplasmic reticulum and enhances its secretion. Mechanistically, the loss of PCSK7/Pcsk7 leads to apoB degradation, triggering an unfolded protein response, autophagy, and β-oxidation, eventually reducing hepatic lipid accumulation. We investigated whether Pcsk7−/− mice could better recover from a NAFLD-inducing 12-weeks high fat/fructose/cholesterol diet when followed by a 4-weeks regular diet. Livers of Pcsk7−/− mice more effectively and safely recovered than those of wild-type mice. These findings were validated following subcutaneous administration of hepatocyte-targeted N-acetylgalactosamine (GalNac)-antisense-oligonucleotides (ASOs) against Pcsk7, strongly supporting the therapeutic intervention of hepatocyte PCSK7 mRNA silencing for NAFLD treatment.
**Introduction**

PCSK7 is the 7th member of the secretory basic amino acid-specific subtilisin-kexin-like proprotein convertases (PCSKs)\(^1\) that cleave precursor proteins at the motif [R/K]-2(Xn)-[R/K]↓, where Xn is either 0, 1, 2 or 3 spacer residues\(^2\). The ubiquitously expressed PCSK7 encodes a type-I membrane-bound protease first synthesized as a ~102-kDa proPCSK7 zymogen in the endoplasmic reticulum (ER), which then undergoes an autocatalytic cleavage to generate an inactive ~92-kDa protein that remains non-covalently associated with its inhibitory N-terminal prodomain\(^1,2\). This complex then traffics to the Golgi and cell surface where PCSK7 is activated in endosomal-like organelles\(^3,4\). The only PCSK7-specific cleavage known is the shedding of human transferrin receptor 1 (TfR1)\(^4,5\), rationalizing the enhanced levels of shed TfR1 in the plasma of humans carrying the single nucleotide polymorphism (SNP) rs236918 in intron 9 of the PCSK7 gene\(^6\). In 2019, Dongiovanni et al. suggested that this SNP is a gain-of-function (GOF) since it increases PCSK7 protein levels and correlates with dyslipidemia and non-alcoholic fatty liver disease (NAFLD)\(^7\).

Exome sequencing of African Americans revealed the association of a coding variant of PCSK7 (p.Arg504His; SNP rs142953140) with ~30% lower plasma triglyceride (TG) levels\(^8\). We showed that PCSK7 non-enzymatically induces the degradation in the ER of apolipoprotein A-V (apoA-V), a co-activator of lipoprotein lipase\(^9\), and provided evidence that the R504H variation is a loss-of-function (LOF), likely due to enhanced Ser\(^{505}\) phosphorylation\(^10\). In contrast, mice overexpressing human PCSK7 in the liver had ~45% higher plasma TG\(^8\). In addition, another study associated other PCSK7 SNPs with TG levels in a Korean population\(^11\). Moreover, a ~50% silencing of PCSK7 in human hepatocellular carcinoma HepG2 cells resulted in ~30% lower lipid droplet (LD) accumulation when cells were incubated with palmitic/oleic acids, supporting the concept that the lack
of PCSK7 protects against TG accumulation in hepatocytes\(^7\). Overall, PCSK7 seems to be the only PCSK that exhibits both enzymatic and non-enzymatic activities, and the latter may be implicated in lipid metabolism. However, the underlying mechanism(s) is/are yet to be unraveled.

In this study, the impact of PCSK7 on lipid metabolism was analyzed in human subjects, cells, and mice. Our present data provide evidence that PCSK7 is a novel regulator of apolipoprotein B100 (apoB100) secretion and liver TG metabolism. ApoB, a key marker for cardiovascular disease risk\(^{12}\), is the only essential scaffolding protein in the assembly of TG-enriched very low-density lipoproteins (VLDL), and a target to treat hyperlipidemia and atherosclerosis\(^{13}\). Furthermore, high apoB\(^{14}\) or very low levels\(^{15}\) were associated with an increased incidence of NAFLD. Our data show that hepatocytes lacking PCSK7 exhibited significantly ~50% reduced intracellular apoB levels and enhanced protective unfolded protein response (UPR) signaling leading to TG degradation possibly via autophagy and fatty acid β-oxidation. Furthermore, mice lacking \(P_{csk7}\) expression (\(P_{csk7}\) KO) exhibited significantly attenuated lipid accumulation in hepatocytes following a 12-weeks high fat/fructose/cholesterol (HFFC) diet that induces NAFLD and a 4-weeks recovery period under a regular diet. This was replicated in wild-type (WT) mice fed an HFFC diet for 17-weeks and then administered trivalent N-acetylgalactosamine (GalNac)-ASOs against \(P_{csk7}\) during an 8-weeks recovery period under a regular diet. Thus, in view of the unexpected role of PCSK7 in enhancing the secretion of apoB and the generation of TG-enriched VLDL from the liver, silencing \(PCSK7\) expression in hepatocytes may be beneficial in the treatment of NAFLD.
Results

Epidemiological, genetic, and biochemical evidence for the association of human PCSK7 with lipid metabolism

Human genetic studies support the association of PCSK7 levels with circulating lipids and apolipoproteins (Table 1A). The heterozygote SNP (G>C; minor allele frequency of ~0.12) is a GOF polymorphism in intron 9 associated with increased PCSK7 shedding activity on TfR1\(^6\) and levels\(^7\). On average, for every additional GOF PCSK7 C-allele, carriers have 0.09% higher plasma TG (p=4.6x10\(^{-119}\)), 0.04 mg/dL higher plasma total cholesterol (TC) (p=1.9x10\(^{-24}\)), 0.54 mg/dL higher plasma apoB (p=8.1x10\(^{-13}\)) and 0.56 mg/dL higher plasma apolipoprotein A1 (p=2.5x10\(^{-10}\)). In contrast, carriers exhibit 0.02 mg/dL lower plasma high-density lipoprotein cholesterol (HDLc; p=2.7x10\(^{-5}\); Table 1A). Moreover, in hereditary hemochromatosis patients, rs236918 carriers are ~5.4 times more likely to develop liver cirrhosis\(^{16}\). These data reaffirm the association of PCSK7 GOF with higher plasma TG and apoB, but lower HDLc, and therefore a higher risk for cardiometabolic disease.

To identify possible carriers of LOF or GOF SNPs in PCSK7, a post hoc analysis was carried out on subjects recruited through a clinical trial at the Montreal Clinical Research Institute (IRCM)\(^{17}\). All subjects with low or high plasma apoB (≤ 25\(^{th}\) or ≥ 75\(^{th}\) percentile for sex and age in a Canadian population) with available DNA were examined (N=8). Among the 5 subjects with low plasma apoB, one woman (subject #2) and one man (subject #6) were identified carrying the PCSK7 LOF variant SNP rs201598301 (G>A; minor allele frequency of ~0.02) in exon 17 leading to the P777L substitution. As shown in Table 1B, these two subjects presented remarkably very low plasma
apoB (< 5th percentile) and plasma TG (= 10th percentile) in a Canadian population (https://www150.statcan.gc.ca/n1/pub/82-626-x/2013001/t039-eng.htm).

**Pcsk7** KO mice exhibit lower circulating and hepatic apoB, along with reduced liver lipid levels

To define the mechanism underlying the above data, we analyzed *Pcsk7*+/+ (WT) and *Pcsk7*−/− (KO) male mice in a pure C57BL/6J background18. In a previous study, we showed that high-fat feeding significantly enhanced TG levels in adipose tissue partly via a non-enzymatic degradation of hepatic apoA-V by PCSK7, leading to higher lipoprotein lipase activity in *Pcsk7* KO mice10. In this study, WT and KO mice fed a regular diet exhibited similar TG and TC levels (Supplementary Fig. 1A). However, Western blot analyses revealed that KO mice exhibit statistically significant ~50% lower levels of circulating and hepatic apoB100, but relatively similar apoB48 levels (Fig. 1A, B), as well as unchanged apoB mRNA levels in the liver (Supplementary Fig. 1B). Since the loss of apoB100 (≤ 25%) has often been reported to lead to hepatic steatosis and NAFLD15, we probed for hepatic lipid accumulation in WT and KO mice. Unexpectedly, KO livers showed a significant reduction in lipid content (Fig. 1C). Image analysis revealed a ~50% lower oil red O (ORO) staining and mean area of LD, with no change in the total number of LD (Fig. 1D). In summary, these data suggest that under regular diet, lack of *Pcsk7* leads to ~50% lower apoB100 levels and reduced lipid content in the liver. In contrast, no changes in apoB, LD, or TG levels were observed under a high-fat diet (Supplementary Fig. 1C, D)

A pulse-chase analysis on primary hepatocytes isolated from WT and KO mice revealed that apoB100 was already reduced by ~50% at time 0 (after a pulse of 15 min) in KO hepatocytes, but
exhibited a similar degradation rate afterwards (Supplementary Fig. 2A). This was associated with lower apoB100 secretion from KO hepatocytes over chase time (Supplementary Fig. 2B).

Enhanced apoB100 degradation in cells lacking PCSK7: evidence for a chaperone/escort-like function of PCSK7 favoring apoB secretion

To confirm the above link between reduced PCSK7 levels and decreased apoB secretion from hepatocytes, endogenous apoB100 levels were analyzed in human HepG2 cells and immortalized human primary hepatocytes (IHH) treated with optimized siRNAs against PCSK7. Importantly, cells were maintained in serum-free media for 6 days. Under these conditions, the absence of endogenous PCSK7 protein was associated with a ~55% decrease in intracellular apoB100 levels in both cell lines (Supplementary Fig. 2C). Complete PCSK7 inactivation in IHH cells using CRISPR-Cas9 (KO cells) also led to ~60% lower levels of intracellular apoB100 protein levels compared to WT IHH cells, when cells were incubated in sugar-and serum-free medium for 1h (Fig. 2A). Importantly, in KO cells the blockade of de novo mRNA translation with cycloheximide revealed a much faster post-translational degradation of apoB100 compared to control IHH cells, especially within the first 15 min (Fig. 2B), suggesting an early degradation by the proteasome. Accordingly, we tested the proteasome inhibitor lactacystin and observed a remarkable accumulation of polyubiquitinated apoB in KO cells compared to WT (Fig. 2C). We further confirmed the implication of the proteasome by showing that only the proteasome inhibitor MG132 (which has a similar effect as lactacystin) reverted apoB levels in KO IHH cells to those in WT cells (Fig. 2D). In contrast, brefeldin A, which blocks exit from the ER/medial Golgi, and 3-methyladenine that inhibits autophagy, did not affect cellular apoB100 levels. Thus,
the absence of PCSK7 likely enhances the co-translational proteasomal degradation of apoB100, as supported by increased polyubiquitination of apoB.

We next analyzed whether PCSK7 interacts with apoB100. Overexpression of an empty vector or a vector expressing V5-tagged PCSK7 in IHH cells treated with MG132 allowed the detection of the co-immunoprecipitation of endogenous apoB100 with PCSK7-V5 (Fig. 2E). This result was confirmed by an inversed protocol whereby a V5-mAb was used to co-immunoprecipitate PCSK7-V5 and apoB100 that were detected by Western blot (Supplementary Fig. 2D). Note that essentially the minor ER-localized proPCSK7 precursor\textsuperscript{3,24} form of the protein best interacts with apoB100, in agreement with the above data showing that an early degradation of apoB100 takes place in the absence of PCSK7. We next generated a shorter secreted N-terminal fragment of apoB (apoB21; aa 1 to 965; 21% of the full-length apoB100) and showed that it is secreted (Fig. 2F, empty vector). Subsequently, we tested whether PCSK7 could escort apoB21 out of WT IHH cells, and since PCSK7 is a secretory serine protease\textsuperscript{24}, we also incubated IHH cells overexpressing PCSK7 and apoB21 with a cell-permeable pan-proprotein convertase inhibitor, decanoyl-RVKR-cmk (RVKR), that blocks PCSK7 shedding activity of TfR\textsuperscript{25}. The data revealed that overexpression of PCSK7 enhanced the levels of intracellular apoB21 by >6-fold and in the medium by ~2-fold (Fig. 2F). A similar effect was observed upon overexpression of PCSK7 in enhancing the endogenous cellular apoB100 level (Supplementary Fig. 2E). The chaperone/escort function of PCSK7 on apoB seems to be independent of its enzymatic activity since it remained insensitive to RVKR, as previously observed for apoA-V\textsuperscript{10} and the major histocompatibility complex-I (MHC-I)\textsuperscript{26}. Altogether, these results indicate that non-enzymatically PCSK7 acts as a chaperone/escort by binding the N-terminal domain of apoB and protecting it from degradation, thereby leading to higher ER exit and secretion.
The PCSK7-P777L variant was identified among subjects with low plasma apoB (Table 1B). We thus tested the ability of this variant to chaperone/escort apoB21 and found that the levels of the latter and PCSK7-P777L are strongly reduced in cells and media (Supplementary Fig. 3A). The Pro to Leu substitution (VP to VL

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introduces a VL

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motif that possibly targets proteins to the lysosomal pathway because of two consecutive aliphatic residues (Leu, Ile, or Val) in their cytosolic tails

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. The levels of both proteins were significantly enhanced upon incubation of IHH cells with the alkanalizing agent NH

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Cl (Supplementary Fig. 3A), suggesting their degradation in acidic compartments, such as lysosomes. Thus, our data indicate that the LOF PCSK7-P777L induces apoB degradation in lysosomes. In support of this conclusion, we observed that the PCSK7-V776L/P777L with an LL

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motif is even more efficient than PCSK7-P777L in enhancing the degradation of apoB21 and reducing its PCSK7-mediated increased secretion (Supplementary Fig. 3B).

Mechanism of hepatic TG reduction in IHH cells and mice lacking PCSK7

In a preliminary step, we analyzed the mRNA expression of 23 liver genes in male mice, known to be involved in fatty acids (FA) oxidation, esterification, lipogenesis, inflammation, and cholesterol metabolism. However, we did not detect any genotype-specific regulation (Supplementary Fig. 4) aside from a minor upregulation (< 2-fold) of Acc2 and Hmgcr expression in KO mice fed a regular diet.

Several hypotheses may explain the unexpected lower levels of liver lipids in KO mice fed a regular diet (Fig. 1C, D): (1) increased secretion of TG-rich lipoproteins; (2) decreased de novo
synthesis of FA; (3) lower availability of circulating albumin-bound non-esterified FA (NEFA) subsequently reducing their internalization and conversion into TG; (4) reduced uptake of TG-enriched lipoproteins; (5) increased lipophagy; or (6) enhanced β-oxidation of FA. Each of these possibilities was addressed below.

First, VLDL secretion was monitored by measuring plasma TG following administration of the detergent poloxamer-407 that blocks TG lipolysis\textsuperscript{28}. After 6h, TG levels were ~50% lower in KO than in WT mice (Fig. 3A), thereby eliminating possibility #1. Second, de novo fatty acid synthesis was monitored in WT and KO primary hepatocytes treated with $^3$H-acetate, revealing that the radioactivity associated with extracted TG\textsuperscript{29} did not differ (Fig. 3B). Third, plasma NEFA levels did not vary between WT and KO plasma (Supplementary Fig. 5A). Fourth, the protein levels of the TG-rich lipoprotein receptor LRP1 (LDL receptor-related protein 1), CD36 a scavenger receptor for free FA and lipoproteins and pyruvate kinase L/R (PKLR) that controls glycolysis were unchanged (Supplementary Fig. 5B). Fifth, we observed a significant stimulation of macroautophagy in KO primary hepatocytes, with higher LC3-II levels both in the absence or presence of chloroquine (Fig. 3C), suggesting enhanced functional lipophagy\textsuperscript{30}, that was reported previously in the absence of apoB\textsuperscript{31}. Finally, to address possibility #6, we were inspired by a report revealing that the complete absence of apoB led to enhanced β-oxidation\textsuperscript{31}. Hence, as previously reported in mouse primary hepatocytes\textsuperscript{29}, we compared the FA oxidation potential of WT or KO IHH cells that were incubated with $^{14}$C-oleate. After 8 and 12h, the released $^{14}$CO$_2$ was captured, thereby revealing enhanced FA β-oxidation in KO IHH cells (Fig. 3D).

The absence of mouse apoB was reported to enhance ER stress due to the accumulation of lipids in the lumen of ER\textsuperscript{31}, which is an established activator of the UPR signaling. To test this possibility, we compared the levels of phosphorylated inositol-requiring enzyme-1α (IRE1α) and protein
kinase RNA-like ER kinase (PERK) in WT and KO IHH cells (Fig. 3E). Elevated levels in the latter suggest that the absence of PCSK7 upregulates the UPR and in turn possibly triggers higher autophagy and lower lipid accumulation via enhanced β-oxidation (Fig. 3E). Finally, to extend our observation of reduced lipid content in the livers of KO mice (Fig. 1C, D) to human cells, we incubated overnight WT or KO IHH cells with oleic acid, washed and then incubated them for another 72h in low glucose-containing medium lacking oleic acid. In the absence of PCSK7, ORO staining of IHH cells revealed an almost complete loss of LD (Fig. 3F).

The absence of PCSK7 favors the loss of fat that accumulated following a 12-weeks-long HFFC diet

We next probed the effect of PCSK7 deficiency on liver fat accumulation in male mice, reported to be more prone to develop NAFLD under regular diet and much more upon feeding an HFFC diet, which promotes a robust NAFLD-like phenotype. Eighty WT and KO mice of 8-weeks of age were fed a regular or HFFC diet for 12-weeks (20 mice of each genotype/diet). At the end of this period, half of the mice were euthanized to assess NAFLD development, while the other half were fed a regular diet for 4-weeks (until 24-weeks of age; Fig. 4A). This strategy was elaborated based on the evidence that under HFFC diet, apoB100 or hepatic lipid levels were similar in WT and KO mice (Supplementary Fig. 1C, D) suggesting that under HFFC diet loss of Pcsk7 does not affect the progression of NAFLD. In agreement, whereas apoB levels were similar in WT and KO mice fed an HFFC diet for 12-weeks (Supplementary Fig. 6), the 4-weeks-recovery under regular diet resulted in > 50% lower apoB100 levels in KO mice than WT both in plasma and liver (Fig. 4B, C). ORO staining of liver sections revealed that the 12-weeks-HFFC diet generated a robust
accumulation of LD, independent of genotype (Fig. 4D, F), suggesting the lack of PCSK7 does not prevent diet-induced NAFLD. In contrast, following the 4-weeks-recovery period, the absence of PCSK7 resulted in > 30% reduction in the ORO-stained area (Fig. 4E, F). The LD number was twice higher, but their mean area decreased by ~70% (Fig. 4F). Thus, the lack of PCSK7 seems to be associated with a lower lipid accumulation, as observed in human IHH cells (Fig. 3F). Interestingly, KO mice fed a regular diet for 16-weeks (12-weeks + 4-weeks recovery) also exhibited a significant ~20% reduction in LD staining compared to WT (Supplementary Fig. 7B). WT and KO mice presented similar body or perigonadal fat weight and liver/body weight ratio when fed a regular or HFFC diet (Supplementary Fig. 8). The only significant difference was a 5-fold enhanced loss of body weight following the 4-weeks recovery period (Supplementary Fig. 8G).

The absence of PCSK7 improves liver pathology in mice recovering from an HFFC diet

We next analyzed the liver pathology generated by a 12-weeks HFFC diet, and its evolution after the 4-weeks recovery on a regular diet. In WT and KO mice, the HFFC diet generated similar grades of steatosis, lobular inflammation, hepatocyte ballooning (Fig. 5A, B), fibrosis (Fig. 5E) and hepatic TG levels (Fig. 5G), all hallmarks of NAFLD-associated pathology. In contrast, the recovery was much better in KO mice, especially for steatosis (Fig. 5C, D) and fibrosis (Fig. 5H). The liver toxicity, as assessed by the levels of circulating alanine aminotransferase (ALT), also returned to normal levels (as seen on regular diet fed mice, Supplementary Fig. 7C) in KO mice (Fig. 5I) compared to the levels after a 12-weeks HFFC diet (Fig. 5F). Finally, liver cholesterol
content did not change after recovery (Supplementary Fig. 7D, E), but in KO mice the hepatic TG levels were ∼25% lower than WT ones (Fig. 5J).

Lipidomic analysis was implemented to address the impact of KO on the hepatic lipidome of mice fed a regular diet and following the recovery period from an HFFC diet (Supplementary Fig. 9). Mass spectrometry (MS) analysis of liver lipids in mice fed a regular diet detected 30 ($p < 0.05$) out of 1168 features that differed between KO versus WT samples, with 12 of them annotated using tandem MS analysis (Supplementary Fig. 9A, right panel). The most significant changes affected TG, with 9 of them downregulated up to -36%, in agreement with our biochemical data. A similar strategy was used to compare KO versus WT after the 4-weeks recovery period. From 1168 features, 149 features were significantly different between KO versus WT samples ($p < 0.018$) with 31 of them annotated by tandem MS analysis (Supplementary Fig. 9B, right panel). Namely, among the upregulated compounds are 6 glycerophospholipids (from 1.44- to 2.64-fold), a sub-class of TG (1.97-fold), and coenzyme Q9 (1.23-fold). Among the downregulated lipids are 3 free FA (0.30-fold), one TG species (0.74-fold) and 19 lysoglycerophospholipids (0.18- to 0.41-fold). The remarkable normalization of the lipid profile upon loss of $Pcsk7$ is visualized by a heatmap (Fig. 5K) that represents the log$_2$-transformed averaged signal intensity (https://software.broadinstitute.org/morpheus). Altogether, our findings show that the loss of $Pcsk7$ expression reverses the hepatic lipid perturbations induced by the HFFC diet.

Finally, except for the mRNA levels of PERK, the levels of IRE1α, GRP78, ATF6, and sXBP1 (prominent markers of UPR signaling) were higher in KO mice after the 4-weeks recovery period (Fig. 6A). The transcript levels of these markers remained unchanged in mice fed a regular diet except modest upregulation in sXBP1 and ATF6 mRNA levels at 12-weeks and 16-weeks regular diet feeding in KO mice (Supplementary Fig. 10A). This prompted us to look at the activation of
UPR signaling via phosphorylation of IRE1α. Indeed, after the 4-weeks recovery period following HFFC feeding KO mice exhibited a higher UPR than WT ones, as indicated by enhanced phospho-IRE1α levels (Fig. 6B). Interestingly, compared to WT, higher UPR signaling was also observed in KO mice fed a regular diet for 12- (modest increase) and 16-weeks (Supplementary Fig. 10B). Notably, a Tunel assay revealed the absence of apoptosis in liver sections from WT and KO mice after the recovery period (Supplementary Fig. 10C), suggesting that the observed upregulated UPR signaling may be physiologically protective. Altogether, our data indicate that early in the ER, PCSK7 interacts with apoB100 independently of its enzymatic activity and then acts as a chaperone/escort that facilitates apoB100 exit from this compartment. The absence of PCSK7 results in an early loss of apoB100 by proteasomal degradation, leading to a protective UPR activation, enhanced autophagy, and lipid β-oxidation, rationalizing the loss of accumulated lipids observed in KO mice that recover from an HFFC diet. A model summarizing these data is schematized in Fig. 6C.

Silencing Pcsk7 expression in hepatocytes accelerates the recovery of mice exhibiting hepatic steatosis.

Because PCSK7 silencing may have clinical applications, e.g., in the treatment of NAFLD, we aimed to inject WT male mice with Pcsk7-specific ASOs during the 8-weeks recovery period that followed an HFFC diet, as above. From the 8 ASOs selected, ASO2 and ASO7 were the most efficient to downregulate Pcsk7 mRNA expression in mouse FL38B hepatocytes (Supplementary Fig. 11A-D). We thus procured the same two ASOs covalently linked to trivalent N-acetyl-Galactosamine (GalNac) moiety (Supplementary Fig. 11E), a modification that allows their selective targeting to hepatocytes via the specific asialoglycoprotein receptor ASGR133,34.
As a first step, WT mice received subcutaneous injections of either modified ASOs at 2, 5, 10 or 15 mg/kg, twice a week for 1 month. At 5 mg/kg, GalNac-ASO7 reduced liver Pcsk7 mRNA levels by >50% (Fig. 7A). In a second step, GalNac-ASO2 and GalNac-ASO7 were injected at 5 mg/kg twice a week for 1 month. Pcsk7 mRNA levels were then analyzed by RT-QPCR in liver samples and primary hepatocytes (Fig. 7B). Again, GalNac-ASO7 reduced more efficiently Pcsk7 mRNA levels, by 69% and ~60% in primary hepatocytes and whole liver, respectively. GalNac-ASO2 induced a ~69% reduction in Pcsk7 mRNA expression in primary hepatocytes versus ~34% in the whole liver (Fig. 7B). Thus, both GalNac-ASOs effectively reduce Pcsk7 mRNA expression in vivo in hepatocytes.

In a final experiment, 48 WT mice of 8-weeks of age were fed an HFFC diet for 17-weeks followed by a regular diet for an 8-weeks recovery period (Fig. 7C). Before the latter, mice were divided into 4 groups of 12: (1) no treatment, (2) GalNac-ASO2, (3) GalNac-ASO7 (both ASOs were subcutaneously injected at 5 mg/kg twice a week), and (4) as a positive control, we used vitamin E (α-tocopherol) supplementation at 500 IU/kg of food, based on the current guidelines of the American Association for the Study of Liver Diseases for the standard of care of NAFLD patients \(^{35,36}\). First, we observed that GalNac-ASO2 and GalNac-ASO7 generated similar reductions of liver Pcsk7 mRNA levels, ~50% and ~60% respectively, while α-tocopherol had an opposite effect with > 30% higher levels of transcripts (Fig. 7D). Second, both GalNac-ASOs achieved a > 55% reduction of plasma apoB levels (Fig. 7E), as previously observed in KO mice (Fig. 4A-C). Since GalNac targets ASOs very efficiently to hepatocytes \(^{37}\), the above results support the notion that apoB levels are primarily regulated by hepatocyte-derived PCSK7.

We then examined the impact of the different treatments on liver pathology. ORO staining revealed that GalNac-ASO7 was more efficient than GalNac-ASO2 to reduce the liver droplet content (-
47%) and mean area (-67%), while GalNac-ASO2 and α-tocopherol had similar and milder effects (Fig. 8A). Similarly, NAFLD phenotypes (inflammation, ballooning, steatosis, and fibrosis), which were graded blindly by a pathologist, were best reduced by GalNac-ASO7. GalNac-ASO2 was not as efficient but was better than α-tocopherol (Fig. 8B, C). Finally, ASO7 significantly reduced liver TG content by ~55% and ASO2 by ~45%, with no effect of α-tocopherol (Fig. 8D). Importantly, none of these treatments led to liver toxicity, as aspartate aminotransferase (AST) or alanine aminotransferase (ALT) plasma levels were stable (Supplementary Fig. 12). In conclusion, Pcsk7 silencing by GalNac-ASOs constitutes a safe and effective approach to reduce NAFLD phenotypes.

Discussion

The nine PCSKs play major roles in various metabolic and dynamic processes during development and in adults. The physiological roles of the ubiquitously expressed PCSK7 are the least understood, even though it is the most conserved member of the family. The little that is known relates to its implication in iron metabolism, as it is the only PCSK able to shed the type-II membrane-bound human Tfr1, and to its regulation of anxiety via activation of pro-brain derived neurotropic factor. Our current study presents compelling in vivo and ex vivo evidence detailing a role of PCSK7 in hepatic lipid homeostasis.

PCSK7 GOF SNP rs236918 (higher protein levels) was linked to higher circulating apoB and, to a lesser extent, plasma TG levels, as well as to the higher incidence of liver cirrhosis in hereditary hemochromatosis patients (Table 1A). Moreover, subjects carrying the LOF SNP rs201598301, encoding a P777L variant of PCSK7 had low plasma TG, apoB, and LDLc levels (Table 1B), reinforcing the hypothesis of a role for PCSK7 in steatosis/steatohepatitis and liver-associated
complications\textsuperscript{7,40}. Finally, mice overexpressing PCSK7 in the liver exhibited \textasciitilde45\% higher plasma TG compared to control ones\textsuperscript{8}. We thus examined the possible link of PCSK7 to liver and plasma lipid metabolism.

PCSK7 KO mice fed a regular diet showed \textasciitilde50\% lower fasting levels of liver and plasma apoB\textsubscript{100} and \textasciitilde50\% lower LD staining, with no apparent change in apoB\textsubscript{48} (Fig. 1), or in circulating TG or TC (Supplementary Fig. 1A). The lack of significant decrease in apoB\textsubscript{48} in the liver and plasma of \textit{Pcsk7} KO mice, under regular diet, may be related to the fact that the latter is not as sensitive to lipidation by microsomal triglyceride transfer protein (MTP) as apoB\textsubscript{100}, likely because hepatic apoB\textsubscript{48} can be secreted as HDL-like particles as previously reported\textsuperscript{41} and therefore is not degraded similarly to apoB\textsubscript{100}. A comparable reduction in apoB\textsubscript{100} (Fig. 2A) and LD staining (Fig. 3F) was seen in human IHH cells lacking PCSK7, rationalized by higher apoB\textsubscript{100} degradation by the proteasome (Fig. 2). In addition, co-expression of human PCSK7 and apoB\textsubscript{21} in IHH cells led to a \textasciitilde2-fold increase in cellular and secreted apoB\textsubscript{21} levels (Fig. 2F), an effect that remained insensitive to a PCSK cell-permeable inhibitor, in agreement with the exclusive presence of proPCSK7 in the ER\textsuperscript{1-3}. Moreover, co-immunoprecipitations showed that PCSK7 binds to apoB\textsubscript{100} in IHH lysates (Fig. 2E). Importantly, PCSK7 did not affect the intracellular levels of MTP\textsuperscript{42} or transmembrane 6 superfamily member 2\textsuperscript{43}, two major ER chaperones implicated in apoB folding and lipidation, and whose inactivation leads to similar apoB phenotypes (Supplementary Fig. 13). Altogether, these data indicate that PCSK7 has a novel chaperone/escort-like effect on apoB\textsubscript{100}. Interestingly, the LOF PCSK9-Q152H variant also has important chaperone properties by increasing the protein levels of the ER-resident chaperones GRP78 and GRP94\textsuperscript{44}. This highlights the importance of PCSK7 and PCSK9 as unique and clinically relevant
non-enzymatic chaperones/escorts that may assist in protein folding/trafficking in the liver and possibly other tissues\textsuperscript{45}.

It was previously reported that apoB/VLDL can be directed from the \textit{trans}-Golgi network (TGN) to lysosomes for degradation following its binding to sortilin\textsuperscript{46}. Interestingly, we demonstrate in this study that the PCSK7-P777L variation targets both PCSK7 and apoB100 for lysosomal degradation. This is likely explained by the introduction of a di-Leu-like motif (Val-Leu\textsubscript{777}) in the cytosolic tail of PCSK7, which possibly targets the complex to lysosomes\textsuperscript{47} and reinforces our hypothesis of a direct apoB-PCSK7 interaction.

Our data reveal that proteasomal apoB degradation is elevated in the absence of PCSK7. In a similar study to ours, Conlon \textit{et al.}\textsuperscript{31} observed higher UPR signaling in mice fed a Western diet and treated with \textit{ApoB} ASOs resulting in the accumulation of LDs in the lumen of ER. Their study also reported enhanced macro-autophagy in response to this lipid accumulation in order to regain ER homeostasis. Notably, the phenomenon of protective autophagy mediated via UPR signaling has also been reported in other previous studies\textsuperscript{48,49}. This prompted us to verify if the absence of PCSK7 would lead to UPR activation and enhanced autophagy. Indeed, we observed elevated phospho-IRE\textsubscript{1}{\alpha} and phospho-PERK levels in KO IHH cells (Fig. 3E). It is plausible that such higher UPR signaling led to enhanced autophagy, and FA $\beta$-oxidation with no change of \textit{de novo} lipogenesis (Fig. 3B, C, D), in line with the observed reduced LD staining in KO IHH cells (Fig. 3F) and mouse KO liver (Fig. 1C, D).

Because WT and KO mice exhibited lower liver lipid levels under a regular diet and loss of \textit{Pcsk7} didn’t seem to affect the progression of NAFLD, this prompted us to induce liver steatosis in both genotypes using an HFFC diet for 12-weeks and then compare their ability to recover from liver steatosis using a regular 4-weeks diet (Fig. 4A). The data showed that compared to WT, KO mice
exhibit a remarkable recovery from the diet-induced NAFLD-like phenotype (Figs. 4 and 5). Interestingly, a heat map representation of the lipidomic analysis of the livers of KO mice recovering from the diet-induced NAFLD revealed a favorable lipid profile very similar to that of WT mice under a regular diet, with no apparent liver phenotype, but very different from WT mice recovering from an HFFC diet (Fig. 5K). Finally, just like human IHH cells (Fig. 3E), KO mice exhibited higher UPR signaling via IRE1α phosphorylation (Fig. 6A, B), which seemingly maintains ER-homeostasis and protects against NAFLD\textsuperscript{50,51}. Notably, in our study LD density remains the same following HFFC feeding. The high lipid influx from the HFFC diet may protect apoB from degradation\textsuperscript{52,53} caused by the loss of Pcsk7 expression. This explains why in our hands, strong differences are only observed after 4- to 8-weeks of recovery under a regular diet, following HFFC feeding.

The above studies revealed that complete KO mice exhibit a safe and effective recovery from an HFFC diet-induced liver steatosis. It was then imperative to prove that the effect observed is primarily due to a lack of Pcsk7 expression in hepatocytes and not elsewhere. Accordingly, we used trivalent (GalNac)\textsubscript{3}-ASOs that target Pcsk7 specifically in hepatocytes due to their selective uptake by ASGR1\textsuperscript{33,34}. Thus, following an HFFC diet for 17-weeks, biweekly subcutaneous injections of the two best GalNac-ASO7 and GalNac-ASO2 were administered during the entire 8-weeks regular diet recovery period (Fig. 7). The results revealed that GalNac-ASO7 and GalNac-ASO2 substantially reduced circulating apoB levels by ~50\% (Fig. 7E), as observed in KO mice. Concomitantly, GalNac-ASO7 and less so GalNac-ASO2 effectively and safely reduced LD accumulation in hepatocytes, liver inflammation, hepatocyte ballooning and fibrosis (Fig. 8). The GalNac-ASO7-induced protective effects were much more impressive than those obtained with a positive control consisting of α-tocopherol in the diet (Fig. 8)\textsuperscript{54,55}, the only available approved
treatment strategy for NAFLD patients\textsuperscript{35}. Notably, while an HFFC diet for 17-weeks is not long enough to promote substantial liver fibrosis\textsuperscript{32}, protection against the observed fibrosis was similar between GalNac-ASO7 and \(\alpha\)-tocopherol (Fig. 8C).

The limitation of our studies relates to their \textit{in vivo} roles in male C57BL/6J mice and human cells. However, because of the differences between mouse and human lipid metabolism\textsuperscript{56}, an extension of these data to human disease will have to await further studies, including analysis of female KO mice to probe for possible sex-dependent effects as observed with \(Pcsk9^{-/-}\) mice\textsuperscript{57-60}, as well as the use of different mouse strains, such as \textit{apobec-1}^{-/-} and \textit{Ldlr}^{-/-} mice\textsuperscript{61,62} that have closer lipid profiles to humans, especially given the correlation between \textit{PCSK7} LOF with lower levels of circulating apoB in human subjects (Table 1B). In conclusion, our data present \textit{Pcsk7} as a novel non-enzymatic early regulator of apoB secretion and that loss of \textit{PCSK7} expression in hepatocytes is associated with reduced hepatic accumulation of apoB and TG. \textit{PCSK7}-mRNA silencing may be a promising new strategy for the treatment of diet-induced NAFLD in humans.
Methods

Association of rs236918 with cardiometabolic traits

A search for genetic associations between the rs236918 variant and twelve cardiometabolic traits was performed in large publicly available GWAS databases (N>100000 for most traits). Cardiometabolic traits included both continuous and dichotomous traits. Continuous traits encompassed circulating lipid fractions (triglyceride, HDL, LDL, and total cholesterol levels), markers of dysglycemia (fasting glucose and insulin levels), and body mass index. Dichotomous traits included risks of type 2 diabetes, hypertension, coronary artery disease, stroke, and chronic kidney disease. Only associations surpassing multiple hypotheses testing were considered statistically significant (P<0.05/12=0.0042). All subjects signed informed consent before the initiation of the studies for rs201598301 SNP analyses. The studies were approved by the Ethics committee at Institut de Recherches Cliniques de Montréal (IRCM).

Human data

To identify possible carriers of LOF or GOF SNPs in PCSK7, a post hoc analysis was carried out on subjects recruited through a clinical trial with hypocaloric diet at the IRCM (Trial registry at https://www.isrctn.com/ISRCTN14476404). The inclusion and exclusion criteria were previously published. In brief, the subjects had a BMI > 27 kg/m², were 45-74 years old, non-smokers, with sedentary lifestyle and low alcohol consumption, and had no chronic disease or medication affecting metabolism (except Synthroid on stable dose). Out of the 82 subjects recruited for the principal study, 32 participated in a substudy and were further approached for the collection of a blood DNA sample for future assessment of genes related to lipid metabolism, among whom DNA was collected from 22 subjects. Eight subjects with low or high plasma apoB (≤ 25th or ≥ 75th
percentile for sex and age in a Canadian population) were then selected for Sanger sequencing of \textit{PCSK7} at the Genomic core of the IRCM and the sequences analyzed with the Sequencher software. All subjects signed consent forms for the participation in the studies and for the collection and preservation of their biological and DNA samples, which were approved by the Human Ethics Board at the IRCM.

\textbf{Animal models}

Mice were maintained in a pathogen-free and climate-controlled facility with a 12h light/dark cycle, free access to water and fed \textit{ad libitum} throughout the experiment. From weaning till 8-weeks of age, all mice were fed \textit{ad libitum} a standard laboratory diet (2018 Teklad Global; Harlan Laboratories). At 8-weeks of age, KO or WT (on a C57B6L/J background, \(\geq\)12 backcrosses) mice were fed with either a regular diet containing 10 \% kcals from fat (commercially available at Research Diets, D09100304), an HFFC diet containing 40 \% kcal Fat, 20 \% kcal Fructose and 2 \% Cholesterol (Research Diets, D09100310) or a regular diet supplemented with 500 IU/kg \(\alpha\)-Tocopherol (Research Diets, D21092803). All diets were continued for 12 or 17-weeks as mentioned in the experimental models. The mice were fasted for 3h before the final bleeding and organ harvesting. Mice for the ASO experiment (WT, C57B6L/J background) were bought from Jackson Laboratories, Maine, United States at 7-weeks of age. The IRCM animal care committee approved all procedures.

\textbf{Cell culture, inhibitors, and transfections}

Immortalized Human Hepatocytes (IHH) cells were procured from Dr. Han Moshage’s laboratory (Department of Gastroenterology and Hepatology, University Hospital Groningen, Groningen), and HepG2 and FL38B cells were bought from ATCC. IHH and HepG2 cells were maintained in DMEM media (Wisent Bio) with 10\% fetal bovine serum (FBS) (v/v), while FL38B cells were
maintained in F12K media (Wisent Bio) +10% FBS (v/v). The cells were cultured at 37°C in a 5% CO₂ humidified incubator. The plates used to culture IHH and HepG2 were coated with 0.1% poly-l-lysine (Sigma Aldrich) for 15 mins before use. When required by the experiment, IHH cells were maintained in no sugar (D-glucose) no lipid (no FBS) media for 1hr. IHH cells were maintained at ≥ 70 % confluency for inhibitor treatments with Brefeldin A (BFA, Ready-made solution 10mg/ml in DMSO, Sigma Aldrich), 3-Methyladenine (re-suspended in culture media, Sigma Aldrich), MG132 (Ready-made solution 10 mM in DMSO, Calbiochem), lactacystin (re-suspended in DMSO, Cayman Chemical) and Decanoyl-RVKR-chloromethylketone (re-suspended in DMSO, Bachem). Transfections for plasmid DNA and siRNA were performed using Fugene HD transfection reagent (Promega) and Dharmafect reagent respectively according to the manufacturer’s instructions. For transfecting plasmid DNA in a 6-well plate, 2 µg plasmid DNA was mixed with opti-MEM media and 7 µl of Fugene reagent. The mixture was vortexed and incubated at room temperature for 15 minutes before adding it drop by drop to the cells. The pIR vector was used to express human PCSK7 with a V5 tag, human PCSK7 P777L with a V5 tag and human apoB21 with a V5 tag. For siRNA transfection in a 6-well plate, 20 µl of scramble or siRNA (Dharmacon, stock solution 5 µM) were added to 180 µl serum-free DMEM (Solution A) and 6 µl of Dharmafect reagent-4 was added to 194 µl serum-free DMEM (Solution B). Both solutions were incubated at room temperature for 5 mins, then mixed and again incubated at room temperature for 20 mins. The mixture was added to the cells at the end of this period. Finally, the wells were topped off by 1.4 ml of fresh complete media.

**Western blot analysis**

Proteins from cells or liver tissue were extracted in 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40 and 0.25% Na deoxycholate (RIPA 1X) buffer with a complete cocktail of
protease inhibitors (Roche). Bradford assay (Bio-Rad) was used to evaluate the protein concentrations. Equal amounts of proteins (30-50 µg) were subjected to SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham) or PVDF (EMD Millipore) membranes by using a Trans-Blot Turbo Transfer System (Bio-Rad) or overnight electro-blotting. Post transfer, the membranes were blocked in 5% skim milk in Tris-buffered saline containing Tween-20 for 1 h and subsequently incubated with primary and secondary antibodies according to the manufacturer’s recommendations. The analysis and quantifications were done using the ChemiDoc imaging system (Biorad) or ImageJ software (NIH). A list of primary and secondary antibodies used in this study are as listed:

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**Co-immunoprecipitation**

For co-IP of PCSK7-apoB100 complex IHH cells were transfected with plasmid DNA coding for human PCSK7 tagged with V5 or an empty vector only encoding for V5 tag. 24h post-transfection, the media was supplemented with 2.5 µM MG132. The cells were lysed 20hr post MG132 supplementation in Pierce non-denaturing IP buffer supplemented with a protease inhibitor cocktail without EDTA (Roche). Lysates containing 0.5 mg total protein were incubated overnight, at 4°C on a rocker, with 1.4 µg apoB antibody (apoB co-immunoprecipitation) or with 50 µl of anti-V5 antibody bound to agarose beads (PC7-V5 co-immunoprecipitation). In the case of apoB
co-immunoprecipitation, 20 μl of A/G PLUS-Agarose were added for an additional hour after the overnight incubation with the antibody. Following three washes with lysis buffer/protease inhibitors, two washes with PBS and elution in 60 μl 2× Laemmli sample buffer, the pull-downs were separated by 6% Tris-glycine SDS-PAGE along with inputs (6% of original material used for co-IP) and analyzed by WB for apoB100 and PCSK7 via V5 antibody. Immunoprecipitation of ubiquitinated apoB100 was performed using an anti-ubiquitin antibody. 50 μl of A/G PLUS-Agarose were added for an additional 1 to 2 h incubation to capture the anti-ubiquitin antibody. Following three washes with PBS and elution in 60 μl Laemmli sample buffer, the pull-downs were separated by 6% Tris-glycine SDS-PAGE along with inputs.

**Cycloheximide assay**

IHH WT or KO cells were seeded (0.5x10^6 cells per well) in a 6-well plate. The following day, the media was changed to no sugar and no lipid media supplemented with 300 μg/ml cycloheximide (re-suspended in DMSO). After the given time points, the media was removed and cells were washed with PBS. Protein lysates were prepared as mentioned above and protein expression was analyzed using WB analysis.

**Isolation and culture of primary hepatocytes**

Hepatocytes were isolated from 12-weeks-old male mice livers using the two-step collagenase perfusion method. After anesthesia of mice by 2% isoflurane inhalation, the peritoneal cavity was opened, and the liver was perfused in situ via the inferior vena cava for 6 min at 37 °C with calcium-free HEPES buffer I (142 mm NaCl, 6.7 mm KCl, 10 mm HEPES, pH 7.6) and for 8 min with calcium-supplemented HEPES buffer II (4.7 mm CaCl2, 66.7 mm NaCl, 6.7 mm KCl, 100 mm HEPES, pH 7.4) containing 0.5 mg/ml collagenase type V (Sigma Aldrich). The perfusion rates were set to 8 and 6 ml/min, respectively. In 3.5-cm Petri dishes coated with fibronectin (0.5
mg/ml, Sigma Aldrich), 0.5x10^6 cells were seeded in Williams' medium E supplemented with 10% fetal bovine serum (Invitrogen). After 2 h, the medium was replaced with hepatozyme medium (Invitrogen) for 12 h before the treatment.

**Lipogenesis and β-oxidation assays**

Lipogenesis and β-oxidation assays were performed as detailed earlier\textsuperscript{29}. Primary hepatocytes were extracted using the method discussed above and seeded (0.5x10^6 cells per well) in a 6-well plate. After 2hr of seeding the cells, the media was changed to serum-free DMEM supplemented with 100 nM Insulin, overnight. The next day, the media was changed to lipogenesis media (Serum-free DMEM + 2 μCi per well [3H]-acetate (Perkin Elmer) + 10 μM cold acetate) for 2 hrs. After the incubation with radioactive media, the cells were washed with PBS and scraped using 500 μl of 0.1 N HCl. 20 μl of this suspension was kept aside for protein estimation using Bradford assay. Lipids were then extracted from the rest of the cell suspension by adding 500 μl 2:1 chloroform-methanol (v/v). This mixture was vortexed for 30 seconds and incubated at room temperature for 5 mins. 250 μl ddH\textsubscript{2}O was then added, followed by vortexing the samples and re-incubating them at room temperature for 5 mins. The samples were centrifuged at 8000 RPM for 10 mins at room temperature and the lower phase was transferred into a fresh tube. Finally, [3H] activity was measured using scintillation fluid.

For the β-oxidation assay, IHH WT or KO cells (0.5x10^6 cells per well) were seeded in 6 well plates. Next day, 2 ml of assay buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}, 0.5 % free fatty acid free BSA) containing 1 μCi per well [1-14C] Oleic Acid were added to the cells for the indicated time points. The Whatman paper was cut into equal small rectangles and folded and placed into the centre well (Thomas Scientific) that was attached to a stopper top (Thomas scientific). After the indicated time points, the assay buffer was transferred into a 15 ml
falcon tube, 250 μl of 2M NaOH was added to the Whatman paper (to capture [14-CO₂] and the tube was sealed using a rubber lid with the centre well inside. 1.5 ml of 6N HCl was then injected into the falcon tube through the stopper top and the tube was allowed to stand overnight. The filter paper was finally taken out and added into a scintillation tube with 1 ml of H₂O, 62 μl 2N NaOH and 10 ml scintillation fluid. The tube was vortexed vigorously to dissolve the [14-C] labelled NaHCO₃ and rested for 2h followed by scintillation counting.

**Blood parameters and lipid analysis**

Blood was collected in heparin-coated Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) or heparinized micro-hematocrit capillaries (Fisher Scientific, Pittsburg, PA), centrifuged for 5 min at 3800g at 4°C, and kept at -80°C until assayed. Plasma TGs (L-Type Triglyceride, Wako), cholesterol (Sobioda), NEFA (Wako) and Alanine Transaminase (Cayman chemical) and Aspartate aminotransferase (Cayman chemicals) levels were measured using commercial kits according to the manufacturer’s instructions. Circulating mouse apoB (Abcam) was measured by ELISA according to the manufacturer’s protocol.

**Hepatic lipid and apolipoprotein analysis**

VLDL production was determined by measuring plasma TG (L-Type Triglyceride, Wako) before and after 0h and 2h, 4h and 6h post intraperitoneal injection of poloxamer 407 (1 g/kg of body weight; Sigma-Aldrich). Lipids content from mice liver were extracted using chloroform, isopropanol, and NP40 (7:11:0.1) lipid extraction buffer. Liver pieces were homogenized using a Dounce homogenizer. The homogenate was centrifuged for 5 mins at 14000 RPM and the supernatant was transferred into a new Eppendorf tube. The supernatant was then dried at 50°C for 60 min (dry bath) and placed in a speed vac for at least 1h (until dry). The pellet was then
dissolved in 0.2 ml of the cholesterol assay buffer (Abcam). Lipids were assayed by measuring cholesterol (Sobioda) and TG (L-Type Triglyceride, Wako)

**Histology and stainings**

Liver pieces (left lateral lobe) were collected and fixed in 4% paraformaldehyde at 4 °C for 24 h, washed in 70% ethanol, and embedded in paraffin. Sections were cut at 5-μm thickness and stained with hematoxylin and eosin (Sigma Aldrich) and Sirius Red (Sigma Aldrich). The visualization of stained liver sections was performed using transmission brightfield microscopy (DM4000 Osteomeasure, Leica). ORO staining was performed on cryopreserved liver sections. Dissected tissue was mounted in OCT embedding compound, snap frozen in isopentane mixed with dry ice, and stored at -80°C. The frozen sections were cut (8 μm thickness) and mounted on gelatin-coated histological slides. The slides were then fixed in 4 % paraformaldehyde for 30 mins and rinsed with cold water (3x). This was followed by 2x washes in isopropanol and 15 min staining in ORO solution (0.4 g ORO powder (Sigma Aldrich)+ 30 mL isopropanol + H2O up to 40 mL). Finally, the slides were washed 2x with isopropanol and 2x with ddH2O and mounted using an aqueous mounting medium (Vector Laboratories). A pathologist assessed simple steatosis, hepatocyte ballooning, lobular inflammation and fibrosis in blinded sections, according to the NAFLD Activity Score-Clinical Research Network (NAS-CRN)71. For ORO staining in the cells, IHH WT or KO cells were incubated with 0.3 mM oleic acid overnight. After 18h, the medium was replaced with EMEM (low sugar) without phenol red + 1% BSA. Finally, after 72h, the cells were washed twice with PBS, fixed and stained with ORO as mentioned above. ORO quantification was performed using a MATLAB program. Tunel staining was performed using a commercial kit (abcam) according to the manufacturer’s protocol on parrafin sections of the liver tissue.
Lipidomics

Lipids from liver samples were processed at the Montreal Heart Institute as previously described\textsuperscript{72}. Briefly, liver samples (40 mg) were all processed in one batch of extraction after spiking with the following lipid standards used to validate the reproducibility of the extraction and measurement: LPC 13:0, PC14:0/14:0, PC 19:0/19:0, PE17:0/17:0, PS12:0/12:0 and PG15:0/15:0 (Avanti Polar Lipids Inc., Alabaster, USA). Lipids were extracted into a methyl tert-butyl ether (MTBE/methanol/H\textsubscript{2}O) mixture, followed by the following steps: i) extraction with ethyl acetate, ii) drying under vacuum, and iii) solubilization in methanol/chloroform by vortexing and sonication. The samples were diluted (1:10 in methanol/chloroform, 2:1) and the diluted samples (2 µl) were injected into a 1290 Infinity HPLC coupled with a 6530 accurate mass QTOF MS system (Agilent Technologies Inc., Santa Clara, USA) via a dual electrospray ionization (ESI) source in positive scan mode. Elution of lipids was assessed on a Zorbax Eclipse plus column (C18, 2.1 x 100 mm, particle size 1.8 µm, Agilent) at 40°C using an 83 min chromatographic gradient of solvent A (0.2% formic acid and 10 mM ammonium formate in water) and B (0.2% formic acid and 5 mM ammonium formate in methanol/acetonitrile/MTBE, 55:35:10 (v/v/v)). A list of MS features, characterized by mass and retention time, was extracted using the Mass Hunter Qualitative Analysis software package (version B.06, Agilent). MS features were first annotated using a public database (Metlin) by alignment with our in-house database containing more than 500 lipids which have been previously identified in human plasma by MSMS analysis for which spectra were manually interpreted, similar to Godzien et al\textsuperscript{73}. The significant MS features were finally validated using MSMS analysis.
Generation of ASOs

Antisense Oligonucleotides (ASOs) used in this study were procured from IDT DNA technologies. ASOs consisted of 20 nucleotides, single-strand DNA. Along with the nucleotides, ASOs were also modified with sugars on their 5’ end or more specifically a trivalent N-acetylgalactosamine attachment for specific delivery to the liver. A phosphorothioate backbone was used along the entire length of the ASO to provide nuclease resistance, while the 2’-O-metoxylethyl (MOE) modification was used exclusively on the first and last 5 nucleotides, leaving the middle 10 nucleotides unmodified at the 2’-sugar position. Sequences of the ASOs used are as listed: ASO2-5’-GalNacCx3-CTCCATATCCTGTCAGTAAGTG-3’ and ASO7-5’-GalNacCx3-GATGTCTCTCTTCTGGGCTCC-3’. The same ASOs were also prepared without the trivalent N-acetylgalactosamine attachment but instead tagged with a Fluorescein (FAM) dye for use in cellular assays.

Immunofluorescence

For immunofluorescence experiments, FL83B cells (0.5x10⁵ cells/well) were plated on poly-L-lysine-coated round microscope coverslips that were placed in a 24-well cell culture plate. Cells were then treated as required (mASO transfection, oleic acid loading, no sugar media incubation). To analyze lipid droplets, the cells were washed twice with PBS and fixed with a solution of 4% paraformaldehyde in PBS (10 min). Samples were then incubated with HCS LipidTOX Red neutral lipid stain (dilution 1/1000 in PBS; Molecular Probes, ref. H34476) for 1h at room temperature. Coverslips were mounted on a glass slide with ProLong Gold antifade reagent with DAPI. Samples were visualized using a Plan-Apochromat 63x 1.4 oil objective of an LSM-710 confocal laser-scanning microscope (Carl Zeiss) with sequential excitation and capture image acquisition with a digital camera. Images were processed with ZEN software. Image analysis to
quantify the fluorescence intensities was accomplished using Volocity®6.0. LipidTOX staining was also performed after Fluorescence-activated cell sorting (FACS). FL38B cells were washed with PBS and trypsinized post ASO transfection and α-Tocopherol treatment. The cells were then suspended in polystyrene tubes containing Ca/Mg free PBS + 1%FBS and immediately introduced into the flow cytometer. For each sample, 10000 events were used for collecting the data. Data about forward scatter (FSC), side scatter (SSC) and green fluorescence (FAM+) was recorded, FSC and SSC data were used to identify viable cells, while FAM+ cells were sorted for further LipidTOX staining as mentioned above.

**ApoB pulse-chase analysis**

Pulse-chase experiment was performed as mentioned earlier. Primary hepatocytes isolated from WT and KO mouse livers were pulsed with [³⁵Methionine/Cysteine] for 15 min and chased in media containing cold methionine and cysteine. Immunoprecipitation of the labelled apoB in cell lysate and cultured media was carried out using rabbit anti-mouse apoB (Meridian Life Science). The gels were dried, and the radioactivity associated with apoB was quantified via autoradiography.

**Gene expression**

Quantitative real-time PCR analyses (qPCR) were performed on samples from mouse liver, as previously described. Total RNA was extracted from frozen liver pieces with TRIzol reagent (Invitrogen, Carlsbad, CA), and reverse transcribed into cDNA using a SuperScript II cDNA reverse transcriptase and RNase OUT inhibitor (Invitrogen). QPCR was performed using PowerUp SYBR Green Master mix (ABI, Fisher Scientific) on Applied Biosystems VIIA 7 Real-Time PCR system. All gene expressions (see Table of primers) were normalized to that of hypoxanthine-
guanine phosphoribosyltransferase (Hprt) or TATA-binding protein (Tbp). List of oligos used in this study:

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**Statistical analysis**

Mouse and cell results values are expressed as mean ± SEM. Normality and equality of variance of the data were checked using the Shapiro-Wilk test and F-test, respectively. The differences between two groups were tested using parametric unpaired Student’s t-test with Welch’s correction for unequal variance or nonparametric Mann-Whitney U test if required. Multi-group comparisons were performed using the non-parametric Kruskal-Wallis test followed by Dunn’s correction when the normality/equal variance test did not pass or using parametric 1-way ANOVA followed by Tukey’s multiple comparisons test when data passed the normality/equal variance tests. GraphPad Prism 8 software was used for all analyses. The number of mice used appears in figure legends.
References


Acknowledgements

This work was supported in part by a CIHR Foundation grant (NGS: # 148363), a Canada Research Chairs in Precursor Proteolysis (NGS: # 950-231335), a CIHR operating grant (MF: # 93581) for the human data, and a Leducq Foundation grant (NGS: # 13 CVD 03). We also thank Univalor/Liphorus Inc: Économie, Sciences et Innovation Québec (PSO3) for supporting this work. We thank Yahya Ashraf for the initial part of this study, Sepideh Mikaeeli and Emmanuelle Girard for the selection of the best mouse ASOs by LipidTox analyses, Suzie Riverin and Manon Laprise for excellent animal care, Sarah Boissel, François Couderc and Myriam Rondeau for DNA sequencing and genotyping, and Jumanah Farah Baig (Faculty of Medicine at UdeM) for her help in the NAFLD pathology scores. We would like to thank Jisca Borgela for her editorial help.

Figure Legends

Table 1. A. Associations between the gain of function PCSK7 SNP (rs236918) and cardiometabolic traits. B. Associations between loss of function PCSK7 SNP (rs201598301) resulting in Proline-777-Leucine and lipid characteristics in humans.

Figure 1: KO mice fed a regular diet exhibit reduced circulating and hepatic apoB100 levels and lower lipid content in the liver. A, B. Western blot analysis of plasma (A) and liver protein extract (B) showing the levels of apoB in KO mice compared to WT mice. The apoE and β-actin immunoblot were used as a loading control for plasma samples and liver protein extracts respectively (N=3). C. Representative images of liver lipids stained by ORO staining are shown (N=8-10) (Scale bar, 100 μm). D. The total area stained, mean count and mean area of the lipid droplets stained in the sections were also quantified. Quantification of the Westernblots is
accompanied where required. Apparent molecular weights are shown with the blots. Statistical comparisons between two groups were conducted using unpaired two-tailed Student’s t-tests, while multiple groups were compared using one-way ANOVAs with the Tukey HSD post-hoc test (exact p values are mentioned where differences are significant).

Figure 2: apoB degradation mechanism and chaperone/escort effect of PCSK7. A. WT or KO IHH cells were maintained in complete growth media overnight. This was followed by incubating the cells in media containing no sugar and no lipids for 1h. The extracted proteins were then subjected to Western blot analysis (N=4). B. WT or KO IHH cells were treated with cycloheximide (final concentration 300 µg/ml, in media containing no sugar and no lipids) for the indicated times. Western blot analysis of the protein lysates prepared from cells at the indicated times (N=3). C. WT or KO IHH cells were treated with Lactacystin (25 µM) for 1h, apoB100 was immunoprecipitated from cell lysates and apoB-100 and ubiquitin were detected by Western blot analysis (N=2). D. WT or KO IHH cells were pre-treated with Brefeldin A (2.5 µg/ml), 3-Methyladenine (10mM), MG132 (10 µM), Lactacystin (25 µM) or DMSO as a control in complete media for 7h. This was followed by repeating the inhibitor treatments in no sugar and no lipid media for 1h. The protein extracts from cells were then subjected to Western blot analysis (N=3-6). E. Naïve IHH cells were used to over-express either V5-tagged PCSK7 or pIRES empty vector (EV) encoding only for the V5 tag. Post-transfection, the cells were incubated with fresh media supplemented with MG132 (25 µM) for 18h. Cell lysate was subjected to co-immunoprecipitation for apoB using anti apoB antibody. Co-immunoprecipitated apoB and PCSK7 along with the inputs were examined via Western blotting with the indicated antibodies (N=3). F. Naïve IHH cells were used to over-express cDNAs of a short form of apoB (apoB21, 965 amino acids) along
with PCSK7 and empty vector as a control. 24h post-transfection, cells were treated with decanoyl-RVKR-cmk (50 uM). Media and cells were collected 18h later and subjected to Western blot analysis (N=3). Quantification of the Western blots is accompanied where required. Apparent molecular weights are shown with the blots. Statistical comparisons between two groups were conducted using unpaired two-tailed Student’s t-tests, while multiple groups were compared using one-way ANOVAs with the Tukey HSD post-hoc test (exact p values are mentioned where differences are significant).

Figure 3: Mechanism behind the reduced hepatic lipid content in absence of PCSK7. A. Hepatic VLDL-TG secretion rates from 16h-fasted WT and KO mice were studied by measuring plasma TG levels using Poloxamer-407 to inhibit plasma lipases (N=5 mice per group). B. De novo synthesized lipids were measured in primary hepatocytes extracted from WT or KO mice (N=3) (B). C. Primary hepatocytes derived from WT or KO mice were utilized to measure LC3 protein expression by Western blotting in the presence or absence of Chloroquine (100 µM). Immunoblotting for β-actin was performed as a loading control and quantification is shown next to the blots (N=2). D. Fatty acid oxidation was quantified in WT or PCSK7 -/- IHH cells by measuring [14C]-CO2 released from the cells (N=2). E. Phospho-IRE1α, Total IRE1α, Phospho-PERK and Total PERK protein expression were analyzed in WT or PCSK7 -/- IHH cells. The cells were incubated with media containing no sugar and no lipids for 2hr followed by lysate preparation and Western blot analysis (N=3). F. WT or PCSK7 -/- cells were stained with ORO, representative images and the quantification are shown (N=6). Quantification of the Western blots is accompanied where required. Apparent molecular weights are shown with the blots. Statistical comparisons between two groups were conducted using unpaired two-tailed Student’s t-tests,
while multiple groups were compared using one-way ANOVAs with the Tukey HSD post-hoc test (exact p values are mentioned where differences are significant).

**Figure 4:** Liver fat accumulation in WT versus KO mice recovering from a high fat/cholesterol/sugar diet. A. Experimental model developed to study the loss of PCSK7 in liver fat accumulation in mice. WT and KO mice (20 mice per diet cohort, per genotype) were either fed a regular diet or a Western diet high in fat, fructose, and cholesterol (HFFC diet) for 12-weeks. At the end of 12-weeks, half of the mice chosen at random were sacrificed to confirm the establishment of NAFLD with the remaining mice given a chance to recover. Following a 4-weeks recovery period under a regular diet, the remaining mice were sacrificed, and analyses were performed. B, C. Western blot analyses of plasma (B) (N=8) and liver protein extract (C) showing levels of apoB in KO mice compared to WT mice after 16-weeks of regular diet or after 12-weeks of HFFC diet + 4-weeks of recovery. α-tubulin was used as a loading control for liver protein extracts (N=3-4). D, E, F. Representative images of liver sections stained with ORO from WT and KO mice are shown after 12-weeks of HFFC diet (D) or after 12-weeks of HFFC diet + 4-weeks of recovery (E) along with the quantification of the total area stained, lipid droplet number and mean area of lipid droplets (F) (N=6-10) (Scale bar, 100 μm). Quantification of the Western blots is shown where required. Apparent molecular weights are indicated with the blots. Statistical comparisons between two groups were conducted using unpaired two-tailed Student’s t-tests, while multiple groups were compared using one-way ANOVAs with the Tukey HSD post-hoc test (exact p values are mentioned where differences are significant). The bar graphs with dotted patterns represent the data corresponding to 12-weeks HFFC fed mice and the bar graphs with
hatching patterns correspond to the data from mice fed 12-weeks of HFFC diet + 4-weeks of recovery.

**Figure 5: The absence of PCSK7 improves liver pathology in mice recovering from a NAFLD-promoting diet.** A, C. Hematoxylin and Eosin (H&E) staining of liver tissue was performed at 12-weeks of HFFC diet feeding (A) and after 12-weeks of HFFC diet feeding + 4-weeks of recovery (C). B, D. Grading of liver steatosis from 0 to 3 (0: <5% steatosis, none; 1: 5%–33%, mild; 2: 34%–66%, moderate; 3: >67%, marked), scoring of hepatocyte ballooning from 0 to 2 (0, none; 1, few balloon cells; and 2, many cells/prominent ballooning), and scoring of lobular inflammation, based on the number of inflammatory foci per 20× field (0, no foci; 1, <2 foci; 2, 2–4 foci; and 3, >4 foci) was performed at 12-weeks of HFFC diet feeding (B) and after 12-weeks of HFFC diet feeding + 4-weeks of recovery (D). E, H. Sirius red staining of liver tissue was performed at 12-weeks of HFFC diet feeding (E) and after 12-weeks of HFFC diet feeding + 4-weeks of recovery (H). Stage of fibrosis using NAFLD activity score-clinical research network (NAS-CRN) scoring (F0, no fibrosis; F1, perisinusoidal zone 3 or portal fibrosis; F2, perisinusoidal and periportal fibrosis without bridging; F3, bridging fibrosis; and F4, cirrhosis) was performed (side panel E, H). F, I. Serum alanine aminotransferase (ALT) levels were measured in WT and KO mice at 12-weeks of HFFC diet feeding (F) and after 12-weeks of HFFC diet feeding + 4-weeks of recovery (I) (N=6-8). G, J. Liver acyl-glycerol content was measured in WT and KO mice at 12-weeks of HFFC diet feeding (G) and after 12-weeks of HFFC diet feeding + 4-weeks of recovery (J) (N=6-8). K. Heat-map of selected lipids significantly changed in KO mice compared to WT and validated using MSMS. Each dot represents a log2-transformed averaged signal intensity (N=6-8). Abbreviations: LPC: lysoglycerophosphatidylcholine; LPE:
lysoglycerophosphatidylethanolamine; PC: glycerophosphatidylcholine; PE: glycerophosphatidylethanolamine; PEO: Ether glycerophosphatidylethanolamine; PG: glycerophosphatidylglycine; PS: glycerophosphatidylserine; TG: triacylglycerol. Statistical comparisons between two groups were conducted using unpaired two-tailed Student’s t-tests, while multiple groups were compared using one-way ANOVAs with the Tukey HSD post-hoc test (exact p values are mentioned where differences are significant).

**Figure 6: The absence of PCSK7 activates UPR signalling.** A. The mRNA levels of the genes encoding for IRE1α and GRP78 in the livers of the WT and KO mice at 12-weeks of HFFC diet feeding and after 12-weeks of HFFC diet feeding + 4-weeks of recovery (A) (N=6-10). B. Western blot analyses of total and phospho-IRE1α expression in livers of WT and KO mice at 12-weeks of HFFC diet feeding and after 12-weeks of HFFC diet feeding + 4-weeks of recovery. The ratio of phospho/total IRE1α has been quantified (N=4). α-tubulin was used as a loading control for liver protein extracts. C. A proposed model for the role of PCSK7 in the liver. PCSK7 acts as an apoB100 chaperone to allow its efficient association with lipids in the ER lumen producing robust VLDL which can then be secreted (left panel). Its absence results in proteasomal degradation of apoB100, possibly resulting in lipid accumulation in the ER lumen. This leads to an increased UPR activation, possibly triggering autophagy and lipid β-oxidation, eventually resulting in reduced lipid accumulation in the liver (right panel). Quantification of the Western blots is shown where required. Apparent molecular weights are indicated with the blots. Statistical comparisons between the two groups were conducted using unpaired two-tailed Student’s t-tests (exact p values are mentioned where differences are significant).
Figure 7: GalNAc Conjugated ASOs effectively knockdown hepatic PCSK7. A. 8-weeks-old mice were injected with 2 mg/kg, 5 mg/kg, 10 mg/kg and 15 mg/kg ASOs namely ASO2 and ASO7 covalently linked to trivalent N-acetyl-Galactosamine (GalNac) residues for 8-weeks subcutaneously. The mice were then sacrificed, their liver harvested and transcript levels of Pcsk7 were measured (N=4 per group). Except for ASO2 at 2 mg/kg, all other values were statistically significant compared to PBS (p <0.001). B. 8-weeks-old mice were injected with 5 mg/kg ASO2 and ASO7 for 8-weeks. This was followed by extracting primary hepatocytes from these mice and transcript levels of Pcsk7 were quantified and compared to the total liver extract. C. The experimental model developed to study the effects of ASO-mediated knockdown of Pcsk7 in mice. Mice were fed an HFFC diet for 17-weeks to induce NAFLD. Following this, the diet was changed to a regular diet and mice were divided randomly into 4 groups of 12. These groups consisted of Non-treated mice, ASO2 injected mice, ASO7 injected mice, and a final group given a 500 IU/kg α-tocopherol supplemented regular diet for 8-weeks. D. Pcsk7 transcript levels were measured after 8-weeks of ASO injections or α-tocopherol diet (N=10-12). E. Plasma apoB levels were measured in mice after 8-weeks of different aforementioned treatments (N=10-12). Statistical comparisons between two groups were conducted using unpaired two-tailed Student’s t-tests, while multiple groups were compared using one-way ANOVAs with the Tukey HSD post-hoc test (exact p values are mentioned where differences are significant).

Figure 8: Hepatic silencing of Pcsk7 expression accelerates the recovery of mice exhibiting hepatic steatosis. A. Representative images of liver lipids stained by ORO staining after 8-weeks of ASO injections/α-tocopherol diet are shown (N=10-12). The total area stained, mean area and mean count of the lipid droplets stained in the sections were also quantified (bottom panel, A). B.
Hematoxylin and Eosin (H&E) staining of liver tissue was performed after 8-weeks of ASO injections/α-tocopherol diet. Grading of liver steatosis from 0 to 3 (0: <5% steatosis, none; 1: 5%–33%, mild; 2: 34%–66%, moderate; 3: >67%, marked), scoring of hepatocyte ballooning from 0 to 2 (0, none; 1, few balloon cells; and 2, many cells/prominent ballooning), and scoring of lobular inflammation, based on the number of inflammatory foci per 20× field (0, no foci; 1, <2 foci; 2, 2–4 foci; and 3, >4 foci) was performed (lower panel, B) (N=10-12). C. Sirius red staining of liver tissue was performed after 8-weeks of ASO injections/α-tocopherol diet. Stage of fibrosis using NAFLD activity score-clinical research network (NAS-CRN) scoring (F0, no fibrosis; F1, perisinusoidal zone 3 or portal fibrosis; F2, perisinusoidal and periportal fibrosis without bridging; F3, bridging fibrosis; and F4, cirrhosis) was performed (c, lower panel) (N=10-12). D. Liver acyl-glycerol content was measured in mice after 8-weeks of ASO injections/α-tocopherol diet (N=10-12) (Scale bar, 100 μm). Statistical comparisons between two groups were conducted using unpaired two-tailed Student’s t-tests, while multiple groups were compared using one-way ANOVAs with the Tukey HSD post-hoc test (exact p values are mentioned where differences are significant).
Author contributions

VS, AP, RSK, NGS conceived the studies and designed the experiments. ASOs were designed by MS and tested on Pcsk7 mRNA by SM, VS, ML, JFL. In vitro studies were conducted by VS, ML, JH, AE, DSR. In vivo studies were conducted VS, AR, ML, DG, JFL, RE, SD. Diets were proposed by JLE. ER stress measurements were partly made by RCA and VS. Lipidomics were performed by MR, IR. The GWAS analysis was done by MC, GP. Human subjects with low apoB and TG were selected by MF. Mouse liver pathology was done by EC, ZHG, VQT. The manuscript was written by NGS, VS, AP and revised by VS, ML, MF, JLE, AP, ESK, RCA, NGS.

Competing interests

There are no competing interests.

Additional information

We have no additional information to report.
### A

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<td>LDLc</td>
<td>mg/dl</td>
<td>0.01</td>
<td>0.003 to 0.02</td>
<td>0.01</td>
<td>237,050</td>
<td>GLGC</td>
</tr>
<tr>
<td>Totalc*</td>
<td>mg/dl</td>
<td>0.04</td>
<td>0.03 to 0.05</td>
<td>1.89x10^-24</td>
<td>237,050</td>
<td>GLGC</td>
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<tr>
<td>Apolipoprotein A1*</td>
<td>mg/dl</td>
<td>0.56</td>
<td>0.41 to 0.73</td>
<td>2.5x10^-10</td>
<td>398,508</td>
<td>UK Biobank</td>
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<tr>
<td>Apolipoprotein B*</td>
<td>mg/dl</td>
<td>0.54</td>
<td>0.42 to 0.68</td>
<td>8.1x10^-13</td>
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<tr>
<td>Lipoprotein(a)</td>
<td>U/L</td>
<td>0.30</td>
<td>-0.05 to 0.64</td>
<td>0.13</td>
<td>348,806</td>
<td>UK Biobank</td>
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<tr>
<td>Alanine Aminotransferase (ALT)</td>
<td>U/L</td>
<td>0.02</td>
<td>-0.05 to 0.09</td>
<td>0.59</td>
<td>494,681</td>
<td>UK Biobank, Finngen, Biobank Japan</td>
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<tr>
<td>Aspartate Aminotransferase (AST)</td>
<td>U/L</td>
<td>0.03</td>
<td>-0.02 to 0.09</td>
<td>0.26</td>
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<td>UK Biobank, Finngen, Biobank Japan</td>
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<tr>
<td>Liver cirrhosis in hereditary hemochromatosis*</td>
<td>Odds Ratio</td>
<td>5.38</td>
<td>2.39 to 12.10</td>
<td>1.02 x 10^-5</td>
<td>759</td>
<td>Stickel et al. (2014)</td>
</tr>
</tbody>
</table>

### B

**PCSK7 LOF rs201598301 (exon 17; P777L)**

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>BMI (kg/m2)</th>
<th>apoB (g/L)</th>
<th>Percentile apoB for age and sex</th>
<th>TG (mmol/L)</th>
<th>Percentile TG for age and sex</th>
<th>LDL (mmol/L)</th>
<th>Percentile LDLLC for age and sex</th>
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<td>2.22</td>
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<tr>
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<td>F</td>
<td>64</td>
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<td>0.71</td>
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<td>= 50th</td>
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<td>0.62</td>
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<td>4</td>
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<td>42.6</td>
<td>1.31</td>
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<td>1.89</td>
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<td>3.92</td>
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<tr>
<td>5</td>
<td>F</td>
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<td>27.2</td>
<td>1.74</td>
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<td>4.99</td>
<td>&gt; 95th</td>
<td>5.33</td>
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<tr>
<td>6</td>
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<td>56</td>
<td>27.8</td>
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<td>&lt; 5th</td>
<td>0.76</td>
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<td>7</td>
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<td>2.60</td>
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<tr>
<td>8</td>
<td>M</td>
<td>59</td>
<td>35.2</td>
<td>1.32</td>
<td>= 75th</td>
<td>1.23</td>
<td>= 95th</td>
<td>3.73</td>
<td>= 90th</td>
</tr>
</tbody>
</table>
Figure 1

A. Western blot analysis of apoB100, apoB48, and apoE in plasma and liver from WT and KO mice fed a regular diet. 

B. Western blot analysis of apoB100, apoB48, and apoE in plasma and liver from WT and KO mice fed a regular diet.

C. Oil red O staining of liver sections from WT and KO mice fed a regular diet.

D. Quantification of Oil red O staining, showing total area stained, number of droplets, and droplet mean area in WT and KO mice fed a regular diet. 

Statistical significance is indicated by * (P < 0.05) and ** (P < 0.01), with P-values provided for each comparison.
Figure 2
**Figure 3**

**A** TG secretion (poloxamer)  

**B** $^3$H-acetate incorporation  

**C** Lysosomal macroautophagy

- **No treatment**
- **Chloroquine**

**D** $^{14}$CO$_2$ release - FA β-oxidation  

**E** Phospho-PERK, Total PERK, Phospho-IRE1α, Total IRE1α  

**F** Total Area Stained
Figure 6

(A) Relative mRNA levels of *Ire1α*, *Grp78*, *Atf6*, *sXbp1*, and *Perk* in WT and KO mice fed with HFFC diet for 12 weeks or HFFC diet + 4-week recovery. 

(B) Western blot analysis of Phospho-IRE1α and Total-IRE1α in WT and KO mice fed with HFFC diet for 12 weeks or HFFC diet + 4-week recovery. 

(C) Schematic representation of the effects of Pcsk7 WT and KO on lipid metabolism.
Figure 7
**Figure 8**

(A) Staining for Oil Red O (a.u.)

Total area stained

Number of droplets

Droplets mean area

(B) Staining for H&E

Liver inflammation

Hepatocytes ballooning

Steatosis

(C) Staining for Sirius red

Liver fibrosis

(D) Liver triglycerides

No treatment

GalNac-ASO2

GalNac-ASO7

α-tocopherol
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.pdf