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BASIC SCIENCE
Lipids

PCSK9 deficiency reduces insulin secretion and promotes glucose intolerance: the role of the low-density lipoprotein receptor

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Aims *PCSK9* loss of function genetic variants are associated with lower low-density lipoprotein cholesterol but also with higher plasma glucose levels and increased risk of Type 2 diabetes mellitus. Here, we investigated the molecular mechanisms underlying this association.

Methods and results *Pcsk9* KO, WT, *Pcsk9*/*Ldlr* double KO (DKO), *Ldlr* KO, albumin *AlbCre*+/*Pcsk9*$^{LoxP/LoxP}$ (liver-selective *Pcsk9* knock-out mice), and *AlbCre*-/*Pcsk9*$^{LoxP/LoxP}$ mice were used. GTT, ITT, insulin and C-peptide plasma levels, pancreas morphology, and cholesterol accumulation in pancreatic islets were studied in the different animal models. Glucose clearance was significantly impaired in *Pcsk9* KO mice fed with a standard or a high-fat diet for 20 weeks compared with WT animals; insulin sensitivity, however, was not affected. A detailed analysis of pancreas morphology of *Pcsk9* KO mice vs. controls revealed larger islets with increased accumulation of cholesteryl esters, paralleled by increased insulin intracellular levels and decreased plasma insulin, and C-peptide levels. This phenotype was completely reverted in *Pcsk9*/*Ldlr* DKO mice implying the low-density lipoprotein receptor (LDLR) as the proprotein convertase subtilisin/kexin Type 9 (PCSK9) target responsible for the phenotype observed. Further studies in albumin *AlbCre*+/*Pcsk9*$^{LoxP/LoxP}$ mice, which lack detectable circulating PCSK9, also showed a complete recovery of the phenotype, thus indicating that circulating, liver-derived PCSK9, the principal target of monoclonal antibodies, does not impact beta-cell function and insulin secretion.

Conclusion PCSK9 critically controls LDLR expression in pancreas perhaps contributing to the maintenance of a proper physiological balance to limit cholesterol overload in beta cells. This effect is independent of circulating PCSK9 and is probably related to locally produced PCSK9.

Keywords PCSK9 • Insulin • Diabetes

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Translational perspective

Mendelian randomization studies have shown that *PCSK9* genetic variants associate with lower low-density lipoprotein cholesterol but higher fasting plasma glucose levels and increased risk of Type 2 diabetes. The investigation of molecular mechanisms responsible for this effect demonstrated that the deficiency of locally but not of circulating proprotein convertase subtilisin/kexin Type 9 (PCSK9) is responsible for increased low-density lipoprotein receptor expression in pancreatic islets, which results in cholesterol accumulation and beta-cell dysfunction. These data suggest that anti-PCSK9 therapies, which target mainly circulating PCSK9, might have a limited impact on beta-cell dysfunction and the incidence of diabetes in contrast to Mendelian randomization analysis, where the effect of global PCSK9 deficiency was investigated.

Introduction

PCSK9 (proprotein convertase subtilisin/kexin Type 9) is a protein, mainly synthesized and secreted by the liver, which binds to specific proteins and escorts them to endosomes/lysosomes compartments for degradation.[1,2] The low-density lipoprotein receptor (LDLR) has been identified as the main target of PCSK9[3]; mice lacking *Pcsk9* exhibit an increased hepatic LDLR expression, resulting in an increased clearance of circulating low-density lipoprotein (LDL) and hypocholesterolaemia,[4] while PCSK9 overexpression induces a two-fold increase in plasma cholesterol levels.[5]

In humans, several mutations in *PCSK9* gene were described, with 'gain-of-function' mutations associated to familial hypercholesterolaemia (FH)[6] and 'loss of function' mutations linked to low-LDL-cholesterol (LDL-C) levels.[7,8]

On these premises, anti-PCSK9 therapies have been developed, and monoclonal antibodies against PCSK9 are available for the treatment of patients with severe hypercholesterolaemia and/or very high cardiovascular risk.

While the liver is the main contributor to circulating PCSK9,[9] other tissues produce PCSK9 pointing to a possible role of this protein beyond the control of LDLR expression in the liver.[1,10]

Of note, the LDLR is abundantly expressed by pancreatic β cells in humans, mice, and rats, where it plays a key role in the uptake of plasma LDL.[11,12] Cholesterol homeostasis is crucial for β cells function and survival; excessive cholesterol accumulation causes a significant reduction in the islets' ability to secrete insulin in response to glucose,[13] and prolonged exposure to high levels of LDL or very low-density lipoprotein could also be lethal for β cells.[12,14] Increased LDLR expression and cholesterol content were observed in the pancreas of *Pcsk9*-deficient mice,[15,16] suggesting also a potential role for the protein in this tissue. Interestingly, in three different Mendelian randomization studies, the analysis of the effects of genetic scores consisting of independently inherited polymorphisms in the *PCSK9* gene resulted into reduced LDL-C levels and cardiovascular events but was also associated with an increased risk of diabetes[17–19]; furthermore, patients with FH (decreased LDLR function) appear to have a lower risk of diabetes.[20]

On this background, we investigated the molecular mechanisms beyond the impaired glucose metabolism observed in PCSK9-deficient state and tested (i) whether the absence of the LDLR rescues the phenotype by studying *Pcsk9*/*Ldlr* double KO (DKO) mice and (ii) the role of circulating/liver-derived PCSK9.

Here, we show that PCSK9 deficiency is associated with impaired glucose tolerance in mice, which appears to be the consequence of decreased insulin secretion from the pancreas rather than insulin resistance. This effect appears to be largely independent from liver-derived PCSK9.

Materials and methods

A detailed description of mice, dietary regimens, glucose tolerance and insulin tolerance tests, pancreatic islet staining, western blot analysis, analysis of cholesterol accumulation in pancreatic islets, bioinformatics, and statistical analysis is presented as the Supplementary material online.

Results

Pcsk9 deficiency is associated with impaired glucose tolerance but not with insulin resistance

To evaluate the effect of PCSK9 on plasma glucose, mice were fed with a standard fat diet (SFD) or a high-fat diet (HFD) for 20 weeks. Despite similar plasma glucose levels at baseline, following glucose tolerance test (GTT), the absence of PCSK9 resulted in a significant delay of glucose clearance in both the SFD (*Figure 1A–D*) or the HFD (*Figure 1E–H*) groups after 12 or 20 weeks of diet. The plasma glucose area under the curve (AUC) in all conditions was significantly higher in *Pcsk9* KO mice compared with WT (*Figure 1C, D, G,* and *H*). The observation that *Pcsk9* KO mice presented glucose intolerance following GTT in spite of similar baseline glucose levels (following overnight fasting) prompted us to perform a fast and refeeding experiment to better understand possible differences in plasma glucose levels under physiological conditions.

After overnight fasting and *ad libitum* refeeding (4 h), *Pcsk9* KO mice presented a significant increase in plasma glucose levels compared with WT littermates (268 ± 6 mg/dL for *Pcsk9* KO compared with 219 ± 30 mg/dL for WT mice, $P = 0.008$) in spite of similar food consumption (2.27 ± 0.43 g/4 h vs. 2.18 ± 0.38 g/4 h). This observation confirmed the presence of impaired glucose tolerance in *Pcsk9* KO mice and set the stage for investigating whether this effect was the consequence of impaired insulin tolerance.

Insulin tolerance test (ITT) was performed in WT and *Pcsk9* KO mice, after a 4 h fast. The animals were injected with insulin (0.2 IU/kg body weight) intraperitoneally. The decrease in plasma glucose levels was similar in WT and *Pcsk9* KO mice fed with an SFD (*Figure 2A* and *B*) or an HFD (see Supplementary material online, *Figure S1A* and *B*) for 12 or 20 weeks, thus excluding the presence of insulin resistance in the *Pcsk9*-deficient state. To further extend this finding, we measured the ratio between phosphorylated Akt vs. total Akt as an index

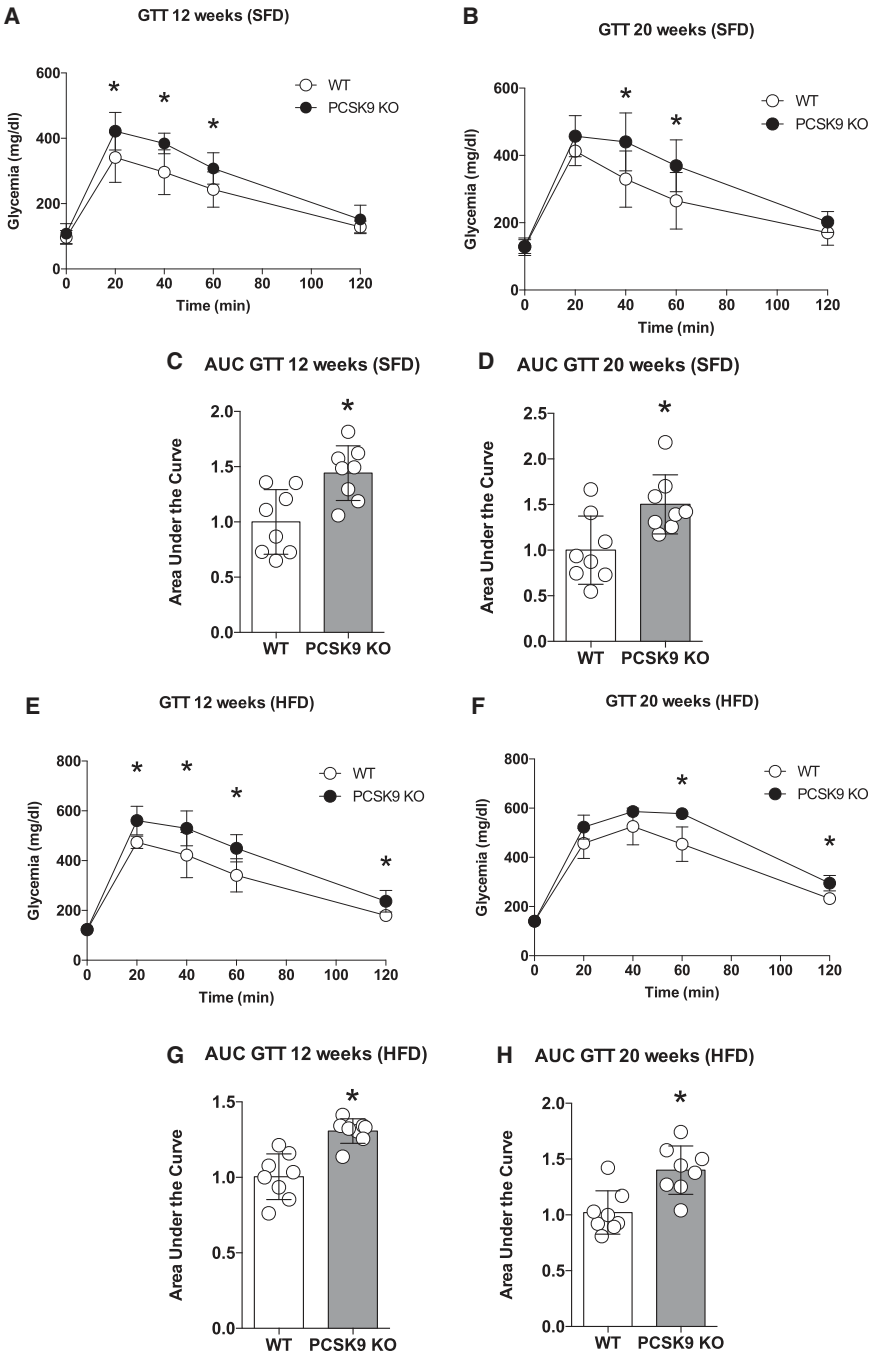


Figure 1 *Pcsk9* deficiency is associated with impaired glucose tolerance. Intraperitoneal glucose tolerance test was performed, and plasma glucose levels were measured at 0, 20, 40, 60, and 120 min. (*A*) Data obtained from mice fed with a standard fat diet for 12 weeks (0 min, $P = 0.426$; 20 min, $^*P = 0.038$; 40 min, $^*P = 0.006$; 60 min, $^*P = 0.028$; 120 min, $P = 0.488$) are shown. (*B*) Data obtained from mice fed with a standard fat diet for 20 weeks (0 min, $P = 0.702$; 20 min, $P = 0.104$; 40 min, $^*P = 0.038$; 60 min, $^*P = 0.041$; 120 min, $P = 0.062$) are shown. (*C*) The AUC of intraperitoneal glucose tolerance test performed on mice fed for 12 weeks with a standard fat diet ($^*P = 0.015$) are shown. (*D*) The AUC of intraperitoneal glucose tolerance test performed on mice fed for 20 weeks with a standard fat diet ($^*P = 0.021$) are shown. (*E*) Data obtained from mice fed with a high-fat diet for 12 weeks (0 min, $P = 0.865$; 20 min, $^*P = 0.013$; 40 min, $^*P = 0.034$; 60 min, $^*P = 0.022$; 120 min, $^*P = 0.012$) are shown. (*F*) Data obtained from mice fed with high-fat diet for 20 weeks (0 min, $P = 0.702$; 20 min, $P = 0.073$; 40 min, $P = 0.125$; 60 min, $^*P = 0.001$; 120 min, $^*P = 0.002$) are shown. (*G*) The AUC of intraperitoneal glucose tolerance test performed on mice fed for 12 weeks with high-fat diet ($^*P = 0.001$) are shown. (*H*) The AUC of intraperitoneal glucose tolerance test performed on mice fed for 20 weeks with high-fat diet ($^*P = 0.003$) are shown. Data are shown as mean ± standard deviation; $n = 8$ mice per all group.

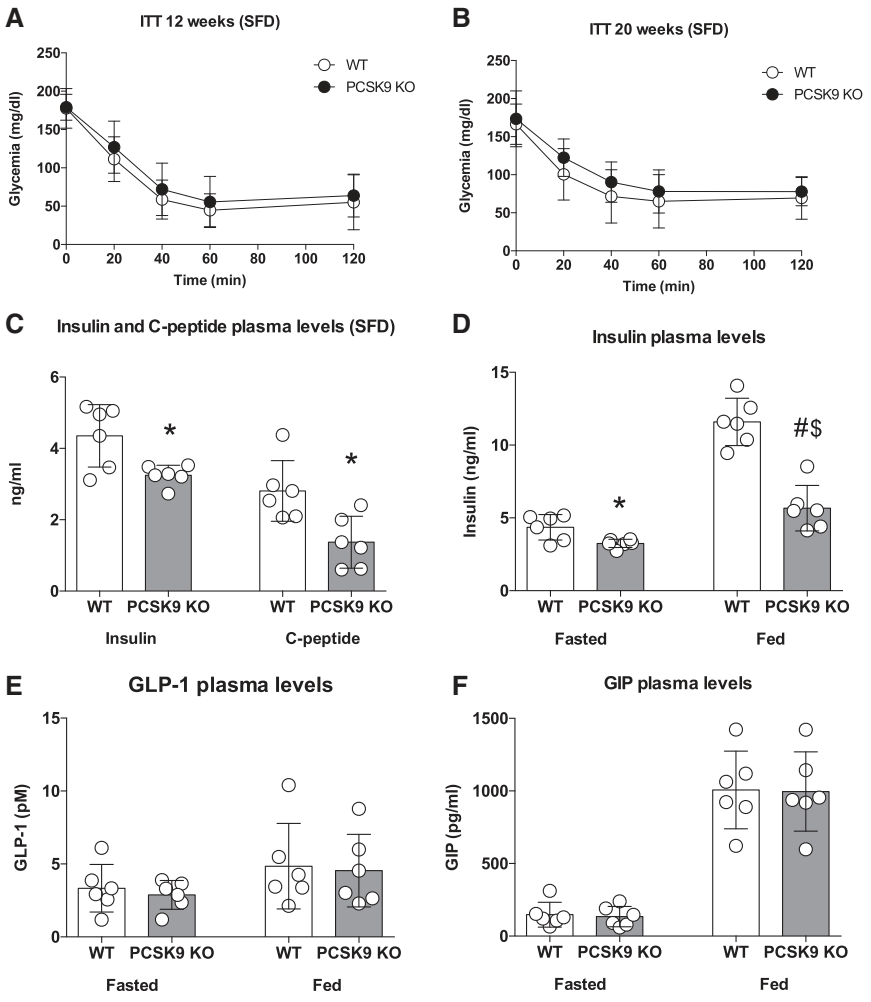


Figure 2 *Pcsk9* deficiency is not associated with insulin resistance but with impaired insulin secretion. Intraperitoneal insulin tolerance test was performed, and plasma glucose levels were measured at 0, 20, 40, 60, and 120 min. (*A*) Data obtained from mice fed with a standard fat diet for 12 weeks (0 min, $P = 0.858$; 20 min, $P = 0.323$; 40 min, $P = 0.495$; 60 min, $P = 0.817$; 120 min, $P = 0.521$) are shown. (*B*) Data obtained from mice fed with a standard fat diet for 20 weeks (0 min, $P = 0.899$; 20 min, $P = 0.206$; 40 min, $P = 0.138$; 60 min, $P = 0.275$; 120 min, $P = 0.524$) are shown. Data are shown as mean ± standard deviation; $n = 8$ mice per group. (*C*) Plasma insulin and C-peptide levels in *Pcsk9* KO compared to WT mice (*$P = 0.047$ and *$P = 0.009$, respectively) are shown. Plasma insulin levels following fasting (overnight) and refeeding (4 h) experiment are presented in (*D*) (*$P = 0.047$ vs. WT fasted; #$P = 0.002$ vs. WT fed, and \$$P = 0.002$ vs. *Pcsk9* KO fasted). (*E* and *F*) Plasma levels of GLP-1 and GIP following fasting (overnight) and refeeding (4 h) are shown. Data are shown as mean ± standard deviation; $n = 6$ mice per group.

of downstream activation of the insulin receptor[21] in the liver and in the soleus muscle before and 30 min after intraperitoneal insulin injection in mice fed with an SFD for 4 months. pAkt S473/Akt ratio or pAktT308/Akt ratio was similar in WT and *Pcsk9* KO mice (see Supplementary material online, *Figure S2A* and *B*) under basal and following IP insulin injection, suggesting that downstream activation of insulin receptor is not affected by *Pcsk9* deficiency.

Pcsk9 deficiency results in impaired insulin secretion and histological abnormalities in pancreatic islets

We then investigated whether *Pcsk9* deficiency affects insulin production and pancreatic β-cell function. Plasma insulin and C-peptide levels were significantly decreased in *Pcsk9* KO mice compared with WT littermates (*Figure 2C*); a similar profile was observed in HFD-fed mice (see Supplementary material online, *Figure S1C*). Following fasting and refeeding experiments, a reduced increment in plasma insulin levels in *Pcsk9* KO mice was observed vs. WT mice (*Figure 2D*). Similarly, following IP glucose injection, plasma insulin levels increased to a lower extent in *Pcsk9* KO mice compared with WT animals (see Supplementary material online, *Figure S1D*). We next evaluated whether the phenotype observed in *Pcsk9* KO mice could also be the consequence of altered incretins production. GLP1 and GIP plasma levels in fasted and refed conditions were similar in WT and *Pcsk9* KO mice (*Figure 2E* and *F*). The same was true when incretin's levels were measured following an oral bolus of glucose (see Supplementary material online, *Figure S1E* and *F*). In contrast to

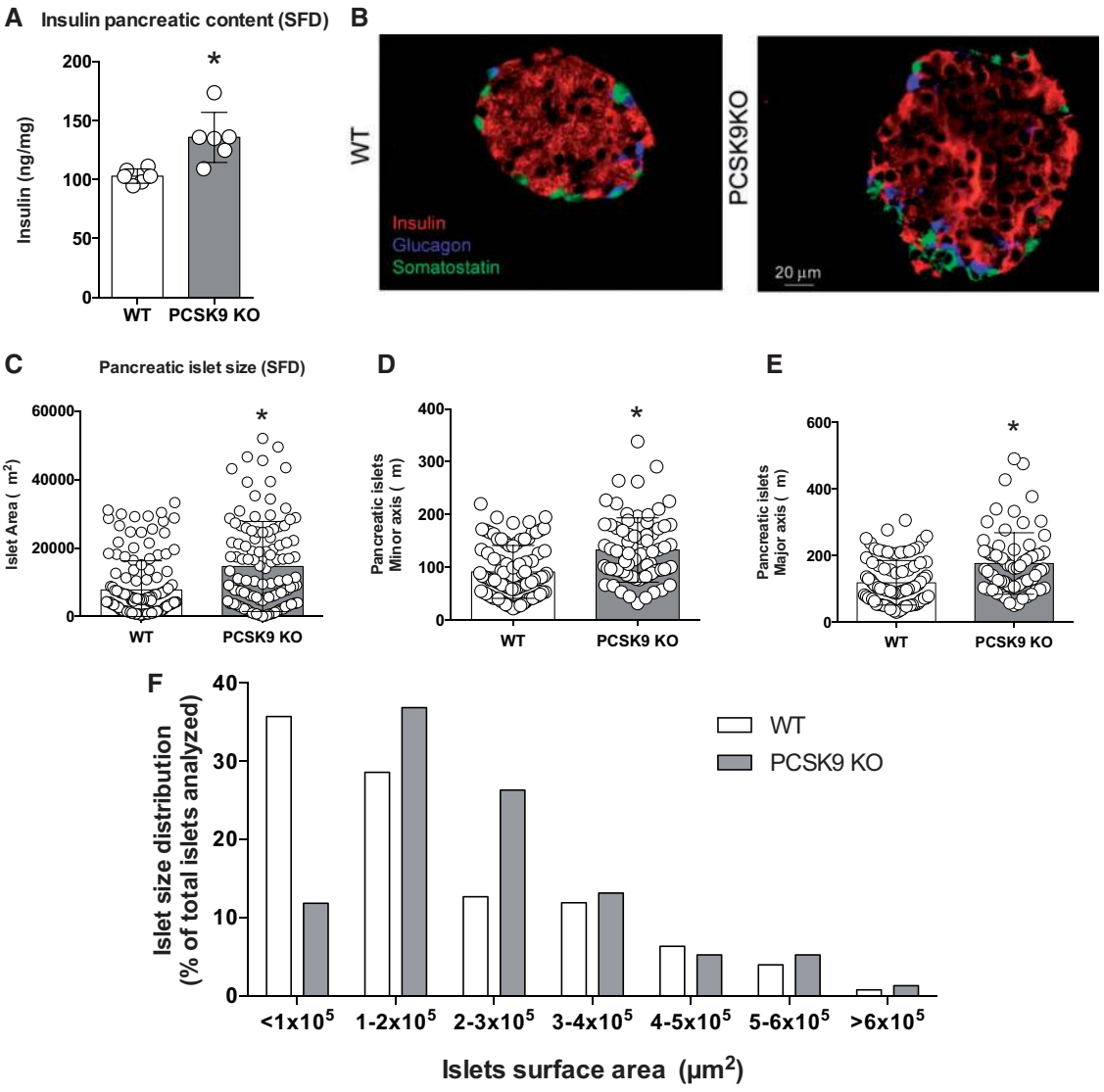


Figure 3 Histological characterization of pancreatic islets from *Pcsk9* KO mice. Pancreatic insulin content in *Pcsk9* KO and WT mice is shown in panel (*A*) (*$P = 0.004$). Data are shown as mean ± standard deviation; $n = 6$ mice per each group. Pancreatic islets morphology shows larger islets in *Pcsk9* KO mice compared with WT (*B–F*). A representative image is presented in panel (*B*) (scale bar: 20 µm). Panel (*C*) shows pancreatic islet size (*$P < 0.001$), while panels (*D* and *E*) show minor and major axis of each islet analysed (*$P < 0.001$ and *$P < 0.001$, respectively). In (*F*), islet size distribution, as percentage of total islets analysed, is shown. Islets were categorized according to the surface area (seven categories: from <10 000 µm^2 to >60 000 µm^2). Data shown in panels (*C–F*) were obtained from the analysis of 24 sections and 100 fields for each pancreas (mean ± standard deviation, $n = 7$ per group).

decreased circulating levels, we observed that pancreatic insulin content was significantly increased in *Pcsk9* KO mice compared with WT mice (*Figure 3A*) (see Supplementary material online, *Figure S1G*). These data suggest that *Pcsk9* mutant KO mice might have a defective insulin secretion, which could lead to an increased accumulation in beta cells. We, therefore, studied pancreatic islets morphology and observed that islets from *Pcsk9* KO mice present with irregular shape (*Figure 3B* and Supplementary material online, *Figure S3A*). A detailed analysis of consecutive pancreatic sections from three different regions of each pancreas (see Supplementary material online, *Figure S3B*) showed larger islet size (*Figure 3C*) together with a significant increase in the major and minor axis of each islet in the pancreas of *Pcsk9* KO mice compared with WT mice (*Figure 3D* and *E*). These findings were paralleled by a different islet size distribution between *Pcsk9* KO and WT mice (*Figure 3F*).

To further extend these data in humans, we investigated the impact of the *PCSK9* 46L variant on the homeostasis model assessment: insulin resistance (HOMA-IR) and beta-cell function (HOMA-BC). HOMA-BC but not HOMA-IR was significantly lower in carriers of the *PCSK9* 46L variant compared with carriers of the wild-type allele

(see Supplementary material online, *Figure S4A* and *B*) indicating that under PCSK9-deficient conditions, impaired β-cell functionality occurs also in humans.

These data suggest a relationship between PCSK9 deficiency and insulin secretion and prompted us to investigate the molecular mechanisms responsible for this effect.

PCSK9 effect on glucose metabolism is dependent on the presence of the low-density lipoprotein receptor

The LDLR is abundantly expressed also on the surface of pancreatic β cells where it plays a key role in the uptake of LDL.[9,10] Increased beta-cell cholesterol levels were associated with decreased insulin secretion.[22–24] The analysis of pancreatic sections from WT and *Pcsk9* KO mice showed that LDLR is present mainly in β cells (*Figure 4A* and Supplementary material online, *Figure S5A*), and as detected by western blot analysis (*Figure 4B and C*) and FACS analysis (*Figure 4D* and Supplementary material online, *Figure S5B*), its expression is increased in pancreatic islets isolated from *Pcsk9* KO animals compared with WT mice.

We next explored whether the increase in LDLR expression might be reflected in changes in pancreatic islets lipid content. Indeed, *Pcsk9* KO mice islets presented higher cholesterol esters levels compared with WT islets, together with significant changes in fatty acid lipidome (*Figure 4E*). This lipid profile was paralleled by the down-regulation of genes involved in cholesterol biosynthesis and uptake, including HMGCoA-R and LDLR, the increase of the expression of ACAT1, which promotes cholesterol esterification (*Figure 4F*) and a non-significant increase in genes involved in cholesterol efflux (*Figure 4F*) and ABCA-1 protein expression (data not shown). Moreover, a similar expression of Annexin V, a marker of apoptosis, was observed in β cells from both animal models (see Supplementary material online, *Figure S5C*) suggesting that, *in vivo* and at the time point analysed, cholesterol accumulation induces β-cell dysfunction before inducing cellular toxicity.

As expected,[25] plasma cholesterol levels were decreased in *Pcsk9* KO mice compared with WT with most of the cholesterol transported on HDL (see Supplementary material online, *Figure S6A* and *B*).

To further test the hypothesis that the observed phenotype in *Pcsk9* KO mice could be dependent on the impact on LDLR, GTT and ITT were evaluated in *Pcsk9*/*Ldlr* DKO mice and *Ldlr* KO animals. The delayed glucose response observed in *Pcsk9*-deficient conditions was not observed in SFD-fed *Pcsk9*/*Ldlr* DKO mice, and, indeed, GTT and ITT curves were superimposable between the two animal groups (*Figure 5A* and *B*) and similar to those observed in WT animals. Also, fast and refeeding experiments resulted in similar plasma glucose levels (190 ± 15 mg/dL for *Pcsk9*/*Ldlr* DKO compared with 191 ± 16 mg/dL for *Ldlr* KO mice).

Moreover, DKO mice and *Ldlr* KO littermates were characterized by similar levels of both plasma and pancreatic insulin levels (5.27 ± 2.78 ng/mL vs. 4.67 ± 3 ng/mL, $P = 0.666$) (71.5 ± 64 ng/mg of tissue vs. 77.7 ± 56.6 ng/mg of tissue, $P = 0.531$) (*Figure 5C* and *D*). Pancreatic islets morphology was similar in both animal groups compared with WT (*Figure 5E* and *F*) as was pancreatic islets cholesterol esters content (see Supplementary material online, *Figure S7A*), indicating that the PCSK9/LDLR axis could explain the phenotype observed in *Pcsk9*-deficient animals.

Circulating PCSK9 does not impact glucose metabolism and beta-cell function

In humans, PCSK9 is mostly produced by the liver but is also synthesized to a relevant amount in the brain, the intestine, the lung, and the pancreas (*Figure 6A*). Pancreatic islets analysis in WT mice showed, in line with previous findings,[26] that PCSK9 is expressed in δ cells but is under the level of detection in α or β cells (*Figure 6B* and Supplementary material online, *Figure S7B*). To dissect out the role of circulating, liver-derived PCSK9[9] from that of locally produced protein, we tested glucose metabolism in SFD-fed liver-specific Pcsk9 KO mice. PCSK9 protein was not detectable in plasma from AlbCre+/Pcsk9LoxP/LoxP, while in AlbCre-/Pcsk9LoxP/LoxP mice, plasma PCSK9 levels were 10.2 ± 4.2 mg/dL. PCSK9 mRNA expression was almost abolished in the liver from AlbCre+/Pcsk9LoxP/LoxP, while mRNA and protein expression in pancreas were similar between AlbCre+/Pcsk9LoxP/LoxP and AlbCre-/Pcsk9LoxP/LoxP mice (*Figure 6C* and *D*), confirming the liver specificity of the KO model. Furthermore, plasma cholesterol levels in AlbCre+/Pcsk9LoxP/LoxP mice were significantly lower compared with AlbCre-/Pcsk9LoxP/LoxP (41.8 ± 7.6 mg/dL vs. 68.5 ± 10.1 mg/dL, $P = 0.013$) and similar to those observed in Pcsk9 KO mice (51.1 ± 14.0 mg/dL), further confirming the key role of liver derived, circulating PCSK9 on plasma cholesterol levels. The LDLR expression (*Figure 6E*, and Supplementary material online, *Figure S8A*) was similar in pancreatic islets from AlbCre+/Pcsk9LoxP/LoxP and AlbCre-/Pcsk9LoxP/LoxP mice as were lipid levels in pancreatic islets (see Supplementary material online, *Figure S8B*). AlbCre-/Pcsk9LoxP/LoxP mice presented similar GTT and ITT curves (*Figure 6F* and *G*), and the same was true for plasma glucose levels following fast and refeeding experiments (194 ± 8 mg/dL for AlbCre+/Pcsk9LoxP/LoxP mice compared with 192 ± 5 mg/dL for AlbCre-/Pcsk9LoxP/LoxP mice). Also pancreatic insulin levels were similar in the two animal models (133.6 ± 47.16 ng/mg of tissue vs. 119.1 ± 43.2 ng/mg of tissue for AlbCre+/Pcsk9LoxP/LoxP and AlbCre-/Pcsk9LoxP/LoxP mice; $P = 0.629$). These data suggest that circulating, liver-derived PCSK9 does not impact LDLR expression in pancreas.

Discussion

Mendelian randomization studies have recently shown that *PCSK9* genetic variants associated with lower LDL cholesterol are also associated with circulating higher fasting glucose concentration and an increased risk of Type 2 diabetes.[17–19] Here, we describe in experimental models, a possible mechanism beyond this causal association and identify LDLR in the pancreatic islets as the potential target of locally produced PCSK9.

The accumulation of cholesterol in pancreatic islets has been associated with reduced glucose induced insulin secretion[13] and cellular toxicity[22–24] pointing to the critical role of cholesterol metabolism in pancreas. Cholesterol influx in β cells mainly results from LDL uptake through the LDLR,[14] the latter is largely expressed in these cells.[11] As a consequence, genetic and acquired conditions increasing LDLR

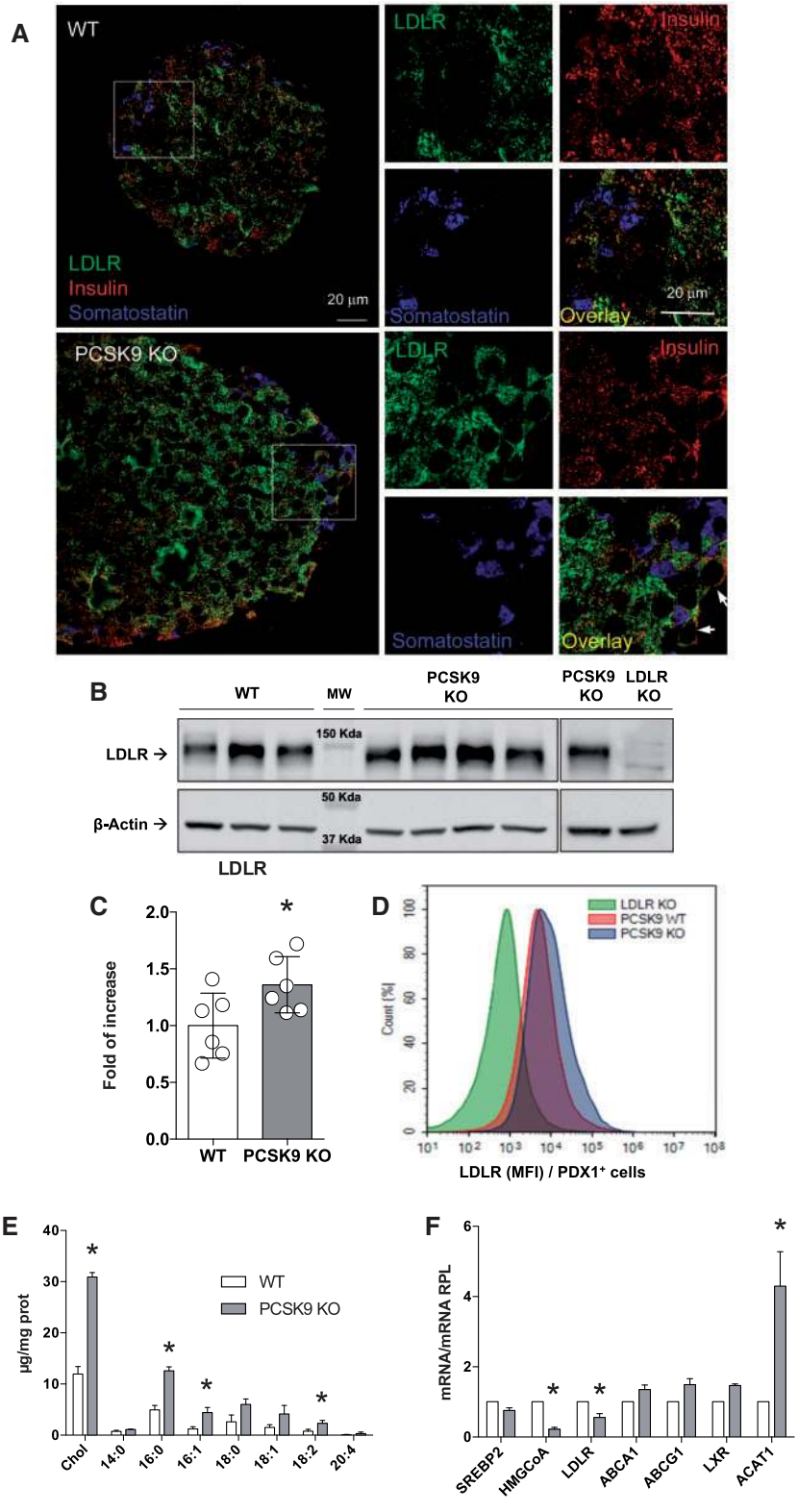


Figure 4 Low-density lipoprotein receptor expression is increased in pancreatic islets from *Pcsk9* KO mice. (*A*) A representative picture of pancreatic islets from WT and *Pcsk9* KO animals stained for insulin (red), somatostatin (blue), and LDLR (green) are shown. The right part of the panel shows a selected area at higher magnification (×2). The insulin staining was used as a marker for β cells and the yellow staining indicates co-expression of insulin and low-density lipoprotein receptor in the same cells, arrowheads highlight the membrane staining

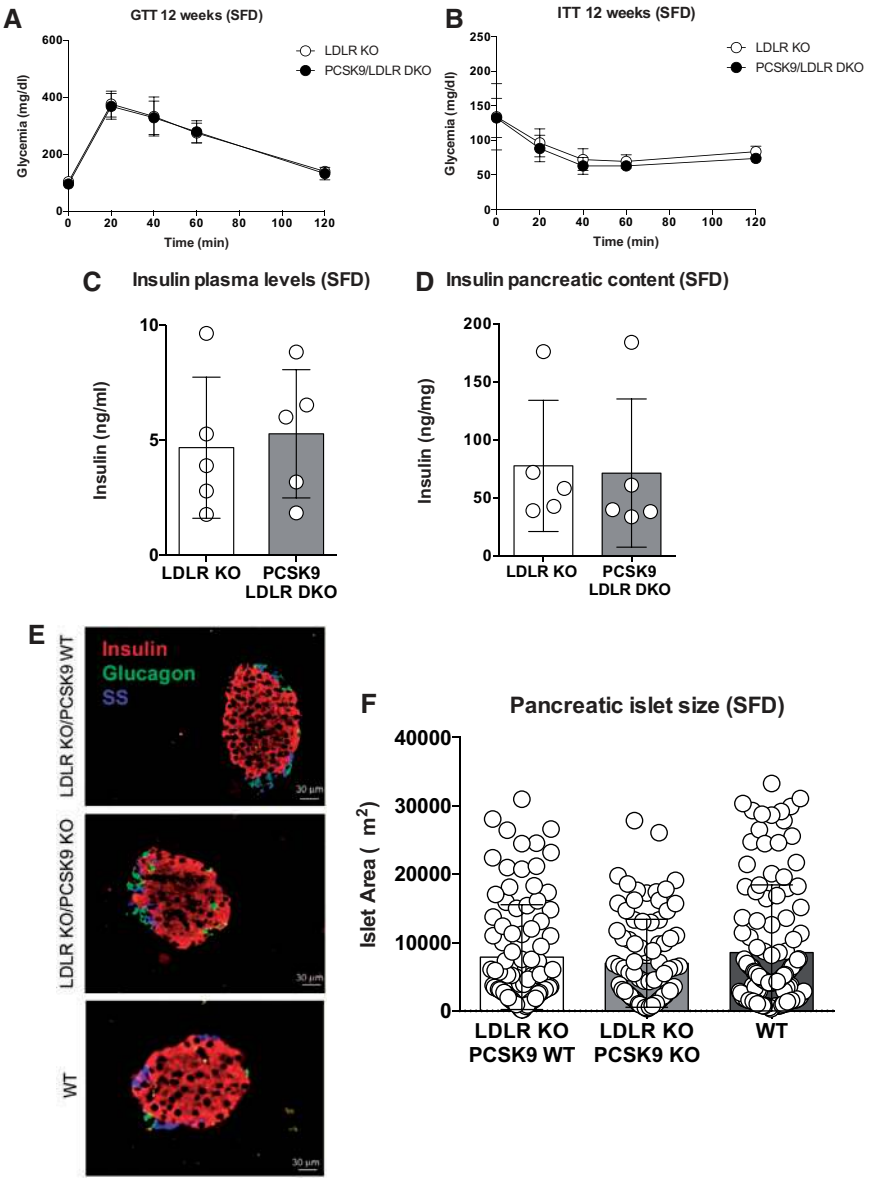


Figure 5 *PCSK9* effect on glucose metabolism is dependent on the low-density lipoprotein receptor. (*A*) Plasma glucose levels in *Pcsk9*/*Ldlr* DKO and in *Ldlr* KO mice following intraperitoneal glucose tolerance test (0, 20, 40, 60, and 120 min) (12 weeks of diet) are shown. (*B*) Plasma glucose levels in *Pcsk9*/*Ldlr* DKO and in *Ldlr* KO mice following intraperitoneal insulin tolerance test (0, 20, 40, 60, and 120 min) are shown. (*C*) Plasma insulin levels and (D) pancreatic insulin levels in *Pcsk9*/*Ldlr* DKO and in *Ldlr* KO mice are shown. (*E*) A representative image of pancreatic islet morphology, while (*F*) presents data on pancreatic islets size are shown. For each mouse, 24 sections and 100 fields were imaged and analysed. Data in panels (*A*–*D*) are shown as mean ± standard deviation; $n = 5$ mice per group (no significant differences were observed between groups $P > 0.05$).

Figure 4 Continued

of low-density lipoprotein receptor in *Pcsk9* KO mice. For each image, the maximum intensity projection of serial confocal sections is shown. Scale bar: 20 µm. (*B*) A representative image of immunoblotting analysis for LDLR in pancreatic islets isolated from *Pcsk9* KO, WT, and *Ldlr* KO mice are shown. (*C*) The quantification of western blot analysis for low-density lipoprotein receptor expression in pancreatic islets isolated from *Pcsk9* KO mice compared with WT mice are shown (data are shown as mean ± standard deviation, $n = 6$ per group, $^*P = 0.047$). (*D*) The mean fluorescence intensity of FACS analysis for low-density lipoprotein receptor expression in pancreatic islets isolated from WT animals (red curve), *Pcsk9* KO mice (blue curve) compared with that of *Ldlr* KO (green curve) are shown. (*E*) Cholesterol esters and fatty acids content in pancreatic islets from *Pcsk9* KO and WT mice (Chol, $^*P = 0.004$; 16: 0, $^*P = 0.011$; 16: 1, $^*P = 0.026$; 18: 2, $^*P = 0.04$; unpaired *t*-test; $n = 3$ mice per group) are shown. (*F*) The expression of genes related to cholesterol biosynthesis, uptake, and efflux, and the expression of ACAT1 in pancreatic islets from *Pcsk9* KO and WT mice (HMGCoA, $^*P = 0.045$; LDLR, $^*P = 0.048$; ACAT1, $^*P = 0.03$ *t*-test; $n = 6$ mice per group) are shown. Data are shown as mean ± standard deviation.

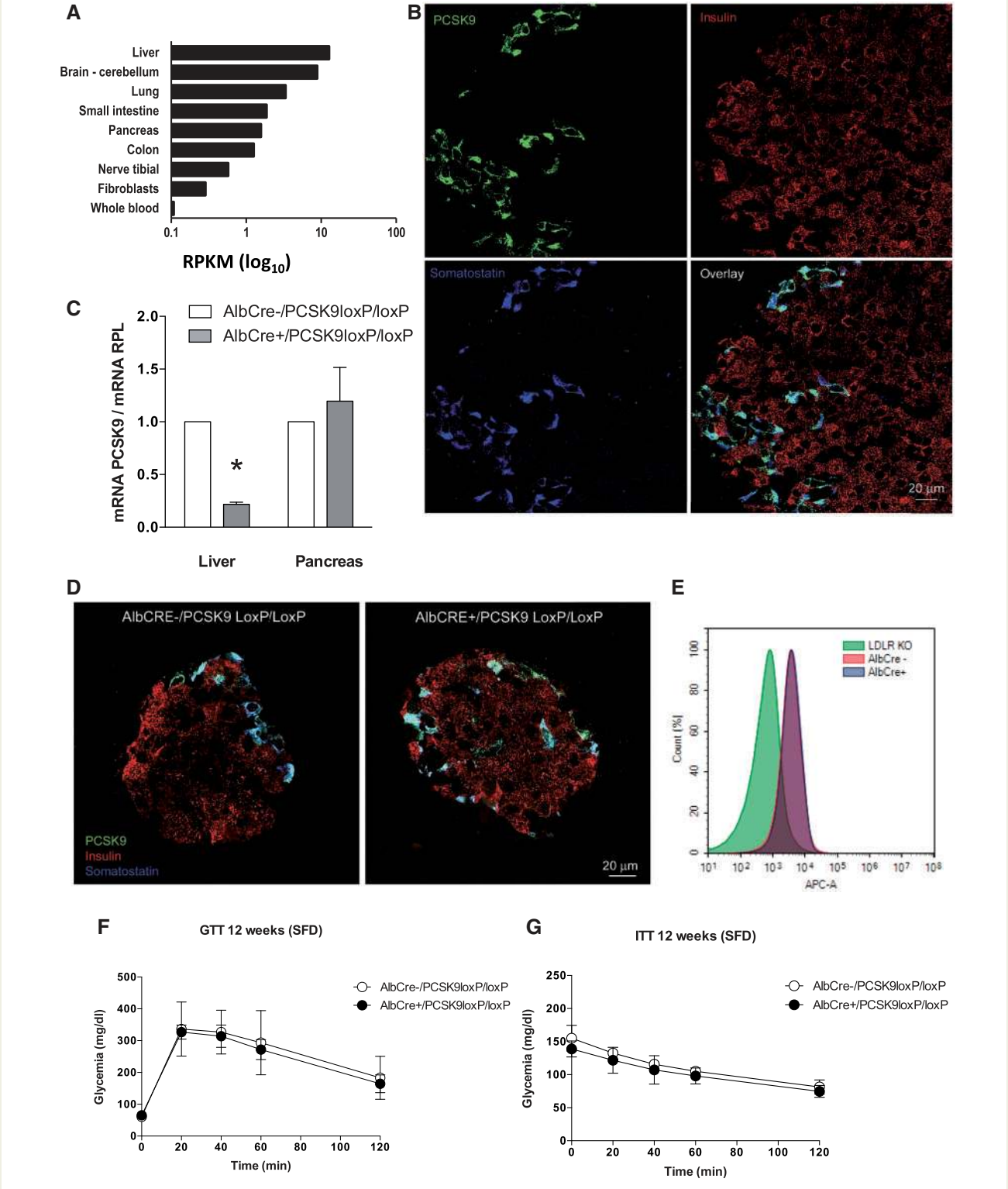


Figure 6 Circulating PCSK9 does not impact glucose metabolism and β-cell function. (*A*) *PCSK9* gene expression in human tissues according to the Genotype–Tissue Expression (GTEx) portal are shown. Data are reported as RPKM (Reads per Kilobase per Million mapped reads): 12.775 RPKM in the liver, 9.098 RPKM in the brain (cerebellum), 3.362 RPKM in the lung, 1.874 in the small intestine, 1.578 in the pancreas, 1.263 in colon (transverse), 0.577 in the nerve (tibial), 0.287 in cells (transformed fibroblasts), and 0.108 in the whole blood. (*B*) A representative image of the immunofluorescence of pancreatic islets from WT mice showing that PCSK9 is expressed in δ cells (somatostatin) (light blue staining in the overlay) are shown. (*C*) PCSK9 mRNA expression in the liver or in the pancreas of *AlbCre+/Pcsk9*$^{LoxP/LoxP}$ and *AlbCre-/Pcsk9*$^{LoxP/LoxP}$ mice are shown (data are shown as

expression should be associated with altered glucose metabolism. Indeed, LOF variants in the HMGCoA reductase which, by limiting cellular cholesterol biosynthesis, favor cholesterol uptake via LDLR are associated with increased risk of developing diabetes,[17] and the same is true for LOF variants in *PCSK9*.[17,18] More interestingly, when the genetic effect of LOF variants in HMGCoA reductase coexists with LOF variants of PCSK9, the risk of developing diabetes is further increased.[17] In line with this, FH subjects bearing a loss of function mutation in the LDLR present a decreased risk of diabetes.[20] Also, pharmacological treatments resulting in increased LDLR expression, such as statins, increase the risk of diabetes,[27] thus indicating that excessive LDLR activity could be the driver of β-cell dysfunction and diabetes. On these premises and given the great interest in anti-PCSK9 therapies, we investigated in detail how PCSK9 deficiency could affect glucose metabolism.

We show here that *Pcsk9* KO mice present with impaired glucose tolerance, independent of the type of diet used (high fat or standard fat) but no insulin resistance. Previous findings on *Pcsk9* KO are contrasting with a work reporting altered glucose metabolism following oral glucose tolerance test in *Pcsk9* KO mice compared to littermates[15] and another reporting no difference between WT and *Pcsk9* KO following intraperitoneal GTT.[26] Here, we take these observations forward by showing that *Pcsk9* KO present decreased plasma insulin and C-peptide levels, a finding paralleled by altered pancreatic morphology and cholesterol esters accumulation, larger islets, and increased insulin content. The increase in cholesterol esters might mirror the intracellular response aimed at mitigating free cholesterol cell toxicity which might result in the long term in β-cell apoptosis.[22–24] This molecular adaptation might occur together with cellular reprogramming to increase cholesterol efflux.

Of note, under hypercholesterolaemic conditions, selective β-cell ABCA-1 deficiency favours cholesterol accumulation in β cells, which associates to impaired insulin secretion.[28] The LDLR plays a key role in determining β-cell dysfunction under hypercholesterolaemic conditions[29] and *Ldlr* KO mice transplanted with WT islets with functional LDL present a normal functionality,[29] suggesting that compensatory mechanisms can maintain islet cholesterol homeostasis in this setting. These observations suggest that both cholesterol uptake and efflux are critical in maintaining a proper β-cell functionality.

It is important to stress that mice transport cholesterol mainly through HDL and that *Pcsk9* KO mice present a significant reduction in cholesterol distribution in HDL[25] coupled with decreased levels of apoE-containing HDL and reduced efflux capacity.[30] It is, therefore, possible that also a decrease in HDL might contribute to cholesterol accumulation in β cells. Furthermore, the differences in lipids and lipoproteins metabolism in mice (based mainly on ApoA-I and ApoE containing liproproteins) compared with humans (based mainly on ApoB containing lipoproteins) suggest the need to further address the crosstalk among these pathways. *Pcsk9* KO presented glucose intolerance when they were fed with either an SFD or an HFD; to limit the implications of insulin resistance driven by HFD, we performed most of the experiments on mice fed with an SFD. This does not exclude the possibility that on a longer time point, the development of metabolic abnormalities, as a consequence of HDF feeding, might mask part of the differences observed.

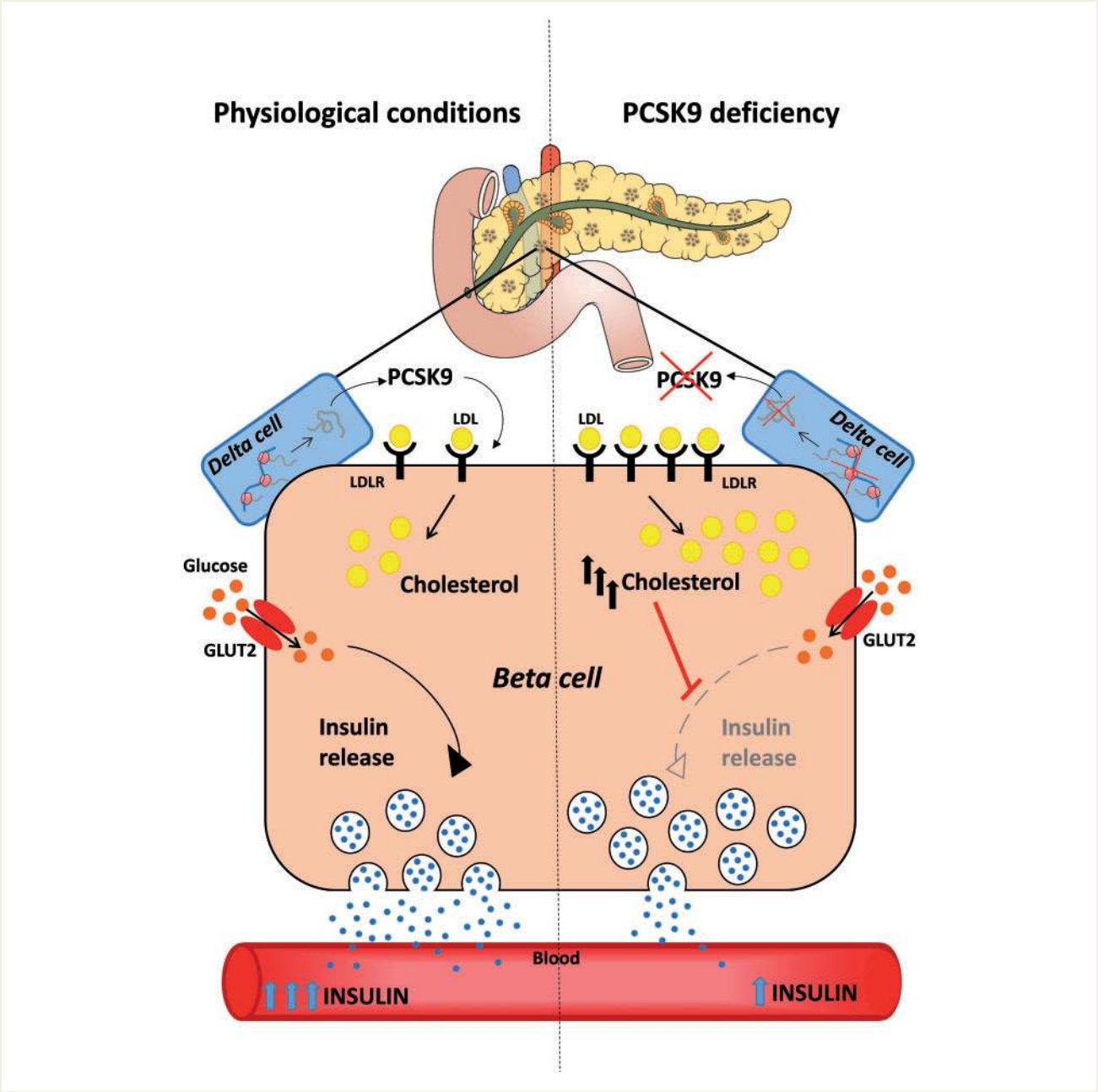
The presence of impaired β-cell function in *Pcsk9* KO mice was further confirmed in humans, where subjects with a *PCSK9* LOF variant presented decreased HOMA-BC but not HOMA-IR compared with age and sex matched carriers of the common allele.

The study of glucose metabolism in *Pcsk9/Ldlr* DKO mice showed that it is the PCSK9-LDLR axis and not the other known targets of PCSK9 such as the very low-density lipoprotein receptor, the apolipoprotein E receptor 2 (ApoER2), or cluster of differentiation 36 (CD36)[31–34] to be responsible for the phenotype observed in *Pcsk9* KO mice.

This observation is critical given that anti-PCSK9 therapies have been recently approved for the treatment of FH and patients at very high cardiovascular risk. It is possible that, while on the one hand anti-PCSK9 therapies will reduce LDL-C levels and related mortality, on the other hand they increase the risk of diabetes. Data available so far with anti-PCSK9 therapies (up to 2.2 years of treatment) do not show an increased incidence of diabetes compared with the group under standard therapy,[35,36] although a recent meta-analysis showed a significant increase in plasma glycaemia and glycated haemoglobin in patients treated with PCSK9 inhibitors that, however, was not sufficient to increase the incidence of diabetes.[37] Whether this effect might appear after longer term treatments remains to be addressed. Our data in PCSK9 tissue selective knock-out mice suggest an alternative explanation. Liver-selective *Pcsk9* KO mice have PCSK9 plasma levels below the detection limit while maintaining PCSK9 production in other tissues including the pancreas. This setting closely mimics the conditions of patients treated with anti-PCSK9 antibodies where PCSK9 is absent the circulation, but it is still produced in extrahepatic tissues, in contrast to the genetic studies with *PCSK9* LOF where all tissues present with a *PCSK9*-deficient condition. Liver-selective *Pcsk9* KO mice present plasma and pancreatic insulin levels, LDLR expression as well as pancreatic islets cholesterol esters levels similar to those of control mice; accordingly, also glucose tolerance and insulin tolerance did not differ. These data indicate that circulating PCSK9 has a minimal if any effect on LDLR in pancreas and suggest the possibility that anti-PCSK9 therapies which target circulating PCSK9 might have a limited impact on LDLR expression in pancreas. Whether this is true also for gene-silencing approaches remains to be addressed.

Figure 6 Continued

mean ± standard deviation, $n = 5$) (*$P < 0.05$), while (*D*) PCSK9 protein expression in the pancreatic islets from the same animals (the maximum intensity projection of serial confocal sections is shown) are shown. Scale bar: 20 μm. Low-density lipoprotein receptor expression in pancreatic β cells is shown in panel (*E*) as overlay of LDLR mean fluorescence intensity in *AlbCre-/Pcsk9*$^{LoxP/LoxP}$, *AlbCre+/Pcsk9*$^{LoxP/LoxP}$, and *Ldlr* KO (12 weeks of diet). (*F*) Plasma glucose levels in *AlbCre+/Pcsk9*$^{LoxP/LoxP}$ and *AlbCre-/Pcsk9*$^{LoxP/LoxP}$ mice following intraperitoneal glucose tolerance test (0, 20, 40, 60, and 120 min) (no significant differences were observed between groups, $P > 0.05$) are shown. (*G*) Plasma glucose levels in *AlbCre+/Pcsk9*$^{LoxP/LoxP}$ and *AlbCre-/Pcsk9*$^{LoxP/LoxP}$ mice following intraperitoneal insulin tolerance test (0, 20, 40, 60, and 120 min) (no significant differences were observed between groups, $P > 0.05$) are shown.



Take home figure Impact of *Pcsk9* deficiency on β-cell function. PCSK9 produced and released from δ cells controls low-density lipoprotein receptor expression in β cells. *Pcsk9* deficiency results in increased expression of low-density lipoprotein receptor in β cells, thus leading to increased accumulation of cholesterol esters which impacts glucose-stimulated insulin secretion, resulting in hyperglycaemia, and impaired glucose tolerance observed.

In summary, PCSK9 critically controls LDLR expression in pancreas perhaps contributing to the maintenance of a proper physiological balance to limit cholesterol overload in β cells. *Pcsk9* deficiency in turn results in increased LDLR expression and cholesterol esters accumulation in pancreatic islets, which impairs insulin secretion (*Take home figure*). Future studies should aim at addressing in humans the long-term safety of targeting circulating PCSK9 on pancreatic β-cell function.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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