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ORIGINAL RESEARCH

Circulating Rather Than Intestinal PCSK9 (Proprotein Convertase Subtilisin Kexin Type 9) Regulates Postprandial Lipemia in Mice

Damien Garçon, François Moreau, Audrey Ayer, Wieneke Dijk, Xavier Prieur, Lucie Arnaud, Anna Roubtsova, Nabil Seidah, Annik Prat, Bertrand Cariou, Cédric Le May

OBJECTIVE: Increased postprandial lipemia (PPL) is an independent risk factor for atherosclerotic cardiovascular diseases. PCSK9 (Proprotein convertase subtilisin kexin type 9) is an endogenous inhibitor of the LDLR (low-density lipoprotein receptor) pathway. We previously showed that PCSK9 inhibition in mice reduces PPL. However, the relative contribution of intracellular intestinal PCSK9 or liver-derived circulating PCSK9 to this effect is still unclear.

APPROACH AND RESULTS: To address this issue, we generated the first intestine-specific *Pcsk9*-deficient (*i-Pcsk9*^{-/-}) mouse model. PPL was measured in *i-Pcsk9*^{-/-} as well as in wild-type and streptozotocin-induced diabetic mice following treatment with a PCSK9 monoclonal antibody (alirocumab). Blocking the circulating form of PCSK9 with alicumab significantly reduced PPL, while overexpressing human PCSK9 in the liver of full *Pcsk9*^{-/-} mice had the opposite effect. Alirocumab regulated PPL in a LDLR-dependent manner as this effect was abolished in *Ldlr*^{-/-} mice. In contrast, *i-Pcsk9*^{-/-} mice did not exhibit alterations in plasma lipid parameters nor in PPL. Finally, PPL was highly exacerbated by streptozotocin-induced diabetes mellitus in *Pcsk9*^{+/+} but not in *Pcsk9*^{-/-} mice, an effect that was mimicked by the use of alicumab in streptozotocin-treated *Pcsk9*^{+/+} mice.

CONCLUSIONS: Taken together, our data demonstrate that PPL is significantly altered by full but not intestinal PCSK9 deficiency. Treatment with an PCSK9 monoclonal antibody mimics the effect of PCSK9 deficiency on PPL suggesting that circulating PCSK9 rather than intestinal PCSK9 is a critical regulator of PPL. These data validate the clinical relevance of PCSK9 inhibitors to reduce PPL, especially in patients with type 2 diabetes mellitus.

Key Words: diabetes mellitus ■ intestine ■ lipid ■ liver ■ mice

Dyslipidemia is one of the major modifiable risk factors for atherosclerotic cardiovascular diseases. Both epidemiological and genetic studies, as well as randomized clinical trials with lipid-lowering therapies, have clearly established that an increased concentration of low-density lipoprotein cholesterol causes atherosclerotic cardiovascular diseases.¹ In addition, it is now widely accepted that elevated nonfasting triglyceride concentrations represent an independent risk factor for atherosclerotic cardiovascular diseases.^{2,3} Postprandial lipemia (PPL) is a natural phenomenon occurring after a meal that is characterized by an increased

concentration of circulating triglyceride-rich lipoproteins. Briefly, PPL comprises increased levels of intestinally produced chylomicrons and their remnants and also of liver-produced VLDLs (very low-density lipoproteins) and their remnants.⁴ The hydrolysis of triglyceride transported in chylomicrons and VLDLs by lipoprotein lipase leads to cholesterol-enriched atherogenic remnants that are catabolized by the liver via the LDLR (LDL receptor), the LRP1 and 5 (LDLR-related protein 1 and 5), and heparan sulfate proteoglycans.⁵ The easy access to highly caloric food has led to a progressive reduction of fasting periods in Western countries, thus increasing the exposure risk

Correspondence to: Cédric Le May, l'institut du thorax, INSERM UMR 1087-CNRS UMR 6291, Nantes, France. Email cedric.lemay@univ-nantes.fr

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Nonstandard Abbreviations and Acronyms

Apo B48	apolipoprotein B48
Apo E	apolipoprotein E
LDLR	LDL receptor
LRP1	LDLR-related protein 1
MTTP	microsomal triglyceride transfer protein
PCSK9	proprotein convertase subtilisin/kexin type 9
PPL	postprandial lipemia
STZ	streptozotocin
TBP	Tata box Binding Protein
TG	triglycerides
VLDL	very low-density lipoprotein

to nonfasting triglyceride concentrations. Furthermore, it has been clearly demonstrated that patients with insulin resistance or type 2 diabetes mellitus display an exacerbated PPL, potentially contributing to the residual cardiovascular risk observed in this population.⁶

Discovered in 2003, PCSK9 (proprotein convertase subtilisin kexin type 9) promotes the lysosomal degradation of the LDLR and thus regulates plasma cholesterol levels (reviewed by Seidah et al⁷). The liver represents the main site of PCSK9 expression and is the only organ that significantly contributes to plasma PCSK9 levels in mice.⁸ However, PCSK9 is also expressed at significant levels in other extrahepatic organs and may play a functional role beyond cholesterol homeostasis (reviewed by Cariou et al⁹). Notably, PCSK9 is highly expressed in intestinal mucosa cells^{8,10} and may act on several intestinal functions. In 2009, we were the first to demonstrate that PCSK9-deficient (*Pcsk9*^{-/-}) mice display a blunted PPL.¹⁰ The molecular mechanisms involved in this effect are not completely understood. On the one hand, some data support a direct action of PCSK9 inside the enterocyte to control apolipoprotein B48 (ApoB48) and chylomicrons secretion.¹⁰⁻¹² On the other hand, the hepatic clearance of chylomicrons and chylomicron remnants is increased in *Pcsk9*^{-/-} mice.¹⁰ Thus, the respective contributions of hepatic and/or intestinal PCSK9 to the regulation of PPL are still unknown.

To address this issue, we used 2 complementary approaches. We generated a unique intestine-specific *Pcsk9*-deficient (*i-Pcsk9*^{-/-}) mouse model to directly investigate the role of intestinal PCSK9 in PPL. In parallel, we investigated the effect of a PCSK9 monoclonal antibody, alirocumab, which specifically inhibits circulating PCSK9 on PPL in wild-type and streptozotocin-induced diabetic mice. Taken together, our data clearly demonstrate that circulating rather than intestinal PCSK9 is the key regulator of PPL in mice.

Highlights

- Inhibition of plasma PCSK9 (proprotein convertase subtilisin/kexin type 9) regulates postprandial lipemia in a LDLR (LDL receptor)-dependent manner.
- Intestinal PCSK9 deficiency does not alter lipid, glucose homeostasis, and postprandial lipemia.
- CSK9 deficiency or inhibition reduces postprandial lipemia in the context of diabetes mellitus.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals and Experimental Design

C57BL6/J mice and LDLR-deficient (*Ldlr*^{-/-}) mice were obtained from Charles River laboratories. Villin-Cre and *Pcsk9*^{fl} C57BL/6 mice were provided by the laboratories of Sylvie Robine and Nabil Seidah, respectively.^{8,13} These mice were crossed to generate *i-Pcsk9*^{-/-}. Due to a higher heterogeneity in the postprandial triglyceride response observed in female mice, we only used male mice for this study. The numbers of mice used for each experiment are indicated in the figure legends. Mice were housed in a 12-hour day/night cycle and had unlimited access to water and food. PPL was measured in overnight fasted mice (14 hours). After an initial blood harvest, mice received an oral gavage of olive oil (200 μ L). Plasma samples were then collected 1, 2, and 4 hours after the gavage. For the fasting-refeeding procedure, plasma was collected at day 1 in random fed condition at 8:00 AM. Mice were fasted for 24 hours. At day 2, plasma from fasted mice were harvested at 8:00 AM and then standard laboratory diet was reintroduced ad libitum to the cage. Plasma samples were then harvested 1, 2, 4, 6, and 24 hours after refeeding. Insulinopenic diabetes mellitus was induced using streptozotocin (Sigma-Aldrich, St Quentin, France) injection. To determine whether intestinal *Pcsk9* deficiency can alter intestinal secretion, the plasma lipase inhibitor, Tyloxapol, was injected intravenously in overnight fasted mice (500 mg/kg body weight diluted in saline solution) before PPL assessment. Streptozotocin was resuspended in citrate buffer (pH 4.5) before injection. Fourteen hours fasted mice received a single high-dose injection of streptozotocin (150 mg/kg, intraperitoneal injection). Blood glucose levels were measured at indicated times using the ONE-TOUCH ULTRA glucometer. Alirocumab (Praluent, Sanofi-Aventis France) was diluted in saline solution (NaCl 0.9%) and administrated subcutaneously at a dose of 10 mg/kg of body weight. All experiments were approved by the "comité d'éthique des Pays de la Loire" (01953.01).

Adenoviral Infection

Pcsk9 adenovirus¹⁴ was intravenously injected at a rate of 5.10⁸ Pi/mouse. Control mice received an intravenous injection of NaCl.

Plasma Lipid Parameters

Cholesterol and triglyceride levels were measured using commercially available enzymatic kits (Sobioda, Montbonnot, France).

mRNA Levels Quantification

Real-time PCR analyses were performed with the MESAGreen PCR Master Mix (Eurogentec, Angers, France). For the oligonucleotide sequences for real-time PCR, please refer to the Major Resources Table. mRNA values were normalized to TBP (Tata box Binding Protein) and TFRC (Transferrin Receptor) for the small intestine and TBP and cyclophilin for the liver, and expressed as mean±SEM.

Western Blots

Mice intestinal mucosa and liver were homogenized, and 75 µg of extracted proteins were resolved on NuPAGE 4% to 12% BisTris gels in MES SDS buffer (Invitrogen, ThermoFisher, St Herblain, France) under reducing conditions for Western blot analysis, as described elsewhere.¹⁵ Antibodies used are listed in the Major Resources Table. Immunoreactive bands were revealed using the ECL plus kit (Biorad, Marnes-la-Coquette, France).

PCSK9 Protein Levels Quantification

Plasma, hepatic, and intestinal PCSK9 were measured using a commercially available ELISA kit (R&D Duoset PCSK9 mouse, Bio-Techne SAS, Noyal Châtillon sur Seiche, France). Samples from plasma, liver, and small intestinal homogenates were respectively diluted 50, 1500, and 20 times in PBS. Measurements were conducted according to the manufacturer's instructions.

In Situ Hybridization

Female and male were bred together for one night and separated at 8 AM the next morning. After 18 days, pregnant females were killed and their embryos were harvested. Embryos were washed in PBS at 4°C, placed in OCT (Tissue-Tek), and frozen in dry ice-cold isopentane. Mouse sense and antisense cRNA probes coding for mouse PCSK9¹⁶ were labeled with [³⁵S]-UTP. Whole mouse cryosections (8–10 µm) obtained at day 18 after pregnancy (E18) were fixed for 1 hour in 4% formaldehyde and hybridized overnight at 55°C. For autoradiography, the sections were dipped in photographic emulsion (NTB-2; Kodak), exposed for 12 days, developed in D19 solution (Kodak), and stained with thionin.

Statistical Analyses

Values are reported as mean±SEM. Statistical significance was determined using a Mann-Whitney *U* test. Values of *P*<0.05 were considered as statistically significant.

RESULTS

Inhibition of Circulating PCSK9 Alters Postprandial Lipemia in a LDLR-Dependent Manner

To determine the functional importance of circulating PCSK9 on PPL, we injected *Pcsk9*^{+/+} mice with a single subcutaneous injection of vehicle or human PCSK9 monoclonal anti-antibody (alirocumab, 10 mg/

kg) to capture circulating PCSK9. As an additional control, we used *Pcsk9*^{-/-} mice injected with vehicle. Three days after injection, we collected plasma samples and assessed plasma cholesterol concentrations. Alirocumab reduced plasma cholesterol levels in *Pcsk9*^{+/+} mice by 20% compared with vehicle, while the total loss of PCSK9 (*Pcsk9*^{-/-}) led to a 36% reduction (Figure 1A and Figure I in the Data Supplement). In contrast, alicumab treatment reduced PPL almost to the same extent as the absence of PCSK9 (Figure 1B).

Given that PCSK9's main function is to regulate LDLR expression, we then verified whether the impact of alicumab on PPL was LDLR-dependent. Confirmatory of an LDLR-dependent effect, the administration of alicumab to *Ldlr*^{-/-} mice altered neither plasma cholesterol concentrations nor PPL (Figure 1C and 1D). To further consolidate the role of circulating PCSK9 in the regulation of PPL, we overexpressed human PCSK9 in *Pcsk9*^{-/-} mice using an adenoviral construction. As expected, human PCSK9 overexpression in the liver led to (1) the appearance of liver-derived human PCSK9 in the circulation (Figure 2A); (2) higher plasma cholesterol levels (Figure 2B); and (3) a loss of hepatic LDLR expression (Figure 2C). In addition, we show that the expression of hepatic human PCSK9 in *Pcsk9*^{-/-} mice increases PPL to a similar extent as observed in *Ldlr*^{-/-} mice (Figure 2D).

Taken together, these results demonstrate that liver-derived circulating PCSK9 is a critical regulator of PPL.

Intestinal PCSK9 Deletion Does Not Alter Metabolic Homeostasis and Postprandial Lipemia

To decipher the role of intracellular intestinal PCSK9 in lipoprotein metabolism, we generated a mouse model with a specific inactivation of *Pcsk9* in intestinal epithelial cells (*i-Pcsk9*^{-/-} mice). These mice were obtained by breeding *Pcsk9*^{fl/fl} mice⁸ with transgenic mice expressing the Cre recombinase under the control of the murine villin promoter.¹³ We observed that *Pcsk9* mRNA levels were significantly reduced in all intestinal segments of *i-Pcsk9*^{-/-} mice compared with *Pcsk9*^{fl/fl} control mice (Figure 3A), although hepatic *Pcsk9* mRNA expression remained unaltered (Figure 3B). In situ hybridization performed in mouse embryos of 18 days of age and using a *Pcsk9* probe confirmed these results by showing a disappearance of labeling only in the small intestine of *i-Pcsk9*^{-/-} mice (Figure 3C). Finally, PCSK9 protein content was undetectable along the intestinal tract of *i-Pcsk9*^{-/-} mice (Figure 3D) but remained unchanged in the liver (Figure 3E), as measured by ELISA. Altogether, these results validate our mouse model of intestinal PCSK9 deficiency.

We next metabolically phenotyped the *i-Pcsk9*^{-/-} mice to assess the consequences of the absence

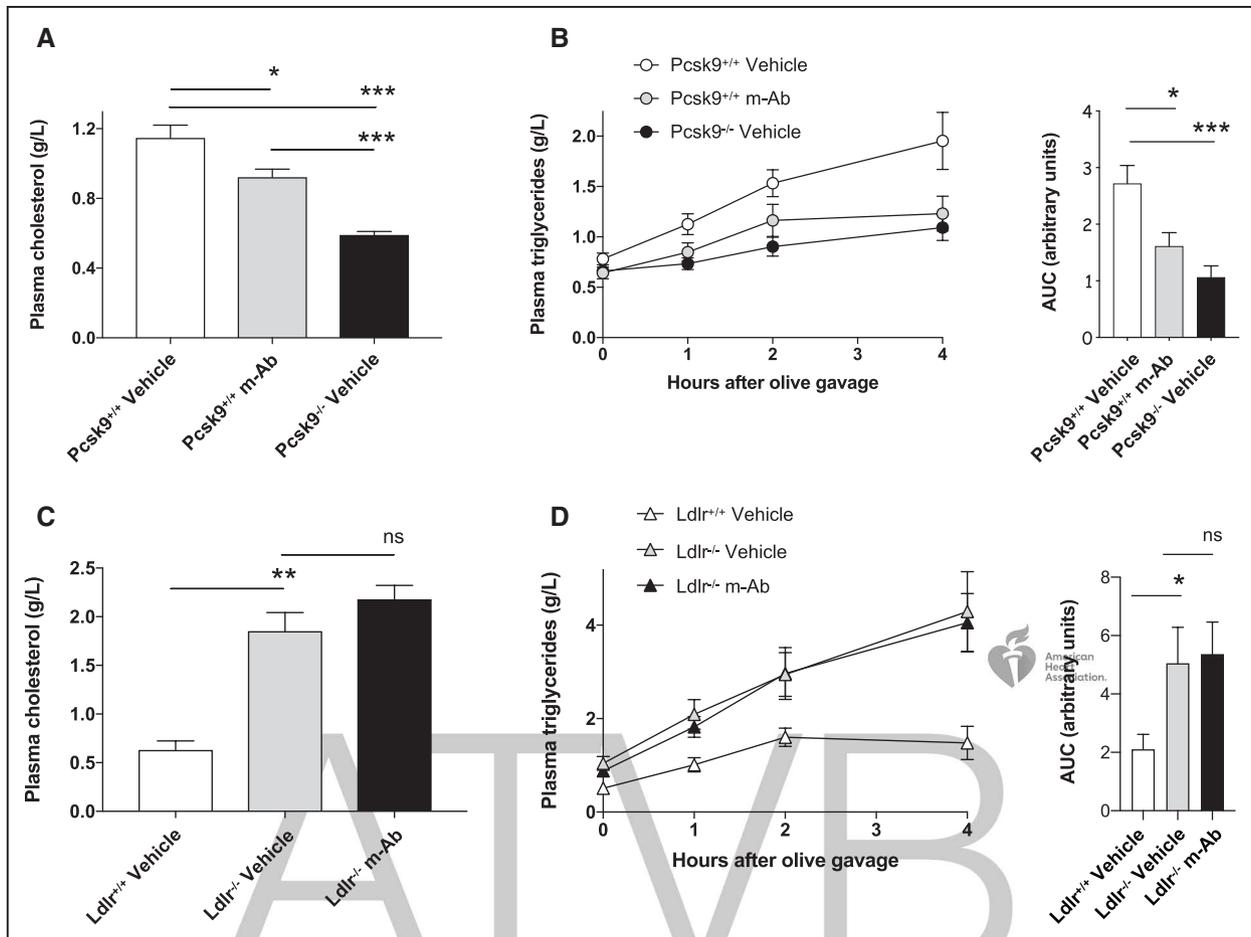


Figure 1. Alirocumab treatment reduces postprandial lipemia in a LDLR (LDL receptor)-dependent manner.

Pcsk9^{+/+} (n=10) and *Pcsk9*^{-/-} mice (n=13) received a single subcutaneous injection of vehicle (saline solution) and *Pcsk9*^{+/+} mice (n=10) a subcutaneous administration of PCSK9 (proprotein convertase subtilisin/kexin type 9) mAb (Alirocumab, 10 mg/kg). Three days later, **A**, Plasma cholesterol levels were assessed after overnight fasting and before the postprandial lipemia test. **B**, Plasma triglyceride concentrations were determined at 0, 1, 2, and 4 h after the olive oil gavage (right panel represents area under the curve). In a second set of experiments, *Ldlr*^{+/+} and *Ldlr*^{-/-} mice (n=8 per group) received a single subcutaneous injection of vehicle (saline solution) or PCSK9 mAb (Alirocumab, 10 mg/kg). **C**, Plasma cholesterol levels and **D** plasma triglycerides were measured as described above. Results are expressed as mean±SEM. Statistical significance was evaluated using Mann-Whitney *U* test, ns indicates nonsignificant; **P*<0.05, ***P*<0.01; ****P*<0.001.

of PCSK9 in the intestinal epithelial cells. First, we measured body weight, plasma glucose, cholesterol, triglyceride, and PCSK9 concentrations during a fasting-refeeding experiment in *i-Pcsk9*^{-/-} and *Pcsk9*^{fl/fl} control mice. As summarized in Figure 4, none of these parameters were significantly altered between *i-Pcsk9*^{-/-} and *Pcsk9*^{fl/fl} control male mice. Similarly, no significant changes in the studied parameters were observed when we compared female *i-Pcsk9*^{-/-} and *Pcsk9*^{fl/fl} control mice (data not shown). To further reveal a potential metabolic phenotype, we challenged the mice by performing (1) an aging experiment (over 52 weeks old); (2) a high-fat diet intervention (60% Kilocalories from fat); and (3) a statin-enriched diet intervention, as *Pcsk9*^{-/-} mice are known to be more sensitive to statins than wild-type mice.¹⁷ However, even under these pathophysiological conditions, we failed to detect any metabolic differences between

i-Pcsk9^{-/-} and *Pcsk9*^{fl/fl} mice (data not shown). Following an oral olive oil gavage, there was no significant change in plasma triglyceride between *i-Pcsk9*^{-/-} and *Pcsk9*^{fl/fl} mice (Figure 4F), indicating that intestinal PCSK9 is not required for the regulation of PPL. Finally, to go further, we intravenously injected the plasma lipase inhibitor, tyloxapol, in control *Pcsk9*^{fl/fl} and *i-Pcsk9*^{-/-} mice and then performed an olive oil oral gavage. As expected, Tyloxapol administration strongly increased plasma triglyceride levels, but the increase was similar in *Pcsk9*^{fl/fl} mice and *i-Pcsk9*^{-/-} mice (Figure 4G) suggesting that intestinal PCSK9 deficiency does not alter intestinal chylomicron secretion. In accordance with these findings, there were no significant changes in the mRNA expression of MTP (microsomal triglyceride transfer protein) and other lipogenic genes in the small intestine of *i-Pcsk9*^{-/-} compared with *Pcsk9*^{fl/fl} mice (Table I in the Data Supplement).

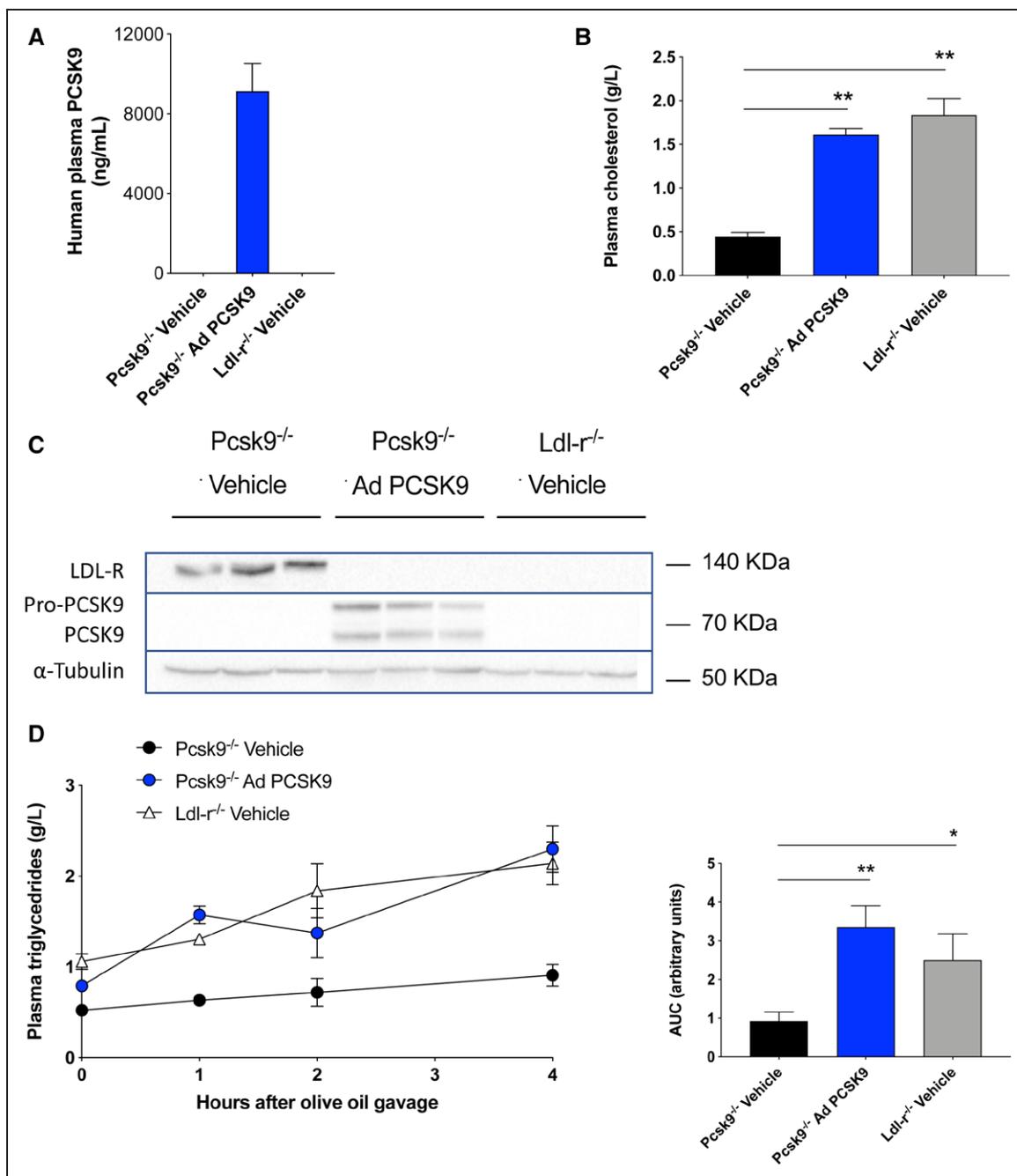


Figure 2. Adenoviral overexpression of PCSK9 (proprotein convertase subtilisin/kexin type 9) reduces hepatic LDLR (LDL receptor) protein content and strongly increases postprandial lipemia.

A, Plasma cholesterol, **(B)** plasma human PCSK9 levels, **(C)** hepatic LDLR, PCSK9, and alpha-tubulin expression assessed by Western-blot analysis, and **(D)** postprandial lipemia measurement in vehicle-treated *Pcsk9*^{-/-} mice (n=5), human PCSK9 adenovirus-treated *Pcsk9*^{-/-} mice (n=5) or vehicle-treated *Ldlr*^{-/-} mice (n=5). Statistical analyses were performed using Mann-Whitney *U* test, **P*<0.05, ***P*<0.01.

Circulating PCSK9 Inhibition Protects Mice Against Diabetes Mellitus-Induced Postprandial Lipemia

As previously mentioned, an exacerbated PPL is frequently observed in patients with diabetes mellitus.¹⁸ To determine whether PCSK9 inhibition might improve PPL in a setting of diabetes mellitus, we used the streptozotocin-induced insulinopenic mouse model of diabetes

mellitus. This model does not recapitulate the progressive insulin resistance and pancreatic beta-cell failure observed in type 2 diabetes mellitus. However, this model does develop a massive postprandial triglyceride response following olive oil gavage, similar to type 2 diabetic patients.¹⁹

In a first set of experiments, we injected a single intraperitoneal dose of a vehicle solution or a high dose of streptozotocin (150 mg/kg) into *Pcsk9*^{+/+} and

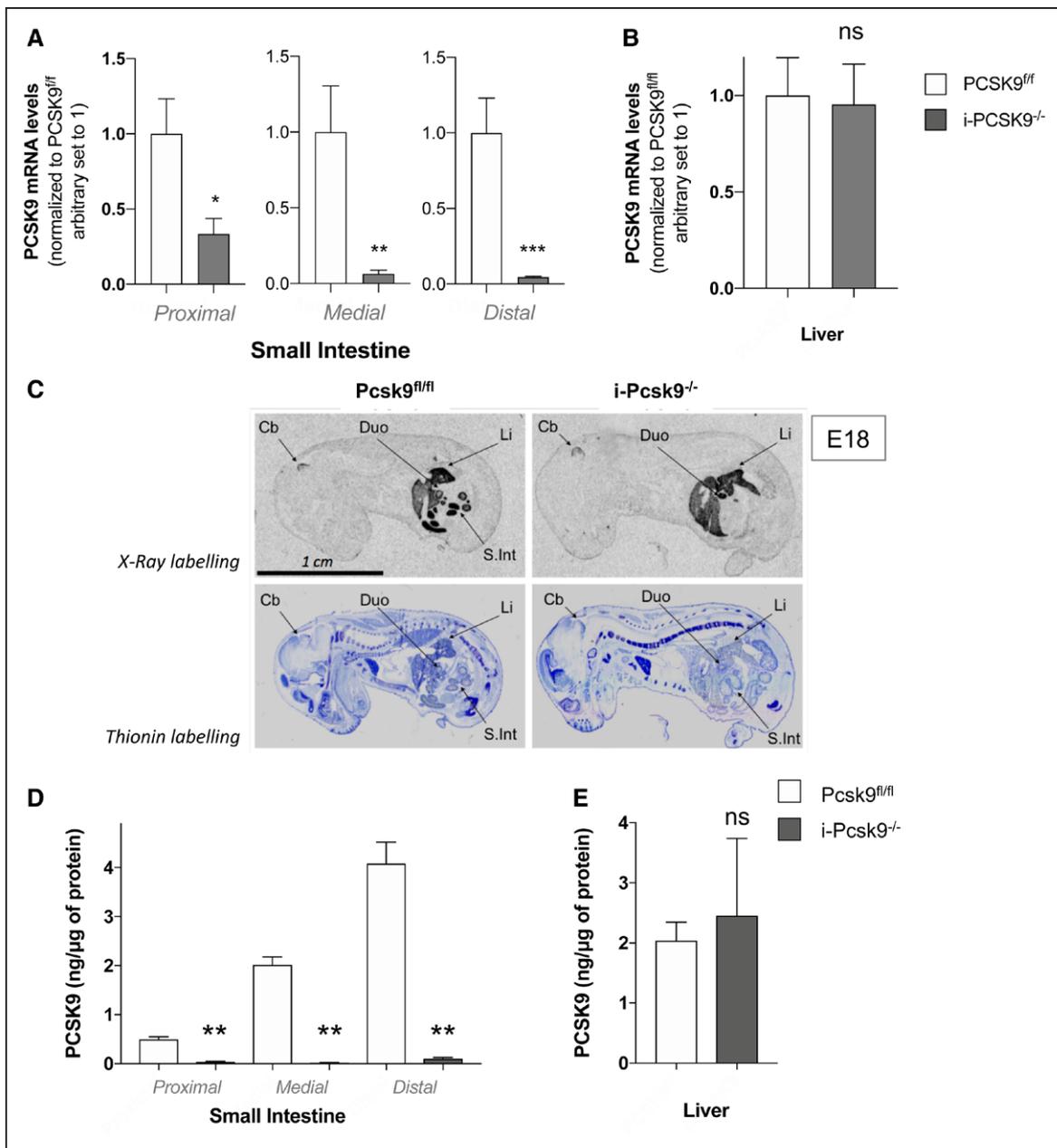


Figure 3. Intestinal PCSK9 (proprotein convertase subtilisin/kexin type 9) expression is reduced in intestinal *Pcsk9*-deficient mouse.

A, mRNA levels of *Pcsk9* in the small intestine of *Pcsk9*^{fl/fl} (n=7) and *i-Pcsk9*^{-/-} mice (n=7). **B**, mRNA levels of *Pcsk9* in the liver of *Pcsk9*^{fl/fl} (n=15) and *i-Pcsk9*^{-/-} mice (n=15). **C**, In situ hybridization of *Pcsk9* mRNA performed on 18-d-old *Pcsk9*^{fl/fl} (left panels) and *i-Pcsk9*^{-/-} embryos (right). Autoradiography and thionin staining were performed successively on the same slide. **D**, Intestinal and **E** hepatic PCSK9 protein levels in *Pcsk9*^{fl/fl} (n=6) and *i-Pcsk9*^{-/-} (n=5) mice were measured by ELISA. Data are expressed as mean±SEM and were analyzed using non parametric Mann-Whitney *U* test, ns indicates nonsignificant, ***P*<0.01, ****P*<0.001.

Pcsk9^{-/-} mice. As expected, 4 days after the injection, streptozotocin induced a strong and comparable hyperglycemia in *Pcsk9*^{+/+} and *Pcsk9*^{-/-} mice (Figure 5A) but did not significantly alter plasma cholesterol levels (Figure II in the Data Supplement). Streptozotocin treatment strongly increased PPL in *Pcsk9*^{+/+} mice but to a lesser extent in *Pcsk9*^{-/-} mice. Indeed, PPL in streptozotocin-treated *Pcsk9*^{-/-} mice was similar to vehicle-injected *Pcsk9*^{+/+} mice¹⁰ (Figure 5B). Thus, PCSK9 deficiency

efficiently protected mice from diabetes mellitus-induced exacerbated PPL (Figure 5B).

In a second set of experiments, we assessed the effect of alirocumab in streptozotocin-treated *Pcsk9*^{+/+} mice. Although the inhibition of circulating PCSK9 did not alter the effect of streptozotocin on blood glucose levels (Figure 5C), it significantly reduced plasma cholesterol levels (-42%; Figure 5D). In agreement with the above data (Figure 1B), alirocumab treatment also

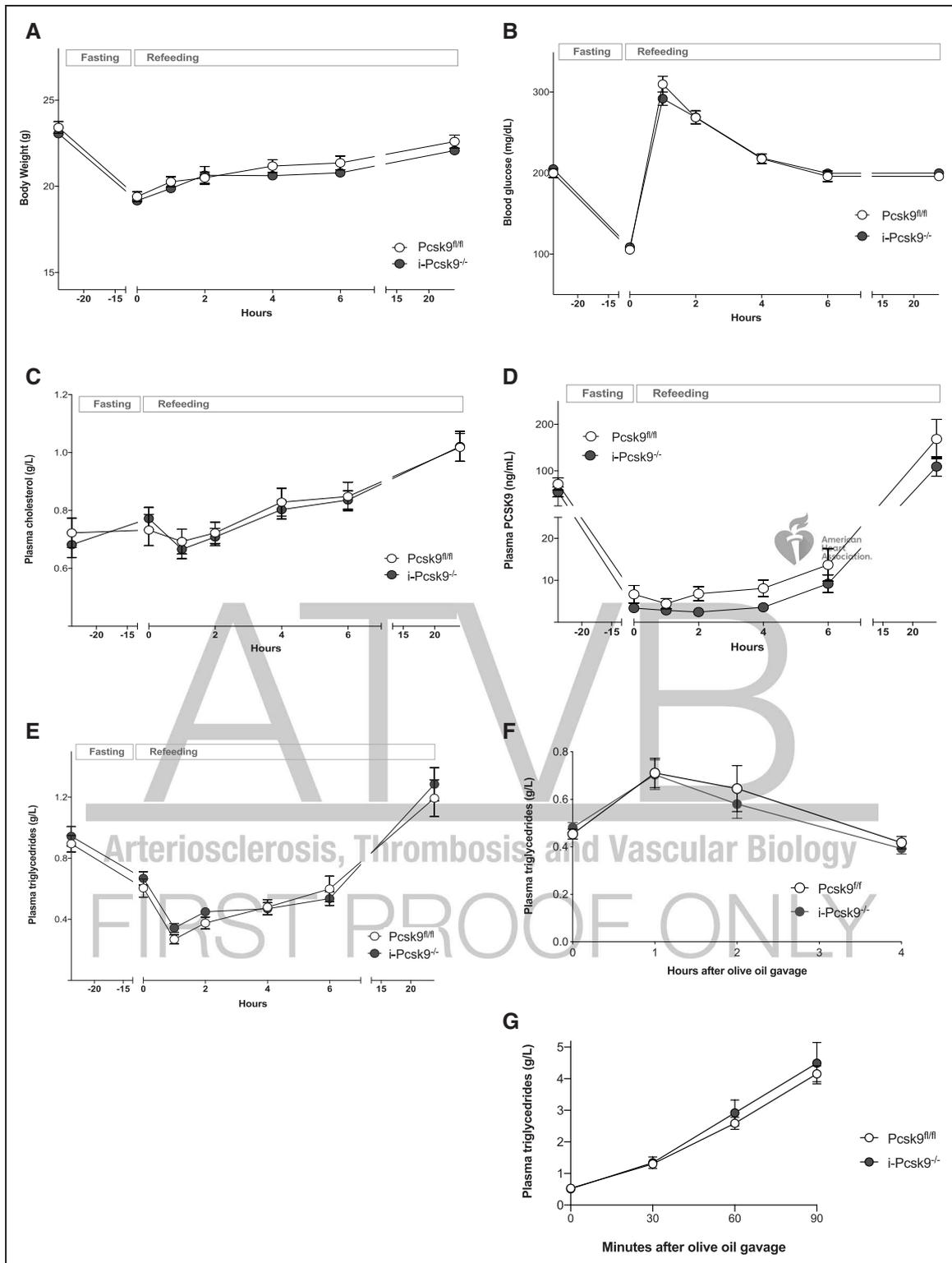


Figure 4. Intestinal PCSK9 (proprotein convertase subtilisin/kexin type 9) deficiency does not alter glucose nor lipid homeostasis in mouse.

A, Body weight, **(B)** blood glucose, **(C)** plasma cholesterol, **(D)** plasma PCSK9, and **(E)** plasma triglycerides were measured in random fed condition, after 24 h of fasting and 1, 2, 4, 6, and 24 h following refeeding in *Pcsk9^{fl/fl}* (n=18) and *i-Pcsk9^{-/-}* mice (n=19). **F**, Postprandial lipemia was measured in 14-h-fasted *Pcsk9^{fl/fl}* and *i-Pcsk9^{-/-}* mice (n=10 per group). **G**, Intestinal triglyceride production was evaluated in 14-h-fasted *Pcsk9^{fl/fl}* (n=9) and *i-Pcsk9^{-/-}* mice (n=6) after intravenous tyloxapol injection and an olive oil gavage. Statistical significance was assessed using Mann & Whitney test.

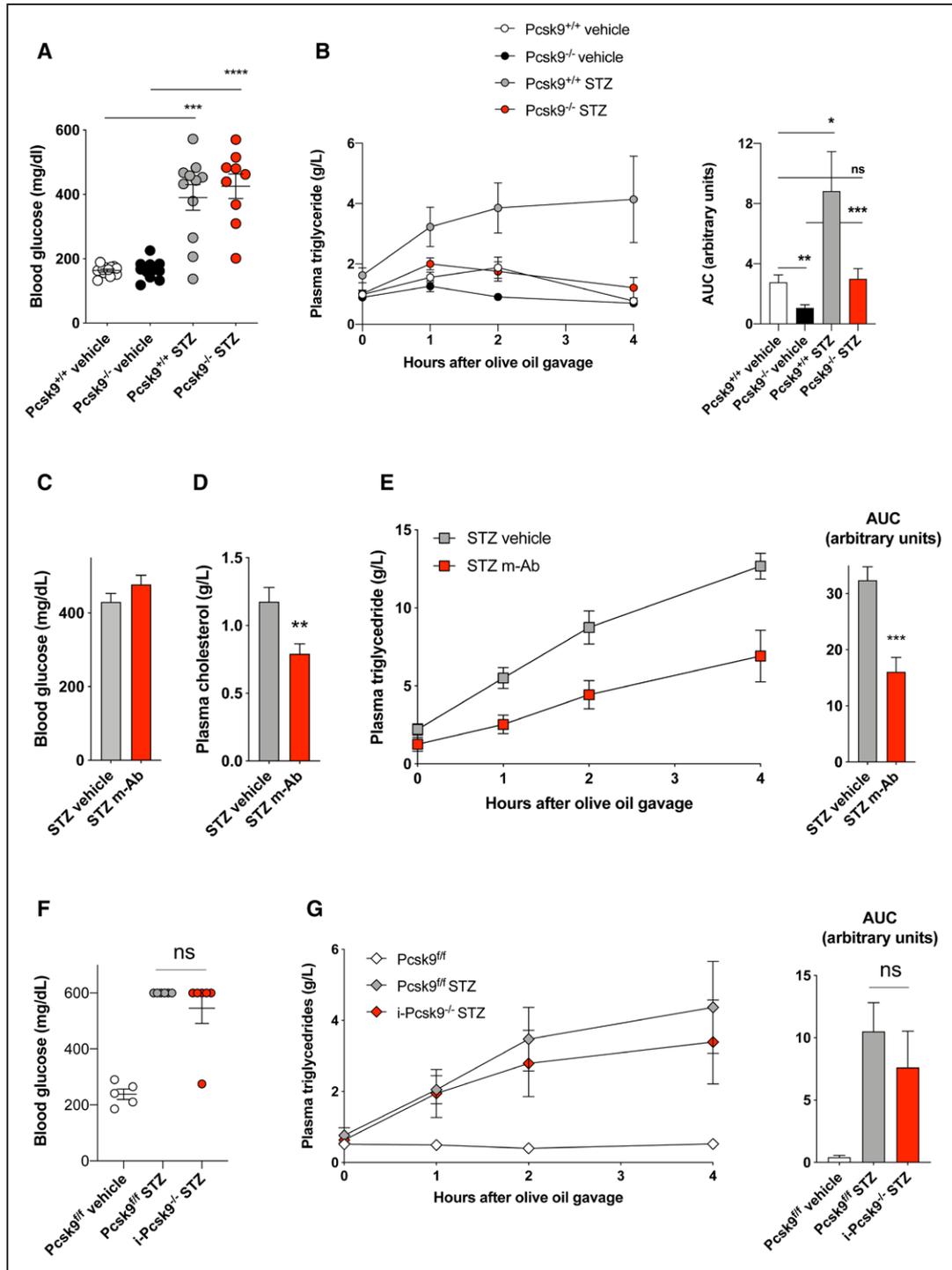


Figure 5. PCSK9 (proprotein convertase subtilisin/kexin type 9) deficiency and inhibition decrease postprandial lipemia in a streptozotocin-induced diabetes model.

Twelve hour-fasted *Pcsk9*^{+/+} and *Pcsk9*^{-/-} mice received an intraperitoneal injection of vehicle (citrate buffer, *Pcsk9*^{+/+} n=12; *Pcsk9*^{-/-} n=11) or 150 mg/kg streptozotocin (*Pcsk9*^{+/+} n=11; *Pcsk9*^{-/-} n=9). Five days later, we measured (A) blood glucose levels, (B) plasma triglyceride levels 0, 1, 2, and 4 h after olive oil gavage (right panel represents area under the curve). Data are represented as scatter plot and mean±SEM and were analyzed using a nonparametric Mann-Whitney U test, ***P<0.001. Twelve hour fasted C57BL/6J mice received an intraperitoneal injection or 150 mg/kg streptozotocin. Two days later, mice received a subcutaneous injection of vehicle (n=10) or 10 mg/kg of monoclonal anti-PCSK9 antibody. At day 5, we measured (C) blood glucose, (D) plasma cholesterol, and (E) plasma triglyceride levels at 0, 1, 2, and 4 h after olive oil gavage (right panel represents area under the curve). Values are mean±SEM and were analyzed using a nonparametric Mann-Whitney U test, **P<0.01; ***P<0.001. Twelve hour fasted *Pcsk9*^{fl/fl} and *i-Pcsk9*^{-/-} mice received an intraperitoneal injection of vehicle (citrate buffer, *Pcsk9*^{fl/fl} n=5) or 150 mg/kg streptozotocin (*Pcsk9*^{fl/fl} n=6; *i-Pcsk9*^{-/-} n=5). Five days later, we measured (F) blood glucose levels, (G) plasma triglyceride levels 0, 1, 2, and 4 h after the olive oil gavage (right panel represents area under the curve). Data are mean±SEM and were analyzed using a nonparametric Mann-Whitney U test, ns indicates nonsignificant.

drastically reduced PPL in streptozotocin-treated mice (−47%; Figure 5E). Finally, intestinal *Pcsk9* deficiency did not protect against the exacerbated PPL induced by streptozotocin (Figure 5F and 5G).

Finally, we tested the effect of pharmacological PCSK9 inhibition on PPL in a mouse model of type 2 diabetes mellitus, the Ob/Ob mice. Although we failed to highlight an exacerbated PPL following olive oil gavage, we did confirm that PCSK9 inhibition significantly reduced PPL in these diabetic mice (Figure III in the [Data Supplement](#)).

Taken together, these data demonstrate that the inhibition of circulating PCSK9 might reduce the exacerbated PPL observed in the context of diabetes mellitus.

DISCUSSION

The present study provides important insights into the role of PCSK9 in PPL regulation. By generating the first intestine-specific PCSK9 knockout mouse model, we demonstrate that the intracellular intestinal form of PCSK9 is not required for the regulation of lipoprotein metabolism. In contrast, liver-derived plasma PCSK9 is a key regulator of PPL. Indeed, hepatic PCSK9 overexpression leads to increased plasma PCSK9 levels and an exacerbated PPL. Conversely, blocking the circulating form of PCSK9 with a PCSK9 monoclonal anti-antibody (ie, alirocumab) reduces PPL. Circulating PCSK9 controls PPL *via* its canonical effect on LDLR protein levels, as its effect is abolished in LDLR-deficient mice. Finally, this study shows for the first time that PCSK9 inhibition efficiently protects mice from the exacerbated PPL observed during diabetes mellitus.

Although the expression of PCSK9 was reported to be substantial in the small intestine of rats in the seminal paper of Seidah et al,¹⁶ its physiological role in this organ has remained largely unknown. In humans, immunohistochemistry shows that PCSK9 is localized in enterocytes and goblet cells in jejunal and ileal biopsies.¹⁰ It was also reported that in obese patients undergoing bariatric surgery, PCSK9 protein expression was lower in the duodenum of insulin-resistant subjects compared with insulin-sensitive ones.²⁰ In accordance with its role in hepatocytes, PCSK9 downregulates intestinal LDLR expression in CaCo-2 cells¹¹ and in the murine intestine.¹⁰

Following an olive oil gavage, *Pcsk9*^{−/−} mice display a blunted PPL compared with their wild-type littermates. We previously showed that this phenotype is not attributable to a defect in fat absorption, gastric emptying, or intestinal transit kinetics.¹⁰ Studies performed in CaCo-2 cells suggested a decreased enterocytic apoB48 secretion following *PCSK9* knock-down. However, although an upregulation of MTTP expression has been implicated, the underlying molecular mechanism remains largely unknown.^{10–12} Importantly, the analysis of our *i-Pcsk9*^{−/−} mice clearly demonstrates that intestinal PCSK9 is not

involved in the regulation of plasma lipids levels and PPL. In accordance with the lack of effect of intestinal deletion of *Pcsk9* on chylomicron production, there was no significant change in MTTP expression in the enterocytes of *i-Pcsk9*^{−/−} mice. Although it has been suggested that PCSK9 silencing decreases MTTP expression *in vitro* in CaCo-2 cells, data from full *Pcsk9*-deficient mice and our *i-Pcsk9*^{−/−} mice suggest that loss of *Pcsk9* does not affect MTTP expression and activity *in vivo*.¹² Although we cannot rule out the existence of compensatory mechanisms in the *i-Pcsk9*^{−/−} mice, such mechanisms have not been observed in liver-specific *Pcsk9*^{−/−} mice that display a pronounced hypocholesterolemic phenotype.⁸ Nevertheless, additional studies are needed to further decipher the function of intestinal PCSK9 beyond the regulation of lipoprotein metabolism.

Several studies have shown that beyond the liver, the small intestine is the second organ expressing PCSK9. The apparent absence of metabolic consequences of intestinal PCSK9 deficiency raises the question of its functional role in this tissue.

Previous studies in the liver have demonstrated an essential role for PCSK9 in hepatocyte proliferation and liver regeneration after hepatectomy.⁸ In the intestine, enterocytes have a short half-life and the intestinal mucosa is in perpetual renewal. Whether the absence of PCSK9 modifies intestinal cell proliferation under stress conditions as well remains, however, unknown. Beyond intestinal proliferation, it might be hypothesized that PCSK9 influences intestinal barrier integrity. Enterocyte cholesterol homeostasis is key to the maintenance of barrier integrity, whereas a loss of intestinal barrier integrity has been associated with food allergy, Non-Alcoholic Fatty Liver Disease/Non-Alcoholic SteatoHepatitis and Alzheimer disease and is influenced by numerous factors (reviewed by Camilleri²¹). Further studies on *i-Pcsk9*^{−/−} mice will be required to determine whether PCSK9 might have a role in intestinal epithelial cell proliferation and/or gut barrier integrity.

By using complementary experimental approaches, we show that liver-derived plasma PCSK9 is key for the regulation of PPL in mice. First, alirocumab greatly reduced PPL in wild-type mice to a similar extent as total *Pcsk9*^{−/−} mice. Interestingly, the effect of alirocumab on PPL is even further exacerbated in the streptozotocin mouse model of diabetes mellitus. Second, the hepatic overexpression of human PCSK9 in wild-type mice markedly increased PPL. Using *in vivo* kinetic studies, we previously demonstrated that *Pcsk9*^{−/−} mice have an increased ability to clear their own chylomicrons compared with wild-type mice.¹⁰ Taken together, these data suggest that PCSK9 deficiency reduces PPL by increasing the clearance of TRL rather than decreasing intestinal chylomicron production. Chylomicron remnants and VLDL (very low-density lipoprotein) remnants are cleared from the circulation by the liver via their binding



to the LDLR, the related LRP1 and LRP5, or syndecan-1 through ApoB or ApoE.^{22,23} Finally, by contrast with Ldlr-deficient mice that show altered plasma lipids concentrations, the single deletion of LRP1 does not affect fasting plasma triglycerides and cholesterol plasma levels.²² However, the double LDLR/ LRP1 deficiency aggravates the dyslipidemia of single Ldlr deficient mice and their ability to clear plasma chylomicron remnants^{22,24} revealing the functional contribution of LRP1 in chylomicron homeostasis. Although PCSK9 can downregulate the expression of LRP1,²³ we demonstrated here that the LDLR is required for mediating the effect of PCSK9 on PPL. Indeed, alirocumab failed to reduce PPL in *Ldlr*^{-/-} mice, indicating that the hepatic catabolism of TRL mainly involves the LDLR pathway.

One important limitation of our study must be acknowledged. Only male mice were used for postprandial lipemia measurements and our results can thus not be extrapolated to female mice. Nevertheless, our findings in mice are in accordance with recent clinical data exploring the link between PCSK9 and PPL regulation in humans. An observational study conducted in 17 obese patients who received an oral fat load highlighted a positive association between plasma PCSK9 levels and the postprandial area under the curve for apoB48, with a negative correlation between circulating PCSK9 and the TRL-apoB48 fractional catabolic rate.²⁴ Genetic data also support a role of PCSK9 in PPL. Indeed, a Canadian study demonstrated that subjects harboring PCSK9 loss-of-function variants display blunted PPL after an oral fat load with reduced postprandial total apoB (-17%), apoB48 (-23%), and triglyceride (-18%) levels compared with noncarrier controls.²⁵ Interventional studies also investigated the effect of PCSK9 inhibition with PCSK9 monoclonal anti-antibody (alirocumab and evolocumab) on PPL. In healthy normolipidemic subjects, in vivo lipoprotein kinetic studies with stable isotopes consistently showed that neither alirocumab²⁶ nor evolocumab²⁷ alter postprandial plasma triglyceride concentrations and apoB48 metabolism. Interestingly, the effect of PCSK9 inhibition on PPL is dependent on the metabolic status. In patients with type 2 diabetes mellitus, the randomized, placebo-controlled BANTING study demonstrated that evolocumab significantly reduced chylomicron triglyceride, chylomicron cholesterol, and VLDL-cholesterol after a mixed-meal test.²⁸ In addition, Taskinen et al. showed that evolocumab treatment reduced postprandial rises in both plasma triglyceride (-21%), apoB48 (-17%), VLDL₁ triglycerides (-15%), and remnant particles (-29%) in a small nonrandomized study conducted in 15 patients with type 2 diabetes mellitus receiving a stable dose of metformin and atorvastatin (20 mg/day). In contrast, evolocumab did not alter the production and the release of chylomicrons from the small intestine.²⁹ Similar findings have been reported by Burggraaf et al

with alirocumab in patients with type 2 diabetes mellitus under insulin therapy.³⁰

In conclusion, our data in mice clearly demonstrate that liver-derived circulating PCSK9 regulates PPL by controlling the catabolism of TRL particles in a LDLR-dependent manner. In accordance with recent interventional studies in humans, these data suggest that blocking circulating PCSK9 with PCSK9 monoclonal anti-antibody represents an efficient strategy to reduce increased PPL, a situation frequently observed in patients with type 2 diabetes mellitus. Thus, PCSK9 inhibition might protect against atherosclerotic cardiovascular diseases beyond its canonical effect on low-density lipoprotein cholesterol lowering.

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Affiliations

From the Institut du thorax, INSERM, CNRS, UNIV NANTES, France (D.G., F.M., A.A., W.D., X.P., L.A., B.C., C.L.); Laboratory of Biochemical Neuroendocrinology, Institut de Recherches Cliniques de Montréal, affiliated to the Université de Montréal, Canada (A.R., A.P.); and L'institut du thorax, Department of Endocrinology, CHU NANTES, France (B.C.).

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