Proprotein Convertase Subtilisin Kexin Type 9 Null Mice Are Protected From Postprandial Triglyceridemia

Cédric Le May, Sanae Kourimate, Cédric Langhi, Maud Chétiveaux, Anne Jarry, Chistine Comera, Xavier Collet, F. Kuipers, Michel Krempf, Bertrand Cariou, Philippe Costet

Objectives—Proprotein convertase subtilisin kexin type 9 (PCSK9) is a natural inhibitor of the low-density lipoprotein receptor (LDLr), and its deficiency in humans results in low plasma LDL-cholesterol and protection against cardiovascular disease. We explored whether PCSK9 expression impacts postprandial triglyceridemia, another important cardiovascular risk factor.

Methods and Results—Real-time PCR and confocal microscopy were used to show that PCSK9 is expressed throughout the entire small intestine and in human enterocytes. On olive oil gavage, PCSK9-deficient mice showed a dramatically decreased postprandial triglyceridemia compared with their wild-type littermates. Lymph analysis revealed that intestinal TG output is not quantitatively modified by PCSK9 deletion. However, PCSK9<sup>−/−</sup> mice present with a significant reduction of lymphatic apoB secretion compared to PCSK9<sup>+/+</sup> mice. Modulating PCSK9 expression in polarized CaCo-2 cells confirmed the relationship between PCSK9 and apoB secretion; PCSK9<sup>−/−</sup> mice consistently secrete larger TG-rich lipoprotein than wild-type littermates. Finally, kinetic studies showed that PCSK9-deficient mice have an increased ability to clear chylomicrons compared to wild-type littermates.

Conclusion—These findings indicate that in addition to its effect on LDL-cholesterol, PCSK9 deficiency might protect against cardiovascular disease by reducing postprandial triglyceridemia. 

Key Words: PCSK9 • chylomicron • postprandial • intestine • CaCo-2

Gain-of-function mutations affecting propprotein convertase subtilisin/kexin type 9 (PCSK9) are associated with autosomal dominant hypercholesterolemia and premature atherosclerosis. It is now established that PCSK9 is a natural inhibitor of the low-density lipoprotein receptor (LDLr), acting posttranscriptionally. Circulating PCSK9 binds to the EGF-A extracellular domain of the hepatic LDLr and prevents its recycling to the cell surface.

A breakthrough study reported that blacks harboring PCSK9 loss-of-function mutations had an 88% risk reduction for coronary heart disease. Although the lower concentrations of LDL cholesterol over one’s lifetime is suggested to be the main reason for this very high level of protection, we hypothesized that PCSK9 deficiency might also affect other risk factors. Low levels of HDL cholesterol (HDL-c) and elevated nonfasted triglycerides (TG) levels are associated with increased risk for cardiovascular disease. No association between PCSK9 loss-of-function mutations and plasma HDL-c levels has been noticed. However, to our knowledge, nonfasting plasma TG were not measured in PCSK9-deficient individuals. They are an important contributor to the risk for cardiovascular disease.

Rashid et al reported the phenotype of PCSK9-deficient mice (PCSK9<sup>−/−</sup>) mice and observed that they exhibit lower plasma cholesterol levels (~50%) and are hypersensitive to statins. The potential role of PCSK9 in the intestine in mice and humans remains unexplored.

During the postprandial period, dietary lipids are actively absorbed into the enterocyte. After intracellular reesterification, long chain fatty acids and cholesterol esters are associated with phospholipids and apolipoproteins, particularly apoB48, to produce TG-rich lipoproteins, mainly chylomicrons.

Chylomicrons are secreted into the mesenteric lymph to eventually deliver lipids to the peripheral tissues and organs. It is believed that the number of chylomicrons does not change during the postprandial phase, but their size increases because of their TG enrichment. Because a single copy of apoB48 is present per chylomicron, the intestinal output of apoB remains constant during the absorptive process. ApoB48 is the preferred protein for the intestine to coat chylomicrons in mice. Chylomicron remnants are cleared from the blood by the LDLr and the LDLr-related protein.

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From the INSERM U915 (C.L.M., S.K., C.L., M.C., M.K., B.C., P.C.), CHU de Nantes, France; Université de Nantes, EA Biometadys (A.J.), Nantes, France; INSERM U563 (C.C., X.C.), Toulouse, France; the Center for Liver, Digestive, and Metabolic Diseases (F.K.), University of Groningen, The Netherlands; Université de Nantes, l’Institut du thorax (M.K., B.C.), Clinique d’Endocrinologie et Nutrition, Nantes, France; the Centre de Recherche en Nutrition Humaine de Nantes (M.K., P.C.), Nantes, France; and INRA UR66 (C.C.), France.
Correspondence to Philippe Costet, INSERM U915, CHU Hôtel Dieu, 3E NORD, 9, Quai Moncousu, 44000, Nantes, France. E-mail Philippe.costet@univ-nantes.fr
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Results

PCSK9 Is Expressed in the Small Intestine of Mice and Humans

Using real-time quantitative PCR, we first examined PCSK9 and the LDLr mRNA quantities along the gastrointestinal tract in mice (Figure 1A). PCSK9 expression in the stomach was virtually undetectable. However, PCSK9 was expressed throughout the small intestine and colon, at a level which did vary significantly along the intestinal cephalo-caudal axis. We next confirmed that the LDLr mRNA quantities along the gastrointestinal tract in mice (Figure 1A). PCSK9 expression in the stomach was virtually undetectable. However, PCSK9 was expressed throughout the small intestine and colon, at a level which did vary significantly along the intestinal cephalo-caudal axis.

Using confocal microscopy, we determined for the first time the localization of PCSK9 protein in frozen sections of normal, human, small intestines. PCSK9 was expressed almost exclusively in the epithelial barrier of the human duodenum and ileum, both in enterocytes and goblet cells (Figure 1B). PCSK9 immunostaining was observed in the cytoplasm of epithelial cells and accumulated at the apical and basolateral sides of the cells (Figure 1B). Blocking of the PCSK9 antibody with an excess of antigen peptide abolished the staining (Figure 1B, upper panel, right), confirming the specificity of the antibody. We also examined the localization of PCSK9 in the human colonic, enterocyte-like cell line, CaCo-2, maintained as polarized by culturing on filters. As shown in Figure 1B (lower panel), the xy image (left) shows a punctuated staining within CaCo-2 cells, and the xz image (right) shows a strong PCSK9 immunostaining mainly in the apical compartment of the cells.

PCSK9 Is Upregulated by Pravastatin in CaCo-2 Cells and the LDLr Expression Is Increased in Intestines of PCSK9−/− Mice

We next examined whether PCSK9 is upregulated by statins in CaCo-2 cells as it has been reported in hepatocytes18,19 (supplemental Figure IA). We exposed the cells for 48 hours to 10 μmol/L pravastatin and observed a 160% increase in PCSK9 mRNA, and a 110% increase in LDLr mRNA (supplemental Figure IA). We next confirmed that the LDLr content is increased in the liver of PCSK9−/− mice compared to wt littermates, and observed a similar phenotype in the medial intestine (+621%, P<0.05; supplemental Figure IB).

PCSK9 Deficiency Protects Mice Against Postprandial Lipemia

Random-fed PCSK9−/− mice had lower total cholesterol than wt littermates (~35% in 3-month-old mice, P<0.001), and similar plasma TG concentrations (supplemental Figure IIA). Plasma TG levels were measured over a 4-hour period in 14 hour–fasted mice after they received an intragastric bolus of olive oil (200 μL; Figure 2). At T0, plasma levels of TG were comparable between PCSK9+/+ and PCSK9−/− mice. Two hours after gavage, PCSK9+/+ mice showed a 209% increase in plasma TG, whereas PCSK9−/− littermates displayed a
strongly attenuated TG postprandial response. Mean TG appearance rates were, respectively, 0.19±0.032 and 0.082±0.022 g/L/h for PCSK9+/+ and PCSK9−/− mice, ie, 62% less (n=12 PCSK9+/+ and n=14 PCSK9−/− mice from to 2 independent experiments).

Fractionation by fast protein liquid chromatography (FPLC) showed that the difference in triglyceridemia observed 2 hours after gavage was attributable to a decrease in the TG content of chylomicrons/VLDL lipoproteins (supplemental Figure IIB and supplemental Methods and Results).

**Gastric Emptying, Intestinal Transit, and Fat Absorption After Oral Oil Challenge**

The defect in postprandial lipemia observed in PCSK9−/− mice could be attributable to (1) decreased intestinal lipid absorption, or chylomicron production, or (2) accelerated lipolysis or clearance of TG-rich lipoproteins. We first measured the radioactivity content of intestinal segments of PCSK9+/+ and PCSK9−/− littersmates 2 hours after a gavage with olive oil containing [3H]triolaen (Figure 3A). Triolein is a triglyceride and unsaturated fat formed from oleic acid, the main constituent of olive oil. This method reflects (1) the esterified or reesterified [3H]-oleate, (3) the reuptake of radiolabeled nascent particles. PCSK9−/− mice had a tendency to accumulate less [3H] in the proximal and medial part of their intestine compared to wt mice, suggesting that lipid uptake could be defective in these segments. To determine intestinal fat absorption, we measured food intake and collected feces every 24 hours for 3 consecutive days. Food intake, weight of excreted feces, and fecal fat excretion were not significantly different between mice (supplemental Table II). PCSK9+/+ and PCSK9−/− mice absorb fat with the same efficiency (Figure 3B) demonstrating that reduced lipemia in PCSK9−/− mice is not attributable to fat malabsorption. As a control, PCSK9+/+ mice fed with orlistat, an inhibitor of pancreatic lipases, showed a 45% decrease in total fat absorption compared to PCSK9+/+ mice under normal chow (Figure 3B).

Altered postprandial lipemia could also be attributable to gastric emptying or intestinal motility defect. We performed an oral gavage with a nonabsorbable 70-KDa FITC conjugated dextran marker diluted in olive oil. After 30 minutes, about 10% of the marker was still present in the stomach of PCSK9+/+ and PCSK9−/− mice (Figure 3C), demonstrating that PCSK9 deficiency does not alter gastric emptying. Intestinal transit was also similar in both genotypes (Figure 3C).

Thus, although there was a trend toward less accumulation of [3H]-triolaen in the proximal and medial intestine of PCSK9−/− mice after an olive oil gavage, they do not show a defect in fat absorption, gastric emptying or intestinal transit.

**Chylomicron Secretion in PCSK9−/− Mice**

To directly explore the impact of PCSK9 deficiency on chylomicron output, we cannulated the lymph ducts from 2 hour–fasted PCSK9+/+ and PCSK9−/− littersmates. After establishing a 30-minute baseline collection, we injected a bolus of olive oil in their duodenums, and collected the lymph at timed intervals for 210 minutes (Figure 4A). Lymph flow was reduced by 20% in knockout mice (0.59 µL/min versus 0.47 µL/min, P<0.05). No difference in cumulative TG contents was observed in the first 1.5 hour. In a second phase, the accumulation tended to be reduced in PCSK9−/− mice, although it did not reach significance (Figure 4A).

Lymph samples collected at various times after olive oil injection were pooled, and equal amounts were loaded on a gel for separation of the proteins by electrophoresis (Figure 4B). As expected, the quantity of apoB was relatively constant during the absorption process within each genotype, and apoB48 was more abundant than apoB100.10 However, apoB100 and apoB48 contents were significantly lower in lymph from PCSK9−/− mice (−39%, P<0.05 and −40%, P<0.01), suggesting that PCSK9 deficiency results in less chylomicrons being secreted. The positive relation between PCSK9 expression and the apoB output was confirmed in CaCo-2 cells (supplemental Figure III and supplemental Methods and Results). We also determined the content of the other apolipoprotein by electrophoresis. No striking difference between genotypes was observed. There was a tendency toward an accumulation of apoE in lymph from PCSK9−/− mice (Figure 4C).

As lymphatic TG output was unchanged and lymphatic apoB secretion was decreased, it suggests that PCSK9−/− mice might secrete larger chylomicrons than wt littersmates. Using dynamic light scattering techniques, we showed that chylomicron diameters from PCSK9−/− mice were increased by 10% compared to wt littersmates (Figure 4D). Assuming that they are spherical particles, PCSK9−/− mice secrete chylomicrons with a volume at least 25% larger than PCSK9+/+ mice (Figure 4D).

**Chylomicron Catabolism in PCSK9+/+ and PCSK9−/− Mice**

We next measured the hepatic TG content after olive oil gavage. There was no change in hepatic TG contents in fed PCSK9+/+ and PCSK9−/− mice (Figure 5A). Fasting in-
duced an accumulation of TG by 100% in both genotypes (P<0.01). Two hours after olive oil gavage, hepatic TG contents were similar to those of PCSK9+/+ mice in the fed state, but remained elevated in PCSK9−/− mice. Consequently, there was 54% more TG in livers of knockout mice after gavage (P<0.05), suggesting that TG originated from olive oil fatty acids. To confirm this, 3H-triolein diluted in olive oil was orally administered to fasted mice, and 2 hours later the amount of hepatic radioactivity was measured. PCSK9−/− mice exhibited almost 200% more radioactivity than wt littermates showing that the hepatic uptake of chylomicrons was increased in these mice (supplemental Figure IV).

We verified whether the clearance of chylomicrons is enhanced in knockout mice (Figure 5B). Chylomicrons obtained by mesenteric lymph duct cannulation of wt and knockout mice (chylol+/- and chylol−/−) were labeled with 125I and injected into the circulatory systems of mice from both genotypes. To mimic the postprandial status, we injected chylol+/- in PCSK9+/- mice and chylol−/− in PCSK9−/− mice. Consistent with the phenotype observed in Figure 2, PCSK9−/− mice cleared chylol−/− 37% faster than PCSK9+/- cleared chylol+/- (P<0.05). We also performed the experiment crossing the donors and receivers. PCSK9−/− mice cleared chylol−/− 21% faster than PCSK9+/- mice did (P<0.01). These results reflect probably the higher hepatic LDLr content observed in PCSK9−/− mice (supplemental Figure IB). Surprisingly, we observed (1) no difference in chylol+/- clearance rates between the genotypes; and (2) PCSK9−/− mice cleared chylol−/− 56% more efficiently than chylol+/- (P<0.001).

Taken together, these data showed that the reduced postprandial lipemia in PCSK9−/− mice is caused in part by increased hepatic uptake.

**Discussion**

This study shows that PCSK9 is highly expressed in enterocytes and plays a critical role in postprandial lipemia in mice. We first determined the expression of PCSK9 in the gastrointestinal tract. We showed that in mice PCSK9 is expressed
throughout the digestive tract and colon at levels equivalent to those found in the liver. In the human intestine, PCSK9 is localized in the cytoplasm probably in the endoplasmic reticulum and the golgi, and it accumulates at the subapical and basolateral compartments of the enterocyte. The heterogeneity of this cellular distribution depending on the intestinal segment considered is puzzling. PCSK9 is expressed at both the apical and basolateral pole in the duodenum, and mainly at the apical pole in the ileum. It is possible that PCSK9 is directed toward both poles of the enterocyte in the upper part of the small intestine, in relation with the absorptive process and the lipoprotein secretion, and only to the apical side in the ileum that secretes less lipoproteins. It appears that CaCo-2 cells would be more representative of an ileal enterocyte, in respect to PCSK9 cellular expression. Indeed, in differentiated CaCo-2 cells, most of the signal was concentrated at the apical side. We observed high levels of expression in the ileum where the bile acid reuptake takes place. Since we recently showed that bile acids repress PCSK9, it is tempting to speculate that PCSK9 might play a role in their metabolism. We also observed some PCSK9 protein in goblet cells, although its function in this cell type remains unknown.

PCSK9-deficient mice clearly exhibited reduced postprandial lipemia after olive oil gavage. A balance study showed that intestinal fat absorption was not significantly different between genotypes, and that PCSK9 deficiency does not alter the gastric emptying and intestinal transit. We have no clear explanation concerning the trend toward a decrease of accumulation of 1H-triolein in the proximal and medial segments of PCSK9−/− mice. It could explain the weak trend observed during lymph cannulation, with PCSK9−/− mice secreting less TG. Thus, it might reflect some aspects of PCSK9 function in the intestine, in relation or not with the strong phenotype observed concerning the intestinal apoB secretion and postprandial lipemia in PCSK9−/− mice. We investigated whether this phenotype could be attributable to chylomicron production or catabolism. We directly collected the mesenteric lymph after olive oil gavage. TG outputs in lymph were virtually similar in both genotypes. We observed a 20% reduction of lymph flow in PCSK9−/− mice. There is a large number of plausible causes for lymph flow reduction, and more work is needed to determine which one is responsible for this phenotype. Direct measurement on lymph samples collected in mice after oil gavage revealed that PCSK9−/− mice secreted less apoB than wt littermates did. The reduction in lymph flow could not account for this, as it was estimated with equal amounts of lymph from both genotypes. Experiments in differentiated CaCo-2 cells confirmed that PCSK9 deficiency results in a decrease in apoB secretion. Interestingly, Rashid et al showed that primary hepatocytes from PCSK9−/− mice exhibit reduced apoB48 secretion. However, on a high-fat diet, transient PCSK9 knockdown in mice led to a decrease in serum apoB100 content and an increase in apoB48 content, probably because of the upregulation of apobec1. Apobec 1 is the enzyme responsible for the production of apoB48 in human intestine and is also expressed in rodent liver. We did not observe a change in the
rich lipoproteins are more avidly recaptured in PCSK9 capture LDL particles.\textsuperscript{17} However, the functional importance of the LDLr in the intestine is unclear. In the liver, Twisk et al showed that patients with familial hypercholesterolemia are characterized by an increased intestinal chylomicron production.\textsuperscript{26} We propose that PCSK9 inhibitors would help manage LDL cholesterol and also postprandial triglyceridemia, 2 important risk factors of cardiovascular disease.

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Disclosures

None.

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Supplement Methods

Materials

The adenovirus vectors coding for the human PCSK9 and a sham control adenovirus (AdPCSK9 and Adnull, respectively) were generated by the Vector Core of the University Hospital of Nantes and described elsewhere.\(^1\) Oleic acid, fatty acid free BSA, cholesterol, sodium taurocholate, pravastatin, DMSO, lentivirus expressing control SHRNA and SHRNA directed against human PCSK9 were purchased from Sigma–Aldrich (St Quentin-Fallavier, France). \(^3\)H-cholesterol, \(^3\)H-triolein and Redivue Pro-mix \(L\)-[^35]S] were provided by GE Healthcare (GE Healthcare, Orsay, France).

Real time PCR

Real time PCR analyses were performed with the SYBR Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France). For the oligonucleotide sequences for real time PCR, please see Supplemental Table I. Values are normalized to cyclophilin (figure 1) or 18S Supplemental Figure S1, S3) and expressed as means ± SEM relative to the proximal intestine or CTRLarbitrarily set at 1.

Triglycerides measurements

Hepatic lipids were extracted with isopropylether/butanol (6/4, v/v). After solvent evaporation, dried TG were sonicated in 0.5ml of the provided kit buffer. Hepatic, plasma,
and lymphatic TG were quantified using the PAP150TG kit (Biomerieux, Marcy l’Etoile, France).

Cell Culture

CaCo-2 cells were cultured in T-75 flasks (Corning Glassworks, Corning, NY) in DMEM (Lonza, Levallois-Perret, France) with 4.5 g/l glucose and supplemented with 10% FBS (Biowest, Nuaillé, France), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Once the flasks reached 80% confluence, the cells were split and seeded at a density of 0.2 X 10^5 cells/well onto polycarbonate micropore membranes (0.4 mm pore size, 24 mm diameter) inserted into Transwells (Costar, Cambridge, MA). Cells were fed every other day and were used 14 days after seeding. Before each experiment, the integrity of the cell monolayer was checked by measuring transepithelial electrical resistance with a voltmeter equipped with a chopstick electrode (Millicell ERS; Millipore, Saint Quentin enYvelines, France).

Immunohistochemistry

PCSK9 immunostaining was performed on frozen sections of human normal small intestine, as well as on filter-grown confluent CaCo-2 cells. Human normal duodenum and distal ileum were obtained from 6 patients undergoing surgery for pancreaticoduodenectomy for cancer (n=3) or right colon carcinoma (n=3), respectively (4 men and 2 women, mean age, 62 years; range, 56-78 years). Patients did not undergo chemotherapy or radiotherapy. Fragments of histologically normal duodenum or distal ileum were taken at about 10 cm downstream of the tumor. The tissue fragments were processed according to the French Guidelines for Research on Human Tissues. Informed patient consent was obtained, according to the French bioethics law. Ten µm cryostat sections of human normal duodenum
and ileum, and filters covered with CaCo-2 were fixed in 4% paraformaldehyde in PBS, and processed for immunofluorescence. Briefly, filters from CaCo-2 cells cultures plates and tissue sections were incubated for 1h with our anti-PCSK9 rabbit polyclonal antibody (dilution: 1:100, IgG directed against the CRSRHLAGASQELQ peptide, an epitope of the C-terminal domain of human PCSK9) in PBS-horse-serum 4%, with or without 4°C overnight preincubation with 1000-fold excess of the immunogenic peptide. After 3 PBS washes, sections were then incubated for 30 min with Alexa Fluor 488-conjugated goat anti-rabbit antibodies (1:100; Molecular Probes). Nuclear staining was performed with DRACQ5 (1µM, Interchim Montluçon, France). Sections were mounted using Prolong antifade medial (Molecular Probe, Eugene, OR., USA). Imaging was performed on a Leica TCS-SP confocal laser-scanning microscope (Leica, Germany) equipped with an argon-krypton laser. Negative controls, performed by omitting the primary antibody, were used to set the parameters of photomultiplicators (PMT). Sections were visualized with x63/1.4 oil objective lens, and image processing performed using TCS-NT software (Leica).

Western Blots

CaCo-2 cells lysates and mice scraped intestinal mucosa were homogenized, and 75 µg of total lysate proteins were resolved on NuPAGE 4–12% BisTris gels in MES SDS buffer (Invitrogen) under reducing conditions for western blot analysis, as described elsewhere.\(^4\) We used antibodies raised against the mouse LDLr (R&D Systems, Lille, France) and cyclophilin A (Millipore, France). Immunoreactive bands were revealed using the ECL plus kit (Amersham Biosciences).

Chylomicron size, lymph apolipoprotein content.

Lymphatic lipoprotein size was determined after lymph collection by dynamic light
scattering techniques using a Zetasizer 3000 HSA (Malvern) at 25°C. Particle diameters were assessed from the volume distribution patterns provided by the analyzer. Mesenteric lymph apolipoprotein content was determined by electrophoresis on a 4-20% Tris-glycine gel, stained with Coomassie blue, on equal amounts of lymph samples (2.5 µL) Purified proteins were used as a positive control (Biodesign, Saco, ME. USA). Relative quantities were estimated by densitometry. The value obtained for apoB100 (Figure 4B) or apoE (Figure 4C) in wild type mice was arbitrarily set at 1.

Chylomicron plasma clearance

Mesenteric ducts from PCSK9\textsuperscript{+/+} and PCSK9\textsuperscript{−/−} mice were cannulated and lymph collected following olive oil gavage. Chylomicrons were isolated by ultracentrifugation, dialyzed for 48h at 4°C against PBS pH7.4 and radiolabeled according to the iodogen method modified by Fraker \textit{et al.}\textsuperscript{5} Briefly, after measurement of protein content, similar amounts of PCSK9\textsuperscript{+/+} and PCSK9\textsuperscript{−/−} chylomicron associated proteins (0.5 mg) were iodinated with \textsuperscript{125}I (0.2 mCi) for 30 minutes. After separation of free \textsuperscript{125}I and \textsuperscript{125}I-labeled chylomicrons, specific activity was measured. Mice fasted for 14 hours received an intravenous injection of radio labeled chylomicron in 0.2 ml of PBS. Blood was collected at indicated times, and plasma radioactivity was measured with a γCounter (1480 Wizard 300 Automatic Gamma Counter, Wallac, Waltham, Massachusetts, USA).

Measurement of newly synthesized and secreted apoB

Differentiated CaCo2 cells plated onto polycarbonate micropore membranes inserted into Transwells were washed twice with PBS and incubated for 1h in methionine/cysteine-free media containing 0.5 mM oleic acid dissolved in fatty acid free BSA (4/1 mol/mol). Cells were then labeled for 24h with 100 µCi/ml [\textsuperscript{35}S] methionine/[\textsuperscript{35}S] cysteine mix (Redivue Pro-
mix L-[\(^{35}\)S]; GE Healthcare, Orsay, France). At the end of the labeling, media were transferred into tubes containing a cocktail of protease inhibitors (Roche Diagnostics). Cells were washed twice with cold PBS and lysed directly in the wells by the addition of a 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.25% sodium deoxycholate buffer containing the protease inhibitor cocktail. Both media and cell lysates were homogenized on a rocking platform for 1 h at 4°C and centrifuged for 10 min at 10,000g to pellet cellular debris. 3X Radioimmunoprecipitation (RIPA) buffer was added to supernatants before immunoprecipitation. Cell extracts and media were incubated with an excess of goat anti-human apoB antibody (Chemicon Europe, Chandlers Ford, UK) and incubated overnight at 4°C. Antigen-antibody complexes were isolated by centrifugation after incubation for 4 h at 4°C with protein A/G agarose plus (Santa Cruz Biotechnology, Le Perray en Yvelines, France). The complexes were subjected to 3 washes with RIPA and 2 with PBS. The tubes were incubated for 10 min at 4°C and centrifuged for 2 min at 4,000 rpm to pellet the complexes between each wash. The immunoprecipitated complexes were boiled for 5 min in 50 µl of lauryl dodecyl sulfate reducing buffer (Invitrogen) and centrifuged, and the supernatant was resolved by gel electrophoresis. The \(^{35}\)S-labeled apoB protein bands were quantified using a phosphorimager.

**Plasma lipid and fast protein liquid chromatography (FPLC) analysis**

Plasma total cholesterol (TC) and triglycerides (TG) were measured using commercial kits (Biomerieux, Marcy l’Etoile, France). Plasma lipoproteins from pooled mouse samples were resolved by FPLC. Briefly, 200µL of plasma were injected into a MV-7 multi-injection loop and separation was performed on 2 superose 6 HR 10/30 columns in series with an elution flow rate of 0.35 mL/min; 0.5mL was collected for each fraction and the entire profile
was completed within 105 minutes. The system was controlled by FPLC DIRECTOR software (Amersham Pharmacia Biotech Inc).

\(^3\)H-triolein intestinal absorption

PCSK9\(^{+/+}\) and PCSK9\(^{-/-}\) mice were fasted for 14 hours and received an intragastric olive oil load (200 µl) containing 15µCi \(^3\)H-triolein. Two hours after gavage, the small intestine was divided into three equal segments (proximal, medial and distal). Intestinal lumina were flushed four times with 5mM taurocholate and the segments were homogenized in lysis buffer and incubated in scintillation fluid (betaplate scint, Perkin Elmer, Courtaboeuf, France). Radioactivity associated with each intestinal fraction was measured with a liquid scintillation analyzer.

Fecal fat balance

PCSK9\(^{+/+}\) and PCSK9\(^{-/-}\) mice were housed in individual cages 3 days prior to the experiment. PCSK9\(^{+/+}\) and PCSK9\(^{-/-}\) mice received a 3.1% fat chow diet (w/w), a third PCSK9\(^{+/+}\) mice group received the same diet supplemented with orlistat (200mg/kg diet). Daily food intake measurement and feces harvesting were performed every 24h for 3 days. Feces were frozen, lyophilized, weighted and mechanically homogenized. An aliquot of homogenized dried feces was resuspended in 0.5ml water and fecal lipids were extracted with 4 ml of isopropylether/butanol (6/4, vol/vol) in glass vials. The mixture was centrifuged at 3500 g for 5 min at 4°C and the supernatant (organic phase) transferred to a new vial and evaporated under N2 at 55 °C. Fecal fat content is the weight difference between the initial empty vial and the vial containing the dried lipid. Daily fat intake was determined by multiplying the daily food intake per 0.031. Total fecal fat excretion was expressed in grams of fat per day. Percentage of total fat absorption was measured using the following
calculation: Total fat absorption (%) = 100% \( \frac{[\text{daily fat intake (g/day)} - \text{daily fat excretion (g/day)}]}{\text{daily fat intake (g/day)}} \).

**Gastric emptying and intestinal transit measurement**

Gastric emptying and intestinal transit were measured by assessing the distribution of a nonabsorbable 70 kDa FITC conjugated dextran marker throughout the gastrointestinal tract of PCSK9\(^{+/+}\) and PCSK9\(^{-/-}\) mice (n=4). Mice were fasted for 12h. Thirty minutes after gavage with 0.1 ml of 5 mg/ml FITC conjugated dextran diluted in olive oil, mice were sacrificed and the stomach and small intestine were carefully removed. The small intestine was divided into 10 segments of equal length. Each segment was homogenized with 3 ml of 5mmol/l taurocholate. Fluorescent activity of each segment was measured using a fluorimeter (Perkin-Elmer, France). Gastric emptying was determined by subtracting the dextran remaining in the stomach from the total dextran (stomach and small intestine) and dividing this value by total dextran. This value was then multiplied by 100 and expressed as per cent. Intestinal transit was analysed using the intestinal geometric centre (IGC) of the distribution of dextran throughout the intestine and was calculated with the equation described by Miller and colleagues

\[
\text{IGC} = \left( \frac{\text{fraction of amount of FITC in each segment}}{ \text{segment number} } \right)
\]

The dextran concentration in each segment was expressed as a fraction of total dextran recovery.

**Supplement Results**

**Fast protein liquid chromatography (FPLC) analysis of plasma from mice 2h after olive oil gavage**

Plasma samples were collected before and 2h after olive oil administration and subjected to fractionation by FPLC (Supplemental Figure IIB). As expected, TG were present
in large lipoproteins (''chylomicron/VLDL TG'' peak). In agreement with the direct measure of total plasma TG, profiles were similar in the fasting state, but olive oil gavage led to a doubling of chylomicrons/VLDL TG in wt littermates and remained unaffected in PCSK9⁻/⁻ mice. As previously reported,⁷ HDL-c levels in PCSK9⁻/⁻ mice were lower and this pattern was maintained during the olive oil challenge (Supplemental Figure IIB).

**PCSK9 expression modulates apoB secretion in CaCo-2 cells**

Since postprandial apoB₄₈ and apoB₁₀₀ outputs are reduced in PCSK9⁻/⁻ (Figure 4B), we checked whether modulating PCSK9 expression in polarized CaCo-2 cells would affect apoB₁₀₀ and apoB₄₈ secretion (Supplemental Figure III).

Unlike primary human enterocytes, CaCo-2 cells secrete both apoB₁₀₀ and apoB₄₈. Interestingly, transient adenoviral overexpression of wild-type human PCSK9 in polarized cells led to increases in basolateral ³⁵S-apoB₁₀₀ and ³⁵S-apoB₄₈ secretions, by 146% and 295% respectively, compared to empty virus-treated cells (Supplemental Figure 3A p<0.001).

Next, we inhibited PCSK9 expression in CaCo-2 cells (Supplemental Figure IIIB). In order to obtain the knockdown of PCSK9 in polarized cells, we produced stable clones using lentivirus-mediated ShRNA delivery. When PCSK9 expression was efficiently knocked down in CaCo-2 cells, a mirror picture of what was observed by overexpression appeared. The results showed a 50% reduction in PCSK9 mRNA quantity (p<0.05) accompanied by an 80% reduction in apoB₁₀₀ (p<0.01) and a 60% reduction in apoB₄₈ output (p<0.05). Taken together, these data support the hypothesis that intestinal apoB₄₈ secretion can be modulated by PCSK9.
Supplement figure legends

Supplement Figure I. PCSK9 upregulation by pravastatin in CaCo-2 cells and intestinal LDLr accumulation in PCSK9−/− mice

A) Differentiated CaCo-2 cells were exposed to pravastatin 10µM (PV) or not (Ctrl) for 48h. RNA were extracted and PCSK9, LDLr, mRNA quantities were analyzed using real time PCR, and standardized using 18S mRNA levels. B) Proteins were extracted from the medial intestine (n=4 mice of each genotype) or the liver (n=2 mice of each genotype) of PCSK9+/+ and PCSK9−/− littermates. LDLr contents were determined by western blot. For the intestine, LDLr value from each lane was normalized based on the corresponding cyclophylin level, and we present the mean of these ratios for each genotype. Data represent means ± SEM (*p<0.05).

Supplement Figure II. Plasma lipids

A) Cholesterol and triglycerides in PCSK9−/− and PCSK9+/+ mice. Plasma levels of triglycerides, cholesterol were determined from non fasted PCSK9+/+ and PCSK9−/− littermates (n=7 mice/group). Values are depicted as means ± SEM (* P≤0.05; ** P≤0.01). B) FPLC analysis of plasma from mice challenged with a fat load. 14h-fasted PCSK9+/+ (white circle, n=12) and PCSK9−/− littermates (black circle, n=14) received an intragastric olive oil load (200 µl). Plasma collected at time 0 and 2h after gavage were subjected to FPLC and TG, cholesterol contents of the fractions determined.

Supplement Figure III. PCSK9 modulates apoB secretion in CaCo-2

Human PCSK9 was overexpressed (AdPCSK9) or not (Adnull) in differentiated CaCo-2 cells using adenoviral infection (A) Clonal populations of cells infected with non targeting or PCSK9 targeting short-hairpin lentiviral construct (SH non targeting and SH
PCSK9) were selected (B). After cells differentiated, we verified by real time PCR the expression of PCSK9, standardized with the 18S mRNA levels. Newly synthesized proteins were labeled with $^{35}$S-methionine and $^{35}$S-apoB secretion was determined by co-immunoprecipitation and gel electrophoresis. Representative blots show apoB100 and apoB48 basolateral secretion. Values are expressed as means ± SEM relative to “non targeting” conditions which are arbitrarily set at 1. *p<0.05, **p<0.01 ***p<0.001.

**Supplement Figure IV. Hepatic $^3$H-triolein content**

PCSK9$^{+/+}$ (white bar, n=5) and PCSK9$^{-/-}$ littermates (black bar, n=5) were fasted for 14h and received an intragastric olive oil load (200 µl) containing 15µCi $^3$H-triolein. Two hours after gavage, mice were euthanized and their liver collected. Values are depicted as means ± SEM. *p<0.05.

**Supplement legends Figure 1**

PCSK9 appears in green, and nuclei in red. PCSK9 is present in the epithelial barrier, in the apical (arrow), and basolateral (arrowhead) compartments of enterocytes, both in the villus (see the duodenum) and crypt (see the ileum). PCSK9 is also expressed by goblet cells (asterisk). The upper right panel (duodenum) shows an absence of immunostaining when the antibody was pre-incubated with an excess of antigen peptide. In CaCo-2 cells maintained on filters, the xy section shows a punctuated distribution of PCSK9, and the xz section shows an accumulation of PCSK9 in the apical compartment (arrow). The filter (basal pole of the cells) appears as a red line (arrowhead, original magnification - x630).
Supplement references


(2) [Recommendations for cryopreservation of cells tumor tissues to be used for molecular analyses]. *Ann Pathol* 2001;21(2):184-201.


Supplement Table I

This table presents the oligonucleotide sequences used in real time PCR analysis for Supplemental Figure S1 and Supplemental Figure S3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Accession Number</th>
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<tr>
<td>mPCSK9</td>
<td>5' - AGG TGG AGG TGG ATC TCT TAG ATA CCA - 3'</td>
<td>5' - CGC TGT TGA AGT CCG TGA TG - 3'</td>
<td>NM_153565</td>
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<tr>
<td>mCyclophilin</td>
<td>5' - TGG CAA ATG CTG GAC CAA A - 3'</td>
<td>5' - GCC ATC CAG CCA TTC ATG CT - 3'</td>
<td>NM_008907</td>
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<tr>
<td>mLDLr</td>
<td>5' - ACC TGC CGA CCT GAT GAA TTC - 3'</td>
<td>5' - GCA GTC ATG TTC ACG GTC ACA - 3'</td>
<td>NM_010700</td>
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<td>18S</td>
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<td>5' - CGA TCC GAG GGC CTC ACT A - 3'</td>
<td>NR_003286</td>
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<tr>
<td>hPCSK9</td>
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<td>5' - AAG TGG ATC AGT CTC ACT AA - 3'</td>
<td>NM_174936</td>
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<td>hLDLr</td>
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<td>5' - CGA ACT GCC GAG AGA TGC A - 3'</td>
<td>NM_000827</td>
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</table>

Supplement Table II. Effect of PCSK9 deficiency and orlistat treatment on food and fat intake, fecal fat excretion, and total fat absorption

Mice, individually housed, had free access to a 3.1% fat chow diet supplemented or not with orlistat 200 mg/kg diet. Food intake and fecal fat excretion were assessed daily for 3 consecutive days. Values shown are means ± SEM. (*p<0.05, ***p<0.01, vs PCSK9+/+ mice under regular diet).

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Orlistat mg/kg diet</th>
<th>Daily Food Intake g/day/mouse</th>
<th>Daily Fat Intake g/day/mouse</th>
<th>Fecal Excretion g/day/mouse</th>
<th>Total Fecal Fat excretion g/day/mouse</th>
<th>Total Fat Absorption %</th>
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<tr>
<td>PCSK9+/+</td>
<td>18</td>
<td>0</td>
<td>5.96 ± 0.24</td>
<td>0.185 ± 0.007</td>
<td>1.162 ± 0.06</td>
<td>0.031 ± 0.004</td>
</tr>
<tr>
<td>PCSK9−/−</td>
<td>18</td>
<td>0</td>
<td>6.00 ± 0.28</td>
<td>0.186 ± 0.009</td>
<td>1.127 ± 0.05</td>
<td>0.035 ± 0.003</td>
</tr>
<tr>
<td>PCSK9−/−</td>
<td>6</td>
<td>200</td>
<td>6.16 ± 0.24</td>
<td>0.191 ± 0.007</td>
<td>1.43 ± 0.1 *</td>
<td>0.104 ± 0.009 ***</td>
</tr>
</tbody>
</table>
Supplement figure IV

Radioactivity (CPM/mg of liver)

+/-

-