Chapitre 16 Les concentrations plasmatiques de PCSK9 sont positivement associées au taux de production des lipoprotéines riches en triglycérides contenant l'apolipoprotéine B-48 chez les hommes

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L'article présenté dans ce chapitre s'intitule :

Plasma PCSK9 correlates with apoB-48-containing TG-rich lipoprotein production in men with insulin resistance

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Résumé

Objectif : L'accumulation plasmatique de LRT contenant l'apo B-48 joue un rôle important dans la pathogénèse de l'athérosclérose chez des sujets avec RI. Dans cette étude, les associations entre les concentrations plasmatiques de PCSK9 et la cinétique intravasculaire des LRT contenant l'apo B-48 a été évaluée dans un grand échantillon d'hommes présentant des degrés variés de RI.

Méthodes : La cinétique des LRT contenan l'apo B-48 a été évaluée suivant l'infusion d'un bolus et d'une perfusion constante de leucine deutérée chez 148 hommes maintenus dans un état postprandial constant. L'expression des gènes clés impliqués dans le métabolisme intestinal du cholestérol et des chylomicrons était évaluée dans un sous-groupe de 71 sujets chez qui une biopsie duodénale était recueillie à jeun.

Résultats : Les concentrations plasmatiques de PCSK9 était positivement associées à la quantité plasmatique postprandiale (r=0,31 ; *P*=0,0002) et au taux de production des LRT contenant l'apo B-48 (r=0,24 ; *P*=0,008), mais pas à leur taux de catabolisme fractionnel (r=-0,04 ; *P*=0,6). Une régression linéaire multiple pas-à-pas a permis d'identifier les concentrations de PCSK9 comme un prédicteur positif du taux de production des LRT contenant l'apo B-48 (β type=+0,20 ; *P*=0,007), indépendamment des concentrations à jeun de TG, d'insuline, de glucose, de C-LDL, de CRP, de l'IMC, de l'âge, de la présence de diabète de type 2 (et prise concomitante de metformine) et de l'apport en gras durant l'étude de cinétique. Des associations positives étaient observées entre l'expression intestinale des gènes *PCSK9* et *HMG-CoAR* (β type=+0,17 ; *P*=0,02), *ACAT2* (β type=+0,55 ; *P*<0,0001) et *R-LDL* (β type=+0,24 ; *P*=0,007).

Conclusions : Les concentrations plasmatiques de PCSK9 sont positivement associées au taux de production des LRT contenant l'apo B-48. Il est suggéré que la PCSK9 contribue à l'accumulation plasmatique des LRT contenant l'apo B-48 en stimulant la sécrétion de ces lipoprotéines chez des hommes présentant différents degrés de RI.

Title page

Plasma PCSK9 correlates with apoB-48-containing TG-rich lipoprotein production in men with insulin resistance

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Running head

Association between PCSK9 and TRL apoB-48 kinetic

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Abbreviations

ACAT2: acetyl-CoA acetyltransferase 2;

ACS1: acetyl-CoA synthetase 1;

Apo: apolipoprotein;

BMI: body mass index;

C: cholesterol;

CRP: C-reactive protein;

FABP2: fatty acid-binding protein 2;

FCR: fractional catabolic rate;

G6PD: glucose-6-phosphate-dehydrogenase;

HMG-CoAR: hydroxy-methyl-glutaryl-CoA reductase;

HNF4α: hepatocyte nuclear factor 4α;

HOMA-IR: homeostasis model assessment of insulin resistance;

IR: insulin resistance;

LDLR: LDL receptor;

LRP-1: LDL receptor-related protein 1;

MTP: microsomal triglyceride transfer protein;

NPC1L1: Niemann–Pick C1-like 1;

PCSK9: Proprotein convertase subtilisin/kexin type 9;

PR: production rate;

PS: pool size;

SAR1B: SAR1 gene homolog B;

SCD1: stearoyl-CoA desaturase 1;

SREBP: sterol regulatory element binding protein;

T2D: type 2 diabetes;

TG: triglyceride;

TRL: triglyceride-rich lipoprotein;

VLDLR: VLDLR receptor.

Abstract

Intestinal TG-rich lipoproteins (TRLs) are important in the pathogenesis of atherosclerosis in insulin resistance (IR). We investigated the association of plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) concentrations with apoB-48–containing TRL metabolism in 148 men displaying various degrees of IR by measuring *in vivo* kinetics of TRL apoB-48 during a constant-fed state after a primed-constant infusion of L-[5,5,5-D3]leucine. Plasma PCSK9 concentrations positively correlated with TRL apoB-48 pool size ($r = 0.31$, $p = 0.0002$) and production rate ($r = 0.24$, $p = 0.008$) but not fractional catabolic rate ($r = -0.04$, $p = 0.6$). Backward stepwise multiple linear regression analysis identified PCSK9 concentrations as a positive predictor of TRL apoB-48 production rate (standard $\beta = +0.20$, $p = 0.007$) independent of BMI, age, type 2 diabetes/metformin use, dietary fat intake during the kinetic study, and fasting concentrations of TGs, insulin, glucose, LDL cholesterol, or C reactive protein. We also assessed intestinal expression of key genes involved in chylomicron processing from duodenal samples of 71 men. Expression of *PCSK9* and *HMG-CoAR* genes was positively associated ($r = 0.43$, $p = 0.002$). These results support PCSK9 association with intestinal secretion and plasma overaccumulation of TRL apoB-48 in men with IR.

Keywords: PCSK9, chylomicron, apolipoprotein B-48, insulin resistance**.**

Introduction

Intestinal triglyceride-rich lipoproteins (TRLs) are important contributors of postprandial hypertriglyceridemia, a major cardiovascular disease risk factor.[1] Although chylomicrons are too large to enter the sub-endothelial space, once hydrolyzed, cholesterol-rich chylomicron remnants are small enough to penetrate the sub-endothelial space and these particles contribute to the formation of foam cells and impair endothelial function.[2,3] In the last years, the link between insulin resistance (IR) and the overproduction and decreased clearance of intestinally derived apolipoprotein (apo) B-48-containing TRLs was extensively described in humans.[4,5] Therefore, given the atherogenicity of intestinal lipoproteins, identifying mechanisms responsible for their overaccumulation in subjects with IR is of great interest.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays a major role in lipoprotein clearance by promoting intracellular lysosomal degradation of hepatic lipoprotein receptors, namely, the LDL receptor (LDLR), the VLDL receptor (VLDLR) and the LDLR-related protein 1 (LRP-1).[6,7] In healthy humans, PCSK9 inhibition with PCSK9 monoclonal antibodies enhances the clearance rates of VLDLs, IDLs and LDLs,[8,9] while the effect on apoB-48-containing lipoprotein clearance remains uncertain.[8] In addition, studies conducted in vitro and in animal models demonstrated that PCSK9 stimulates the secretion of both hepatic and intestinal TRLs.[10-13] However, no in vivo human study have corroborated these observations so far.[8,14]

Considering that accumulating evidence now suggests that subjects with IR exhibit increased concentrations of PCSK9,[15-21] one can propose that PCSK9 contributes to the overaccumulation of apoB-48-containing lipoproteins by enhancing their secretion and reducing their clearance in IR subjects. However, the extent to which PCSK9 concentrations influence TRL apoB-48 metabolism has been mostly evaluated in limited samples of healthy humans and remains to be thoroughly characterized in the IR state.[8,14]

The general objective of the present study was to gain further insight on the association between PCSK9 and intestine-derived TRL metabolism in humans. More specifically, the relationship between total plasma concentrations of PCSK9 and TRL apoB-48 production rate (PR), fractional catabolic rate (FCR) and pool size (PS) was evaluated in a large sample of men with various degrees of IR. We hypothesized that total plasma PCSK9 concentrations are positively associated with TRL apoB-48 PS and PR and inversely associated with TRL apoB-48 FCR. We also evaluated the association between the intestinal expression of *PCSK9* and the expression of other key genes involved in cholesterol and chylomicron metabolism. We hypothesized that *PCSK9* intestinal mRNA levels are positively associated with the intestinal expression of *apoB* and microsomal triglyceride transfer

protein (*MTP)*, as previous studies on intestinal Caco-2/15 cells demonstrated that PCSK9 has a direct transcriptional effect on intestinal expression of these two genes.[10,11]

Material and methods

This study was a cross-sectional analysis of data from male subjects who participated in in vivo tracer kinetic studies in our laboratory.[22-30] Only data from subjects who were either on no particular treatment,[22, 28] a control diet[26, 27, 29] or on a placebo[23-25, 30] in the previous studies were used for the present analyses. These studies were approved by the Laval University Ethical Review Committee, and written consent was obtained from all subjects.

Study Subjects

Male subjects (n=148) with various degrees of IR compose the current sample. None of the subjects had symptomatic cardiovascular disease, monogenic hyperlipidemia, an acute inflammatory state (evidenced by the presence of fasting CRP levels > 10 mg/dL),[30] type 1 diabetes, insulin therapy, acute hepatic or renal disease, cancer history, uncontrolled arterial hypertension, and recent history of drug or alcohol abuse. Subjects with type 2 diabetes (T2D) (n=28), as defined by the American Diabetes Association,[31] were receiving stable dose of metformin for at least 3 months prior to the clinical assessments. All subjects were withdrawn from lipid-lowering medication for at least 6 weeks prior to kinetic studies.

Biochemical measurements

Fasting blood samples were collected after a 12 hour fast prior to the beginning of the kinetic study in tubes containing disodium EDTA and benzamidine (0.03%).[32] Blood lipids were measured using enzymatic methods and ultracentrifugation as previously described.[33] Glucose levels were measured using colorimetry, and insulin levels were examined using electrochemiluminescence (Roche Diagnostics, Indianapolis, IN, USA). Commercial enzyme-linked immunosorbent assay kits were used to measure plasma levels of CRP (Biocheck Inc., Foster City, CA, USA) and PCSK9 (Circulex, CycLex, Nagano, Japan).

Experimental Protocol for In Vivo Stable Isotope Kinetic Study

Subjects underwent a primed constant infusion of L-[5,5,5-D_3]leucine while they were maintained in a constant fed state. Starting at 7:00 AM, the subjects received 30 small, identical snacks every half hour for 15 hours, each containing 1/30th of their estimated daily food intake based on the Harris-Benedict equation. The following three types of snacks were used during the experimental protocol: 1) low-fat (22.4% of total caloric intake from fat), 2) moderate-fat (35.1% of total caloric intake from fat) or 3) high-fat (41.1% of total caloric intake from fat). At 10:00 AM, L-[5,5,5-D_3]leucine (10 μmol/kg body weight) was injected as a bolus intravenously and then by continuous infusion (10 μmol/kg body

weight/h) over a 12 h period. Blood samples were collected at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 11, and 12 h.

Quantification and Isolation of apoB-48

In 105 subjects, VLDL apoB-100 and TRL apoB-48 were isolated from plasma by ultracentrifugation (d < 1.006 g/mL). VLDL apoB-100 and TRL apoB-48 were subsequently separated using SDS-PAGE according to standardized electrophoresis procedures. Densitometry was used to measure the relative proportion of apoB-48.[34,35] Three different time points were scanned to estimate the average concentration of apoB-48 and to confirm steady states.

In 43 subjects, TRL apoB-48 concentration was determined using a noncompetitive enzyme-linked immunosorbent assay kit (Shibayagi Co. Ltd., Gunma, Japan).[36] The assay was calibrated according to manufacturer's instructions. The within assay variation was 3.5% and the between assay variation was 2.8-8.6%. Three different time points during the infusion protocol were also used to estimate the average concentration of apoB-48 and to confirm steady states.

The two methods are well correlated (r=0.59, P<0.01).[38] Sensitivity statistical analyses were conducted to confirm that current observations were not modified by the method used to quantify apoB-48.

Isotopic Enrichment Determinations

The isotopic enrichment of leucine in apoB-48 was determined using gas chromatography-mass spectrometry in 105 subjects (including n=12 with T2D) and using liquid-chromatography with multiple reaction monitoring in 43 subjects (including n=16 with T2D). These two procedures have been previously described and are highly correlated (r=0.99, P<0.0001).[30] Sensitivity statistical analyses were conducted to confirm that current observations were not modified by the method used to determine the isotopic enrichment of leucine in apoB-48.

Kinetic Analysis

The TRL apoB-48 FCR (pools/d) was derived using a multi-compartmental model previously described.[39] We assumed a constant enrichment of the precursor pool and used the TRL apoB-48 plateau tracer/tracee ratio data as the forcing function to drive the appearance of tracer into apoB-48.[39] Assuming that each subject remains in steady state with respect to apoB-48 metabolism during the study as previously shown,[39] the FCR is equivalent to the fractional synthetic rate. The apoB-48 PR was determined using the following formula: PR (mg/kg/d) = [FCR ● apoB-48 concentration (mg/dL) ● plasma volume (L)] / body weight (kg).[40] The plasma volume was estimated at 4.5% of body weight. The SAAM II program (SAAM Institute, Seattle, WA, USA) was used to fit the model to the observed tracer data.

Intestinal biopsies and extraction and quantification of total RNA

In a subgroup of 71 nondiabetic subjects, duodenal biopsies were collected from the second portion of the duodenum during gastroduodenoscopy after a 12 h fast in a delay ranging from 24 to 48 h of the kinetic study. The procedure was conducted in the fasting state and limited to the second portion of the duodenum to avoid complications (e.g. pulmonary aspiration, perforation).

Samples (3 x 3 mm) were collected using single-use biopsy forceps, immediately flash frozen in liquid nitrogen and stored at -80°C before RNA extraction. Intestinal tissue samples were homogenized in 1 mL of Qiazol (Qiagen, Hilden, Germany). RNA was extracted using a RNeasy kit (Qiagen, Hilden, Germany). To eliminate any contaminating DNA, biopsies were treated with RNase-free DNase. Total RNA extraction and quantitative real-time polymerase chain reaction (PCR) were performed using standard procedures as previously described.[27] Primer sequence and gene description are provided in **Supplemental Table 1**. Expression of the house-keeping gene, glucose-6-phosphate-dehydrogenase (*G6PD*), was used as reference. Quantitative real-time PCR measurements were performed by the CHU de Québec-Université Laval Research Center Gene Expression Platform (Quebec, Canada).

Minimal detectable association calculation

Minimal detectable association calculation was first conducted on the expected association between plasma PCSK9 concentrations and TRL apoB-48 FCR as the primary outcome. Our calculation indicated that a sample size of 148 subjects would allow us to detect a correlation coefficient of 0.232 or greater with a power of 80% at a two-sided 0.05 significance level, with the conservative assumption that the standard deviation of plasma PCSK9 concentrations and TRL apoB-48 FCR is 50% of the mean. This calculation is consistent with the previous study by Chan et al.[14] where an inverse association between PCSK9 levels and TRL apoB-48 FCR (standard β: -0.589) was observed in 17 obese subjects.

Using similar assumption, calculation on the expected association between intestinal mRNA of *PCSK9* and *MTP* was also conducted. A sample size of 71 subjects would allow us to detect a correlation coefficient of 0.337 or greater with a power of 80% at a two-sided 0.05 significance level. No data from previous human study was available to compare with this calculation.

Statistical Analyses

Statistical analyses were conducted using SAS software (Studio University Edition v3.5, SAS Institute Inc., Cary, NC, USA). Non-normally distributed variables were transformed and normalized prior to Pearson's correlation analyses. The adaptive Holm-Bonferroni method was used to adjust Pearson's correlation *P* values for multiple testing. This sequential stepdown approach is a rigorous and recognized technique to control for a family-wise error rate.[41] The method ensures that the probability

of ≥1 false discovery under the null hypothesis is fixed at the α level while maintaining high statistical power. *P* values are adjusted and evaluated against α<0.05.

Backward stepwise multiple linear regression models were used to evaluate independent associations between plasma PCSK9 levels and TRL apoB-48 FCR, PR and PS and between intestinal mRNA level of PCSK9 and mRNA levels of key genes involved in cholesterol and chylomicron metabolism. For models with kinetic parameters as dependent variable, sensitivity analyses were conducted on selected models by adjusting for the method used to quantify apoB-48 and to determine the isotopic enrichment of leucine in apoB-48 in order to validate that results are independent of these methods. For models with intestinal gene expression as dependent variable, the presence of multicollinearity among independent variables was tested by calculating the tolerance and the variance inflation factor.

Statistical analyses on intestinal gene expression data (Pearson's correlations and multiple linear regression models) were systematically adjusted for the intestinal mRNA level of the *G6PD* house keeping gene to limit the heterogeneity between duodenal samples.

Results

Anthropometric and fasting biochemical characteristics of the subjects are presented in **Table 1**. Subjects exhibited features of IR with obesity, high homeostasis model assessment of IR (HOMA-IR) index, hypertriglyceridemia and low HDL-C concentrations as a group. Fasting total plasma PCSK9 concentrations were positively correlated with body mass index (BMI) (r=0.19, *P*=0.05) and fasting plasma levels of LDL-C (r=0.36, *P*=0.0003) (**Figure 1**). No association was observed between PCSK9 concentrations and age (r=0.07, *P*=0.4), TG (r=0.19, *P*=0.07), HDL-C (r=0.01; *P*=0.9), insulin (r=0.10, *P*=0.2) and glucose concentrations (r=0.09, *P*=0.3) as well as the HOMA-IR index (r=0.11, *P*=0.2).

Among the 148 subjects, total plasma PCSK9 concentrations were positively correlated with TRL apoB-48 PS (**Figure 2**). PCSK9 concentrations were not associated with TRL apoB-48 FCR but were positively associated with TRL apoB-48 PR (**Figure 2**).

These associations were further confirmed using backward stepwise multiple linear regression analyses (**Table 2**). Fasting PCSK9 concentrations were found to be a significant correlate of TRL apoB-48 PS (standard β=+0.15, *P*=0.006) and PR (standard β=+0.20, *P*=0.007) independent of fasting levels of TGs, insulin, glucose, LDL-C, CRP, BMI, age, T2D/therapy with metformin, and the fat content of the kinetic snacks. The association between PCSK9 concentrations and TRL apoB-48 PR was not modified by insulin levels ($P_{\text{interaction PCSK9 * insulin}}$=0.7), T2D/therapy with metformin ($P_{\text{interaction PCSK9 * T2D}}$=0.6) or LDL-C concentrations ($P_{\text{interaction PCSK9 * LDL-C}}$=0.4). In the selected model, subsequent adjustment for the method used to quantify apoB-48 and to determine the isotopic

enrichment of leucine in apoB-48 slightly attenuated the association between PCSK9 and TRL apoB-48 PR without altering its significance (PCSK9 standard β=+0.16, *P*=0.3).

Pearson's correlation tests showed that intestinal mRNA levels of *PCSK9* were not correlated with age (r=-0.18, *P*=0.9), BMI (r=-0.21, *P*=0.6), HOMA-IR (r=0.03, *P*=0.9), plasma concentrations of insulin (r=-0.01, *P*=0.9), glucose (r=0.17, *P*=0.9), TGs (r=-0.02, *P*=0.9), LDL-C (r=-0.16, *P*=0.9), HDL-C (r=-0.08, *P*=0.9) and PCSK9 (r=0.13, *P*=0.9) (Figure 1). However, intestinal mRNA levels of *PCSK9* were positively correlated with the intestinal expression of sterol regulatory element-binding protein 2 (*SREBP2*) (r=0.35, *P*=0.003), hydroxy-methyl-glutaryl-CoA reductase (*HMG-CoAR*) (r=0.43, *P*=0.002), *LDLR* (r=0.51, *P*=0.0004) and acetyl-CoA acetyltransferase 2 *(ACAT2*) (r=0.74, *P*<0.0001) (**Figure 3**). Inverse association was observed between the intestinal expression of *PCSK9* and Niemann-Pick C1-like 1 (*NPC1L1*) (r=-0.33, *P*=0.006), and no association was found with the expression of *MTP* (r=0.11, *P*=0.4), *ApoB* (r=-0.14, *P*=0.2) or SAR1 gene homolog B (*SAR1B*) (r=0.14, *P*=0.2).

Stepwise backward multiple linear regression analysis showed that the intestinal expression of *PCSK9* was a significant correlate of the intestinal expression of *HMG-CoAR* (standard β=+0.17, *P*=0.02), *ACAT2* (standard β=+0.55, *P*<0.0001), *LDLR* (standard β=+0.24, *P*=0.007), and *NPC1L1* (standard β=-0.56, *P*<0.0001), independent of the expression of *SREBP2*, plasma levels of TGs, insulin, glucose, CRP and LDL-C, BMI and age (**Table 3**).

Intestinal mRNA levels of *PCSK9* were not associated with TRL apoB-48 PS (r=0.09, *P*=0.5) or PR (r=-0.11, *P*=0.3), but an inverse association with TRL apoB-48 FCR was found (r=-0.24, *P*=0.04). The association between intestinal mRNA levels of *PCSK9* and TRL apoB-48 FCR was not significant (*P*=0.5) following backward stepwise multiple linear regression analysis including plasma levels of TGs, insulin, glucose and CRP, BMI, age and the fat content of the kinetic snacks as independent covariates (data not shown).

Discussion

The association between PCSK9 and TRL apoB-48 metabolism was assessed in a large sample of men displaying various degrees of IR. TRL apoB-48 kinetics were measured using a primed constant infusion of deuterated leucine while subjects were maintained in a constant fed state. The intestinal expression of *PCSK9* and other key genes involved in cholesterol and chylomicron metabolism was assessed in duodenal biopsies obtained in the fasting state. Fasting PCSK9 concentrations were not associated with TRL apoB-48 FCR but were independent positive correlates of TRL apoB-48 PR and PS. The intestinal expression of *PCSK9* was positively associated with genes involved in *de novo* cholesterol synthesis (*HMG-CoAR* and *ACAT-2*) and the uptake of cholesterol-rich lipoproteins

(*LDLR*). This study demonstrated that PCSK9 is associated with intestinal secretion and plasma overaccumulation of TRL apoB-48 in men displaying various degrees of IR.

The identification of determinants of the secretion and the clearance of intestinal TRLs are key issues to develop effective therapy targeting the overaccumulation of these atherogenic particles in IR subjects. Since the first identification of PCSK9 in the early 2000s, accumulating evidence suggests that the role of this proprotein in lipoprotein metabolism goes far beyond the lysosomal intracellular degradation of LDLR.[42] In that regard, previous in vitro and animal studies have reported a relationship between PCSK9 and intestinal and hepatic apoB-containing lipoprotein secretion.[10, 11, 13, 43] To our knowledge, the current study is the first to report a positive association between plasma PCSK9 concentrations and intestinal TRL secretion in humans. This finding may involve a local effect of intestinal-derived PCSK9 or, alternatively, a downstream effect of circulating PCSK9, which is mainly derived from liver.[44]

In intestinal Caco-2/15 cells incubated with oleic acid, treatment with PCSK9 increased cellular and secreted apoB-48.[10, 11] More specifically, PCSK9 was shown to upregulate mRNA expression and protein levels of both apoB-48 and MTP, leading to chylomicron secretion.[10, 11] In the current study, no relationship was found between intestinal mRNA levels of *PCSK9, apoB* or *MTP*, and between fasting intestinal *PCSK9* expression and TRL apoB-48 PR. Considering the important inhibitory effect of insulin on the intestinal expression of *MTP*, one cannot exclude that the effect of PCSK9 on the expression of *MTP* was blunted by hyperinsulinemia in the fasting state in the present study.[28] Moreover, it is likely that the effect of PCSK9 on intestinal expression of *MTP* and *apoB* occurs mainly in the postprandial state as protein levels of PCSK9 and other lipogenic enzymes are highly modulated by feeding.[45, 46] However, this specific aspect of chylomicron metabolism remains challenging to evaluate in humans as conducting gastroduodenoscopy in the postprandial state exposed patients to pulmonary aspiration. Thus, although we observed a positive association between plasma PCSK9 levels and TRL apoB-48 PR in the current sample of men displaying various degrees of IR, we could not relate this relationship to a direct upregulation of intestinal expression of *apoB* or *MTP* mediated by intestinal-derived PCSK9.

Another mechanism underlying the stimulatory effect of PCSK9 on apoB-48 secretion could be related to a downstream impact of circulating PCSK9 on intestinal LDLR abundance.[10, 11] In Caco-2/15 cells, it was observed that the intracellular cholesterol depletion induced by PCSK9-mediated LDLR degradation stimulates cholesterol uptake from the intestinal lumen and activates chylomicron synthesis and secretion pathways.[10, 11] In humans, PCSK9 inhibition with evolocumab has no or modest effect on the fractional enterocytic cholesterol absorption measured in the fasting state.[9, 47] In the present study, the intestinal expression of *PCSK9* was negatively associated with the intestinal expression of *NPC1L1* and positively associated with *ACAT2* expression, two proteins critically

involved in intestinal cholesterol absorption.[48, 49] It remains unclear if the positive association between plasma PCSK9 and TRL apoB-48 secretion is related to PCSK9-stimulated intestinal cholesterol absorption in the current sample of men with various degrees of IR.[10, 11] Nonetheless, one must keep in mind that NPC1L1 and ACAT2 are both regulated by intra-cellular cholesterol content.[50, 51] In this context, we cannot exclude that the hypothesis where PCSK9 stimulates TRL apoB-48 secretion via enhanced intestinal cholesterol absorption may be more fully manifested in the fed state. Finally, previous report from Caco-2 cells suggested that PCSK9-associated reduction of intestinal LDLR abundance stimulates endogenous cholesterol synthesis.[52] On the one hand, studies conducted with evolocumab in insulin-sensitive healthy humans have reported only modest effects of PCSK9 on cholesterol synthesis.[9, 47] On the other hand, intestinal mRNA levels of *PCSK9* were positively correlated with intestinal mRNA levels of *HMG-CoAR* in the present study. In this context, one can propose that the positive association between plasma PCSK9 concentrations and apoB-48-containing TRL secretion observed in the current study is associated with an increased endogenous intestinal cholesterol synthesis.[9, 47] This remains only a hypothesis at this stage. In sum, although exact mechanisms remain unclear, the present study provides evidence demonstrating a positive association between total plasma PCSK9 concentrations and TRL apoB-48 PR in men with various degrees of IR.

One could hypothesize that the association between plasma PCSK9 levels and TRL apoB-48 PR was derived from the binding interaction between PCSK9 and apoB-48 in the circulation, considering that plasma PCSK9 levels correlated with TRL apoB-48 PR and PS but not FCR, and that TRL apoB-48 PR is calculated from the PS. The binding interaction of PCSK9 within the N-terminal region of apoB, i.e. apoB-18, has been previously investigated.[13, 43, 53] However, Kosenko et al.[53] demonstrated that PCSK9 binds to apoB in LDLs but not in TG-rich VLDLs. In VLDLs, some epitopes of the apoB protein moiety are masked by lipids and not accessible.[53, 54] In this context, the construct where the association between plasma PCSK9 levels and TRL apoB-48 PR is derived from the binding interaction between PCSK9 and apoB-48 remains unlikely. The current data points toward an association between PCSK9 and chylomicron secretion, even though the binding interaction between PCSK9 and apoB-48 in intestinal TRLs remains to be thoroughly evaluated.

Both newly synthesized intracellular PCSK9 and secreted PCSK9 promote the intracellular lysosomal degradation of lipoprotein receptors, mainly LDLR as well as VLDLR and LRP-1.[6,7,52] It is well established that chylomicron remnants are primarily cleared from circulation by hepatic LDLRs.[53] In the absence of LDLR, the uptake and endocytosis of chylomicron remnants are alternatively mediated by LRP-1 or by binding with hepatic lipase independent of hepatic receptors or apoE.[53] Chan et al.[14] provided the first data suggesting that PCSK9 is involved in TRL apoB-48 clearance in humans. More specifically, these authors observed that plasma PCSK9 concentrations are inversely associated with TRL apoB-48 FCR in 17 obese subjects.[14] we found no association between total plasma

concentrations and intestinal expression of PCSK9 and TRL apoB-48 FCR in the sample of 148 male subjects with various degrees of IR in the present study. These observations suggest that alternative pathways involved in the clearance of intestinal lipoproteins effectively compensate the PCSK9-induced reduction in LDLR abundance. It is also plausible that alterations in lipoprotein clearance induced by IR are more important than those induced by PCSK9 in mediating apoB-48-containing lipoprotein clearance.[4] Overall, the current observations do not support a major role of PCSK9 in TRL apoB-48 clearance in IR men.

It has been recently reported that alirocumab, a PCSK9 monoclonal antibody, has no impact on the postprandial TG and apoB-48 response in healthy subjects.[8] In another study, subjects with loss-of-function mutation in the *PCSK9* gene exhibited lower postprandial lipemia than normal subjects.[58] In a third study conducted in patients with familial hypercholesterolemia, no association was found between plasma levels of PCSK9 and apoB-48, although LDL-R deficiency is associated with increased plasma concentrations of these two proteins.[59] Finally, the current study supports the notion that plasma PCSK9 is associated with the overaccumulation of TG-rich particles in men displaying different degrees of IR. Discrepancies between these studies conducted in different types of patients highlight that intra- and extracellular PCSK9 may have distinct effects on intestinal TRL metabolism and that PCSK9 effect on TRL metabolism may be dependent on the abundance or the activity of LDLR.[60, 61] These elements needs to be investigated to elucidate the relationship between PCSK9 and intestinal TRL metabolism and to delineate the presence of a causality between high plasma PCSK9 levels and exaggerated postprandial lipemia.[60]

The main strength of this study was that we beneficiated from our long-standing investigations on intestinal lipoprotein metabolism to conduct the present assessment using a large sample size of subjects that provided important statistical power. The main limitation of the study was that two methods were used for the estimation of TRL apoB-48 kinetics. Although densitometry is a standardized method, this procedure remains less precise than ELISA to determine apoB-48 concentrations. Nonetheless, we stress that the two methods are highly correlated.[27, 30, 38] and that statistical analyses were conducted to confirm that current observations were not modified by the method used to determine the isotopic enrichment of leucine in apoB-48. Another important limitation was that duodenal biopsies were collected in the fasting state. Gastroduodenoscopy remains an invasive procedure that may be conducted, at best, few hours after ingestion of a meal. We also acknowledge that total plasma PCSK9 concentrations do not directly reflect PCSK9 biological activity.[42] Approximately 40% of circulating PCSK9 is bind to LDLs and this binding interaction reduces PCSK9 activity.[42] Another important proportion of plasma PCSK9 is cleaved and inactivated by hepatic furins.[42] The diverse sources of plasma PCSK9 (liver, gut, and kidney) potentially also blunted associations with intestinally derived lipoproteins.[7] Finally, the generalization of the results is

also limited by the fact that only men were included in the present study. A similar assessment in women is required.

In conclusion, plasma PCSK9 levels are associated with the secretion rate, but not the clearance, of intestinal lipoproteins in men displaying various degrees of IR. It is suggested that PCSK9 contributes to the overaccumulation of intestinal TRLs in IR subjects primarily by stimulating the secretion of these particles, although exact mechanisms remain unclear. Extensive investigations on the impact of PCSK9 inhibition on TRL apoB-48 metabolism in IR subjects are required to corroborate the present observations.

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Disclosures

All authors declare that they have no relevant conflicts of interest.

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Tables

Table 16-1 Anthropometric and fasting biochemical characteristics of the subjects (n=148)

Characteristics		Range
Age (y)	42.3 ± 12.3	21.0 - 64.0
Weight (kg)	94.5 ± 15.4	66.4 - 140.1
BMI (kg/m^2)	30.5 ± 4.3	22.0 - 42.5
Insulin (ρmol/L)	120 ± 66	18 - 403
Glucose (mmol/L)	5.8 ± 1.5	3.9 - 11.5
HOMA-IR	4.7 ± 3.8	0.5 - 29.7
Type 2 diabetes,		
Yes	28 (18.9)	
No	120 (81.1)	
Total-C (mmol/L)	5.14 ± 0.83	3.15 - 7.72
TG (mmol/L)	2.08 ± 1.08	0.42 - 5.62
LDL-C (mmol/L)	3.27 ± 0.76	1.60 - 5.67
HDL-C (mmol/L)	1.04 ± 0.22	0.60 - 1.69
Total-C/HDL-C ratio	5.15 ± 1.24	2.28 - 8.78
CRP (mg/L)	2.91 ± 2.35	0.03 - 9.68
PCSK9 (ng/mL)	284 ± 76	144 - 527

[1] Data are presented as the mean ± standard deviation of the mean or frequency (percent). Range is presented as minimum - maximum. [2] BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; C: cholesterol; TG: triglyceride; CRP: C-reactive protein; PCSK9: proprotein convertase subtilisin/kexin type 9.

Table 16-2 Significant independent predictors of TRL apoB-48 kinetics

Dependent variables	Independent variables	Determination coefficient (R^2)	Standard β	*P*
TRL apoB-48 PS	Fasting TG concentrations	45.9	+0.60	<0.0001
	Fat content of the kinetic snacks	7.4		
	Low fat vs high fat		-0.23	<0.0001
	Moderate fat vs high fat		+0.00	0.9
	BMI	4.9	+0.20	0.0004
	Plasma PCSK9 concentrations	2.9	+0.15	0.006
	Total	61.1		<0.0001
TRL apoB-48 FCR	Fasting TG concentrations	14.0	-0.39	<0.0001
	Fat content of the kinetic snacks	10.0		
	Low fat vs high fat		-0.26	0.0006
	Moderate fat vs high fat		+0.19	0.01
	Total	23.9		<0.0001
TRL apoB-48 PR	Fat content of the kinetic snacks	17.7		
	Low fat vs high fat		-0.36	<0.0001
	Moderate fat vs high fat		+0.14	0.048
	Fasting TG concentrations	9.2	+0.30	<0.0001
	Plasma PCSK9 concentrations	3.9	+0.20	0.007
	Total	30.9		<0.0001

[1] Backward stepwise multiple linear regression analyses were conducted among the 148 subjects of the study. Models initially included fasting concentrations of PCSK9, TGs, insulin, glucose, CRP, LDL-C, BMI, age, fat content of the kinetic snacks, and type 2 diabetes as independent variable. [2] TRL: triglyceride-rich lipoprotein, apo: apolipoprotein, PR: production rate, TG: triglyceride, PCSK9: proprotein convertase subtilisin/kexin type 9, FCR: fractional catabolic rate, PS: pool size.

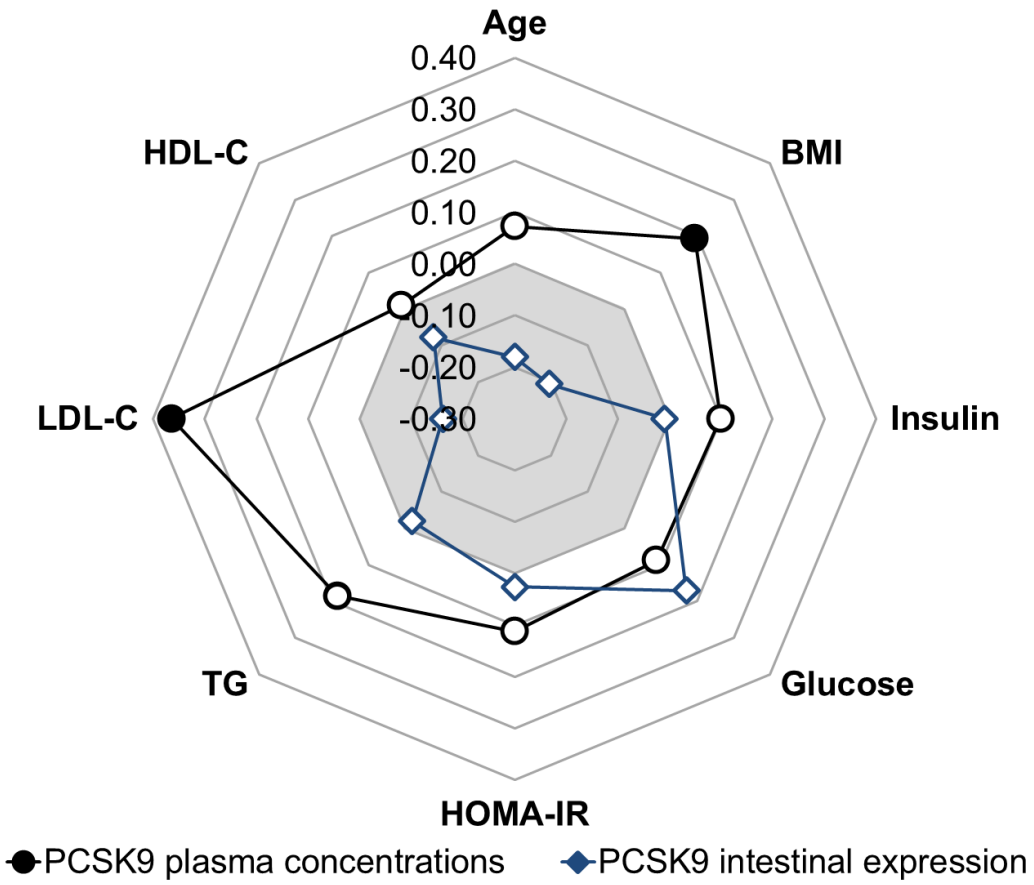
Table 16-3 Independent correlates of the intestinal expression of key genes involved in cholesterol and chylomicron metabolism

Dependent variables	Independent variables	Determination coefficient (R^2)	Standard β	*P*
HMG-CoAR expression	*SREBP2* expression	62.3	+0.78	<0.0001
	Plasma TG concentrations	3.5	+0.18	0.01
	PCSK9 expression	2.9	+0.17	0.02
	Total	68.7		<0.0001
ACAT2 expression	*PCSK9* expression	39.0	+0.55	<0.0001
	SREBP2 expression	18.2	+0.38	<0.0001
	G6PD expression	15.2	-0.35	<0.0001
	Plasma insulin concentrations	2.5	+0.14	0.03
	Total	75.0		<0.0001
LDLR expression	*SREBP2* expression	27.1	+0.51	<0.0001
	G6PD expression	21.1	+0.44	<0.0001
	PCSK9 expression	5.8	+0.24	0.007
	BMI	3.1	-0.17	0.04
	Total	57.2		<0.0001
NPC1L1 expression	*G6PD* expression	28.3	+0.68	<0.0001
	PCSK9 expression	17.7	-0.56	<0.0001
	SREBP2 expression	14.4	+0.51	<0.0001
	Total	60.4		<0.0001

[1] Backward stepwise multiple linear regression analyses were conducted among the 71 subjects of the study who provided a duodenal biopsy. Models initially included the intestinal expression of *SREBP2*, *PCSK9* and *G6PD* (reference gene), plasma concentrations of TGs, insulin, glucose, CRP, LDL-C, BMI and age as independent variables. [2] *HMG-CoAR*: hydroxy-methyl-glutaryl-CoA reductase; *SREBP2*: sterol regulatory element-binding protein 2; TG: triglyceride; *PCSK9:* proprotein convertase subtilisin/kexin type 9; *LDLR:* LDL receptor; *G6PD*: glucose-6-phosphate dehydrogenase: BMI: body mass index; *NPC1L1:* Niemann–Pick C1-like 1*ACAT2:* acetyl-CoA acetyltransferase 2.

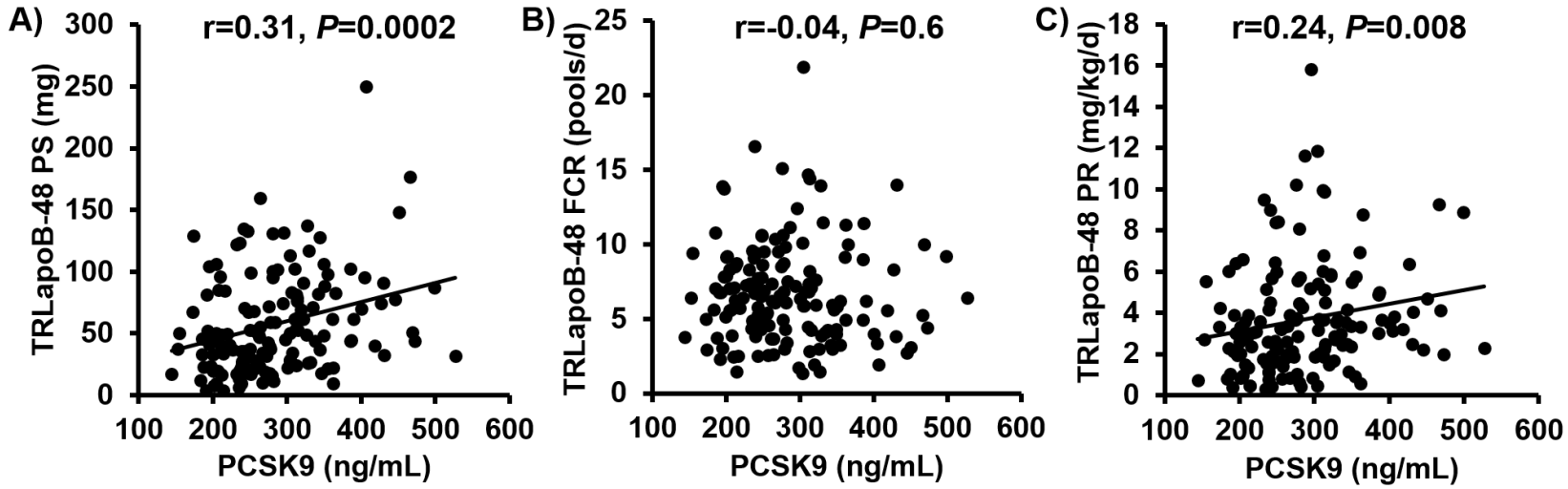
Figures

Figure 16-1 Radar plot presenting the associations between PCSK9 and characteristics of the subjects



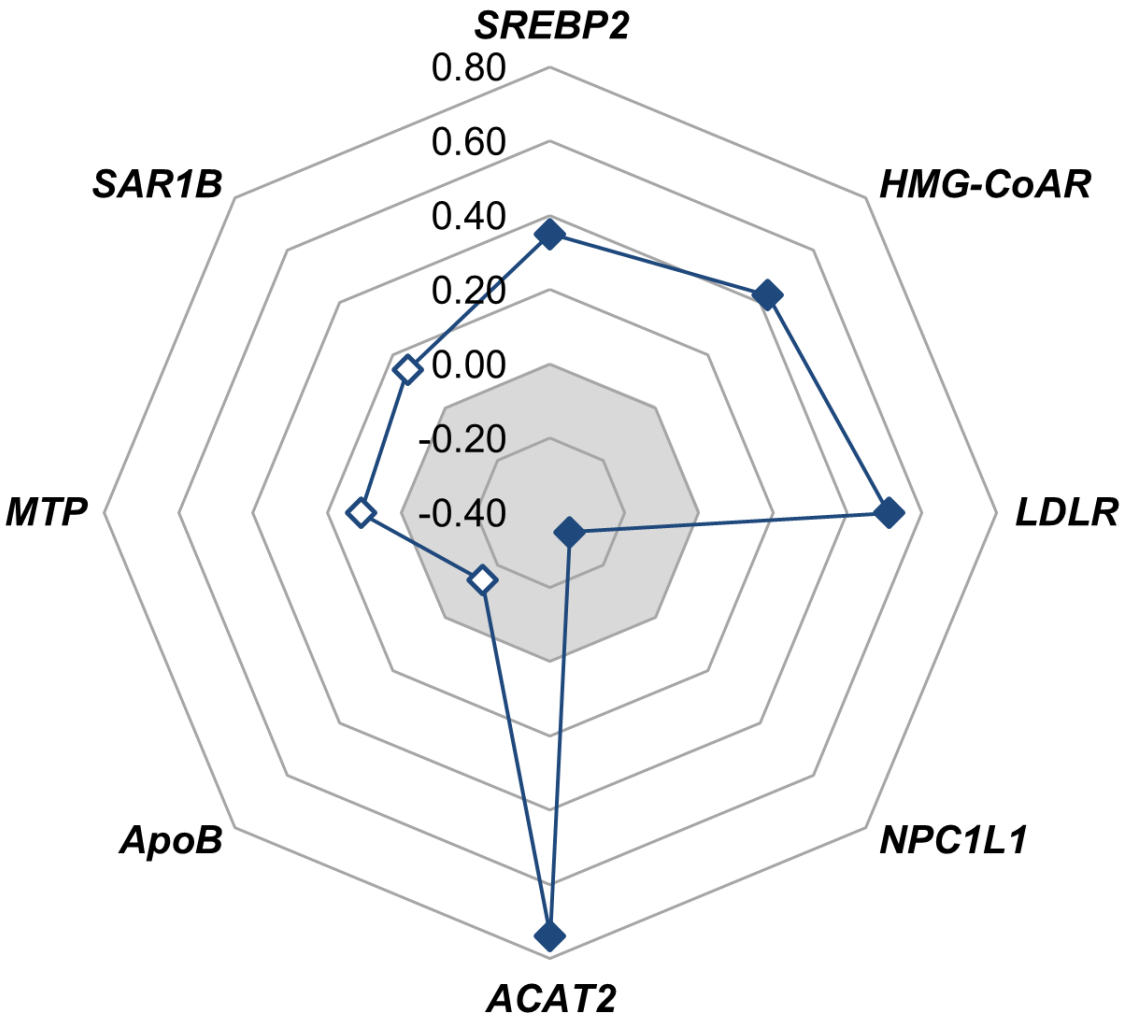
Radar plot presenting the associations between PCSK9 (●: plasma concentrations, n=148; ♦: intestinal expression, n=71) and anthropometric and fasting biochemical characteristics of the subjects. Radar lines represent Pearson's correlation coefficient. Correlations between intestinal expression of *PCSK9* and anthropometric and fasting biochemical characteristics of the subjects were adjusted for the expression of the reference gene *G6PD*. All *P* values were adjusted for multiple testing using the adaptive Holm-Bonferroni method. Black-filled marks identify significant association ($P<0.05$), and white-filled marks represent non-significant association ($P>0.05$). BMI: body mass index; C: cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; TG: triglyceride; PCSK9: proprotein convertase subtilisin/kexin type 9.

Figure 16-2 Correlations between fasting PCSK9 concentrations and TRL apo B-48 kinetic parameters



Pearson's correlations between fasting PCSK9 concentrations and postprandial TRL apoB-48 A) pool size (PS), B) fractional catabolic rate (FCR), and C) production rate (PR) in n=148 men with various degrees of insulin resistance. *P* values were adjusted for multiple testing using the adaptive Holm-Bonferroni method.

Figure 16-3 Radar plot presenting the associations between the intestinal expression of PCSK9 and intestinal expression of key genes involved in cholesterol and chylomicron metabolism in n=71 men



Radar lines represent Pearson's correlation coefficient. Correlations were adjusted for the expression of the reference gene *G6PD*. *P* values were adjusted for multiple testing using the adaptive Holm-Bonferroni method. Blue-filled marks identify significant association ($P<0.05$), and white-filled marks represent non-significant association. *SREBP2*: sterol regulatory element-binding protein 2; *HMG-CoAR*: hydroxy-methyl-glutaryl-CoA reductase; *LDLR:* LDL receptor; *NPC1L1:* Niemann–Pick C1-like 1; *ACAT2:* acetyl-CoA acetyltransferase 2; *ApoB*: apolipoprotein B; *MTP*: microsomal triglyceride transfer protein; *SAR1B*: SAR1 gene homolog B.

Supplemental material

Supplemental table 16-1 Sequence primers and gene description

Gene Symbol	Description	GenBank	size (pb)	Primer sequence 5'→3' S/AS
ACAT2	Homo sapiens acetyl-Coenzyme An acetyltransferase 2 (ACAT2), 2 transcripts	NM_005891	267	CTGTGGCTCCGGAAGATGTGT/CTCCTGTTCTCAAGTAAGCCAAGTG
APOB	Homo sapiens apolipoprotein B (APOB)	NM_000384	274	CTGCGCAACGAGATCAAGACA/CATGCTGGGAATCGACTTGTGA
G6PD	Homo sapiens glucose-6-phosphate dehydrogenase (G6PD), nuclear gene encoding mitochondrial protein	NM_000402	121	GATGTCCCCTGTCCCACCAACTCTG/GCAGGGCATTGAGGTTGGGAG
HMGCoAR	Homo sapiens 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), 2 transcripts	NM_000859	195	GGGACCAACCTACTACCTCAG/CGACCTGTTGTGAATCATGTGACTT
LDLR	Homo sapiens low density lipoprotein receptor (LDLR)	NM_000527	193	GCCGTAAGGACACAGCACACAACC/GGAGCACGATGGGGAGGACAAT
MTP	Homo sapiens microsomal triglyceride transfer protein (MTTP), 2 transcripts	NM_000253	210	CAGGGTGGTCTAGCTATTGATATTTC/TGGGTACTGAGAAAACTGCACTGT
NPC1L1	Homo sapiens NPC1-like 1 (NPC1L1), 2 transcripts	NM_013389	273	GCTGCTGTTTCTCGCCCTGTT/GGGAACTCTGTGGCATACTGGATCT
PCSK9	Homo sapiens proprotein convertase subtilisin/kexin type 9 (PCSK9), 2 trancripts	NM_174936	172	CAGGGGAGGACATCATTGGTG/TTGGCAGAGAAGTGGATCAGTC

SAR1B	Homo sapiens SAR1 homolog B (S. cerevisiae)	NM_016103	203	TGGCATTGTATTTCTGGTGGATTGTG/TCCCCTTTCCTGTTGTCTGACCATATA
SREBP2	Homo sapiens sterol regulatory element binding transcription factor 2 (SREBF2), 2 transcripts	NM_004599	206	AGGAGAAAGGCGGACAACCCATAATA/CCAGCTTCAGCACCATGTTCTC
ADNg	Homo sapiens 3-beta-hydroxysteroid dehydrogenase/delta-5-delta-4-isomerase (3-beta-HSD) gene (intron)	M38180	260	GAAGGGCAGAGGTGGAACTAGAA/AACAAAGACCAAAGACCAGTGAGA