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

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Abstract Intestinal triglyceride (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 the 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 β = +0.20, P = 0.007) independent of BMI, age, T2D/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.—Drouin-Chartier, J-P.; Tremblay, A. J.; Hogue, J. C.; Lemelin, V.; Lamarche, B.; and Couture, P. Plasma PCSK9 correlates with apoB-48-containing triglyceride-rich lipoprotein production in men with insulin resistance. J. Lipid Res. 2018. 59: 1501–1509.

Supplementary key words proprotein convertase subtilisin/kexin type 9 • apoB-apoB • cholesterol

Intestinal triglyceride (TG)-rich lipoproteins (TRLs) are important contributors of postprandial hypertriglyceridemia, a major cardiovascular disease risk factor (1). Although chylomicrons are too large to enter the subendothelial space, once hydrolyzed, cholesterol-rich chylomicron remnants are small enough to penetrate the subendothelial 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 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), whereas 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 has corroborated these observations so far (8, 14).

Considering that accumulating evidence now suggests that subjects with IR exhibit increased concentrations of

Abbreviations: CRP, C-reactive protein; FCR, fractional catabolic rate; G6PD, glucose-6-phosphate-dehydrogenase; HDL-C, HDL cholesterol; HMG-CoAR, HMG-CoA reductase; HOMA-IR, homeostasis model assessment of insulin resistance; IR, insulin resistance; LDL-C, LDL cholesterol; LDLR, LDL receptor; LRP-1, LDL receptor-related protein 1; MTP, microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick Cl-like 1; PCSK9, proprotein convertase subtilisin/kexin type 9; PR, production rate; PS, pool size; SARC1, SARC1 gene homolog B; TG, triglyceride; TRL, triglyceride-rich lipoprotein.

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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 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).

MATERIALS 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 C-reactive protein (CRP) levels > 10 mg/dl) (31), T1D, insulin therapy, acute hepatic or renal disease, cancer history, uncontrolled arterial hypertension, or recent history of drug or alcohol abuse. Subjects with T2D (n = 28), as defined by the American Diabetes Association (32), were receiving a 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 h fast prior to the beginning of the kinetic study in tubes containing disodium EDTA and benzamidine (0.03%) (33). Blood lipids were measured using enzymatic methods and ultracentrifugation as previously described (34). Glucose levels were measured using colorimetry, and insulin levels were examined using electrochemiluminescence (Roche Diagnostics, Indianapolis, IN). Commercial ELISA kits were used to measure plasma levels of CRP (Biocheck Inc., Foster City, CA) 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-D3] 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 h, 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-D3]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 (35, 36). 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 by using a noncompetitive ELISA kit (Shibayagi Co. Ltd., Gunma, Japan) (37). 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 GC/MS 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 per day) was derived using a multicompartamental model as 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) 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 or perforation).

Samples (3 × 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). To eliminate any contaminating DNA, biopsies were treated with RNase-free DNase. Total RNA extraction and quantitative real-time PCR were performed using standard procedures as previously described (27).

Primer sequence and gene description are provided in supplemental Table S1. Expression of the housekeeping 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 (Québec, 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 0.292 or greater with a power of 80% at a two-sided 0.05 significance level, with the conservative assumption that the SD 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 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 the α level while maintaining high statistical power. That assumption that the SD of 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 0.292 or greater with a power of 80% at a two-sided 0.05 significance level, with the conservative assumption that the SD 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 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 studies were available to compare with this calculation.

Statistical analyses

Statistical analyses were conducted using SAS software (Studio University Edition version 3.5, SAS Institute Inc., Cary, NC). Nonnormally 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 were 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 a 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 housekeeping gene to limit the heterogeneity between duodenal samples.

RESULTS

Anthropometric and fasting biochemical characteristics of the subjects are presented in Table 1. As a group, subjects exhibited features of IR with obesity, high homeostasis model assessment of IR (HOMA-IR) index, hypertriglyceridemia, and low HDL cholesterol (HDL-C) concentrations. Fasting total plasma PCSK9 concentrations were positively correlated with BMI (r = 0.19, P = 0.05) and fasting plasma levels of LDL cholesterol (LDL-C) (r = 0.36, P = 0.0003) (Fig. 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, Pearson’s correlation tests showed that total plasma PCSK9 concentrations were positively correlated with TRL apoB-48 PS (Fig. 2). PCSK9 concentrations were not associated with TRL apoB-48 FCR, but were positively associated with TRL apoB-48 PR (Fig. 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 (PinteractionPCS9*insulin = 0.7), T2D/therapy with metformin (Pinteraction PCS9*T2D = 0.6) or LDL-C concentrations.

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Fig. 1. Radar plot presenting the associations between PCSK9 (●, PCSK9 plasma concentrations, n = 148; ○, PCSK9 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 nonsignificant association (P > 0.05). C, cholesterol.

Interaction $\beta_{\text{PCSK9} \times \text{LDL-C}} = 0.4$). 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 $\beta = +0.16$, $P = 0.03$).

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 ($r = 0.13$, $P = 0.9$) (Fig. 1). However, intestinal mRNA levels of PCSK9 were positively correlated with the intestinal expression of SREBP2 ($r = 0.35$, $P = 0.003$), HMG-CoA reductase (HMG-CoAR) ($r = 0.43$, $P = 0.002$), LDLR ($r = 0.51$, $P = 0.0004$), and ACAT2 ($r = 0.74$, $P < 0.0001$) (Fig. 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 SARI gene homolog B (SARI8) ($r = 0.14$, $P = 0.2$).

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 $\beta = +0.17$, $P = 0.02$), ACAT2 (standard $\beta = +0.55$, $P < 0.0001$), LDLR (standard $\beta = +0.24$, $P = 0.007$), and NPC1L1 (standard $\beta = -0.56$, $P < 0.0001$), 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 correlated 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 developing 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

### Table 2. Significant independent predictors of TRL apoB-48 kinetics

| Dependent Variables | Independent Variables | Determination Coefficient ($R^2$) | Standard $\beta$ | $p$ |
|---------------------|-----------------------|----------------------------------|-----------------|-----|
| TRL apoB-48 PS      | Fasting TG concentrations | 45.9                            | +0.60           | <0.0001 |
|                     | Fat content of the kinetic snacks | 7.4                       | -0.23           | <0.0001 |
|                     | Low fat vs. high fat     | -0.26                           | 0.0006          | 0.95 |
|                     | Moderate fat vs. high fat | +0.19                           | 0.01            | 0.0001 |
|                     | 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                  | -0.26           | 0.0006 |
|                     | Low fat vs. high fat     | -0.26                           | 0.0006          | 0.95 |
|                     | Moderate fat vs. high fat | +0.19                           | 0.01            | 0.0001 |
|                     | Total                   | 25.9                            | -               | <0.0001 |
| TRL apoB-48 PR      | Fat content of the kinetic snacks | 17.7                    | -0.36           | <0.0001 |
|                     | Low fat vs. high fat     | -0.36                           | 0.0001          | 0.48 |
|                     | Moderate fat vs. high fat | +0.14                           | 0.048           | 0.0001 |
|                     | Fasting TG concentrations | 9.2                             | +0.30           | <0.0001 |
|                     | Plasma PCSK9 concentrations | 3.9                       | +0.20           | 0.007 |
|                     | Total                   | 30.9                            | -               | <0.0001 |

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 T2D as independent variables.
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 whether the positive association between plasma PCSK9 and TRL apoB-48 secretion observed in the present 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.

PCSK9 stimulation of TRL apoB-48 PR is derived 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 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.

**TABLE 3. Independent correlates of the intestinal expression of key genes involved in cholesterol and chylomicron metabolism**

| Dependent Variables | Independent Variables | Determination Coefficient (R²) | 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 concentations | 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 |

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. No multicollinearity was detected in the models.
Both newly synthesized intracellular PCSK9 and secreted PCSK9 promote the intracellular lysosomal degradation of lipoprotein receptors, mainly LDLR, as well as VLDLR, LRP-1, and apoER2 (6, 7, 55, 56). It is well established that chylomicron remnants are primarily cleared from circulation by hepatic LDLRs (57). 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 (57). 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). However, 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 present 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 intracellular and extracellular PCSK9 may have distinct effects on intestinal TRL metabolism and that the PCSK9 effect on TRL metabolism may be dependent on the abundance or the activity of LDLR (60, 61). These elements need 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 benefited from our longstanding 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, a 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 bound 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 blunt 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. This study supports the notion that PCSK9 is associated with increased intestinal secretion and plasma overaccumulation of TRL apoB-48 in men displaying various degrees of IR, although underlying 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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REFERENCES
1. Nordestgaard, B. G., M. Benn, P. Schnohr, and A. Tybjaerg-Hansen. 2007. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. JAMA. 298: 300–308.
2. Pal, S., K. Semorine, G. F. Watts, and J. Mamo. 2003. Identification of lipoproteins of intestinal origin in human atherosclerotic plaque. Clin. Chem. Lab. Med. 41: 792–795.
3. Doi, H., K. Kugiyama, M. Ohgushi, S. Sugiyama, T. Matsumura, Y. Ohita, T. Nakano, K. Nakajima, and H. Yasse. 1998. Remnants of chylomicron and very low density lipoprotein impair endothelium-dependent vasorelaxation. Atherosclerosis. 137: 341–349.
4. Hogue, J. C., B. Lamarche, A. J. Tremblay, J. Bergeron, C. Gagne, and P. Couture. 2007. Evidence of increased secretion of apolipoprotein B-48-containing lipoproteins in subjects with type 2 diabetes. J. Lipid Res. 48: 1336–1342.
5. Ducruy, H., B. Lamarche, K. D. Uelfman, R. Valero, J. S. Cohn, and G. F. Lewis. 2006. Hyperinsulinemia is associated with increased production rate of intestinal apolipoprotein B-48-containing lipoproteins in humans. Arterioscler. Thromb. Vasc. Biol. 26: 1357–1363.
6. Camuel, M., X. Sun, M. C. Asselin, E. Paramithiotis, A. Prat, and N. G. Seidah. 2013. Proprotein convertase subtilisin/kexin type 9 (PCSK9) can mediate degradation of the low density lipoprotein receptor-related protein 1 (LRP-1). PLoS One. 8: e64149.
7. Seidah, N. G., and A. Prat. 2012. The biology and therapeutic targeting of the proprotein convertases. Nat. Rev. Drug Discov. 11: 367–383.
8. Reyes-Soffer, G., M. Pavlyha, C. Ngai, T. Thomas, S. Holleran, R. Ramakrishnan, W. Karmally, R. Nandakumar, N. Fontainez, J. Obunike, et al. 2012. Effects of PCSK9 inhibition with alirocumab on lipoprotein metabolism in healthy humans. Circulation. 135: 352–362.
Adding MUFA to a dietary portfolio of cholesterol-lowering foods reduces apoA1 fractional catabolic rate in subjects with dyslipidaemia. Br. J. Nutr. 110: 426–436.

27. Tremblay, A. J., B. Lamarache, M. E. Labonte, M. C. Lepine, V. Lemelin, and P. Couture. 2014. Dietary medium-chain triglyceride supplementation has no effect on apolipoprotein B-48 and apolipoprotein B-100 kinetics in insulin-resistant men. Am. J. Clin. Nutr. 99: 54–61.

28. Couture, P., A. J. Tremblay, I. Kelly, V. Lemelin, A. Droit, and B. Lamarache. 2014. Key intestinal genes involved in lipoprotein metabolism are downregulated in dyslipidemic men with insulin resistance. J. Lipid Res. 55: 128–137.

29. Drouin-Chartrier, J. P., A. J. Tremblay, M. C. Lepine, V. Lemelin, B. Lamarache, and P. Couture. 2018. Substitution of dietary omega-6 polyunsaturated fatty acids for saturated fatty acids decreases LDL apolipoprotein B-100 production rate in men with dyslipidaemia associated with insulin resistance: a randomized controlled trial. Am. J. Clin. Nutr. 107: 26–34.

30. Tremblay, A. J., B. Lamarache, I. Kelly, A. Charest, M. C. Lepine, A. Droit, and P. Couture. 2014. Effect of sitagliptin therapy on triglyceride-rich lipoprotein kinetics in patients with type 2 diabetes. Diabetes Obes. Metab. 16: 1223–1229.

31. Pearson, T. A., G. A. Mensah, R. W. Alexander, J. L. Anderson, R. O. Cannon 3rd, M. Criqui, Y. F. Fadl, S. P. Fortmann, Y. Hong, G. L. Marcus, et al. 2003. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 107: 499–511.

32. American Diabetes Association 2010. Diagnosis and classification of diabetes mellitus. Diabetes Care. 33(Suppl 1): 66–76.

33. Cardin, A. D., K. R. Wint, J. Chao, H. S. Margolius, V. H. Donaldson, and R. L. Jackson. 1984. Degradation of apolipoprotein B-100 of human plasma low density lipoproteins by tissue and plasma kalirenes. J. Biol. Chem. 259: 8522–8528.

34. Zilverstrijt, D. B., and T. M. Shea. 1989. Quantitation of apoB-48 and apoB-100 by gel scanning or radioimmunodetection. J. Lipid Res. 30: 1639–1646.

35. Kinoshita, M., M. Koijima, T. Matsushima, and T. Teramoto. 2005. Determination of apolipoprotein B-48 in serum by a sandwich ELISA. Clin. Chim. Acta. 351: 115–120.

36. Borén, J., N. Matikainen, M. Adiels, and M. R. Taskinen. 2014. Postprandial hypertriglyceridaemia as a coronary risk factor. Clin. Chim. Acta. 431: 131–142.

37. Welty, F. K., A. H. Lichtenstein, P. H. Barrett, G. G. Dolnikowski, O. Cannon 3rd, M. Criqui, Y. Y. Fadl, S. P. Fortmann, Y. Hong, G. L. Marcus, et al. 2003. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 107: 499–511.

38. Borén, J., N. Matikainen, M. Adiels, and M. R. Taskinen. 2014. Postprandial hypertriglyceridaemia as a coronary risk factor. Clin. Chim. Acta. 431: 131–142.

39. Welty, F. K., A. H. Lichtenstein, P. H. Barrett, G. G. Dolnikowski, O. Cannon 3rd, M. Criqui, Y. Y. Fadl, S. P. Fortmann, Y. Hong, G. L. Marcus, et al. 2003. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 107: 499–511.

40. Kinoshita, M., M. Koijima, T. Matsushima, and T. Teramoto. 2005. Determination of apolipoprotein B-48 in serum by a sandwich ELISA. Clin. Chim. Acta. 351: 115–120.

41. Borén, J., N. Matikainen, M. Adiels, and M. R. Taskinen. 2014. Postprandial hypertriglyceridaemia as a coronary risk factor. Clin. Chim. Acta. 431: 131–142.

42. Lagace, T. A. 2014. PCSK9 and LDLR degradation: regulatory mechanisms. Arterioscler. Thromb. Vasc. Biol. 34: 2121–2127.
Circulating proprotein convertase subtilisin kexin type 9 has a diurnal rhythm synchronous with cholesterol synthesis and is reduced by fasting in humans. *Arterioscler. Thromb. Vasc. Biol.* **30**: 2666–2672.

47. Peach, M., R. Xu, D. Fitzpatrick, L. Hamilton, R. Somaratne, R. Scott, S. M. Wasserman, and C. S. Djedjos. 2016. Effect of evolocumab on cholesterol synthesis and absorption. *J. Lipid Res.* **57**: 2217–2224.

48. Buhman, K. K., M. Accad, S. Novak, R. S. Choi, J. S. Wong, R. L. Hamilton, S. Turley, and R. V. Farese, Jr. 2000. Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat. Med.* **6**: 1341–1347.

49. Altmann, S. W., H. R. Davis, Jr., L. J. Zhu, X. Yao, L. M. Hoos, G. Tetzloff, S. P. Iver, M. Maguire, A. Golovko, M. Zeng, et al. 2004. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science.* **303**: 1201–1204.

50. Pramfalk, C., B. Angelin, M. Eriksson, and P. Parini. 2007. Cholesterol regulates ACAT2 gene expression and enzyme activity in human hepatoma cells. *Biochem. Biophys. Res. Commun.* **364**: 402–409.

51. Jia, L., J. L. Betters, and L. Yu. 2011. Niemann-pick C1-like 1 (NPC1L1) protein in intestinal and hepatic cholesterol transport. *Annu. Rev. Physiol.* **73**: 239–259.

52. Leblond, F., N. G. Seidah, L. P. Precourt, E. Delvin, M. Dominguez, and E. Levy. 2009. Regulation of the proprotein convertase subtilisin/kexin type 9 in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **296**: G805–G815.

53. Kosenko, T., M. Golder, G. Leblond, W. Weng, and T. A. Lagace. 2013. Low density lipoprotein binds to proprotein convertase subtilisin/kexin type-9 (PCSK9) in human plasma and inhibits PCSK9-mediated low density lipoprotein receptor degradation. *J. Biol. Chem.* **288**: 8279–8288.

54. Marcel, Y. L., M. Hogue, P. K. Weech, J. Davignon, and R. W. Milne. 1988. Expression of apolipoprotein B epitopes in lipoproteins. Relationship to conformation and function. *Arteriosclerosis.* **8**: 832–844.

55. Poirier, S., G. Mayer, V. Poupon, P. S. McPherson, R. Desjardins, K. Ly, M. C. Asselin, R. Day, F. J. Duclos, M. Witmer, et al. 2009. Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route. *J. Biol. Chem.* **284**: 28856–28864.

56. Poirier, S., G. Mayer, S. Benjannet, E. Bergeron, J. Marcinkiewicz, N. Nassoury, H. Mayer, J. Nimpf, A. Prat, and N. G. Seidah. 2008. The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. *J. Biol. Chem.* **283**: 2363–2372.

57. Havel, R. J. 1998. Receptor and non-receptor mediated uptake of chylomicron remnants by the liver. *Atherosclerosis.* **141(Suppl 1)**: S1–S7.

58. Ooi, T. C., J. A. Krysa, S. Chaker, H. Abujrad, J. Mayne, K. Henry, M. Cousins, A. Raymond, C. Favreau, M. Taljaard, et al. 2017. The effect of PCSK9 loss-of-function variants on the postprandial lipid and ApoB-lipoprotein response. *J. Clin. Endocrinol. Metab.* **102**: 3452–3460.

59. Drouin-Chartier, J. P., J. C. Hogue, A. J. Tremblay, J. Bergeron, B. Lamarche, and P. Couture. 2017. The elevation of plasma concentrations of apoB-48-containing lipoproteins in familial hypercholesterolemia is independent of PCSK9 levels. *Lipids Health Dis.* **16**: 119.

60. Dijk, W., C. Le May, and B. Cariou. 2018. Beyond LDL: what role for PCSK9 in triglyceride-rich lipoprotein metabolism? *Trends Endocrinol. Metab.* **29**: 420–434.

61. Druce, I., H. Abujrad, and T. C. Ooi. 2015. PCSK9 and triglyceride-rich lipoprotein metabolism. *J. Biomed. Res.* **29**: 429–436.