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# Impact of PCSK9 inhibition with evolocumab on the postprandial responses of triglyceride-rich lipoproteins in type 2 diabetic subjects

**Running title:** Impact of evolocumab on postprandial TRL

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## ABSTRACT

**Background**—Monoclonal antibodies to proprotein convertase subtilisin/kexin type 9 (PCSK9) significantly lower the levels of LDL and very low-density lipoproteins, but their effect on postprandial lipoprotein metabolism in dyslipidemic subjects is unclear.

**Objective—**To investigate the effects of evolocumab on postprandial lipid responses, ectopic fat depots, whole body cholesterol synthesis, hepatic lipogenesis, and fat oxidation in patients with type 2 diabetes.

**Methods**—The trial was a single-phase, nonrandomized study of 12-week treatment with evolocumab 140 mg s.c. Q2W in 15 patients with type 2 diabetes on background statin therapy. Cardiometabolic responses to a high-fat mixed meal were assessed before and at the end of the intervention period.

**Results**—Evolocumab treatment reduced significantly postprandial rises in plasma total triglyceride (by 21%, P<0.0001) and $VLDL_1$-triglyceride (by 15%, P=0.018), but the increase in chylomicron-triglyceride following the meal was not significantly perturbed (P=0.053). There were reduced postprandial responses in plasma total apoC-III (by 14%, P<0.0001), apoB48 concentration (by 17%, P=0.0046) and in 'remnant-like particles (RLP)' cholesterol (by 29%, P<0.0001) on the PCSK9 inhibitor. Treatment reduced the steady state (i.e. fasting and postprandial) concentrations of $VLDL_2$ cholesterol by 50% (P<0.0001) and $VLDL_2$ triglyceride by 29% (P<0.0001), in addition to the 78% reduction of LDL cholesterol (P<0.001). The changes in apoC-III associated significantly with reduction in postprandial responses of RLP-cholesterol and TRL-cholesterol. Evolocumab therapy did not influence liver fat accumulation, hepatic *de novo* lipogenesis or fasting β-hydroxybutyrate, but did increase total body cholesterol synthesis (P<0.01).

**Conclusion**—Evolocumab treatment improved postprandial responses of TRLs and measures of cholesterol-enriched remnant particles in type 2 diabetic subjects. These results indicate that postprandial phenomena need to be taken into account in assessing the full range of actions of PCSK9 inhibitors in dyslipidemic individuals.


**Key words:** apoB, Postprandial lipids, Remnant lipoproteins, Apolipoprotein C3, Atherogenic dyslipidaemia, De novo lipogenesis, Evolocumab, PCSK9, Liver fat

**ABBREVIATIONS**

| | |
|---|---|
| ACS | acute coronary syndrome |
| ALT | alanine aminotransferase |
| ANGPTL3 | angiopoietin-like protein 3 |
| ANOVA | analysis of variance |
| apo | apolipoprotein |
| APPI | atmospheric pressure photoionization |
| AST | liver transaminases aspartate transaminase |
| AUC | area under the curve |
| BMI | body mass index |
| CABG | coronary artery bypass grafting |
| CM | chylomicrons |
| DNL | *de novo* lipogenesis |
| eGFR | estimated glomerular filtration rate |
| ELISA | enzyme-linked immunosorbent assay |
| HbA1c | hemoglobin A1c |
| HDL | high density lipoproteins |
| HL | hepatic lipase |
| HSPG | heparan sulphate proteoglycans |
| IDL | intermediate density lipoproteins |
| LDL | low density lipoproteins |
| LRP | LDL receptor-related protein |
| LPL | lipoprotein lipase |
| MI | myocardial infarction |
| NYHA | New York Heart association functional classification |
| PCI | percutaneous coronary intervention. |
| PCSK9 | proprotein convertase subtilisin/kexin type 9 |
| RLP | remnant-like particle |
| TRL | triglyceride-rich lipoproteins |
| T2D | type 2 diabetes |
| VLDL | very low density lipoproteins |

## INTRODUCTION

Despite major advances in therapy, atherosclerotic cardiovascular disease (ASCVD) and its clinical manifestations such as myocardial infarction and ischaemic stroke are still the leading causes of mortality and morbidity globally.[1] Both epidemiological and genetic studies have established that an increased level of cholesterol-rich LDL is the principal risk factor for developing ASCVD.[1] However, in recent years there has been a growing awareness of the atherogenic potential and hence clinical importance of postprandial lipoproteins based on the findings of epidemiological studies that have identified postprandial hypertriglyceridemia as an independent risk for coronary atherosclerosis.[2-11]

The hypertriglyceridemia that develops following dietary lipid absorption comprises increased levels of intestinally derived apoB48-containing chylomicrons (CM) and their remnants, and liver derived apoB100-containing very low density lipoproteins (VLDL) and their remnants.[12] Chylomicrons transport dietary lipids to adipose, cardiac, and skeletal muscle tissue, where their triglyceride components are hydrolysed by lipoprotein lipase (LPL), releasing the fatty acids for uptake by tissues.[13] When a large portion of the triglycerides has been hydrolysed, cholesterol-enriched chylomicron remnants are formed. These remnant particles are cleared by the liver via the LDL receptor (LDL-R), the LDL receptor-related protein (LRP) and heparan sulphate proteoglycans (HSPG).[13] Angiopoietin-like protein 3 (ANGPTL3) and apoC-III have emerged as important metabolic regulators of the metabolism of triglyceride-rich lipoproteins (TRLs) and novel candidate targets for intervention to correct the dyslipidaemia and ameliorate CVD risk. The impact of apoC-III on TRL metabolism and atherogenesis has been linked to both LPL-mediated mechanisms and indirect mechanisms, such as increased secretion of TRLs, proinflammatory responses in vascular cells and impaired LPL-independent hepatic clearance of TRL remnants.[14]

As humans spend most of the waking day in the postprandial state, effective lipid lowering therapies must take account of the potential impact on all atherogenic lipoproteins, including the components of postprandial hypertriglyceridemia. Importantly, the postprandial triglyceride response in type 2 diabetic subjects is considerably increased and prolonged relative to non-diabetic subjects.[15, 16] This component of the diabetic dyslipidemia likely contributes to the residual risk in statin-treated type-2 diabetic subjects. Indeed, genetic studies suggest that not only LDL, but also TG-rich remnants are directly causal in ASCVD, independent of LDL-C levels.[10, 17] Therefore, there is need for aggressive lipid lowering in people with type 2 diabetes and very high-risk.[18, 19] Statins by upregulating LDL receptors, and potentially other hepatic receptors, increase clearance of both LDL particles and of CM and VLDL remnants. These drugs have been shown to lower fasting and postprandial plasma triglyceride and apoB48 concentrations.[20-22] Monoclonal antibodies to proprotein convertase subtilisin/kexin type 9 (PCSK9) significantly lower LDL and

fasting TRL levels,[23-26] but their effect on postprandial lipid metabolism has not yet been elucidated fully for individuals with dyslipidaemia. Plasma PCSK9 concentration associates with postprandial TRL metabolism,[27] and recent results from the BANTING study in type 2 diabetics gave an indication that inhibition of PCSK9 with monoclonal antibodies reduced levels of postprandial lipids and apoproteins including VLDL-cholesterol, chylomicron triglyceride and apoB48.[28] In contrast, kinetic studies with stable isotopes in normolipemic subjects found no impact of PCSK9 inhibition on postprandial plasma triglycerides or apoB48 concentrations.[29] Likewise, Chan et al recently reported that this therapy, again in subjects with normal plasma triglyceride levels, had no significant effect on the response to a fat meal challenge in postprandial plasma triglycerides and apoB48.[22]

The end-products of cholesterol utilization are the bile acids.[30] In fact, the synthesis of bile acids is the major route of cholesterol catabolism in mammals. Although several of the enzymes involved in bile acid synthesis are active in many cell types, the liver is the only organ where their complete biosynthesis can occur.[30] Interestingly, despite the marked effect on cholesterol homoeostasis, statins do not alter plasma bile acid levels. PCSK9 inhibition markedly augments the LDL lowering action of statins, and it has been hypothesized that combined PCSK9 and HMG-CoA reductase inhibition may alter bile acid metabolism.[31] However, studies in mice indicate that hepatocyte cholesterol and bile acid homeostasis is maintained with combined anti-PCSK9 and statin intervention through efficient liver enzymatic conversion of LDL-derived cholesterol into bile acids and excretion of both, with undisturbed enterohepatic recycling.[31] Studies in humans are still lacking.

In the present study, we examined in detail in type 2 diabetics the postprandial effects of PCSK9 inhibition using evolocumab. In addition, we investigated the action of the drug on cholesterol and bile acid synthesis.

## METHODS

**Study Design—**This study was designed as a nonrandomized clinical trial in which subjects were examined before and after 12 weeks' treatment with evolocumab. The study participants were 15 type 2 diabetics recruited between October 2016 and June 2017 from previous studies and by using an online recruitment service provided by Clariness GmbH, Germany. After an initial telephone interview of 195 subjects, 77 were invited for a screening visit. Of these, 63 subjects were excluded because of failure to meet inclusion criteria. All study subjects used daily stable doses of metformin and statin throughout the study period. The protocol comprised two periods: a run-in period and a treatment period. The run-in period started at the screening visit when the eligibility to enter the treatment period was evaluated. If the subjects were not using statin, or used another statin, atorvastatin was initiated at dose of 20 mg/day and this statin run-in period was 4 weeks after the change in statin or statin dose. The treatment regimen was continued throughout the study. Evolocumab at a dose of 140 mg subcutaneously every 2 weeks (Q2W) was added to the statin therapy at the last day of the run-in period and continued over a 12-week treatment period.

Each study subject underwent a fat-rich mixed meal tolerance test and abdominal magnetic resonance imaging before and after the 12-week treatment with evolocumab. Weight and height were measured in the study centre after an overnight fast (with subjects barefoot, wearing underwear). Waist circumference was recorded at the midpoint between the lower rib margin and the iliac crest. Three consecutive readings were taken, and the mean was recorded. Subjects were instructed to avoid alcohol and strenuous exercise for 72 hours before each study visit. The volunteers also visited the study centre at weeks 2, 4, and 8 for safety blood samples. One study subject was excluded during the evolocumab treatment period because of worsening of asthma symptoms resulting in oral prednisolone use. Thus, 14 out of the 15 study subjects finished the planned protocol.

The study protocol was approved by ethics committee of Helsinki University Hospital and the National Agency of Medicines. Helsinki, Finland (Eruct 2016-00176-30). The trial was undertaken in accordance with the Declaration of Helsinki and the European Medicines Agency Note for Guidance on good clinical practice. Study subjects signed informed consent before study procedures were initiated.

**Inclusion Criteria—**Male or female (non-fertile or using medically approved birth control method) overweight/obese subjects with type 2 diabetes (T2D) treated with lifestyle counselling and a stable dose of metformin (any dose) and atorvastatin (20 mg /day) were eligible for the study. Additional inclusion criteria were HbA1c value <=9.0%, BMI between 25–40 kg/$m^2$, age 18–77 yrs. and plasma triglycerides between 1.0–4.5 mmol/l and LDL cholesterol >1.8, but < 4.0 mmol/L (on atorvastatin 20 mg/day).

**Exclusion Criteria—**Exclusion criteria included type 1 diabetes, apoE2/2 phenotype, elevated liver enzymes (ALT/AST > 3×UNL), eGFR<60ml/min, clinically significant TSH outside the normal range, BMI > 40 kg/m$^2$, HbA1c > 9.0 %, fasting triglyceride >4.5 mmol/l, total cholesterol > 7.0 mmol/L, positive urine or serum pregnancy test, any other diabetes medication except diet or metformin, untreated or inadequately treated hypertension (>160 mmHg systolic and/or >105 mmHg diastolic), use of thiazide diuretics at a dose of ≥25 mg/day, lipid-lowering drugs other than statins within 3 months, use of estrogen therapy and history/diagnosis of diabetes nephropathy/retinopathy.

Additional exclusion criteria were history of MI, ACS or coronary revascularization (PCI or CABG) within the last 6 months, planned revascularization (e.g., CABG, PCI, carotid or peripheral revascularization procedures) within 3 months of screening, NYHA class III/IV congestive heart failure persisting despite treatment, history of haemorrhagic stroke, hypersensitivity to evolocumab or any of the excipients used in the drug formulation, current use of antithrombotic or anticoagulant therapy, known bleeding tendency, and history of cancer within the past 5 years.

**Fat-rich Mixed Meal—**Participants were served a fat-rich mixed meal in the morning after an overnight fast. The meal consisted of bread, butter, cheese, ham boiled eggs, fresh red pepper, low fat milk (1 %), orange juice and tea or coffee and served with a fat-rich cocoa emulsion containing 40 g olive oil (Amway, Firenze). The total energy content of the meal averaged 1032 Kcal with 59.8 % from fat (68.5 grams), 24.4 % from carbohydrate (63.0 grams) and 15.5 % from protein (40.1 grams). Blood samples were obtained at fasting and at 0.5, 1, 2, 3, 4, 6 and 8 hours after the meal. During the test only water was allowed *ad libitum* and the participants remained physically inactive.

On the evening before the study day, subjects received 2 g/kg deuterated water ($^2H_2O$; Larodan Fine Chemicals, Sweden), which was consumed between 18:00 and 22:00 to assess the contribution of hepatic *de novo* lipogenesis (DNL) to fatty acids in VLDL. A blood sample was drawn as a background sample in the week before the meal test day. *De novo* lipogenesis was analysed at 0 h and was calculated from enrichment of deuterated water.[32, 33]

**Proton magnetic resonance Spectroscopy—**Proton magnetic resonance spectroscopy was performed using a 1.5-T whole-body device to determine liver fat content,[34] and subcutaneous abdominal and intra-abdominal fat.[35] All analyses of the imaging results were performed by one person (AH). Subjects were advised to fast for 4 hours before imaging.

**Biochemical Analyses—**Lipoprotein fractions [chylomicrons (Sf >400), large $VLDL_1$ particles (Sf 60–400) and smaller $VLDL_2$ particles (Sf 20–60)] from blood samples drawn before and during the fat-rich mixed meal test were separated by density gradient ultracentrifugation.[36] Triglyceride and

cholesterol concentrations in total plasma and lipoprotein fractions were analyzed using the Konelab 60i analyser (Thermo Fisher Scientific, Finland) TRL cholesterol and remnant-lipoprotein cholesterol were analyzed using automated direct assays (Denka Seiken Co., LTD, Tokyo, Japan). ApoB48 levels in total plasma were measured by ELISA (Shibayagi, Shibukawa, Japan). Concentrations of plasma glucose were measured using the hexokinase method and insulin with sandwich immunoassays (Roche Diagnostic, Germany). Plasma levels of apoC-III were measured immunoturbidometrically (Kamiya Biomedical Company, Seattle, WA, USA) and β-hydroxybutyrate concentrations were measured by an enzymatic method with β-hydroxybutyrate FS kit (DiaSys Diagnostic Systems, Holzheim, Germany) on a Konelab 60i analyzer (Thermo Fisher Scientific, Finland). Plasma non-esterified fatty acids (NEFA) were analyzed with an automated enzymatic colorimetric method (Wako Chemicals, Neuss, Germany). An immunochemical method was used for the selective measurement of lipoprotein lipase (LPL) and hepatic lipase (HL) activities in postheparin plasma.[37]

**Analyses of postprandial synthesis and intestinal absorption of cholesterol and of bile acid synthesis—**Lipids from plasma samples were extracted by a slightly modified BuMe method,[38] evaporated to dryness under a stream of nitrogen, dissolved in a small volume of methanol and 1 µl was injected on the mass spectrometer system. Analysis was performed by supercritical chromatography tandem mass spectrometry with atmospheric pressure photoionisation (SFC-APPI-MS/MS) on a Waters UPC2 chromatograph (Waters Inc, USA) connected to a Xevo TQ-S mass spectrometer (Waters, Manchester, UK). Column was a Waters BEH Si 2.1 x 100 mm with 1.7 µm particles. The column was held at Flow was 1.7 ml/min and gradient was from 0% MeOH in CO2 to 6% MeOH in CO2 at 5 min then back to 0% in 0.1 min and equilibrate for 0.5 min. The make-up flow was MeOH 0.3 ml/min. The analytes and their respective internal standards were ionized by APPI and detected by the following mass spectrometric transitions (M-OH)+: C4, 401.4 => 177.1 CE = 20, d7-7-ketocholesterol (I S), 407.2 => 175.1, lanosterol, 409.2 => 191.1 CE = 12, d3-Lanosterol 412.3 => 191.1 CE = 12, sitosterol, 397.2 => 161 CE=22, d7-Sitosterol, 404.2 => 161 CE = 22. Calibration was performed by standards in 6 concentrations ranging from C4, 3.5 to 706 nM, Lanosterol, 11.7 to 2340 nM and Sitosterol, 109 to 22000 nM.

The lanosterol segment of the cholesterol pathway comprises the rate-limiting and committed steps of cholesterol biosynthesis, and lanosterol/cholesterol is an established surrogate marker of total body cholesterol synthesis.[39] Sitosterol is an abundant plant sterols not synthesized in the human body and sitosterol/cholesterol is an established surrogate marker of cholesterol absorption.[40] The plasma oxysterol 7α-hydroxy-4-cholesten-3-one (C4)/cholesterol is used as an index of hepatic bile acid synthesis.[41] Kinetics of postprandial increase in cholesterol synthesis was indirectly followed by generating for each patient quadratic functions in which variables were the ratios

lanosterol/cholesterol and time (0, 4 and 8 hours), respectively. The index of the rate of increase in cholesterol synthesis was calculated using the first derivative of quadratic function at the given time point (4 and 8 hours).

**Statistical Analyses—**Statistical analyses were performed using the GraphPad Prism, version 7. A repeated measures ANOVA test was used to examine the differences in postprandial concentrations of analytes (**Figures 1 and 2**) before and at the end of the evolocumab intervention. T-tests were performed to investigate differences (before *vs* after intervention) in individual time-points after the fat-rich mixed meal. To control for multiple testing, false discovery rate was analysed using the two-stage step-up method of Benjamini, Krieger and Yekutieli. The significance of treatment associated changes in **Table 1** were assessed using paired difference t-test; the results did not differ whether the test was performed on untransformed data or following transformation to logarithmic values. Correlation coefficients and associated p-values were calculated using the non-parametric Spearman test. P-values in **Table 2** were calculated using paired t-test. Differences in postprandial kinetics of synthesis and intestinal absorption of cholesterol and of bile acid synthesis were calculated using the Wilcoxon signed-rank test.

## RESULTS

### Demographics, body fat depots and glycaemia

**Table 1** presents the characteristics and cardiometabolic biomarker profiles of the 14 type 2 diabetic subjects who completed the protocol. We observed no change in body weight, visceral or subcutaneous fat depots, or liver fat content following 12 weeks of evolocumab therapy. There were no significant changes in fasting glucose, insulin concentration, indexes of insulin sensitivity/insulin resistance or HbA1c. Likewise, we did not observe any effect of evolocumab on hepatic *de novo* lipogenesis, fasting β-hydroxybutyrate, or NEFA concentrations.

### Fasting and postprandial lipids

As expected, evolocumab add-on therapy resulted in marked reductions in fasting total cholesterol (-42%), LDL cholesterol (-78%) and apoB (-53%) (**Table 1**). We did not find any significant changes in fasting plasma triglycerides, apoB48 or apoC-III levels. Interestingly, evolocumab therapy resulted in a significant 47% reduction of fasting triglyceride-rich-lipoprotein (TRL)-cholesterol (measured as the d<1.006 g/ml fraction) but no significant reduction was seen in fasting remnant-like particle (RLP)-cholesterol (this assay measures the concentration of particles that do not bind to anti-apoA-I [HDL] or anti-apoB100 [LDL, IDL and apoB100-containing VLDL]). Fasting apoC-III levels were associated with both RLP-cholesterol and TRL-cholesterol values before and during evolocumab therapy (r=0.75, p=0.0044 and r=0.79, p=0.0021 respectively).

HDL cholesterol and apoA1 were increased modestly by the addition of evolocumab (**Table 1**). No significant changes of postheparin plasma activities of LPL or HL were induced by evolocumab (**Table 1**).

Despite no change in fasting plasma triglycerides or apoC-III on evolocumab therapy, we observed significantly reduced postprandial responses (analysed as area under the curve [AUC]) in plasma total triglycerides, $VLDL_1$-triglycerides, apoC-III and apoB48 (**Figure 1**). There was a trend to reduced AUC for chylomicron triglyceride on evolocumab but this failed to reach significance (P=0.053, **Figure 1**). It was notable that the reductions in postprandial response, where they occurred, were in the main seen 4-6 hours after ingestion of the fat-rich mixed meal. Evolocumab therapy also reduced the steady state levels of $VLDL_2$-triglycerides (29% decrease, P=0.006) throughout the postprandial period (**Figure 1**). Evolocumab therapy significantly reduced by 44% the levels of TRL-cholesterol (P<0.0001), $VLDL_2$ cholesterol level by 50% (P<0.0001), and LDL cholesterol by 78% (P<0.0001) both in the fasting state and during the postprandial period (**Figure 2**). Despite the lack of impact on mean fasting levels, PCSK9 inhibition significantly reduced the postprandial responses of RLP cholesterol (29% reduction in AUC, P<0.0001), and $VLDL_1$ cholesterol (23% reduction in AUC, P=0.0002). Change in plasma apoC-III on evolocumab showed a strong correlation with change in plasma TG values (r= 0.82, P= 0.0008) as well as with change in fasting RLP-C and TRL-C (r= 0.81, P=0.0014; and r=0.61, P= 0.030, respectively) (**Figure 3** and **Supplementary Figure S1**).

**Effect of evolocumab on total body cholesterol biosynthesis, intestinal cholesterol absorption, and bile acid synthesis.**

To investigate whether evolocumab treatment was associated with postprandial changes in cholesterol and bile acid metabolism, biomarkers of cholesterol synthesis (lanosterol), intestinal cholesterol absorption (sitosterol) and bile acid synthesis (7α-hydroxy-4-cholesten-3-one; C4) were measured and normalized to plasma cholesterol concentration (**Table 2**). PCSK9 inhibition increased the lanosterol/cholesterol ratio in the fasting and postprandial states indicating that the evolocumab treatment led to an increase in total body cholesterol synthesis (**Table 2)**. Interestingly, analysis of dynamics of the postprandial increase of cholesterol synthesis showed higher rates at 4 and 8 hours after evolucumab treatment (P<0.01 and P<0.05, respectively) (**Figure 4**). No changes were observed in either fasting or postprandial biomarkers for intestinal cholesterol absorption (sitosterol/cholesterol ratio; **Table 2**), or bile acid synthesis (C4/cholesterol ratio; **Table 2**) either at baseline or after evolocumab treatment.

## DISCUSSION

In this study of the effects of PCSK9 inhibition on lipid and lipoprotein responses to a mixed, fat-rich meal in type 2 diabetic patients, we showed that 12 weeks' treatment with evolocumab in this dyslipidemic condition perturbed postprandial lipid metabolism in a way that was distinct from the action of statins,[20-22] and from what has been observed when PCSK9 inhibitors are given to normal subjects.[22, 29] Evolocumab treatment reduced significantly the postprandial rise in plasma total triglyceride, $VLDL_1$-triglyceride, TRL cholesterol, RLP cholesterol, apoC-III and apoB48, while the increment in chylomicron triglyceride exhibited a trend that did not achieve significance. It was notable that the greatest impact of the drug appeared to occur 4 to 6 hours after meal ingestion; the initial postprandial rise (i.e. in the first 4 hours) was virtually identical before and on evolocumab therapy. Fasting levels of plasma triglyceride, RLP-C, apoC-III and apoB48 were not influenced by treatment. There were, however, substantial significant decreases in the 'steady state' – fasting and postprandial levels - of $VLDL_2$ triglyceride and cholesterol, LDL cholesterol and apoB.

In interpreting these findings, it is important to note that the impact of evolocumab was studied on a background of moderate dose statin therapy. Statins themselves have effects on post-prandial lipid metabolism - reducing the degree of alimentary lipemia, as well as on fasting VLDL levels[20-22]. We propose that the addition of evolocumab had two principal actions in these diabetic subjects as shown in **Figure 5**, first an effect on small VLDL ($VLDL_2$) and LDL particles that leads to a marked reduction in the circulating concentrations of these lipoprotein classes throughout the day. Second, an impact on the removal of postprandial TRL particles and their remnants including apoB48-containing particles, and $VLDL_1$ remnants which may contain apoB100 or apoB48.[42] Evolocumab therapy appeared not to influence the production and release of chylomicrons from the intestine, as evidenced by the lack of effect on the total chylomicron response, and especially the initial rise. The changes in TRL metabolism were associated with levels of plasma apoC-III, an apoprotein cofactor that is known to influence the lipolysis and clearance of TRL particles.[14, 43-46] It is clear from these findings that a full appreciation of the actions of PCSK9 inhibitors needs to take into account the impact of these drugs on triglyceride-rich lipoprotein particles as well as cholesterol transporting LDL.

The effects of PCSK9 inhibition on postprandial lipoprotein metabolism has been reported for individuals with normal plasma lipid levels (plasma triglyceride <1.0 mmol/l)[22, 27], and in part in type 2 diabetics with raised fasting plasma triglyceride[28] (mean of 2.0 mmol/l) where a 2- to 3-hour post-meal window was examined. Reyes-Soffer et al[29] examined the impact of alirocumab on the apoB-containing lipoprotein spectrum and while the action of the drug on VLDL and LDL kinetics was clearly in line with its known mechanism of action, there was no impact on the response in plasma triglyceride and apoB48 to a fat test meal. The study of Chan et al,[22] again in healthy volunteers, compared directly statin therapy which induced a substantial reduction in postprandial

plasma triglyceride, VLDL cholesterol and apoB48 to evolocumab treatment which had no discernible impact on these parameters. They reported further on the kinetics of apoB48, documenting an increased clearance rate on statin therapy but again no change on evolocumab. The BANTING study[28] in type 2 diabetic subjects found no significant effect of evolocumab on fasting plasma triglyceride but a 13.4% to 17.0% decrease respectively in the 2-hour and 3-hour AUC in this lipid after a fat meal. Likewise, there was a decrease in chylomicron triglyceride AUC of 7.4-12.8% and of 8.5 to 14.5% in apoB48 AUC; these modest changes on PCSK9 inhibition were recorded as being of variable statistical significance. Overall, the results of the BANTING trial[28] are in accord with the findings of the present study; the detection of early changes may be attributable to the greater subject numbers in the former. However, the short time window employed may have limited the ability to detect the more notable effects of the drug which occurred, as mentioned above, later after meal ingestion.

Evolocumab treatment in our type 2 diabetic group was associated with increased levels of biomarkers of cholesterol synthesis both in fasting and postprandial state. These findings suggest that PCSK9 inhibition decreases the intracellular cholesterol pool (secondary to prolonged exposure to very low levels of cholesterol in plasma), and as a consequence, tissues, primarily intestine and liver (which account for the majority of whole-body cholesterol synthesis) upregulate cholesterol de novo production in order to sustain cellular cholesterol homeostasis. The increase in postprandial cholesterol biosynthesis might be necessary to support lipoprotein assembly and secretion of both chylomicrons (during absorption of the fat-rich meal) and VLDL (as lipolysed fatty acid are taken up by the liver). In contrast, no changes were observed in markers of intestinal cholesterol absorption or bile acid synthesis. This observation is in line with the concept that bile acid synthesis is privileged and maintained also in condition of low levels of intracellular cholesterol as previously described in gallstone patients treated with high doses of statins in the presence or absence of ezetimibe.[47, 48]

Advanced bioinformatics investigating liver-specific genes that are co-expressed with fatty acid synthase revealed that PCSK9 is linked to NAFLD pathogenesis;[49] hepatic PCSK9 expression has been shown to be associated with steatosis severity and activation of genes regulating hepatic lipogenesis in patients at risk of NASH.[50] Data also indicate that circulating PCSK9 levels associate with hepatic lipogenesis and liver fat accumulation in humans[50, 51] and mice.[52] However, it is still unclear whether circulating PCSK9 is causatively linked to increased hepatic fat content, or if the association merely reflect coordinated expression. Here we found no changes in hepatic fat accumulation after evolocumab therapy.

The limitations of the present metabolic study are the size of the cohort, its design as an open label trial, and the fact that for ethical and practical reasons all subjects were placed on a standard

background regimen of 20 mg/d atorvastatin. In this context it is encouraging to see that our findings were in accord with those of the larger BANTING trial.[28] Comparing the two studies of type 2 diabetic subjects given evolocumab in addition to statin therapy, it can be seen that the effects of the PCSK9 inhibitor were similar for plasma triglyceride, a 12.6% reduction in BANTING[28] versus 14% in Table 1, and for LDL a 65% decrease[28] versus 78% in Table 1.

In summary, 12 weeks' treatment with evolocumab in type 2 diabetics improved specific aspects of the postprandial response of triglyceride-rich lipoprotein to a mixed, fat-rich meal. As depicted in **Figure 5**, the data are consistent with a decrease in the circulating levels of remnant lipoproteins as well as small VLDL and LDL. This combined action could lead to enhanced reduction in risk of an ASCVD event since remnants are considered in an emerging consensus have an atherogenic potential equal to that of LDL. The findings of this study emphasize the importance of taking into account not only alterations in fasting values but also postprandial responses when evaluating the clinical benefits of novel drug therapies for hyperlipidaemia. It appears also that the action of PCSK9 inhibitors on postprandial lipoprotein metabolism relates primarily to the level of remnants generated during dietary fat absorption which is in turn determined by the prevailing plasma triglyceride concentration and efficiency of lipolysis.

## CONFLICT OF INTEREST

The authors report no conflicts of interest related to this work.

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## AUTHOR CONTRIBUTIONS

The authors contributed to the present work as follows: MRT, and JB contributed to conception and design, LA, JK, NM, SS, KP, AH, NL, RN and MS to the acquisition of data or analysis, and MRT, EB, MA, PP, CP and JB to the interpretation of data. MRT, EB, CP and JB drafted the original and revised manuscripts and all authors approved the final approval of the version to be published.

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**Table 1.** Characteristics of the subjects before and after evolocumab treatment. Data are indicated as mean ± SD. Mean absolute changes from baseline to week 12 are shown with ± SD and mean percentage changes are indicated in parenthesis. P-values were calculated using a paired difference t-test and p-values <0.05 are indicated in bold.

| | Before | After | Change | P-value |
|---|---|---|---|---|



BP, blood pressure; HOMA, VAT, visceral adipose tissue, SAT, subcutaneous adipose tissue; HOMA, homeostatic model assessment; NEFA, non-esterified fatty acids; β-OHB, β-hydroxybutyric acid; LPL, lipoprotein lipase; HL, hepatic lipase

| | | | | |
|---|---|---|---|---|
| Weight (kg) | 82.7 ± 16 | 82.8 ± 15 | 0.1 ± 1.3 (0.15%) | 0.74 |
| BMI (kg/m2) | 29.4 ± 4 | 29.4 ± 3.7 | 0.1 ± 0.4 (0.18%) | 0.68 |
| Waist (cm) | 104 ± 9.9 | 104 ± 9.6 | -0.04 ± 2.6 (-0.037%) | 0.96 |
| Systolic BP (mmHg) | 138 ± 16 | 135 ± 16 | -3.7 ± 13 (-2.7%) | 0.31 |
| Diastolic BP (mmHg) | 83.8 ± 6.8 | 79.7 ± 6.6 | -4.2 ± 7.4 (-5%) | 0.064 |
| Heart rate (bpm) | 66.5 ± 10 | 69.4 ± 10 | 2.8 ± 8.3 (4.3%) | 0.24 |
| VAT ($cm^3$) | 2420 ± 960 | 2450 ± 1100 | -13.3 ± 230 (-0.55%) | 0.84 |
| SAT ($cm^3$) | 3900 ± 1700 | 4110 ± 1600 | 8.5 ± 120 (0.22%) | 0.81 |
| ***Glucose homeostasis*** | | | | |
| P-Glucose (mmol/l) | 6.3 ± 0.4 | 6.4 ± 0.8 | 0.1 ± 0.5 (1.8%) | 0.39 |
| HbA1c (%) | 5.9 ± 0.4 | 6.1 ± 0.5 | 0.2 ± 0.3 (3.3%) | 0.061 |
| Insulin (µU/ml) | 8.9 ± 3.6 | 10.3 ± 7 | 1.4 ± 4.9 (16%) | 0.31 |
| HOMA2-%B | 66.9 ± 18 | 65.4 ± 17 | -1.5 ± 17 (-2.2%) | 0.75 |
| HOMA2-%S | 98.8 ± 56 | 99.6 ± 53 | 0.8 ± 41 (0.83%) | 0.94 |
| HOMA2-IR | 1.4 ± 0.98 | 1.2 ± 0.5 | -0.2 ± 0.7 (-14%) | 0.30 |
| ***Lipids and lipoproteins*** | | | | |
| Cholesterol (mmol/l) | 3.9 ± 0.5 | 2.2 ± 0.6 | -1.7 ± 0.4 (-42%) | **<0.001** |
| HDL-C (mmol/l) | 1.4 ± 0.4 | 1.6 ± 0.4 | 0.2 ± 0.2 (16%) | **<0.001** |
| Triglycerides (mmol/l) | 1.6 ± 0.5 | 1.3 ± 0.5 | -0.2 ± 0.6 (-14%) | 0.18 |
| LDL-C (mmol/l) | 1.7 ± 0.4 | 0.4 ± 0.2 | -1.4 ± 0.4 (-78%) | **<0.001** |
| ApoC-III (mg/dl) | 12.2 ± 4.3 | 10.4 ± 5.2 | -1.9 ± 3.7 (-15%) | 0.093 |
| ApoB (mg/dl) | 75.9 ± 15 | 35.3 ± 11 | -40.5 ± 8.8 (-53%) | **<0.001** |
| ApoB48 (mg/l) | 6.1 ± 3.8 | 5.3 ± 3.3 | -0.8 ± 2.5 (-13%) | 0.27 |
| ApoA-I (mg/dl) | 142 ± 25 | 151 ± 26 | 9.5 ± 11 (6.7%) | **0.011** |
| TRL-C (mg/dl) | 33.4 ± 11 | 17.8 ± 6.5 | -15.6 ± 7.8 (-47%) | **<0.001** |
| RLP-C (mg/dl) | 8.5 ± 4.3 | 7.0 ± 4.7 | -1.4 ± 4.4 (-17%) | 0.27 |
| ***Hepatic lipid metabolism*** | | | | |
| DNL (µmol/l) | 17.6 ± 13 | 17.1 ± 12 | -0.48 ± 13 (-2.7%) | 0.90 |
| NEFA (µmol/l) | 615 ± 210 | 608 ± 180 | -7.2 ± 190 (-1.2%) | 0.89 |
| β-OHB (mg/dl) | 0.67 ± 0.24 | 0.73 ± 0.32 | 0.05 ± 0.34 (7.9%) | 0.59 |
| Liver fat (%) | 6.6 ± 6.6 | 6.3 ± 7.2 | -0.2 ± 1 (-2.5%) | 0.59 |
| LPL activity (mU/ml) | 134 ± 52 | 122 ± 44 | -12 ± 53 (-1.9%) | 0.44 |
| HL activity (mU/ml) | 227 ± 110 | 220 ± 89 | -9 ± 65 (4.5%) | 0.64 |

**Table 2.** Lanosterol, sitosterol, C4 and their relations to total plasma cholesterol before and 4h and 8h after fat-rich mixed meal (FRMM). The analyses were performed before and after the 12 weeks evolocumab intervention. Lanosterol, sitosterol and C4 were quantified in nmol/L concentrations, and total cholesterol in mmol/L concentration. Thus, data are reported as mmol/mol. P-value is calculated using a paired t-test, p-values < 0.05 are bold.

| | Time point after FRMM | Before intervention (mean ± SD) | After intervention (mean ± SD) | P-value |
|---|---|---|---|---|
| **Lanosterol/Tot chol** | 0h | 43.3 ± 15 | 75.2 ± 27 | **<0.0001** |
| | 4h | 73.2 ± 23 | 134 ± 66 | **<0.001** |

| | | | | |
|---|---|---|---|---|
| | 8h | 65.5 ± 23 | 118 ± 38 | **<0.0001** |
| **C4/Tot chol** | 0h | 15.7 ± 12 | 18.2 ± 10 | 0.15 |
| | 4h | 16.8 ± 11 | 17.1 ± 11 | 0.94 |
| | 8h | 9.94 ± 5.7 | 9.89 ± 4.2 | 0.94 |
| **Sitosterol/Tot chol** | 0h | 819 ± 220 | 871 ± 270 | 0.18 |
| | 4h | 898 ± 230 | 931 ± 390 | 0.69 |
| | 8h | 904 ± 260 | 961 ± 270 | 0.06 |

**Figure 1. Postprandial responses of triglycerides, apoB48 and apoC-III before (●) and after evolocumab treatment (○).** Area under the curve (AUC) was normalized so that 100 % corresponds to the AUC before treatment; subsequently the percentage after treatment is in reference to this value. AUC-values were calculated using the trapezoidal rule. P-values refer to a repeated measures ANOVA analysis with time point (before or after evolocumab) as factor. Stars indicate significant difference (FDR-adjusted t-test) before vs after treatment at a particular time point after the meal.

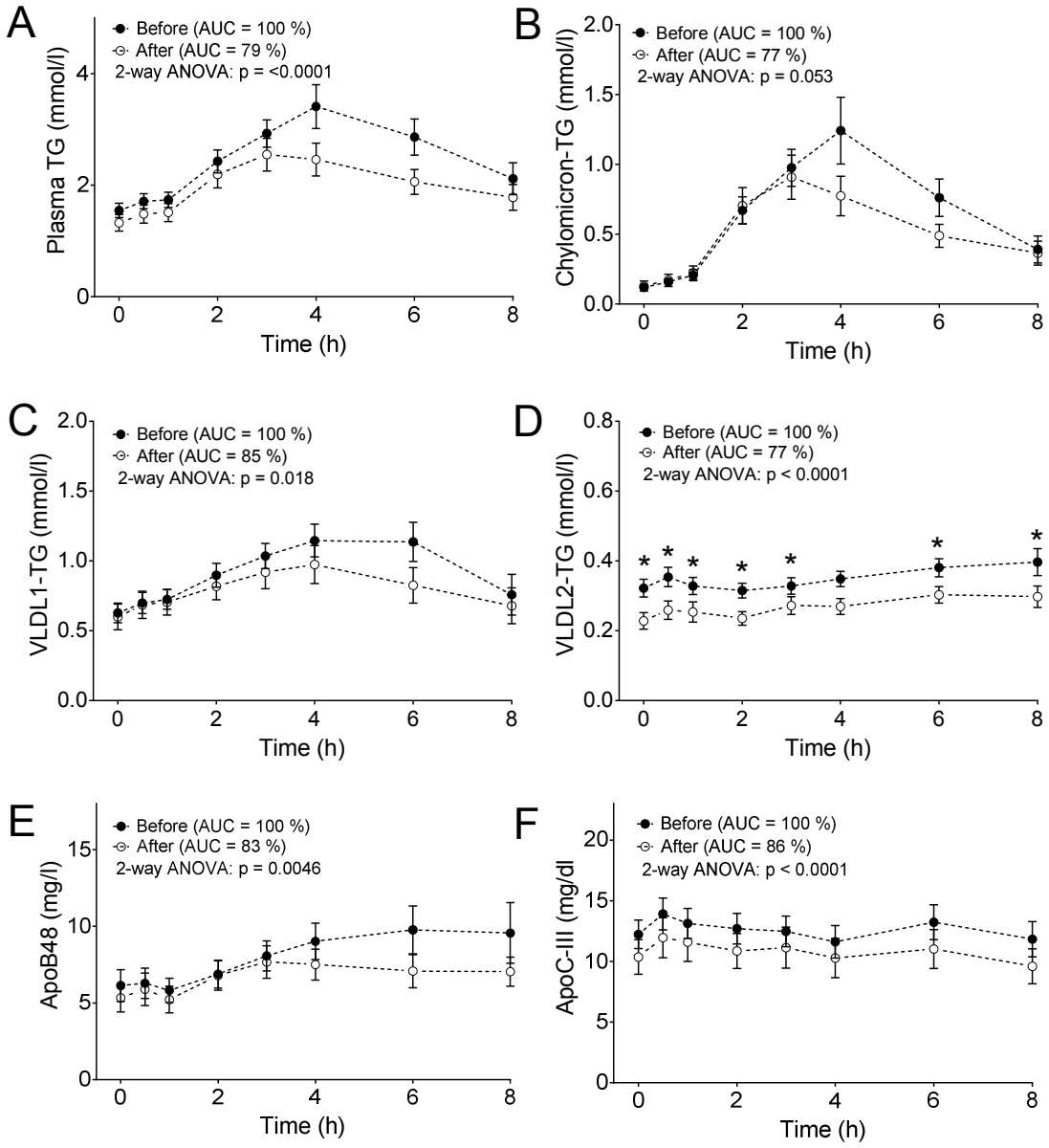


**Figure 2. Postprandial responses of cholesterol in different fractions and total apoB before (●) and after evolocumab treatment (○).** P-values refer to a repeated measures ANOVA analysis with time point (before or after evolocumab) as factor. Area under the curve (AUC) was normalized so that 100 % corresponds to the AUC before treatment; the percentage after treatment is in reference to this value.

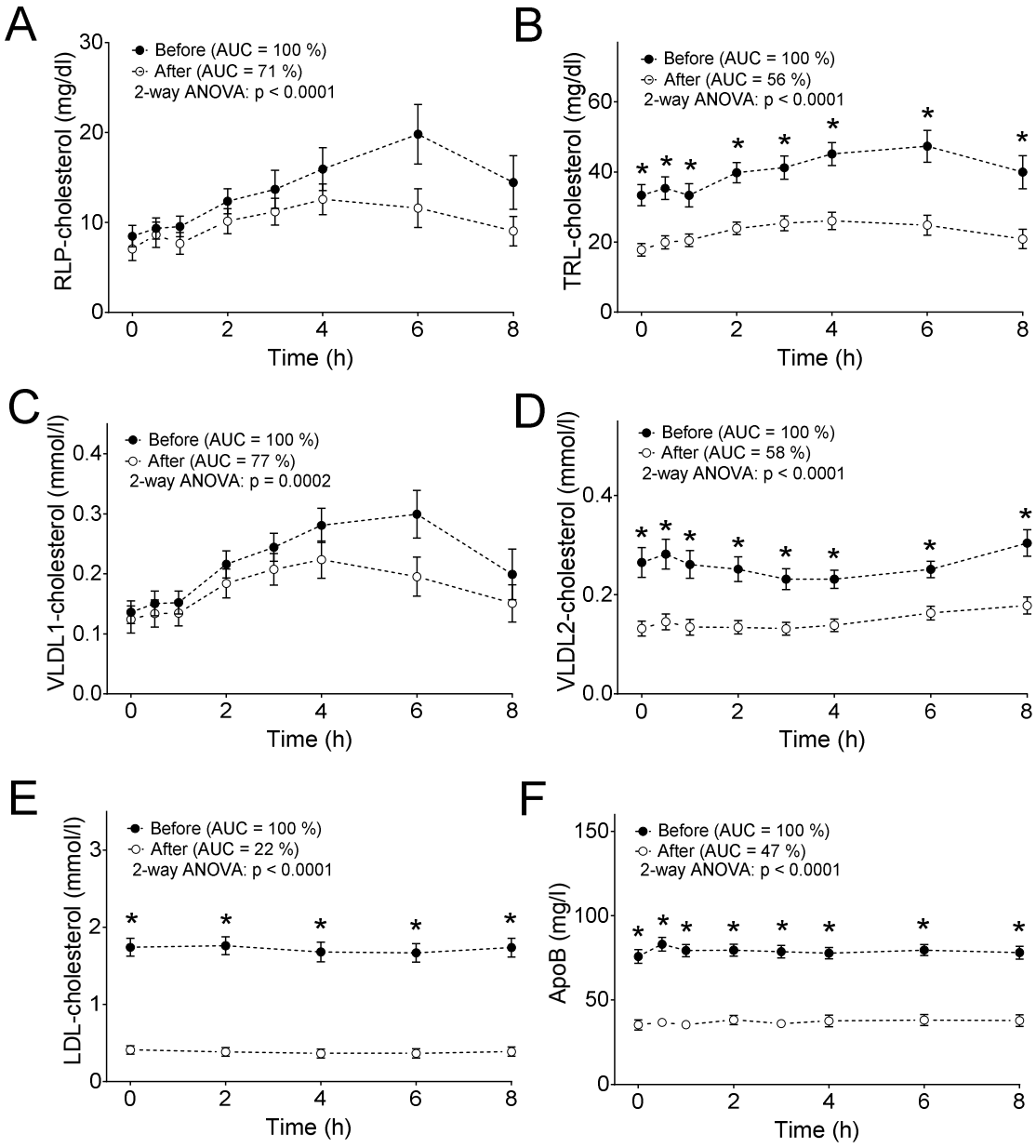


**Figure 3**. Correlation of change in apoC-III after evolocumab intervention with changes in (A) plasma triglycerides, (B) RLP-cholesterol and (C) TRL-cholesterol. R-values refer to Spearman correlation coefficients.

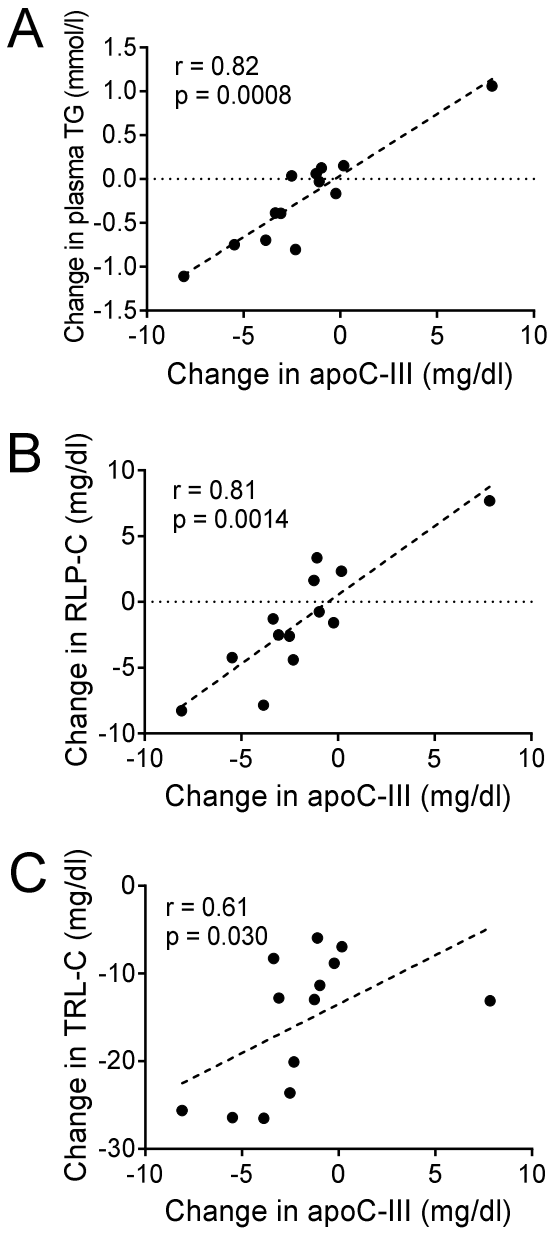


**Figure 4. Postprandial rates of increase in cholesterol synthesis.** The kinetics were calculated by generating for each patient a quadratic function. The rate of cholesterol synthesis was calculated using the first derivative of quadratic function at the given time point (4 and 8 hours). Differences in postprandial indexes of cholesterol synthesis were calculated by using the Wilcoxon signed-rank test.

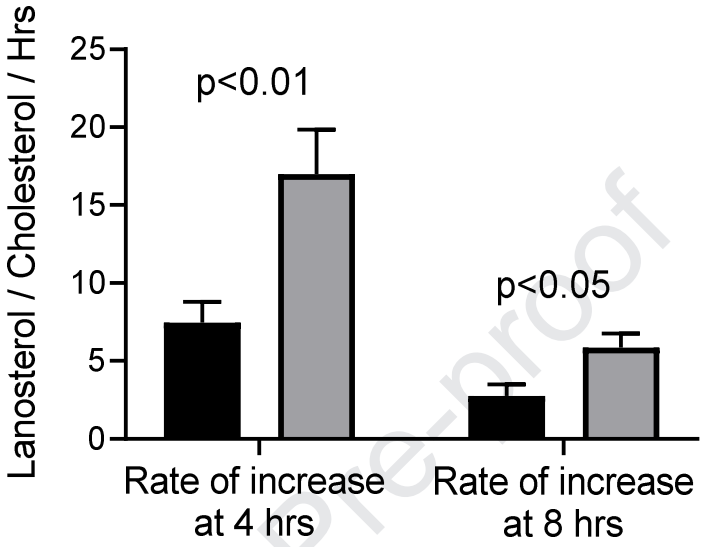
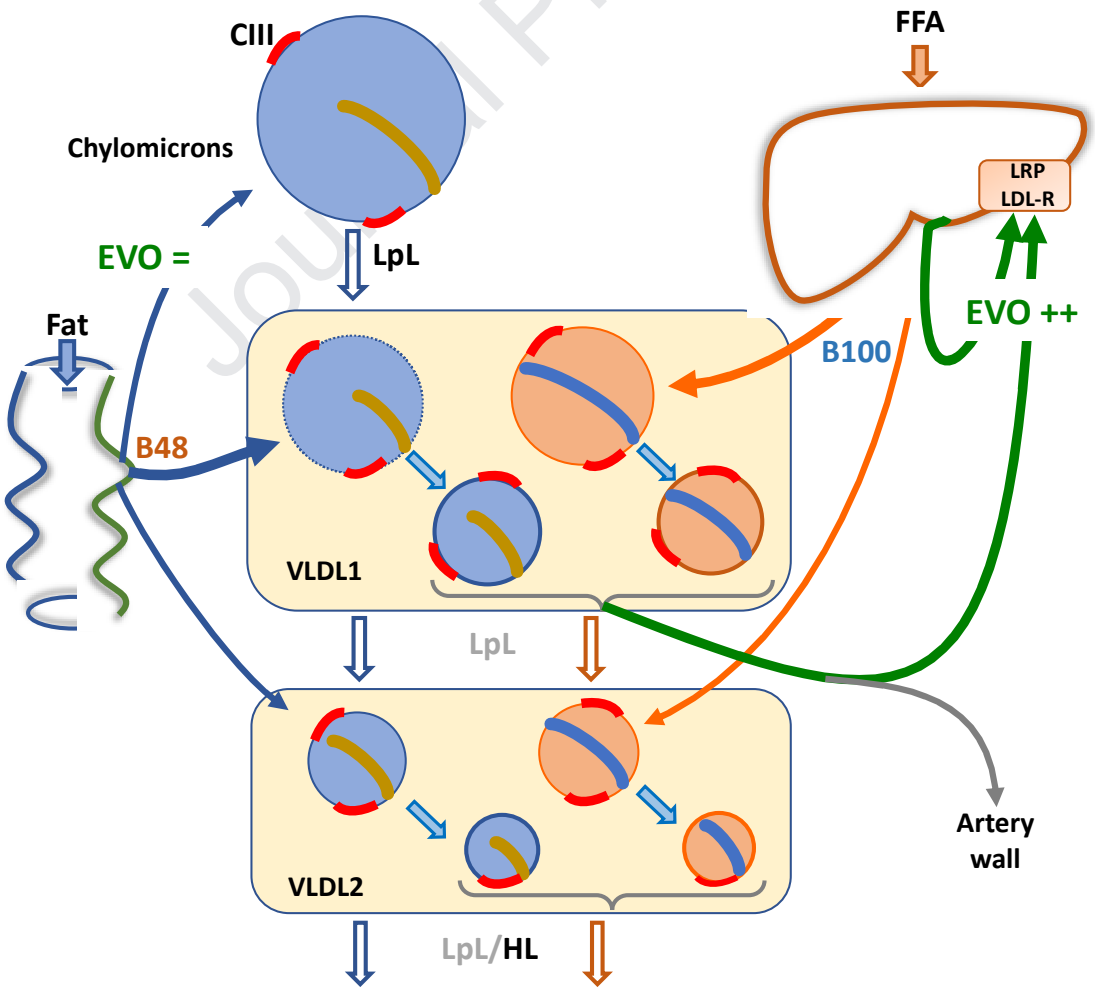


**Figure 5. Potential mechanism of action of evolocumab on postprandial lipoprotein metabolism.** In this scheme which is based on a previous report on the integrated metabolism of chylomicrons and VLDL during dietary lipid absorption,[42] it is envisaged that the intestine secretes apoB48-containing particles into the chylomicron and VLDL density ranges while the liver releases apoB100-containing $VLDL_1$ and $VLDL_2$. Remnant particles, the product of partial lipolysis, can form in each density interval. Evolocumab treatment (Evo) does not appear to affect chylomicron release from the intestine (**Evo=).** It is known that PCSK9 inhibition substantially increases the expression of lipoprotein receptors (especially in the liver) and this leads to accelerated clearance of LDL and VLDL particles). Here it is proposed that on evolocumab an abundance of hepatocyte LDL receptors (**Evo++)** increases clearance of remnant particles from the $VLDL_1$ and $VLDL_2$ density ranges leading to the decreases seen in the later phase of the postprandial period. Further, there is the possibility that small $VLDL_2$ secreted from the liver are immediately taken up again (maybe in the Space of Disse), and this leads to the drop in steady state $VLDL_2$ levels. ApoC-III present on TRL particles will be reduced as particle clearance is accelerated by the superabundance of receptors.



**HIGHLIGHTS**

- Evolocumab treatment improves postprandial responses of TRLs in type 2 diabetics
- Evolocumab treatment decreases remnant lipoproteins as well as small VLDL and LDL
- This reduces ASCVD risk as the atherogenic potential of remnants equal that of LDL
- It is important to test postprandial responses when evaluating novel drug therapies