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Arteriosclerosis, Thrombosis, and Vascular Biology

CLINICAL AND POPULATION STUDIES

Effects of Evolocumab on the Postprandial Kinetics of Apo (Apolipoprotein) B100- and B48-Containing Lipoproteins in Subjects With Type 2 Diabetes

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OBJECTIVE: Increased risk of atherosclerotic cardiovascular disease in subjects with type 2 diabetes is linked to elevated levels of triglyceride-rich lipoproteins and their remnants. The metabolic effects of PCSK9 (proprotein convertase subtilisin/kexin 9) inhibitors on this dyslipidemia were investigated using stable-isotope-labeled tracers.

APPROACH AND RESULTS: Triglyceride transport and the metabolism of apos (apolipoproteins) B48, B100, C-III, and E after a fat-rich meal were investigated before and on evolocumab treatment in 13 subjects with type 2 diabetes. Kinetic parameters were determined for the following: apoB48 in chylomicrons; triglyceride in $VLDL_1$ (very low-density lipoprotein) and $VLDL_2$; and apoB100 in $VLDL_1$, $VLDL_2$, IDL (intermediate-density lipoprotein), and LDL (low-density lipoprotein). Evolocumab did not alter the kinetics of apoB48 in chylomicrons or apoB100 or triglyceride in $VLDL_1$. In contrast, the fractional catabolic rates of $VLDL_2$-apoB100 and $VLDL_2$-triglyceride were both increased by about 45%, which led to a 28% fall in the $VLDL_2$ plasma level. LDL-apoB100 was markedly reduced by evolocumab, which was linked to metabolic heterogeneity in this fraction. Evolocumab increased clearance of the more rapidly metabolized LDL by 61% and decreased production of the more slowly cleared LDL by 75%. ApoC-III kinetics were not altered by evolocumab, but the apoE fractional catabolic rates increased by 45% and the apoE plasma level fell by 33%. The apoE fractional catabolic rates was associated with the decrease in $VLDL_2$- and IDL-apoB100 concentrations.

CONCLUSIONS: Evolocumab had only minor effects on lipoproteins that are involved in triglyceride transport (chylomicrons and $VLDL_1$) but, in contrast, had a profound impact on lipoproteins that carry cholesterol ($VLDL_2$, IDL, LDL).

REGISTRATION: URL: https://www.clinicaltrials.gov; Unique identifier: NCT02948777.

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Key Words: apolipoprotein ■ cardiovascular diseases ■ chylomicrons ■ evolocumab ■ kinetics

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Type 2 diabetes (T2DM) is associated with increased risk of ischemic cardiovascular events even when current standard of care is in place, and atherosclerotic cardiovascular disease continues to be the leading cause of morbidity and mortality in subjects with T2DM.[1,2] An important component of the elevated risk is believed to be the dyslipidemia that develops in T2DM, which is characterized by increased levels of both fasting and postprandial TRLs (triglyceride-rich lipoproteins: VLDLs [very low-density lipoproteins], and chylomicrons) and their remnants, accumulation of small dense LDLs (low-density lipoproteins), and low levels of HDLs (high-density lipoproteins).[3] There is clear evidence of the benefit of lowering LDL-cholesterol (LDL-C) levels in subjects

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Nonstandard Abbreviations and Acronyms

ANGPTL-3	angiopoietin-like protein 3
Apo	apolipoprotein
FCR	fractional catabolic rate
HDL	high-density lipoprotein
IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein-cholesterol
Lp(a)	lipoprotein(a)
PCSK9	proprotein convertase subtilisin/kexin 9
T2DM	type 2 diabetes
TRL	triglyceride-rich lipoprotein
TRL-C	TRL-cholesterol
VLDL	very low-density lipoprotein

Highlights

- Anti-PCSK9 (proprotein convertase subtilisin/kexin 9) monoclonal antibodies had profound impact on the cholesterol-carrying lipoprotein species ($VLDL_2$ [very low-density lipoprotein], IDL [intermediate-density lipoprotein], and LDL [low-density lipoprotein]), but little effect on triglyceride transport, both in the post-prandial and in the fasting state.
- The marked reduction of LDL was linked to the appearance of metabolic heterogeneity in this lipoprotein fraction.
- Anti-PCSK9 monoclonal antibodies did not influence apoC (apolipoprotein C)-III kinetics but the fractional catabolic rate of apoE increased 45%, and the plasma level of apoE (apolipoprotein E) fell 33%. Associations were evident between apoE fractional catabolic rate and the decrease in $VLDL_2$ and IDL apoB100 (apolipoprotein B100) concentrations.
- Increase in the number of LDL receptors through PCSK9 inhibition has multiple and complex effects on the $VLDL_2$-IDL-LDL metabolic cascade resulting in a decrease of these atherogenic lipoproteins, which are linked to atherosclerotic cardiovascular disease risk.

with T2DM,[4] but there is also an emerging consensus that TRL remnants, as independent causative agents in atherogenesis, should also be targeted.[5,6]

Statins are the first-line therapy for LDL-C lowering in subjects with T2DM as well as in the general population at risk.[4,7,8] The benefits of profound reductions in LDL-C have been explored in large-scale trials of combination therapies with statin plus ezetimibe[9–11] or PCSK9 (proprotein convertase subtilisin/kexin 9) inhibitors,[12–16] and it is clear that in subjects with T2DM on maximally tolerated statin dose, both add-on treatments lead to additional reduction in cardiovascular events.[17,18] Exploration of the wider impact of PCSK9 inhibitors on the plasma lipoprotein profile reveals that these agents produce moderate reductions in fasting triglyceride levels in the general at-risk population[13,19] and in those with T2DM.[20–22] To date, there is limited information on the effects of PCSK9 inhibitors on postprandial lipoprotein metabolism. The drugs seem to have little or no impact on alimentary lipemia in healthy people with low plasma triglyceride levels.[23,24] However, this may not be the whole picture because investigations using model systems indicate that PCSK9 is highly expressed in intestinal cells, that genetically modified mice lacking PCSK9 have reduced postprandial triglyceridemia,[25,26] and that PCSK9 potentially has a central role in chylomicron synthesis in intestinal cells.[27]

Postprandial lipid responses are related strongly to plasma triglyceride levels,[28–31] and this has led to studies by ourselves and others into the effects of PCSK9 inhibitors on alimentary lipemia in subjects with T2DM and moderately elevated triglyceride.[32,33] It was observed that these drugs to a degree did reduce the postprandial increments in plasma and chylomicron triglyceride levels.[34,35] The aim of the present study was to examine in greater detail the effects of the PCSK9 inhibitor evolocumab on triglyceride transport and postprandial lipid metabolism in subjects with T2DM. By using a novel integrated non–steady-state model,[36] we evaluated the impact of evolocumab on the kinetics of apoB48- and apoB100-containing lipoproteins. Our goal was to shed further light on the benefits of PCSK9 inhibitors and the extent to which this drug class alters remnant lipoprotein metabolism in addition to its LDL-C-lowering action.

METHODS

Data Availability

Data that support the findings of this study but are not included in the article or in the Data Supplement are available from the corresponding author upon reasonable request. We are not able, however, to share detailed individual data that can be used to identify the study subjects.

Study Design and Subjects

The outline design, description of the participating subjects, and changes in postprandial responses as assessed by increments in plasma concentration have previously been reported.[33] The present investigation explores the changes in apoprotein kinetics using stable-isotope-tracer technology and multicompartmental modeling.

Briefly, inclusion criteria were a diagnosis of T2DM (average duration, 4.8 years), current hemoglobin A1c <9.0% (11.7 mmol/L); body mass index, 25 to 50 kg/m^2; age, 18 to 77 years; plasma triglyceride, 1.0 to 4.5 mmol/L, and LDL-C, 1.8 to 4.0 mmol/L on a standardized lipid-lowering regimen of atorvastatin 20 mg/day. Fourteen subjects with T2DM were

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recruited and 13 subjects completed the investigation. One subject was excluded from the analyses because of oral prednisone use (an exclusion criterion) because of worsening of asthma symptoms. The 13 subjects in this study were included also in the investigations presented in our earlier report.[33]

The study had a sequential, nonrandomized design with all subjects undergoing a 4-week run-in period to establish uniform background medication (statin plus metformin, which was continued throughout the whole study). If a subject was not on atorvastatin, this treatment was initiated at a dose of 20 mg/day. The first metabolic investigation was then performed. Evolocumab at a dose of 140 mg was then administered subcutaneously every 2 weeks and continued for 12 weeks. At the end of this treatment period, the second metabolic investigation was undertaken.

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.

Metabolic Protocol

Subjects were admitted to the clinical research unit of the Helsinki University Central Hospital on the test-meal day (day 0) having fasted for 12 hours overnight before and after evolocumab treatment. To investigate the kinetics of apo (apolipoprotein) B100, apoB48, apoC-III, apoE, and triglyceride, we used stable-isotope-labeled leucine and glycerol as described (Figure I in the Data Supplement).[36,37] The tracers, administered as a bolus at 8.00 AM (0-hour time point), were deuterated leucine (5,5,5-D3 Euriso-Top, d3-leucine) at a dose of 7 mg/kg body weight, and 500 mg of deuterated glycerol (1,1,2,3,3-D5 Euriso-Top, d5-glycerol). At the 2-hour time point, subjects consumed a standard fat-rich meal that contained 927 kcal (59.5% from fat [68 g], 24.4% from carbohydrate [63 g], and 15.5% from protein [40 g]) and consisted of bread, cheese, ham, boiled eggs, fresh red pepper, a cocoa emulsion containing 40 g olive oil (Carapelli, Firence), orange juice, and tea or coffee. During the kinetic study, only water was allowed (ad libitum) and the participants remained physically inactive.

Blood samples were obtained before (at 0 hour) and after tracer injection. A 24-hour blood sample was obtained at 8.00 AM the next day when the subject returned to the ward, again in the fasting state. The samples collected at 30, 45, 60, 75, 90, 120, and 150 minutes and 3, 4, 5, 6, 8, 10, and 24 hours were used for the measurement of d3-leucine enrichment in apoB48 and apoB100 and d5-glycerol enrichment in triglyceride in $VLDL_1$ and $VLDL_2$. D3-leucine enrichment was measured in IDL (intermediate-density lipoprotein)- and LDL-apoB100 at these time points and in further blood samples collected at 8.00 AM (in the fasting state) on days 2, 3, 4, and 7.

Measurement of De Novo Lipogenesis

On the evening before the test-meal day, subjects were given 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 to triglyceride palmitate in VLDL. Background enrichment of deuterium in VLDL triglyceride palmitate was determined from a blood sample drawn in the week before the test-meal day. De novo lipogenesis was estimated at the 0-hour time point.[38]

Quantification of apoB48 and Tracer Enrichment

The amount of apoB48 (in milligrams) and its tracer enrichment in chylomicrons, $VLDL_1$, and $VLDL_2$ was determined as previously described.[36] Briefly, isotopically labeled internal apoB48-peptide standards were added to 21 µL plasma before digestion with trypsin. An antipeptide antibody technique (mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies) was used to enrich the sample because of the low abundance of apoB48. Enrichment and quantification were performed using liquid chromatography–mass spectrometry.[36]

Determination of d3-Leucine Enrichment in Plasma Leucine and apoB100 and d5-Glycerol Enrichment in Triglyceride

D3-leucine tracer enrichment in plasma was measured at all time points during the first 24 hours. $VLDL_1$ and $VLDL_2$ were separated by density gradient ultracentrifugation and enrichment of apoB100 and triglyceride-glycerol from the fractions were analyzed as described.[39]

Determination of d3-Leucine Enrichment in apoC-III and apoE

Enrichment of apoC-III was analyzed as previously described.[40] In the same protocol, enrichment of apoE was analyzed as described in the Data Supplement.

Multicompartmental Modeling and Parameter Estimation

The base compartmental model describing nonsteady-state (postprandial) kinetics of apoB48- and apoB100-containing lipoproteins has recently been published.[37] It was adapted to include IDL- and LDL-apoB100 kinetics as described in Figure I in the Data Supplement. The apoB-triglyceride model was implemented in SAAMII (simulation, analysis, and modeling software II),[41] and all subjects were modeled individually. ApoC-III kinetics were derived using a previously described model in Monolix (Lixoft SAS, 2018; Antony, France) using population modeling.[40] ApoE kinetics were modeled using a similar approach to that of apoC-III.

The apoB48/apoB100/triglyceride multicompartmental model[36,37] included nonsteady-state elements to analyze the change in concentration in key lipoprotein fractions during absorption of the fat-rich meal. For triglyceride in chylomicrons, $VLDL_1$ and $VLDL_2$, apoB48 in chylomicrons, $VLDL_1$ and $VLDL_2$, and apoB100 in $VLDL_1$, the change in concentration was included in the model along with the tracer enrichment data (Figure II in the Data Supplement). The modeling procedure was

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required to achieve the best possible fit to both sets of observations: mass change and enrichment (tracer/tracee ratios). Input of dietary triglyceride was a constant because this was known (from the composition of the fat-rich meal) and was the same for all subjects both before and on evolocumab. Using the fat load as a constraint for the non–steady-state analysis reduces ambiguity in the model and permits valid rate constants to be derived. Both production rates and fractional catabolic rates (FCR) for apoB48 were permitted to vary and a good fit was obtained to the observed data (as seen in Figure 1 and Figure II in the Data Supplement). The VLDL system in the model was constructed as a traditional steady-state system with one addition: during lipid absorption, the FCR for VLDL was permitted to decrease to allow the $VLDL_1$ concentration to increase (as was observed). This feature as explained previously[36,37] reflects the known competition of VLDL and chylomicrons for lipoprotein lipase with chylomicrons being the preferred substrate.

Lipoprotein Preparation and Biochemical Analyses

Lipoprotein fractions (chylomicrons, Sf >400; large $VLDL_1$ particles, Sf 60–400; and smaller $VLDL_2$ particles, Sf 20–60) were prepared from blood samples density gradient ultracentrifugation.[42] IDL and LDL fractions were isolated from 2 mL EDTA plasma by sequential centrifugation in a TI 50.4 rotor; VLDL was removed first following centrifugation for 18 hours, IDL was then separated after a further centrifugation at density of 1.035 g/mL, and finally LDL was obtained following centrifugation at a density of 1.063 g/mL. Triglyceride and cholesterol concentrations in total plasma and lipoprotein fractions (chylomicrons, $VLDL_1$, $VLDL_2$, IDL, and LDL) were analyzed using the Konelab 60i analyzer (Thermo Fisher Scientific, Finland). TRL-cholesterol (TRL-C; ie, the cholesterol in the aggregate of chylomicron remnants, VLDL and IDL) and remnant-lipoprotein cholesterol (ie, cholesterol in lipoproteins not binding to anti-apoAI and anti-apoB100), were analyzed using automated direct assays (Denka Seiken, Tokyo, Japan).[2] ApoB48 levels in total plasma were measured by ELISA (Shibayagi, Shibukawa, Japan). Concentrations of plasma glucose were measured using the hexokinase method and insulin using sandwich immunoassays (Roche Diagnostic, Germany). Plasma levels of apoC-III were measured immunoturbometrically (Kamiya Biomedical Company, Seattle, WA). ELISAs were used to measure serum apoE (STA-367, Cell Biolabs, San Diego, CA) and ANGPTL-3 (angiopoietin-like protein 3; RD191092200R, BioBendor, Brno, Czech Republic). 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 nonesterified fatty acids were analyzed with an automated enzymatic colorimetric method (Wako Chemicals, Neuss, Germany).

Determination of Lipoprotein(a) Concentrations and Apo(a) Isoforms

The analyses were performed by ELISA and Western blotting as described previously.[43] A polyclonal affinity-purified rabbit anti-human apo(a) antibody was used for coating and the horseradish-peroxidase-conjugated monoclonal anti-apo(a) antibody 1A2 for detection.[44] The lower detection limit of the assay is 0.1 mg/dL. For Western blotting, 150 ng Lp(a) (lipoprotein[a]) of each sample were loaded on a 1.46% agarose gel with 0.08% SDS (18 hour, 0.04 A constant current). A size standard containing 5 plasma samples with only one apo(a) isoform of 13, 19, 23, 27, 35 KIV (plasminogen kringle 4 homologue) repeats was applied in every seventh well of the gel. The gel was blotted semi-dry to a PVDF (polyvinylidene difluoride) membrane and blocked with 1% BSA, 85 mmol/L NaCl, 10 mmol/L TRIS, 0.2% Triton X-100 (30 minutes, 37 °C). After incubation with horseradish-peroxidase-conjugated 1A2 antibody, the membrane was washed, ECL substrate (Western-Bright Chemilumineszenz Spray, Biozym, Vienna, Austria) was added, and signals were recorded (Amersham Hyperfilm ECL, GE Healthcare, Chicago, IL).

Lipase Measures

The heparin test was performed after an overnight fast, and the subjects received a bolus injection of 75 IU/kg heparin intravenously. An immunochemical method was used for the selective measurement of lipoprotein lipase and hepatic lipase activities in postheparin plasma.[34]

Determination of Intra-Abdominal Fat Depot

Proton magnetic resonance spectroscopy was performed using a 1.5-T whole-body device to determine liver fat content[45] and subcutaneous abdominal and intra-abdominal fat.[46] All analyses of the imaging results were performed by one person (A. Hakkarainen). Subjects were advised to fast for 4 hours before imaging.

Statistical Methods

Statistical analyses were performed using R version 3.6.3. *P* values were calculated using the Wilcoxon Signed-rank test. R values refer to Spearman rank correlation coefficient. Repeated measures ANOVA were performed using the packages *lme4* and *lmerTest*. Repeated measures correlation analyses were performed using the package rmcorr.

RESULTS

Demographics, plasma lipid, and apolipoprotein concentrations, and indices of hepatic lipid metabolism for the 13 subjects with T2DM in this study are given in Table 1. Further characteristics in terms of the response of cardiometabolic risk factors to evolocumab have been reported earlier.[33] Evolocumab therapy added to background statin treatment resulted in a 42% fall in total plasma cholesterol and a profound, near 80% decrease in LDL-C. Plasma triglyceride, which was within the normal range before addition of evolocumab, did not show a significant change. Total plasma apoB fell 53%, but there was no change in fasting apoB48 levels. Plasma apoC-III was reduced moderately (15%), and there was a more marked decrease in apoE

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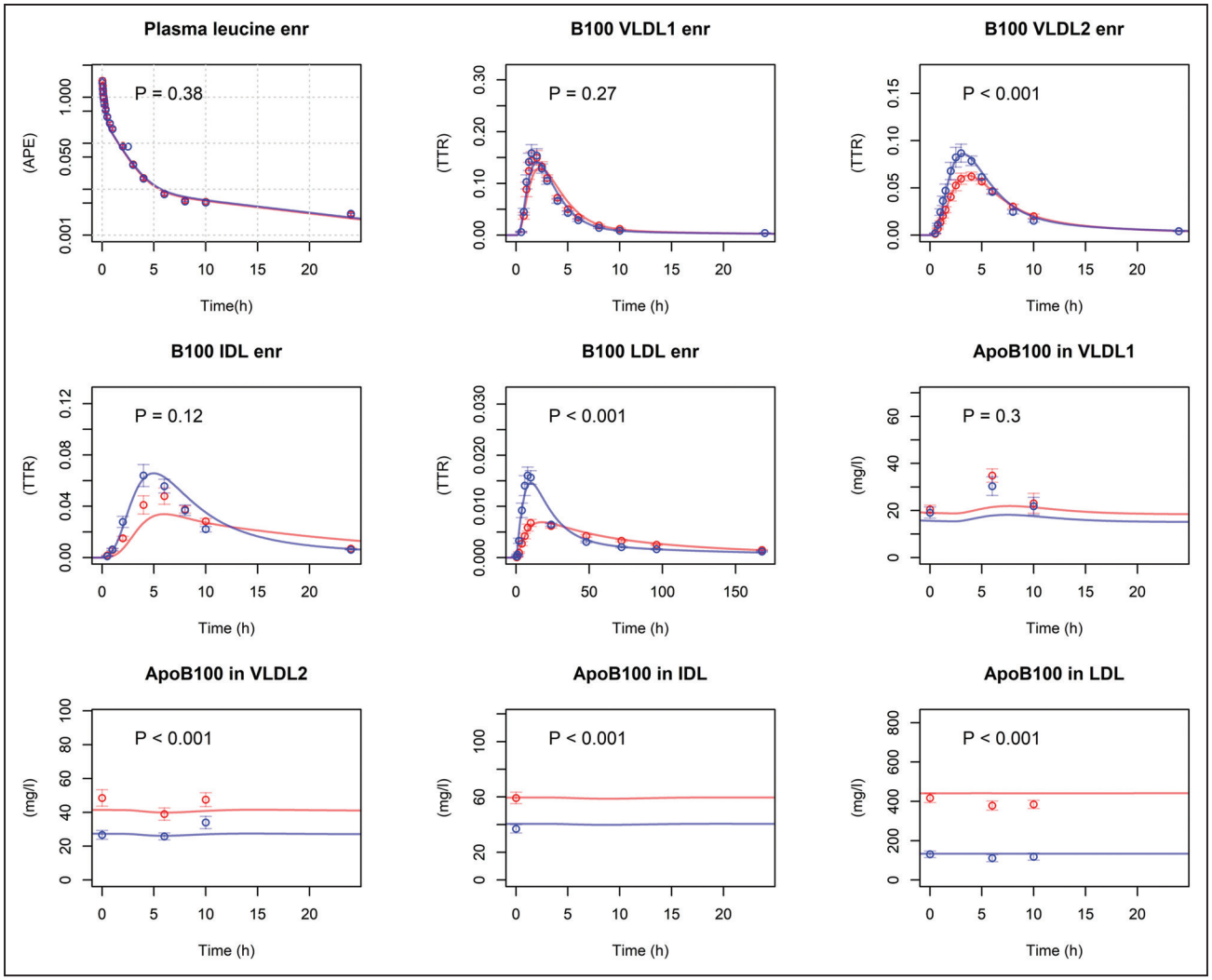


Figure 1. ApoB100 (apolipoprotein B100) kinetics in subjects with type 2 diabetes before and on evolocumab.
Mean (±SEM) observed values for subjects (n=13) before (red points) and on (blue points) evolocumab therapy. ApoB48 (apolipoprotein B48) and apoB100 concentrations in each lipoprotein fraction are in milligrams per liter, enrichments are tracer-to-tracee ratios (TTR), or for plasma leucine atom percent excess (APE). *P* value refers to repeated measures ANOVA except for IDL (intermediate-density lipoprotein) pool size, which refers to Wilcoxon signed-rank test. Solid lines are model fits to the observed data. LDL indicates low-density lipoprotein; and VLDL, very low-density lipoprotein.

concentration (33%, $P<0.001$). Lp(a) fell 24%, in line with expectations.[47–50] TRL-C was substantially reduced, but remnant-lipoprotein cholesterol was not altered significantly. There was no change in markers of liver lipid metabolism or liver fat on evolocumab.

Effects of Evolocumab on apoB100 Metabolism

Parameters describing apoB48 and apoB100 kinetics were derived using a compartmental model for chylomicron and VLDL metabolism that has been described in detail in earlier publications[36,37] and was expanded for the present study to include IDL- and LDL-apoB100 sub-systems (Figure I in the Data Supplement). Inputs to the model were (1) concentrations of apoB48 and triglyceride in chylomicrons, concentrations of apoB48, apoB100, and triglyceride in $VLDL_1$ and $VLDL_2$, and concentration of apoB100 in IDL and LDL; (2) the enrichment curves for apoB48 in chylomicrons, $VLDL_1$ and $VLDL_2$, and for apoB100 in $VLDL_1$, $VLDL_2$, IDL, and LDL; and (3) enrichment curves for triglyceride in $VLDL_1$ and $VLDL_2$. Figure 1 and Figure II in the Data Supplement provide mean values for the full data set.

$VLDL_1$- and $VLDL_2$-apoB100 and -Triglyceride Kinetics

Evolocumab treatment had little effect on the pool sizes and tracer enrichment curves for apoB100 in $VLDL_1$ (Figure 1) and the derived production rates and FCR for apoB100 in this fraction were virtually identical in the 2 phases of the study (Table 2). Similarly, triglyceride

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Table 1. Baseline Characteristics and Changes on Evolocumab in Subjects With T2DM

	Before evolocumab n=13	On evolocumab n=13	Change	*P* value
Weight, kg	82.7±15.9	82.8±15	0.1±1.3 (+0.1%)	0.31
BMI, kg/m^2	29.4±4	29.4±3.7	0.1±0.4 (+0.2%)	0.31
Lipids and lipoproteins				
Cholesterol, mmol/L	3.9±0.5	2.3±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%)	0.0033
Triglycerides, mmol/L	1.6±0.5	1.3±0.5	−0.2±0.6 (−14%)	0.19
LDL-C, mmol/L	1.7±0.4	0.4±0.2	−1.4±0.4 (−78%)	<0.001
LDL size, nm	25.6±1.5	25.7±1.3	−0.1±0.9 (−0.4%)	0.62
ApoC-III, mg/dL	12.2±4.3	10.4±5.2	−1.9±3.7 (−15%)	0.022
ApoB, mg/dL	75.9±14.5	35.3±10.9	−40.5±8.8 (−53%)	<0.001
ApoB48, mg/L	6.1±3.8	5.3±3.3	−0.8±2.5 (−13%)	0.38
Lp(a), mg/dL	29.8±29.9	22.7±25.7	−7.2±6.4 (−24%)	<0.001
ApoE, mg/dL	3.0±0.7	2.0±0.5	−1±0.6 (−33%)	<0.001
ApoA-I, mg/dL	142±25.1	151±25.7	9.5±11.3 (+7%)	0.011
TRL-C, mg/dL	33.4±10.9	17.8±6.5	−15.6±7.8 (−47%)	<0.001
RLP-C, mg/dL	8.5±4.3	7.1±4.7	−1.4±4.5 (−17%)	0.24
ANGPTL3, ng/mL	240±45	246±58	−5.6±48 (+2%)	0.79
Hepatic lipid metabolism				
DNL, %	17.6±12.8	17.1±11.7	−0.5±13 (−3%)	0.95
NEFA, μmol/L	615±207	608±181	−7.2±190 (−1%)	0.64
β-OHB, mg/dL	0.7±0.2	0.7±0.3	0.1±0.3 (+8%)	0.59
Liver fat, %	6.6±6.6	6.3±7.2	−0.2±1.1 (−3%)	0.43

Data are shown as mean±SD. *P* values were calculated using the Wilcoxon signed-rank test. Biomarkers were analyzed in fasting samples taken on the day of the kinetic study, before, and after the 12-wk evolocumab treatment period. Data have partly been published earlier (see study by Taskinen et al[33]). ANGPTL3 indicates angiopoietin-like protein 3; ApoB, apolipoprotein B; ApoB48, apolipoprotein B48; ApoC, apolipoprotein C; ApoE, apolipoprotein E; BMI, body mass index; DNL, de novo lipogenesis; HDL-C, high-density lipoprotein-cholesterol; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein-cholesterol; Lp(a), lipoprotein(a); NEFA, nonesterified fatty acid; OHB, β-hydroxybutyrate; RLP-C, remnant-like particle-cholesterol; T2DM, type 2 diabetes; and TRL-C, TRL-cholesterol.

kinetics within $VLDL_1$ were the same before and on evolocumab treatment (Table 2).

In contrast, there was a clear impact of evolocumab on $VLDL_2$-apoB100 and -triglyceride kinetics (Figure 1; Table 2). The enrichment curve for both $VLDL_2$-apoB100 and -triglyceride rose and fell more sharply on evolocumab than it did before treatment, consistent with a higher turnover rate. The model-derived kinetic parameters revealed that while the $VLDL_1$ to $VLDL_2$ transfer rate and direct production rates for $VLDL_2$-apoB100 and -triglyceride were not affected by evolocumab, the FCRs for $VLDL_2$-apoB100 and -triglyceride both increased by about 45% (P=0.0034, P=0.013, respectively; Table 2). The net result was that the pool sizes for $VLDL_2$-apoB100 and -triglyceride fell 28% and 31%, respectively (P=0.013 and P=0.0017; Table 2).

IDL- and LDL-apoB100 Kinetics

On evolocumab, the overall LDL-apoB100 pool size was reduced 70% (P<0.001) due to a 144% increase in the FCR (P<0.001) and a 31% fall in the amount of IDL converted to LDL (from a mean of 450 to 310 mg/day, P=0.027; Table 2). There was no change in direct LDL production.

We noted during the modeling process that subjects on evolocumab exhibited metabolic heterogeneity within the LDL density range. The decay component of the enrichment curve was biexponential, indicating the presence of 2 metabolically distinct entities (Figure 2). Up to 50 hours after administration of the tracer, there was relatively rapid catabolism of LDL, and thereafter the decay curve was dominated by LDL that was catabolized more slowly. To account for these observations, it was necessary to introduce 2 compartments (LDL-A and LDL-B) into the LDL sub-system in the model (Figure I in the Data Supplement) to achieve a satisfactory fit to the experimental data; this was applied to subjects before and on evolocumab treatment.

To discount the possibility that the slowly decaying LDL component was due to the presence of Lp(a)-associated apoB100, we measured the amount of Lp(a) in the LDL fraction before and on evolocumab therapy. In all subjects in both study periods, Lp(a) was present at negligible levels within the LDL fraction, representing <0.2% of the apoB100.

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Table 2. Effects of Evolocumab on apoB100, apoB48, and Triglyceride Metabolism in Subjects With T2DM

	Before evolocumab n=13	On evolocumab n=13	*P* value
ApoB100 metabolism			
$VLDL_1$ production, mg/d	790±230	750±230	0.61
$VLDL_1$ pool, mg	61±36	53±45	0.34
$VLDL_1$ FCR, pools/d	17±8.6	20±11	0.68
$VLDL_1$ to $VLDL_2$ transfer, mg/d	530±300	510±210	0.64
$VLDL_2$ direct production, mg/d	270±91	230±96	0.11
$VLDL_2$ pool, mg	130±47	93±58	0.013
$VLDL_2$ FCR, pools/d	6.6±3.3	9.5±4.2	0.0034
$VLDL_2$ to IDL transfer, mg/d	650±210	620±270	0.84
IDL direct production, mg/d	47±67	39±48	0.96
IDL pool, mg	190±50	130±49	0.0034
IDL FCR, pools/d	3.5±1.3	5.4±3.2	0.15
IDL to LDL transfer, mg/d	450±190	310±140	0.027
LDL direct production, mg/d	110±23	110±21	0.89
LDL pool, mg	1500±470	460±270	<0.001
LDL FCR, pools/d	0.32±0.15	0.78±0.34	<0.001
Triglyceride metabolism			
$VLDL_1$ production, g/d	34±18	33±13	0.91
$VLDL_1$ pool, g	1.8±1	1.8±1.5	0.95
$VLDL_1$ FCR, pools/d	38±27	37±23	1
$VLDL_2$ total production, g/d	8.8±3.8	8.5±2.7	0.68
$VLDL_2$ direct production, g/d	2.4±0.83	2.1±0.86	0.31
$VLDL_2$ pool, g	1.1±0.43	0.75±0.43	0.0017
$VLDL_2$ FCR, pools/d	10±6.3	15±8.7	0.013
ApoB48 metabolism			
Total production, mg/d	570±60	580±75	0.31
Postprandial CM prod, mg/d	240±50	230±43	0.91
CM-apoB48 FCR, pools/d	37±24	46±32	0.27
CM-TG production, g/d	66.5±0	66.5±0	NA
CM-TG FCR, pools/d	41±27	54±31	0.27

Data are shown as mean±SD. *P* values were calculated using the Wilcoxon signed-rank test. ApoB48 indicates apolipoprotein B48; ApoB100, apolipoprotein B100; FCR, fractional catabolic rate; IDL, intermediate-density lipoprotein; T2DM, type 2 diabetes; TG, triglyceride; and VLDL, very low-density lipoprotein.

Individual production rates and FCRs were then derived for the 2 LDL metabolic species as shown in Figure 3. The FCR for LDL-B was the same before and on evolocumab at about 0.19 pools/day (as reflected in the parallel decay curves in Figure 2 about 70 hours after tracer administration), while the FCR for LDL-A showed a 61% increase on evolocumab (from 1.80 to 2.90 pools/day, P=0.008; Figure 3). Using this modification to the model, we were able to derive pool sizes for the 2 LDL species and showed that LDL-A decreased 55% and LDL-B 77% (P<0.001) on evolocumab treatment (Figure 3).

To further explore the cause of this metabolic heterogeneity in LDL, we examined the kinetics of apoB100 in the precursor IDL. Here, while we had pools sizes for all subjects, enrichment curves were available for only 6 of the group. The model fitting process, however, was able to simulate IDL-apoB100 production and clearance rates for all (Table 2) and for the 6 where the comparison could be made, there was good agreement of the simulated kinetic parameters with those obtained by fitting the experimental data (data not shown). IDL-apoB100 pool size was reduced by 33% on evolocumab (Table 2, Figure 3). There was no change in IDL direct production or the amount transferred from $VLDL_2$ on evolocumab, and so the fall in pool size was likely because of a 54% rise in the overall FCR (although this did not achieve significance; Table 2). The amount of IDL converted to LDL-A (about 260 mg/day) was not altered by evolocumab (Figure 3). However, evolocumab increased the fractional rate of direct catabolism of IDL (from 1.3 to 2.7 pools/day) and the amount of IDL apoB100 cleared from the circulation (from 190 to 290 mg/day), although these changes did not achieve statistical significance. The 70% decrease in the fractional transfer rate from IDL to LDL-B and in the amount of IDL converted to LDL-B (from 200 to 50 mg/day, P<0.001) was the most marked perturbation in apoB100 flux on evolocumab (Figure 3).

EFFECTS OF EVOLOCUMAB ON APOB48, APOC-III, AND APOE METABOLISM

ApoB48 in Chylomicrons and VLDL

Postprandial responses to the test meal showed a small but significant decrease in $VLDL_1$-triglyceride (as reported previously[33]) and a borderline decrease in chylomicron triglyceride (Figure II in the Data Supplement). Differences between the values before and on evolocumab were most evident in the later phase of alimentary lipemia (at the 6- and 8-hour time points). Likewise, there was a trend on evolocumab to reduce apoB48 levels in the chylomicron and $VLDL_1$ fractions at these time points (Figure II in the Data Supplement). Production and clearance rates for apoB48 in response to the fat-rich meal were derived from the pools sizes and enrichment curves for chylomicrons, $VLDL_1$, and $VLDL_2$ (Figures I and II in the Data Supplement). Evolocumab had no significant impact overall on these kinetic parameters (Table 2), although there was a tendency for the FCR for apoB48 and triglyceride in chylomicrons to increase.

ApoC-III and apoE Kinetics

Mean plasma apoC-III levels were reduced moderately on evolocumab therapy (Table 1), but we did not observe any significant changes in the production or FCR of apoC-III (Table 3). In contrast, evolocumab induced a more marked decrease in mean plasma apoE levels

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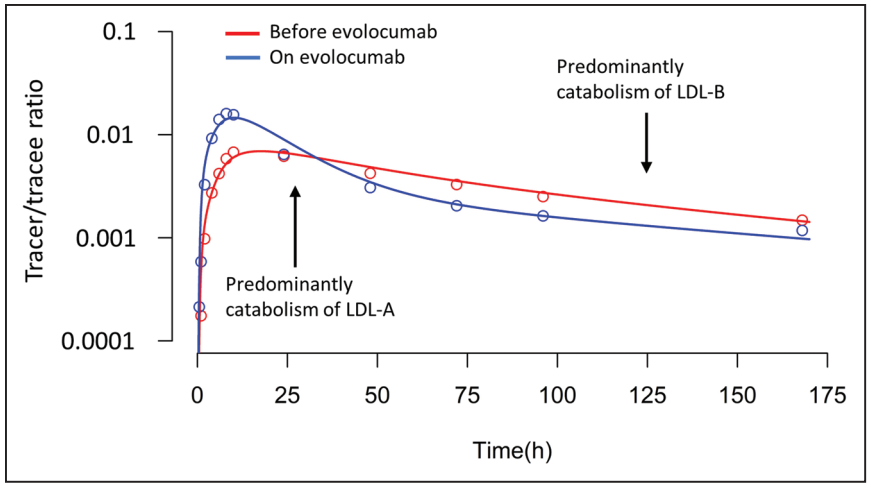


Figure 2. LDL (low-density lipoprotein)-apoB100 (apolipoprotein B100) kinetics in subjects with type 2 diabetes before and on evolocumab. On evolocumab, the LDL-enrichment curve displays a bi-exponential pattern. To fit the LDL-enrichment data on evolocumab, a model with 2 pools of LDL (pool A and B) with differing kinetic properties was required. The same model structure was applied before and on evolocumab. Data points refer to the mean of all subjects and lines refer to the mean of the model fits before and on evolocumab.

(Table 1), which was attributable to a 48% increase in the FCR ($P<0.001$; Table 3).

Given that apoE has a role in the receptor-mediated metabolism of apoB-containing lipoproteins and that apoE catabolism was increased by evolocumab (Table 1, Figure III in the Data Supplement), we explored interrelationships between the apoE FCR and circulating levels of $VLDL_1$-, $VLDL_2$-, IDL-, and LDL-apoB100 (Figure 4). The pool size of $VLDL_1$-apoB100 (which was not altered by evolocumab) was not related to the apoE FCR (Figure 4A). $VLDL_2$- and IDL-apoB100 pool sizes appeared to show moderate, continuous inverse associations with (log) apoE FCR both before and on evolocumab (repeated measures: $VLDL_2$, $r=-0.44$, $P=0.034$; IDL, $r=-0.48$, $P=0.022$; Figure 4B and 4C). This pattern was similar to the relationship between the FCR and pool size of apoE itself (Figure III in the Data Supplement) LDL-apoB100 was inversely related to apoE FCR in a repeated measures correlation analysis ($P=0.029$), but the association was not continuous and was evident mainly on evolocumab (Figure 4D).

DISCUSSION

PCSK9 inhibitors are now integrated into clinical guidelines as highly effective LDL-C-lowering agents. Although their mode of action—increasing the number of LDL receptors—is well established, their wider effects on lipoprotein metabolism are still to be determined. Here, we investigated the effects of the PCSK9 inhibitor

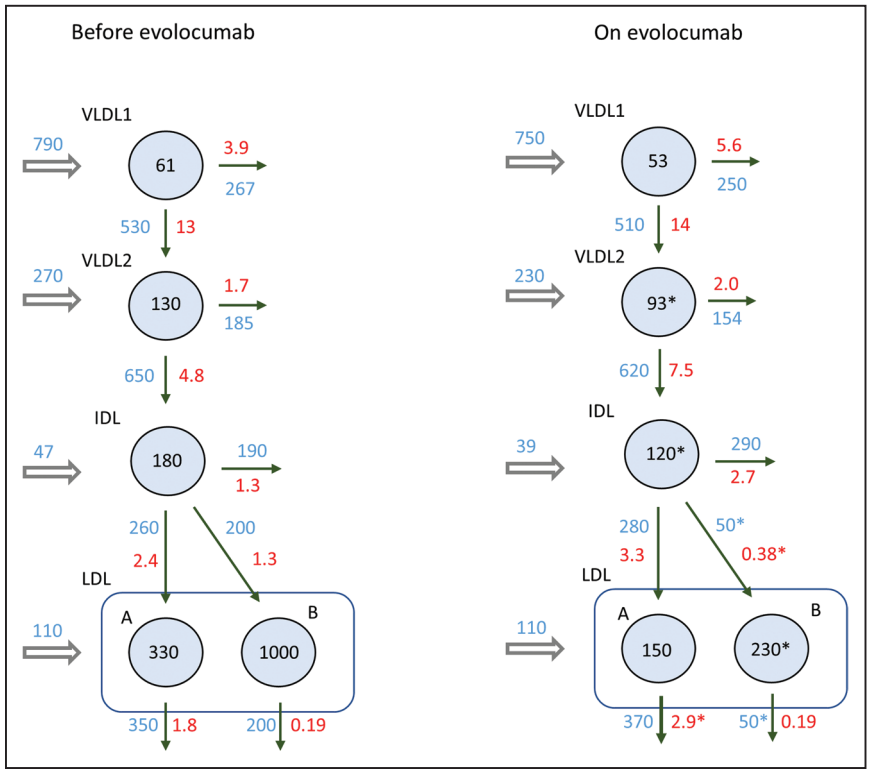


Figure 3. Schematic representation of the kinetics of apoB100 (apolipoprotein B100) in $VLDL_1$ (very low-density lipoprotein), $VLDL_2$, IDL (intermediate-density lipoprotein), and LDL (low-density lipoprotein) before and on evolocumab. Blue numbers indicate absolute fluxes (mg/d), red numbers indicate fractional catabolic rates (pools/d), and black numbers indicate pool sizes (mg). *$P<0.05$ (Wilcoxon signed-rank test).

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Table 3. Effects of Evolocumab on ApoC-III and ApoE Metabolism in Subjects With T2DM

	Before evolocumab, n=12	After evolocumab, n=12	*P* value
ApoC-III			
Production rate, mg/d	531±188	519±339	0.48
Pool size, mg	433±209	389±334	0.35
FCR, pools/d	1.31±0.34	1.47±0.33	0.20
ApoE			
Production rate, mg/d	816±140	769±316	0.089
Pool size, mg	134±30.4	86.9±37.3	0.0045
FCR, pools/d	6.20±0.66	9.17±1.78	<0.001

Data for one study subject was excluded because of technical reason. Data are shown as mean±SD. *P* values were calculated using the Wilcoxon signed-rank test. ApoC indicates apolipoprotein C; ApoE, apolipoprotein E; FCR, fractional catabolic rate; and T2DM, type 2 diabetes.

evolocumab on the kinetics of apoB48- and apoB100-containing lipoproteins in subjects with T2DM. We found that the impact of evolocumab therapy on the 2 major VLDL subfractions differed. Metabolism of large, triglyceride-rich $VLDL_1$ was not altered significantly by treatment with the PCSK9 inhibitor, whereas the FCR for both apoB100 and triglyceride in smaller $VLDL_2$ was increased, and as a result the concentration of this lipoprotein species fell. In terms of the postprandial response to a test meal, there was a consistent tendency for chylomicron- and $VLDL_1$-triglyceride and -apoB48 levels to be marginally lower in the latter phase of alimentary lipemia on evolocumab, but this was of borderline significance and possibly attributable to the reduction in plasma apoC-III on evolocumab (which would be expected to enhance lipolysis). Overall, chylomicron-apoB48 kinetics were not altered significantly by evolocumab in these subjects. Thus, it appears that evolocumab, in contrast to its substantial impact on the cholesterol-carrying lipoprotein species ($VLDL_2$, IDL and LDL), does not perturb to any great extent the main pathways of triglyceride transport arising in the liver (production and lipolysis of $VLDL_1$) or the intestine (production and lipolysis of chylomicrons).

LDL-C was reduced dramatically by evolocumab as expected, and this was attributable to a marked increase in the overall FCR of LDL-apoB100 (Table 2). However, the changes in LDL kinetics were more nuanced than expected. LDL-apoB100 exhibited distinct metabolic heterogeneity on evolocumab treatment that was attributed to the presence of 2 metabolically distinct species: a rapidly removed component (here termed LDL-A) whose FCR increased on evolocumab, and a slowly catabolized component (LDL-B) with a markedly decreased production rate but no apparent change in FCR on evolocumab (Figure 3). Evolocumab altered the relative proportion of these 2 LDL species, an effect that was linked to alterations in the metabolism of IDL (more IDL was catabolized directly and less converted to LDL-B). Further, we showed relationships between the pool sizes of apoB100 in $VLDL_2$, IDL (and to an extent LDL), and the metabolism of apoE. These associations can be understood in light of the role of apoE as a ligand for LDL-receptor mediated pathways in $VLDL_2$ and IDL metabolism[51] and the increase in the number of LDL receptors on evolocumab treatment. This more detailed picture of the effects of PCSK9 inhibitors on the whole spectrum of apoB-containing lipoproteins provides new insight into the precise mechanism of action of the drugs and how they may be best applied in the management of dyslipidemia.

Previous investigations of the effects of PCSK9 inhibitors on lipoprotein metabolism reveal a consistent pattern of action.[24,52] These studies showed an approximate doubling of LDL-apoB on alirocumab[24] or evolocumab,[52] and reduced conversion of IDL to LDL in both. The PCSK9 inhibitors promoted an increase in IDL-apoB FCR of about 50%.[24,52] However, the effect on VLDL-apoB kinetics was less marked, with an increase in FCR that did not reach significance in one of the studies[24] but was significant in the larger cohort used in the other study.[52] Our results are in accord with these earlier findings and provide more detailed insight into the actions of PCSK9 inhibitors in the VLDL and LDL density ranges. Both of the earlier kinetic studies were conducted in healthy volunteers with low plasma triglyceride and LDL-C levels. Our investigations widen the subject profile to include those with T2DM and more elevated triglyceride concentrations. There is general agreement on the effect of PCSK9 inhibitors on chylomicron-apoB48 metabolism: we saw very modest changes in the postprandial response and previous studies reported no influence of the drugs on triglyceride and apoB48 plasma concentration following a fat-load test.[24,53] Further, both we and Chan et al[53] found no significant effect of PCSK9 inhibitor therapy on the production rate or FCR of apoB48.

PCSK9 is a major regulator of LDL receptors.[54] Given the observed increase in LDL FCR on evolocumab (Table 2), it is reasonable to estimate that activity of the LDL receptor pathway at least doubles when PCSK9 action is inhibited with antibodies. The LDL receptor recognizes not only apoB100 on LDL but also apoE, which can facilitate lipoprotein binding and uptake by cells.[35,55] It thus follows that direct catabolism of lipoproteins via LDL receptors could be accelerated across the VLDL-IDL-LDL spectrum if particles are able to access this upregulated pathway. Our finding that the FCRs for $VLDL_2$, IDL and LDL, but not $VLDL_1$, are enhanced by evolocumab suggests that $VLDL_1$ particles are not metabolized to any great extent by the LDL receptor pathway. In healthy subjects, $VLDL_1$ is principally metabolized by lipoprotein lipase, which catalyzes hydrolysis of triglyceride in the particle, resulting in the formation of $VLDL_2$.[51,56] The LDL receptor is not involved in this lipolytic process. However, as plasma triglyceride levels rise and lipolysis becomes

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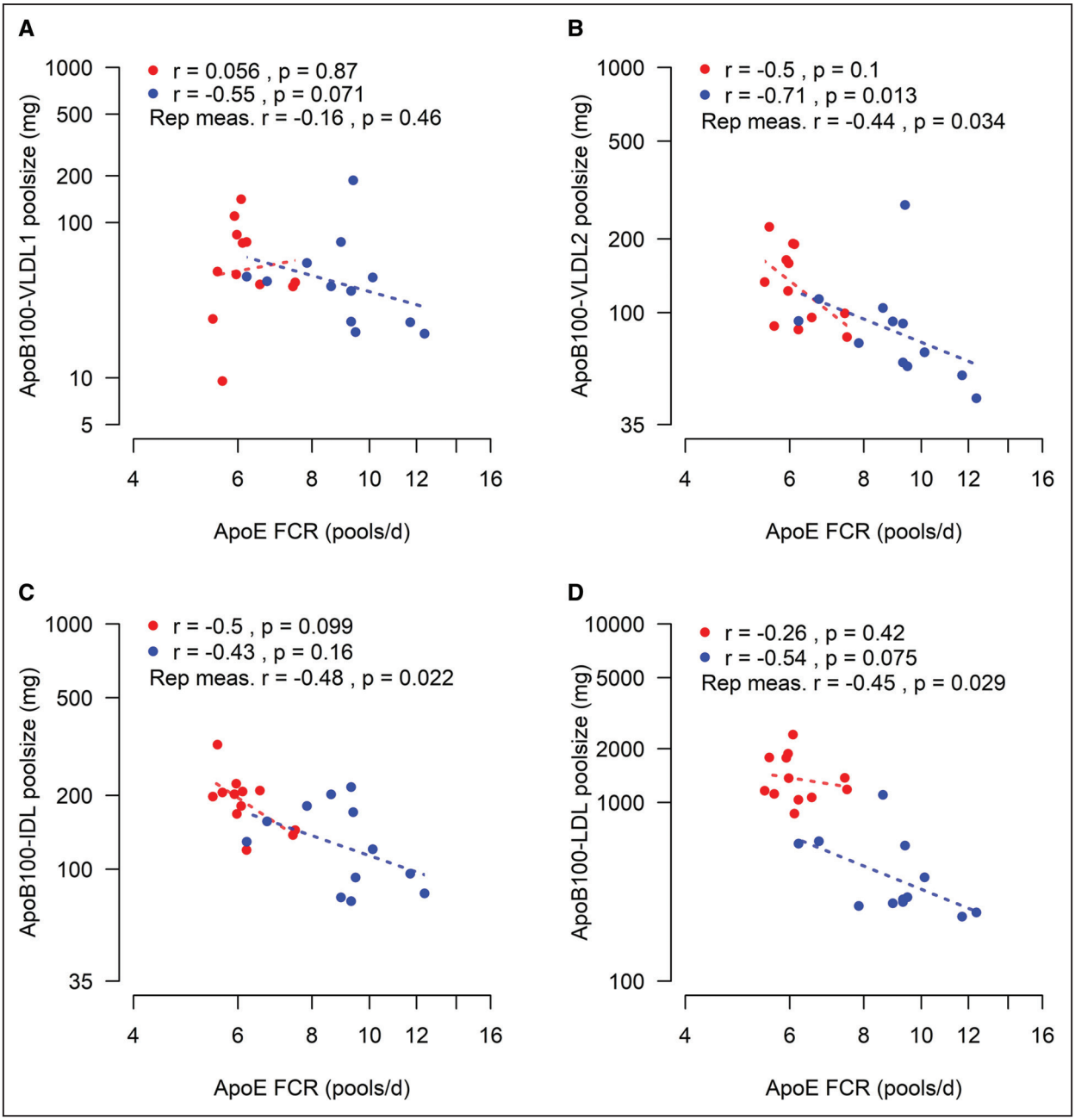


Figure 4. Relationships between apoB100 (apolipoprotein B100) pool sizes and apoE fractional catabolic rate (FCR) before and on evolocumab.

R values refer to Spearman correlation coefficients, or repeated measures ANOVA (Rep meas). Red indicates before evolocumab and blue indicates on evolocumab. IDL indicates intermediate-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very low-density lipoprotein.

less efficient, direct removal of $VLDL_1$ particles from the circulation can occur, possibly via a receptor-mediated pathway.[37,51] The identity of the receptor responsible is unknown. However, it is noteworthy that (1) statins have a more marked effect than evolocumab on plasma triglyceride and total VLDL-apoB100 FCR,[52] (2) we previously found statin therapy to have a significant impact on $VLDL_1$ metabolism,[57] and (3) in subjects with homozygous familial hypercholesterolemia (who lack LDL receptors), clearance of $VLDL_1$ proceeds at a normal rate but that of $VLDL_2$, IDL and LDL is substantially delayed.[58] It is likely, therefore, that one of the other members of the lipoprotein receptor superfamily facilitates the clearance of $VLDL_1$ particles[51]; one potential contender is the LDL receptor-related protein 1, which is known to be upregulated by statins.[59,60] Together, the evidence indicates that statins accelerate the metabolism of $VLDL_1$ and $VLDL_2$ whereas PCSK9 inhibitors affect only the kinetics of $VLDL_2$. This distinction provides a plausible explanation for the different impact of the drug classes on overall VLDL-apoB kinetics and for the more modest triglyceride lowering on PCSK9 inhibitors compared with statins,[61,62]

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and has implications for their relative utility in treating hypertriglyceridemia.

Our observations of the effects of evolocumab on LDL-apoB100 kinetics offer further insight into the detailed action of PCSK9 inhibitors. Our initial hypothesis was that stimulation of LDL receptor activity on evolocumab would be reflected in an increased FCR, and this indeed was seen as shown in Table 2. However, the appearance of a clear bi-exponential decay for the tracer in LDL-apoB100 could not be explained by simple accelerated clearance. As discussed above, the metabolic heterogeneity was explained by the presence of 2 lipoprotein species (LDL-A and LDL-B) with different clearance rates (we ruled out the possibility that the slowly catabolized apoB100 was present in Lp[a]). Previous kinetic studies using radio-iodinated LDL revealed that LDL is metabolically heterogeneous in most people.[51,63] LDL particles within the same density range include those that are actively removed by LDL receptors (FCR of about 0.6 pools/day in healthy subjects) and those that are more slowly catabolized, possibly as a result of decreased affinity for the receptor (FCR of about 0.2 pools/day, similar to the rate observed in the present investigation).[51,63] Stable-isotope-tracer studies do not usually provide enough discrimination to be able to distinguish between the metabolism of LDL subpopulations. However, in our current study, the underlying heterogeneity became evident because of the increased number of LDL receptors on treatment with a PCSK9 inhibitor. Thus, evolocumab not only decreased the concentration of LDL overall but also altered the type of particles in this density range. PCSK9 inhibition had the following 2 effects on LDL metabolism: (1) receptor-mediated clearance of LDL-A was upregulated and (2) the proportion of slowly metabolized, and hence potentially more atherogenic,[51] LDL-B was reduced markedly. This was not because of accelerated catabolism but rather to a profound decrease in the generation of this LDL metabolic species from IDL.

We also further explored the effects of evolocumab on IDL kinetics and its relationship to apoE metabolism. ApoE acts as a ligand for the LDL receptor and lipoprotein particles carrying this apoprotein are able to access the LDL receptor-mediated endocytic pathway. There appeared to be significant inverse relationships between the pool sizes of $VLDL_2$ and IDL and the FCR of apoE across the 2 phases of the study. This suggests that when receptor numbers are increased by PCSK9 inhibition, the fall in the circulating pools of $VLDL_2$ and IDL might at least partly be explained by accelerated metabolism via apoE-mediated pathways. It is thus tempting to speculate that the increased number of LDL receptors on evolocumab led to (1) accelerated clearance of IDL containing apoE, which (2) increased the rate of direct IDL catabolism, (3) lowered the circulating pool of IDL, and (4) decreased the amount of IDL converted into LDL, possibly specifically LDL-B. The inverse association of LDL-apoB100 with apoE FCR on evolocumab can likewise be attributed to accelerated catabolism of the precursor IDL. The altered fate of IDL (Figure 3) with its consequences for the metabolism of LDL adds to understanding of the influence of PCSK9 on lipoprotein metabolism.[24,52] It should be noted that IDL also displays structural heterogeneity and can be fractionated into larger, triglyceride-rich and smaller, cholesterol-rich particles containing variable amounts of apoE and apoC.[64] However, it is not possible at present to connect this compositional variation with the metabolic fate of IDL particles.

The key findings in this study—a differential effect of evolocumab on VLDL subfractions, increased IDL direct clearance, and the appearance of a distinct metabolic heterogeneity in LDL—can be set in the framework of metabolic channeling that is present throughout the VLDL-LDL delipidation cascade.[51,63,65] Based mainly on investigations with radiolabeled tracers, we found that $VLDL_1$ delipidation gave rise to IDL and LDL that were more slowly cleared from the circulation than IDL and LDL derived from directly secreted $VLDL_2$; as noted above, these observations are concordant with the results of kinetic experiments using radiolabeled LDL. The slower clearance of LDL and IDL formed from $VLDL_1$ delipidation was postulated to be due to apoB100 on these particles having a conformation that did not express the high-affinity site for binding to the LDL receptor, although the lower-affinity binding domain(s) was present.[51,63,66] These lipoproteins are thought to still undergo receptor-mediated catabolism but less efficiently than their counterparts derived from $VLDL_2$.[63,65] This concept is supported by the long-standing observation that isolated LDL subfractions have differing abilities to bind to the LDL receptor: smaller, denser species that accumulate in subjects with raised plasma triglyceride exhibit reduced binding.[67–69] In our subjects with T2DM and moderately raised plasma triglyceride, background statin treatment is likely to have already accelerated the clearance of lipoprotein species capable of binding to the LDL receptor, and hence the low abundance of LDL-A relative to LDL-B (Figure 3). Statins do not seem to alter the amount of IDL converted to LDL[52,57] whereas this seems to be a consistent feature of PCSK9 inhibitor action (Table 2),[24,52] an intriguing discordancy given that both agents increase receptor number. We propose that the very low levels of LDL achieved on evolocumab allowed other lipoprotein species to access the hyper-stimulated LDL receptor-mediated catabolic pathway; this included the $VLDL_1$-derived IDL that have apoE (despite having apoB100 with a low-affinity conformation). However, the alternate explanation that PCSK9 itself plays a role in determining the fate of IDL (clearance versus conversion to LDL) cannot be discounted.

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The principal limitations of the present study lie in the number of subjects examined (as is usual for a metabolic investigation) and the constraints on the degree of blood sampling and hence the data on which to derive the kinetic parameters. The design as a sequential-treatment, unblinded study should also be borne in mind when evaluating the less dramatic effects of the drug. Unfortunately, while we had IDL pool sizes for all participants, IDL-apoB100 enrichments were determined for only 6 subjects and we had to rely on modeling to generate a full set of values for this lipoprotein fraction. The multicompartmental model we used had both steady-state and non–steady-state elements. Caution must be exercised when using non–steady-state models because they are subject to ambiguity. Accordingly, we introduced specific constraints into the present model to allow the reliable derivation of kinetic rate constants. Finally, models for analysis of apoprotein kinetics were developed separately for apoE, apoC-III, and apoB48/100.

In conclusion, this study of the effects of evolocumab on triglyceride transport and apoB100 metabolism in subjects with T2DM offers further insight into the detailed mechanism of action of evolocumab and, by extrapolation, the role of PCSK9 inhibitors in lipoprotein metabolism. PCSK9 inhibitors have little impact on triglyceride transport in either the post-prandial or the fasting state. This is distinct from the action of statins and has implications for the utility of PCSK9 inhibitors in correcting hypertriglyceridemia. The profound effects of evolocumab on LDL concentration and metabolism were attributable to hyperstimulation of the LDL receptor pathway affecting the entire $VLDL_2$-IDL-LDL delipidation cascade. The change in the fate of IDL and the altered metabolic properties of LDL provided additional novel insight into how PCSK9 inhibitors impact on the metabolism, and potential atherogenicity, of cholesterol-carrying lipoproteins.

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