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Original Research

Composition and distribution of lipoproteins after evolocumab in familial dysbetalipoproteinemia. A randomized controlled trial

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KEYWORDS
Familial dysbetalipoproteinemia;
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Fasting;
PCSK9 monoclonal antibodies;
Clinical trial;
Apolipoprotein B;
Lipoproteins

Background: PCSK9 monoclonal antibodies (mAbs) reduce fasting and post fat load cholesterol in non-HDL and IDL in familial dysbetalipoproteinemia (FD). However, the effect of PCSK9 mAbs on the distribution and composition of atherogenic lipoproteins in patients with FD is unknown.

Objective: To evaluate the effect of the PCSK9 mAb evolocumab added to standard lipid-lowering therapy in patients with FD on fasting and post fat load lipoprotein distribution and composition.

Methods: Randomized placebo-controlled double-blind crossover trial comparing evolocumab (140 mg subcutaneous every 2 weeks) with placebo during two 12-week treatment periods. Patients received an oral fat load at the start and end of each treatment period. (Apo)lipoproteins were measured with ultracentrifugation, gradient gel electrophoresis, retinyl palmitate and SDS-PAGE.

Results: PCSK9 mAbs significantly reduced particle number of all atherogenic lipoproteins, with a stronger effect on smaller lipoproteins than on larger lipoproteins (e.g. IDL-apoB 49%, 95%CI 41–59 and VLDL-apoB 33%, 95%CI 16-50). Furthermore, PCSK9 mAbs lowered cholesterol more than TG in VLDL, IDL and LDL (e.g. VLDL-C 48%, 95%CI 29–63%; and VLDL-TG 20%, 95%CI 6.3–41%). PCSK9 mAbs did not affect the post fat load response of chylomicrons.

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Conclusion: PCSK9 mAbs added to standard lipid-lowering therapy in FD patients significantly reduced lipoprotein particle number, in particular the smaller and more cholesterol-rich lipoproteins (i.e. IDL and LDL). PCSK9 mAbs did not affect chylomicron metabolism. It seems likely that the observed effects are achieved by increased hepatic lipoprotein clearance, but the specific working mechanism of PCSK9 mAbs in FD patients remains to be elucidated.

Introduction

Approximately 0.7% of the general population is homozygous for the ε2 allele in the *APOE* gene.[1] Since about 10-18% of these ε2ε2 individuals develop the specific dysbetalipoproteinemia phenotype,[2,3] the estimated prevalence of familial dysbetalipoproteinemia (FD) is 1 in 850 to 3500 individuals, making FD the second most common monogenic lipid disorder after familial hypercholesterolemia.[4] FD is associated with a very high risk of cardiovascular disease (CVD), due to accumulation of atherogenic cholesterol-enriched triglyceride-rich lipoproteins (TRLs) and generally displays a (very) low concentration of low-density lipoprotein cholesterol (LDL-C).[5,6] Treatment options for FD include dietary changes, statins and fibrates, although with these treatment options the non-high-density lipoprotein-cholesterol (non-HDL-C) treatment goal is only attained in 40% of FD patients.[7] To explore the effects of proprotein convertase subtilisin kexin 9 (PCSK9) monoclonal antibodies (mAbs) added to standard lipid-lowering therapy in FD patients, the EVOLVE-FD trial was conducted. In this randomized crossover trial the effect of evolocumab was investigated in 28 FD patients. The trial showed large reductions in fasting and post fat load exposure to non-HDL-C, intermediate-density lipoprotein-cholesterol (IDL-C) and apolipoprotein B (apoB), with reductions in fasting non-HDL-C, IDL-C and apoB of 51% (95%CI 43 –57%), 44% (95%CI 30 –55%) and 48% (95%CI -42 –53%), respectively.[8]

The aim of this study was to evaluate the effect of evolocumab on top of standard lipid-lowering treatment, compared with placebo, on fasting and post fat load lipoprotein distribution and composition in FD patients.

Methods

Patients and study design

Details regarding in- and exclusion criteria and study design of the EVOLVE-FD study (Effects of EVOLocumab VErsus placebo added to standard lipid-lowering therapy on fasting and post fat load lipids in patients with FD) have been reported elsewhere.[8] In brief, patients between 18 and 80 years who were genetically diagnosed with FD and had a dysbetalipoproteinemia phenotype, were eligible to participate in the study. A dysbetalipoproteinemia phenotype was defined as either apoB/total cholesterol (TC) ratio <0.15 mmol/g,[9] TC >5 mmol/L and TG >3 mmol/L[10] or non-HDL-C/apoB >3.69 mmol/g,[11] with or without lipid-lowering medication. An FD genotype was defined as an ε2ε2 genotype or a heterozygous dominant *APOE* variant known to associate with an FD phenotype, confirmed by genotyping or isoelectric focusing. When patients were using lipid-lowering medication the dose must have remained stable for at least 12 weeks. Non-HDL-C levels had to be >1.6 mmol/L and fasting triglycerides <10 mmol/L. Main exclusion criteria were uncontrolled T2DM (defined as HbA1c >69 mmol/mol), morbid obesity (BMI >40 kg/m^2), uncontrolled blood pressure ($>180/110$ mmHg), significant kidney or liver disease, premenopausal status, and excessive alcohol consumption (>21 units per week for men and >14 units per week for women).

The EVOLVE-FD study had a randomized, double-blind, placebo-controlled, crossover design (Supplementary Fig. 1). Evolocumab 140 mg or matching placebo were administered subcutaneously every 2 weeks during two 12-week treatment periods in a random order (both supplied by Amgen, Breda, the Netherlands). There was an 8-week wash-out period between the two treatment periods.

All patients signed written informed consent. The study was approved by the Medical Ethics Review Committee of the UMC Utrecht and by the competent authority of the Netherlands. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The EVOLVE-FD study was registered at www.clinicaltrials.gov (NCT03811223).

Oral fat load and retinyl palmitate

At the start and at the end of both treatment periods, patients visited the medical center in the morning after a $\geq$12 h fast. At every visit patients underwent a standardized protocol and received an oral fat load. The oral fat load consisted of unsweetened fresh cream (Albert Heijn, Zaandam, the Netherlands) with a fat content of 35% (mass/volume). Cream was administered at a dose of 110 g of fat per m^2 of body surface area, with a maximum of 500 mL and ingested within 10 min. The fresh cream was mixed with 2 mL of retinyl palmitate (50.000 IE/mL, waterig DMB, pharmacy 'de magistrale bereider'), used to investigate exogenous lipoprotein metabolism in the post fat load phase. Venous blood samples were obtained before (at 0 h) and 1, 2, 4, 6 and 8 h after the oral fat load. During these eight hours,

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only drinking water was allowed. Other details of the study procedures and methods have been described elsewhere.[8]

Laboratory measurements

Density gradient ultracentrifugation

Before, 4 and 8 h after the oral fat load the levels of cholesterol, TG and apoB were determined in the chylomicron, VLDL, IDL and LDL fractions isolated by density gradient ultracentrifugation.[12] Briefly, KBr (0.35 g/mL plasma) was added to plasma to achieve a density of 1.26 g/mL. One mL of plasma was placed in an ultracentrifuge tube followed by 1.9 mL of KBr solutions (of 1.21, 1.10, 1.063, 1.04 and 1.02 g/mL) in physiological saline and 1 mL of water. Samples were centrifuged for 30 min at 20 °C at 207.000 g with an SW41 rotor in an Optima XPN-80 ultracentrifuge (Beckman Instruments, Indianapolis, IN, USA). After this, chylomicrons were isolated from the top 1 mL of the tube and this volume was replaced with 1 mL water before further centrifugation for 18 h at 4 °C at 207.000 g, using the same rotor and centrifuge. After centrifugation, fractions of 250 µL were eluted from the bottom of the tube in which cholesterol, TG and total apoB (including apoB48 and apoB100) were measured using a Selectra E (DDS Diagnostic system, Istanbul, Turkey). Lipoproteins were separated based on density. Chylomicrons were above fraction 46, VLDL was found in fractions 41–46. Fractions 34–40 were designated as IDL, and LDL as <34, in line with the corresponding standard density ranges in g/mL from ultracentrifugation. Density gradient ultracentrifugation was performed at the Laboratory of Vascular Medicine in Erasmus Medical Center, Rotterdam, the Netherlands.

Polyacrylamide gradient gel electrophoresis

The preparation of non-denaturing polyacrylamide gradient gels is described elsewhere.[13] In short, neutral lipids (cholesterol and TG) were prestained with Sudan Black. One gel was made for each visit, consisting of 6 lanes (at time point 0, 1, 2, 4, 6, 8 h after the oral fat load) of the patient. The gels were calibrated with markers of ultracentrifugationally prepared VLDL1, VLDL2, IDL and LDL. Gels were placed in a photographic chamber and images were captured by a mounted video camera. The image was digitized for densitometric analysis in ImageJ.[14] After this, the lanes, which were converted to density plots in ImageJ, were analyzed with RStudio statistical software (version 3.5.1; R foundation for Statistical Computing, Vienna, Austria). The migration range (in inches) of the lipoprotein fractions was standardized and presented as a retardation factor (Rf), for which the beginning of the separation gel was defined as zero and the end of small dense LDL as one. The cut-offs of the markers were automatically defined for each gel separately, and were set when the relative intensity of the next marker was higher than that of the previous marker. The total area under the curve (AUC) for total staining and the AUC for the separate fractions (CM, VLDL1, VLDL2, IDL and LDL) were calculated. The relative AUCs of the separate fractions were compared with the total AUC and expressed as percentage of the total staining. Thus, this method did not allow quantification of lipoprotein concentrations in absolute terms, but did allow insight into the relative distribution of neutral lipid among the different lipoprotein fractions. Polyacrylamide gradient gel electrophoresis (PGGE) was performed at the Laboratory of Chemical Pathology at the University of Cape Town, South Africa.

Retinyl palmitate analyses

Examination of retinyl palmitate levels in plasma was derived from a previous publication[15] and performed with high-performance liquid chromatography (HPLC). Plasma samples (100 µL) were prepared by a protein precipitation with ethanol and then liquid-liquid extraction followed with hexane. The hexane extract was evaporated under nitrogen and reconstituted with injection solvent containing butylated hydroxytoluene as a preservative. Fifty µL of reconstituted sample was injected. Quantification was done by preparing calibration standards with pooled plasma that was spiked with known concentrations of certified reference standards of retinyl palmitate. Quality control (QC) samples were prepared in a similar manner to the calibrators – both the calibrators and QCs were extracted as described above.

The extracted standards, QCs and patient samples were analysed with an Agilent 1260 Infinity High Performance Liquid Chromatography system, with a Diode Array Detector (DAD). Reverse phase chromatography was used, and separation was achieved on an Agilent Poroshell C18 column held at 40 °C. A 12 min gradient elution was used with mobile phases A and B set up as water and Methanol: Acetonitrile (80:20, v/v) adjusted to pH 5 with acetic acid, respectively. The DAD was set to 325nm for analyte detection. Data acquisition and quantitation was done using MassHunter software. Linear calibration curves with weighted regression were used to quantify patient samples in µgram/L. Retinyl palmitate analyses were performed by the Laboratory of Chemical Pathology at the University of Cape Town, South Africa.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Each lipoprotein fraction (CM, VLDL, IDL and LDL) was analyzed for (apolipo)protein composition by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% gradient gels (mini-PROTEAN TGX Precast Gels, product 4568096, Biorad). Proteins were stained overnight with Pageblue Protein Staining Solution (product 24620, Thermo Scientific) after washing with demineralized water. Gels were scanned with an Amersham Imager 600+ (Cytiva). Analysis was performed with ImageQuant V8.2. All separate proteins were calculated as percentage of total protein in the fraction, and expressed in µg/mL. SDS-PAGE was performed at the Laboratory of Vascular Medicine in Erasmus Medical Center, Rotterdam, the Netherlands.

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Statistical analyses

Baseline characteristics were presented as means with standard deviations (SD) or medians with interquartile range (IQR) when appropriate. Categorical variables were shown as number with percentage. The post fat load increase was expressed as incremental AUC (iAUC), which is based on the AUC that was calculated with the trapezoidal rule. The iAUC was calculated after adjustment for fasting lipid levels by subtracting eight (hours)*(value at time point 0) from the AUC. Since the data on the difference for every patient between placebo and evolocumab was very skewed, median absolute (for fasting levels and iAUC) and percentage difference (for fasting levels) between two treatment arms were calculated. Bootstrapping (1000 samples with replacement) was used to obtain robust confidence intervals (CIs) with corresponding p-values. The lipid composition (cholesterol vs TG) per lipoprotein fraction after placebo and after evolocumab was compared. Also, particle size was expressed as (cholesterol+TG)/apoB ratio. There were no missing biochemical variables, but in three patients the retinyl palmitate data were removed due to an analysis error (one patient had extremely high retinyl palmitate concentrations in the fasting state, and two patients had lipemic samples). Carry-over and period effects were assessed with an independent samples *t*-test and no carry-over (p=0.65) or period effect (p=0.13) were observed. All p-values were two-tailed and $p<0.05$ was considered statistically significant. All analyses were performed with R statistical software (version 3.5.1; R foundation for Statistical Computing, Vienna, Austria).

Table 1 Baseline characteristics.

	Patients (n=28)
Age (years)	62 ± 9
Female sex	12 (43)
APOE genotype	
- ε2ε2	26 (93)
- Dominant *APOE* variant	3 (11)
Cardiovascular disease	7 (25)
- Coronary heart disease	2 (7)
- Peripheral vascular disease	1 (4)
- Cerebrovascular disease	3 (11)
- Abdominal aortic aneurysm	1 (4)
Diabetes mellitus type 2	9 (32)
Hypertension	22 (79)
Metabolic syndrome	21 (75)
Family history of premature CVD	7 (25)
Lipid-lowering treatment	26 (93)
- Statin only	6 (21)
- Ezetimibe only	2 (7)
- Fibrate only	1 (4)
- Statin + ezetimibe	8 (29)
- Statin + fibrate	8 (29)
- Statin + ezetimibe + fibrate	1 (4)
High intensity statin	7 (25)
Body mass index (kg/m^2)	29.5 ± 3.6
Waist circumference (cm)	107 ± 11
Laboratory measurements	
- Total cholesterol (mmol/L)	4.9 ± 1.9
- Triglycerides[a] (mmol/L)	2.8 (1.8 - 3.5)
- Non-HDL-cholesterol (mmol/L)	3.6 ± 1.7
- HDL-cholesterol (mmol/L)	1.3 ± 0.4
- Apolipoprotein B (g/L)	0.8 ± 0.2
- Lipoprotein (a)[a] (mg/dL)	8.2 (3.3 – 31.2)
- Glucose (mmol/L)	6.1 ± 1.5

Data are shown as n (%), mean ± standard deviation, or when not-normally distributed as median (interquartile range), indicated by [a].

Results

Baseline characteristics

In total, 31 patients were randomized, and 28 patients completed the study. The patient disposition and reasons for screening failure and dropout are described in Supplementary Table 1. The baseline characteristics of the 28 patients whose data were used for the analyses are shown in Table 1. The mean age was 62 ± 9 years and 57% were male. Most patients had an ε2ε2 genotype (93%), two patients had a dominant variant in the *APOE* gene and one patient had an ε2ε2 genotype and a dominant variant in *APOE*. Twenty-five percent of the patients had a history of CVD and 32% had T2DM. Most patients (93%) were on lipid-lowering medication, mostly a combination of a statin and ezetimibe (29%) or a statin and a fibrate (29%). One in four patients used high-intensity statins at baseline. Mean cholesterol level was 4.9 ± 1.9 mmol/L, mean non-HDL-C level was 3.6 ± 1.7 mmol/L and median triglyceride level was 2.8 (IQR 1.8–3.5) mmol/L.

Number and distribution of lipoproteins

The particle number (expressed as total apoB concentration) was significantly reduced for all lipoproteins using ultracentrifugation. Of total apoB, 5% was CM-apoB, 42% was VLDL-apoB, 22% was IDL-apoB and 31% was LDL-apoB after placebo. Median reductions after evolocumab were 29% (95%CI 10–61%), 33% (95%CI 16–50%), 49% (95%CI 41–59%) and 58% (95%CI 50–73) for fasting CM-apoB, VLDL-apoB, IDL-apoB and LDL-apoB, respectively (Fig. 1 and Supplementary Table 2). There were no significant changes in 8 h post fat load increase (iAUC) of apoB (Supplementary Table 3).

Results from the PGGE gels are shown in Supplementary Fig. 2. The relative amount of neutral lipid in larger lipoproteins (i.e. CM, VLDL1 and VLDL2) was higher after treatment with evolocumab compared to placebo. For example, 34% of all neutral lipid was present in VLDL2 after placebo and 38% after evolocumab (p=0.008). In contrast, the relative contribution of neutral lipid was lower in the smaller lipoproteins (IDL and LDL) after treatment with evolocumab compared to placebo. IDL neutral lipid significantly decreased from 20% of all neutral lipid after placebo to 15% after evolocumab (p=0.005). For LDL this

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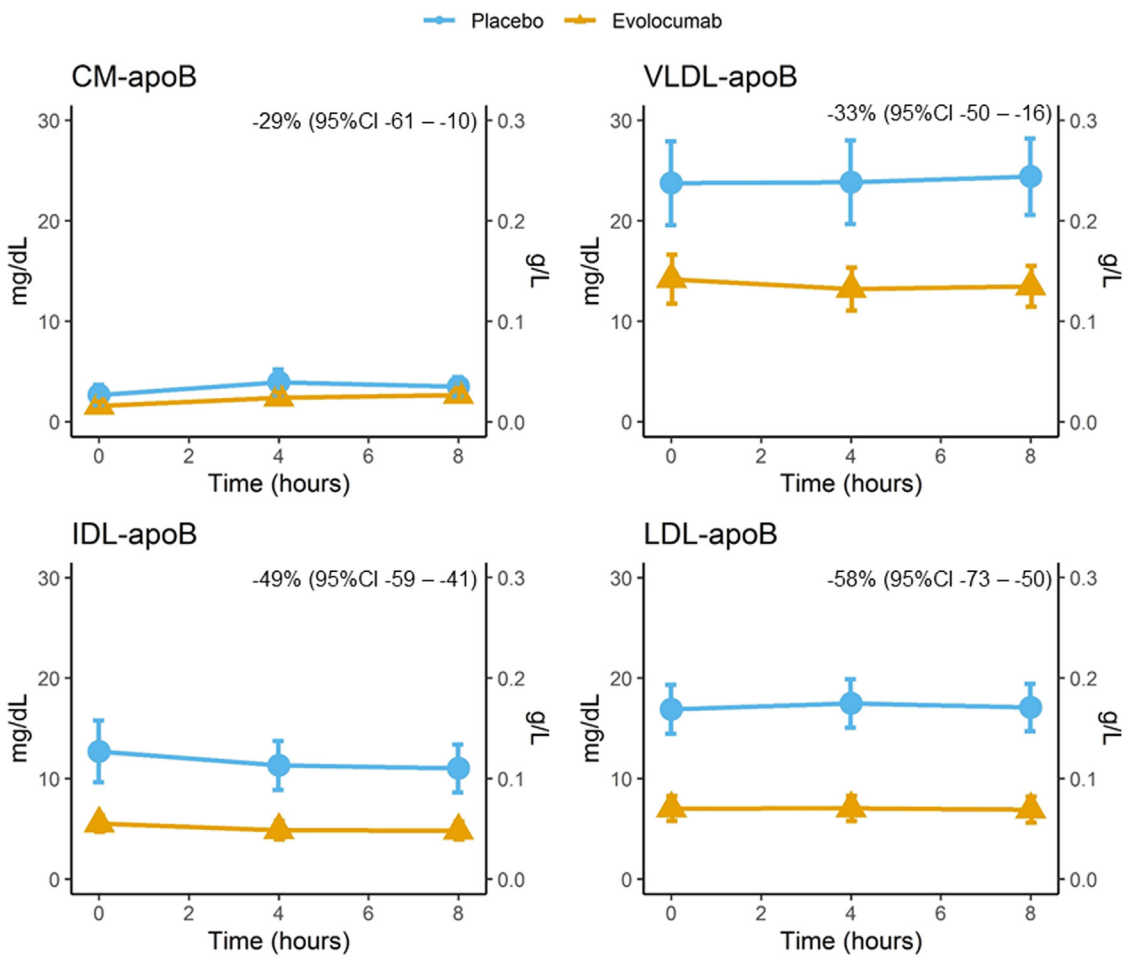

Fig. 1 Effect of evolocumab on fasting and 8 h post fat load apoB levels in lipoproteins. Fasting and 8 h post fat load apoB levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue). The top right of each graph shows the median percentage difference in fasting value after treatment with evolocumab compared with placebo.
Abbreviations: CM-apoB = chylomicron-apolipoprotein B, VLDL-C very-low density lipoprotein- apolipoprotein B, IDL-C = intermediate-density lipoprotein- apolipoprotein B, LDL-C = low-density lipoprotein- apolipoprotein B.

was 13% and 10% after placebo and evolocumab, respectively (p=0.09).

Composition of lipoproteins

In absolute terms, compared to placebo, cholesterol levels in all lipoproteins (CM, VLDL, IDL and LDL) were significantly reduced after 12 weeks treatment with evolocumab. The median reductions in fasting CM-C, VLDL-C, IDL-C and LDL-C were 58% (95%CI 36–71%); 48% (95%CI 29–63%); 53% (95%CI 36–64%) and 52% (36–65%), respectively (Fig. 2 and Supplementary Table 4). After treatment with evolocumab, fasting levels of CM-TG were significantly reduced by 26% (95%CI 20–40%) and fasting VLDL-TG were reduced by 20% (95%CI -6.3–41%). Finally, although absolute TG levels in these lipoproteins were very low, IDL-TG and LDL-TG were also significantly reduced by 33% (95%CI 19–42) and 50% (95%CI 31–60%), respectively (Fig. 3 and Supplementary Table 5). There were no changes in 8 h post fat load increase (iAUC) of cholesterol and TG (Supplementary Table 3).

Treatment with evolocumab reduced cholesterol levels more than TG levels in all lipoproteins. For example, VLDL consisted of 49% cholesterol and 51% TG when using placebo, and 39% cholesterol and 61% TG after evolocumab (Fig. 4).

In line with this the lipoprotein size (expressed as (TG plus cholesterol)/apoB-ratio) increased for VLDL (3.5% (95%CI -11.2–16.3)), IDL (9.8% (95%CI -4.6–19.4) and LDL (12% (95%CI 6.9 – 32.9)) (Supplementary Table 6), indicating a greater reduction in particle number (apoB) than in lipid content.

Fasting and post fat load chylomicron response

Ultracentrifugation showed a significant reduction in fasting CM-apoB, CM-C and CM-TG and an unchanged post fat load iAUC of CM-apoB, CM-C and CM-TG after evolocumab compared to placebo. However, the 8 h post

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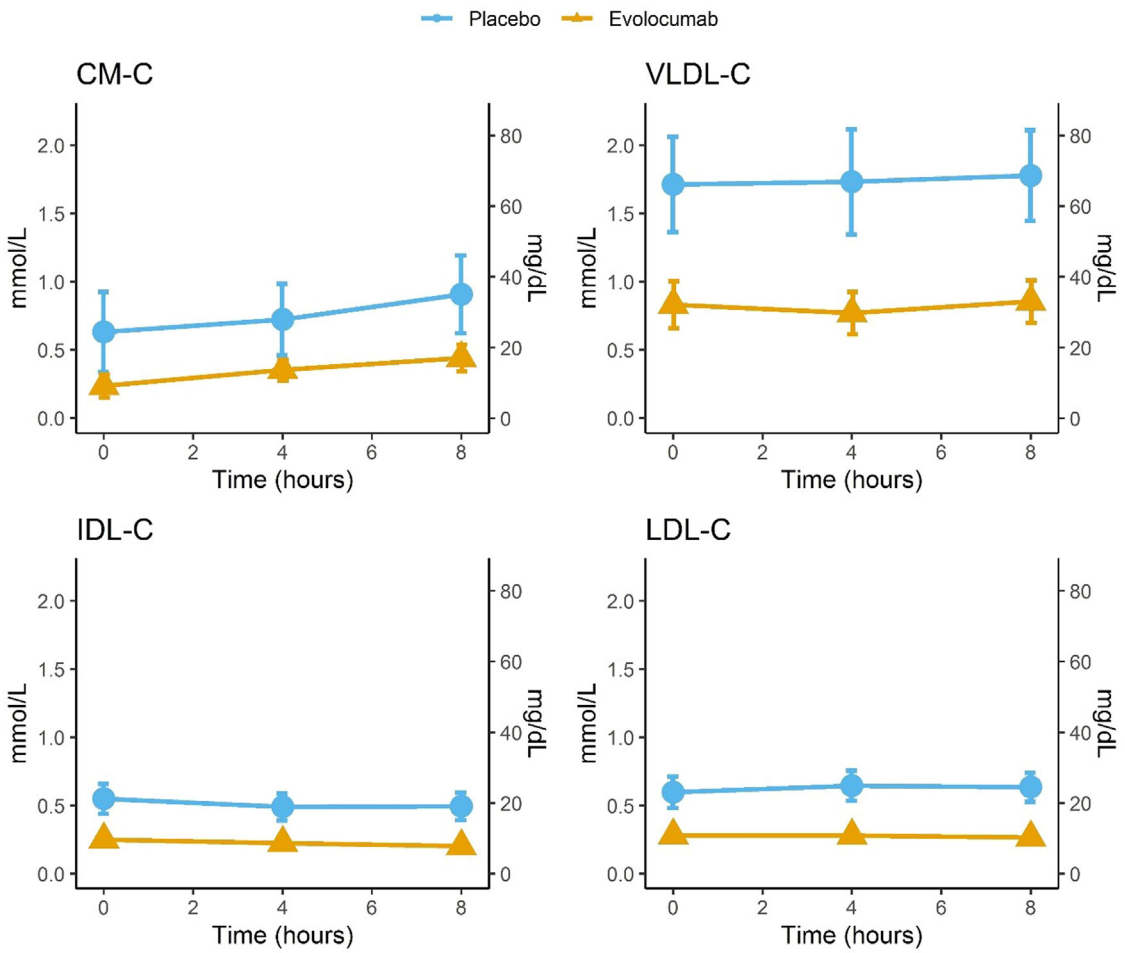

Fig. 2 Effect of evolocumab on fasting and 8 h post fat load cholesterol levels in lipoproteins. Fasting and 8 h post fat load cholesterol levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue). The top right of each graph shows the median percentage difference in fasting value after treatment with evolocumab compared with placebo.
Abbreviations: CM-C = chylomicron-cholesterol, VLDL-C very-low density lipoprotein-cholesterol, IDL-C = intermediate-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein-cholesterol.

fat load retinyl palmitate iAUC was not significantly different after evolocumab (7%, 95%CI -15–22%) compared to placebo (Supplementary Fig. 3 and Supplementary Table 7). The results of SDS-PAGE showed that the lipoproteins in the ultracentrifugation chylomicron fraction mainly consisted of buoyant VLDL particles, based on the presence of mostly apoB100 proteins and hardly any apoB48 proteins in the CM subfraction (Supplementary Fig. 4).

Discussion

In this study in 28 patients with genetically confirmed FD, several effects of the PCSK9 mAbs evolocumab on lipoprotein distribution and composition were observed. First, in absolute terms, VLDL, IDL and LDL particle numbers were significantly reduced after treatment with PCSK9 mAbs. This reduction was larger for the smaller lipoprotein particles (IDL and LDL) compared to the larger VLDL particles. Second, absolute levels of cholesterol and TG in VLDL, IDL and LDL were reduced, with a larger reduction in cholesterol than in TG. Third, VLDL, IDL and LDL particles increased in size. Fourth, PCSK9 mAbs did not affect the number or composition of chylomicrons in the fasting or non-fasting state.

The present study showed that evolocumab reduced the number of smaller particles (-49% and -58% for IDL and LDL, respectively) more than the number of larger particles (-33% for VLDL), compared to placebo. A stable isotope study with alirocumab 150 mg every 2 weeks in 18 healthy individuals showed that IDL-apoB was reduced by 30% and LDL-apoB by 56% after 10-week treatment.[16] In contrast to the present findings, no effect on VLDL-apoB was found in that study, and the effect on IDL was smaller (30% versus 49%). These differences could be due to the fact that the study included healthy individuals with remnants in the normal range, compared to FD patients that have remnant accumulation. However, another study in 80 healthy men found that a high dose of evolocumab (420 mg every 2 weeks) significantly reduced fasting (22%) and post fat load exposure (15%) VLDL-apoB levels.[17,18] In patients with T2DM, who have a lipoprotein phenotype that is more similar to FD (but

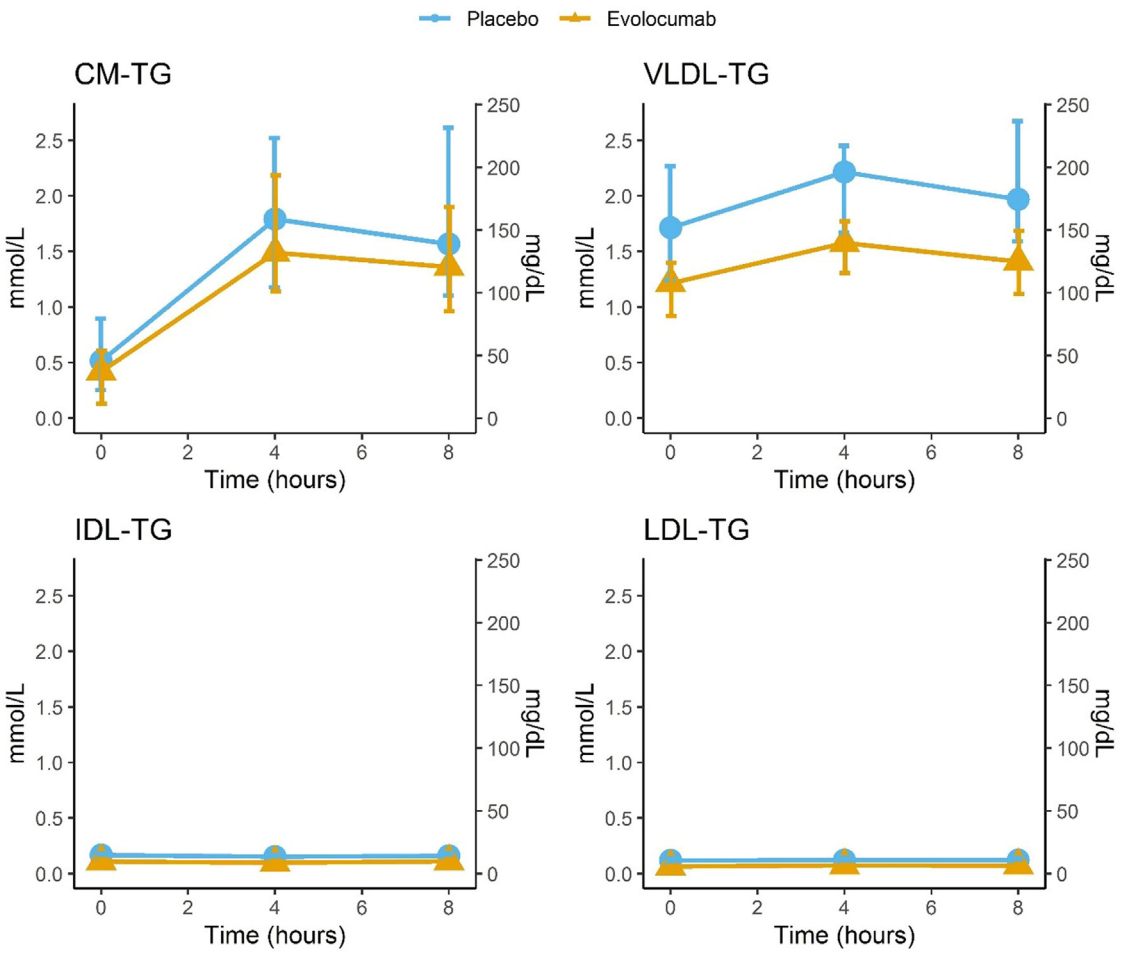

Fig. 3 Effect of evolocumab on fasting and 8 h post fat load triglyceride levels in lipoproteins. Fasting and 8 h post fat load triglyceride levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue). Triglyceride concentrations in medians, with bootstrapped 95% CI. The top right of each graph shows the median percentage difference in fasting value after treatment with evolocumab compared with placebo.

Abbreviations: CI = confidence interval, CM-TG = chylomicron-triglycerides, VLDL-C very-low density lipoprotein-triglycerides, IDL-C = intermediate-density lipoprotein-triglycerides, LDL-C = low-density lipoprotein-triglycerides.

compared to patients with FD have relatively high LDL-C levels), a stable isotope study showed that evolocumab had little effect on the larger VLDL1-apoB100 concentration, but did lead to a significant reduction in the smaller VLDL2-apoB100 concentration.[19] In conclusion, PCSK9 mAbs seem to reduce particle number of LDL, IDL and probably VLDL2 in all populations, although the effects on VLDL and IDL might be larger in FD.

The present study showed that PCSK9 mAbs reduce cholesterol and TG in VLDL, IDL and LDL and that the reduction in cholesterol was more pronounced than the reduction in TG. This is likely due to the fact that smaller and more cholesterol-rich lipoproteins were cleared preferentially compared to the larger TG-rich lipoproteins, rather than a preferential clearance of cholesterol relative to triglycerides in a specific lipoprotein. Furthermore, the reduction in lipids was smaller than the reduction in lipoprotein particle number, leading to an increase in particle size for all lipoproteins including LDL. An observational longitudinal study showed that after treatment with PCSK9 mAbs, VLDL particle size was increased, indicating accelerated degradation of smaller (more cholesterol-enriched) VLDL particles.[20] However, the increase in LDL size is not in line with previous studies, that found that the LDL size decreased after treatment with PCSK9 mAbs.[18,20] The difference might be explained by the fact that ultracentrifugation can include small remnants in the LDL fraction, which is especially true for FD because remnants are in abundance. Therefore the increase in LDL particle size might in reality reflect an increase in the number of remnants in the LDL fraction.

The observed changes in composition and distribution of apoB carrying particles could be clinically relevant since smaller, cholesterol-rich particles (IDL and LDL), and cholesterol-enriched larger particles (such as beta-VLDL) are more atherogenic compared to other larger apoB lipoproteins such as large TG-rich VLDL and CMs.[6] Therefore PCSK9 mAbs are likely associated with a decreased risk of atherosclerosis.

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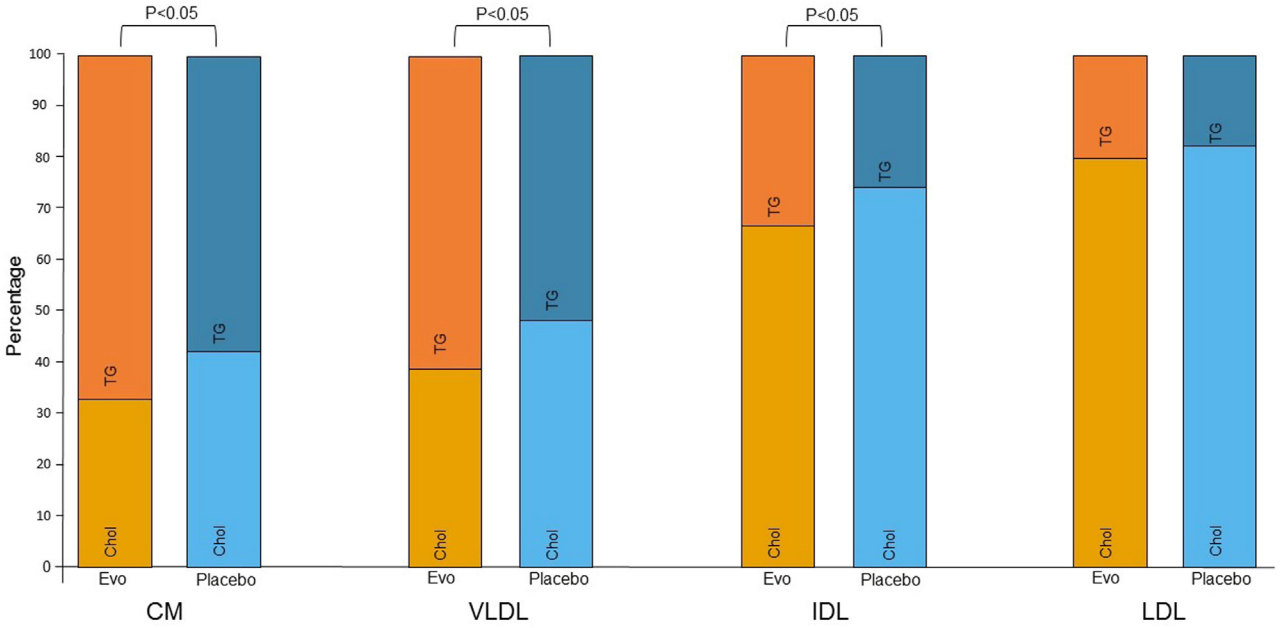

Fig. 4 Cholesterol and TG distribution per lipoprotein particle after evolocumab, compared with placebo in the fasting state. Distribution of cholesterol (bottom) and triglycerides (top) per lipoprotein particle after evolocumab (orange) and after placebo (blue). The figure shows that after PCSK9 mAbs the TG content increases and the cholesterol content decreases in CM, VLDL and IDL, relative to placebo. P-value for difference in cholesterol in each fraction between evolocumab and placebo.

Given that all patients with FD have dysfunctional apoE, that has a greatly reduced affinity (<2%)[3] for the LDL-R, it is intriguing that LDL-R upregulation (the primary mode of action of PCSK9 mAbs) leads to significant reductions in apoB lipoproteins in this FD population. This might be due to an effect on IDL conversion; lipoprotein clearance by other hepatic receptors than LDL-R; or clearance by the LDL-R despite reduced apoE affinity.

The first possibility is that PCSK9 mAbs stimulate the conversion of IDL to LDL. Usually IDL is converted to LDL through lipolysis by lipoprotein lipase (LPL) and hepatic lipase (HL). However, previous studies showed that PCSK9 mAbs did not affect LPL or HL activity.[19,21] In addition, kinetic studies with stable isotopes in healthy subjects and patients with T2DM showed that there was a decreased conversion from IDL to the LDL fraction after treatment with PCSK9 mAbs.[16,17,19] Taken together, the existing evidence does therefore not support increased conversion from IDL to LDL as an explanation for the effects of PCSK9 mAbs in patients with FD.

The second possibility is that PCSK9 mAbs enhance IDL clearance through other receptor systems than the LDL-R. There are three other IDL clearing pathways, namely HSPG, VLDL-receptor (VLDL-R) and LDL receptor-related protein 1 (LRP1). Although it has been shown that the number of LRP1 receptors is not affected by PSCK9 mAbs,[22] and the effects of PCSK9 mAbs on HSPGs are unknown, in theory, LDL reduction by PCSK9 mAb might create more space for (small) IDL in the space of Disse, leading to easier uptake of IDL by the HSPG and LRP1 systems. Recently, it was shown that the VLDL-R also plays a role in IDL clearance, and that its expression is regulated by PCSK9.[23,24] However, this receptor is located in peripheral tissues and not in the liver, and is therefore most likely not involved in lipoprotein clearance.[25] In conclusion, evidence for the role of other receptor systems in explaining the effect of PCSK9 mAbs on IDL reduction in FD is presently unclear.

The final explanation is that PCSK9 mAbs increase direct hepatic clearance of IDL particles through the LDL-R, despite the reduced affinity of apoE2. Upregulation of LDL-R by PCSK9 mAbs can lead to rapid clearance of LDL and therefore to less competition with apoE on IDL to bind to the LDL-R. In line with this, a stable isotope study in healthy subjects and in patients with T2DM found an increased direct clearance of IDL by the liver after PCSK9 mAb treatment. Although it is unknown by which receptor this clearance was mediated, the authors suggest that very low levels of LDL achieved with PCSK9 mAbs allow other lipoproteins (with less affinity for the LDL-R) to enter the extremely upregulated LDL-R pathway.[19] A previous study in ApoE3*Leiden.CETP mice (in which mice express both mouse apoE and the human mutant apoE3*Leiden) it was found that PCSK9 mAbs resulted in a significant reduction of cholesterol (-45%) and TG levels (-36%),[26] while PCSK9 mAbs had no effect in mice without any functional ApoE. This shows that some apoE binding to the LDL-R is necessary for PCSK9 mAbs to function, but that a reduced affinity is probably enough. This notion is further supported by the fact that statins, whose main mechanism of action is upregulation of the LDL-R, have been shown to reduce particle number, and cholesterol and TG content in all apoB100 particles, including IDL, in patients with FD.[27–29] Taken together

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these findings seem to indicate that the reduced affinity of apoE in FD is enough for clearance through the LDL-R when upregulated by PCSK9 mAbs in humans.

The present study found no effect of PCSK9 mAbs on chylomicron metabolism. The post fat load CM-TG response (iAUC) after treatment with evolocumab was identical to placebo and there was no change in retinyl palmitate concentrations after evolocumab, suggesting that there was no effect on chylomicron clearance. Although the results of the ultracentrifugation did show a significant reduction in CM particle number and content (CM-C and CM-TG), SDS-PAGE showed that these "CM" lipoproteins mainly consisted of large VLDL particles. In FD, larger, more buoyant VLDL particles may be seen,[30] due to reduced lipolysis. The absence of an effect on CM is in line with previous studies, demonstrating that PCSK9 mAbs do not substantially affect chylomicron metabolism, including chylomicron formation in the intestine, transport in the thoracic duct, and lipolysis of TG in the circulation and chylomicron-remnant clearance in the liver.[16,17,19,21] Large particles such as CMs do not enter the space of Disse but the smaller remnant lipoproteins can reach receptors expressed on hepatocytes that can efficiently process chylomicron-remnants in normal persons. ApoB48, although being derived from apoB100, is lacking the LDL-R binding domain.[31] Therefore, CMs depend on apoE for clearance by the LDL-R as well as other receptors. The entry into the space of Disse by remnants containing apoB100 could result in their uptake by apoE binding to the LDL-R or other receptors as well as their conversion to LDL by HL for uptake by apoB100, especially when LDL-R are upregulated. The main strength of this study is that it is part of the largest RCT conducted in patients with FD, with extensive determination of lipoprotein fractions, both in the fasting and post fat load state.

Some limitations need to be considered. First, since FD is a typical IDL (remnant) disease with extremely elevated IDL particles and relatively low LDL-C levels, it is noteworthy that LDL-C levels measured with ultracentrifugation were higher than IDL-C levels (LDL-C 0.6 ± 0.3 mmol/L *vs* IDL-C 0.5 ± 0.3 mmol/L). This is probably due to the method of ultracentrifugation itself, because, in FD, small IDL may contaminate LDL fractions.[32] Likewise, large VLDL ended up in the CM fraction. This might have led to an overestimation of LDL and CM and an underestimation of IDL and VLDL. Furthermore, we were unable to differentiate which part of the IDL fraction was cleared by hepatic receptors directly and which part was converted to LDL. Studies with stable isotopes in patients with FD are needed to assess this. Finally, the size of the lipoproteins was expressed as a ratio and was not directly measured.

In conclusion the present study showed that PCSK9 mAbs significantly reduced the number of all atherogenic apoB100-containing lipoproteins (VLDL, IDL and LDL) in FD patients; and increased them in size. Through these effects, evolocumab is expected to reduce CVD risk in this high-risk population.[33] These findings highlight the potential of PCSK9 mAbs, not only with respect to LDL-C lowering, but also with respect to IDL-C lowering, in patients with and without FD.

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Author contribution

Britt Heidemann: Writing – Original draft, Formal analysis, Visualization, Project administration, Investigation
David Marais: Writing – Review & Editing, Supervision, Methodology, Laboratory analyses
Monique Mulder: Writing – Review & Editing, Laboratory analyses, Investigation
Frank Visseren: Writing – Review & Editing, Supervision, Methodology, Recruitment of participants, Investigation, Funding acquisition
Jeanine Roeters van Lennep: Writing – Review & Editing, Recruitment of participants
Erik Stroes: Writing – Review & Editing, Recruitment of participants, Investigation
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Ethical statement

All patients signed written informed consent. The study was approved by the Medical Ethics Review Committee of the UMC Utrecht and by the competent authority of the Netherlands. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice.

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Supplementary materials

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