ORIGINAL RESEARCH ARTICLE

Effects of PCSK9 Inhibition With Alirocumab on Lipoprotein Metabolism in Healthy Humans

BACKGROUND: Alirocumab, a monoclonal antibody to proprotein convertase subtilisin/kexin type 9 (PCSK9), lowers plasma low-density lipoprotein (LDL) cholesterol and apolipoprotein B100 (apoB). Although studies in mice and cells have identified increased hepatic LDL receptors as the basis for LDL lowering by PCSK9 inhibitors, there have been no human studies characterizing the effects of PCSK9 inhibitors on lipoprotein metabolism. In particular, it is not known whether inhibition of PCSK9 has any effects on very low-density lipoprotein or intermediate-density lipoprotein (IDL) metabolism. Inhibition of PCSK9 also results in reductions of plasma lipoprotein (a) levels. The regulation of plasma Lp(a) levels, including the role of LDL receptors in the clearance of Lp(a), is poorly defined, and no mechanistic studies of the Lp(a) lowering by alirocumab in humans have been published to date.

METHODS: Eighteen (10 F, 8 mol/L) participants completed a placebo-controlled, 2-period study. They received 2 doses of placebo, 2 weeks apart, followed by 5 doses of 150 mg of alirocumab, 2 weeks apart. At the end of each period, fractional clearance rates (FCRs) and production rates (PRs) of apoB and apo(a) were determined. In 10 participants, postprandial triglycerides and apoB48 levels were measured.

RESULTS: Alirocumab reduced ultracentrifugally isolated LDL-C by 55.1%, LDL-apoB by 56.3%, and plasma Lp(a) by 18.7%. The fall in LDL-apoB was caused by an 80.4% increase in LDL-apoB FCR and a 23.9% reduction in LDL-apoB PR. The latter was due to a 46.1% increase in IDL-apoB FCR coupled with a 27.2% decrease in conversion of IDL to LDL. The FCR of apo(a) tended to increase (24.6%) without any change in apo(a) PR. Alirocumab had no effects on FCRs or PRs of very low-density lipoproteins-apoB and very low-density lipoproteins triglycerides or on postprandial plasma triglycerides or apoB48 concentrations.

CONCLUSIONS: Alirocumab decreased LDL-C and LDL-apoB by increasing IDL- and LDL-apoB FCRs and decreasing LDL-apoB PR. These results are consistent with increases in LDL receptors available to clear IDL and LDL from blood during PCSK9 inhibition. The increase in apo(a) FCR during alirocumab treatment suggests that increased LDL receptors may also play a role in the reduction of plasma Lp(a).

CLINICAL TRIAL REGISTRATION: URL: http://www.clinicaltrials.gov. Unique identifier: NCT01959971.

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Clinical Perspective

What Is New?

- In this study of healthy volunteers, we demonstrated that the marked reductions in low-density lipoprotein (LDL) cholesterol levels observed when proprotein convertase subtilisin/kexin type 9 (PCSK9) is inhibited by the monoclonal antibody, alirocumab, are caused by a dramatic increase in the efficiency of removal of atherogenic LDL particles from the circulation.
- We also observed reduced production of LDL-apolipoprotein B resulting from increased efficiency of removal of intermediate density lipoproteins and, therefore, less production of LDL.
- These findings are the first human data supporting earlier studies in cells and mice that showed increased LDL receptor (LDLR) activity when PCSK9 is inhibited.

What Are the Clinical Implications?

- The role of the LDLR in regulating blood levels of atherogenic apolipoprotein B lipoproteins, particularly LDL, is clear, as are the benefits of increasing the capacity of the LDLR pathway for cardiovascular disease risk.
- The discovery that PCSK9 reduces LDLRs on the surface of cells by targeting the receptor for degradation stimulated development of PCSK9 inhibitors.
- Our study, which demonstrates that inhibition of PCSK9 with alirocumab increases the removal of LDL from the circulation by the LDLR, increases confidence that PCSK9 inhibitors will confer clinical benefit in the ongoing cardiovascular outcome trials.

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Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted member of the subtilase family of serine proteases. PCSK9 binds to hepatic low-density lipoprotein receptors (LDLRs) and targets them, together with bound LDLs for degradation rather than recycling. As a result, PCSK9 reduces the number of functional LDLRs on cell surfaces, thereby increasing levels of LDL-cholesterol (LDL-C) in the blood.[1,2] Alirocumab is a fully human monoclonal antibody that specifically binds to PCSK9 and inhibits PCSK9 binding to, and PCSK9-mediated degradation of, LDLRs. As a result, alirocumab treatment substantially decreases blood levels of LDL-C and apolipoprotein B (apoB).[3–5]

Based on the mechanism of PCSK9 action, it has been postulated that alirocumab lowers blood levels of LDL-C and apoB by increasing LDLRs in the liver, leading to increased efficiency of the removal of LDL particles from the circulation. However, apoB enters the circulation from the liver on very low-density lipoproteins (VLDLs), which are converted first to intermediate-density lipoproteins (IDLs) and, subsequently, to LDL. During this process,

both VLDL and IDL particles may leave the bloodstream by interacting with LDLRs, particularly in the liver.[6–8] Thus, drugs that reduce blood levels of LDL-C can mediate their effects through pathways other than the classical LDLR-mediated uptake of LDL, such as by reducing the initial secretion of VLDL or the conversion of VLDL and IDL to LDL. The purpose of this study was to evaluate the effects of alirocumab on the flux of apoB from its entry into the circulation as VLDL, through IDL and LDL. In addition, we determined the effects of PCSK9 inhibition on the metabolism of VLDL triglyceride (TG) and levels of TG-rich lipoproteins during the postprandial (PP) period.

An unexpected finding in early clinical trials was a reduction in lipoprotein (a) levels with inhibition of PCSK9 activity.[9,10] The role of the LDLR in the removal of Lp(a) from the circulation remains controversial. For example, statins, which increase LDLRs significantly, do not reduce or may even increase plasma Lp(a) levels.[11] We also, therefore, conducted an exploratory study of the effects of inhibition of PCSK9 on the metabolism of Lp(a).

METHODS

Study Subjects

We enrolled 20 healthy volunteers, 18 to 65 years of age, with LDL-C levels >90 mg/dL. Subjects were recruited from internal clinical trial databases, advertisements, and postings at the Columbia University Medical Center (CUMC). Participants were not receiving lipid-lowering medications. All subjects of childbearing capacity agreed to use birth control for the duration of the study and had a negative serum pregnancy test when applicable. This study was conducted in accordance with the principles of Good Clinical Practice and was approved by the CUMC Institutional Review Board. All study subjects provided written informed consent.

Study Design

This was a Phase 1, placebo-controlled, single-blind, single-sequence, 2-period study (http://www.clinicaltrials.gov. Unique identifier: NCT01959971; for the study design, see online-only Data Supplement Figure I). All subjects were instructed on adherence to the American Heart Association Heart Healthy Diet guidelines during the study and were regularly checked for dietary compliance. Participants received 2 subcutaneous injections of placebo every 2 weeks followed immediately by 5 subcutaneous injections of alirocumab, 150 mg every 2 weeks. Studies of PP lipemia were conducted 2 weeks after the first placebo injection and again immediately after 5 doses of alirocumab. Lipoprotein kinetic studies were performed 2 weeks after completion of placebo treatment and 2 weeks after the last alirocumab dose. For each participant, a comparison of study outcomes across the 2 treatment periods (placebo vs alirocumab) was performed. Thus, each person served as his or her own control.

Safety

Alirocumab is a US Food and Drug Administration–approved lipid-altering treatment. The safety of alirocumab and its

effects on blood lipids have been previously reported for men and women.[5,10] Safety data were closely monitored with clinical and laboratory assessments throughout the study (see online-only Data Supplement for further details).

Biochemical Assays

Blood was collected at the end of each treatment period after a 12-hour fast. Plasma total cholesterol, TG, high-density lipoprotein cholesterol (HDL-C), apoC-III, and apoE were measured by Human Elisa Kits (ab154131 [apoC3]; ab108813 [apoE], Abcam, Cambridge, MA). Plasma LDL-C levels were estimated using the Friedewald formula. Cholesterol and TG were also measured enzymatically in VLDL, IDL, LDL, and HDL isolated by ultracentrifugation.[12] ApoB in plasma and in VLDL, IDL, and LDL was measured using an apoB enzyme-linked immunosorbent assay (ELISA) kit (AlerCheck, Inc.). Plasma Lp(a) levels were measured using a monoclonal antibody-based ELISA method validated to accurately measure Lp(a) independently of the number of repeats in Kringle IV type 2.[13] Apo(a) isoforms were assayed with methodology previously described,[14] in which the isoform size visualized on agarose gel electrophoresis is directly proportional to the number of Kringle IV type 2 repeats. In some subjects, only 1 apo(a) isoform is expressed. In most individuals, however, apo(a) is expressed by both alleles, with 1 isoform more prevalent than the other. Particle sizes and number were determined by ion mobility analysis.[15] Lathosterol, campesterol, and β-sitosterol were measured by gas chromatography as previously described.[16]

Serum levels of anti-alirocumab antibodies (ADA) and free PCSK9 were assayed by Regeneron Pharmaceuticals, Inc., Tarrytown, NY (see online-only Data Supplement). Serum samples were assayed for ADAs using a validated immunoassay developed by Regeneron Pharmaceuticals, Inc. In brief, this titer-based, bridging immunoassay used a Meso Scale Discovery electrochemiluminescence instrument (Rockville, MD). The assay comprised 3 different stages: an initial screening assay to identify potentially ADA-positive samples, a confirmation assay based on competition with exogenously added alirocumab, and a determination of the titer of ADA for confirmed positive samples.

Free PCSK9 levels in serum were determined using specific validated enzyme-linked immunosorbent assays developed by Regeneron Pharmaceuticals, Inc. The lower limit of detection of free PCSK9 was 31.2 ng/mL.

Hepatic Lipase (HL) and Lipoprotein Lipase (LpL) Activity Measurements

Post-heparin plasma was collected 15 minutes after an i.v. injection of heparin (60 U/kg body weight [BW]) on 2 occasions: after subjects had received 2 doses of placebo and after they had received 5 doses of alirocumab (see online-only Data Supplement).

Stable Isotope Kinetic Studies

Participants were admitted to the inpatient unit of the Irving Institute for Clinical and Translational Research (IICTR) at CUMC for 2.5 days. On day 1, fasting bloods were obtained, and meals for the day were provided. At 1 am on the morning of day 2, constant feedings of an isocaloric, 18% fat diet began

and continued every 2 hours for 32 hours. At 9 am, boluses of $^2H_3$-L-leucine (10 μmol/kg BW), Ring-$^{13}C_6$-L-phenylalanine (29.4 μmol/kg BW), and $^2H_5$-glycerol (100 μmol/kg BW) were administered over a 10-minute period, followed by a constant infusion of $^2H_3$-L-leucine (10 μmol/kg BW/hr) over 15 hours. Blood samples were collected at 0 (prebolus), 20, and 40 minutes and at 1, 2, 4, 6, 8, 10, 12, 14, 15, 15.5, 16, 18, 21, 24, and 48 hours and processed to isolate VLDL, IDL, LDL, and HDL.[12,17]

Determination of Stable Isotopic Enrichment of apoB and TG

The lipoprotein fractions isolated during each turnover study were used to determine stable isotopic enrichments of apoB in VLDL, IDL, and LDL with $^2H_3$-L-leucine (10 μmol/kg BW) and Ring-$^{13}C_6$-L-phenylalanine (29.4 μmol/kg BW). Additionally, kinetic analysis of TG in VLDL was performed with $^2H_5$-glycerol (100 μmol/kg BW). ApoB was isolated from VLDL, IDL, and LDL by SDS gel electrophoresis. Enrichment of leucine and phenylalanine in apoB lipoproteins and plasma-free leucine and phenylalanine enrichment was measured by gas chromatography–mass spectrometry using an Agilent 6890 gas chromatograph and a 5973 mass spectrometer using negative chemical ionization.[12] The enrichment of TG-glycerol was determined by gas chromatography–mass spectrometry using positive chemical ionization after derivatization of glycerol to triacetin.[12]

Mathematical Modeling of the apoB and TG Enrichment Data

A multicompartmental model was used to estimate apoB and TG rate constants and fluxes from the enrichment data.[18] ApoB and TG were required to have the same pool structure for VLDL and the same rate constants for the VLDL pools but with different mass distributions. The minimum number of pools needed to fit the 9 sets of data (2 tracers, leucine and phenylalanine, in VLDL-, IDL-, and LDL-apoB and plasma amino acids, and 1 tracer, glycerol, in VLDL-TG simultaneously was chosen for the final model).[17] The data were fitted by least squares using a computer program, Poolfit,[19] which solves the differential equations in closed form and computes the fits and parameter sensitivities as sums of exponentials. The fits yielded FCRs of apoB in VLDL, IDL, and LDL and of TG in VLDL. The model also estimated rates of conversion of apoB between VLDL and IDL, and IDL and LDL. PRs were calculated by multiplying FCRs by the appropriate lipoprotein pool sizes of apoB, which were estimated as each lipoprotein's concentration of apoB in mg/dL multiplied by plasma volume (taken as 45 mL/kg BW).

Determination of Stable Isotope Enrichment of apo(a)

Apo(a) enrichment with $^2H_3$-L-leucine was measured as described by Zhou et al.[20] In brief, equal volumes (100 μL) of ultracentrifuged fractions of LDL and HDL, which together hold 95% of plasma Lp(a), were combined, desalted, reduced with DTT, alkylated with iodoacetamide, and digested using trypsin. A multiple reaction monitoring method was used to monitor the following precursor product ion transitions to a peptide specific to apo(a) (LFLEPTQADIALLK): 786.7>1069.7 (M0) and

788.2>1069.7 (M3); 2 µL of the digested samples were analyzed using a nanoAcquity ultraperformance liquid chromatography system coupled with an ionKey source integrated to a Xevo TQS triple quad tandem mass spectrometer (Waters, Milford, MA). The separation was achieved using an iKey Peptide BEH C18 separation device (130 Å, 1.7 µm, 150 µm X 100 mm) maintained at 60°C. The gradient was 90% A (0.1% formic acid in water)/10% B (0.1% formic acid in acetonitrile) ramped linearly to 10% A at 6 minutes, held for 3 minutes, and then re-equilibrated to initial conditions (total run time: 12 minutes; flow rate: 3 µL/min). The multiple reaction monitoring transitions were monitored with a collision energy of 24 eV.

Apo(a) Modeling

The apo(a) enrichment data were modeled as previously described for apoC-III.[17] Briefly, we estimated apo(a) FCR by fitting the leucine enrichment in the apo(a) protein with a single-pool model, with the precursor enrichment set at the same level as found with the apoB model described earlier. Apo(a) PR in nM/kg/d was calculated as the product of apo(a) FCR and the molar pool size per kg BW of apo(a), which is the apo(a) concentration (nM) multiplied by the plasma volume (taken as 45 mL/kg BW).

Post-Prandial Lipemia Studies

PP TG and apoB48 levels were measured after consumption of a standard high-fat liquid meal.[21] Details of the protocol are found in the online-only Data Supplement.

Statistical Analysis

The corresponding authors had full access to all the data and take responsibility for its integrity and the data analysis. The primary end point was the change in LDL-apoB FCR from the end of placebo treatment to the end of alirocumab treatment. With 18 subjects completing the protocol, the study had 80% power to see a 35% change in LDL-apoB FCR, assuming an FCR of 0.42 pools/day at the end of the placebo treatment period using a 2-sided paired *t* test with an alpha of 0.05. Prespecified secondary analyses included FCRs of VLDL- and IDL-apoB, PRs of VLDL-, IDL-, and LDL-apoB, conversion of VLDL to LDL and IDL to LDL, FCR and PR of VLDL-TG, plasma concentrations of Lp(a), FCR and PR of apo(a), lipoprotein size and number, and postheparin plasma LpL and HL activities at the end of placebo and alirocumab treatment periods. Exploratory analyses included levels of plant sterols, apoCIII, and apoE at the end of each treatment period. All secondary and exploratory analyses are presented with nominal *P*-values uncorrected for multiple comparisons. Exploratory outcomes analyses were performed at CUMC and were not included in the original Sanofi study statistical analysis plan.

RESULTS

Eight males (mean age 39.9±9.9 years) and 10 females (mean age 47.0±12.2 years) completed the study. Two subjects decided to leave the study for personal reasons. One dropped out during the placebo period and 1 at the end of the placebo period; neither received alirocumab (online-only Data Supplement Figure II). No serious ad-

Table 1. Baseline Characteristics: 8 Healthy Men and 10 Healthy Women Completed the Study

Parameter	Value
Sex, n (%)	
Male	8 (44.4)
Female	10 (55.6)
Age, y (mean±SD)	
Male	39.9±9.9
Female	47±12.3
Race, n (%)	
White	3 (17)
Black	13 (72)
Unknown/mixed race/other	2 (11)
BMI, kg/m², mean (±SD)	28.1±4.2

BMI indicates body mass index.

verse events occurred during the study (see online-only Data Supplement). Baseline characteristics are shown Table 1. Participants had normal plasma glucose levels and normal hepatic and renal function. Five doses of alirocumab treatment resulted in significant reductions of fasting plasma levels of total cholesterol (34.4%), calculated LDL-C (57.9%), and apoB (46.6%), with no significant effects on plasma HDL-C and TG levels (Table 2).

Alirocumab reduced mean apoB levels in ultracentrifugally isolated IDL (29.9% $P\leq0.0001$) and LDL (56.3% $P\leq0.0001$); no significant effect was found on the concentration of VLDL-apoB (Table 2). In addition, alirocumab treatment resulted in decreased mean levels of cholesterol and TG concentrations in IDL (41.1%, $P\leq0.0001$ and 20.9%, $P=0.0005$, respectively) and LDL (55.1%, $P\leq0.0001$ and 35.5%, $P\leq0.0001$, respectively) without affecting lipid levels in VLDL (Table 2). The reductions in IDL- and LDL-apoB levels measured by ELISA were in accord with similar reductions in IDL and LDL particle numbers determined by ion mobility (online-only Data Supplement Table I). The reduction in LDL particle number was associated with a shift toward smaller particles after alirocumab treatment (online-only Data Supplement Table II).

Reductions in IDL- and LDL-apoB concentrations were caused by increases in the mean FCRs of these lipoproteins (46.1%, $P<0.001$ and 80.4%, $P\leq0.0001$, respectively) (Figure 1). No significant change occurred in the mean VLDL-apoB FCR during alirocumab administration. In addition to the marked increase in LDL-apoB FCR, mean LDL-apoB PR was reduced by 23.9% ($P<0.0001$). The decrease in the PR of LDL-apoB was caused by the significant increase in the FCR of IDL-apoB noted earlier coupled with a mean 27.2% decrease in the conversion of IDL-apoB to LDL-apoB ($P<0.005$). No significant changes were found in the mean VLDL- or IDL-apoB PRs.

Table 2. Effects of 10 Weeks of Alirocumab Treatment* on Plasma Lipids and Lipoproteins

	Placebo	Alirocumab	Change (%)	P Value
Plasma Levels: mg/dL (SD)				
Total cholesterol	193.1 (32.7)	126.1 (24.6)	−34 (9.1)	<0.0001
HDL-C	58.6 (15.7)	62.7 (14.1)	8.8 (12.3)	NS
Triglycerides	72.1 (41.4)	63.7 (35.8)	−8.2 (24.0)	NS
LDL-C†	120.1 (25.0)	50.8 (19.1)	−57.9 (12.2)	<0.0001
ApoB	100.9 (20.2)	53.6 (13.5)	−46.6 (10.6)	<0.0001
ApoCIII‡	8.8 (2.4)	8.2 (1.9)	−4.2 (24.1)	NS
ApoE‡	12.0 (3.7)	8.6 (2.4)	−26.9 (10.9)	<0.0001
Lipoprotein apoB and lipid levels¶: mg/dL (SD)				
VLDL-apoB	8.0 (5.9)	6.0 (3.5)	−12.3 (43.0)	NS
IDL-apoB	4.0 (1.9)	2.6 (1.1)	−29.9 (22.2)	<0.0001
LDL-apoB	71.2 (20.6)	30.4 (9.2)	−56.3 (10.2)	<0.0001
VLDL-C	12.7 (7.3)	9.1 (4.9)	−17.4 (48.7)	0.1
IDL-C	5.6 (2.6)	3.0 (1.1)	−41.1 (27.8)	<0.0001
LDL-C	89.3 (18.3)	39.8 (11.9)	−55.1 (10.7)	<0.0001
VLDL-TG	55.0 (33.9)	48.6 (33.8)	−4.1 (52.0)	NS
IDL-TG	6.9 (3.1)	5.1 (1.6)	−20.9 (20.8)	0.0005
LDL-TG	13.5 (3.9)	8.6 (2.2)	−35.5 (10.4)	<0.0001

All data are presented as absolute means, % change, and standard deviations (SD). apoB indicates apolipoprotein B; apoCIII, apolipoprotein CIII; apoE, apolipoprotein E; HDL-C, high-density lipoprotein-cholesterol; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDL-C, low-density lipoprotein cholesterol; NS, not significant TG, triglycderides; and VLDL, very low density lipoprotein.

*Alirocumab administered every 2 weeks.

†Calculated using the Friedewald equation.

‡apoCIII, apoE, and sterol measurements were only measured in N=14 participants who consented to future research use of their samples.

¶Lipoproteins isolated by ultracentrifugation.[10]

Consistent with alirocumab lack of effects on VLDL-TG levels or VLDL-apoB metabolism, no changes occurred in mean FCR or PR of VLDL-TG (Figure 2).

Significant correlations were found between baseline PCSK9 levels and changes in levels of both LDL-C (R^2 =0.42; P=0.003) and LDL-apoB (R^2=0.22; P=0.05) (Figure 3).

Apo CIII levels were not different between the 2 study periods (8.8±2.4 vs 8.2±1.9 mg/dL), but a 26.9±10.9% decrease (P=0.0034) in plasma apo E levels (12.0±3.7 vs 8.6±2.4 mg/dL) did occur at the end of alirocumab administration (Table 2). Plasma levels of sitosterol tended to increase (113.0±37.9 vs 128.4±31.4 µmol X 10^2/mmol cholesterol; P=0.07), whereas levels of lathosterol (81.0±33.5 vs 90.4±39.6 µmol X 10^2/mmol cholesterol) and campesterol (167.3±54.9 vs

182.8±53.9 µmol X 10^2/mmol cholesterol) were unaffected by alirocumab treatments.

Inhibition of PCSK9 caused a reduction in the median level of plasma Lp(a) of 18.7% compared with placebo (P<0.01). These reductions were associated with a trend for an increase in the median FCR for apo(a) (24.6%; P=0.09) with no change in the median apo(a) PR (−9.0%; P=0.6) (Table 3). No significant correlations were found between baseline Lp(a) levels and changes in Lp(a) levels or apo(a) FCR after alirocumab treatment. However, a positive correlation did occur between Lp(a) isoform size and changes in apo(a) FCR on alirocumab (R^2=0.25; P=0.04) (data not shown).

The last 10 subjects enrolled participated in a substudy of the effects of alirocumab on PP lipemia. Inhibition of PCSK9 activity by alirocumab had no effects on the mean incremental area under the curve above baseline of either TG or apoB48 (a marker of chylomicrons) during the 8 hours after consumption of a high-fat meal (Figure 4). In accord with the absence of effects of PCSK9 inhibition on either fasting or PP TG metabolism, treatment with alirocumab did not alter the mean levels of postheparin HL (0.96±0.6 vs 1.05±0.62 µmol ffa/mL plasma/hr) or LpL activities (1.26±0.93 vs1.35±0.90 µmol ffa/mL plasma/hr) compared with placebo.

DISCUSSION

In this study of the effects of alirocumab, a fully human monoclonal antibody against PCSK9, we demonstrated that 55% reductions in LDL-C and LDL-apoB were caused by a dramatic increase in the efficiency of clearance of LDL particles, as evidenced by an 80% increase in the FCR of LDL-apoB. We also found a 24% reduction in LDL-apoB PR caused by increased efficiency of removal of IDL particles and, therefore, less conversion of IDL to LDL. Both of these results are fully consistent with marked increases in LDLR mediated by inhibition of PCSK9.

The efficacy of alirocumab and other antibodies directed against PCSK9[22] is compatible with the ability of the protein to target the complex of the LDL/LDLR to the lysosome for degradation, thereby reducing the recycling of the LDLR that normally occurs after delivery of LDL to that organelle.[1,2] Earlier studies in cells and animals supported a model in which inhibition of PCSK9 activity leads to increased LDLRs on the surface of the liver and increased efficiency of LDL particles removal from the circulation (Figure 5). Our results provide evidence in humans confirming those preclinical investigations.

The importance of the present results derives from the possibility that mechanisms other than more efficient LDLR-mediated removal of LDL could account for some of the significant reductions in plasma LDL-C and LDL-apoB levels observed during alirocumab treatment. This study explored several potential alternative effects of
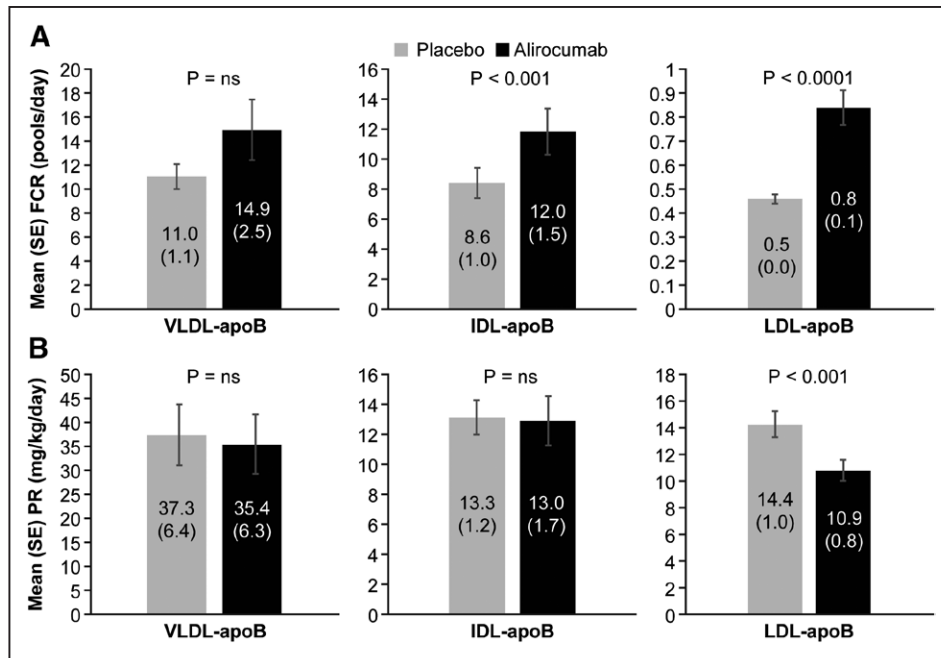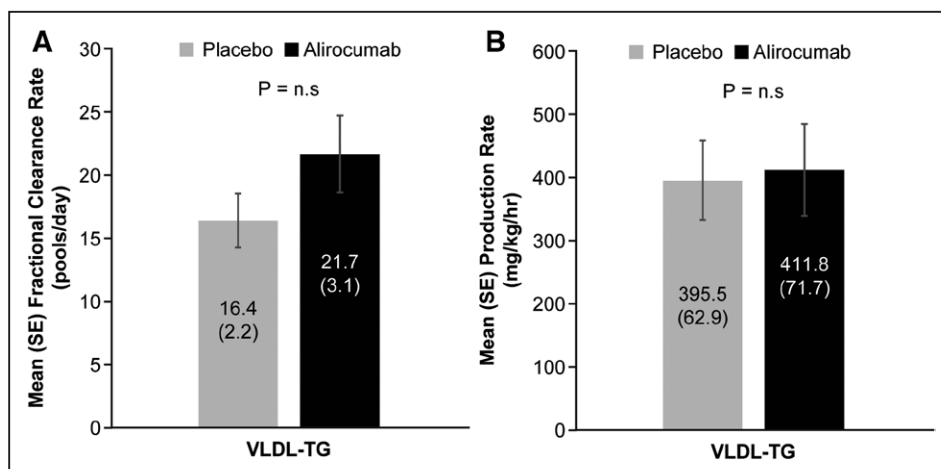
Figure 1. Effects of alirocumab on VLDL-, IDL-, and LDL–apoB fractional clearance rates (A) and production rates (B).
FCRs of VLDL, IDL, and LDL-apoB (mean±SE) were determined by stable isotopic enrichment of apoB in each lipoproteins. PRs (mean±SE) were calculated using the FCRs and plasma pool size of apoB in each lipoprotein. Alirocumab treatment significantly increased the FCRs of IDL and LDL apoB and reduced the PR of LDL apoB compared with placebo. No significant effects of alirocumab on VLDL apoB metabolism were found. apoB indicates apolipoprotein B; FCR, fractional clearance rate; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; ns, not significant; PR, production rate; SE, standard error; and VLDL, very low-density lipoprotein.

alirocumab, including decreased secretion of VLDL (the major precursor of LDL), increased removal of VLDL or its remnants without conversion to IDL and then LDL, or, more likely, increased removal of IDL, a lipoprotein close to LDL in size and composition, without conversion to LDL. Importantly, statins, which increase the number

of LDLRs by inhibiting hydroxymethylglutaryl-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, have been demonstrated to have multiple effects on the metabolism of VLDL, IDL, and LDL.[23,24] Thus, it seemed possible that, depending on the background lipid metabolism present in any individual, one or more of the

Figure 2. Effect of alirocumab on VLDL-TG fractional clearance rates (A) and production rates (B).
FCR of VLDL-TG (mean±SE) was determined by stable isotopic enrichment of glycerol in each sample of isolated VLDL over 48 hours. VLDL-TG PR (mean±SE) was calculated using the FCR and the plasma pool size of TG in VLDL. Alirocumab treatment did not affect either the FCR or the PR of VLDL-TG compared with placebo. FCR indicates fractional clearance rate; PR, production rate; TG, triglycerides; and VLDL, very low-density lipoprotein.
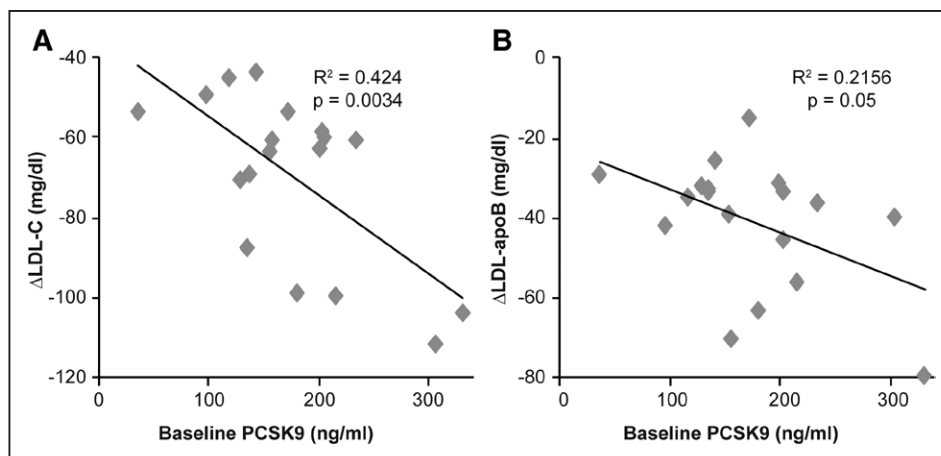
Figure 3. Correlation between baseline serum-free PCSK9 levels and changes in (A) LDL-C and (B) LDL-apoB levels.
Baseline levels of serum-free PCSK9 were examined for correlation with alirocumab-induced changes in LDL-C and LDL-apoB in all 18 subjects. Changes in both LDL-C and LDL-apoB were related to PCSK9 levels at baseline. apoB indicates apolipoprotein B; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; and PCSK9, proprotein convertase subtilisin/kexin type 9.

scenarios described previously might be important in the lowering of LDL-C by inhibition of PCSK9.

In this study of healthy volunteers, it is clear that the major effect of alirocumab was to enhance the efficiency of LDL particle clearance from the circulation; the FCR for LDL-apoB increased by 80.4%. Because only 1 apoB per LDL particle exists, this increase in the FCR translates to nearly twice as many LDL particles cleared per day during alirocumab treatment compared with placebo. Because LDLRs account for at least 80% of the clearance of LDL particles from the circulation, the near doubling of the FCR for LDL-apoB almost certainly resulted from a similar increase in available LDLRs on the surface of the liver.[25] We also demonstrated a significant increase in the FCR of IDL-apoB, compatible with the known ability of LDLRs to bind and internalize a range of apoB-lipoproteins. This increase in IDL-FCR was accompanied

by greater direct removal of IDL-apoB, which resulted in a reduced LDL-apoB PR. Thus, two related mechanisms for the marked reductions in LDL-C and LDL-apoB levels occurred during alirocumab therapy. An additional or alternative explanation for the increase in LDL-apoB FCR after alirocumab treatment could have been that changes in the physical-chemical characteristics of LDL particles would increase their affinity for the LDLR. For example, alirocumab might directly increase LDL particle size, and larger LDL particles have a greater affinity for the LDLR.[26] However, our finding that alirocumab treatment resulted in a shift in the distribution of LDL toward smaller particles is compatible with greater removal of larger LDL particles by increased numbers of LDLR rather than a primary treatment-mediated shift in LDL toward larger particles.

In contrast to the findings for IDL and LDL, we saw no significant effect of alirocumab on VLDL-apoB and VLDL-TG metabolism. Because VLDL-apoB or its remnants can interact with the LDLR, resulting in direct removal of VLDL particles from the circulation without conversion to LDL,[6,8,27] the possibility existed that PCSK9 inhibition would be associated with an increased flux of VLDL particles through that pathway. The probability that VLDL will be converted to IDL and LDL by lipolysis rather than being internalized by LDLRs depends on the quantity of TG in each VLDL particle, with TG-rich VLDL particles more likely to be removed directly from plasma compared with smaller, TG-poor VLDL particles.[28] We studied healthy individuals with normal plasma TG levels, and, therefore, lipolytic conversion to IDL and LDL was probably rapid, reducing the possibility of an interaction between VLDL and LDLRs in the hepatic bed. Lipolysis, which would be the dominant determinant of the fate of relatively TG-poor VLDL particles entering the circulation in our healthy vol-

Table 3. Effects of Alirocumab on Lp(a) Plasma Levels and the Kinetics of apo(a)

	Placebo	Alirocumab	Change (%)	P Value
Lp(a) nmol/l	50.4 (22.2, 79.1)	35.7 (15.1, 74.9)	−18.7 (-30.6, -11.2)	<0.01
apo(a) FCR pools/d*	0.16 (0.13, 0.23)	0.20 (0.15, 0.25)	+24.6 (-22.4, 59.6)	0.09
apo(a) PR nmol/kg/d*	0.41 (0.15, 0.71)	0.40 (0.23, 0.61)	−9.0 (-33.7, 24.1)	0.6

Lp(a) levels are expressed as medians with interquartile ranges for the percent changes (N=18).

apo(a) indicates apolipoprotein(a); FCR, fractional clearance rate; Lp(a), lipoprotein(a); and PR, production rate.

*Lp(a) kinetics studies were performed on only 17 subjects because 1 subject had levels of Lp(a) too low for liquid chromatography/mass spectrometry determination of enrichment.
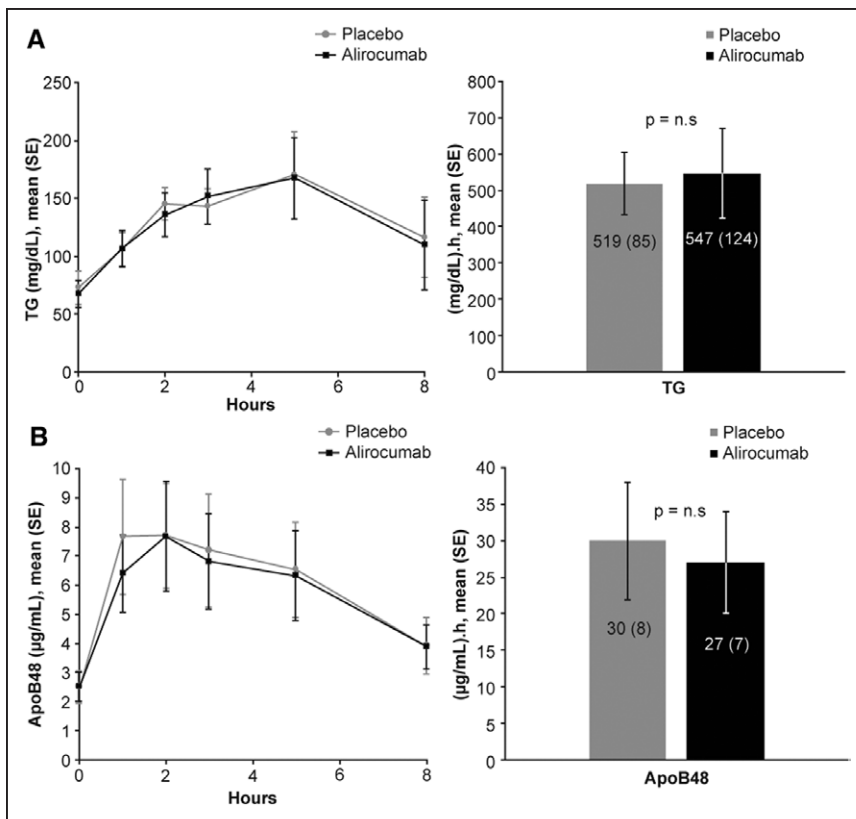
Figure 4. Postprandial levels of TG (A) and ApoB48 (B) after a high-fat meal.
(**Left**) Mean concentrations over time. (**Right**) Incremental area-under-the-curve (IAUC). Ten participants had blood samples just before and at 1, 2, 3, 5, and 8 hours after consuming a high-fat liquid meal providing 1237 Kcal per 2 m^2 body surface area from 75% fat, 10% protein, and 15% carbohydrate The data are presented as individual timepoints (mean±SE) and area under the curve above baseline (IAUC) (mean±SE) of plasma TG and apoB48 concentrations. Alirocumab had no effects on postprandial levels of TG and apoB48 compared with placebo. apoB indicates apolipoprotein B; and TG, triglycerides.

unteers, was not affected by alirocumab, as indicated by unchanged postheparin lipolytic activities and apoCIII levels. The decrease in apoE levels was most likely the result of increased fractional removal of apoE-containing IDL and LDL particles from the blood. However, lower apoE levels could have contributed to the lack of an increase in direct removal of VLDL despite increased LDLR availability.

In addition to the potential for increased removal of VLDL by hepatic LDLRs, the effects of PCSK9 on the secretion of apoB-lipoproteins have also been demonstrated. Overexpression of PCSK9 in either the liver or the intestine increases secretion of apoB-lipoproteins,[29,30] whereas knockdown of PCSK9 has the opposite effect.[30,31] Additionally, individuals with gain-of-function mutations in PCSK9 were found to have increased secretion of VLDL-, IDL-, and LDL-apoB.[32] Our finding that alirocumab did not affect either VLDL PR or PP lipemia is, therefore, in contrast to these reports, but it is possible that these differences also reflect the absence of underlying abnormalities in either lipolysis- or receptor-mediated removal of apoB48 particles in the individuals we studied. Alternatively, pharmacological interference with the action of circulating PCSK9 does not affect intracellular actions of PCSK9 on VLDL or chylomicron secretion. Finally, studies with alirocumab and other monoclonal antibodies directed against PCSK9 in populations with elevated fasting TG levels may produce different results.

A theoretical concern related to the alirocumab-mediated increase in LDL clearance from the circulation is an increase in the flux of LDL-C into the liver, with the potential for steatosis or cholesterol gallstone formation. However, after an initial increase in hepatic uptake of IDL and LDL particles immediately after the first dose of alirocumab, a stable increase in the FCR of a smaller circulating pool of LDL particles will result in little or no change in the absolute rate of cholesterol uptake by the liver in a new steady state. The absence of significant changes in lathosterol, a measure of cholesterol synthesis, or either sitosterol or campesterol, markers of cholesterol absorption, indicate hepatic cholesterol homeostasis was maintained during chronic alirocumab therapy.[33,34]

The metabolism of Lp(a) remains poorly defined 50 years after its discovery.[35,36] Although plasma levels appear to be closely related to PR,[36,37] understanding of the pathways involved in the clearance of Lp(a) remains modest at best. Indeed, some studies indicate that neither the LDLR nor apoB plays a role in the removal of Lp(a) from the circulation,[38,39] whereas others support a central role for the LDLR in the uptake of Lp(a) by hepatocytes.[40] Additional uncertainty arises from the observations that although Lp(a) levels are increased in individuals with familial hypercholesterolemia,[41] a disorder with reduced levels of LDLR, statins, which increase the FCR of LDL-apoB by 25% to 40%, do not lower Lp(a) levels.[11] It must be noted, however, that statins raise both the number of LDLR and
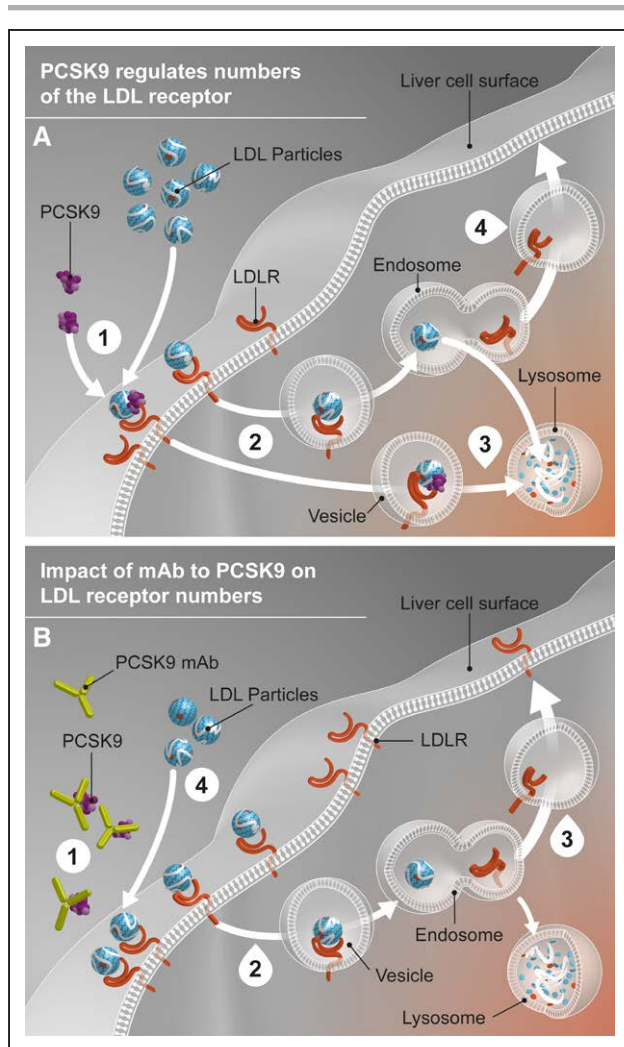
Figure 5. Role of PCSK9 in LDL metabolism and impact of PCSK9 monoclonal antibody.
(A) (1) LDLR binds to LDL particle at the liver cell surface. PCSK9 can also bind to the LDLR. (2) The LDL particle–LDLR complexes with or without PCSK9 bound are internalized in the liver cell by endocytosis. (3) LDLR not bound to PCSK9 release the LDL particle, which goes to a lysosome for digestion, whereas the LDLR is recycled to the cell surface. (4) LDLR bound to PCSK9 is digested in the lysosome along with the LDL particle. **(B)** (1) PCSK9 mAb binds PCSK9 in the circulation, preventing it from binding the LDLR. (2) The LDL particle–LDLR complexes are internalized in the liver cell. (3) In the absence of PCSK9 binding, increased LDLR receptor recycling and more LDLR on the liver cell surface to bind result. (4) Circulating LDL particle levels are reduced. LDL indicates low-density lipoprotein; LDLR, low-density lipoprotein receptor; mAb, monoclonal antibody; and PCSK9, proprotein convertase subtilisin/kexin type 9.

plasma concentrations of PCSK9, with the latter effect negating, at least partially, the increase in LDLR.[42] Treatment of homozygous familial hypercholesterolemia patients with evolocumab was associated with reductions in Lp(a) levels, suggesting non-LDLR clearance of Lp(a), but

only 1 of the 49 subjects reported was homozygous for a null mutation in the LDLR.[43]

Our results for Lp(a), which are suggestive and not conclusive, certainly do not rule out other complementary or alternative pathways for Lp(a) removal from the circulation. We also acknowledge studies of the simultaneous kinetics of both apo(a) and apoB demonstrating different FCRs for apo(a) and apoB, suggesting that they are not always linked on a single Lp(a) particle.[44,45] However, in a third study, it appeared that the 2 proteins were linked throughout their time in the circulation.[46] Although further work is required to fully define the stability of the association between apo(a) and apoB in Lp(a), this potential complexity would not impact our findings because the FCR of apo(a) was determined in a similar fashion in our study and the others. Indeed, the FCRs of apo(a) in all 3 of those published reports were similar to those we determined in the present investigation. Finally, a study in which FCRs of apo(a) were determined by the rate of rise of plasma levels after physical removal of Lp(a) by apheresis reported rates nearly identical to the FCRs we found at baseline.[47]

In conclusion, inhibition of PCSK9 activity with alirocumab treatment of healthy, normolipidemic volunteers reduced LDL-C and LDL-apoB concentrations by 55% and 56%, respectively, in association with a nearly doubling of the efficiency with which LDL particles were removed from the circulation. A significant fall in LDL-apoB PR caused by increased direct removal of IDL particles from the circulation was also found. Both of these findings are consistent with increased availability of LDLR on the surface of the liver, the main organ for LDL clearance. Lp(a) concentrations were reduced significantly, with a strong trend toward an increased FCR, suggestive of a role for the LDLR in the clearance of Lp(a). Alirocumab did not have an effect on Lp(a) PR.

Of note, the potent effect of inhibition of PCSK9 activity on the FCR of LDL-apoB observed in this study indicates that individuals without dyslipidemia continually lose ≥50% of LDLRs to lysosomal degradation because of the actions of PCSK9. The number of LDLRs on cell surfaces is exquisitely regulated at the transcriptional level by effects of the concentration of hepatic-free cholesterol on the sterol response element binding protein-2,[48] raising the interesting question as to why we evolved with PCSK9 as a parallel regulator of LDL uptake.

AFFILIATIONS

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