**Brief Report**

**Proprotein Convertase Subtilisin Kexin Type 9 Inhibition for Autosomal Recessive Hypercholesterolemia**

Aurélie Thedrez, Barbara Sjouke, Maxime Passard, Simon Pramart-Fauvet, Alexis Guédon, Mikael Croyal, Geesje Dallinga-Thie, Jorge Peter, Dirk Blom, Milco Ciccarese, Angelo B. Cefalù, Livia Pisciotta, Raul D. Santos, Maurizio Averna, Frederick Raal, Paolo Pintus, Maria Cossu, Kees Hovingh, Gilles Lambert

**Objective**—Proprotein convertase subtilisin kexin type 9 (PCSK9) inhibitors lower low-density lipoprotein (LDL) cholesterol in the vast majority of patients with autosomal dominant familial hypercholesterolemia. Will PCSK9 inhibition with monoclonal antibodies, in particular alirocumab, be of therapeutic value for patients with autosomal recessive hypercholesterolemia (ARH)?

**Approach and Results**—Primary lymphocytes were obtained from 28 genetically characterized ARH patients and 11 controls. ARH lymphocytes treated with mevastatin were incubated with increasing doses of recombinant PCSK9 with or without saturating concentrations of alirocumab. Cell surface LDL receptor expression measured by flow cytometry and confocal microscopy was higher in ARH than in control lymphocytes. PCSK9 significantly reduced LDL receptor expression in ARH lymphocytes albeit to a lower the extent than in control lymphocytes (25% versus 76%, respectively), an effect reversed by alirocumab. Fluorescent LDL cellular uptake, also measured by flow cytometry, was reduced in ARH lymphocytes compared with control lymphocytes. PCSK9 significantly lowered LDL cellular uptake in ARH lymphocytes, on average by 18%, compared with a 46% reduction observed in control lymphocytes, an effect also reversed by alirocumab. Overall, the effects of recombinant PCSK9, and hence of alirocumab, on LDL receptor expression and function were significantly less pronounced in ARH than in control cells.

**Conclusions**—PCSK9 inhibition with alirocumab on top of statin treatment has the potential to lower LDL cholesterol in some autosomal recessive hypercholesterolemia patients. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.116.307493.)

**Key Words:** alirocumab • hypercholesterolemia • proprotein convertase subtilisin kexin type 9, human • receptors, LDL • therapeutics

Proprotein convertase subtilisin kexin 9 (PCSK9) inhibition with monoclonal antibodies is a promising strategy to lower low-density lipoprotein cholesterol (LDL-C) by >50% in dyslipidemic patients. Homozygous familial hypercholesterolemia (HoFH), the most severe genetic disorder of lipoprotein metabolism, is inadequately treated with existing lipid-lowering medications. Because PCSK9 enhances the intracellular degradation of the LDL receptor (LDLR), its inhibition does not lower LDL-C in HoFH patients with mutations that completely abolish LDLR function. However, in carriers of LDLR mutations with reduced, but not absent, LDLR function, PCSK9 inhibition lowered LDL-C by 40%. Besides LDLR defects, the HoFH phenotype can also result from mutations in both alleles of the LDLR adaptor protein (LDLRAP1), a disorder known as autosomal recessive hypercholesterolemia (ARH). LDLRAP1 connects the LDLR with the clathrin machinery, allowing LDL endocytosis in cells such as hepatocytes and lymphocytes, but not in other cell types such as dermal fibroblasts.

The aim of the present study was to evaluate ex vivo the LDL-C–lowering potential of PCSK9 inhibition in ARH.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARH</td>
<td>autosomal recessive hypercholesterolemia</td>
</tr>
<tr>
<td>FH</td>
<td>familial hypercholesterolemia</td>
</tr>
<tr>
<td>HoFH</td>
<td>homozygous FH</td>
</tr>
<tr>
<td>LDL-C</td>
<td>low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
</tr>
<tr>
<td>LDLRAP1</td>
<td>LDLR adaptor protein 1</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>PCSK9</td>
<td>proprotein convertase subtilisin kexin type 9</td>
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Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

The demographic and clinical characteristics of ARH patients are summarized in Table I in the online-only Data Supplement. Mean LDL-C was 278±38 mg/dL, despite maximal tolerated doses of statins and apheresis in 89% of patients. Circulating PCSK9 levels were extremely elevated in ARH at 526±31 ng/mL.15,12

We first assessed cell-surface LDLR expression in lymphocytes isolated from control individuals and ARH patients by flow cytometry. After mevastatin treatment, Δ mean fluorescence intensity (MFI) levels of cell surface LDLR expression reached a maximum of 635±56 arbitrary unit (AU) in control lymphocytes. In ARH lymphocytes, cell surface LDLR levels were 3× more elevated at ΔMFI levels of 1595±215 AU (P<0.01 versus controls). PCSK9 dose dependently reduced LDLR expression down to ΔMFI levels of 150±19 AU (−76%) and 1192±96 AU (−25%), whereas alirocumab restored LDLR expression at ΔMFI levels of 548±96 AU and 1573±212 AU in control and ARH lymphocytes, respectively (Figure [A]). Total LDLR expression in control and ARH lymphocytes was ascertained by confocal microscopy, and followed expression patterns of cell surface LDLR levels measured by flow cytometry (Figure [B]).

We next assessed the cellular uptake of fluorescent LDL in control and ARH lymphocytes. In sharp contrast with LDLR expression patterns, LDL uptake was significantly reduced in ARH lymphocytes compared with controls. Maximal LDL uptake was observed in mevastatin-treated control lymphocytes with ΔMFI levels of 818±85 versus 532±57 AU in ARH lymphocytes (P<0.01 versus controls). PCSK9 significantly reduced LDL cellular uptake down to ΔMFI levels of 440±48 AU (−46%) and 435±44 AU (−18%) in control and ARH lymphocytes, whereas alirocumab restored LDL uptake at ΔMFI levels of 722±78 and 496±58 AU, respectively (Figure [C]). Noteworthy, the levels of LDL uptake and the effects of PCSK9 treatment on LDL uptake were larger in ARH lymphocytes than in lymphocytes isolated from a receptor defective/negative HoFH patient, used as negative control (Figure I in the online-only Data Supplement). We also ascertained that LDL uptake in lymphocytes was not altered by alirocumab alone (ie, in the absence of PCSK9; not shown).

Finally, we performed a similar series of experiments in skin fibroblasts from 1 control and 1 ARH patient, as previously described.4,12 We showed that ARH fibroblasts respond almost just as well to statins, PCSK9, and alirocumab than control fibroblasts, in terms of cell surface LDLR expression (Figure IIA in the online-only Data Supplement) and LDL cellular uptake (Figure IIB in the online-only Data Supplement).

Discussion

In the present study, we showed that despite much higher cell surface LDLR expression levels, ARH lymphocytes display sharply reduced LDL uptake capacities compared with control lymphocytes. We also showed that ARH lymphocytes were 3× less sensitive to PCSK9-mediated LDLR degradation than control lymphocytes. Consequently, ARH lymphocytes were found less responsive to alirocumab than control lymphocytes in absolute terms, suggesting that the clinical potential of PCSK9 inhibitors to lower LDL-C in ARH may be limited.

Together, our in vitro results in lymphocytes and fibroblasts are fully consistent with data from the literature showing that LDLRAP1 is required for LDLR-mediated LDL internalization in clathrin-coated pits of lymphocytes and hepatocytes but not of dermal fibroblasts.9,10 This rules out the use of dermal fibroblasts to test specifically the clinical potential of PCSK9 inhibition with monoclonal antibodies in ARH.

Despite the central role played by the LDLRAP1 in LDLR turnover in lymphocytes, we found that exogenous PCSK9 was able to slightly but significantly reduce LDLR expression and LDL uptake in ARH lymphocytes, albeit to a lower extent than in control lymphocytes. A small proportion of cell surface LDLR bound to PCSK9 might, for instance, be endocytosed by a mechanistic pathway not requiring clathrin and the LDLRAP1.8 This certainly merits further investigation.

Noteworthy, just like what was observed in HoFH fibroblasts with identical LDLR defective mutations,6 ARH lymphocytes responded variably to PCSK9 (−4% to −31% LDL uptake), a variation of greater relative magnitude than that observed in control lymphocytes (−35% to −54%). In this respect, we have not been able to identify any association between lymphocytes response to PCSK9 and the various LDLRAP1 mutations or any other demographic of clinical parameters of the ARH lymphocytes donors (not shown).

Despite the inherent limitation of lymphocytes as a proxy for hepatocytes, which account for the bulk of plasma LDL clearance in humans, our results suggest that PCSK9 inhibition may be beneficial for ARH patients by lowering LDL-C further, at least for some of them. In that respect, repeated injections of alirocumab 150 mg every 2 weeks were performed in 2 of our patients. There was some significant reduction in LDL-C for 1 patient (−1.1 mmol/L, ie, −11.3%) but not for the other (−0.1 mmol/L, ie, −1.8%). Another ARH patient reported in the literature failed to respond to PCSK9 inhibition with evolocumab 420 mg every month (+0.4 mmol/L, ie, +3.5%).6 Pharmacological reductions in LDL-C of bigger magnitude (−50%) were observed in 1 ARH patient treated with the microsomal transfer protein inhibitor lomitapide.11,15 However, given that most ARH patients are currently receiving LDL apheresis treatments every 2 weeks, that they all display very high circulating PCSK9 levels, and that, unlike lomitapide, monoclonal antibodies targeting PCSK9 seem to cause little side effects,1 it is probably worth testing these new therapies in ARH patients.
**Sources of Funding**

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**Disclosures**

Drs Blom, Santos, Averna, Raal, and Hovingh received clinical trial funding from Sanofi-Regeneron and Amgen. Dr Lambert had received honoraria and research funding from Sanofi-Regeneron, Amgen and Pfizer Inc. The other authors report no conflicts.

**References**


5. Stein EA, Honarpour N, Wasserman SM, Xu F, Scott R, Raal FJ. Effect of the proprotein convertase subtilisin/kexin 9 monoclonal antibody,


• We evaluated ex vivo whether proprotein convertase subtilisin kexin type 9 inhibition with monoclonal antibodies could be of therapeutic value for patients with autosomal dominant hypercholesterolemia.

• Autosomal dominant hypercholesterolemia lymphocytes were overall less responsive to proprotein convertase subtilisin kexin type 9 and proprotein convertase subtilisin kexin type 9 inhibition than control lymphocytes.

• The clinical potential of proprotein convertase subtilisin kexin type 9 inhibitors to sharply lower low-density lipoprotein-cholesterol in autosomal dominant hypercholesterolemia may therefore be limited but nevertheless worth being tested in those patients at extreme cardiovascular risk.
Proprotein Convertase Subtilisin Kexin Type 9 Inhibition for Autosomal Recessive Hypercholesterolemia

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MATERIAL AND METHODS

**Patients** - Blood samples were collected from 11 normolipemic controls (6 men and 5 women, 18 to 47 years old) and 28 genetically confirmed ARH patients, including fourteen c.432insA homozygotes, four c.65G>A homozygotes, five compound heterozygotes for the two previous mutations on two different alleles, two c.65G>A / c.432insA double heterozygotes, one compound heterozygote for c.65G>A and c.89-1G>C mutations on two different alleles, one c.72insG homozygote and one c.406C>T homozygote, all mutations on LDLRAP1 resulting in a premature stop codon. Lymphocytes isolated from a defective/negative compound heterozygote FH patient (i.e LDLR-D206E / LDLR-V408M) were used as negative controls. Plasma lipids and lipoproteins were measured in accredited laboratories. Prior to lymphocyte isolation, an aliquot of plasma was used to measure circulating PCSK9 levels by ELISA (R&D Systems, Lille, France) and apolipoprotein (a) molar concentration by liquid chromatography mass spectrometry.

**Lymphocytes** - Lymphocytes were isolated using Ficoll Paque Plus (Sigma-Aldrich, Saint Quentin-Fallavier, France) and subsequently frozen at -80°C in RPMI culture medium (Life Technologies, Saint Aubin, France) containing 10% dimethylsulfoxide (DMSO) and 70% fetal calf serum (FCS) until use.

**LDLR expression in lymphocytes** - Freshly thawed lymphocytes were seeded in flat bottom 96-well plates (2x10^5 cells per well) in RPMI containing 10mM Hepes, 1mM Sodium Pyruvate and 0.5% FCS for 2 hours at 37°C. The culture medium was subsequently supplemented with 0 or 10µg/mL mevastatin (Sigma) for 24h. Increasing doses of recombinant gain of function PCSK9-D374Y [0, 150, 600ng/mL] (Cyclex Co, Nagano, Japan) were added to the medium for an additional 4h, as previously described. In a subset of experiments, alirocumab (stock solution 25mg/mL in 10mM Na2HPO4) was added concomitantly into the wells at a final concentration of 19.2µg/mL. For cell surface LDLR expression analysis by flow cytometry, lymphocytes were subsequently washed twice in ice cold phosphate buffered saline (PBS) containing 1% bovine serum albumin (PBS-1% BSA), and incubated with an allophycocyanin-conjugated antibody against the human LDLR (clone 472413) or an IgG1 (clone 11711) isotype control (R&D Systems) at 0.625µg/mL for 20min at room temperature in the dark. Cells were next incubated with a phycoerythrin-conjugated antibody against the human CD3 (clone BW264/56) (Miltenyi Biotec, Paris, France) diluted at 1/40 in PBS-1% BSA for 10min on ice in the dark. Lymphocytes were then washed twice in ice-cold PBS-1% BSA and once in ice cold PBS before fixation with PBS containing 0.5% paraformaldehyde (Sigma-Aldrich). Cells were analyzed by flow cytometry using a LSRII cytometer (Beckton Dickinson, Le Pont de Claix, France). Forward scatter versus side-scatter gates were set to include only viable cells. A minimum of 2500 CD3 positive cells were analysed for LDLR expression using the FlowJo software (Tree Star, Ashland, OR, USA). Mean fluorescence intensity (MFI) of cells incubated with the isotype control fluorescent
antibody (non-specific binding) was subtracted from the MFI of cells incubated with a specific anti-LDLR fluorescent antibody to determine specific MFI levels [ΔMFI] of LDLR cell surface expression. ΔMFI are expressed in arbitrary units (AU) throughout.

For total LDLR expression analysis by confocal microscopy, lymphocytes were grown as above but seeded in wells from a poly-L-lysine coated 8-wells Millicell EZ slide (Merck-Millipore, Fontenay Sous Bois, France) for the final 2h of the incubation period. Cells were gently washed once in PBS before fixation with PBS containing 4% paraformaldehyde for 15min at room temperature. Lymphocytes were then washed twice in PBS and incubated with PBS containing 5% goat serum (Life Technologies) and 0.3% saponine (Sigma-Aldrich) for 1h at room temperature and subsequently incubated overnight at 4°C with a monoclonal antibody against the LDLR (clone C7) (SantaCruz Biotech., Heidelberg, Germany) at 4µg/mL in permeabilization buffer (PBS-1% BSA containing 0.3% saponine). Lymphocytes were washed twice in permeabilization buffer and incubated with a biotin conjugated goat anti-mouse IgG secondary antibody (Life technologies) diluted at 1/1000 in permeabilization buffer for 45min at room temperature. Cells were washed twice in permeabilization buffer and incubated with Alexa 568-conjugated streptavidin (Life technologies) for 30min at room temperature in the dark. Finally, cells were washed three times in PBS, slides were mounted with coverslides in Prolong anti-fade reagent containing DAPI (Life Technologies) and visualized on a confocal A1 RSi microscope (Nikon, Melville, USA).

**LDL cellular uptake in lymphocytes** - Lymphocytes were grown in RPMI containing 0.5% FCS with or without mevastatin, subsequently supplemented with or without recombinant PCSK9-D374Y and alirocumab, as above. For the final three hours of the incubation time, LDL-Bodipy (Life Technologies) was added to the medium at a 10µg/mL final concentration. Cells were washed twice in ice-cold PBS-1% BSA, once in ice-cold PBS and re-suspended in ice-cold PBS supplemented with 0.2% trypan blue (Sigma) to quench cell surface-bound fluorescent LDL prior to flow cytometry analysis. Background fluorescence was measured in lymphocytes incubated without LDL-bodipy and subtracted from the fluorescence measured in cells incubated with LDL-Bodipy. MFI of cells incubated without fluorescent lipoproteins (autofluorescence) was subtracted from the MFI of cells incubated with fluorescent lipoproteins (LDL-bodipy) to determine the specific MFI levels [ΔMFI] of LDL uptake in those cells, expressed in arbitrary units (AU) throughout.

**Fibroblasts** - Normal human dermal fibroblasts (NHDF) were purchased from PromoCell (Heidelberg, Germany). ARH fibroblasts (GM0696) were purchased from the Corriel Cell Repository (Camden, NJ). Fibroblasts were grown in DMEM #31966 (Life Technologies) containing 20% FCS.

**LDLR expression in fibroblasts** - Fibroblasts were seeded in flat bottom 96-well plates (35000 cells/well) in DMEM containing 0.5% FCS for 24h at 37°C and subsequently supplemented with 0 or 20µg/mL mevastatin (Sigma-Aldrich) for 24h. Increasing doses (0, 150 and 600ng/mL) of recombinant PCSK9-D374Y (Cyclex) with or without Alirocumab (at 19.2µg/mL) were added for an additional 4h. Fibroblasts were then washed gently with PBS, lifted with Accutase (Sigma-Aldrich) and re-suspended in ice cold PBS. Cells were subsequently washed twice in ice-cold PBS-1% BSA, and incubated with an allophycocyanin-conjugated antibody against the human LDLR (clone 472413) or an IgG1 (clone 11711) isotype control (R&D Systems) at 0.625µg/mL for 20min at room temperature in the dark. Cells were then washed twice in ice-cold PBS-1% BSA and once in ice cold PBS before fixation with PBS containing 0.5% paraformaldehyde (Sigma-Aldrich) and analysis by flow cytometry.

**LDL cellular uptake experiments in fibroblasts** - We used fibroblasts grown as above but for the final three hours of the incubation time, LDL-Bodipy (Life Technologies) was added to the medium at a 10µg/mL final concentration. Cells were washed twice in ice-cold PBS-1% BSA, once in ice-cold PBS and re-suspended in PBS supplemented with 0.2% trypan blue prior to flow cytometry analysis.

**Statistical analyses** - Assuming 25% differences between groups in LDLR expression as well as LDL uptake levels, we determined that 10 individuals per group would yield 95% power to detect a significant difference in terms of response to PCSK9 between ARH and controls. Comparisons
between controls and ARH groups were performed using a Mann Whitney test. All treatments were systematically tested on the same plate for each patient and control individual. Comparisons between treatments were done using a Wilcoxon matched-pairs signed rank test. All results in the text and figures are presented as mean ± SEM.

REFERENCES

SUPPLEMENTAL MATERIAL

PCSK9 inhibition for Autosomal Recessive Hypercholesterolemia

Aurélie Thedrez PhD¹, Barbara Sjouke MD², Maxime Passard MSc¹, Simon Prampart-Fauvet, BSc¹, Alexis Guédon BSc¹, Mikael Croyal MSc¹, Geesje Dallinga-Thie PhD², Jorge Peter PhD², Dirk Blom MD-PhD², Milco Ciccarese MD³, Angelo B. Cefalu MD⁵, Livia Pisciotta MD⁵, Raul D. Santos MD-PhD⁷, Maurizio Averna MD⁵, Frederick Raal MD-PhD⁵, Paolo Pintus MD⁸, Maria Cossu MD⁴, Kees Hovingh MD-PhD², and Gilles Lambert PhD¹,¹⁰,¹¹,¹².
SUPPLEMENTAL TABLE I - Demographic and clinical characteristics of 28 ARH patients included in the study. Data are presented as mean ± SEM. Plasma lipids and lipoproteins are pre-apheresis values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age</td>
<td>35 ± 3 years</td>
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<tr>
<td>Sex</td>
<td>16 males / 12 females</td>
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<tr>
<td>Total Cholesterol (TC)</td>
<td>350 ± 30 mg/dL</td>
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<tr>
<td>Triglycerides (TG)</td>
<td>85 ± 33 mg/dL</td>
</tr>
<tr>
<td>HDL Cholesterol (HDL-C)</td>
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<tr>
<td>LDL Cholesterol (LDL-C)</td>
<td>278 ± 38 mg/dL</td>
</tr>
<tr>
<td>Apo(a)</td>
<td>70 ± 10 nmol/L</td>
</tr>
<tr>
<td>PCSK9</td>
<td>526 ± 31 ng/mL</td>
</tr>
<tr>
<td>Statin w/ or w/o ezetimibe</td>
<td>100 %</td>
</tr>
<tr>
<td>Apheresis (every fortnight)</td>
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SUPPLEMENTAL FIGURE I: Fluorescent LDL uptake in control, ARH and receptor defective/negative HoFH lymphocytes. ∆MFI is the mean fluorescence intensity difference between cells incubated with and without fluorescent lipoproteins. Histograms represent the mean ± SEM.
SUPPLEMENTAL FIGURE II: Similar effect of PCSK9 and Alirocumab on LDLR expression and function in control and ARH fibroblasts. (A) Cell surface LDLR expression in control and ARH fibroblasts assessed by flow cytometry. ΔMFI = difference of mean LDLR fluorescence with isotype control staining (AU). (B) Fluorescent LDL uptake in control and ARH fibroblasts. ΔMFI = difference of mean fluorescence intensity with cell auto-fluorescence (AU). Histograms represent the mean ± SEM of at least three independent experiments. ns, * , ** and *** : p>0.05, p<0.05, p<0.01 and p<0.001, respectively, with Mann Whitney tests.
PCSK9 lowers LDL cellular uptake in primary lymphocytes isolated from patients with autosomal recessive hypercholesterolemia (ARH) by 18%, compared with a 46% reduction in control lymphocytes. These effects were reversed by alirocumab, a monoclonal antibody against PCSK9. Since the effects of recombinant PCSK9 on LDL receptor function were significantly less pronounced in ARH than in control lymphocytes, we anticipate that alirocumab has the potential to lower LDL cholesterol levels in some but not all ARH patients.