Effect of open-label infusion of an apolipoprotein A-I-containing particle (CER-001) on reverse cholesterol transport and artery wall thickness in patients with familial hypoalphalipoproteinemia.

**Authors:** Ruud S. Kootte¹, Loek P. Smits¹, Fleur M. van der Valk¹, Jean-Louis Dasseux², Constance H. Keyserling², Ronald Barbaras², John F. Paolini², Raul D. Santos¹, Theo H. van Dijk⁴, Geesje M. Dallinga¹, Aart J. Nederveen², Willem J.M. Mulder¹, G. Kees Hovingh¹, John J.P. Kastelein¹, Albert K. Groen⁴, Erik S. Stroes¹.

**Affiliations authors:** ¹Department of Vascular Medicine and Experimental Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands; ²Cerenis Therapeutics Holding, Toulouse, France; ³Lipid Clinic Heart Institute, Sao Paulo, Brazil; ⁴Department of Laboratory Medicine, University Medical Center Groningen, Groningen, the Netherlands; ⁵Department of Radiology, Academic Medical Center, Amsterdam, the Netherlands

**Institution:** Academic Medical Center, Amsterdam, The Netherlands.

**Running title:** CER-001 in familial hypoalphalipoproteinemia

**Address for correspondence:** E.S.G. Stroes, dept. of Vascular Medicine, AMC Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel: +31 20 5666612; fax: +31 20 6968833; email: e.s.stroes@amc.nl.

**Conflicts of interest:**

J-L.D., C.C.K., R.B. and J.F.P. are employed by Cerenis.

R.D.S. has received consultant and lecturing fees from Astra Zeneca, Amgen, Aegerion, Biolab, Boehringer Ingelheim, Bristol Myers Squibb, Genzyme, Pfizer, Novartis, Eli Lilly, Sanofi, Regeneron and Unilever.

G.K.H has received lecturing fees from Amgen, Sanofi, MSD, Eli Lilly and Cerenis. He participates in the MODE study (involving CER001)

E.S.G.S. has received lecturing fees from Amgen, Sanofi, Eli Lily and Torrent. He served as principal investigator for this study on CER001 infusion in patients with hypoalphalipoproteinemia.

J.J.P.K. is a consultant to and receives honoraria from Cerenis, Medicines company, CSL-Behring, as well as from Dezima Pharmaceuticals, Eli Lilly, MSD, Isis Pharmaceuticals and Boehringer Ingelheim. He was also member of the steering committee of the CHI-SQUARE study (involving CER-001).

**Word count:** 5887 (excluding title page, abstract, abbreviations, references, tables and figure legends).
Individual authors contributions:

Ruud S. Kootte Substantial contributions to the conception and design; acquisition, analysis and interpretation of the data; drafting the article; final approval of the version to be published.

Loek P. Smits. Substantial contributions to the conception and design; acquisition, analysis and interpretation of the data; drafting the article; final approval of the version to be published.

Fleur M. van der Valk. Substantial contributions to the conception and design; acquisition, analysis and interpretation of the data; drafting the article; final approval of the version to be published.

Jean-Louix Dasseux. Substantial contributions to the conception and design; final approval of the version to be published.

Constance H. Keyserling. Substantial contributions to the conception and design.

Ronald Barbaras Substantial contributions to the conception and design.

John F. Paolini Substantial contributions to the conception and design; final approval of the version to be published.

Raoul D. Santos Substantial contributions to final approval of the version to be published.

Theo H. van Dijk Substantial contributions to analysis and interpretation of the data; final approval of the version to be published.

Geesje M. Dallinga-van Thie Substantial contributions to acquisition, analysis and interpretation of the data; final approval of the version to be published.

Aart J. Nederveen Substantial contributions to the acquisition, analysis and interpretation of the data; final approval of the version to be published.

Willem M. Mulder Substantial contributions to the conception and design; acquisition, analysis and interpretation of the data; final approval of the version to be published.

G. Kees Hoving Substantial contributions to the conception and design; acquisition, analysis and interpretation of the data; drafting the article; final approval of the version to be published.

John J. Kastelein Substantial contributions to the conception and design; drafting the article; final approval of the version to be published.

Albert K. Groen Substantial contributions to the conception and design; acquisition, analysis and interpretation of the data; final approval of the version to be published.

Erik S. Stroes Substantial contributions to the conception and design; acquisition, analysis and interpretation of the data; drafting the article; final approval of the version to be published.
Abstract

Background Reverse cholesterol transport (RCT) contributes to the anti-atherogenic effects of High-density lipoprotein (HDL). Patients with the orphan disease familial hypoalphalipoproteinemia (FHA) are characterized by decreased tissue cholesterol removal and an increased atherogenic burden. We performed an open-label, uncontrolled proof-of-concept study to evaluate the effect of infusions with a human apolipoprotein-AI containing HDL-mimetic particle (CER-001) on RCT and the arterial vessel wall in FHA.

Methods Subjects received 20 infusions of CER-001 (8mg/kg) during 6 months. Efficacy was assessed by measuring (apo)lipoproteins, plasma-mediated cellular cholesterol efflux, fecal sterol excretion (FSE) and carotid artery wall dimension by MRI and artery wall inflammation by FDG-PET/CT scan.

Results We included seven FHA patients (HDL-c 13.8 [1.8 – 29.1] mg/dl; apoA-I 28.7 [7.9 – 59.1] mg/dl). Following 9 infusions in 1 month, apoA-I and HDL-c increased directly after infusion by 27.0 mg/dl and 16.1 mg/dl (p=0.018). CER-001 induced a 44% relative increase (p=0.018) in in vitro cellular cholesterol efflux with a trend towards increased FSE (p=0.068). After nine infusions of CER-001, carotid mean vessel wall area decreased compared to baseline from 25.0 to 22.8 mm² (p=0.043) and target-to-background ratio from 2.04 to 1.81 (p=0.046).

Conclusion In FHA-subjects, CER-001 stimulates cholesterol mobilisation and reduces artery wall dimension and inflammation, supporting further evaluation of CER-001 in FHA patients.

Word count: 209
Abbreviations

ACS: acute coronary syndrome

(S)AE: (serious) adverse event

ALT: alanine transaminase

ApoA-I: apolipoprotein A-I

AST: aspartate transaminase

CPK: creatine phosphokinase

CETP: cholesteryl ester transfer protein

CRO: contract research organization

CV: cardiovascular

DPPG: dipalmitoylphosphatidylglycerol

ELISA: enzyme-linked immunosorbent assay

FDA: food and drug administration

FDG: \(^{18}\)F-fluorodeoxyglucose

FHA: familial hypoalphalipoproteinemia

FID: flame ionization detector

(F)NS: (fecal) neutral sterols

FSE: fecal sterol excretion

GCP: good clinical practice

HDL-c: HDL-cholesterol

IQR: interquartile range
IVUS: intravenous ultrasonography

LCAT: lecithin:cholesterol acyl transferase

LDH: lactate dehydrogenase

LDL-c: LDL-cholesterol

MVWA: mean vessel wall area

(3T) MRI: (3.0 Tesla) magnetic resonance imaging

PET/CT: positron emission tomography/computed tomography

ROI: region of interest

RCT: reverse cholesterol transport

SD: standard deviation

SPSS: Statistical Package for the Social Sciences

SUSAR: suspected unexpected serious adverse reactions

SUV_{max}: maximal standardized uptake value

TBR(max): (maximal) target-to-background ratio

ULN: upper limit of normal

VLDL-c: VLDL-cholesterol
Introduction

The large residual burden of cardiovascular (CV)-disease in patients receiving guideline-based medical treatment, underscores the need for additional therapeutic interventions (1). Strategies aimed at increasing HDL-cholesterol (HDL-c) have been pursued as a promising target in CV-prevention for more than 2 decades (2,3), albeit without a clear CV-benefit. Both the cholesteryl ester transfer protein inhibitors (4) and nicotinic acid derivatives (5), increasing HDL-c on top of standard-of-care by 25-40%, have failed to reduce CV-risk. The expectation of HDL-c as an anti-atherogenic target was further jeopardized by the Mendelian randomization studies, which revealed that common genetic variations affecting HDL-c levels were not associated with CV-risk (6). In retrospect, the strongest benefit for HDL-increasing strategies in experimental atherosclerosis were confined to interventions increasing apolipoprotein A-I (apoA-I) levels, the major protein constituent of the HDL particle. ApoA-I has been shown to play a key role in the initial step of reverse cholesterol transport (RCT) (7,8). Tissue cholesterol efflux (TCE) was decreased in patients with the orphan disease of genetically-determined low HDL-c (Familial Hypoalphalipoproteinemia (FHA)) (9), which coincided with accelerated atherogenesis (10,11). Early results from infusion experiments with reconstituted HDL corroborated a therapeutic potential, showing increased fecal cholesterol excretion (12,13) as well as a reduction in the cholesterol content in human atherosclerotic lesions (14). IVUS trials in patients with acute coronary syndrome (ACS)(15,16), however, failed to show a significant benefit of apoA-I infusion on coronary atheroma volume versus placebo, although apoA-I infusion was associated with a regression versus baseline. In line, the CHI-SQUARE study, testing the effect of short-term infusion of CER-001 in patients after an ACS, also showed regression compared to baseline without a significant change compared to placebo infusion (17). A common factor in all these studies was, however, that all participants had normal HDL-c levels.

In the present proof-of-concept study we hypothesized that infusions with CER-001, a discoidal HDL-mimetic particle containing recombinant human apoA-I in a complex with two natural phospholipids would promote cholesterol mobilization from the artery wall in patients with FHA, characterized by clear reductions in apoA-I levels. To this end, we selected patients with molecularly diagnosed FHA (apoAI, ABCA1, or LCAT deficiency) and low apoA-I levels, who received a total of 20 infusions during 6 months. The efficacy was assessed by measuring plasma (apo)lipoprotein changes, plasma-mediated cellular cholesterol efflux and fecal sterol excretion (FSE). Both at baseline and after 6 months, we also measured the effect of CER-001 on arterial wall dimensions and inflammation.
Methods

This Phase II study (International Clinical Trials Registry Platform, WHO, EUCTR2011-006188-23-NL) was conducted in accordance with the Declaration of Helsinki and in compliance with current Good Clinical Practices (eGCPs; ICH E6) and the requirements of the US Food and Drug Administration (FDA; 21 CFR 312). The study (SAMBA) was designed jointly by the academic investigators and the Sponsor (Cerenis™ Therapeutics, S.A., France), and was executed at the Academic Medical Center in Amsterdam, The Netherlands. The protocol was approved by the local institutional review board and all participants provided written informed consent.

Subjects

We recruited adult subjects (≥ 18 yrs.) with an HDL-c deficiency based on hetero- or homozygosity for a mutation affecting at least one of three genes known to affect HDL-c concentration: apoA-I, ATP-binding cassette transporter A1 (ABCA1) and lecithin:cholesterol acyl transferase (LCAT). If applicable, subjects were on stable lipid-lowering therapy at least 6 weeks before study initiation. Exclusion criteria included major renal (serum creatinine > 2.0 mg/dL) and hepatic (ALT/AST > twice upper limit of normal (ULN)) dysfunction.

Design and Intervention

The study scheme is depicted in Figure 1. At baseline, we subjected eligible FHA-patients to 3T MRI and ¹⁸F-fluorodeoxyglucose (FDG) PET/CT-scanning of the carotid arteries. Following the baseline examinations, the first of in total 20 CER-001 infusions was administered (8mg in apoA-I equivalents/kg body weight, infused over 60 minutes). CER-001 (Cerenis, France) is a negatively charged HDL-mimetic, consisting of recombinant human apolipoprotein A-I and a combination of two naturally occurring phospholipids. The apoA-I component is expressed in mammalian Chinese Hamster Ovary cells and purified by a three-step column chromatography process. The phospholipid component consists of egg sphingomyelin and 1,2-dihexadecanoyl-sn-glycero-3-phospho-(1-rac-glycerol)(dipalmitoylphosphatidyl-glycerol, DPPG) in a 97:3 weight ratio. The addition of DPPG, a negatively charged phospholipid, in the CER-001 particle has been shown to minimize fusion of CER-001 with endogenous lipoproteins, thereby retaining the functional properties of the CER-001 particles after infusion (Goffinet M. ATVB conference; April, 2011; Chicago, USA). The ratio of protein to total phospholipids
in the CER-001 complex is 1:2.7 weight/weight. The drug product is a solution of the CER-001 complexes in phosphate buffered sucrose/mannitol solution (10mM phosphate buffer, 4.0% sucrose, 2.0% mannitol, pH 8.0). The concentration of CER-001 complexes in the formulation is expressed as the concentration of the apoA-I component. Intravenous infusions of CER-001 were administered over 1 hour. One week after the first infusion, the second to ninth infusion of CER-001 were given every 3 days over a three-week period after which another 11 infusions were given every 2 weeks. For the first, ninth (day 29) and last infusion (month 6), we withdrew blood prior to and at 1, 4, 8, and 24 hours after the start of CER-001 infusion to evaluate lipid and lipoprotein parameters. Subjects remained fasting until the 4 hour time point. Additionally, plasma-mediated cellular cholesterol efflux was measured in vitro in the blood samples obtained after the first infusion. After the 9th and 20th infusion, the 3T-MRI of the carotid arteries was repeated. The FDG-PET/CT-scan was only repeated after the 9th infusion because of cumulative exposure to ionizing radiation (total exposure of 9.6 mSv per patient). Fecal sterol excretion was determined at baseline and after the first CER-001 infusion in a subset of 4 subjects consenting to this additional analysis.

**Plasma lipids, lipoprotein profiles and apoA-I**

In all study participants, lipoprotein profiles were determined by HPLC using a Sepharose 6 column and enzymatically detected in-line for total cholesterol and unesterified cholesterol as performed by a CRO (Amatsi, Fontenille, France). The area under each of the peaks corresponding to lipoproteins with the sizes of VLDL, LDL and HDL was integrated. Cholesterol ester levels were determined by subtracting the unesterified cholesterol from the total cholesterol in each fraction. The assays of total plasma and unesterified cholesterol were performed on the automated biochemical automator Pentra 400 (Horiba ABX Diagnostics, Montpellier, France) using cholesterol-oxidase enzymatic reaction. The assay of apoA-I was performed using a commercial ELISA kit (Assay Pro, CA, USA). Intra-assay coefficient variance was 4.8% with an inter-assay coefficient variance of 7.21%.

**Plasma-mediated cellular cholesterol efflux**

Plasma-mediated cellular cholesterol efflux was quantified in vitro using whole plasma samples collected prior to and at the different time-points following initial administration of CER-001. Briefly, cultured J774 macrophages were labelled with $[^3]$H]-cholesterol (2 µCi/ml) for 24h. Following overnight equilibration, $[^3]$H]-cholesterol release was measured after 4h incubation with plasma. All assays were performed in triplicate.
Plasma-mediated cellular cholesterol efflux was expressed as the percentage of the radioactivity released from cells into the medium relative to the total radioactivity in cells and medium (18).

**Fecal sterol excretion**

Before and after CER-001 infusion, FSE was determined for a total of eight days in a subpopulation of 4 subjects. At the beginning of the experiment, a blood and fecal sample was collected. Subsequently, participating FHA-patients were asked to adhere to a cholesterol-restricted diet (<250mg cholesterol/day), to keep a dietary record and to ingest 3mg D₄-sitostanol thrice daily until the end of the experiment. Sitostanol was used to correct for variations in fecal flow, as it is not absorbed by the intestines (19). For the assessment of FSE, consisting of fecal neutral sterols ((F)NS) and bile acids, daily frozen fecal samples were thawed and homogenized with distilled water (1:1; w/w) from which 20 ml was dispensed into a 30 ml plastic tube. FNS and bile acids were extracted as previously described (20,21). Sterols were analyzed by capillary gas chromatography (Agilent 6890, Amstelveen, The Netherlands), equipped with a flame ionization detector (FID) and a CP Sil 19 capillary column (25m x 0.25mm x 0.2µm; Chrompack BV, Middelburg, The Netherlands). Fecal D₄-sitostanol was analysed in its acetate derivatives by GC/MS (Agilent 7890A, Amstelveen, The Netherlands), using a ZB-5ms capillary column (30m x 0.25mm x 0.25µm; Phenomex, Utrecht, The Netherlands). The daily excretion of NS and bile acids was calculated as relative amounts compared to the daily excretion of D₄-sitostanol. Ultimately, daily sterol excretion was added together to determine total sterol excretion during the experiment.

**3T MRI**

MRI-scans of both carotid arteries were obtained on a 3T MRI scanner (Philips Medical Systems, Best, The Netherlands) using a dedicated, bilateral carotid coil (Shanghai Chenguang Medical Technologies, Shanghai, China). MRI-scans were acquired according to a standardized protocol (22). In short, axial T1-weighted turbo-spin echo images were acquired in end-diastole from both common carotid arteries, using double inversion recovery preparation as black blood technique. In total, twelve 2mm slices were acquired from each common carotid artery, with the most cranial slice being 8 mm below the carotid flow divider. Vessel wall dimensions were analyzed using dedicated measurement software (Vessel-Mass, Leiden University Medical Center, Leiden, The Netherlands). Before blinding, baseline and repeat scans were co-registered based on vessel wall morphology and distance from the carotid flow divider. One blinded reader performed all analyses. Lumen and
outer wall contours were drawn manually in Vessel-Mass, after which mean vessel wall area (MVWA) was calculated.

FDG-PET/CT

Patients were subjected to FDG-PET/CT imaging at baseline and after the 9th CER-001 treatment. PET/CT imaging of the carotid arteries was performed as previously described (23,24). In brief, PET/CT scans (Philips Gemini, Philips, Best, the Netherlands) of the neck region were obtained ninety minutes after FDG infusion (~200 MBq, 5.5 mCi). FDG uptake in both the left and right carotid arterial wall was assessed by one blinded reader as follows; at least 5 regions of interest (ROIs) delineating the artery were drawn. Within each ROI the maximal arterial standardized uptake value (SUV\text{max}) was obtained as the maximal pixel activity within the ROI. For both the left and the right carotid artery, the mean SUV\text{max} was derived by averaging the SUV\text{max} of all ROIs. The maximal arterial target-to-background ratio (TBR\text{max}) was calculated by correcting the mean SUV\text{max} for the mean background blood activity in the venous blood pool, which was derived from the average of at least 5 ROIs within the jugular veins. The index vessel which was used as read-out parameter reflects the carotid artery with the highest FDG uptake at baseline (25).

Safety

For safety monitoring, blood withdrawals were performed directly prior to and 24 hours after start of the 1st, 2nd, 4th, 7th, 9th, 13th, 16th and 20th infusion (e.g. complete blood count, alkaline phosphatase, ALT, AST, bilirubin, LDH, CPK). Anti-apoA-I antibody production was tested in samples taken prior to the 1st, 4th, 9th, 13th, 16th and 20th infusion. An electrochemiluminescent detection method was used for detection of anti-apoA-I antibodies with a sensitivity of 0.15 ng/ml. In case a subject tested positive for antibodies, the neutralizing potential of antibodies was determined by a cell-based assay, utilizing the ABCA1-mediated cholesterol efflux to CER-001 in J774 macrophages, as described previously. Inhibitory potential of antibodies was quantified as a decrease in cholesterol efflux capacity. Additionally, the occurrence of adverse events and medication changes was checked at every infusion and to test for hemodynamic changes, blood pressure was determined prior to and 2 hours after the infusions.

Statistical analyses

The academic authors had full access to the data and performed all statistical analyses (R.S.K., L.P.S.). The manuscript was written by the academic authors, after which the employees from Cerenis (J.L.D., J.P., C.K.,
R.B.) were allowed to review and provide feedback. The final responsibility of the manuscript lies with the corresponding author (E.S.G.S). All authors vouch for the validity and completeness of the data. Depending on the distribution, data are presented either as medians with interquartile ranges (IQR) or means with standard deviations (SD). For comparison of outcome data prior to versus after treatment, the Wilcoxon signed-rank test was used. In case of repeated measurements, we used the Friedman test to assess whether there was a significant difference in overall lipid parameters over time. If significance was found, post-hoc analyses were performed using the Wilcoxon signed-rank test to do paired testing between all individual post-infusion lipid values and the pre-infusion lipid values. Statistical analyses were performed using SPSS statistics version 20.0 (IBM, Chicago, IL, USA). A p-value of <0.05 was considered statistically significant.

Endpoints

The primary endpoint of the study comprised changes in apoA-I plasma concentration and plasma lipids. Secondary endpoints included changes in carotid MRI (MVWA) and FDG-PET/CT (TBRmax) and changes in FSE upon treatment. Adverse events (AEs) and laboratory parameters (including antibody-development) were the key safety endpoints.

Results

Baseline characteristics

Baseline characteristics are listed in Table 1. Seven FHA-patients (5 male, 2 female) were included in whom the following mutations were identified: a homozygous mutation in 2 patients (for apoA-I and ABCA1 genes respectively), double heterozygosity for apoA-I and ABCA1 genes in 1 patient, and 4 patients with a heterozygous mutation (for ABCA1 in 2 patients; for apoA-I and LCAT in 1 patient) (Supplementary Table 1). Four subjects had coronary artery disease: two subjects had suffered from multiple myocardial infarctions, one subject underwent prophylactic coronary artery bypass grafting and one subject had received percutaneous coronary interventions for angina pectoris. Five patients were using lipid-lowering medication. The baseline lipid profile showed a median HDL-cholesterol of 13.8 mg/dl and a median apoA-I concentration of 28.7 mg/dl.
Lipoprotein and apoA-I changes

Figure 2 depicts the lipid profile changes after one month of CER-001 infusion. CER-001 increased apoA-I and HDL-c for at least 8 hours after the start of the infusion with a peak in HDL-c directly after the completion of the infusion at \( t= 1 \) hour (median +16.1 mg/dl versus pre-treatment; \( p=0.018 \)) and for apoA-I levels 4 hours after the start of the infusion (median +27.0 mg/dl; \( p=0.018 \)). Unesterified cholesterol in the HDL fraction increased markedly (+11.5 mg/dl; \( p=0.018 \)), in combination with a modest increase of esterified cholesterol in the HDL fraction (median +3.6 mg/dl; \( p=0.018 \)). Analysis of VLDL-c and LDL-c revealed small post-dose increases predominantly in the unesterified cholesterol content, whereas esterification in these lipoproteins did not increase upon treatment. Six months of therapy resulted in similar post-dose changes in lipoprotein- and apoA-I levels (Supplementary Figure 1).

Plasma-mediated cellular cholesterol efflux and fecal sterol excretion

In vitro plasma-mediated cellular cholesterol efflux increased significantly upon CER-001 infusion. Plasma drawn 4 hours after the start of the first CER-001 infusion showed an absolute increase of 1.9 ± 0.6% compared to baseline (Figure 3), corresponding to a relative increase of 44 ± 26% (\( p=0.018 \); Figure 3). FSE was measured in a subset of 4 participants, who were all males with mutations either in the gene encoding for apoA-I and/or ABCA1 (Supplementary Table 1). Characteristics such as weight, lipid profile and cholesterol intake did not change between the baseline and post-CER-001-infusion measurements. After CER-001 treatment there was a trend towards increased FSE (from 8.94 [6.43 – 11.76] to 10.11 [6.81 – 12.91] g; \( p=0.068 \)), corresponding to an extra 0.68 [0.38 – 1.65] g of sterols being excreted during 8 days after treatment. This trend was caused by enhanced neutral sterol excretion (\( p=0.068 \)), whereas bile acid excretion was unaffected (\( p=0.715 \)). FSE increased in all four patients studied following CER-001 infusions, and we observed no apparent relation between genotype and effect of CER-001 infusion on FSE (Supplementary Figure 2).

Carotid artery wall imaging

At baseline, median carotid MVWA was 25.0 mm\(^2\) [21.4 – 27.0] (Figure 4A/B). None of the patients had overt atherosclerotic plaques at baseline, but MVWA was increased compared to that observed in healthy controls (22). Following nine infusions of CER-001 within a one-month time frame, carotid MVWA decreased by a mean 4.6 ± 4.4% to 22.8 mm\(^2\) [20.8 – 25.7] (\( p=0.043 \)). After 6 months of infusions, the MVWA was 21.8 [20.7 – 25.3]
(p=0.018 vs. baseline; p=0.128 versus month 1), corresponding to a total mean 6.7 ± 4.5% decrease versus baseline. With respect to the inflammatory activity, we found a baseline median TBRmax in the index vessel of 2.04 [1.51 – 2.21] (Figure 4C/D). One month of CER-001 infusions reduced the TBRmax to 1.81 [1.49 – 1.92] (p=0.046), corresponding to a mean percent reduction from baseline of 8.9 ± 12.7%.

Safety

No SAEs or SUSARs were reported during this study. CER-001 infusions did not invoke blood pressure changes. Safety lab results showed no Hy’s law (26) cases and one 3x ULN elevation in AST in one patient while on treatment, who already had elevated AST at baseline. In 4 of the 7 subjects, anti-apoA-I antibodies were absent throughout the study period. In one subject, with a homozygous apoA-I mutation, anti-apoA-I antibodies were present prior to CER-001 infusion. Another subject, with a heterozygous LCAT-mutation, tested positive for antibodies in study week 18, whereas antibodies were absent at all measurements prior to and after this time point. The third testing positive for antibodies had a heterozygous apoA-I mutation, tested negative for antibodies at baseline, was positive for antibodies in study week 20 and 26. In none of these cases, the antibodies affected cholesterol efflux capacity to CER-001, as a marker of the neutralizing properties of anti-apoA-I antibodies.

Discussion

In FHA-patients, infusion of the HDL-mimetic CER-001 resulted in a significant increase in plasma apoA-I and HDL-c, with changes in both unesterified and esterified cholesterol content. These changes were accompanied by a significant increase in plasma-mediated cellular cholesterol efflux capacity in vitro with a concomitant trend towards increased total sterol excretion in the feces. After nine infusions of CER-001 within one-month, MVWA of the carotid artery decreased significantly compared to baseline, which persisted after an additional 11 infusions during the subsequent 5 months. The TBRmax of the carotid artery, reflecting arterial wall inflammation, was also reduced after 9 infusions of CER-001. Collectively, the results of this proof-of-concept study imply that CER-001 stimulates RCT in orphan-disease FHA-patients, leading to a reduction in both arterial wall thickness as well as arterial wall inflammation.
Plasma lipid changes following CER-001 infusion

Following infusion of CER-001, plasma apoA-I levels increased significantly with a return to baseline levels within 24-hours, most likely reflecting the low dose administered. The increase in plasma HDL-c levels mirrored the increase in plasma apoA-I concentration, which is consistent with the stability of the CER-001 complex and different from previously reported apoA-I agents (12,13). The increase in the unesterified cholesterol content and subsequent increase in esterified cholesterol content within the HDL-fraction following CER-001 infusion indicates that the apoA-I within the CER-001 particle is recognized by endogenous LCAT, responsible for cholesterol esterification in plasma (27). However, the increased HDL-cholesterol following CER-001 infusion consisted predominantly of unesterified cholesterol, whereas normally the HDL-fraction of cholesterol contains more esterified than unesterified cholesterol (28). These findings imply suboptimal esterification of the cholesterol mobilized by CER-001. Future studies are needed to determine whether the affinity of the recombinant apoA-I or the phospholipid composition of CER-001 contribute to a suboptimal cholesterol esterification.

Although the initial cholesterol mobilization induced by CER-001 is partitioned specifically to the HDL fraction, at later time points CER-001 infusion also induced a modest increase in unesterified cholesterol content in the VLDL and LDL fraction, whereas esterified cholesterol levels were not elevated. Similar effects have been reported upon apoA-I infusion in experimental models (29–31). The VLDL-c increase in response to apoA-I infusion has been attributed to both increased CETP-mediated cholesterol exchange between HDL-c and VLDL-c leading to higher VLDL-c as well as to enhanced hepatic VLDL-production reflecting an increased cholesterol flux towards the liver (29,31). However, the observations that the increase in VLDL-c was confined to unesterified cholesterol in our participants, together with previous observations that CER-001 also increased VLDL-c in mice which are naturally CETP-deficient ((30)(32), argue against a CETP-driven mechanism, and hint towards an increased VLDL secretion The mechanism by which CER-001 increases hepatic VLDL-c secretion remains to be elucidated.

Plasma-mediated cellular cholesterol efflux in vitro and fecal sterol excretion

We observed a mean baseline plasma-mediated cellular cholesterol efflux in vitro of 5.2% in FHA-patients, which is lower compared to the previously reported 8 to 11% in healthy volunteers (33,34). As expected, plasma-mediated cellular cholesterol efflux after CER-001 infusion showed a relative increase of 44%,
indicating an excess capacity of the plasma to promote cholesterol efflux induced by CER-001 (30,35). Recently, Rader and colleagues showed that the in vitro cholesterol efflux was inversely related to CVD events in two different, large cohorts (34,36), lending support to a potentially beneficial effect of CER-001 in CVD. The ultimate step of RCT is elimination of cholesterol as bile acids and neutral sterols into the feces. We observed a trend towards an increased FSE after infusion of CER-001, which resulted in a mean additional 900 mg sterol excretion. An increase in sterol excretion following apoA-I infusion is supported by previous studies, in which infusion of higher dosages of apoA-I increased fecal neutral sterol excretion by 39% to 54% (12,13). The additional 901 mg of sterol excretion observed over 8 days after infusion of a mean dose of 750 mg CER-001 is comparable (per gram of apoA-I infused) to that achieved after administration of a dose of 4 grams apoA-I complexed with phosphatidylcholine, which induced an extra 5g of sterol excretion over 9 days (12).

Collectively, these data support the notion that CER-001 has a stimulatory effect on RCT. The preferred sources for the mobilisation of cholesterol can however not be determined in the present study. It is therefore difficult to separate the amount of cholesterol removed from the atherosclerotic vessel wall from that removed from other cholesterol compartments (37).

**Carotid artery wall imaging**

Following 9 infusions of CER-001, a mean reduction of 4.6% in MVWA of the carotid arteries was observed compared to baseline. Combined with the plasma lipid changes and observed cholesterol fluxes, the reduction in MVWA presumably reflects the mobilization of cholesterol from the arterial wall. Previous data on experimental animal studies using apoA-I infusions support this mechanism (30,38). It has proven more challenging, however, to demonstrate an effect of infusing apoA-I containing particles on atherosclerosis in humans. A single reconstituted HDL-infusion significantly reduced the lipid content in atherosclerotic plaques (14). In line, multiple infusions of apoA-I-Milano complexes, reconstituted apoA-I complexes, CER-001 or re-infusion of autologous HDL after extra-corporal HDL-c delipidation, also resulted in regression of coronary atheroma volume over baseline in post-ACS patients (15–17,39). None of these studies, however, reached a significant change compared to placebo.

Several distinct differences between these studies and our study deserve closer attention. First, we treated patients with severely lowered HDL-c levels (mean 15.5 mg/dl), whereas previous IVUS trials included post-ACS patients with mean HDL-c baseline levels of approximately 40 mg/dl (15–17). The potential impact of the absolute HDL-c level on CV-risk is supported by epidemiological studies showing the steepest association
between CV-risk and HDL-c levels in patients with markedly lowered HDL-c levels (2,3). More recently, we substantiated the impact of low HDL-c on tissue cholesterol efflux particularly in patients with very low HDL-c levels (9), which is compatible with the concept that the best therapeutic result of apoA-I infusion can be obtained in those patients with the lowest apoA-I levels. As the limited number of subjects included in the present study does not allow for subgroup analysis, further studies are needed to determine to what extent the response to apoA-I treatment depends upon the baseline apoA-I levels and/or baseline genotype. Second, we used a high dosing frequency of nine infusions within a one-month period, compared to only 4-6 weekly infusions in previous ApoA-I infusion trials in ACS patients (15–17). The return to baseline of plasma apoA-I within 24 hours after the infusion of 8 mg/kg CER-001 could imply that a higher dosing frequency might have a stronger impact. The lack of further improvement in carotid MVWA following 5 months of treatment in a lower dosing frequency (bi-weekly infusions) lends further support to this concept.

In addition, repetitive infusion of CER-001 resulted in a significant 8.9% mean decrease in arterial wall FDG uptake (TBRmax) in the index carotid artery. In the context of atherosclerosis, FDG uptake is used as an estimate of arterial wall inflammation (23). Potent anti-inflammatory effects of apoA-I/HDL have been reported previously. In experimental models, pre-β-HDL administration markedly attenuated the inflammatory responses in a collar-induced atherosclerosis model in rabbits (38) and LDL-receptor knock-out mice (30). In line, Shaw et al. observed a marked decrease in the number of macrophages and inflammatory markers in plaques from patients with peripheral artery disease treated with reconstituted HDL (14). The mechanisms contributing to this anti-inflammatory effect have recently been extensively reviewed (40,41). The anti-inflammatory effect of CER-001 infusions may also relate to the removal of cholesterol from the arterial wall, as it was shown that acute LDL-c lowering had the capacity to reduce inflammatory activity even in advanced atherosclerotic lesions within a 2-week timeframe (42). In support, we corroborated a direct anti-inflammatory effect of LDL-c lowering in patients with familial hypercholesterolemia, in whom LDL-apheresis was associated with a 14% reduction of arterial TBR (43). A third explanation for the anti-inflammatory effect of CER-001 treatment could relate to the scavenger effect of the HDL particle for pro-inflammatory intermediates in which the HDL particle extracts pro-inflammatory oxidized sterols from the arterial wall (44).
**Safety**

CER-001 infusions were generally well tolerated and study-drug-related AEs were mild. No hemodynamic changes were observed. Laboratory safety parameters did not reveal any clinically relevant signal. Treatment-emergent antibodies to apoA-I were observed in two subjects. The antibodies in 1 of these 2 subjects spontaneously reverted to negative prior to the last dose of CER-001. The other subject, with a heterozygous mutation for apoA-I, tested positive for antibodies intermittently. Additional testing however showed none of the antibodies to have neutralizing properties.

**Study limitations**

The major limitation of the present study relates to the limited sample size, combined with the heterogeneity of genetic mutations of included subjects. The limited number of participants is, however, a direct consequence of the rare prevalence of genetic mutations resulting in severe apoA-I/HDL-deficiency (45–47), precluding the inclusion of larger numbers of patients with the orphan disease FHA. Second, the present study did not include a separate placebo infusion. In this respect, it should be noted that in previous trials evaluating the effect of apoA-I mimetics on atherosclerotic burden (15–17), a beneficial effect of the compound was observed compared to baseline, but not to placebo. In the present study, however, placebo infusion could not be implemented in view of the rarity of the disease combined with the intensity of the study protocol. Third, the data on the fecal sterol excretion should be interpreted with caution. Only four patients consented to participate in this intensive sub-study, whereas one patient also continued the use of ezetimibe, which is known to affect FSE (48). In addition, the patients were studied in an outpatient setting, resulting in imprecise control of dietary cholesterol intake, which may also impact FSE.

Notwithstanding these limitations, the results from our proof-of-concept study do support further clinical evaluation of the HDL-mimetic CER-001 in patients with FHA using a randomized, placebo-controlled design. Awaiting such trial(s), the present data have provided a basis for the European Medicines Agency to grant two orphan designations for the use of CER-001 in the treatment of patients with apoA-I deficiency or ABCA1 deficiency (August 2014).

**Conclusion**
The present proof-of-concept study shows that CER-001, a recombinant human apoA-I-containing HDL-mimetic particle, stimulates RCT in FHA patients. Given the observed reduction in carotid MVWA and arterial wall inflammation, our data imply that the mobilized cholesterol may originate at least partly from the atherosclerotic vessel wall. Collectively, these findings support further clinical evaluation of the effect of CER-001 in larger trials in patients with FHA.

Acknowledgements

The authors would like to thank A.W.M. Schimmel for her work on plasma lipid analysis and R. Boverhof for his work on the fecal sterol excretion experiments. The authors thank R. Snoeks, A.M. van der Berg-Faaij and W.M. de Jong for their assistance with performing the MRI- and FDG-PET/CT-scans. Part of the research was supported by a grant from the Netherlands Heart Foundation (2011-B019: generating the best evidenced based pharmaceutical targets for atherosclerosis (GENIUS)).
References


Figure legends

**Figure 1. Study scheme**

Fecal sterol excretion studies were performed if the subject consented to these additional analyses.

Abbreviations: 3T MRI = 3.0 Tesla magnetic resonance imaging; FDG-PET/CT = 18F-fluorodeoxyglucose positron emission tomography/computed tomography

**Figure 2. Lipoprotein profile changes and apoA-I kinetics after one month of treatment**

Plasma was obtained at baseline and 1, 4, 8 and 24 hours after start of the ninth infusion. Row A depicts changes in plasma cholesterol levels, row B in HDL-cholesterol, row C in LDL-cholesterol, row D in VLDL-cholesterol and row E in apoA-I following CER-001 infusion. Data represent baseline-corrected medians with interquartile ranges. Values at every time point were compared to baseline. A p-value < 0.05 was considered statistically significant and is depicted with an asterisk.

Abbreviation: apoAI = apolipoprotein AI; IQR = interquartile range

**Figure 3. Plasma-mediated cellular cholesterol efflux**

Plasma-mediated cellular cholesterol efflux was analysed in vitro using J774 macrophages. Cholesterol efflux capacity from plasma derived 1, 4, 8 and 24 hours after infusion was compared to baseline efflux capacity. Data represent baseline-corrected medians with interquartile ranges. A p-value < 0.05 was considered statistically significant and is depicted with an asterisk.
Figure 4. Imaging results

Mean vessel wall area (MVWA) and target to background ration (TBRmax) of the carotid arteries, as assessed by MRI- and FDG-PET/CT-scan respectively, were compared between baseline and after one month of 9 CER-001 infusions. MVWA was also measured after 6 months with 11 additional CER-001 infusions. For TBRmax, the index vessel was chosen.

Representative pre- and post-treatment 3T MRI- and FDG-PET/CT-scans are depicted in A and C. In case of the MRI, the original images and the ROI is shown. Figures 5B and 5D show the results of both scans. Data represent medians with interquartile ranges. A p-value < 0.05 was considered statistically significant.

3T MRI = 3.0 Tesla magnetic resonance imaging; FDG = 18F-fluordexoyglucose; PET/CT = positron emission tomography/computed; ROI = region of interest.
Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Subject (n=7)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex, n (%)</td>
<td>2 (29)</td>
</tr>
<tr>
<td>Age, years</td>
<td>51.1 [47.0 – 55.4]</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.4 [26.8 – 32.3]</td>
</tr>
<tr>
<td>CAD, n (%)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>Lipid-lowering medication, n (%)</td>
<td>5 (71)</td>
</tr>
<tr>
<td>Statin, n (%)</td>
<td>5 (71)</td>
</tr>
<tr>
<td>Ezetimibe, n (%)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Niacin, n (%)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>135 [107 – 143]</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>82 [70 – 96]</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.9 [4.7 – 6.1]</td>
</tr>
<tr>
<td>TChol, mg/dl</td>
<td>123.6 [93.8 – 163.3]</td>
</tr>
<tr>
<td>HDL-c, mg/dl</td>
<td>13.8 [1.8 – 29.1]</td>
</tr>
<tr>
<td>LDL-c, mg/dl</td>
<td>77.1 [53.5 – 101.0]</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>118.7 [87.7 – 298.5]</td>
</tr>
<tr>
<td>ApoA-I, mg/dl</td>
<td>28.7 [7.9 – 59.1]</td>
</tr>
</tbody>
</table>

Data represent medians with interquartile ranges.

Abbreviations: ApoA-I = apolipoprotein A-I; BMI = body mass index; CAD = coronary artery disease; DBP = diastolic blood pressure; HDL-c = HDL-cholesterol; LDL-c = LDL-cholesterol; SBP = systolic blood pressure; TChol = total cholesterol
Figure 1. Study flow-chart.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Outcome measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 infusion</td>
<td>Cholesterol efflux</td>
</tr>
<tr>
<td>CER-001 8 mg/kg (day 0)</td>
<td>3T MRI scan (day 30)</td>
</tr>
<tr>
<td>8 infusions</td>
<td>Fecal sterol excretion (2)</td>
</tr>
<tr>
<td>CER-001 8 mg/kg (q3 days)</td>
<td>FDG-PET/CT scan (day 30)</td>
</tr>
<tr>
<td>11 infusions</td>
<td>Fecal sterol excretion (1)</td>
</tr>
<tr>
<td>CER-001 8 mg/kg (q2 weeks)</td>
<td>3T MRI scan (week 27)</td>
</tr>
</tbody>
</table>

- Pre-treatment: Day 0 - 7
- Initial dosing period: Day 8 - 29
- Induction period: Week 6 - 26
Figure 2. Lipoprotein profile changes and apoA-I kinetics after one month of treatment

A. Plasma cholesterol

B. Plasma HDL-cholesterol

C. Plasma LDL-cholesterol

D. Plasma VLDL-cholesterol

E. Plasma Apolipoprotein A-I
Figure 3. Plasma-mediated cellular cholesterol efflux
Figure 4. Imaging results