Clinical and Population Studies

High-Density Lipoprotein Subfractions and Cholesterol Efflux Capacities After Infusion of MDCO-216 (Apolipoprotein A-IMilano/Palmitoyl-Oleoyl-Phosphatidylcholine) in Healthy Volunteers and Stable Coronary Artery Disease Patients

Herman J. Kempen, Bela F. Asztalos, Matthijs Moerland, Elias Jeyarajah, James Otvos, David G. Kallend, S. Eralp Bellibas, Peter L.J. Wijngaard

Objective—To determine effects of single ascending doses of MDCO-216 on high-density lipoprotein (HDL) subfractions in relation to changes in cholesterol efflux capacity in healthy volunteers and in patients with stable angina pectoris.

Approach and Results—Doses of 5- (in volunteers only), 10-, 20-, 30-, and 40-mg/kg MDCO-216 were infused during 2 hours, and plasma and serum were collected during 30 days. Plasma levels of HDL subfractions were assessed by 2-dimensional gel electrophoresis, immunoblotting, and image analysis. Lipoprotein particle concentrations and sizes were also assessed by proton magnetic resonance (1H-NMR). There was a rapid dose-dependent increase of total apolipoprotein A-I (apoA-I) in pre-β1, α-1, and α-2 HDL levels and decrease in α-3 and α-4 HDL. Using a selective antibody apoA-IMilano was detected in the large α-1 and α-2 HDL on all doses and at each time point. ApoA-IMilano was also detected at the α-4 position but only at high doses. 1H-NMR analysis similarly showed a rapid and dose-dependent shift from small- to large-sized HDL particles. The increase of basal and ATP-binding cassette transporter A1–mediated efflux capacities reported previously correlated strongly and independently with the increase in pre-β1-HDL and α-1 HDL, but not with that in α-2 HDL.

Conclusions—On infusion, MDCO-216 rapidly eliminates small HDL and leads to formation of α-1 and α-2 HDL containing both wild-type apoA-I and apoA-IMilano. In this process, endogenous apoA-I is liberated appearing as pre-β1-HDL. In addition to pre-β1-HDL, the newly formed α-1 HDL particle containing apoA-I Milano may have a direct effect on cholesterol efflux capacity. (Arterioscler Thromb Vasc Biol. 2016;36:736-742. DOI: 10.1161/ATVBAHA.115.307052.)

Key Words: apolipoprotein A-I ■ cholesterol ■ healthy volunteers ■ immunoblotting ■ stable angina

Epidemiological data show an inverse relationship between plasma level of high-density lipoprotein (HDL)-cholesterol or apolipoprotein A-I (apoA-I) and coronary heart disease.1-3 The protective effects of HDL are assumed to be related in large part to its ability to promote the efflux of cholesterol from cholesterol-laden foam cells in the atherosclerotic plaque and so mediate the transport of this cholesterol to the liver for catabolism or excretion.4-6

In animal studies, intravenous infusion of homologous HDL7 or raising hepatic apoA-I production by inserting the gene for apoA-I8 or using adenoviral apoA-I gene transfection9 led to impressive reduction of atherosclerotic plaques. These observations encouraged efforts to find drugs to increase HDL- or apoA-I–related parameters to reduce atherosclerotic plaque burden and coronary heart disease in humans.

One of these approaches consists of intravenous infusion of apoA-I Milano–phospholipid complex, based on the observation that carriers of this apoA-I variant have a reduced incidence of atherosclerosis and cardiovascular events.10 More than 10 years ago, repeated infusion of a complex of recombinant ApoA-I Milano with palmitoyl-oleoyl-phosphatidylcholine (at the time named ETC-216) was shown to cause rapid regression of atherosclerotic plaque burden as measured by intravascular ultrasound.11 More recently this complex (now called MDCO-216 after improvements related to purity and yield in the manufacturing process) was shown to strongly enhance ATP-binding cassette transporter A1 (ABCA1)–mediated cholesterol efflux capacity after drug infusion in cynomolagus monkeys12 and in humans.13

Received on: December 16, 2015; final version accepted on: February 12, 2016.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.115.307052
We recently demonstrated that short incubation of MDCO-216 with whole human serum or plasma at 37°C led to a rapid increase in pre-\(\beta\)-1, \(\alpha\)-1, and \(\alpha\)-2 HDL and a decrease in \(\alpha\)-3 and \(\alpha\)-4 HDL. In parallel, ABCA1-mediated cholesterol efflux capacity was synergistically increased, that is, the increase was greater than the sum of the increases caused by MDCO-216 alone or by serum alone.\(^{14}\)

This article reports the effects on HDL subfractions after MDCO-216 infusion in healthy volunteers and in patients with coronary artery disease (CAD), measured by immunoblotting after 2-dimensional (2D)-PAGE as well as by proton nuclear magnetic resonance (\(^1\)H-NMR). A marked and prompt increase of pre-\(\beta\)-1-HDL was found by 2D-PAGE, whereas both methods showed an increase in larger (\(\alpha\)-1 and \(\alpha\)-2) HDL particle concentration and decrease in smaller (\(\alpha\)-3 and \(\alpha\)-4) HDL particles, similar to what was seen after in vitro incubation.\(^{14}\) The increase in pre-\(\beta\)-1 and \(\alpha\)-1 HDL both correlated independently with the increase in basal and ABCA1-mediated efflux capacities.

### Materials and Methods

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

### Results

#### ApoA-I and apoA-IMilano in HDL Subfractions, as Assessed by 2D-Polyacrylamide Gel Electrophoresis (2D-PAGE)

The effect of MDCO-216 infusion on total apoA-I levels in whole plasma were reported in our previous article,\(^{13}\) but are presented in Figure III in the online-only Data Supplement for reference purposes. As shown in Figure 1, infusion of MDCO-216 into healthy volunteers led to a rapid increase in pre-\(\beta\)-1-HDL and \(\alpha\)-1 HDL, with a decrease in \(\alpha\)-3 and \(\alpha\)-4 HDL. The increases in pre-\(\beta\)-1 and \(\alpha\)-1 HDL were greater than the sum of the increases caused by MDCO-216 alone or by serum alone.\(^{14}\)

![Figure 1. Plasma concentrations of total apolipoprotein A-I in high-density lipoprotein subfractions after infusion of increasing doses of MDCO-216 or placebo in healthy volunteers. Error bars represent SD, except for the 10-mg/kg dose where it represents range. Infusion duration was 2 hours.](http://figshare.com/)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Plasma Concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo (n=8)</td>
<td>10 mg/kg (n=2)</td>
</tr>
<tr>
<td>20 mg/kg (n=4)</td>
<td>30 mg/kg (n=4)</td>
</tr>
<tr>
<td>40 mg/kg (n=4)</td>
<td>10 mg/kg (n=2)</td>
</tr>
</tbody>
</table>

Figure 1. Plasma concentrations of total apolipoprotein A-I in high-density lipoprotein subfractions after infusion of increasing doses of MDCO-216 or placebo in healthy volunteers. Error bars represent SD, except for the 10-mg/kg dose where it represents range. Infusion duration was 2 hours.
MDCO-216 in healthy volunteers caused rapid dose-dependent increases of apoA-I in pre-β1-HDL and in α-2 and α-1 and decreases of apoA-I in α-3. ApoA-I in α-4 HDL also decreased after infusion of 5 to 30 mg/kg but initially increased after infusion of 40-mg/kg dose, whereafter it also decreased below baseline. ApoA-I in pre-β1-HDL returned to baseline at 24 hours, whereas the other changes returned to baseline at 168 hours. Placebo-treated subjects showed little or no change in these HDL subfractions.

Infusion of MDCO-216 in patients with CAD yielded similar changes in HDL subfractions as shown for healthy volunteers, except that for α-4 HDL the initial increase was observed at the doses of 30 and 40 mg/kg.

Of note, the values obtained by 2D-PAGE correlated significantly with the values obtained by ELISA, at baseline and at 4 hours after infusion of MDCO-216 in both the cohorts (Table I in the online-only Data Supplement). Although at baseline, the pre-β1-HDL concentrations measured by 2D-PAGE are clearly higher than those for pre-β1-HDL measured by ELISA reported earlier, after infusion of MDCO-216 the values obtained by the 2 methods are becoming quantitatively more similar (Table I in the online-only Data Supplement).

The total plasma concentrations of apoA-IMilano measured using a Mab 17F3 (which only recognizes apoA-I Milano) are presented in Figure IV in the online-only Data Supplement. As shown in Figure 2, on 2D-electrophoresis, the majority of the A-IMilano was detected in the α-1 and α-2 spots at all time points after infusion of 10-mg/kg MDCO-216. After infusion of the 40-mg/kg dose, some A-IMilano was also detected at α-4 position but only at 2 and 4 hours after start of infusion, probably representing low levels of the original MDCO-216 drug product, which has about the same size and mobility as native α-4 particles. Notably, with the 40-mg/kg dose the increase in apoA-IMilano content was much stronger in the α-2 fraction than in α-1, whereas the opposite was observed for the 10-mg/kg dose. Notably, apoA-IMilano was not detected in any of the pre-β fractions.

HDL Subfractions Assessed by 1H-NMR
Lipoprotein particle concentrations and sizes were measured by 1H-NMR in all subjects at baseline and all time points after start of infusion. This enabled us to cross-check the findings obtained with 2D-PAGE by measuring the particle concentrations of small, medium, and large HDL (see Materials and Methods section of this article for size ranges of these subfractions and Table II in the online-only Data Supplement for correlation between the baseline values for these subfractions with those measured by 2D-PAGE). As shown in Figure 3, infusion of MDCO-216 caused an immediate and dose-dependent decrease in the plasma level of small HDL particles and a similar-sized increase in the concentration of medium-sized HDL particles. A small increase in the level of large HDL was observed only at the highest dose.

Dose–Response Curves Based on AUEC0-24
We calculated the response to MDCO-216 as area under the effect curve between 0 and 24 hours (AUEC0–24) for all efflux and HDL subfraction parameters for each individual subject. Time and dose responses on serum efflux capacities and for pre-β1-HDL as measured by ELISA were reported before.

As evident from Figure 4, the responses for basal and ABCA1-mediated efflux capacities increased over the whole dose range. Also, the responses for basal and ABCA1 effluxes did not significantly differ between volunteers and patients at any dose.

Figure 5 shows that responses for pre-β1-HDL (ELISA) and α-1 HDL in healthy volunteers increased across the 0- to 40-mg/kg dose range, whereas the responses for the other HDL subfractions reach a plateau. The response for pre-β1-HDL (ELISA) was significantly lower in volunteers than in patients with CAD at all doses and the response for α-1 HDL was higher in volunteers than in patients with CAD at the 40-mg/kg dose.

Finally, the response of small-sized HDL and of medium-sized HDL particle concentrations reached a plateau at 30 mg/kg, whereas the response of large HDL particle concentration was much smaller and inconsistent (Figure 6).

Correlations of Responses of HDL Subfraction With Responses of Efflux
We then explored whether the basal and ABCA1-mediated efflux responses were correlated with the various HDL subfraction responses. For this calculation, we used the responses of all 32 subjects for which efflux capacities were measured because volunteers and patients did not differ with respect to efflux response (Figure 4). As shown in Table, responses (calculated as AUEC0–24) of basal and ABCA1-mediated effluxes correlated positively with responses of pre-β1-HDL and α-1 HDL, and inversely with those of small HDL but not significantly with any other HDL subfraction.
Coefficients for pre-β1-HDL (ELISA) and α-1 HDL remained significant in various runs of multiple regression analysis in which these 2 predictors were entered together with various other lipid and lipoprotein responses (not shown).

**Discussion**

MDCO-216 infusion to healthy volunteers and patients with CAD rapidly and markedly increased levels of apoA-I in pre-β1-HDL, α-2, and α-1 HDL and decreased levels of α-3 HDL. Alpha-4 HDL showed a decrease after infusion of lower doses of MDCO-216 but showed an initial increase after infusion of 30- and 40-mg/kg doses. Because apoA-IMilano was found at this α-4 position too (Figure 2), we conclude that this initial increase of total apoA-I at the α-4 position at the higher doses represents small amounts of unconverted MDCO-216, which has a mobility similar to endogenous α-4 HDL.14

Figure 3. Effect of MDCO-216 infusion at increasing doses on high-density lipoprotein (HDL) particle concentrations in healthy volunteers. HDL-S, HDL-M, and HDL-L designate particle concentrations of small, medium, and large HDL, respectively. Error bars represent SD (n=8 for placebo and n=4 for all other dose groups).

Figure 4. Dose–response curves for increasing doses of MDCO-216 on basal and ATP-binding cassette transporter A1 (ABCA1)–mediated efflux capacities, in healthy volunteers (HV) and in patients with coronary artery disease (CAD). Responses are area under the effect curve from t=0 to t=24 hours (AUEC<sub>0–24</sub>) calculated as described in Materials and Methods section of this article. Data points represent means and error bars represent SEM.
Comparing the changes observed with 2D-PAGE with those seen with 1H-NMR, the drop in α-3 and α-4 HDL corresponds with the strong drop in small HDL. The strong increase in α-2 corresponds with that in medium-sized HDL. Likewise, an increase in apoA-I in α-1 HDL was apparent only at the doses of 30 and 40 mg/kg, in line with the lack of effect on large-sized HDL concentration at the lower doses as observed by 1H-NMR. Apparently, the majority of the apoA-IMilano accumulates in particles that cannot increase much in size, in line with an earlier report that incorporation of apoA-IMilano allows for a smaller particle diameter expansion as compared with apoA-I wild-type (WT).15

The findings presented in Figures 1 and 2 suggest that MDCO-216 rapidly fuses with plasma α-3 and α-4 particles, thus depleting these subfractions and causing increases in the concentration of large-sized α-2 and α-1 particles carrying both apoA-I WT and apoA-IMilano. A speculative mechanism for the increase in pre-β1-HDL is outlined in Figure 7, suggesting that on fusion of MDCO-216 with the small HDL particles a substantial part of apoA-I WT is displaced from the fusion partners and seems as free apoA-I or pre-β1-HDL. As described by Miyazaki et al,16 the Mab, developed for quantitation of pre-β1-HDL as used in this study, likely reacts with free apoA-I. These changes also occur when MDCO-216 is incubated at 37°C with plasma or serum in vitro,14 suggesting that they are completely physicochemical in nature. The displacement is most pronounced at the 40-mg/kg dose, where at 8 hours the level of apoA-IM in α-2 is about equal to that of apoA-I WT in pre-β1. At the 10-mg/kg dose the displacement is less marked, with apoA-I WT remaining mostly in α-2 particles.

Asztalos et al17 reported previously on the correlations between HDL subfractions measured by 2D-PAGE and the various cholesterol efflux capacities in a population of 105 healthy male subjects. In that study, pre-β1-HDL and α-2 HDL were both significantly correlated with ABCA1-mediated efflux. In this study, we find that the response in ABCA1-mediated efflux correlated with the response in both pre-β1 and α-1 HDL (Table) but not with other subfractions. It must be realized that α-1 HDL in our case contains both

![Figure 5. Dose–response curves for pre-β1 high-density lipoprotein (HDL), α-1 HDL, α-2 HDL, α-3 HDL and α-4 HDL in healthy volunteers (HV) and patients with coronary artery disease (CAD). Responses are area under the effect curve from t=0 to t=24 hours (AUEC0–24) calculated as described in Materials and Methods section of this article. Data points represent means and error bars represent SEM.](http://atvb.ahajournals.org/)

*Figure 5.* Dose–response curves for pre-β1 high-density lipoprotein (HDL), α-1 HDL, α-2 HDL, α-3 HDL and α-4 HDL in healthy volunteers (HV) and patients with coronary artery disease (CAD). Responses are area under the effect curve from t=0 to t=24 hours (AUEC0–24) calculated as described in Materials and Methods section of this article. Data points represent means and error bars represent SEM.
apoA-I WT and apoA-I Milano, and it may well be that this novel particle has a higher effluxing capacity than α-1 HDL containing only apoA-I WT. Dedicated experiments would be needed to test this directly.

As shown in our earlier report, at the 10-mg/kg dose the increase in ABCA1 cholesterol efflux capacity closely coincides in time with the increase in pre-β1, both returning to baseline after 24 hours. After the 40-mg/kg dose, however, ABCA1-mediated efflux is not yet back at baseline at 24 hours, at which time α-1 HDL is still above baseline while pre-β1-HDL is back to baseline (Figure 1), in line with the hypothesis that the new α-1 HDL particle containing apoA-I Milano has a direct effect on ABCA1-mediated efflux capacity.

The observation that responses of basal and ABCA1-mediated effluxes did not differ between healthy volunteers and patients with CAD likely is explained by the lower pre-β1 response being compensated by a greater response in α-1 HDL in the healthy volunteers.

Higher levels of pre-β1-HDL have been reported before in patients with CAD and in subjects with hypertriglyceridemia. Indeed, in our study the patients with CAD also had higher pre-β1-HDL and triglyceride levels at baseline when compared with the healthy volunteers and we found a significant correlation between baseline plasma levels of triglyceride and pre-β1-HDL ELISA in both the cohorts (r=0.48, P=0.018 in healthy volunteers and r=0.54, P=0.006 in patients with CAD). The greater response in pre-β1-HDL ELISA to drug infusion in our patients with when compared with the healthy volunteers (Figure 5) may, therefore, possibly be related to the stronger increase in plasma triglyceride observed in the patients when compared with the healthy volunteers. However, there was no correlation between the response in pre-β1-HDL and that in triglyceride (r=0.35, n=32, P=NS), arguing against this hypothesis.

A final comment is in order with respect to the clinical significance of the findings reported here and in our previous article. The transient increase in triglyceride observed after MDRO-216 infusion is considered to be a pharmacodynamic response to MDRO-216 and have no specific adverse clinical relevance; increased triglyceride levels are noted in carriers of the ApoA-IMilano variant who nevertheless seem to be protected against atherosclerotic cardiovascular disease. Repeated infusion of ETC-216 the predecessor product of MDRO-216 actually resulted in regression of plaque volume, and it is tempting to now explain this regression by the prominent increase in cholesterol efflux capacity because of prompt and marked increases in pre-β1-HDL and α-1 HDL.

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**Table. Pearson Correlations Coefficients of Responses in Basal and ABCA1-Mediated Cholesterol Efflux With Responses in HDL Subfractions, for Volunteers and Patients With CAD Combined (n=32)**

<table>
<thead>
<tr>
<th></th>
<th>AUEC₀⁻二十四 Basal Efflux</th>
<th>AUEC₀⁻二十四 ABCA1-Mediated Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUEC₀⁻二十四 (Pre-β₁ Elisa)</td>
<td>0.72</td>
<td>0.74</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (Pre-β₁ 2D)</td>
<td>NS</td>
<td>0.47</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (α-1 2D)</td>
<td>0.68</td>
<td>0.76</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (α-2 2D)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (α-3 2D)</td>
<td>−0.41</td>
<td>NS</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (α-4 2D)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (large HDL-P)</td>
<td>0.55</td>
<td>NS</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (medium HDL-P)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AUEC₀⁻二十四 (small HDL-P)</td>
<td>−0.55</td>
<td>−0.53</td>
</tr>
</tbody>
</table>

Responses are expressed as AUEC₀⁻二十四 calculated as explained in Materials and Methods section of this article. 2D indicates 2-dimensional; ABCA1, ATP-binding cassette transporter A1; AUEC, area under the effect curve; CAD, coronary artery disease; HDL, high-density lipoprotein; and NS, not significant.
were employed at LabCorp (formerly Liposcience Inc).

Gomaraschi, University of Milan, for help with the figures.

Acknowledgments

Joannes Reijers and Jacobus Burggraaf are acknowledged for their contributions to the meticulous execution and design of the Phase I study at CHDR Leiden, The Netherlands. We thank Monica Gomaraschi, University of Milan, for help with the figures.

Disclosures

H.J. Kempen, D.G. Kallend, and P.L.J. Wijngaard were employed at The Medicines Company (Schweiz) GmbH. E. Jeyarajah and J. Otvos were employed at LabCorp (formerly Liposcience Inc.).

References


Significance

Infusion of MDCO-216, a complex of dimeric apolipoprotein A-I Milano with palmitoyl-oleoyl-phosphatidylcholine, in healthy volunteers and patients with coronary artery disease, causes immediate dose-dependent decreases in small high-density lipoprotein (HDL) and reciprocal increase in medium- and large-sized HDL particles. MDCO-216 as such is short lived in plasma after infusion, not detectable at all after 10-mg/kg dose and disappearing at 8 hours after the 40-mg/kg dose. In contrast, apolipoprotein A-I Milano in α-1 and α-2 HDL in plasma has a half-life of at least 24 hours and is still detectable at 168 hours. The responses of basal and ATP-binding cassette transporter A1–mediated efflux, integrated during 24 hours after start of infusion of the 40-mg/kg dose, correlate with both the response of pre-β1-HDL and that of α-1 HDL. The newly formed α-1 HDL particle containing apolipoprotein A-I Milano may have a direct effect on ATP-binding cassette transporter A1–mediated efflux capacity.
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Arterioscler Thromb Vasc Biol. 2016;36:736-742; originally published online February 25, 2016;
doi: 10.1161/ATVBAHA.115.307052

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Materials and Methods

Study Design, randomization

The study design and protocol were described in detail previously (1). Briefly, the study protocol was developed by the Sponsor, The Medicines Company, and organized and executed by the Centre for Human Drug Research (CHDR) in Leiden, Netherlands, in compliance with the Declaration of Helsinki. The protocol was approved by accredited local (BEBO, Assen, The Netherlands) and national (CCMO, The Hague, The Netherlands) independent medical ethics committees. After signing informed consent and meeting all inclusion and exclusion criteria, 24 healthy volunteers and 24 patients with stable CAD were randomized between February and October 2013 to receive a two hour infusion of MDCO-216 or placebo.

Description of drug product

MDCO-216 is a complex of highly-purified dimeric recombinant apoA-IMilano and palmitoyl-oleoyl-phosphatidylcholine (POPC). The production of the recombinant protein in E.coli and its purification has been described (2). Complexation with POPC was performed using a high-pressure homogenization procedure. The final product (stock solution) contained 13 mg/ml protein, 14 mg/ml POPC, 1.3 mg/ml Di-Na. hydrogen phosphate heptahydrate, 0.178 mg/ml Na. dihydrogen phosphate dehydrate, 62 mg/ml sucrose, 8.2 mg/ml mannitol, pH 7.5.

Randomization and blinding

Subjects were assigned in a 2:1 ratio to MDCO-216 or matching placebo to maintain blinding. Healthy volunteers received single doses of 5, 10, 20, 30 or 40 mg/kg (mg based on protein component of drug product) and stable CAD patients received doses of 10, 20, 30 or 40 mg/kg. Dose escalation was only permitted after approval from a Safety Review Committee consisting of specialists in Cardiology, Vascular Medicine, the Principal Investigator and Medical Director from the sponsor following the review of safety data to day 7. In the first two cohorts of healthy volunteers (5 mg/kg and 10 mg/kg) 2 subjects received MDCO-216 and 1 subject received placebo. In the remaining cohorts of both healthy volunteers and CAD patients 4 subjects received MDCO-216 and 2 received placebo. Altogether, 8 healthy volunteers and 8 CAD patients received placebo.

Study conduct; blood sampling

Participants received a standard meal on the evening of day -1. In the morning of day 1, participants got a standardized continental breakfast, 60-90 minutes before dosing. Subsequently, participants remained fasted until approximately 4 hours after start of drug
administration, when a light lunch was taken. Water, fruit juice (except grapefruit juice) or decaffeinated drinks were allowed on request.

Blood samples were taken just prior to start on infusion (t=0 or baseline), and then at 30 min, 2 h (end of infusion) 4 h, 8 h, 24 h, 48 h, 7 days and 30 days after start of infusion. Catheters were kept patent by intermittent flushing with normal saline or a continuous intravenous drip infusion of 0.9% NaCl at low rate. For plasma, blood was collected in a 6 mL K₂EDTA tube (vacutainer). The vacutainers were precooled by immersion in ice water, and also after the collection they were kept immersed in ice water until further processing. After centrifuging the sample for 15 minutes at 2000G at 4°C the plasma was immediately distributed over 6 labelled 2 mL cryotubes and stored at -40°C until shipment on dry ice. For serum, blood was collected in 2 mL serum (plain) tube (vacutainer). Blood was allowed to clot at room temperature for 15-30 minutes, after which the vacutainers were centrifuged for 15 minutes at 4°C at 2000G. Without delay, the serum was equally divided over two cryotubes which were stored at -80°C until shipment on dry ice.

Plasma samples were shipped on dry ice from the Clinical Trial center to a central lab who split the samples for the different assays described hereunder, requiring one thaw and refreeze. The aliquot taken for prebeta-1 HDL ELISA analysis was diluted 100-fold in 50% sucrose according to the manufacturer’s recommendation, and then kept frozen at -80°C until analysis.

**Laboratory analyses**

Plasma concentrations of total apoA-I were measured as described before (1), whereas plasma concentrations of apoA-I Milano were determined using an ELISA with monoclonal antibody 17F3 (Mabtech, Nacka, Sweden) recognizing only apoA-I Milano. Full details of this assay will be provided in a separate publication (Bellibas et al, manuscript in preparation).

ApoA-I in various HDL subfractions were determined in plasma by non-denaturing two-dimensional agarose-polyacrylamide gel electrophoresis (2D-PAGE), immunoblotting and image analysis, as described in detail before (3). In brief, after electrophoresis the gels were blotted on nitrocellulose membrane, which were incubated with a monospecific goat polyclonal antibody against human apoA-I also recognizing apoA-I Milano, or with a monoclonal antibody 17F3 (Mabtech, Nacka, Sweden) recognizing only apoA-I Milano. Subsequently the membranes were incubated with ¹²⁵I-labeled immunopurified rabbit F(Ab')₂ fraction against goat IgG. After washing membranes several times the bound ¹²⁵I-label in the various fractions were quantified in a Fluorolmager (Molecular Dynamics) yielding the % label in each HDL subfraction.prebeta-1, prebeta-2, prebeta-3, alpha-1, alpha-2, alpha-3, alpha-4, prealpha-1 prealpha-2, prealpha-3.
Concentration of total apoA-I or apoA-I-Milano in individual HDL particles were calculated by multiplying percentiles with plasma concentration of those proteins. The amount of apoA-I in prebeta-2, prebeta-3 and the various pre-alpha fractions together added up to less than 5% of total apoA-I and did not significantly change after infusion of MDCO-216 and therefore are not reported here. Mean sizes of the major subfractions are as follows: preβ-1 5.6 nm, α-4 7.5 nm, α-3 7.8 nm, α-2 9.2 nm, α-1 11.0 nm.

Prebeta-1 HDL in plasma was also measured using an ELISA specifically measuring apoA-I in prebeta-1HDL without detecting any of the other apoA-I containing lipoproteins (4). The results of this measurement was reported in our previous paper (1).

HDL subfraction particle concentrations were assessed in plasma by 1H-NMR as previously described (5). Particle concentrations are reported for small HDL (diameter 7.3-8.2 nm), medium HDL (diameter 8.2-9.4 nm) and large HDL (diameter 9.4-14 nm).

It should be noted that 2D_PAGE and 1H-NMR quantify HDL subfractions by different principles: 2D-PAGE measures apoA-I in HDL particles, while the 1H-NMR signal quantitates the terminal methyl group protons of the different lipoprotein lipids in whole plasma. (Since prebeta-1 HDL is very lipid-poor, 1H-NMR does not detect this HDL subfraction.) For a more detailed description and comparison of the two methods the reader is referred to Rosenson et al (6) and Arsenaut et al (7). As an internal validation we looked at the correlation of the particle concentrations of small, medium and large HDL with the concentrations of alpha4, alpha3, alpha 2 and alpha1 HDL in the baseline samples. As shown in Table II of the Data Supplement, the values for the large particles and also for the small particles as measured by the two methods correlate well with each other.

Basal and ABCA1-mediated cholesterol efflux capacities were determined in full serum (i.e. without prior removal of apoB-containing lipoproteins) as described previously (1, 8). Serum was shipped frozen on dry ice from the Clinical Trial center in The Netherlands to the cholesterol efflux assay lab (Vascular Strategies, College Meeting, PA, USA) without any in-between thawing. Basal and ABCA1-mediated cholesterol efflux was measured using J774 mouse macrophage cells. The cells were pre-incubated with 3H-cholesterol and ACAT inhibitor 58-035 (but not preloaded with mass cholesterol) during 18 h with or without Cpt-cAMP. After washing the cells were incubated for 4 h with the serum samples added at 2% (by volume). [³H]-cholesterol released to the medium after incubation with cells for 4 h was measured by liquid scintillation counting. Cholesterol efflux was expressed as the radiolabel released as a percentage of [³H]-cholesterol within cells before addition of serum. All efflux values were corrected by subtracting the small amount of radioactive cholesterol released from cells.
incubated with serum-free medium. The cholesterol efflux from J774 cells preincubated with Cpt-cAMP is denoted as global efflux while the efflux in the absence of Cpt-cAMP is denoted as basal efflux. ABCA1-dependent efflux from J774 cells was calculated as difference between global and basal efflux.

Results were reported already in detail in our previous paper (1), but were used in this manuscript to calculate responses (as explained below) and correlations with HDL subfraction measurements.

**Calculations and statistics**

Responses to drug administration were calculated for each subject and each parameter as area-under-the effect curve in the period from 0 to 24 h after start of infusion (AUEC0-24). The time period of 24 h was chosen because as reported separately (1) the effects on cholesterol efflux pathways and prebeta-1 HDL (determined by ELISA) were mostly complete at 24 h. For this calculation the baseline value for each parameter was first subtracted from the values at the time points 0.5h, 2h, 4h 8h and 24h after start of infusion, yielding effect sizes at each time point. The AUEC0-24 then is the sum of the effect areas within the time intervals 0-0.5, 0.5-2, 2-4, 4-8, and 8-24, with each effect area calculated as (interval length in h) * (effect size at t + effect size at t-1) * 0.5.

Means and standard deviations as well as basic comparative statistics and correlations and multiple regressions were calculated using the formulas provided in Excel.

References:


Supplementary data

Fig. I
Changes in HDL-subfractions after 40 mg/kg MDCO-216 for subject 153 (volunteer) before (-5) and 2h and 4 h after starting infusion of MDCO-216. 2D-PAGE was run of whole plasma; membranes were reacted with an antibody recognizing both wild-type ApoA-I and apoA-I Milano.

Fig. II
Changes in HDL-subfractions after 40 mg/kg MDCO-216. 2D-PAGE of whole plasma; membranes were reacted with an antibody (Mab 17F3) recognizing only apoA-IMilano.
Fig. III.

Plasma levels of total apoA-I after infusion of increasing doses of MDCO-216 in healthy volunteers or CAD patients. (Error bars are range for doses 5 and 10 mg/kg in healthy volunteers, SD for all others). These figures are provided as reference to Fig. 1 of the manuscript; detailed data were reported in Ref. 13.
Fig. IV

Plasma levels of apoA-I Milano after infusion of increasing doses of MDCO-216 in healthy volunteers or CAD patients. (Error bars are range for doses 5 and 10 mg/kg in healthy volunteers, SD for all others). These figures are provided as reference to Fig. 2 of the manuscript; full data will be reported separately (A. Bellibas et al, manuscript in preparation).
Table I. Prebeta-1 HDL concentrations measured by 2D-PAGE or by ELISA.

Baseline values and increase above baseline at 4 h after start of MDCO-216 infusion. Values are means (SD) and represent mg/dl based on apoA-I

A. For healthy volunteers

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>4 h after 20 mg/kg</th>
<th>4 h after 30 mg/kg</th>
<th>4 h after 40 mg/kg</th>
<th>4 h after 20-40 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=24)</td>
<td>n=4</td>
<td>n=4</td>
<td>n=4</td>
<td>N=12</td>
<td></td>
</tr>
<tr>
<td>Prebeta-1 HDL 2D-PAGE</td>
<td>13.9 (4.8)</td>
<td>12.7 (4.4)</td>
<td>19.2 (2.1)</td>
<td>24.9 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Prebeta-1 HDL ELISA</td>
<td>4.1 (1.4)</td>
<td>8.5 (1.3)</td>
<td>13.0 (2.7)</td>
<td>21.7 (6.1)</td>
<td></td>
</tr>
<tr>
<td>Pearson correlation between methods</td>
<td>0.71</td>
<td></td>
<td></td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

B. For CAD patients

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>4 h after 20 mg/kg</th>
<th>4 h after 30 mg/kg</th>
<th>4 h after 40 mg/kg</th>
<th>4 h after 20-40 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=24</td>
<td>n=4</td>
<td>n=4</td>
<td>n=4</td>
<td>N=12</td>
<td></td>
</tr>
<tr>
<td>Prebeta-1 HDL 2D-PAGE</td>
<td>17.7 (4.6)</td>
<td>15.5 (3.6)</td>
<td>17.4 (0.9)</td>
<td>19.4 (15.0)</td>
<td></td>
</tr>
<tr>
<td>Prebeta-1 HDL ELISA</td>
<td>6.5 (2.5)</td>
<td>5.8 (2.5)</td>
<td>10.0 (2.9)</td>
<td>15.1 (4.4)</td>
<td></td>
</tr>
<tr>
<td>Pearson correlation between methods</td>
<td>0.43</td>
<td></td>
<td></td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>
Table II. Pearson correlation coefficients between baseline values for HDL subfractions measured by 2D-PAGE and those measured by 1H-NMR.

A. For healthy volunteers (N=24)

<table>
<thead>
<tr>
<th></th>
<th>large HDL-P</th>
<th>med HDLP-P</th>
<th>small HDL-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-1</td>
<td>0.923*</td>
<td>0.266</td>
<td>-0.425*</td>
</tr>
<tr>
<td>alpha-2</td>
<td>0.580*</td>
<td>0.591*</td>
<td>-0.339</td>
</tr>
<tr>
<td>alpha-3</td>
<td>-0.093</td>
<td>0.248</td>
<td>0.448*</td>
</tr>
<tr>
<td>alpha-4</td>
<td>-0.068</td>
<td>-0.042</td>
<td>0.066*</td>
</tr>
<tr>
<td>alpha3+4</td>
<td>-0.091</td>
<td>0.146</td>
<td>0.578*</td>
</tr>
</tbody>
</table>

B. For CAD patients (N=24)

<table>
<thead>
<tr>
<th></th>
<th>large HDL-P</th>
<th>med HDLP-P</th>
<th>small HDL-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-1</td>
<td>0.936*</td>
<td>0.118</td>
<td>-0.382</td>
</tr>
<tr>
<td>alpha-2</td>
<td>0.656*</td>
<td>0.485*</td>
<td>-0.140</td>
</tr>
<tr>
<td>alpha-3</td>
<td>-0.248</td>
<td>-0.117</td>
<td>0.391</td>
</tr>
<tr>
<td>alpha-4</td>
<td>0.190</td>
<td>-0.082</td>
<td>0.371</td>
</tr>
<tr>
<td>alpha3+4</td>
<td>-0.069</td>
<td>-0.046</td>
<td>0.437*</td>
</tr>
</tbody>
</table>

*: p<0.05