Chapter 5

Restoration of endothelial function by increasing HDL in subjects with isolated low-HDL

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Abstract

**Background** Loss-of-function mutations in the ATP-binding cassette (ABCA)-1 gene locus are the underlying cause for familial hypoalphalipoproteinemia (FHA), providing a human isolated low-HDL model. In these FHA subjects, we evaluated the impact of isolated low-HDL on endothelial function as well as the vascular effects of an acute increase in HDL.

**Methods and Results** In nine ABCA1 heterozygotes and nine controls vascular function was assessed using venous occlusion plethysmography. Forearm blood flow (FBF) responses to the endothelium-dependent and -independent vasodilators serotonin (5HT) and sodium nitroprusside (SNP), respectively, and the inhibitor of nitric oxide synthase L-NMMA, were measured. Dose-response curves were repeated after systemic infusion of apolipoprotein A-I/ phosphatidylcholine discs (apoA-I/PC). At baseline, ABCA1 heterozygotes had decreased HDL levels (0.4±0.2 mmol/L; p<0.05), and their FBF responses to both 5HT (max 49.0±10.4 %) and L-NMMA (max −22.8±22.9 %) were blunted compared to controls (both p<0.005). Infusion of apoA-I/PC discs increased plasma HDL to 1.3±0.4 mmol/L in ABCA1 heterozygotes, which resulted in complete restoration of vasomotor responses to both 5HT and L-NMMA (both p≤0.001). Endothelium-independent vasodilation remained unaltered throughout the protocol.

**Conclusions** In ABCA1 heterozygotes isolated low-HDL is associated with endothelial dysfunction, attested by impaired basal and stimulated NO bioactivity. Strikingly, both parameters were completely restored after a single, rapid infusion of apoA-I/PC. These findings indicate that besides its long-term role within reverse cholesterol transport, HDL per se also exerts direct beneficial effects on the arterial wall.
Introduction

Despite a clinically significant reduction in coronary event rates following the use of statins, both primary and secondary prevention trials indicate that 60-70% of major cardiovascular events cannot be prevented with current therapeutic strategies. The latter has prompted the search for novel drug targets to further improve cardiovascular outcome. Amongst these options, strategies aiming at increasing high-density lipoprotein (HDL) levels hold great promise\(^1\). In this respect, a strong inverse relationship has been established between HDL levels and the incidence of coronary artery disease (CAD)\(^4\). In line, the prevalence of HDL deficiency (<35 mg/dL) in men with premature CAD reaches up to 40%\(^5\). Nevertheless, the implications of increasing HDL levels for cardiovascular outcome remain to be determined. The fact that low HDL is often associated with other risk factors such as enhanced triglyceride levels and insulin resistance (comprising the metabolic syndrome) suggests that those, instead of low HDL per se, might be predominantly responsible for the observed relationship with cardiovascular events. This has incited the search for human models with an isolated low-HDL-phenotype in which the consequences of isolated low-HDL as well as pharmacological modalities targeting HDL on endothelial phenotype and cardiovascular outcome can be studied.

A proper model is familial hypoalphalipoproteinemia, which was recently found to result from loss-of-function mutations in the ABCA1 gene. Heterozygous carriers are characterized by a moderate to severe decrease of HDL but have normal levels of apoB-containing lipoproteins, thus enabling assessment of the role of isolated low-HDL \textit{in vivo}\(^7\). Since the initial step in the reverse cholesterol transport (RCT) pathway is impeded in these individuals, cell-derived cholesterol accumulates within the arterial wall, clinically manifested by advanced carotid intima-media thickening (IMT) and markedly increased susceptibility to atherosclerosis\(^8\). Endothelium-derived nitric oxide (NO) has emerged as a pivotal antiatherogenic mediator. Diminished NO-bioavailability is a hallmark of (early) atherosclerosis, which can be measured in patients indirectly as impaired endothelium-dependent vasomotor response \(^10\). In this respect, endothelial vasodilator dysfunction has been demonstrated in subjects with established coronary risk factors even before the onset of morphological changes, whilst most studies have indicated its reversibility after risk factor exclusion\(^11\). More recently, the predictive value of endothelial vasodilator dysfunction for future cardiovascular events has been underscored by several research groups\(^15\). In the present study, we evaluated the consequences of isolated low-HDL for vascular function in subjects carrying heterozygous ABCA1 gene mutations, compared to normolipidemic, sex- and age-matched controls. Previous data in these individuals, including accelerated carotid atherogenesis suggested a \textit{a priori} compromised endothelial function. Subsequently, we determined whether an acute increase in HDL level (upon infusion of apoA-I/PC discs) would translate into an improvement of vascular function.
Patients and methods

Subjects
Nine ABCA1 heterozygotes (6 men and 3 women; mean [±SD] age, 42.9±13.9) and nine age- and sex-matched, normolipidemic controls (6 men and 3 women; mean [±SD] age, 46.1±13.5) were enrolled in the study. Affected subjects were selected from available family members of four Dutch kindreds with known mutations on the ABCA1 gene locus, as described previously\(^1\). At baseline, their HDL cholesterol levels were 0.5±0.2 versus 1.2±0.3 mmol/L (p<0.05) in the control group. Four subjects were smokers, equally divided over both groups (Table 1). All subjects had normal plasma levels of apo B-containing lipoproteins (TC, LDL or TG below 95\(^{th}\) percentile), whereas none of the subjects had diabetes mellitus, hypertension (defined as systolic blood pressure above 140 mmHg and/or diastolic blood pressure above 90 mmHg) or congestive heart failure. Before entering the study, all lipid-lowering medication was discontinued for a period of at least 6 weeks. All subjects refrained from alcohol, tobacco, and caffeine-containing drinks for more than twelve hours prior to the study. The study protocol was approved by the Institutional Review Board at the Academic Medical Center, University Hospital of Amsterdam. All subjects gave written informed consent.

Study protocol
Assessment of vascular function was performed at baseline and after apoA-I/PC infusion using venous occlusion, strain-gauge plethysmography (EC4; Hokanson Inc). All experiments were performed in a quiet, and air-conditioned room (temperature 22 to 24°C), having the subjects in a supine position throughout the study. The non-dominant brachial artery was cannulated with a 20-gauge polyethylene catheter under local anesthesia and blood was drawn for baseline measurements. Insertion was followed by a twenty minutes interval for the artery to re-establish baseline conditions. Thereafter, forearm blood flow (FBF), expressed in milliliters per minute per 100 mL of forearm tissue volume (FAV), was measured simultaneously in both arms. During each measurement blood pressure cuffs around both upper arms were inflated (40 mm Hg) using a rapid cuff inflator. Synchronously, bilateral wrist cuffs were inflated to above-systolic blood pressure in order to exclude hand circulation (200 mm Hg). Intra-arterial blood pressure and heart rate were continuously monitored. Next, FBF response to cumulative doses of the endothelium-dependent vasodilator serotonin (SHT, Sigma; 0.6, 1.8, and 6 ng.100 mL FAV\(^{-1}\).min\(^{-1}\)), the endothelium-independent vasodilator sodium nitroprusside (SNP, Spruyt Hillen; 6, 60, 180, and 600 ng.100 mL FAV\(^{-1}\).min\(^{-1}\)) and the competitive inhibitor of endothelial nitric oxide synthase (eNOS) N\(^\text{G}\)-mono-methyl-L-arginine (L-NMMA, Kordia; 50, 100, 200, and 400 μg.100 mL FAV\(^{-1}\).min\(^{-1}\)) was measured. All agents were administered intra-arterially for 6, 4 and 8 minutes at each dose respectively, using a constant-rate infusion pump. Six measurements during the last two minutes (steady state) were averaged to determine mean FBF. The three different infusion blocks proceeded after a 15-minute rest period, or until FBF had returned to baseline. Then, a venous catheter was inserted in the contralateral arm for systemic administration of apolipoprotein A-I/phospha-
tidylcholine discs at a dose of 80 mg/kg body weight over 4 hours (ZLB Bioplasma AG). Subsequently, all three infusion blocks were repeated.

**Biochemical analysis**

Blood samples were drawn from the subjects after a 12 hour overnight fast, immediately and three hours after apoA-I/PC infusion, respectively. After centrifugation within 1 hour after collection, aliquots were snapfrozen in liquid nitrogen and stored at -80°C until the assays were performed. All measurements were performed at the vascular and clinical laboratory of the Academic Medical Center, University Hospital of Amsterdam. Liver transaminases were measured with standard laboratory methods. Plasma triglycerides, total cholesterol, LDL and HDL levels were determined using high performance gel permeation chromatography (HPGC). In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser, a FP-920 fluorescence and UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line cholesterol (or triglyceride) PAP enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at 0.1 mL/min. EDTA plasma was diluted 1:1 with Tris buffered saline and 30 μL sample/buffer mixture was loaded on a Superose 6 HR 10/30 column (Pharmacia Biotech) for lipoprotein separation at a flow rate of 0.31 mL/min. Plasma levels of apoB and apo-AI were assayed on stored plasma by rate nephelometry. The serum concentration of malondialdehyde modified LDL was assayed by a murine monoclonal antibody 4E6-based sandwich ELISA (Mercodia AB, Uppsala, Sweden), as has been described previously.

**Statistical analysis**

All results of clinical parameters, including plethysmographic data, are expressed as mean ±SD. Descriptive statistics between both groups were compared by means of 2-tailed paired or unpaired Student’s t test. Forearm blood flow (FBF) was averaged over six consecutive recordings during the last two minutes of each infusion. Statistical analysis of FBF measurements for individual subjects between both groups was performed using two-way ANOVA for repeated measures. A probability value of <0.05 was considered significant and <0.01 as highly significant.

**Results**

Baseline clinical characteristics of the ABCA1 heterozygotes and control subjects are summarized in table 1. HDL levels were significantly lower in ABCA1 heterozygotes compared to controls (p<0.05). Other lipid parameters, as well as systolic and diastolic blood pressures and body-mass index were not significantly different. After apoA-I/PC infusion plasma HDL cholesterol increased from 0.4±0.3 to 1.3±0.4 mmol/L and 1.0±0.3 to 2.1±0.5 mmol/L in ABCA1 heterozygotes and controls, respectively (table 2).
Table 1. Demographic and Baseline Parameters of the Study Cohort

<table>
<thead>
<tr>
<th></th>
<th>ABCA1 heterozygotes (n=9)</th>
<th>Controls (n=9)</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>42.9 ± 13.9</td>
<td>46.1 ± 13.5</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>3/6</td>
<td>3/6</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.1 ± 2.7</td>
<td>25.8 ± 3.5</td>
</tr>
<tr>
<td>Smoking</td>
<td>2/9</td>
<td>2/9</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>1/9</td>
<td>0/9</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>131.6 ± 23.4</td>
<td>130.1 ± 13.4</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>79.0 ± 8.9</td>
<td>78.1 ± 5.9</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>62.2 ± 9.0</td>
<td>60.1 ± 10.5</td>
</tr>
<tr>
<td>Basal FBF, ml.100 mL FAV⁻¹.min⁻¹</td>
<td>3.2 ± 1.0</td>
<td>2.6 ± 0.6</td>
</tr>
</tbody>
</table>

Values represent mean ± SD. BMI indicates body mass index; HR, heart rate; FBF, forearm blood flow, FAV, forearm tissue volume.

Table 2. Laboratory Parameters of the Study Cohort

<table>
<thead>
<tr>
<th></th>
<th>ABCA1-A1 heterozygotes before HDL increase (n=9)</th>
<th>after HDL increase (n=9)</th>
<th>Controls before HDL increase (n=9)</th>
<th>after HDL increase (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>4.3 ± 1.5</td>
<td>5.6 ± 1.5</td>
<td>5.4 ± 1.2</td>
<td>6.5 ± 1.4</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>0.4 ± 0.2*</td>
<td>1.3 ± 0.4†</td>
<td>1.0 ± 0.3</td>
<td>2.1 ± 0.5†</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.5 ± 1.0</td>
<td>3.5 ± 0.8</td>
<td>4.1 ± 1.1</td>
<td>3.7 ± 1.2</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.6 ± 0.9</td>
<td>2.9 ± 2.3</td>
<td>1.2 ± 0.6</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>Apo A-I (g/L)</td>
<td>0.7 ± 0.3*</td>
<td>2.3 ± 0.5†</td>
<td>1.2 ± 0.2</td>
<td>2.6 ± 0.4†</td>
</tr>
<tr>
<td>Apo B (g/L)</td>
<td>1.0 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>oxLDL (U/L)</td>
<td>46.1 ± 16.1</td>
<td>40.2 ± 16.0</td>
<td>44.0 ± 25.7</td>
<td>37.8 ± 20.1</td>
</tr>
</tbody>
</table>

Values represent mean ± SD. TC indicates total cholesterol; HDL, high-density cholesterol; LDL, low-density cholesterol; TG, triglycerides; apo A-I, apolipoprotein AI; apo B, apolipo-protein B; oxLDL, oxidized LDL. * p<0.05 vs normocholesterolemic controls. † p<0.05 vs baseline.

Effects of apoA-I/PC infusion on eNOS activity

At baseline, the vasoconstrictor response to L-NMMA, reflecting basal NO activity, was blunted in ABCA1 heterozygotes compared to controls (p=0.001; figure 1). After a single rapid infusion of apoA-I/PC discs, the L-NMMA constrictor response was increased significantly (p=0.001; figure 1), reaching levels comparable to control subjects. In contrast, apoA-I/PC infusion had no effect on L-NMMA response in control subjects (figure 1).

The intraarterial infusion of the endothelium-dependent vasodilator serotonin (5HT) increased FBF in a dose-dependent manner in both groups. At baseline, the FBF response to 5HT was impaired significantly in ABCA1 heterozygotes compared to controls (p<0.0001; figure 2). After apoA-I/PC infusion, FBF response to 5HT increased significantly to levels comparable with control responses (p<0.001; figure 2). In line with L-NMMA data, apoA-I/PC infusion had no effect on 5HT induced vasodilator responses in control subjects (figure 2).
Figure 1. L-NMMA induced vasoconstriction before and after HDL increase FBF dose-response curves to the inhibitor of nitric oxide synthase NG-monomethyl-L-arginine (L-NMMA) before (O) and after (●) apoA-I/PC infusion in ABCA1 heterozygotes and normocholesterolemic controls (before △; after ▲). L-NMMA-induced vasoconstriction at baseline was blunted in ABCA1 mutation carriers compared to control subjects (* p=0.001). After HDL increase by apoA-I/PC infusion, the FBF response to L-NMMA in the ABCA1 heterozygotes completely normalized († p=0.001).

Figure 2. Serotonin induced vasodilation before and after HDL increase FBF dose-response curves to the endothelium-dependent vasodilator serotonin (5-HT) before (O) and after (●) apoA-I/PC infusion in ABCA1 heterozygotes and normocholesterolemic controls (before △; after ▲). Endothelium-dependent vasodilation to 5-HT at baseline was attenuated in ABCA1 heterozygotes compared to control subjects (* p<0.0001). After HDL increase by apoA-I/PC infusion, the FBF response to 5HT in the ABCA1 mutation carriers completely normalized († p<0.001).

Figure 3. Sodium Nitroprusside induced vasodilation before and after HDL increase FBF dose-response curves to the endothelium-independent vasodilator sodium nitroprusside (SNP) before and after apoA-I/PC infusion in ABCA1 heterozygotes and normocholesterolemic controls. At baseline, SNP induced-vasodilator response was not different between ABCA1 heterozygotes and controls (p=0.30) and remained unaltered by apoA-I/PC infusion.
At baseline, endothelium-independent vasodilation in response to SNP was not different between ABCA1 heterozygotes and controls, whereas apoA-I/PC infusion likewise showed no effect on the SNP vasodilator response in either group (figure 3).

**Biochemistry**

Circulating levels of oxidized LDL (oxLDL) were not significantly different between ABCA1 heterozygotes and controls. Also, the levels of these parameters remained unaltered after apoA-I/PC infusion.

**Discussion**

In the present study, we show that both basal as well as stimulated NO-activity is sharply reduced in ABCA1 heterozygotes when compared to age- and sex-matched controls. Strikingly, an increase in HDL by a single, rapid infusion of apoA-I/PC discs results in complete recovery of endothelial vasomotor response. These novel data indicate that isolated low-HDL in ABCA1 heterozygotes is associated with endothelial dysfunction, which is entirely reversible upon HDL increase.

The ABCA1-transporter facilitates apoA-I-mediated transfer of cellular cholesterol and phospholipids from the plasma membrane to poorly lipidated (apo)lipoproteins. Loss of ABCA1 function leads to defective lipidation, resulting in enhanced clearance of the HDL precursor fraction with ensuing low plasma HDL levels\(^\text{23,24}\). We recently demonstrated that heterozygous carriers of ABCA1 gene mutations are characterized by advanced arterial wall thickening and an increased risk for early-onset CAD. The correlation between their cholesterol efflux rates and arterial wall thickness suggests a critical role for the impairment of the apoA-I/RCT-pathway in mediating these changes in vascular morphology. Currently, we additionally show an impairment in both basal and stimulated NO bioavailability, which is completely reversible upon HDL increase. The observed vascular effects shortly after HDL increase, seem less likely to be related to the RCT-pathway for two reasons. First, ABCA1 heterozygotes are characterized by a significantly impaired RCT-pathway, in which the apoA-I concentration is not the rate limiting factor\(^\text{25}\). Second, vascular effects as a result of modulating the apoA-I/RCT-pathway are not expected to occur that soon, since cholesterol is primarily mobilized from easily mobilizable stores, e.g. liver and spleen \(^\text{26,27}\). Hence, these data suggest that HDL by itself is a potent regulator of the endothelial NO pathway \textit{in vivo}.

The interactions between (low) HDL and endothelial NO-bioavailability can be explained by several mechanisms. First, as the localization of eNOS in cholesterol-rich microdomains of the plasma membrane (including caveolae) is critical for its activation, caveolar disruption has a profound effect on endothelial NO release \(^\text{28}\). Recently, caveolar processing was shown to be disturbed in ABCA1-defective human fibroblasts, resulting in a substantial decrease in the
number of caveolae on the plasma membrane \(^{29}\). Given that ABCA1 is also present in endothelial cells, similar processes are likely to contribute to the impaired eNOS functionality \(^{30,31}\). Next, low HDL may be associated with increased vascular oxidative stress, secondarily contributing to impaired endothelial NO-bioavailability. Partially, due to the presence of the enzymes paraoxonase and platelet-activating factor hydrolase, HDL has been found to exert potent anti-oxidant effects \(^{32,33}\). In line, infusion of apoA-I/PC discs, similar to our protocol, resulted in a decreased susceptibility to oxidation of LDL in humans. However, in the present study, we were unable to find support for a pro-oxidant state in ABCA1 heterozygotes, since oxLDL levels were not significantly higher, nor were they altered upon apoA-I/PC infusion. Thirdly, recent data have elegantly shown a direct stimulatory effect of HDL on endothelial NO-synthase via an SR-B1-dependent pathway\(^{34-36}\). Particularly, the low basal NO-activity in the ABCA1 heterozygotes may support a physiological role of HDL as a direct regulator of eNOS activity in vivo.

Infusion of apoA-I/PC promotes intravascular generation of nascent HDL which have a natural course in humans\(^{37}\). Subsequently, HDL increase may contribute to improvement of endothelial vasomotor function by several mechanisms. First, recent data have shown acute stimulatory effects of apoA-I on intracellular cholesterol trafficking from the Golgi apparatus to the cell-surface in human fibroblasts\(^{38}\), resulting in a rapid increase in the number of membrane caveolae. Theoretically, in these ABCA1 heterozygotes apoA-I may have initiated restoration of intracellular events involving caveolar processing with subsequent reshutteling of eNOS to caveolar regions in endothelial cells. Finally, along with aforementioned direct eNOS-regulating mechanisms, an increase in apoAI-containing HDLs may have resulted in enhanced eNOS activity, as particularly attested by augmented basal NO bioavailability.

**Clinical perspectives**

In the present study, we show that low-HDL by itself can be independently responsible for adverse cardiovascular effects, assessed as endothelial dysfunction. Importantly, the reversibility of vascular dysfunction in low-HDL patients upon acute HDL increase constitutes a powerful stimulus for ongoing efforts targeting HDL increase as a tool in cardiovascular prevention. The current findings not only underscore the beneficial cardiovascular potential with regard to the endothelium, but also indicate that endothelial function testing is a suitable intermediate endpoint in future trials evaluating the effects of HDL increase on cardiovascular disease progression.
Acknowledgments

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