Restoration of Endothelial Function by Increasing High-Density Lipoprotein in Subjects With Isolated Low High-Density Lipoprotein

Radjesh J. Bisoendial, MD; G. Kees Hovingh, MD; Johannes H.M. Levels, PhD; Peter G. Lerch, PhD; Irmgard Andresen, MD, PhD; Michael R. Hayden, MB, ChB, PhD; John J.P. Kastelein, MD, PhD; Erik S.G. Stroes, MD, PhD

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

Methods and Results—In 9 ABCA1 heterozygotes and 9 control subjects, vascular function was assessed by venous occlusion plethysmography. Forearm blood flow responses to the endothelium-dependent and -independent vasodilators serotonin (5HT) and sodium nitroprusside, 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 (apoA-I/PC) disks. At baseline, ABCA1 heterozygotes had decreased HDL levels (0.4\(\pm\)0.2 mmol/L; \(P<0.05\)), and their forearm blood flow responses to both 5HT (maximum, 49.0\(\pm\)10.4\%) and L-NMMA (maximum, 22.8\(\pm\)22.9\%) were blunted compared with control subjects (both \(P<0.005\)). Infusion of apoA-I/PC disks increased plasma HDL to 1.3\(\pm\)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 to 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 in addition to its long-term role within reverse cholesterol transport, HDL per se also exerts direct beneficial effects on the arterial wall. (Circulation. 2003;107:2944-2948.)

Key Words: lipoproteins \(\oplus\) apolipoproteins \(\oplus\) endothelium \(\oplus\) nitric oxide

Despite a clinically significant reduction in coronary event rates after the use of statins, both primary and secondary prevention trials indicate that 60% to 70% of major cardiovascular events cannot be prevented with current therapeutic strategies. This has prompted the search for novel drug targets to further improve cardiovascular outcome. Among these options, strategies aiming at increasing HDL levels hold great promise.\(^1\)\(^{-}\)\(^3\) In this respect, a strong inverse relationship has been established between HDL levels and the incidence of coronary artery disease.\(^4\) In line with this, the prevalence of HDL deficiency (<35 mg/dL) in men with premature coronary artery disease reaches up to 40%.\(^5\)\(^6\) 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 (composing the metabolic syndrome), suggests that those, instead of low HDL per se, might be primarily responsible for the observed relationship with cardiovascular events. This has promoted the search for human models with an isolated low-HDL phenotype in which the consequences of isolated low HDL and pharmacological modalities targeting HDL for 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 ATP-binding cassette (ABCA)-1 gene. Heterozygous carriers are characterized by a moderate to severe decrease of HDL but have normal levels of apolipoprotein B (apoB)--containing lipoproteins, thus enabling...
assessment of the role of isolated low HDL in vivo.7 Because 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 and markedly increased susceptibility to atherosclerosis.8,9 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 indirectly in patients as impaired endothelium-dependent vasomotor response.10 In this context, endothelial vasodilator dysfunction has been demonstrated in subjects with established coronary risk factors even before the onset of morphological changes, although most studies have indicated its reversibility after risk factor exclusion.11–14 More recently, the predictive value of endothelial vasodilator dysfunction for future cardiovascular events has been underscored by several research groups.15–18

In the present study, we evaluated the consequences of isolated low HDL for vascular function in subjects carrying heterozygous ABCA1 gene mutations compared with normolipidemic sex- and age-matched control subjects (controls). Previous data in these individuals, including accelerated carotid atherogenesis, suggested a priori compromised endothelial function. Subsequently, we determined whether an acute increase in HDL level (on infusion of apolipoprotein A-I/phosphatidylcholine [apoA-I/PC] disks) would translate into an improvement of vascular function.

### Methods

#### Subjects
Nine ABCA1 heterozygotes (6 men and 3 women; mean [±SD] age, 42.9±13.9 years) and 9 age- and sex-matched normolipidemic controls (6 men and 3 women; mean [±SD] age, 46.1±13.5 years) were enrolled in the study. Affected subjects were selected from available family members of 4 Dutch kindreds with known mutations on the ABCA1 gene locus, as described previously.11 At baseline, 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 between the 2 groups (Table 1). All subjects had normal plasma levels of apoB-containing lipoproteins (total cholesterol, LDL, or triglycerides below the 95th percentile), whereas none of the subjects had diabetes mellitus, hypertension (defined as systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg), or congestive heart failure. Before entering the study, subjects discontinued all lipid-lowering medication for a period of ≥6 weeks. All subjects refrained from alcohol, tobacco, and caffeine-containing drinks for more than 12 hours before 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 by use of venous occlusion strain-gauge plethysmography (EC4; Hokanson Inc). All experiments were performed in a quiet, air-conditioned room (temperature, 22°C to 24°C), with the subjects in the supine position throughout the study. The nondominant 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 20-minute interval for the artery to reestablish 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) by use of a rapid cuff inflator. Simultaneously, bilateral wrist cuffs were inflated to above-systolic blood pressure to exclude hand circulation (200 mm Hg). Intra-arterial blood pressure and heart rate were monitored continuously. Next, FBF response to cumulative doses of the endothelium-dependent vasodilator serotonin (5HT, Sigma; 0.6, 1.8, and 6 ng·100 mL FAV−1·min−1), the endothelium-independent vasodilator sodium nitroprusside (SNP, Spryit Hillen; 6, 60, 180, and 600 ng·100 mL FAV−1·min−1), and the competitive inhibitor of endothelial NO synthase (eNOS) Nω-nomonomethyl-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, with a constant-rate infusion pump. Six measurements during the last 2 minutes (steady state) were averaged to determine mean FBF. The 3 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 apoA-I/PC disks at a dose of 80 μg/kg body weight over a period of 4 hours (ZLB Bioplasma AG).15,20 Subsequently, all 3 infusion blocks were repeated.

#### Biochemical Analysis
Blood samples were drawn from the subjects after a 12-hour overnight fast, immediately and 3 hours after apoA-I/PC infusion. After centrifugation within 1 hour after collection, aliquots were snap-frozen 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, and LDL and HDL levels were determined with high-performance gel permeation chromatography.21 In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and an FP-920 fluorescence and UV-975 UV/VIS detector (Jasco). An extra P-50 pump (Pharmacia Biotech) was used for addition of in-line cholesterol (or triglyceride) PAP enzymatic reagent (Biomerieux) at 0.1 mL/min. EDTA plasma was diluted 1:1 with Tris-buffered saline, and 30 μL of a 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 apoA-I and apoA-I 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) as described previously.22

#### Statistical Analysis
All results of clinical parameters, including plethysmographic data, are expressed as mean±SD. Descriptive statistics between the 2

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**TABLE 1.** Demographic and Baseline Parameters of the Study Cohort

<table>
<thead>
<tr>
<th></th>
<th>ABCA1 Heterozygotes (n=9)</th>
<th>Control Subjects (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>42.9±13.9</td>
<td>46.1±13.5</td>
</tr>
<tr>
<td>Sex, M/F</td>
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<td>3/6</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
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<td>25.8±3.5</td>
</tr>
<tr>
<td>Smoking, n</td>
<td>2/9</td>
<td>2/9</td>
</tr>
<tr>
<td>Cardiovascular disease, n</td>
<td>1/9</td>
<td>0/9</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>131.6±23.4</td>
<td>130.1±13.4</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</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.
Results

Baseline clinical characteristics of the ABCA1 heterozygotes and control subjects are summarized in Table 1. HDL levels were significantly lower in ABCA1 heterozygotes than in 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 from 1.0±0.3 to 2.1±0.5 mmol/L in ABCA1 heterozygotes and controls, respectively (Table 2).

Effects of ApoA-I/PC Infusion on eNOS Activity

At baseline, the vasodilator response to L-NMMA, reflecting basal NO activity, was blunted in ABCA1 heterozygotes compared with controls (P<0.05; Figure 1). After a single rapid infusion of apoA-I/PC disks, the L-NMMA constrictor response was increased significantly (P=0.001; Figure 1), reaching levels comparable to those of control subjects. In contrast, apoA-I/PC infusion had no effect on L-NMMA response in control subjects (Figure 1).

Intra-arterial infusion of the endothelium-dependent vasodilator 5HT increased FBF in a dose-dependent manner in the 2 groups. At baseline, the FBF response to 5HT was impaired significantly in ABCA1 heterozygotes compared with controls (P<0.0001; Figure 2). After apoA-I/PC infusion, FBF response to 5HT increased significantly to levels comparable to those of 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).

At baseline, endothelium-independent vasodilation in response to SNP was not different between ABCA1 heterozygotes and controls, and apoA-I/PC infusion likewise showed no effect on the SNP vasodilator response in either group (Figure 3).

Biochemistry

Circulating levels of oxidized LDL 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 and stimulated NO activity is sharply reduced in ABCA1 heterozygotes compared with age- and sex-matched controls. Strikingly, an increase in HDL by a single, rapid infusion of apoA-I/PC disks 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 on 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.\(^{7,8}\) We recently demonstrated that heterozygous carriers of ABCA1 gene mutations are characterized by advanced arterial wall thickening and an increased risk for early-onset coronary artery disease. 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.

In this study, we also show an impairment in both basal and stimulated NO bioavailability, which is completely reversible on HDL increase. The observed vascular effects shortly after HDL increase seem less likely to be related to the RCT pathway, for 2 reasons. First, ABCA1 heterozygotes are characterized by a significantly impaired RCT pathway in which the apoA-I concentration is not the rate-limiting factor.\(^{23}\) Second, vascular effects as a result of modulating the apoA-I/RCT pathway are not expected to occur that soon, because cholesterol is mobilized primarily from easily mobilizable stores, eg, liver and spleen.\(^{8,24}\) Hence, these data suggest that HDL by itself is a potent regulator of the endothelial NO pathway in vivo.

The interactions between (low) HDL and endothelial NO bioavailability can be explained by several mechanisms. First, because 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.\(^{25}\) 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.\(^{26}\) Given that ABCA1 is also present in endothelial cells, similar processes are likely to contribute to the impaired eNOS functionality.\(^{27,28}\) Next, low HDL may be associated with increased vascular oxidative stress, secondarily contributing to impaired endothelial NO bioavailability. Partially because of the presence of the enzymes paraoxonase and platelet-activating factor hydrolase, HDL has been found to exert potent antioxidant effects.\(^{29,30}\) In line with this, infusion of apoA-I/IPC disks, as in 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 to oxidation of LDL in humans. In the present study, we were unable to find support for a pro-oxidant state in ABCA1 heterozygotes, because oxidized LDL levels were not significantly higher, nor were they altered on apoA-I/IPC infusion. Third, recent data have elegantly shown a direct stimulatory effect of HDL on eNOS via a scavenger receptor, class B, type I–dependent pathway.\(^{31–33}\) In particular, 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/IPC promotes intravascular generation of nascent HDL, which has a natural course in humans.\(^{24}\) 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,\(^{34}\) 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 reshuttling of eNOS to caveolar regions in endothelial cells. Finally, along with the aforementioned direct eNOS-regulating mechanisms, an increase in apoA-I–containing HDLs may have resulted in enhanced eNOS activity, as attested to in particular by augmented basal NO bioavailability.

**Clinical Perspectives**

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

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References

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