HDL and Coronary Artery Disease

A Novel ApoA-I Mutation (L178P) Leads to Endothelial Dysfunction, Increased Arterial Wall Thickness, and Premature Coronary Artery Disease

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OBJECTIVES
We investigated the consequences of an apolipoprotein A-I (apoA-I) gene defect with regard to lipid metabolism, endothelial function, arterial wall thickness, and coronary artery disease (CAD) risk.

BACKGROUND
Due to limited numbers of carriers of the apoA-I defects, data on the consequences of such defects have remained inconclusive.

METHODS
Lipids and lipoproteins were measured in 54 apoA-I (L178P) carriers and 147 nonaffected siblings. Flow-mediated dilation (FMD) was assessed in 29 carriers and 45 noncarriers, and carotid intima-media thickness (IMT) could be determined in 33 heterozygotes and 40 controls. Moreover, CAD risk was evaluated for all apoA-I mutation carriers.

RESULTS
Heterozygotes exhibited lower plasma levels of apoA-I (~50%; p < 0.0001) and high-density lipoprotein cholesterol (~63%; p < 0.0001). In addition, carriers had impaired FMD (p = 0.012) and increased carotid IMT (p < 0.001), whereas multivariate analysis revealed that heterozygotes had a striking 24-fold increase in CAD risk (p = 0.003).

CONCLUSIONS
Heterozygosity for a novel apoA-I mutation underlies a detrimental lipoprotein profile that is associated with endothelial dysfunction, accelerated carotid arterial wall thickening, and severely enhanced CAD risk. Importantly, the extent of atherosclerosis in these subjects was similar to the burden of premature arterial wall abnormalities seen in patients with familial hypercholesterolemia. These data illustrate the pivotal role in humans of apoA-I in the protection against CAD. (J Am Coll Cardiol 2004;44:1429–35) © 2004 by the American College of Cardiology Foundation

Prospective epidemiologic studies have shown that high-density lipoprotein (HDL) cholesterol plasma levels are inversely related to coronary artery disease (CAD) risk (1). This agrees with the finding that low plasma levels of HDL cholesterol are a common form of dyslipidemia in patients suffering from premature CAD (2). Moreover, the importance of increasing HDL cholesterol levels is illustrated by a 2% to 3% reduction of major cardiovascular events associated with a 1 mg/dl (0.026 mmol/l) increase of HDL cholesterol on five years of fenofibrate treatment (3). The atheroprotective role of HDL is partly ascribed to its role in reverse cholesterol transport, by which HDL transports cholesterol from peripheral cells to the liver and steroidogenic organs (4). In addition, HDL displays significant anti-oxidant and anti-inflammatory properties (5). Apolipoprotein A-I (apoA-I), the major protein constituent of the HDL particle, is critical to HDL metabolism. It provides HDL with structural integrity and is required for normal HDL function. Human apoA-I is expressed in the liver and small intestine, and secretion into plasma results in de novo HDL production. In this process, apoA-I is lipidated through adenosine triphosphate (ATP)-binding cassette AI transporter-mediated cholesterol efflux, which results in the formation of disc-shaped pre-beta, HDL particles (6). Lecithin/cholesterol acyltransferase, an enzyme that uses apoA-I as a cofactor, subsequently esterifies free cholesterol on the nascent HDL particle, which leads to the formation of larger and spherical HDL. Finally, apoA-I has been shown to play an important role in one of the last steps of the reverse cholesterol transport process. As a ligand of the scavenger receptor B-I, it facilitates the specific uptake of cholesterol esters from HDL by the liver, albeit in mice (7). It is not known whether, in
Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>apoA-I</td>
<td>apolipoprotein A-I</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>FMD</td>
<td>flow-mediated dilation</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>IMT</td>
<td>intima-media thickness</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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humans, apoA-I also binds to the recently described novel hepatic receptor (ectopic beta chain of ATP synthase) to enable holo-HDL particle uptake (8).

To date, over 40 apoA-I gene defects have been described, which may have severe consequences for HDL metabolism (9). The risk of CAD associated with these apoA-I gene defects, however, has not been elucidated. This is primarily due to limited numbers of carriers of apoA-I defects, and, as a consequence, inconclusive data are provided by the literature. Some apoA-I mutations supposedly underlie CAD (L159P) (10), whereas others show no effect (L144R) (11) or even protection against CAD (R173C) (12).

We identified a novel apoA-I mutation (L178P) in Dutch families suffering from familial hypo-alpha-lipoproteinemia. We report on the consequences of this apoA-I mutation for lipid metabolism in a large group of heterozygotes compared with family controls. As surrogate end points for CAD, we subsequently measured endothelial function by flow-mediated dilation (FMD) and carotid intima-media thickness (IMT). Finally, cardiovascular end points in the affected and control individuals were scored and assessed by multivariate analyses.

Methods

Study population. The subjects were recruited from a Dutch population-based study to identify genes that control HDL cholesterol levels (performed in collaboration with the Center for Molecular Medicine and Therapeutics at the University of British Columbia, Canada; and Xenon Genetics Inc., British Columbia, Canada). The population comprised 94 probands who met the following inclusion criteria: 1) HDL cholesterol plasma level below the fifth percentile for age and gender; 2) absence of other primary or secondary lipid disorders (i.e., diabetes, alcohol abuse); and 3) high likelihood of inherited low HDL cholesterol (defined as an HDL cholesterol below the fifth percentile for age and gender and absence of other primary or secondary lipid disorders in at least one first-degree family member). Informed consent was obtained from all subjects for plasma sampling, storage, genetic analysis, and vascular tests, under a protocol approved by the ethics committee of the Academic Medical Center in Amsterdam. Past medical history, presence of cardiovascular risk factors, use of medication, and information on geographic origin of the probands were assessed by a questionnaire.

DNA analysis and mutation detection. DNA was extracted from peripheral blood leukocytes, as described previously (13). Genotyping, linkage analysis, haplotyping, and sequencing were used in an attempt to localize the molecular defects in families segregating low HDL. In one of the families (Family #11), this exercise resulted in the identification of a novel C/T point mutation at nucleotide 643 in exon 4 of the ApoA-I gene (using Genbank entry GI4557320 as reference sequence), predicting the exchange of a leucine for a proline residue at position 178 (L178P) in the mature ApoA-I protein. The leucine residue at position 178 is highly conserved with the exception of the pig, where this residue is instead a phenylalanine (14). The variant was found on the affected haplotype and fully co-segregated with hypo-alpha-lipoproteinemia in this family. In support of the causal nature of this mutation, the sequence variant was not detected in 374 control chromosomes (data not shown).

Using polymerase chain reaction-restriction fragment length polymorphism analysis, we identified five additional heterozygous carriers in the remaining 93 low HDL probands (Family #8, #12, #28, #61, and #94). It is of note that all six probands originate from the same geographic region in the Netherlands, suggesting common ancestry. A Markov Chain Monte Carlo with bayesian integration approach (15) (described under Statistical Analysis) was used for the estimation of age of the mutation.

Laboratory analysis. Blood samples were drawn after an overnight fast. Total plasma cholesterol and triglycerides were determined by an enzymatic colorimetric procedure (CHOD-PAP, Boehringer, Mannheim, Germany); HDL cholesterol was measured as cholesterol remaining after precipitation of apoB-containing lipoproteins by MnCl2. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. Plasma apoA-I and apoB concentrations were determined by immunonephelometry. Immunoelectrophoresis was used to quantify LpAI (Sebia, Isy-les Moulineaux, France), and LpAI:AII was calculated as the difference between total apoA-I and LpAI concentrations (16).

Flow-mediated dilation. Flow-mediated vasodilation was measured as previously described (17). In short, FMD was assessed after 10 h of fasting. A blood pressure cuff was placed below the elbow of the right arm. After a 10-min rest, the diameter of the brachial artery was visualized in the antecubital fossa using a 7.5-MHz transducer. Reactive hyperemia was induced by inflating the blood pressure cuff to 220 mm Hg for 4 min. After release of the cuff, reactive vasodilation was monitored at 30-s intervals during 5 min. Wall track measurements were performed and digitally stored by the same sonographer, who was blinded as to the genetic status of the participants. Baseline vessel luminal diameter was calculated as the average of three baseline measurements. The FMD results were calculated using the following equation: (maximal lumen after hyperemia — mean diameter at baseline)/diameter at baseline × 100%.
Intima-media thickness. The IMT measurements were performed as earlier described (18). In short, high-resolution B-mode ultrasound images were acquired using an Acuson 128XP/10v (Acuson Corp., Mountain-view, California), with a 7.0-MHz linear array transducer. Segments of 10 mm of the following predefined wall segments were scanned: the common carotid artery, the carotid bulb, and the internal carotid artery. The acquired images were saved as JPEG image files. One image reader, blinded as to the genetic status of the patient, measured the IMT of the far wall of those segments. The mean combined IMT of these three segments was used to compare carriers and noncarriers.

Statistical analysis. A Markov Chain Monte Carlo with a bayesian integration approach was applied using DMLE+2.14 (15) to estimate the age of the Apo-A-I (L178P) mutation. The six pedigree disease haplotypes and 46 independent control haplotypes taken from the pedigrees, genotyped at 10 microsatellite markers spanning 12 cM, were used for the estimation. Program parameters were set to 1,000,000 replications; average population growth was calculated to 0.18 per generation for the last 300 years; and the haplotype fraction sampled in the Northern region of the Netherlands (Friesland) was set to 0.012.

Continuous outcomes were compared between affected and unaffected subjects using the general linear mixed model with pedigree number as a random factor to account for the association between subjects from the same pedigree. For dichotomous outcomes, a similar generalized linear mixed model was applied with pedigree as random factor. The Cox proportional hazard model extended with a frailty parameter per pedigree was used to determine event-free survival rates for affected and unaffected subjects. The proportional hazard conditions were met. Analyses were performed using the Statistical Program for the Social Sciences (Version 10.0, SPSS Inc., Chicago, Illinois) and S-plus for the Cox regression with frailty parameter. The significance level was set at p < 0.05 (two-tailed).

RESULTS

Demographic and genetic characteristics of study cohort. Active recruitment in six kindreds yielded 54 heterozygotes for the apo-A-I defect and 147 family controls. The number of cases and controls in the different pedigrees were distributed as follows—Family #11: 12 carriers and 28 controls; Family #8: 7 carriers and 4 controls; Family #12: 13 carriers and 49 controls; Family #28: 3 carriers and 11 controls; Family #61: 6 carriers and 5 controls; and Family #94: 13 carriers and 50 controls.

All individuals were Caucasian of Dutch descent and lived in an isolated part in the Northern region of the Netherlands. The demographic data of carriers and noncarriers are given in Table 1. Mean age, age range, male/female ratios, and the prevalence of hypertension (defined by the use of antihypertensive medication) and smoking were similar in both groups.

Univariate linear regression revealed that the effects of the apo-A-I mutation described in subsequent sections were not different among the six families, suggesting homogeneity of the total population. Genotyping and haplotyping of these families with genetic markers in close proximity to the Apo-A-I gene demonstrated that they shared a 2.67-cM haplotype segment, suggestive of common ancestry. Two kindreds even shared a 7.62-cM haplotype. The age of the ApoAI L178P mutation was estimated at 20 generations, with the 95% confidence interval (CI) ranging from 12 to 35 generations.

Lipids, lipoproteins, and apolipoproteins. Compared with control subjects, heterozygotes for the apo-A-I defect presented with a 52% mean decrease of apo-A-I levels (0.70 vs. 1.47 g/l, p < 0.0001) (Table 2) and a 62% decrease of HDL cholesterol levels (0.44 vs. 1.22 mmol/l, p < 0.0001). These reductions of HDL cholesterol were reflected by a significant decrease of HDL containing only apoA-I (LpA-I: 0.31 vs. 0.60 g/l, p < 0.0001) and HDL containing both apoA-I and apoA-II (LpA-I:A-II: 0.39 vs. 0.87 g/l, p = 0.0004). In the absence of effects on triglycerides and LDL cholesterol, the reduction of HDL cholesterol was

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<tr>
<th>Table 1. Demographic Characteristics of Heterozygotes for the apo-A-I (L178P) Defect and Unaffected Family Controls</th>
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<tr>
<td><strong>ApoAI (L178P)</strong></td>
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<tr>
<td><strong>Mean age (yrs)</strong></td>
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<tr>
<td><strong>Age range</strong></td>
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<tr>
<td><strong>Males/females</strong></td>
</tr>
<tr>
<td><strong>Smoker (%)</strong></td>
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<tr>
<td><strong>Hypertension (%)</strong></td>
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<tr>
<td><strong>BMI (kg/m²)</strong></td>
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All data are expressed as the mean value ± SD or number (%). Differences between carriers and noncarriers were not significant.

BMI = body mass index.

<table>
<thead>
<tr>
<th>Table 2. Lipids, Lipoproteins, and Apolipoproteins of Carriers and Noncarriers of Apo-A-I Mutation (L178P)</th>
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<tr>
<td><strong>ApoAI (L178P)</strong></td>
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<tr>
<td>ApoA-I (g/l)</td>
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<tr>
<td>HDL cholesterol (mmol/l)</td>
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<tr>
<td>LpA-I (g/l)</td>
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<tr>
<td>LpA-I:A-II (g/l)</td>
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<td>TC (mmol/l)</td>
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<tr>
<td>TG (mmol/l)</td>
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<tr>
<td>LDL cholesterol (mmol/l)</td>
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<tr>
<td>Lipoprotein(a) (mg/l)</td>
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<tr>
<td>ApoB (g/l)</td>
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All data are given as the mean value ± SD.

ApoA-I = apolipoprotein A-I; apoB = apolipoprotein B; HDL = high-density lipoprotein; LpA-I = HDL containing apoA-I; LpA-I:A-II = HDL containing apoA-I and apoA-II; LDL = low-density lipoprotein; TC = total cholesterol; TG = triglycerides.
mirrored by a decrease of total cholesterol levels (4.20 vs. 4.72 mmol/l, \( p = 0.001 \)) in apoA-I (L178P) heterozygotes. Lipoprotein(a) levels were not different in affected and unaffected subjects.

**Flow-mediated vasodilation.** Flow-mediated dilation could be assessed in 29 carriers (18 men and 11 women) and 45 family controls (24 men and 21 women). Controls were significantly younger than carriers (29.2 vs. 40.7 years, \( p = 0.01 \)). We found no association between age and FMD in this cohort (\( p = 0.18 \)). In addition, smoking habits and the prevalence of medically treated hypertension did not differ among both groups. The median FMD was significantly reduced in apoA-I (L178P) carriers compared with family controls (3.5% vs. 5.5%, \( p = 0.012 \)) (Fig. 1). This difference remained significant after exclusion of heterozygotes (\( n = 5 \)) with from CAD.

**IMT.** The IMT measurements were performed in 33 heterozygotes (12 women and 21 men) and 40 family controls (18 women and 22 men). The mean carotid IMT of mutation carriers was significantly increased compared with that of family controls (0.79 vs. 0.56 mm, \( p < 0.0001 \)). The cross-sectional data were subsequently plotted against age, which suggested that the progression of carotid wall thickening over time differed significantly between carriers and controls. Our data show a 0.0047- or 0.0082-mm (combined) carotid IMT increase per year of age in controls and heterozygotes, respectively (\( p < 0.001 \)) (Fig. 2). The impact of the apoA-I (L178P) mutation is clear when considering similar rates of carotid wall thickening in a cohort of 215 patients with familial hypercholesterolemia (Fig. 2) (19).

![Figure 1](image1.png)

**Figure 1.** Log-transformed (logFMD) results are significantly lower in carriers of the apoA-I (L178P) mutation compared with family controls. **Inverted triangles** = apoA-I (L178P) carriers; **circles** = family controls.

![Figure 2](image2.png)

**Figure 2.** Mean combined carotid intima media thickness (IMT) increase per year of age is significantly higher in heterozygotes compared with controls. The rate of progression of carotid wall thickening over time is similar in L178P heterozygotes and familial hypercholesterolemia (FH) patients. **Solid circles** = ApoA-I (L178P) carriers; **open diamonds** = patients with FH; **+** = family controls.
Table 3. Multivariate Analysis of Risk of Coronary Artery Disease Attributable to the L178P Mutation Carrier Status

<table>
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<tr>
<th>Controlled for Age, Gender, and Pedigree</th>
<th>n</th>
<th>OR (95% CI)</th>
<th>p Value</th>
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<tbody>
<tr>
<td>Including pedigree probands</td>
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<tr>
<td>CAD risk (all ages)</td>
<td>201</td>
<td>24.1 (3.2–179.1)</td>
<td>0.0009</td>
</tr>
<tr>
<td>CAD risk (age 20 yrs and over)</td>
<td>167</td>
<td>23.7 (3.2–174.6)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Excluding pedigree probands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD risk (all ages)</td>
<td>195</td>
<td>18.9 (2.3–153.8)</td>
<td>0.0030</td>
</tr>
<tr>
<td>CAD risk (age 20 yrs and over)</td>
<td>161</td>
<td>18.5 (2.3–148.0)</td>
<td>0.0030</td>
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CAD = coronary artery disease; CI = confidence interval; OR = odds ratio.

Cardiovascular events. Among the 54 L178P heterozygotes, eight subjects (15%) had CAD (i.e., acute myocardial infarction, coronary artery bypass graft operation, or coronary angioplasty), of which six occurred at a premature age (before the age of 55 and 60 years in men and women, respectively). In contrast, only two of 147 control subjects (1.4%) suffered from an event, of which none occurred prematurely. Using univariate analysis, this difference was highly statistically significant (p < 0.0001; data not shown).

On multivariate analysis, controlling for age, gender, and pedigree, the apoA-I (L178P) carrier status was found to convey a 24-fold increase in the risk of developing CAD (Table 3). The odds ratio to develop CAD when carrying the apoA-I gene mutation was 18.9 (CI 2.3 to 153.8, p = 0.0030) after excluding the six probands. The difference in event-free survival between carriers and controls, as shown in Figure 3, further illustrates the severe consequences of this mutation (p = 0.008).

DISCUSSION

This report shows the detrimental consequences of a novel apoA-I (L178P) mutation on HDL metabolism, endothelial function, carotid arterial wall IMT and CAD risk in a population comprising 54 heterozygous carriers. The novel defect was identified in six families, all originating from the Northeastern part of the Netherlands. Evidence of a founder effect for L178P was obtained from haplotype analysis, which demonstrated that all carriers share a 2.67-cM haplotype surrounding the gene.

apoA-I (L178P): effects on apoA-I levels, HDL cholesterol, and triglycerides. Although we observed a 50% decrease of apoA-I levels in apoA-I (L178P) carriers, heterozygotes for other apoA-I defects have been associated with even greater apoA-I reductions (up to 80%) (10,20). This has been attributed to dominant negative effects of some mutations, and, possibly, L178P does not exhibit such properties. In this respect, it is of interest to note that we were not able to detect apoA-I (L178P) in plasma by means of mass spectrometry of the apoA-I protein moiety of isolated HDL (data not shown). This suggests that the L178P variant is not properly processed in the cell, resulting in absent or greatly diminished secretion into the circulation. Alternatively, the mutant protein may be hypercatabolized on secretion (thereby rendering plasma levels too low for detection), an effect that has been described for other apoA-I variants (21,22). These data imply that the effects observed can be solely attributed to half normal levels of wild-type apoA-I.

Heterozygous carriers of the apoA-I (L178P) defect presented with 62% reductions in HDL-C. Similar reductions have been described for other apoA-I mutations (23,24) although some reports describe HDL-C reductions up to 80% of normal (24). Finally, we noted no effects on TG levels, which is in contrast with a study that revealed higher TG levels in men suffering from an apoA-I (delta K107) defect (25).

apoA-I (L178P): cardiovascular disease. Although murine studies have unequivocally shown that apoA-I protecst against atherosclerosis (26,27), and epidemiologic data in humans clearly indicate that apoA-I levels are a strong predictor of CAD (28), susceptibility for CAD has been shown to vary significantly between carriers of apoA-I defects. Ten of 22 apoA-I–deficient subjects (13 different families) have been described to suffer from CAD to various degrees (29–33).

It is of interest to note that two publications described mutations at the same amino acid position. Two heterozygous carriers for the Leu178His defect were shown to suffer from low HDL cholesterol and amyloidosis, but no signs of CAD were reported (34). Interestingly, no amyloidosis was observed in our L178P mutation carriers. The majority of ApoA-I mutations associated with amyloidosis change the isoelectric point of the protein (14). This might underlie the difference in clinical phenotype between L178H and L178P. The L178H introduces a positively charged residue, whereas L178P does not. Also, it should be noted that the L178H protein was identified in the plasma of its carriers, while this was not the case in heterozygous carriers of the L178P defect. During the preparation of this manuscript, Ikewaki et al. (35) reported on a 51-year-old female homozygote for a premature stop codon at residue 178 underlying HDL cholesterol deficiency (0.08 mmol/l) and premature heart failure. In this case, the number of carriers...
(n = 3) left no opportunity to investigate the direct correlation between the mutation and the risk for CAD. Taken together, the relatively small number and young age of individuals with apoA-I gene defects have until now compromised detailed studies into CAD risk.

Some of these difficulties can be overcome by using surrogate markers to assess atherosclerotic burden, such as FMD and carotid artery IMT (17,36,37). Both FMD and IMT correlate with established risk factors and have been shown to have predictive value for future vascular events (17). It is clear from our data that heterozygotes for apoA-I (L178P) suffer from endothelial dysfunction as compared with family controls, which is likely related to the low levels of HDL cholesterol in these patients. This hypothesis is supported by experiments in which infusion of reconstituted HDL acutely improved endothelial function in patients with isolated low HDL cholesterol levels, due to ABCA1 defects (38). It is well recognized that impaired endothelium-dependent vasomotor response, caused by diminished nitric oxide bioavailability, precedes structural arterial wall alterations. This, in turn agrees with our finding of an increased carotid IMT in affected subjects compared with family controls. However, we needed to account for the difference in age between the two groups. We therefore calculated the rate of progression of arterial wall thickening by plotting the mean carotid artery wall thickness against age. The rate of progression in affected subjects was found to be significantly increased.

Finally, the size of our cohort allowed us to not only correlate apoA-I (L178P) with low HDL cholesterol, reduced FMD, and increased IMT progression, but also with a definite increased risk for cardiovascular complications. Multivariate analysis revealed a dramatic 24-fold increase in CAD, as defined by myocardial infarction, coronary artery bypass graft surgery, and percutaneous transluminal coronary angioplasty.

Together, our data illustrate that the L178P defect is deleterious. This brings us to address IMT measurements in carriers of another interesting mutation in apoA-I denoted as apoA-I-Milano (R173C). In contrast to all other apoA-I defects, apoA-I-Milano has been described to be beneficial despite the fact that carriers present with a 60% reduction in HDL cholesterol (39). It has been described that this variant has increased potential to promote cellular cholesterol efflux (40). Others have recently shown that infusions of ApoA-I-Milano/phospholipid complexes in CAD patients result in a significant regression of coronary plaque (41). Regarding IMT, 21 heterozygous carriers of the apoA-I-Milano (R173C) mutation were found to have similar average thicknesses of carotid segments compared with control subjects (n = 42), further underlining the distinct properties of this peculiar Milano variant (39).

Conclusions. We unambiguously show that isolated low HDL cholesterol due to heterozygosity for apoA-I (L178P) is marked by endothelial dysfunction, accelerated carotid arterial wall thickening, and an increased incidence of premature vascular events compared with their family controls. In this cohort, genetically defined low HDL cholesterol appears equally detrimental as hereditary high LDL cholesterol with respect to IMT progression. The data underscore the pivotal importance of normal apoA-I and HDL cholesterol levels as a defense against all stages of cardiovascular disease and furthermore indicate that therapeutic intervention to raise HDL cholesterol may be equally effective to reduce CAD risk as drugs that lower LDL cholesterol.

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