High-density lipoprotein and C-reactive protein, friend and foe in cardiovascular disease
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Citation for published version (APA):
Bisoendial, R. J. (2006). High-density lipoprotein and C-reactive protein, friend and foe in cardiovascular disease
A Novel ApoA-I Mutation (L178P) leads to endothelial dysfunction, increased arterial wall thickness and premature coronary artery disease

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Abstract

Objectives and background. We here report on a large group of heterozygotes for a novel apoA-I mutation (L178P) that enabled us to assess for the first time to investigate the consequences of an apoA-I gene defect with regards to lipid metabolism, endothelial function, arterial wall thickness as well as coronary artery disease (CAD) risk.

Methods. Lipids and lipoproteins could be measured in 54 apoA-I (L178P) carriers and 147 non-affected siblings. Flow-mediated dilatation (FMD) was also assessed in 29 carriers and 45 non-carriers 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 HDL-C (-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 familial hypercholesterolemia patients. These data illustrate the pivotal role in humans of apoA-I in the protection against CAD.
Introduction

Prospective epidemiological studies have shown that high-density lipoprotein cholesterol (HDL-c) plasma levels are inversely related to coronary artery disease (CAD) risk. This agrees with the finding that low plasma levels of HDL-C are a common form of dyslipidemia in patients suffering from premature CAD. Moreover, the importance of increasing HDL-C levels is illustrated by a 2-3% reduction of major cardiovascular events associated with a 1mg/dL (0.026 mmol/L) increase of HDL-C upon 5 years of fenofibrate treatment. The atheroprotective role of HDL is in part ascribed to its role in reverse cholesterol transport (RCT), by which HDL transports cholesterol from peripheral cells to the liver and steroidogenic organs. In addition, HDL displays significant anti-oxidant and anti-inflammatory properties.

ApolipoproteinA-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, whereas secretion into plasma results in the novo HDL production. In this process, apoA-I is lipidated through ATP binding cassette Al transporter-mediated cholesterol efflux, which results in the formation of disc-shaped pre-β1HDL particles. Lecithin:cholesterol acyltransferase (LCAT), 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 RCT process. As a ligand of the scavenger receptor B-I, it furthermore facilitates the specific uptake of cholesteryl esters from HDL by the liver, albeit in mice. Whether, in humans, apoA-I also binds to the recently described novel hepatic receptor (ectopic B-chain of ATP synthase), to enable holo-HDL particle uptake is not known.

To date, over 40 apoA-I gene defects have been described, that may have severe consequences for HDL metabolism. The risk for 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 is provided by the literature. Some apoA-I mutations supposedly underlie CAD (L159P), whereas others show no effect (L144R) or even protection against CAD (R173C). We identified a novel apoA-I mutation (L178P) in Dutch families, suffering from familial hypoalphalipoproteinemia. We here report on the consequences of this apoA-I mutation for lipid metabolism in a large group of heterozygotes compared to family controls. As surrogate endpoints for CAD we subsequently measured endothelial function by flow-mediated dilatation (FMD) and carotid intima media thickness (IMT). Finally, cardiovascular endpoints in the affected and control individuals were scored and assessed by multivariate analyses.
Material and methods

Study Population
The subjects were recruited from a Dutch population-based study to identify genes that control HDL-c 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-C plasma level below the 5th percentile for age and sex, 2) absence of other primary or secondary lipid disorders (i.e. diabetes, alcohol abuse) and 3) high likelihood of inherited low HDL-C (defined as an HDL-C below the 5th percentile for age and sex 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 regarding 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. 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. The variant was found on the affected haplotype and fully co-segregated with hypoalphalipoproteinemia 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 PCR-RFLP analysis, we identified five additional heterozygous carriers in the remaining 93 low HDL probands (families 8, 12, 28, 61 and 94). It is of note that all six probands originate from the same geographical region in the Netherlands, suggesting common ancestry. A Markov Chain Monte Carlo with Bayesian integration approach (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 (TC) and triglycerides (TG) were determined by an enzymatic colorimetric procedure (CHOD-PAP, Boehringer I Mannheim), HDL-c was measured as cholesterol remaining after precipitation of apoB-containing lipoproteins by MnCl₂. Low-density lipoprotein-cholesterol (LDL-c) was calculated using the Friedewald formula. Plasma apoA-I and apoB concentrations were determined
by immunonephelometry. Immunelectrophoresis was used to quantify LpAl (Sebia, Isy-les Moulineaux, France), and LpAl:AII was calculated as the difference between total apoA-I and LpAl concentrations.

Flow-Mediated Dilatation (FMD)
Flow-mediated vasodilatation was measured as previously described. In short, FMD was assessed after 10 hours of fasting. A blood pressure cuff was placed below the elbow of the right arm. After a 10 minutes 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 minutes. After release of the cuff, reactive vasodilatation was monitored at 30 seconds intervals during 5 minutes. Wall track measurements were performed and digitally stored by the same sonographer, who was blinded for the genetic status of the participants. Baseline vessel luminal diameter was calculated as the average of three baseline measurements. FMD results were calculated using the following equation: (maximal lumen after hyperemia – mean diameter at baseline) / diameter at baseline x 100%.

Intima Media Thickness (IMT)
IMT measurements were performed as earlier described. In short, high-resolution B-mode ultrasound images were acquired using an Acuson 128XP/10v (Acuson Corporation, Mountainview, CA), with a 7.0 MHz linear array transducer. Segments of 10mm of the following predefined wall segments were scanned: the common carotid artery (CCA), the carotid bulb (CB) and the internal carotid artery (ICA). The acquired images were saved as JPEG image files. One image reader, blinded for 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 non-carriers.

Statistical Analysis
A Markov Chain Monte Carlo with Bayesian integration approach was applied using DMLE+2.14 to estimate the age of the ApoA-I(L178P) mutation. The six pedigree disease haplotypes and 46 independent control haplotypes taken from the pedigrees, genotyped at ten 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 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, IL, USA) 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 the Study Cohort

Active recruitment in six kindreds yielded fifty-four heterozygotes for the apoA-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 North the Netherlands. The demographic data of carriers and non-carriers 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 effects of the apoA-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 ApoA-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 ApoAl L178P mutation was estimated at 20 generations, with the 95% confidence interval ranging from 12 to 35 generations.

### Table 1. Demographic Characteristics of Heterozygotes for the apoA-I (L178P) Defect and unaffected Family Controls

<table>
<thead>
<tr>
<th></th>
<th>ApoAI(L178P) heterozygotes (n=54)</th>
<th>Non-carriers (n=147)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age mean in years</td>
<td>37.6 ± 19.2</td>
<td>37.9 ± 16.8</td>
</tr>
<tr>
<td>Age range</td>
<td>3-81</td>
<td>0.8 – 90</td>
</tr>
<tr>
<td>M / F</td>
<td>32 / 22</td>
<td>68 / 79</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>9 (17.6)</td>
<td>48 (30.6)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>4 (7.4)</td>
<td>9 (6.1)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.9 ± 5.0</td>
<td>25.0 ± 5.3</td>
</tr>
</tbody>
</table>

All values are given as mean ± SD. Percentages are given between brackets. M/F= Male/Female ratio, BMI= Body Mass Index. Differences between carriers and non-carriers were not significant.

### Lipids, Lipoproteins and Apolipoproteins

Compared to control subjects, heterozygotes for the apoA-I defect presented with a 52% mean decrease of apoA-I levels (0.70 vs. 1.47 g/L; p<0.0001; Table 2) and a 62% decrease
Table 2. Lipids, Lipoproteins and Apolipoproteins of Carriers and Non-carriers of apoA-I Mutation (L178P)

<table>
<thead>
<tr>
<th></th>
<th>ApoAl(L178P) carriers (n=54)</th>
<th>Non-carriers (n=147)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoA-I (g/L)</td>
<td>0.70 ± 0.31</td>
<td>1.47 ± 0.33</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL-c (mmol/L)</td>
<td>0.44 ± 0.22</td>
<td>1.22 ± 0.36</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LpA-I (g/L)</td>
<td>0.31 ± 0.10</td>
<td>0.60 ± 0.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LpA-I:A-II (g/L)</td>
<td>0.39</td>
<td>0.87</td>
<td>0.0004</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.20 ± 1.06</td>
<td>4.72 ± 0.99</td>
<td>0.001</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.31 ± 0.88</td>
<td>1.19 ± 1.28</td>
<td>0.09</td>
</tr>
<tr>
<td>LDL-c (mmol/L)</td>
<td>3.16 ± 0.92</td>
<td>2.98 ± 0.91</td>
<td>0.23</td>
</tr>
<tr>
<td>Lipoprotein(a) (mg/L)</td>
<td>182.78 ± 209.0</td>
<td>213 ± 165.9</td>
<td>0.45</td>
</tr>
<tr>
<td>apoB (g/L)</td>
<td>0.99 ± 0.26</td>
<td>0.89 ± 0.20</td>
<td>0.21</td>
</tr>
</tbody>
</table>

All values are given as mean ± SD. ApoA-I= apolipoprotein A-I; HDL-c= High-density lipoprotein cholesterol; LpA-I= HDL containing apoA-I; LpA-I:A-II= HDL containing apoA-I and apoA-II; TC= Total Cholesterol; TG= Triglycerides; LDL-c = Low-density lipoprotein cholesterol; apoB= apolipoprotein B.

of HDL-C levels (0.44 mmol/l vs. 1.22 mmol/l; p<0.0001). These reductions of HDL-C 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 absence of effects on TG, and LDL-C the reduction of HDL-C was mirrored by a decrease of TC 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 Vasodilatation

FMD 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 years, 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 prevalence of medically-treated hypertension did not differ amongst both groups. The median FMD was significantly reduced in apoA-I(L178P) carriers compared to family controls (3.5 % vs. 5.5%; p=0.012)(Figure 1). This difference remained significant after exclusion of heterozygotes (n=5) that suffered from CAD.

**Intima Media Thickness**

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 to family controls (0.79 mm 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) (Figure 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 (FH) 20.

![Figure 2](image)

**Figure 2.** Mean combined carotid intima media thickness (IMT) increase per year of age is significantly higher in heterozygotes compared to controls. The rate of progression of carotid wall thickening over time is similar in L178P heterozygotes and FH patients.

**Cardiovascular Events**

Amongst the 54 L178P heterozygotes, eight subjects (15%) suffered from CAD (i.e. acute myocardial infarction, coronary artery bypass 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 out 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). In multivariate analysis, controlling for age, gender and pedigree, the apoA-I(L178P) carrier status was found to convey a 24-fold increase in risk for developing CAD (Table 3). The odds ratio to develop CAD when carrying the apoA-I gene mutation was 18.9 (CI=2.3-153.8; p=0.003) 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).

Table 3. Multivariate Analysis of Risk of CAD attributable to the L178P Mutation Carrier Status

<table>
<thead>
<tr>
<th>Controlled for age, gender and pedigree</th>
<th>n</th>
<th>Odds Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Including pedigree probands</td>
<td></td>
<td></td>
<td></td>
</tr>
<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 (aged 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 (aged 20 yrs and over)</td>
<td>161</td>
<td>18.5 (2.3-148.0)</td>
<td>0.0030</td>
</tr>
</tbody>
</table>

Figure 3 Event free survival is significantly lower in apoA-I (L178P) carriers, compared to controls.

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 6 families, all originating from the North Eastern part of Netherlands. Evidence of a founder effect for L178P was obtained from haplotype analysis, which demonstrated that all carriers share a 2.67cM haplotype surrounding the gene.
ApoA-l (L178P): effects on apoA-l levels, HDL-C and TG

While we observed a 50% decrease of apoA-l levels in apoA-l(L178P) carriers, heterozygotes for other apoA-l defects have been associated with even greater apoA-l reductions (up to 80%)\textsuperscript{21,22}. 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-l(L178P) in plasma by means of mass spectrometry of the apoA-l 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 upon secretion (thereby rendering plasma levels too low for detection), an effect that has been described for other apoA-l variants \textsuperscript{23,24}. These data imply that the effects observed can be solely attributed to half normal levels of wild-type apoA-l.

Heterozygous carriers of the apoA-l (L178P) defect presented with 62% reductions in HDL-C. Similar reductions have been described for other apoA-l mutations\textsuperscript{25,26} although some reports describe HDL-C reductions up to 80% of normal.\textsuperscript{27} 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 other apoA-l (delta K107) defect\textsuperscript{28}.

ApoA-l (L178P): cardiovascular disease

While murine studies have unequivocally shown that apoA-l protects against atherosclerosis\textsuperscript{29,30}, and epidemiological data in humans clearly indicate that apoA-l levels are a strong predictor of CAD\textsuperscript{31}, susceptibility for CAD has been shown to vary significantly between carriers of apoA-l defects. Ten out of 22 apoA-l deficient subjects (13 different families) have been described to suffer from CAD to various degrees.\textsuperscript{32-35}

It is of interest to note that two publications described mutations at the same at the same amino acid position. Two heterozygous carriers for the Leu178His defect were shown to suffer from low HDL-c and amyloidosis, but no signs of CAD were reported.\textsuperscript{37} Interestingly, no amyloidosis was observed in our L178P mutation carriers. The majority of ApoA-l mutations associated with amyloidosis change the isoelectric point of the protein\textsuperscript{38}. This might underlie the difference in clinical phenotype between the 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 and colleagues reported on a 51 year old woman, homozygote for a premature stop codon at residue 178 underlying HDL-c deficiency (0.08 mmol/L) and premature heart failure.\textsuperscript{39} 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-l 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 flow-mediated dilation (FMD) and carotid artery intima media thickness.
Both FMD and IMT correlate with established risk factors and have been shown to have predictive value for future vascular events. It is clear from our data that heterozygotes for apoA-I(L178P) suffer from endothelial dysfunction when compared to family controls, which is likely related to the low levels of HDL-C 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.

It is well-recognized that impaired endothelium-dependent vasomotor response, caused by diminished NO-bioavailability, precedes structural arterial wall alterations. This in turn agrees with our finding of an increased carotid IMT in affected subjects compared to 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-C, 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 MI, CABG and PTCA.

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-C.

It has been described that this variant has increased potential to promote cellular cholesterol efflux. Others have recently shown that infusions of ApoA-I Milano/phospholipids complexes in CAD patients result in a significant regression of coronary plaque. Regarding IMT, 21 heterozygous carriers of the apoA-I Milano (R173C) mutation were found to have similar average thicknesses of carotid segments compared to control subjects (n=42) further underlining the distinct properties of this peculiar Milano variant.

In conclusion, we unambiguously show that isolated low HDL-c 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 to their family controls. In this cohort, genetically defined low HDL-C appears equally detrimental as hereditary high LDL-C with respect to IMT progression. The data underscore the pivotal importance of normal apoA-I and HDL-C levels as a defense against all stages of cardiovascular disease and furthermore indicate that therapeutic intervention to raise HDL-C may be equally effective to reduce CAD risk as drugs that lower LDL-C.
Acknowledgements

John J.P. Kastelein is an established investigator of the Netherlands Heart Foundation (2000D039). G. Kees Hovingh and Jan Albert Kuivenhoven are supported by the Netherlands Heart Foundation (2000.115 and 1998T011, respectively). Michael R. Hayden is holder of a Canada Research Chair in Human Genetics. This work was supported by grants from the CIHR to Michael R. Hayden and from Xenon Genetics to Michael R. Hayden. We thank A. Smit, J. Gort and P. Rol for their assistance in the vascular studies; and A.H.E.M. Klerkx for laboratory support.

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