ABCA1 mutation carriers with low high-density lipoprotein cholesterol are characterized by a larger atherosclerotic burden

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Received 13 June 2012; revised 22 August 2012; accepted 15 October 2012; online publish-ahead-of-print 7 November 2012

Aims
Low HDL-C is a potent risk factor for cardiovascular disease (CVD). Yet, mutations in ABCA1, a major determinant of circulating HDL-C levels, were previously not associated with CVD risk in cohort studies. To study the consequences of low plasma levels of high-density lipoprotein cholesterol (HDL-C) due to ATP-binding cassette transporter A1 (ABCA1) dysfunction for atherosclerotic vascular disease in the carotid arteries.

Methods and results
We performed 3.0 Tesla magnetic resonance imaging (MRI) measurements of the carotid arteries in 36 carriers of high impact functional ABCA1 mutations and 36 normolipidemic controls. Carriers presented with 42% lower HDL-C levels ($P = 0.001$), a larger mean wall area (18.6 $\pm$ 6.0 vs. 15.8 $\pm$ 4.3 mm$^2$; $P = 0.02$), a larger mean wall thickness (0.82 $\pm$ 0.21 vs. 0.70 $\pm$ 0.14 mm; $P = 0.005$), and a higher normalized wall index (0.37 $\pm$ 0.06 vs. 0.33 $\pm$ 0.04; $P = 0.005$) compared with controls, retaining significance after adjustment for smoking, alcohol consumption, systolic blood pressure, diabetes, body mass index, history of CVD, LDL-C, and statin use ($P = 0.002$).

Conclusion
Carriers of loss of function ABCA1 mutations display a larger atherosclerotic burden compared with age and sex-matched controls, implying a higher risk for CVD. Further studies are needed to elucidate the full function of ABCA1 in the protection against atherosclerosis. These data support the development of strategies to up-regulate ABCA1 in patients with established CVD.

Keywords
HDL • ABCA1 • Carotid MRI • Lipoproteins • Atherosclerosis

Introduction
Prospective studies have consistently shown that high-density lipoprotein cholesterol (HDL-C) is inversely correlated with cardiovascular risk.1–3 As a consequence, HDL-increasing strategies have been studied intensively to reduce the residual cardiovascular risk in statin-treated patients.4–6 The lack of specific and potent HDL-increasing compounds, however, has precluded us from answering the question whether HDL-C is a truly causal factor in atherogenesis.7 In fact, Briel et al.8 reported the absence of a correlation between HDL-C increase and CVD risk in a recent meta-analysis comprising data from $\sim$300,000 subjects having received lipid-modulating therapies, whereas low-density lipoprotein cholesterol (LDL-C) decrease was invariably associated with a decrease in cardiovascular risk. None of these lipid-modulating therapies, however, had as its primary objective to raise HDL-C. The absolute increases in circulating HDL-C levels in these studies were modest, raising the possibility of a power problem of the analyses. Moreover, the lack of effect of CETP inhibition,6,9 and nicotinic acid has increased the concerns regarding HDL-C as
a suitable target for the prevention of CVD, although these trials were troubled by either off-target effects or design issues. Combined with the fact that HDL-C levels are confounded by other risk factors, such as body mass index (BMI), triglycerides (TG), and smoking, this has cast doubt on the causal role of the HDL particle in atherogenesis. The most widely characterized mechanism by which HDL-C protects against atherosclerosis is reverse cholesterol transport. In this pathway, pivotal steps comprise the ABCA1 (adenosine triphosphate-binding cassette transporter A1)-mediated cholesterol efflux (GenBank No. AF275948) followed by esterification of cholesterol via lecithin–cholesterol acyltransferase (LCAT). Heterozygous ABCA1 mutation carriers are characterized by half-normal HDL-C levels. Schaefer et al. reported that 19% of ABCA1 heterozygotes and 45% of ABCA1 homozygotes had evidence of coronary artery or cerebrovascular disease, compared with 4% in the control population. In line, van Dam et al. reported the loss of efflux capacity in ABCA1 mutation carriers with a correspondingly increased carotid intima-media thickness (cIMT). In contrast, Frikke-Schmidt et al. reported that in 109 ABCA1 mutation carriers compared with 41 BS2 controls, the multifactorially adjusted OR for ischaemic heart disease was 0.93 (95% CI: 0.53–1.62) for heterozygous ABCA1 mutation carriers compared with controls.

We, therefore, decided to assess the effects of loss of function ABCA1 mutations on atherosclerosis. We performed carotid 3.0 Tesla magnetic resonance imaging (MRI) as well as cIMT imaging in ABCA1 mutation carriers and controls. Functionality of each ABCA1 mutation was verified using in vitro cholesterol efflux assays.

Methods
Study design
Subjects with low HDL-C levels, defined as HDL-C < 5th percentile, were selected from a cohort of hypopalphalipoproteinaemia patients and screened for ABCA1 mutations. Family members of ABCA1 mutation carriers were actively recruited. Carriers of functional ABCA1 gene mutations (n = 36) and controls (n = 36) matched for age and gender were enrolled in this study. Index patients were excluded if CVD was present at the time of referral. Furthermore, blood was obtained from 36 unaffected family members for lipid analysis. All participants provided written informed consent. The study was conducted at the Academic Medical Center in Amsterdam, the Netherlands from March 2010 to November 2011. The study protocol was approved by the Institutional Review Board.

The presence of cardiovascular risk factors, the use of medication, and a family history of CVD were assessed by a questionnaire. Blood pressures were measured using an oscillometric blood pressure device (Omron 705IT, Hoofddorp, the Netherlands). The BMI was calculated from weight and length. The HOMA index was calculated as (glucose × insulin)/22.5. Blood was obtained after an overnight fast and stored at −80°C. Plasma cholesterol, LDL-C, HDL-C, and TG were analysed using commercially available kits (Randox, Antrim, UK and Wako, Neuss, Germany). Plasma apolipoprotein AI and apolipoprotein B were measured using a commercially available turbidometric assay (Randox, Antrim, UK). All analyses were performed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

Genotyping
Mutation detection was performed as published previously. In short, the sequence reactions were performed using a BigDye terminator ABI prism kit (Applied Biosystems, Foster City, CA, USA). Sequences were analysed with the Sequencer package (Gene Codes Co, Ann Arbor, MI, USA).

Cholesterol efflux assays
ABCA1 mutation functionality was tested using skin fibroblasts (passage number 5–15), cultured in 24-well plates until 80% confluency. The cells were loaded with media containing 0.2% BSA, 30 μg/mL cholesterol, and 0.5 μCi/mL 3H-cholesterol for 24 h. After washing, the cholesterol efflux was started by the addition of 10 μg/mL apoA-I. After 4 h, the medium was collected and the amount of 3H-cholesterol was quantified by liquid scintillation counting. Cellular concentrations of 3H-cholesterol were measured after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the counts in the efflux medium by the sum of the counts in the medium plus the cell extract.

Carotid magnetic resonance imaging
Scans were performed as described previously. In short, scans were obtained in a 3.0 Tesla Philips whole-body scanner (Philips, Best, the Netherlands), using a single-element microcoil (Philips, Hamburg, Germany). Ten slices were scanned of the distal 3.0 cm of the left and right common carotid artery. A total of 20 images were obtained per scan. Images were saved in DICOM format using standardized protocols. Quantitative image analysis was performed using the semi-automated measurement software (VesselMass, Leiden University Medical Center, the Netherlands). One trained reader, with excellent scan–rescan and intra-observer variability assessed all the images using standardized protocols for reading and rating images combined with dedicated semi-automated software, blinded for all data of the participants. The mean wall thickness (MWT), lumen area (LA), outer wall area (OWA), and total wall volume (TWV) were measured. The normalized wall index (NWI) was calculated as: NWI = MWA/OWA. The mean wall area (MWA) is calculated as: MWA = meanOWA – meanLA. The prevalence of plaque components (PC) and the total PC volume (mm3) were also assessed. Plaque component was defined as an area with lower signal intensity within the arterial wall on a T1-weighted image, representing either lipid-rich tissue or calcification. The prevalence of PC was reported as the percentage of the total number of images that showed PC. The volume of PC’s was reported as the sum of all PC volumes of all subjects per group. A total of 46 slides, corresponding to 3% of the total, were excluded from the PC analysis due to insufficient quality.

Carotid ultrasound imaging
Carotid B-mode ultrasound scans of the left and right common, bulb, and internal carotid arterial far walls were assessed as previously published. One reader analysed all the images, blinded for group and any other data of the participants. The ultrasound parameter was the mean common carotid intima-media thickness (CIMT), defined as the average far wall IMT of the left and right distal 1 cm of the common carotid artery.

Outcome parameters
The NWI was the primary outcome parameter of the study. Secondary MRI outcome parameters were MWA (mm2), MWT (mm), and TWV (mm3). The secondary ultrasound parameter was CIMT (mm). Plaque
component analysis, expressed as mean ± standard deviations, unless otherwise specified. Possible differences in demographic, biometrical, and biochemical parameters between carriers of ABCA1 mutations and controls were assessed using unpaired Student’s t-tests, or Mann–Whitney U test, where appropriate. Differences in carotid imaging parameters between ABCA1 mutation carriers and controls were assessed using unpaired Student’s t-tests, unless otherwise specified. In addition, a linear regression model was used, in which carriership, smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of cardiovascular disease (CVD), LDL-C, and statin use were indicated as independent variables and NWI, MWA, MWT, TWV, and CcIMT were indicated as dependent variables. The authors had full access to the raw data and take responsibility for its integrity.

Statistical analysis
Continuous variables are expressed as means ± standard deviations, unless otherwise specified. Possible differences in demographic, biometrical, and biochemical parameters between carriers of ABCA1 mutations and controls were assessed using unpaired Student’s t-tests, or Mann–Whitney U test, where appropriate. Differences in carotid imaging parameters between ABCA1 mutation carriers and controls were assessed using unpaired Student’s t-tests, unless otherwise specified. In addition, a linear regression model was used, in which carriership, smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of cardiovascular disease (CVD), LDL-C, and statin use were indicated as independent variables and NWI, MWA, MWT, TWV, and CcIMT were indicated as dependent variables. The authors had full access to the raw data and take responsibility for its integrity.

Results
Baseline characteristics
ABCA1 mutation carriers from 14 separate families were included, comprising 2 homozygous, 2 compound heterozygous, and 32 heterozygous patients. Subjects were carriers of the following mutations: c.6401+2T>C, p.Ser930Phe, p.Ser824Leu, p.Arg587Trp, p.Thr929Ile, p.Asn935Ser, c.3535+1G>C, p.Asp571Gly, p.Asn1800his, p.Leu1056Pro, p.Gln1038Ter, p.Asn935Ser, c.3535+1G>C, p.Arg579Gln, and p.Phe1760Valfs*21. Controls from the general population were matched for age and gender (Table 1). Carriers displayed a 16% lower total cholesterol (P = 0.004; Table 1), largely due to a 42% reduction in HDL-C levels (P < 0.001). Apo B levels were higher by 10% in carriers (P = 0.19), while carriers had 32% lower apo A-I levels (P < 0.001; Table 1). Other parameters were not significantly different (Table 1).

Lipid profiles were measured in 36 unaffected family members of ABCA1 mutation carriers, matched for age and gender. Total cholesterol (4.35 ± 1.31 vs. 5.23 ± 1.10 mmol/L, P = 0.003) and HDL-C (0.84 ± 0.38 vs. 1.57 ± 0.41 mmol/L, P < 0.001) were lower in carriers compared with unaffected relatives, whereas LDL-C was comparable (3.11 ± 1.04 vs. 3.23 ± 0.92 mmol/L, P = 0.60). Triglycerides were higher in carriers compared with unaffected relatives [1.06 (0.78–1.39) vs. 0.77 (0.57–1.06) mmol/L, P = 0.002].

Cholesterol efflux assays
Fourteen mutations were found in the carriers. Five of these mutations have already been shown to have a significant impact on ABCA1 function (p.Asn1800his, p.Thr929Ile, p.Arg587Trp, p.Leu1056Pro, and p.Phe1760Valfs*21). The efflux capacity of the remaining nine mutations: p.Asn935Ser, c.3535+1G>C, p.Ser824Leu, p.Ser930Phe, p.Gln1038Ter, c.1195-1G>C, p.Arg579Gln, and p.Arg579Gln. The efflux capacity was assessed in heterozygotes, except for mutation p.Gln1038Ter which was tested in homozygous patient. Efflux measured in fibroblasts from a heterozygous p.Cys1477Arg carrier was used as a positive control since the efflux capacity has consistently been shown to be impaired. One patient compound heterozygous for mutation p.Arg579Gln and p.Val771Met was included. In the view of contradictory statements on functionality of mutation p.Val771Met, we assumed that the major part of the efflux impairment of the p.Arg579Gln and p.Val771Met combination is attributable to p.Arg579Gln.

Carotid magnetic resonance imaging and ultrasound
Magnetic resonance imaging data are shown in Table 2. The NWI was significantly higher in carriers compared with controls (P = 0.005) (Figure 2). Adjustment for differences in smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of CVD, LDL-C, and statin use resulted in an even stronger statistical significance (P = 0.002). The mean wall area, MWT, and TWV were also significantly higher in carriers compared with controls (P = 0.02, 0.005 and 0.02, respectively) and retained significance after adjustment for the above mentioned risk factors (P = 0.03, 0.002, and 0.03, respectively). Plaque components related to lipid-rich tissue or calcification (PC prevalence) were 2.5 times more prevalent in carriers compared with controls (P = 0.01, Figure 3) with a concomitant higher total PC volume (Table 2).

Ultrasound CcIMT was not higher in carriers compared with controls.

Discussion
In the present study, we show that carriers of the loss of function ABCA1 mutations exhibit more carotid artery wall thickening as assessed by MRI compared with age- and sex-matched controls. Both normalized wall index and MWA as well as thickness and TWV were significantly higher in carriers compared with controls. These differences retained significance after adjustment for traditional risk factors. In support, PCs were more prevalent in carriers compared with controls. Collectively, these findings support the concept that functional ABCA1 mutations, resulting in lower cholesterol efflux capacity, lead to more atherosclerotic vascular disease.

Our results show a higher burden of carotid atherosclerosis in ABCA1 mutation carriers. As expected, carriers presented with decreased cholesterol efflux capacity, which has been shown to be negatively correlated with an atherosclerotic burden. Carriers also presented with more arterial wall thickening. Carotid artery wall thickening is associated with a higher risk of cardiovascular events. In support, carriers were characterized by a higher prevalence of PCs. Recent data revealed that information on plaque composition in the carotid artery has a higher predictive value for CVD compared with a thickened cIMT per se.

In contrast, cIMT assessed using ultrasound was not different between carriers and controls. This discrepancy between carotid MRI and carotid ultrasound measurements most likely reflects the lower sensitivity and higher variability of cIMT measurements when compared with carotid 3.0-T MRI. We recently showed that heterozygous carriers of LCAT gene mutations also have
more carotid atherosclerosis using MRI, whereas cIMT did not reveal any difference. It should be noted that carotid ultrasound measures the wall thickness in a two-dimensional way, whereas atherosclerosis is a three-dimensional disease. Consequently, carotid MRI has been shown to yield superior power compared with ultrasound measurements, enabling smaller sample sizes to detect differences in the wall thickness.

The absence of an IMT difference contradicts earlier findings by van Dam et al. In their control group, IMTs were lower compared with our controls (0.67 vs. 0.73), one-third in our control group compared with our controls. Taking into account an IMT progression of 0.0047 mm/year, the calculated IMT of their controls at the age of 51 (0.69 mm) approximates the value observed in our control group (0.67 mm). With respect to the lower mean IMT in our ABCA1 mutation carriers (0.67 vs. 0.73), one-third of our carriers used statins when compared with the absence of statin use in the carriers reported by van Dam et al., whereas statins are known to reduce IMT progression by ≏13 years lower in their control group compared with our controls. Taking into account an IMT progression of 0.0047 mm/year, the calculated IMT of their controls at the age of 51 (0.69 mm) approximates the value observed in our control group (0.67 mm). With respect to the lower mean IMT in our ABCA1 mutation carriers (0.67 vs. 0.73), one-third (n = 11) of our carriers used statins when compared with the absence of statin use in the carriers reported by van Dam et al., whereas statins are known to reduce IMT progression by 0.02 mm within the first 6–12 months followed by a decreased IMT progression at a longer follow-up.

Genome-wide association studies revealed that ABCA1 correlates with HDL-C and total cholesterol, but not with incidence of CVD. A potential pitfall of GWAS studies, however, pertains to the fact that the effect of single nucleotide polymorphisms in a single gene on HDL-C levels is often small. This may result in an underestimation of the association between gene defects and CHD risk. Moreover, HDL is most strongly associated
with CHD risk in the case of low HDL-C levels. In GWAS studies, the extreme tails of HDL-C levels are typically under-represented. In most cases, SNPs result in small changes in HDL-C levels within the normal range. Consequently, no effect of the SNP on CHD risk is observed. Moreover, it is often unknown whether SNPs give rise to biologically relevant changes in ABCA1 function. As a consequence, findings from GWAS studies regarding the effect of ABCA1 on CVD risk have, only limited value, and cannot provide definite conclusions regarding gene function and associated CVD risk.

An alternative approach is the assessment of effects of genetic variation in ABCA1 using a Mendelian randomization approach as performed by Frikke-Schmidt et al.42 In line with our results, they report that in a prospective cohort comprising $\approx 9000$ individuals, heterozygosity for the ABCA1 mutation p.Lys776Asn led to a two-to-three-fold higher risk of ischaemic heart disease. They also reported that three genetic variations in ABCA1 predict risk of ischaemic heart disease.31 In a more recent prospective cohort study, they reported that heterozygosity for loss-of-function mutations was not associated with a higher risk of ischaemic heart disease.20 Several factors may have contributed to the contrasting findings when comparing their results to our data. First, the discrepancy may relate to methodological differences. Frikke-Schmidt et al.43 assessed functionality of the mutations using cholesterol efflux assays in transfected HeLa cells, mimicking homozygosity for the ABCA1 mutation. An efflux capacity of 79% was considered indicative of compromised efflux capacity, whereas in the case of heterozygosity, the efflux is expected

**Figure 1** Normalized cholesterol efflux from cultured skin fibroblasts to apoA-I. % Efflux is shown as mean ± SD, n = 3 separate experiments. Fibroblasts from a healthy control were used as a control.

**Figure 2** Normalized wall index in ATP-binding cassette transporter A1 mutation carriers vs. controls, corrected for smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of cardiovascular disease, low-density lipoprotein cholesterol, and statin use. Data are shown as mean ± SD.

**Table 2** Carotid 3.0 Tesla magnetic resonance imaging and B-mode ultrasound parameters for ATP-binding cassette transporter A1 mutation carriers and controls

<table>
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<tr>
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<th>ABCA1 mutation carriers (n = 36)</th>
<th>Controls (n = 36)</th>
<th>P^1</th>
<th>Adjusted (P^2)</th>
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<tr>
<td>3.0 Tesla MRI</td>
<td></td>
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<tr>
<td>NWI</td>
<td>0.37 (0.06)</td>
<td>0.33 (0.04)</td>
<td>0.005</td>
<td>0.002</td>
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<tr>
<td>MWA (mm^2)</td>
<td>18.6 (6.0)</td>
<td>15.8 (4.3)</td>
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<td>MWT (mm)</td>
<td>0.82 (0.21)</td>
<td>0.70 (0.14)</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>TWV (mm^3)</td>
<td>1116 (363)</td>
<td>946 (255)</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Plaque composition analysis</td>
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<tr>
<td>PC presence (%)</td>
<td>32/670 (5)</td>
<td>16/716 (2)</td>
<td>0.01</td>
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<tr>
<td>Total PC volume (mm^3)</td>
<td>230.8</td>
<td>74.4</td>
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<tr>
<td>B-Mode ultrasound</td>
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<tr>
<td>CcIMT (mm)</td>
<td>0.67 (0.22)</td>
<td>0.67 (0.16)</td>
<td>0.98</td>
<td>0.50</td>
</tr>
</tbody>
</table>

P^1 for the unadjusted model, P^2 for multivariate model adjusting for smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of cardiovascular disease (CVD), LDL-C, and statin use. CcIMT is the mean common carotid intima-media thickness. PC presence is reported as total number of images showing PC. Total PC volume is the sum of all PC volumes of all subjects per group. ABCA1, ATP-binding cassette transporter; NWI, normalized wall index; MWA, mean wall area; TWV, total wall volume; MWT, mean wall thickness; LA, lumen area; PC, plaque component.
to be 20–30%. This may have led to an inclusion of non-pathogenic mutations. In contrast, the efflux assays in our study were performed using patients’ fibroblasts, representing the actual biology of a heterozygous ABCA1-deficient cell. Since variability of the assay is considerable, we performed the experiments in triplicate. The inclusion of relatively mild mutations in the prospective cohort study of Frikke-Schmidt is further reflected by the fact that the HDL-C levels in carriers compared with controls is only 29% lower. HDL-C levels in the present study were 42% lower, which is in line with the earlier reported HDL-C levels in heterozygous ABCA1 mutation carriers.

**Limitations**

Several aspects of our study deserve closer attention. First, we assessed a surrogate endpoint for CVD, which precludes us from drawing final conclusions with regard to cardiovascular event risk. The low incidence of mutation carriers makes it impossible to perform prospective outcome studies in ABCA1 heterozygotes. In spite of the relatively low number of subjects, the present study does provide evidence of an adverse effect of ABCA1 dysfunction on the arterial wall. Secondly, we cannot exclude the potential impact of indirect referral bias. Although we excluded all index cases referred for CVD from the analysis, a positive family history for CVD is a strong predictor for CVD. Hence, we cannot exclude that family members of affected probands are characterized by thicker carotid artery walls due to factors beyond their ABCA1 mutation carriage.

**Clinical implications**

The present study shows that carriers of ABCA1 mutations display more carotid atherosclerosis compared with controls implying a higher CVD risk. These findings suggest that early and aggressive CVD preventive measures are warranted in ABCA1 mutation carriers. Collectively, our findings lend support to the concept that up-regulation of ABCA1 is an attractive target for future CVD risk reduction.

**Acknowledgements**

The authors would like to thank C.A.M. Koch and J.F. Los for their assistance in expanding the pedigrees, J. Peter for his role in identification of ABCA1 mutation carriers and A.W.M. Schimmel for her work on the cholesterol efflux experiments. The authors thank J.A. Kuivenhoven for his scientific advice and A.H. Zwinderman for his statistical advice.

**Funding**

This work was supported by the Dutch Heart Foundation (grant number 2009B027) and by an educational grant by Merck Sharp Dohme (MSD, Haarlem, the Netherlands). Prof. Kastelein is a recipient of The Lifetime Achievement Award of The Dutch Heart Foundation (2011, the Hague, the Netherlands).

**Conflict of interest:** none declared.

**References**


