Increased Systemic and Plaque Inflammation in \textit{ABCA1} Mutation Carriers With Attenuation by Statins

Andrea E. Bochem, Fleur M. van der Valk, Sonia Tolani, Erik S. Stroes, Marit Westerterp,* Alan R. Tall*

\textbf{Objective}—We previously demonstrated that subjects with functional \textit{ATP-binding cassette (ABC) A1} mutations have increased atherosclerosis, which has been attributed to the role of \textit{ABCA1} in reverse cholesterol transport. More recently, a proinflammatory effect of \textit{Abca1} deficiency was shown in mice, potentially contributing to atherogenesis. In this study, we investigated whether \textit{ABCA1} deficiency was associated with proinflammatory changes in humans.

\textbf{Approach and Results}—Thirty-one heterozygous, 5 homozygous \textit{ABCA1} mutation carriers, and 21 matched controls were studied. \textsuperscript{18}Fluorodeoxyglucose positron emission tomography with computed tomographic scanning was performed in a subset of carriers and controls to assess arterial wall inflammation (target:background ratio). Heterozygous \textit{ABCA1} mutation carriers had a 20\% higher target:background ratio than in controls (target:background ratio; $P=0.008$). In carriers using statins (n=7), target:background ratio was 21\% reduced than in nonstatin users (n=7; $P=0.03$). We then measured plasma cytokine levels. Tumor necrosis factor \textalpha, monocyte chemoattractant protein-1, and interleukin-6 levels were increased in heterozygous and homozygous \textit{ABCA1} mutation carriers. We isolated monocytes from carriers and controls and measured inflammatory gene expression. Only \textit{TNF} \textit{α} mRNA was increased in monocytes from heterozygous \textit{ABCA1} mutation carriers. Additional studies in THP-1 macrophages showed that both \textit{ABCA1} deficiency and lipoprotein-deficient plasma from \textit{ABCA1} mutation carriers increased inflammatory gene expression.

\textbf{Conclusions}—Our data suggest a proinflammatory state in \textit{ABCA1} mutation carriers as reflected by an increased positron emission tomography–MRI signal in nonstatin using subjects, and increased circulating cytokines. The increased inflammation in \textit{ABCA1} mutation carriers seems to be attenuated by statins. (\textit{Arterioscler Thromb Vasc Biol.} 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.304959.)

\textbf{Key Words:} genetics ■ lipids ■ positron-emission tomography

High-density lipoprotein cholesterol (HDL-C) levels are inversely correlated with cardiovascular risk. The atheroprotective effects of HDL have traditionally been attributed to its role in reverse cholesterol transport. The \textit{ATP-binding cassette transporter A1} (\textit{ABCA1}) plays a crucial role in mediating cholesterol efflux from peripheral cells, including arterial wall macrophages, to lipid-poor apolipoprotein A1 or pref HDL particles. Homozygous \textit{ABCA1} mutation carriers display near absent HDL-C levels, whereas heterozygous carriers are characterized by half-normal HDL-C. Single nucleotide polymorphisms in the \textit{ABCA1} gene have variously been reported to have no impact on cardiovascular disease, or to be associated with an increased cardiovascular disease risk. However, studies in \textit{ABCA1} mutation carriers, displaying marked defects in cholesterol efflux and profound decreases in HDL levels, showed increased arterial wall thickness, and cardiovascular disease risk in carriers than in controls.

The paradigm that a macrophage-dominated inflammatory process, initiated by the deposition of cholesterol-rich lipoproteins in the arterial wall, is central to atherosclerosis has been widely accepted. The molecular mechanisms linking defective cholesterol homeostasis to increased inflammation are not well understood. Recent studies have implicated defective cellular cholesterol efflux pathways in increased inflammatory gene expression in monocytes and macrophages, as well as the increased production of inflammatory cells such as monocytes and neutrophils. Deficiency of \textit{ABCA1} or \textit{ABCG1} is associated with a proinflammatory phenotype in mouse peritoneal macrophages, as well as in the macrophages of atherosclerotic plaques. Whether \textit{ABCA1} deficiency in humans represents a proinflammatory state is presently unknown.
In this study, we assessed whether ABCA1 mutation carriers exhibit proinflammatory changes in the arterial wall as measured by 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography with computed tomography (PET/CT). We also measured plasma cytokine levels and assessed inflammatory gene expression in ABCA1-deficient monocytes/macrophages in vitro.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.¹⁷

Results
Baseline Characteristics
Baseline characteristics of study participants are listed in the Table. Thirty-six ABCA1 mutation carriers from 14 separate families were included, comprising 3 homozygous, 2 compound heterozygous, and 31 heterozygous patients. Homozygous and compound heterozygous subjects had Tangier Disease. Subjects were carriers of the following mutations: p.Leu1056Pro, p.Asn1800his, p.Thr929Ile, c.3535+1G>C, c.6401+2T>C, p.Asn1800his, p.Ser930Phe, p.Phe1760Valfs*21, p.Ser824Leu, p.Gln1038Ter, p.Thr929Ile, p.Arg587Trp, p.Asn935Ser, and p.Arg579Gln. Heterozygosity for these mutations has been shown to impair cholesterol efflux by 40% to 85%.¹⁷⁻²⁰ Fourteen of the 36 ABCA1 mutation carriers were on statin therapy, including 3 homozygous subjects. Statin users were excluded by 40% to 85%.¹⁷⁻²⁰ Fourteen of the 36 ABCA1 mutation carriers from 14 separate families were included, comprising 3 homozygous, 2 compound heterozygous, and 31 heterozygous patients. Homozygous and compound heterozygous subjects had Tangier Disease. Subjects were carriers of the following mutations: p.Leu1056Pro, p.Asn1800his, p.Thr929Ile, c.3535+1G>C, c.6401+2T>C, p.Asn1800his, p.Ser930Phe, p.Phe1760Valfs*21, p.Ser824Leu, p.Gln1038Ter, p.Thr929Ile, p.Arg587Trp, p.Asn935Ser, and p.Arg579Gln. Heterozygosity for these mutations has been shown to impair cholesterol efflux by 40% to 85%.¹⁷⁻²⁰

Vascular 18F-FDG PET/CT
18F-FDG PET/CT (18F-FDG PET/CT) scanning was performed in a random subset of heterozygous ABCA1 mutation carriers (n=14) and controls (n=15). In the whole group, the target: background ratio (TBR) was not significantly different in heterozygous ABCA1 mutation carriers than in controls (data not shown). However, the average mean TBR of the left and right carotid was 20% higher in nonstatin using heterozygous ABCA1 mutation carriers than in statin using heterozygous ABCA1 mutation carriers (P=0.03 for left mean TBR; P=0.006 for right mean TBR; Figure 1). After excluding the heterozygous ABCA1 mutation carriers using statins, the mean TBR in the left and right carotid was higher in heterozygous ABCA1 mutation carriers than in controls (P=0.06 and 0.02, respectively; Figure 1). Maximum TBR was significantly higher for the right carotid (P=0.008; Figure 1) and showed a trend to an increase in the left carotid (P=0.06). In Figure II in the online-only Data Supplement, representative images of CT and 18F-FDG PET/CT right carotid arteries of heterozygous ABCA1 mutation carriers, controls, and heterozygous ABCA1 mutation carriers using statins are displayed.

Because the TBR signal depends on glucose uptake in macrophages in the arterial wall¹¹ and ABCA1 has been reported to have a role in glucose uptake,²² we evaluated a potential direct effect of ABCA1 expression on macrophage glucose uptake. Macrophage-glucose uptake did not differ between heterozygous ABCA1 mutation carriers and controls (Figure III in the online-only Data Supplement), indicating that the differences in TBR signal cannot be explained by a direct effect of ABCA1 on glucose uptake.

Systemic Inflammatory Phenotype in ABCA1 Mutation Carriers
To assess whether the apparent inflammatory phenotype in the arterial wall of heterozygous ABCA1 mutation carriers also manifested itself systemically, plasma cytokines were measured in both heterozygous and homozygous ABCA1 mutation carriers. Plasma levels of tumor necrosis factor α (TNFα) were significantly higher in homozygous ABCA1 mutation carriers versus controls (Figure 2A). In line with the PET/CT data, TNFα levels were significantly higher in nonstatin using ABCA1 heterozygous mutation carriers than in statin using heterozygous carriers who had levels similar to controls (Figure 2A). TNFα levels also seemed to be higher in the nonstatin using homozygous carriers than in statin using homozygous carriers. Plasma levels of monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) were increased in heterozygous ABCA1 mutation carriers and homozygous ABCA1 mutation carriers than in controls (Figure 2B). In contrast to TNFα, no effect of statin use was observed (Figure 2B and 2C).

Inflammatory Gene Expression in Monocytes From Heterozygous ABCA1 Carriers
To assess whether monocytes showed increased inflammatory gene expression, we isolated CD14+ monocytes from heterozygous ABCA1 mutation carriers and controls and measured TNFα, IL-1β, MCP-1, and IL-6 mRNA expression.

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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</thead>
<tbody>
<tr>
<td>ABCA1/G1</td>
</tr>
<tr>
<td>18F-FDG</td>
</tr>
<tr>
<td>HDL-C</td>
</tr>
<tr>
<td>IL-1β/6</td>
</tr>
<tr>
<td>MCP-1</td>
</tr>
<tr>
<td>PET/CT</td>
</tr>
<tr>
<td>TBR</td>
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<tr>
<td>TNFα</td>
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¹¹ Materials and Methods are available in the online-only Data Supplement.
TNFα mRNA was increased in heterozygous ABCA1 mutation carriers than in controls (2.5-fold; \( P<0.01 \)) (Figure 3). Interestingly, statin use did not affect the increase in TNFα mRNA. Expression of \( \text{IL-1β, MCP1, and IL-6} \) was not different from controls (Figure 3).

### ABCA1 Deficiency and Macrophage Inflammation

To determine whether the increased plasma inflammatory cytokines and decreased HDL from \( \text{ABCA1} \) mutation carriers could contribute to enhanced macrophage inflammation, we added polyethylene glycol supernatant (apolipoprotein B-depleted plasma still containing HDL) from controls, heterozygous, and homozygous \( \text{ABCA1} \) mutation carriers to THP-1 macrophages. Only the apolipoprotein B-depleted plasma from homozygous \( \text{ABCA1} \) mutation carriers increased mRNA expression of inflammatory cytokines (Figure 5A). This could reflect the virtual absence of HDL in the plasma from homozygous subjects, whereas the half normal levels of HDL in the heterozygous subjects may have been sufficient to suppress inflammatory cytokine production. To test this idea further, we added pooled lipoprotein-deficient serum from controls, heterozygous, and homozygous \( \text{ABCA1} \) mutation carriers to THP-1 macrophages. Lipoprotein-deficient serum from heterozygous \( \text{ABCA1} \) mutation carriers increased TNFα, \( \text{IL-1β, IL-6} \), and \( \text{MCP-1} \) mRNA expression, whereas lipoprotein-deficient serum from homozygous \( \text{ABCA1} \) mutation carriers caused a more marked widespread increase in mRNA

### Proinflammatory Effects of Apolipoprotein B-Depleted Plasma and Lipoprotein-Deficient Plasma From ABCA1-Deficient Subjects

#### Table.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=21)</th>
<th>No Statin Tot (n=22)</th>
<th>No Statin Het (n=20)</th>
<th>No Statin Hom (n=2)</th>
<th>Statin Tot (n=14)</th>
<th>Statin Het (n=11)</th>
<th>Statin Hom (n=3)</th>
<th>P value2</th>
<th>P value1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>51.0±11.3</td>
<td>50.2±13.1</td>
<td>50.7±12.6</td>
<td>41.9±22.9</td>
<td>54.6±14.6</td>
<td>54.6±16.0</td>
<td>54.7±10.5</td>
<td>0.37</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>Male, n (%)</strong></td>
<td>9 (43)</td>
<td>11 (48)</td>
<td>9 (45)</td>
<td>1 (50)</td>
<td>6 (43)</td>
<td>5 (46)</td>
<td>1 (33)</td>
<td>0.77</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Body mass index, kg/m²</strong></td>
<td>24.1±3.1</td>
<td>25.8±3.8</td>
<td>26.1±3.8</td>
<td>22.4±1.5</td>
<td>25.8±4.2</td>
<td>27.9±4.5</td>
<td>24.5±7.1</td>
<td>1.00</td>
<td>0.10</td>
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<tr>
<td><strong>Smokers, n (%)</strong></td>
<td>2 (10)</td>
<td>5 (25)</td>
<td>5 (25)</td>
<td>0 (0)</td>
<td>1 (8)</td>
<td>1 (9)</td>
<td>0 (0)</td>
<td>0.21</td>
<td>0.52</td>
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<tr>
<td><strong>Diabetes mellitus, n (%)</strong></td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>1 (8)</td>
<td>1 (9)</td>
<td>0 (0)</td>
<td>0.74</td>
<td>0.80</td>
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<tr>
<td><strong>Statin use, n (%)</strong></td>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>14 (100)</td>
<td>11 (100)</td>
<td>3 (100)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

**Blood pressure**

<table>
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<tr>
<th>Parameters</th>
<th>Values</th>
<th>P value1</th>
<th>P value2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systolic, mm Hg</strong></td>
<td>129 (122–138)</td>
<td>0.11</td>
<td>0.75</td>
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<tr>
<td><strong>Diastolic, mm Hg</strong></td>
<td>80 (74–85)</td>
<td>0.86</td>
<td>0.74</td>
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<tr>
<td><strong>Hypertension, n (%)</strong></td>
<td>3 (14)</td>
<td>0.09</td>
<td>0.60</td>
</tr>
</tbody>
</table>

**Lipid metabolism**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
<th>P value1</th>
<th>P value2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol, mmol/L</strong></td>
<td>3.59±0.82</td>
<td>0.007</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>LDL-cholesterol, mmol/L</strong></td>
<td>3.49±0.79</td>
<td>0.009</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>HDL-cholesterol, mmol/L</strong></td>
<td>1.53±0.40</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/L)</strong></td>
<td>1.01 (0.64–1.42)</td>
<td>0.96</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Apolipoprotein B, mg/dL</strong></td>
<td>110.6±21.44</td>
<td>0.37</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Apolipoprotein A-I, mg/dL</strong></td>
<td>161.99±19.87</td>
<td>0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Vessel wall thickness</strong></td>
<td><strong>Values</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NWI</strong></td>
<td>0.32±0.03</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>MWT, mm</strong></td>
<td>0.66±0.11</td>
<td>0.43</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Values are indicated as mean±SD unless otherwise indicated. P value for student t-test, than in control, unless otherwise specified. P value1 tests ABCA1 mutation carriers vs controls; P value2 tests ABCA1 mutation carriers not on statins vs ABCA1 mutation carriers on statins. HDL indicates high-density lipoprotein; Het, heterozygous subjects; hom, homozygous subjects; LDL, low-density lipoprotein; MWT, mean wall thickness; N.a., not applicable, and NWI, normalized wall index.**

*Parameters tested by means of \( \chi² \) test.

†Parameters for which median and interquartile range are given and testing was performed by Mann–Whitney U test.
expression of inflammatory cytokines (TNFα, IL-1β, MCP-1, IL-8, and inducible nitric oxide synthase) (Figure 5B). These experiments suggest that in ABCA1 carriers half normal HDL levels may be sufficient to counteract the effect of proinflammatory cytokines in plasma, whereas in homozygous subjects the near absence of HDL is permissive for the proinflammatory effect. Thus, in heterozygotes, partial ABCA1 deficiency (comparable with the partial knockdown in Figure 4) in monocytes and macrophages may make a key contribution to inflammatory cytokine expression.

Discussion

ABCA1 mutation carriers displayed both increased vessel wall inflammation as assessed by 18F-FDG PET/CT and increased systemic inflammation as reflected by a proinflammatory plasma cytokine profile and increased inflammatory gene expression in circulating monocytes. In vitro experiments with human THP-1 macrophages revealed a proinflammatory effect of lipoprotein-deficient serum from heterozygous and homozygous ABCA1 mutation carriers, most likely secondary to increased levels of plasma cytokines, as well as a cell intrinsic effect of ABCA1 deficiency. Both cellular ABCA1 deficiency and reduced levels of plasma HDL may contribute to increased monocyte and macrophage inflammatory responses; however, our findings suggest that in heterozygotes cellular ABCA1 deficiency may have the predominant role, whereas in homozygous subjects, both cellular ABCA1 deficiency and the absence of plasma HDL may also contribute. Our findings are consistent with studies in mouse macrophages, in which genetic deficiency of Abca1 leads to enhanced inflammatory gene expression.13-16 This has been attributed to increased plasma membrane lipid raft formation promoting signaling via toll-like receptors 2, 3, and 4.14-16,23 Our data show an association between ABCA1 deficiency and increased systemic and plaque inflammation in humans, probably contributing to the increased atherosclerotic plaque volume that has been observed in ABCA1 mutation carriers.10,11

18F-FDG PET/CT has emerged as a reliable noninvasive technique for visualization of metabolic activity in the arterial wall in humans.12 Metabolic activity likely reflects the inflammatory state of the arterial wall because the arterial uptake of 18F-FDG has been shown to correlate with circulating inflammatory biomarkers,25 inflammatory gene expression,26 cardiovascular disease risk factors,27 and the number of plaque macrophages.28 We showed that the 18F-FDG uptake in the arterial wall of nonstatin using heterozygous ABCA1 mutation carriers was increased than in matched controls, paralleling an increase in vessel wall thickness. Although suspected based on studies in macrophages from Abca1 mice,13-16 this is the first confirmation of an in vivo role for ABCA1 in the suppression of inflammation in humans.

The finding of increased vessel wall inflammation in heterozygous ABCA1 mutation carriers is likely to contribute to their increased cardiovascular risk,5,9 because carotid arterial wall 18F-FDG uptake has been associated with increased cardiovascular risk, independent of the degree of stenosis.29,30

Interestingly, the increased inflammatory status in the vessel wall of heterozygous ABCA1 mutation carriers was manifested systemically because plasma levels of TNFα, MCP-1, and IL-6 were also increased. This is consistent with previous reports, showing that ABCA1 suppresses secretion of IL-1β, IL-6, and TNFα.15,16,31,32 Furthermore, TNFα mRNA expression was increased in circulating monocytes, consistent with a systemic proinflammatory state. These findings are also consistent with reports that plasma C-reactive protein levels are

![Figure 1](image1.png)

**Figure 1.** Increased vessel wall inflammation in heterozygous ABCA1 mutation carriers. Vessel wall inflammation was assessed by positron emission tomography with computed tomography in controls (n=15), and heterozygous ABCA1 mutation carriers without (n=7) and with statin (n=7) treatment. Data are presented as means±SEM. P-values are indicated. TBR indicates target: background ratio.

![Figure 2](image2.png)

**Figure 2.** Proinflammatory cytokines in plasma of controls, heterozygous (het), and homozygous (hom) ABCA1 mutation carriers. Plasma was isolated from controls, heterozygous ABCA1 mutation carriers not using (ABCA1 het) or using a statin (ABCA1 het statin), and homozygous ABCA1 mutation carriers not using (ABCA1 hom) or using a statin (ABCA1 hom statin). Levels of proinflammatory cytokines were assessed using ELISA. A, Tumor necrosis factor α (TNFα), B) monocyte chemotactic protein-1 (MCP-1), and (C) interleukin-6 (IL-6). In all graphs, each datapoint represents 1 patient or control. The mean is indicated. *P<0.05, **P<0.01, and ***P<0.001.
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Increased Inflammation in \(ABCA1\) Mutation Carriers

Inflammatory mediators of plaque inflammation are associated with ABCA1 mRNA levels in human peripheral monocytes. The cross-sectional design of this study precludes us from answering whether plaque inflammation is causal or secondary to atherosclerosis. However, because of the increases in inflammatory mediators in our study are secondary to genetic changes in ABCA1, and knock-down of ABCA1 increases inflammatory gene expression, it is reasonable to conclude that excessive plaque inflammation contributes to increased atherosclerotic burden. Our findings suggest an anti-inflammatory effect of statin treatment in humans as determined by both reduced \(^{18}\)F-FDG uptake in the arterial wall and decreased circulating levels of cytokines. Although the effect of statins on \(^{18}\)F-FDG PET/CT signal in atherosclerotic subjects is in line with previous publications, the ≈20% decrease in TBR in statin users in our study is larger than the ≈10%\(^{14}\) and ≈9%\(^{36}\) decreases in other reports. This may be explained by the short treatment period of 3 to 6 months in these intervention trials versus long-term use in our patients or the increased inflammatory status of \(ABCA1\) mutation carriers.

Interestingly, among the inflammatory cytokines measured, \(TNF\alpha\) mRNA levels were clearly increased in monocytes of \(ABCA1\) heterozygotes and \(TNF\alpha\) protein levels were increased in plasma; however, statins lowered \(TNF\alpha\) plasma levels while appearing not to affect monocyte \(TNF\alpha\) mRNA levels. \(TNF\alpha\) is an important inflammatory cytokine that has...
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Figure 5. Effect of apolipoprotein B (apoB)–depleted plasma and lipoprotein-deficient plasma from ABCA1 mutation carriers on macrophage inflammation. A, ApoB was peg-precipitated from plasma and apoB-depleted plasma was incubated overnight with THP-1 macrophages (n=13 per group; n=4 for homozygous ABCA1 mutation carriers). B, Plasma samples (n=10 per group; n=4 for homozygous ABCA1 mutation carriers) were pooled and lipoprotein-deficient serum was obtained after ultracentrifugation of ADAM17 (TACE).37 This raises the intriguing possibility of proatherogenic effects especially on the endothelium and smooth muscle cells. TNFα is synthesized as a type 2 membrane protein and released from cells as a result of the activity of Adam17 (TACE).37 This raises the intriguing possibility of an independent effect of statins on TNFα processing.

In conclusion, our data demonstrate a proinflammatory state in heterozygous and homozygous ABCA1 mutation carriers as reflected by increased circulating cytokines. This is attributed to a cellular effect of ABCA1 deficiency with an additional contribution of lower HDL levels especially in homozygous subjects. Our findings suggest that the increased inflammation documented in ABCA1-deficient cells and animal models14–16,23 is also present in humans. The increased inflammation in ABCA1 mutation carriers, documented by an increased carotid 18F-FDG PET/CT signal, seems to be attenuated by statins, as shown by normalization of 18F-FDG PET/CT and plasma cytokine levels.

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Disclosures
A.R. Tall is a consultant to Amgen, Arisaph, and CSL. The other authors report no conflicts.

References


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Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2015/02/19/ATVBAHA.114.304959.DC1

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**Supplemental Figure I.** Vessel wall thickness in controls and heterozygous ABCA1 mutation carriers. Vessel wall thickness was assessed by 3.0 Tesla magnetic resonance imaging (3T-MRI) in controls (n=21), heterozygous ABCA1 mutation carriers without (n=14) and with (n=7) statin treatment. TBR denotes target to background ratio; NWI normalized wall index, and MWT mean wall thickness. Data are presented as mean ± SEM and p-values for student’s t-test are indicated.
Supplementary Figure II. Representative CT and \(^{18}\)F-FDG PET/CT images of the right carotid artery. Top: CT images. A white circle delineates the arterial wall of the right carotid artery. Bottom: matching \(^{18}\)F-FDG PET/CT images of the right carotid artery indicated by a white arrow in a control subject (left), a non-statin using heterozygous \(ABCA1\) mutation carrier (middle) and a statin-using heterozygous \(ABCA1\) mutation carrier (right). Arterial wall \(^{18}\)F-FDG uptake is increased in non-statin using \(ABCA1\) mutation carriers. Scale bars indicate 2 cm.
Supplementary Figure III. 2-NB Deoxyglucose uptake in macrophages from controls and heterozygous \textit{ABCA1} mutation carriers. Peripheral blood mononuclear cells were isolated from controls (n=9) and heterozygous \textit{ABCA1} mutation carriers (n=9) and differentiated into macrophages. Uptake of the glucose analogue 2-NBDG was measured using flow cytometry. MFI indicates mean fluorescent intensity. Each datapoint represents one condition. N.s. is non significant.
Supplementary Figure IV. ABCA1 siRNA knock-down efficiency in THP-1 macrophages. ABCA1 siRNA was used to knock-down ABCA1 in human THP-1 macrophages and a suitable scrambled siRNA was used as a control. After 48 h, ABCA1 (A) and ABCG1 mRNA expression (B) was assessed and corrected for the housekeeping gene cyclophilin. n=12. (C) ABCA1 protein expression was assessed by Western blot. (D) Quantification of ABCA1 protein, corrected for β-actin. n=5. Data are presented as mean ± SEM. **P<0.01, ***P<0.001.
SUPPLEMENTAL METHODS

Study participants

Subjects with low HDL-C levels, defined as HDL-C < 5\textsuperscript{th} percentile, were selected from a cohort of hypoalphalipoproteinemia patients\textsuperscript{1} and screened for ABCA1 (GenBank No. AF275948) mutations. Family members of ABCA1 mutation carriers were recruited. Carriers of functional ABCA1 gene mutations and controls matched for age and gender were enrolled in this study. Non-carrier family members or spouses were included if they could be matched for age with a carrier. Because insufficient numbers of unaffected family controls volunteered, we complemented the control group with unrelated controls recruited by advertisement. Body mass index was calculated from weight and length. Hypertension was defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg or use of antihypertensive medication. Blood was obtained after an overnight fast and stored at -80 °C. All participants provided written informed consent. The study protocol was approved by the Institutional Review Board at the AMC, The Netherlands.

Genotyping

Mutation detection was performed as published previously.\textsuperscript{1} 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 Sequencher package (Gene Codes Co, Ann Arbor, Mi, USA).

Plasma processing

Blood samples were collected from all subjects after 12-hour fasting. Plasma cholesterol, LDL-C, HDL-C and triglycerides (TG) were measured using commercially available kits (Randox, Antrim, United Kingdom and Wako, Neuss, Germany). Plasma apolipoprotein AI and apolipoprotein B were measured using a turbidometric assay (Randox, Antrim, United Kingdom). All analyses were performed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland). For experiments with the combined HDL and lipoprotein deficient plasma (referred to as LPDS) fraction, to precipitate apoB, 100 µl plasma from each patient was incubated with 40 µl of 20% polyethyleneglycol (PEG, Sigma P-2139 in 200mM glycine, pH10) solution. This mixture was incubated at room temperature for 15 min. After this incubation, the solution was centrifuged at 4000 rpm for 10 min. 50 µl of the supernatant was used per reaction. For experiments with LPDS alone, plasma from 10 controls or ABCA1 mutation carriers, or from 4 TD patients, was pooled, and LPDS was isolated by ultracentrifugation according to Havel and co-workers.\textsuperscript{2} LPDS was dialyzed against PBS before use in experiments.

Carotid $^{18}$F-FDG PET-CT

Carotid $^{18}$F-FDG PET-CT scanning was performed in 14 ABCA1 mutation carriers and 15 controls. Seven of the ABCA1 mutation carriers were using statin therapy. Scans were performed on a Gemini time-of-flight multidetector helical PET/CT scanner (4 min/bed position) (Philips, Best, the Netherlands) as reported previously.\textsuperscript{3,5} Subjects fasted for at least 6 h before i.v. injection of $^{18}$F-FDG (200 MBq, 5.5 mCi). After 90 minutes of $^{18}$F-FDG circulation time, subjects underwent PET/CT imaging according to a previously validated acquisition and reconstruction protocol.\textsuperscript{4,5} Mean and maximum standardized uptake values (SUV) were measured in both carotids at 5 mm intervals on axial slices. SUV is a widely used PET quantifier, calculated as a time-corrected and dose-corrected ratio of tissue radioactivity divided by body weight. Then, target to background ratio (TBR) was
calculated from the ratio of arterial SUV of right and left common carotid artery compared with the background activity in the jugular veins. Both TBR$_\text{max}$, the mean of maximum SUV values and TBR$_\text{mean}$, the average of mean TBR values derived from every axial section of the vessel were calculated. Images were analysed by two experienced readers, blinded for patient data.

**In vitro glucose uptake**

Monocytes from controls and ABCA1 mutation carriers were treated with 100nM phorbol myristate acetate (PMA) for 72 h to stimulate differentiation into macrophages. Cells were cultured with 40 µM 2-NBDG (a fluorescently-labeled deoxyglucose analog) and washed. Cellular 2-NBDG uptake was measured by flow cytometry.

**Plasma cytokine measurements**

Plasma levels of tumor necrosis factor α (TNFα), monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) were measured using ELISA (R&D systems, Minneapolis, USA).

**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 semi-automated measurement software (VesselMass, Leiden University Medical Center, the Netherlands). One trained reader, with excellent scan-rescan and intraobserver variability analyzed all the images using standardized protocols for reading and rating images, combined with dedicated semi-automated software, blinded for all data of the participants. Mean wall thickness (MWT), lumen area (LA) and outer wall area (OWA) were measured. Mean wall area (MWA) is calculated as meanOWA minus meanLA. Normalized wall index (NWI) was calculated as: NWI= MWA / OWA.

**mRNA expression levels**

Total RNA was extracted from human monocytes or macrophages using an RNeasy Micro or Mini kit (Qiagen), respectively. For monocytes, RNA was synthesized using SuperScript VILO and for macrophages using M-MLV (Invitrogen). mRNA levels of TNFa, IL-1β, MCP-1, IL-6, IL-8, and iNOS were assessed using qPCR on a Stratagene Mx3000P (Agilent Technologies), and initial differences in RNA quantity were corrected for using the housekeeping gene cyclophilin.

**Monocyte isolation**

Blood was collected in a BD vacutainer CPT (REF 362753) after an o/n fast. Whole blood was centrifuged through a Ficoll gradient, and peripheral blood mononuclear cells (PBMCs) were collected from the buffy coat, washed, and frozen. From the PBMC population, CD14+ monocytes were isolated using CD14+ coated beads (Miltenyi Biotec). After isolation, CD14+ monocytes were lysed immediately in RLT buffer and RNA was extracted, cDNA synthesized, and mRNA expression assessed as described above.
THP-1 cell culture

THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% pen-strep at 37°C in 5% CO₂. For experiments, cells were plated at a concentration of 5*10⁵ cells/ml and incubated with 100nM PMA for 72 h to stimulate differentiation into macrophages. Macrophages were then washed, and incubated o/n with RPMI 1640 medium containing 2.5% LPDS from controls, heterozygous ABCA1 mutation carriers, or TD patients. Cells were lysed and RNA was extracted, cDNA synthesized, and mRNA expression assessed as described above. In other experiments, ABCA1 was knocked down in THP-1 macrophages by use of siRNA. ABCA1 and scrambled siRNA were from Thermo Scientific. For knockdown experiments, THP-1 macrophages were transfected with 80 nM siRNA in OPTIMEM using Lipofectamine RNAiMAX (Invitrogen). At 48 h after transfection, cells were treated o/n with or without reconstituted HDL (rHDL) at 50 µg/ml, which was kindly provided by CSL Australia. After the incubation, cells were lysed and RNA was extracted. Inflammatory gene expression was assessed as described above.

Statistics

All data are presented as means ± SEM or SD. Student’s t-test was used to test for differences between two datasets. To define differences between more datasets, One-way Analysis of Variance (ANOVA) was used with a Bonferroni multiple comparison post-test. The criterion for significance was set at \( P<0.05 \). In the Table, p-value for \( \chi^2 \) test is given for male sex, smokers, diabetes, statin use and hypertension. For non-normally distributed data (systolic blood pressure, diastolic blood pressure, triglycerides) median and interquartile range are given and testing was performed by Mann Whitney U test. Statistical analyses were performed using GraphPad Prism version 5.01 (San Diego, CA, USA) and PASW statistics 18 (Chicago, IL, USA).
References


