Identification and targeting of genes in atherosclerosis
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ABCA8 regulates cholesterol efflux and high density lipoprotein cholesterol levels


Arterioscler Thromb Vasc Biol 2017; epub Sept 7, 2017
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ABSTRACT

Objective: High-density lipoproteins (HDL) are considered to protect against atherosclerosis in part by facilitating the removal of cholesterol from peripheral tissues. However, factors regulating lipid efflux are incompletely understood. We previously identified a variant in ATP binding cassette transporter A8 (ABCA8) in an individual with low HDL cholesterol (HDLc). Here we investigate the role of ABCA8 in cholesterol efflux and in regulating HDLc levels.

Approach and results: We sequenced ABCA8 in individuals with low and high HDLc and identified, exclusively in low-HDLc probands, three predicted deleterious heterozygous ABCA8 mutations (p.Pro609Arg (P609R), IVS17-2 A>G and p.Thr741Stop (T741X)). HDLc levels were lower in heterozygous mutation carriers compared to first degree family controls (0.86 ± 0.34 vs. 1.17 ± 0.26 mmol/L, p = 0.005). HDLc levels were significantly decreased by 29% (p = 0.01) in Abca8b⁻/⁻ mice on a high cholesterol diet compared to wild-type mice, whereas hepatic over-expression of human ABCA8 in mice resulted in significant increases in plasma HDLc and the first steps of macrophage-to-feces reverse cholesterol transport. Over-expression of wild-type but not mutant ABCA8 resulted in a significant increase (1.8-fold, p = 0.01) of cholesterol efflux to ApoA-I in vitro. ABCA8 co-localizes and interacts with ABCA1 and further potentiates ABCA1-mediated cholesterol efflux.

Conclusions: ABCA8 facilitates cholesterol efflux and modulates HDLc levels in humans and mice.
INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death worldwide. Prospective epidemiological studies have established a robust inverse correlation between high-density lipoprotein cholesterol (HDLc) levels and risk for CVD. However, simply raising HDLc levels may be insufficient to protect against coronary artery disease (CAD). These seemingly counterintuitive findings underscore the crucial need for greater understanding of HDL biology.

Atherosclerosis, characterized by the accumulation of lipids and cholesterol-filled macrophages in the arterial wall, is the pathological process underlying CVD. Prevention of intracellular cholesterol accumulation through decreasing uptake and/or increasing efflux of cholesterol to extracellular lipoproteins is necessary to maintain macrophage lipid homeostasis. Cholesterol efflux is an early step in the reverse cholesterol transport (RCT) pathway, a process by which HDL particles transport cholesterol from extra-hepatic tissues to the liver, for subsequent excretion in bile. Indeed, this aspect of HDL functionality is strongly and inversely correlated with coronary heart disease in many, but not all studies. Two major transport proteins have been shown to facilitate cholesterol efflux and play a role in RCT, the ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1). ABCA1 plays a critical role as a transporter of intracellular free cholesterol and phospholipids to the extracellular acceptor apolipoprotein AI (ApoA-I), to form nascent HDL. Mature HDL particles act as acceptors for ABCG1-mediated cholesterol efflux.

Family and twin studies estimate that HDLc has a heritability of between 40 and 60%, and a substantial portion of HDLc heritability remains to be elucidated. Recent genome-wide association studies have identified numerous genetic loci that associate with significant changes in plasma HDLc levels across large populations. However, few of these loci have been functionally investigated. One of these variants, rs4148008 in ABCA8 was reported to be significantly associated with an average 0.42 mg/dL decrease in HDLc levels. The rs4148008 variant is localized in intron 30 of ABCA8, and has a global minor allele frequency (MAF) of 0.42 in dbSNP and 0.29 in Hapmap central Europeans. In support of this association, we previously identified a single proband with low HDLc who carries a predicted loss of function mutation, p.Thr741Stop, in ABCA8. Although the function of ABCA8 remained to be elucidated, it belongs to the ABC transporter family, suggesting it might play a role in HDL metabolism and cholesterol efflux in a similar fashion as the canonical cholesterol efflux proteins ABCA1 and ABCG1. Here, we identify ABCA8 as a new protein involved in cholesterol efflux and characterized its role in RCT and in modulating HDLc levels.
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Materials and methods

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

Results

*ABCA8* mutations are found in probands with low HDLc

To determine if mutations in *ABCA8* could result in low plasma HDLc levels, we sequenced *ABCA8* in 80 probands with HDLc < 10th percentile in whom mutations in *LCAT*, *APOA1* and *ABCA1* were previously excluded. As controls, 120 probands with HDLc ≥ 90th percentile were sequenced. Sequencing of the 39 exons and exon-intron boundaries of *ABCA8* resulted in the identification of 2 probands exclusively in the low HDLc cohort with potential mutations. These variants are p.Pro609Arg (P609R, rs144777539, chromosome 17:66914289 G>C) and c.IVS17-2 A>G (Chromosome 17:66899693, genome build 37.3) (Fig. 1A). We also previously identified an individual with HDLc ≤ 5th percentile, harboring a nonsense mutation, p.Thr741Stop (T741X), (Chromosome 17:66902243). These three probands, who were heterozygous carriers of three different *ABCA8* variants, formed the basis for further studies.

To investigate whether these three variants are deleterious, we first determined their frequencies in the dbSNP, 1000 genome and exome variant server (ESP) databases. The P609R variant is rare (MAF: 0.001 in the ESP population), while the c.IVS17-2 A>G and T741X variants were not found. Mutation functional prediction algorithms predicted the P609R variant to be “probably damaging”, while c.IVS17-2 A>G was predicted to abolish an essential splice site. T741X results in the truncation of more than half the protein, including ATP binding cassette domain 2, which is likely to cause a large functional defect (Fig. 1A). Across vertebrate genomes, both Pro609 and the nucleotide A in IVS17-2 A>G are conserved. Together, the *in silico* data suggest that all three variants are likely to be deleterious.

HDL cholesterol levels are decreased in *ABCA8* mutation carriers

To investigate whether these mutations may underlie reduced HDLc levels, we first assessed their segregation with HDLc levels in 44 family members of the 3 probands (pedigrees in Supplementary Fig. I). Heterozygous *ABCA8* mutation carriers showed significantly reduced plasma HDLc levels compared to first-degree relative controls (0.86 ± 0.34 mmol/L, n = 15 vs. 1.17 ± 0.26, n = 32; p = 0.005) (Fig. 1B). Statistical significance remained when the original probands were excluded from the analyses (carriers: 0.95 ± 0.28 mmol/L, n = 12; p = 0.018), indicating that plasma HDLc levels are significantly decreased in *ABCA8* mutation carriers.
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<table>
<thead>
<tr>
<th></th>
<th>Heterozygous ABCA8 mutation carriers</th>
<th>First-degree relative controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>50.2 ± 17.6</td>
<td>48.1 ± 24.1</td>
<td>0.8</td>
</tr>
<tr>
<td>% male</td>
<td>73.3</td>
<td>46.9</td>
<td>0.1</td>
</tr>
<tr>
<td>HDLc (mmol/L)</td>
<td>0.86 ± 0.34</td>
<td>1.17 ± 0.26</td>
<td>0.005</td>
</tr>
<tr>
<td>HDLc percentile</td>
<td>15.5 ± 18.1</td>
<td>34.8 ± 21.4</td>
<td>0.001</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.49 ± 1.19</td>
<td>4.77 ± 0.93</td>
<td>0.5</td>
</tr>
<tr>
<td>LDLc (mmol/L)</td>
<td>2.94 ± 0.85</td>
<td>3.12 ± 0.70</td>
<td>0.6</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.65 ± 1.60</td>
<td>1.24 ± 0.60</td>
<td>0.08</td>
</tr>
<tr>
<td>ApoA-I (mmol/L)</td>
<td>0.039 ± 0.009</td>
<td>0.0523 ± 0.008</td>
<td>-</td>
</tr>
<tr>
<td>ApoB (mmol/L)</td>
<td>0.0014 ± 0.0002</td>
<td>0.002 ± 0.0004</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.2 ± 3.8</td>
<td>25.0 ± 5.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 1. Basic characteristics, and plasma lipid and lipoprotein levels in ABCA8 mutation carriers and first-degree relative controls.
The p-value for age was calculated using t-test and Fisher’s exact chi-square test was used to calculate the p-value for % male. All other p-values were generated using a mixed linear model to adjust for correlation between individuals in the same family. Endpoint p-values are controlled for age and sex. TG values were log transformed before statistical analyses. Data are presented as mean±SD and statistically significant values are in bold.

We next assessed plasma lipids and apolipoproteins. Plasma levels of apoA-I were, on average, 26.4% lower in mutation carriers compared to controls (carriers: 0.039 ± 0.009 mmol/L, n = 2; controls: 0.0523 ± 0.008, n = 9) (Table 1), although few samples were measured due to limited availability of human plasma samples. No significant differences were observed in LDLc, triglycerides, total cholesterol and ApoB levels (Table 1).

ABCA8 mutations associate with reduced large HDL particle concentration, and HDL particle size

The association of ABCA8 mutations with HDL particle number, size and composition was analyzed by means of nuclear magnetic resonance (NMR) spectroscopy and lipidome analyses. HDL derived from heterozygous ABCA8 mutation carriers was significantly smaller than HDL from first-degree relative controls (carriers: 8.7 ± 0.2 nm, n = 9; controls: 9.2 ± 0.4 nm, n = 17; p = 0.004) (Fig. 1C), which in turn was associated with lower large HDL particle concentration (carriers: 2.7 ± 1.3, n = 9; controls: 5.1 ± 2.6 µmol/L, n = 17; p = 0.015) (Fig. 1D). No significant differences in total HDL particle concentration, LDL or VLDL particle size and concentration were observed.
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**Figure 1.** Mutations in ABCA8 result in low HDL cholesterol, HDL particle size and concentration in families.

(A) Predicted topographical model of ABCA8 with identified mutations. (B) Plasma HDLc levels in heterozygous ABCA8 mutation carriers and first-degree relative controls. (C) Mean HDL particle size, and (D), concentration of large HDL particles as assessed by NMR spectroscopy. Averages and standard errors are shown.

**Plasma HDLc levels are reduced in Abca8b knockout mice**

To validate the direct relationship between ABCA8 and HDLc levels, we generated mice with a targeted deletion of *Abca8b*. Two tandem gene orthologs exist for *ABCA8* in mice, *Abca8a* and *Abca8b*. *Abca8b* shows 75% identity with *ABCA8*, whereas *Abca8a* shows 68% identity. Thus, *Abca8b* was selected for initial knock-out mouse generation. Absence of *Abca8b* expression in tissues including the liver was confirmed by RT-PCR in *Abca8b−/−* mice (Supplementary Fig. II). No changes in plasma HDLc levels were observed in *Abca8b−/−* mice compared to littermate controls on a chow diet (WT mice: 1.53 ± 0.46, *Abca8b−/−*: 1.44 ± 0.35 mmol/L; p-value: 0.577). However, when placed on a high cholesterol diet, *Abca8b−/−* mice showed a significant, 29% lower plasma HDLc level compared to wild-type controls (*Abca8b+/+: 4.46 ± 0.35; Abca8b−/−: 3.17 ± 0.31 mmol/L; p = 0.01) (Fig. 2A). In addition, total cholesterol, LDLc and triglyceride levels were also reduced in *Abca8b−/−* mice (Table 2). No significant changes in the expression of hepatic *Abca8a* and *Abca1* were observed (Supplementary Fig. II).
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Figure 2. ABCA8 expression modulates plasma HDLc levels.

(A) Plasma HDLc levels in Abca8b/− and wild-type mice fed a high cholesterol diet. Data are presented as mean±SEM. (B-E) Adenoviral human ABCA8 was delivered by tail vein to wild-type mice, and liver specific expression was observed by (B) RT-PCR, and (C) western immunoblotting 72h after injection. HDLc (D), and total cholesterol (E) levels in control and ABCA8 over-expressing mice 24 hours after injection.


Table 2. Plasma lipid levels in Abca8b⁻/⁻ mice on high cholesterol diet

<table>
<thead>
<tr>
<th></th>
<th>Abca8b⁺⁺</th>
<th>Abca8b⁻/⁻</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>4.74 ± 0.37</td>
<td>3.39 ± 0.34</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL Cholesterol</td>
<td>4.46 ± 0.35</td>
<td>3.17 ± 0.31</td>
<td>0.01</td>
</tr>
<tr>
<td>LDL Cholesterol</td>
<td>0.88 ± 0.09</td>
<td>0.58 ± 0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.80 ± 0.04</td>
<td>0.69 ± 0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

TC = Total cholesterol; HDLc = HDL cholesterol; LDLc = LDL cholesterol; TG = triglycerides. Data are presented as mean±SD in mmol/L.

Hepatic ABCA8 over-expression in mice significantly increases plasma HDLc levels

We next determined the tissue distribution of human ABCA8 and the mouse orthologs Abca8a and Abca8b. We found high human ABCA8 mRNA in the heart, as well as in the liver and skeletal muscle (Supplementary Fig. IIIA), in line with previous observations. Mouse Abca8a and Abca8b expression was highest in the liver, and was also abundant in heart and skeletal muscle (Supplementary Fig. IIIB-C), in agreement with the human tissue distribution profile and with previous observations. Since mutations in ABCA8 are associated with lower plasma HDLc levels, and both human and mouse ABCA8 genes are highly expressed in the liver, we hypothesized that hepatic ABCA8 over-expression would significantly increase HDLc levels. Hepatic over-expression of human ABCA8 in wild-type mice via adenoviral (Ad) injection resulted in the expression of human ABCA8 predominantly in the liver (Fig. 2B-C). Both plasma HDLc (Fig. 2D) and total cholesterol (Fig. 2E) were significantly increased 24 hours after Ad-ABCA8 infection compared to baseline (23.1%, p = 0.007, and 13.8%, p = 0.024, respectively). 48h post-infection, HDLc levels normalized. No significant changes in non-HDLc levels were observed.

Early steps of macrophage-to-feces RCT are significantly increased in mice with hepatic ABCA8 over-expression

As liver-specific over-expression of ABCA8 resulted in significantly increased plasma HDLc levels in mice, we investigated whether an increase in macrophage-to-feces RCT occurred when human ABCA8 was over-expressed in the liver of wild-type mice. Following injection of [³H]-cholesterol-loaded macrophages, a significant 50% increase in plasma [³H] counts was observed in mice with hepatic ABCA8 over-expression (ABCA8: 3.9 ± 0.2, controls: 2.6 ± 0.2, % of injected dose, p < 0.01 at 48 hrs, Fig. 3A). To compare the ability of ABCA1 to facilitate RCT in the same model, we also determined if increased macrophage-to-feces RCT occurred when ABCA1 was adenovirally over-expressed in the liver. As with ABCA8, increased plasma [³H] counts were also observed in mice over-expressing hepatic ABCA1 (ABCA1: 4.2±0.2, controls: 2.6 ± 0.2, % of injected dose, p<0.001 at 48 hrs,
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Figure 3. Hepatic ABCA8 and ABCA1 increase the early steps of in vivo reverse cholesterol transport. (A) Significantly increased plasma \[^{3}H\] counts at 48h in mice with hepatic over-expression of human ABCA8 or human ABCA1. (B) Significantly increased liver \[^{3}H\] counts in the liver-specific ABCA8 or ABCA1 overexpressing mice. (C) Unchanged fecal \[^{3}H\] counts in mice with ABCA8 or ABCA1 liver-specific overexpression. Averages and standard errors are shown.

Fig. 3A). Liver \[^{3}H\] counts were also significantly increased in the mice over-expressing either hepatic ABCA8 (ABCA8: 2.4 ± 0.1, controls: 1.7 ± 0.2, % of injected dose, p < 0.01 at 48 hrs, Fig. 3B), or ABCA1 (ABCA1: 2.1±0.1, controls: 1.7 ± 0.2, % of injected dose, p<0.05 at 48 hrs, Fig. 3B). However, despite the increased plasma and liver counts, no significant changes in fecal bile acid (BA) or neutral sterol (NS) counts were observed (Fig. 3C), indicating that in our model, hepatic over-expression of either ABCA8 or ABCA1 affects the early steps of the RCT pathway.

ABCA8 localizes to the plasma membrane and endoplasmic reticulum and facilitates cholesterol efflux to lipid-free ApoA-I

Since mutations in ABCA8, like ABCA1, are associated with low plasma HDLc levels, and ABCA1 is a well-established plasma membrane (PM) localized lipid efflux protein, we hypothesized that ABCA8 might also localize at the PM and regulate cellular cholesterol transport. When expressed in COS-7 cells, ABCA8 was indeed localized at the PM and also co-localized with calnexin, indicating endoplasmic reticulum (ER) localization (Supplementary Fig. IV). In contrast, ABCA8 harboring the P609R mutation was almost exclusively identified intracellularly, and co-localized with calnexin, indicating defective cell-surface expression (Supplementary Fig. IV). We failed to detect T741X (Supplementary Fig. IV), suggesting that the full-length T741X protein is not expressed. To determine if the first 741 amino acids in T741X are expressed, we generated T741-V5-X, with the V5 tag replacing the mutant stop codon. Like P609R, T741-V5-X co-localized almost exclusively with calnexin in the ER, and was not detected at the PM (Supplementary Fig. IV). Thus, ABCA8 encoding P609R or T741X fails to translocate to the cell surface. IVS17-2 A>G was not generated because we utilized a cDNA construct.

The ability of ABCA8 to localize at the PM suggests it could play a role in cholesterol efflux. Indeed, wild-type ABCA8 increased cholesterol efflux to ApoA-I by 181% when
transfected into COS-7 cells (Fig. 4A). In contrast, transfection of P609R or T741X resulted in efflux comparable to empty-vector controls (Fig. 4A). Thus, ABCA8 facilitates cholesterol efflux to ApoA-I, which is abolished by both P609R and T741X. To further confirm the role of ABCA8 in efflux, and to compare its efflux capacity to ABCA1, we assessed ApoA-I mediated cholesterol efflux in fibroblasts isolated from ABCA8 and ABCA1 mutation carriers. Indeed, fibroblasts from heterozygous ABCA8 mutation carriers showed a 20-43% reduction in cholesterol efflux (Fig. 4B). In comparison, cholesterol efflux from heterozygous ABCA1 mutation carrier fibroblasts was reduced by 43% on average (Fig. 4B). The mutations in the ABCA1 carriers are IVS24+1 G>C, p.Asp575Gly and IVS48+2 T>C, and are loss of function mutations identified in patients with Tangier disease or Familial Hypoalphalipoproteinemia.\textsuperscript{21,22}

Our data suggest that ABCA1 is a more potent facilitator of cholesterol efflux compared to ABCA8, at least in fibroblasts. To confirm this difference in efflux capacity between ABCA1 and ABCA8, we over-expressed similar amounts of V5-ABCA8 and V5-ABCA1 as assessed by anti-V5 immunoblots. ABCA1 showed a 1.8-fold increase in efflux capacity compared to ABCA8 (Fig. 4C), confirming that ABCA1 is a more potent cholesterol efflux protein. Moreover, co-transfection of the two proteins resulted in further enhanced cholesterol efflux (Fig. 4C), suggesting that ABCA8 and ABCA1 together further augment cholesterol efflux to ApoA-I. To further determine the influence of ABCA8 on ABCA1 activity, we assessed cholesterol efflux to ApoA-I in fibroblast cultures established from control, ABCA8 heterozygote, ABCA1 heterozygote, and ABCA1 homozygote individuals in the presence/absence of the LXR agonist TO-901317. TO-901317 induces the expression of ABCA1 but not ABCA8 (Supplementary Figure V). We observed that the ABCA1-specific cholesterol efflux decreased by 49% in fibroblasts from ABCA8 heterozygotes, and a similar 52% decrease was observed in fibroblasts from ABCA1 heterozygotes (Fig. 4D). These findings indicate that the loss of a single ABCA8 allele has the same impact on ABCA1-specific efflux as the loss of a single ABCA1 allele, and suggest that ABCA1 and ABCA8 work together to regulate cholesterol efflux to ApoA-I.

**ABCA8 co-localizes with and interacts with ABCA1**

Since ABCA8 and ABCA1\textsuperscript{23} both localize at the PM and ER, facilitate cholesterol efflux to ApoA-I, and when expressed together, further enhance cholesterol efflux compared to each alone, and since a reduction in ABCA8 affects ABCA1-specific efflux, we hypothesized that they might interact. We first determined the subcellular co-localization of ABCA8 and ABCA1 by co-expressing both proteins with different tags (ABCA8-V5 and ABCA1-GFP) in HEK293T cells. Indeed, ABCA8 co-localizes completely with ABCA1 at the PM and intracellularly (Fig. 4D). In addition, ABCA8 and ABCA1 co-immunoprecipitate when both proteins are co-expressed (Fig 4E). Thus, ABCA8 and ABCA1 act together in regulating cholesterol efflux.
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**Figure 4.** ABCA8 facilitates cholesterol efflux to lipid-free ApoA-I. (A) Efflux of cholesterol to lipid-free ApoA-I in COS-7 cells transfected with wild-type and mutant human ABCA8. (B) Efflux of cholesterol to lipid-free ApoA-I in fibroblasts isolated from ABCA8 and ABCA1 heterozygous mutation carriers and control individuals. (C) Efflux of cholesterol to lipid-free ApoA-I in COS-7 cells transfected with wild-type human ABCA8, ABCA1 or ABCA8 + ABCA1 together. (D) Co-localization of human wild-type ABCA8-V5 and ABCA1-GFP overexpressed in HEK293T cells, and visualized with an anti-V5 and anti-GFP antibodies. (E) Co-immunoprecipitation of human ABCA1 and ABCA8-V5 in HEK293T cells.
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DISCUSSION

We describe here the identification and validation of ABCA8 as a cholesterol efflux protein that influences HDL metabolism in humans. Loss of function mutations in ABCA8 result in significantly lower plasma HDLc levels compared to non-carrier relative controls. In mice, hepatic over-expression of human ABCA8 resulted in a significant selective increase in plasma HDLc levels, whereas targeted deletion of the mouse ortholog Abca8b resulted in significantly reduced plasma HDLc levels.

ABCA8 shows several similarities with ABCA1, a protein with a well-established role in lipid efflux and HDL metabolism. Both proteins are found in the liver. Subcellularly, ABCA1 is localized to the plasma membrane (PM), ER, and endocytic vesicles. Its localization at the PM is essential for its role in cholesterol efflux, and mutations disrupting this PM localization result in significantly reduced cholesterol efflux. A similar subcellular distribution pattern and impact on cholesterol efflux is observed for ABCA8. Both ABCA8 and ABCA1 facilitate the efflux of cholesterol to lipid-free ApoA-I, and our data suggest that ABCA1 is the more efficient cholesterol efflux protein. A previous study found that ABCA8 was also capable of significantly increasing cholesterol efflux, albeit not specifically to either ApoA-I or ApoE.

In humans, mutations in either ABCA1 or ABCA8 result in significantly lower plasma HDLc. While some pedigrees lacked statistical power to assess Mendelian segregation of mutations with HDLc levels, across all pedigrees we observed a significant 27% lower plasma HDLc levels in heterozygous ABCA8 mutation carriers, which is comparable to our previous observations of ~40% lower HDLc in heterozygous ABCA1 mutation carriers. Mutations in ABCA8, similar to ABCA1, result in decreased large HDL particle concentration and reduced HDL particle size. Complete deletion of mouse Abca1 results in a 99.5% reduction in HDLc levels, while a 29% decrease in HDLc was observed in Abca8b-/- mice on a high cholesterol diet. The difference in the HDLc level between Abca8b-/- and Abca1-/- mice might be explained by ABCA8’s lower relative efflux capacity. In addition, in mice, two orthologous genes exist for human ABCA8, Abca8a and Abca8b, and it is possible that in the Abca8b-/- mice, Abca8a might contribute to plasma HDLc levels. Abca8b-/- mice on a high cholesterol diet also show lower LDLc levels compared to wild-type mice, whereas ABCA8 mutation carriers present a very specific decrease only in HDLc, but not in LDLc. The lower LDLc levels in the Abca8b-/- mice might be due to an accelerated LDL catabolism, similar to previous observations in hepatic Abca1-/- mice.

When either ABCA8 or ABCA1 are over-expressed in the liver of mice, the movement of labeled cholesterol from macrophages to the plasma and liver, the early steps in RCT, is significantly elevated. Similarly, systemic increases in Abca1 expression stimulate macrophage-to-feces RCT in mice, while the absence of Abca1 in mice leads to decreased...
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RCT. Since $ABCA8$ and $ABCA1$ were over-expressed exclusively in liver and not in macrophages, our data suggest that liver ABCA8, like liver ABCA1, facilitates the generation of a particle with the ability to take up lipids from macrophages. However, this increase in the initial steps of RCT did not result in changes in fecal counts. While the reasons for this observation warrant further study, liver-specific $Abca1$ deletion also did not decrease macrophage to feces RCT.

Based on the similarities between ABCA1 and ABCA8, the specific functions of each protein remain a key question. HDLc levels are extremely low in the absence of $ABCA1$, both in TD patients and $Abca1^{-/-}$ mice, suggesting that $ABCA8$ does not compensate for the absence of $ABCA1$. This, together with our finding that ABCA8 and ABCA1 interact, suggest that these two proteins are unlikely to be operating in completely independent pathways to modulate HDLc levels, but rather, may act via overlapping pathways.

One possibility for the overlapping pathway hypothesis is that ABCA1 and ABCA8 act together as a complex or regulate each other’s function. This hypothesis is not without precedent. ABCA12, a transporter of glucosylceramide, interacts with, and regulates ABCA1’s cholesterol transporter function in macrophages, and ABCA12 deficiency results in impaired RCT and macrophage foam cell formation. It is thought that ABCA12 modulates ABCA1 function via its binding to ABCA1 and increasing the protein levels and stability of ABCA1. If ABCA8 plays a similar role, then a similar phenotype would be expected in either the absence of ABCA1 or ABCA8. However, reduced ABCA8 has a milder impact on plasma HDLc levels and cholesterol efflux when compared to reduced ABCA1 levels. Thus, another possibility for the regulation of ABCA1 function by ABCA8 could be that ABCA8 transports lipids, perhaps sphingomyelin, to or in the PM, to form specific membrane domains, thus contributing to the lipid composition of these membrane domains and creating regions from which ABCA1 can then transport lipids to ApoA-I.

This model also may explain the increased cholesterol efflux capacity of ABCA1 compared with ABCA8. Of the two proteins, ABCA1 may be the primary cholesterol transporter, and the absence of ABCA1 results in a large reduction in cholesterol efflux. ABCA8 may affect the cholesterol pool size available for ABCA1 mediated efflux via the transport of another lipid species. Thus, in the absence of ABCA8, a smaller impact on cholesterol efflux is observed. This model also fits with the observation of additional enhancement in cholesterol efflux when both ABCA8 and ABCA1 are over-expressed.

There are some differences between mouse and human lipoprotein metabolism. For example, mice carry most of their plasma cholesterol in HDL particles, whereas humans carry most of their plasma cholesterol in LDL particles, due to a lack of cholesteryl ester transfer protein (CETP) in mice. CETP transfers cholesterol esters from HDL to LDL or VLDL particles. This and other differences are limitations to translating our observations directly from mice to humans and vice versa. Indeed, we do observe some differences between the
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results in our human or mouse models. A humanized mouse such as CETP transgenic mice crossed to our Abca8b−/− mice would facilitate the translation of our findings.

The identification of deleterious mutations in ABCA8 as a novel cause of reduced plasma HDLc in humans adds a piece to the intriguing puzzle of HDL metabolism. Our data indicate that ABCA8 interacts with ABCA1 and regulates its efflux capacity. Whether this has an impact on atherosclerosis progression remains to be seen.

Acknowledgments

We thank all study participants. We also thank K. Los for the genetic fieldwork, and J. Peter, Amber Anushree Eliathamby and Tricia Chua for technical assistance. Microscopy images were acquired at the SBIC-Nikon Imaging Centre at A*STAR.

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Disclosures

Chris Radomski is an employee and Michael Hayden is on the board at Xenon Pharmaceuticals.
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27. Tietjen I et al. Increased risk of coronary artery disease in Caucasians with extremely low HDL cholesterol due to mutations in ABCA1, APOA1, and LCAT. Biochim Biophys Acta 2012;1821(3):416–24.
SUPPLEMENTARY MATERIAL AND METHODS

Sequencing of *ABCA8* in extreme HDL cohort

A previously described cohort consisting of 80 unrelated Dutch Caucasian probands with HDLc≤10th percentile (LHDL) and as controls, 120 unrelated probands of Dutch Caucasian ancestry with HDLc≥90th percentile (HHDL) was utilized. Study protocols were approved by the Ethics Committee of the Academic Medical Center, Amsterdam. All subjects provided written informed consent. Plasma apoA-I and apoB were measured from fresh plasma using a commercially available turbidimetric assay (Randox) and analysed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland) as previously described. Cholesterol and triglyceride levels were determined in fresh plasma at density d<1.006 g/mL obtained after preparative ultracentrifugation, before and after precipitation with dextran manganese. Other co-variables such as age, sex, BMI, medical history, alcohol intake, and smoking history were available for all individuals. Genomic coordinates for the exons of *ABCA8* were compiled and sequencing of each exon and at least 50bp of adjacent intron was performed using next generation paired-end read sequencing (Illumina, San Diego, CA) as previously described. Sequence changes were identified by alignment of sequence data to the human genome (NCBI Build 36.1) and classified as synonymous, missense, nonsense or splice site variants.

Sequence changes of interest were confirmed by standard fluorescent dye terminator chemistry sequencing (Beckman Coulter Genomics, MA, and SeqWright, TX) and analyzed using Sequencher v4.7 (Gene Codes Corporation, MI). Exon primer sequences are available upon request.

Frequency of the *ABCA8* variants were determined in the dbSNP, 1000 genome and exome variant server (ESP) databases (https://www.ncbi.nlm.nih.gov/SNP; http://phase-3browser.1000genomes.org/index.html; http://evs.gs.washington.edu/EVS; respectively). Mutation functional prediction was assessed using the prediction algorithms Polyphen2.0 (http://genetics.bwh.harvard.edu/pph2) and Spliceview (http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html).

Predicted topological model generation

Protein topology was predicted utilizing biological knowledge (for example, the ATP-binding cassettes should have an intra-cellular localization in order to be functional), the predicted topology of known full ABC transporters, as well as compiling topological prediction from several topology and transmembrane domain prediction algorithms (TMPred (http://www.ch.embnet.org); PredictProtein (https://www.predictprotein.org); HMMTOP (http://www.enzim.hu/hmmtop/); TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/
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Segregation analysis of mutations in families

The families of the three unrelated probands with ABCA8 mutations were ascertained and pedigrees generated. A total of 44 family members of the probands were genotyped using standard Sanger sequencing techniques described above. Only the variant found in a specific proband was genotyped in the proband’s family.

Lipoprotein particle analysis

Size and concentration of the lipoprotein particle subclasses present in plasma from ABCA8 heterozygous carriers and their family controls were determined by Nuclear magnetic resonance (NMR) spectroscopy at LipoScience, Inc. (Raleigh, NC).3

Generation of Abca8b−/− mice

Sperm was obtained from Abca8b−/− mice (Abca8b^{tm1a(EUCOMM)Wtsi}, UC Davis-KOMP repository) and mice generated on the C57BL/6J background. Loss of Abca8b expression was assessed by RT-PCR in tissues. Chow diet (Altromin 1324_mod) or high cholesterol diet (HCD, Research Diets D12089, modified from Western Diet D12079B + 1% cholesterol) were provided ad libitum. Male mice were fed with either chow or HCD for 2 months beginning at 2 months of age. Blood was withdrawn from saphenous vein and plasma isolated to quantify plasma lipid levels using the Cobas c311 system (Roche Diagnostics). As above, all animal work was approved by the Institutional Animal Care and Use Committee at A*STAR.

RNA isolation and quantitative RT-qPCR

Total RNA was isolated from tissues using the RNeasy Mini kit (Qiagen, CA). cDNA was generated using random hexamers and SuperScriptII Reverse Transcriptase kit (Invitrogen, CA). qPCR was performed using SYBR Select Master Mix (Applied Biosystems, CA). Tissue distribution analysis was performed using the Human Multiple Tissue cDNA panel (Clontech, CA). Reactions were performed in technical triplicates using specific primers (hABCA8: Fw-TTCATGTTGGCATTTGACACTTG, Rv-GGATCGGATCCATTCTAT; hHPRT1: Fw-TGACACAGAGGCAAGTGAA, Rv-CACATCACAGCTCCCCACT; mRPL37: Fw-CTGATGGGCGTTGAGAAGT, Rv-CTGATTGGGATACAGGCCAGAGC, Rv- CAAATCTCCCTGAAGT; mAbca8a: Fw-CGTGGGCCTTATTGTGCAAGA, Rv-CAGGTCCACATCAGGCTG; mAbca8b: Fw-ATAAGTGTGCGCCAACAAACT, Rv-TGACAGGCGTGTACCCTATCA; mAbca1: Fw-TCCCGAGCAATGCTTCTTC, Rv-GCGCTCAACTTTTACGAAGGC). Relative quantification
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of gene expression was performed with the internal controls ribosomal protein L37 (RPL37; for mouse liver), and with the geometric mean of human hypoxanthine phosphoribosyltransferase 1 and TATA binding protein (HPRT1 and TBP, respectively; for tissue distribution panel). qPCR for hepatic biliary transport genes (Abcg5, Abcg8 and Abcb11) was performed using TaqMan® Fast Gene Expression Master Mix (Applied Biosystems, CA) and acidic ribosomal protein (36b4) as internal control. qPCR results were analyzed by the comparative Ct method.4

Western blot

Mouse liver was homogenized in 300 µl of lysis buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP40, 0.5% sodium deoxycholate) supplemented with complete protease inhibitors (Roche, Mannheim, Germany). Samples were centrifuged and supernatants used for protein quantification. For transfected cells, pellets were resuspended in 1ml of lysis buffer (0.5 mM Na₂HPO₄, 0.1mM EDTA, pH=7.0, complete protease inhibitors (Roche)) and sonicated. Cell lysates were centrifuged and the pellet was re-suspended in 40-60 µl of lysis buffer. Protein concentration was determined by the Bradford assay (Bio-Rad, CA) and 50 µg of protein was resolved by electrophoresis on 6% SDS-PAGE gels and transferred to PVDF membranes, blocked with 5% skim milk, and probed with anti-V5, anti-calnexin or anti-beta tubulin (Sigma Aldrich, MO) antibodies, followed by incubation with horseradish peroxidase conjugated secondary antibodies and membranes were developed using Chemiluminescence (Thermo Scientific, IL).

Generation of wild-type and mutant ABCA8 cDNA

Human liver QUICK-clone™ cDNA (Clontech, CA) was utilized to amplify the ABCA8 gene using gene specific primers. PCR fragments were inserted into pcDNA3 and Sanger sequenced to confirm the clones. A clone matching the Genbank cDNA gi572882596 (RefSeq NM_001288985.1) was used for all further experiments. The ABCA8 cDNA clone was also generated with a c-terminal V5 (P and V proteins of the paramyxovirus of simian virus 5) epitope tag. The sequence of the human liver cDNA amplified ABCA8 clone contained an additional 40 amino acids that were absent from the RefSeq sequence NM_007168.2 or NP_009099, but were present on the Genbank sequence gi572882596 or RefSeq sequence NM_001288985.1. The mutations Pro609Arg (P609R) and Thr741X (T741X) were generated in both the V5 tagged and untagged ABCA8 cDNA clones using standard site directed mutagenesis methodology,5 and were sequence confirmed. Since our ABCA8 clones contained a c-terminal V5 tag, an additional T741X clone, T741-V5-X, was generated where the mutant stop codon in T741X was replaced with the V5 tag, which was then followed by a stop codon.
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Adenoviral over-expression of ABCA8 in mice

The V5-tagged ABCA8 cDNA clone was inserted into an adenoviral vector, amplified, and PFU titred (SignaGen, MD). Expression of ABCA8 from the adenovirus was confirmed by infection of Hela cells, followed by Western immunoblotting. Four month-old male C57BL/6J mice were purchased from the Biological Resource Centre, Singapore. All animal work was approved by the Institutional Animal Care and Use Committee of the Biological Resource Centre at A*STAR, Singapore and conformed to National Institutes of Health guidelines and public law. Mice were pre-injected with $5 \times 10^8$ pfu of adeno-AP (alkaline phosphatase) to inactivate Kupffer cells, and tail vein injections were performed as previously described. $1 \times 10^9$ pfu of adeno-ABCA8 or adeno-AP was delivered via tail veins. Blood was collected at baseline, 24, 48 and 72 hours post-infection from mice fasted for 4h, for plasma cholesterol quantification using an enzymatic commercial kit (Infinity cholesterol kit, Thermo Fisher Scientific, MA). Mice were sacrificed 72 hours post adenoviral administration, and tissues collected for expression analyses.

In vivo reverse cholesterol transport assay

In vivo reverse cholesterol transport (RCT) assays were performed as previously described. Briefly, wild-type C57BL/6J donor mice were injected intraperitoneally with 1.0 ml of 4% Brewer thioglycollate medium (Becton Dickinson, Le Point de Claix, France). On day 4 after thioglycollate injection, peritoneal macrophages were harvested, plated, and allowed to adhere for 4 h at 37°C under 5% CO$_2$ humidified air. Macrophages were loaded with 50 μg/ml acetylated LDL and 3 μCi/ml $[^3H]$cholesterol (Perkin Elmer, Boston, MA) for 24 h, and equilibrated for 18 h in RPMI 1640 medium containing penicillin (100 U/ml)/streptomycin (100 μg/ml) and 2% BSA (Sigma). Immediately before injection, cells were harvested, re-suspended, and 2 million cells per mouse were injected intra-peritoneally into individually housed recipient mice that had been injected 6 hours before with $1 \times 10^9$ pfu of adeno-null, adeno-ABCA8 or adeno-ABCA1 via the tail vein as detailed above. Plasma was collected at baseline, 24 and 48 hours after macrophage injection. At 48 h, livers were harvested, and stored at −80°C. Feces were collected continuously up to 48 h and subsequently pooled. Counts in plasma were assessed by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT). Counts from the liver were determined following solubilization of the tissue (Solvable, Packard) and were normalized to total liver mass. Fecal samples were dried, weighed, and thoroughly ground. Aliquots were separated into bile acid (BA) and neutral sterol (NS) fractions as previously published. Counts recovered from the BA and NS aliquots were normalized to the total amount of feces produced over the whole experimental period. All obtained counts were expressed relative to the administered tracer dose.
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Cell culture and transfection

HEK-293T and/or COS-7 cells were maintained at 37°C and 5% CO2 in Dulbecco’s Modified Minimal Essential Medium (DMEM) (Sigma, MO) supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare, Buckinghamshire, United Kingdom), 4 mM glutamine and 1% penicillin/streptomycin. One day after seeding, cells were transiently transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Life Technologies, CA).

Immunofluorescence

HEK-293T cells were plated at a density of 37,500 cells/cm² in a 12-well plate. Twenty-four hours after transfection, cells were washed with PBS, fixed with 500 µL cold MeOH and permeabilized with 500 µL 0.3% TritonX-100 in PBS. Blocking was performed with 4% normal goat serum (NGS, Life Technologies, CA) in PBS for 1h followed by incubation with anti-V5 (1:200, Invitrogen, CA) and anti-calnexin (1:200, Sigma Aldrich, MO) antibodies or anti-V5 and anti-GFP (Abcam) antibodies in 2% NGS overnight at 4°C. Cells were washed with 1% BSA in PBS and incubated with goat anti-rabbit Alexa Fluor 555 and goat anti-mouse Alexa Fluor 480 (1:200, Invitrogen, CA) in 2% NGS in PBS for 1h at RT, and stained with DAPI (10 µg/ml, Sigma Aldrich, MO) in PBS for 10 minutes. Cover slips were mounted in 5µL ProlongGold (Invitrogen). Images were obtained using the Nikon A1R’si Confocal Microscope.

Cholesterol efflux assays

COS-7 cells were transiently transfected with ABCA8, ABCA8-P609R, ABCA8-T741X or/and ABCA1 plasmids using Xtremegene 9 (Roche). Twelve hours later, confluent cells were loaded with 2 µCi/ml of [3H] cholesterol (Perkin-Elmer) for 20 hrs. Cells were washed and equilibrated with DMEM containing 1mg/ml of fatty acid free BSA for 1 hr. DMEM + 1 mg/ml fatty acid free BSA ±15 µg/ml human apolipoprotein A-I (ApoA-I) was added to the cells for 4 hrs. Radioactivity was measured in supernatants and in cell lysates after lysis with 0.1N NaOH. Similarly, fibroblasts isolated from control individuals or ABCA8 and ABCA1 mutation carriers were loaded with 30 µg/ml cholesterol and 0.5 µCi/ml of [3H] cholesterol in media containing 2 mg/ml BSA for 20h ± 10 µg/ml TO-901317 (Sigma Aldrich). Cells were washed and DMEM containing 2mg/ml of BSA ±15 µg/ml human apolipoprotein A-I (ApoA-I) was added to the cells for 4 hrs. Radioactivity was measured in supernatants and in cell lysates after lysis with isopropanol. ApoA-I dependent efflux was calculated as the percentage of radioactivity in the supernatant compared to the total counts (cells+supernatant) in the wells incubated with ApoA-I minus the wells without ApoA-I.5
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Co-immunoprecipitation

For the co-IP assay, HEK293T cells (ATCC) were co-transfected with human-V5-ABCA8-pcDNA3.1 and human-ABCA1-pcDNA3.1. 24 hours after transfection, cells were lysed (10mM Tris-HCl, 0.1% Triton, 150mM NaCl, pH8.0, 1X protease inhibitor cocktail (Roche)) and centrifuged at 14,000 g for 20mins at 4°C. Protein A sepharose beads 4 Fast flow (50 μl; GE healthcare) were incubated for two hours at 4°C with 2 μg of anti-V5 antibody (Invitrogen) or 25 μg AC10 antibody (Santa Cruz). The lysates (500 μg) were incubated with the mixture overnight at 4°C. Unspecific binding was checked using control beads incubated without protein lysate. The beads were washed three times in cold homogenization buffer containing 0.4% Tween 20 with protease inhibitors and the bound proteins were detached and collected by adding Laemmli sample buffer containing 200 mM dithiothreitol and heating the samples to 100°C for 10 mins. The samples were then subjected to Western blotting to detect human ABCA8 with mouse anti-V5 (Invitrogen) and AC10 antibody to detect human ABCA1.

Statistical analyses

Students t-test and repeated measures ANOVA were used for the experiments. For segregation analyses, after testing for endpoints normality, a linear mixed model was used, which generalizes the standard linear model. The mixed model takes into account the data correlation structure due to familial relationships incorporated into the random effect. We applied the Satterthwaite procedure to adjust for the small sample size.9 The statistical analyses were performed with the MIXED procedure in SAS 9.4.

7. Singaraja RR et al. Both hepatic and extrahepatic ABCA1 have discrete and essential functions in the maintenance of plasma high-density lipoprotein cholesterol levels in vivo. Circulation 2006;114(12):1301–9.
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SUPPLEMENTARY MATERIAL

Supplementary Figure I A

ABCA8 P609R

Supplementary Figure I B

ABCA8 IVS17-2 A>G
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Supplementary Figure I

Pedigrees of probands with mutations in ABCA8 showing segregation with low HDLc.

Representative segregation of (A) P609R, (B) IVS17-2 A>G, and (C) T741X with reduced HDLc. For each individual, the individual ID, HDLc (in mmol/L) and [HDLc percentile], and genotype are shown. Squares, Males; Circles, Females; Arrow, Proband. Filled shape, HDLc ≤5th percentile; empty shape, HDLc >5th percentile. Slash = deceased; Genotype in brackets = imputed genotype.

Supplementary Figure II

Abca8b-/- mice have decreased Abca8b and unaltered Abca8a and Abca1 expression levels in liver. Hepatic Abca8b, Abca8a and Abca1 gene expression was assessed by RT-PCR and normalized to the housekeeping gene RPL37. Data are presented as mean±SEM.
Supplementary Figure III. Human ABCA8 and mouse Abca8a and Abca8b tissue distribution. mRNA levels were assessed by RT-PCR and normalized to the geometric mean of two housekeeping genes (HPRT1 and TBP for hABCA8; HPRT1 and RPL37 for mAbca8a/8b). Brain tissue was used as calibrator (2^(-ΔΔCt)). Data are presented as mean±SEM. (A) Human ABCA8 mRNA levels measured in human multiple tissue cDNA panels consisting of pooled RNA from several individuals. (B,C) Mouse Abca8a and Abca8b mRNA levels were measured in 4-10 mice.
Supplementary Figure IV. ABCA8 localizes to the plasma membrane and endoplasmic reticulum. Human wild-type or mutant ABCA8 cDNAs were tagged with the V5 epitope, transfected into HEK293T cells, and visualized with an anti-V5 antibody. The ER marker, calnexin, was used to assess intra-cellular localization.