ABCA8 Regulates Cholesterol Efflux and High-Density Lipoprotein Cholesterol Levels


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 adenosine triphosphate–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, 3 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 with first-degree family controls (0.86±0.34 versus 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 with wild-type mice, whereas hepatic overexpression of human ABCA8 in mice resulted in significant increases in plasma HDLc and the first steps of macrophage-to-foeces reverse cholesterol transport. Overexpression of wild-type but not mutant ABCA8 resulted in a significant increase (1.8-fold; P=0.01) of cholesterol efflux to apolipoprotein AI in vitro. ABCA8 colocalizes and interacts with adenosine triphosphate–binding cassette transporters A1 and further potentiates adenosine triphosphate–binding cassette transporters A1–mediated cholesterol efflux.

Conclusions—ABCA8 facilitates cholesterol efflux and modulates HDLc levels in humans and mice. * (Arterioscler Thromb Vase Biol. 2017;37:00-00, DOI: 10.1161/ATVBAHA.117.309574.)

Key Words: atherosclerosis • cholesterol, HDL • heterozygous • macrophage • mutations • reverse cholesterol transport

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. 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 extrahepatic 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 cholesteryl efflux and play a role in RCT, the adenosine triphosphate–binding cassette transporters A1 (ABCA1) and G1 (ABCG1).11 ABCA1 plays a critical role as a transporter of intracellular-free cholesteryl and phospholipids to the extracellular acceptor apolipoprotein A1 (ApoA-I), to form nascent HDL.12 Mature HDL particles act as acceptors for ABCG1-mediated cholesteryl efflux.11

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.13 Recent genomewide association studies have identified numerous genetic loci that associate with significant changes in plasma HDLc levels across large populations.13 However, few of these loci have been functionally investigated. One of these variants, rs4148008 in ABCA8, has a global minor allele frequency of 0.42 in dbSNP and 0.29 in Hapmap central Europeans. In support of this association, rs4148008 in ABCA8 was reported to be significantly associated with an average 0.42 mg/dL decrease in HDLc levels.14 The rs4148008 variant is localized in intron 30 of ABCA8, and has a global minor allele frequency 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 ABCA8.15 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 cholesteryl efflux in a similar fashion as the canonical cholesterol efflux proteins ABCA1 and ABCG1. Here, we identify ABCA8 as a new protein involved in cholesteryl efflux and characterized its role in RCT and in modulating HDLc levels.

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.16 As controls, 120 probands with HDLc≥20th 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) (Figure 1A). We also previously identified an individual with HDLc≤5th percentile, harboring a nonsense mutation, p.Thr741Stop (T741X) (Chromosome 17:66902243).15 These 3 probands, who were heterozygous carriers of 3 different ABCA8 variants, formed the basis for further studies.

To investigate whether these 3 variants are deleterious, we first determined their frequencies in the dbSNP, 1000 genome, and exome variant server databases. The P609R variant is rare (minor allele frequency: 0.001 in the exome variant server 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 adenosine triphosphate–binding cassette domain 2, which is likely to cause a large functional defect (Figure 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 3 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 Figure I in the online-only Data Supplement). Heterozygous ABCA8 mutation carriers showed significantly reduced plasma HDLc levels compared with first-degree relative controls (0.86±0.34 mmol/L, n=15 versus 1.17±0.26, n=32; P=0.005; Figure 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.

We next assessed plasma lipids and apolipoproteins. Plasma levels of apoA-I were, on average, 26.4% lower in mutation carriers compared with controls (carriers: 0.039±0.009 mmol/L, n=21; controls: 0.052±0.008, n=9; Table 1), although few samples were measured because of 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 spectroscopy17 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; Figure 1C), which in turn was associated with lower large HDL particle concentration (carriers: 2.7±1.3, n=6; controls: 5.1±2.6 mmol/L, n=7; P=0.015; Figure 1D). No significant differences in total HDL particle concentration, LDL or VLDL particle size and concentration were observed.

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 knockout mouse generation. Absence of Abca8b expression in tissues including the liver was confirmed by reverse transcription polymerase chain reaction in Abca8b−/− mice (Figure II in the online-only Data Supplement). No changes in plasma HDLc levels were observed in Abca8b−/− mice compared with littermate controls on a chow diet (Abca8b+/+: 1.53±0.46; Abca8b−/−: 1.44±0.35 mmol/L; P=0.577). However, when placed on a high-cholesterol diet, Abca8b−/− mice showed a significant, 29% lower plasma HDLc level compared with wild-type controls (Abca8b+/+: 4.46±0.35; Abca8b−/−: 3.17±0.31 mmol/L; P=0.01; Figure 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 (Figure II in the online-only Data Supplement).

**Hepatic ABCA8 Overexpression 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 (Figure IIIA in the online-only Data Supplement), in line with previous observations. Mouse Abca8a and Abca8b expression was highest in the liver, and was also abundant in heart and skeletal muscle (Figure IIIB, C in the online-only Data Supplement), in agreement with the human tissue distribution profile and with previous observations. Since this is the first report studying Abca8b−/− mice and the expression of Abca8b is high in the heart and skeletal muscle, we assessed the organ/body mass ratio and performed gross pathology of these 2 tissues. No macroscopic differences were observed in heart or skeletal muscle from Abca8b−/− compared with wild-type mice (data not shown). Because 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 overexpression would significantly increase HDLc levels. Hepatic overexpression of human ABCA8 in wild-type mice via adenoviral (Ad) injection resulted in the expression of human ABCA8 predominantly in the liver (Figure 2B, 2C). Both plasma HDLc (Figure 2D) and total cholesterol (Figure 2E) were significantly increased.
24 hours after Ad\textit{A}B\textit{C}A\textit{A}8 infection compared with baseline (23.1\%, \textit{P}=0.007, and 13.8\%, \textit{P}=0.024, respectively). Forty hours postinfection, 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 \textit{ABCA8} Overexpression

As liver-specific overexpression of \textit{ABCA8} resulted in significantly increased plasma HDLc levels in mice, we investigated whether an increase in macrophage-to-feces RCT occurred when human \textit{ABCA8} was overexpressed in the liver of wild-type mice. After injection of [\textit{1}H]-cholesterol-loaded macrophages, a significant 50% increase in plasma [\textit{1}H] counts was observed in mice with hepatic \textit{ABCA8} overexpression (\textit{ABCA8}: 3.9±0.2, controls: 2.6±0.2, % of injected dose, \textit{P}<0.01 at 48 hours, Figure 3A). To compare the ability of \textit{ABCA1} to facilitate RCT in the same model, we also determined if increased macrophage-to-feces RCT occurred when \textit{ABCA1} was adenovirally overexpressed in the liver of wild-type mice. As with \textit{ABCA8}, increased plasma [\textit{1}H] counts were also observed in mice overexpressing hepatic \textit{ABCA1} (\textit{ABCA1}: 4.2±0.2, controls: 2.6±0.2, % of injected dose, \textit{P}<0.001 at 48 hours, Figure 3A). Liver [\textit{1}H] counts were also significantly increased in the mice overexpressing either hepatic \textit{ABCA8} (\textit{ABCA8}: 2.4±0.1, controls: 1.7±0.2, % of injected dose, \textit{P}<0.01 at 48 hours, Figure 3B) or \textit{ABCA1} (\textit{ABCA1}: 2.1±0.1, controls: 1.7±0.2, % of injected dose, \textit{P}<0.05 at 48 hours, Figure 3B).

ABCA8 Localizes to the Plasma Membrane and Endoplasmic Reticulum and Facilitates Cholesterol Efflux to Lipid-Free ApoA-I

Since mutations in \textit{ABCA8}, like \textit{ABCA1}, are associated with low plasma HDLc levels, and \textit{ABCA1} is a well-established plasma membrane (PM) localized lipid efflux protein, we hypothesized that \textit{ABCA8} might also localize at the PM and regulate cellular cholesterol transport. When expressed in COS-7 cells, \textit{ABCA8} was indeed localized at the PM and also colocalized with calnexin, indicating endoplasmic reticulum localization (Figure IV in the online-only Data Supplement). In contrast, \textit{ABCA8} harboring the P609R mutation was almost exclusively identified intracellularly, and colocalized with calnexin, indicating defective cell surface expression (Figure IV in the online-only Data Supplement). Thus, \textit{ABCA8} encoding P609R or T741X fails to translocate to the cell surface. IVS17-2 A>G mutation was not generated because we utilized a cDNA construct.

The ability of \textit{ABCA8} to localize at the PM suggests it could play a role in cholesterol efflux. Indeed, wild-type \textit{ABCA8} increased cholesterol efflux to ApoA-I by 181\% when transfected into COS-7 cells (Figure 4A). In contrast, transfection of P609R or T741X resulted in efflux comparable to empty vector controls (Figure 4A). Thus, \textit{ABCA8} facilitates cholesterol efflux to ApoA-I, which is abolished by both P609R and T741X. To further confirm the role of \textit{ABCA8} in efflux, and to compare its efflux capacity to \textit{ABCA1}, we assessed ApoA-I–mediated cholesterol efflux in fibroblasts isolated from \textit{ABCA8} and \textit{ABCA1} mutation carriers. Indeed, fibroblasts from heterozygous \textit{ABCA8} mutation carriers showed a 20\% to 43\% reduction in cholesterol efflux (Figure 4B). In comparison, cholesterol efflux from heterozygous \textit{ABCA1} mutation carrier fibroblasts was reduced by 43\% on average (Figure 4B). The mutations in the \textit{ABCA1} carriers are IVS24+1 G>C, p.Asp575Gly, and IVS4+8+2 T>C, and are loss of function mutations identified in patients with Tangier disease or Familial Hypoalphalipoproteinemia. Our data suggest that \textit{ABCA1} is a more potent facilitator of cholesterol efflux compared with \textit{ABCA8}, at least in fibroblasts. To confirm this difference in efflux capacity between \textit{ABCA1} and \textit{ABCA8}, we overexpressed similar amounts of \textit{V5-ABCA8} and \textit{V5-ABCA1} as assessed by anti-V5 immunoblots. \textit{ABCA1} showed a 1.8-fold increase in efflux capacity compared with \textit{ABCA8} (Figure 4C), confirming that \textit{ABCA1} is a more potent cholesterol efflux protein. Moreover, cotransfection of the 2 proteins resulted in further enhanced cholesterol efflux (Figure 4C), suggesting that \textit{ABCA8} and \textit{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, which induces the expression of ABCA1 but not ABCA8. 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 (Figure 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 Colocalizes With and Interacts With ABCA1**

Because ABCA8 and ABCA1 both localize at the PM and endoplasmic reticulum, facilitate cholesterol efflux to ApoA-I, and when expressed together, further enhance cholesterol efflux compared with each alone, and because a reduction in ABCA8 affects ABCA1-specific efflux, we hypothesized that they might interact. We first determined the subcellular colocalization of ABCA8 and ABCA1 by coexpressing both

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Adenosine triphosphate–binding cassette transporters A8 (ABCA8) expression modulates plasma high-density lipoprotein cholesterol (HDLc) levels. A, Plasma HDLc levels in Abca8b<sup>−/−</sup> 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) reverse transcription polymerase chain reaction (n=4–14), and (C) western immunoblotting 72 h after injection. HDLc (D) and total cholesterol (E) levels in control and ABCA8 overexpressing mice 24 hours after injection.
proteins with different tags (ABCA8-V5 and ABCA1-GFP) in HEK293T cells. Indeed, ABCA8 colocalizes completely with ABCA1 at the PM and intracellularly (Figure 4D). In addition, ABCA8 and ABCA1 communoprecipitate when both proteins are coexpressed (Figure 4E). Thus, ABCA8 and ABCA1 act together in regulating cholesterol efflux.

**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 with noncarrier relative controls. In mice, hepatic overexpression 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.24 Subcellularly, ABCA1 is localized to the PM, endoplasmic reticulum, and endocytic vesicles.25 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.25 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.26

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.27 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 on a western-type diet,29 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, 2 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 showed lower LDLc levels compared with 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 because of an accelerated LDL catabolism, similar to previous observations in hepatic *Abca1−/−* mice.30

When either *ABCA8* or *ABCA1* are overexpressed 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,31 while the absence of *Abca1* in mice leads to decreased RCT.32 Because ABCA8 and ABCA1 were overexpressed 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.34

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Hepatic Adenosine triphosphate–binding cassette transporters A8 (ABCA8) and Adenosine triphosphate–binding cassette transporters A1 (ABCA1) increase the early steps of in vivo reverse cholesterol transport. A. Significantly increased plasma [3H] counts at 48 h in mice with hepatic overexpression of human ABCA8 or human ABCA1. B. Significantly increased liver [3H] counts in the liver-specific *ABCA8* or *ABCA1* overexpressing mice. C. Unchanged fecal [3H] counts in mice with *ABCA8* or *ABCA1* liver-specific overexpression. n=7 each. Mean and standard errors are shown.

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**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>TC</td>
<td>4.74±0.37</td>
<td>3.39±0.34</td>
<td>0.02</td>
</tr>
<tr>
<td>HDLc</td>
<td>4.46±0.35</td>
<td>3.17±0.31</td>
<td>0.01</td>
</tr>
<tr>
<td>LDLc</td>
<td>0.88±0.09</td>
<td>0.58±0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>TG</td>
<td>0.80±0.04</td>
<td>0.69±0.02</td>
<td>0.03</td>
</tr>
</tbody>
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Data are presented as mean±SD in mmol/L. HDLc indicates high-density lipoprotein cholesterol; TC, total cholesterol; LDLc, low-density lipoprotein cholesterol; and TG, triglycerides.
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,35 suggesting that ABCA8 does not compensate for the absence of ABCA1. This, together with our finding that ABCA8 and ABCA1 interact, suggest that these 2 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.36,37 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 with reduced ABCA1 levels. Thus, another possibility for the regulation of ABCA1 function by ABCA8 could be that ABCA8 transports lipids, perhaps sphingomyelin,26 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 2 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 overexpressed.

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, because of a lack of cholesteryl ester transfer protein in mice.38 Cholesteryl ester transfer protein 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 results in our human or mouse models. A humanized mouse such as CETP transgenic mice crossed to our Abca8b−/− mice would further 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.

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Disclosures
C. Radomski is an employee and M.R. Hayden is on the board at Xenon Pharmaceuticals.

References


Highlights

- Adenosine triphosphate–binding cassette transporters A8 regulates high-density lipoprotein cholesterol levels in humans and mice.
- Adenosine triphosphate–binding cassette transporters A8 facilitates cholesterol efflux to lipid-free apolipoprotein A1.
- Adenosine triphosphate–binding cassette transporters A8 interacts with adenosine triphosphate–binding cassette transporters A1, and further potentiates cholesterol efflux.
ABCA8 Regulates Cholesterol Efflux and High-Density Lipoprotein Cholesterol Levels

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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 (1). 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 (2). 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 (3). Other co-variables such as age, sex, BMI, medical history, alcohol intake, and smoking history were available for all individuals (1). 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 (1). 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 (1). 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) (1). 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://phase3browser.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/TMHMM/); OCTOPUS (http://octopus.cbr.su.se); NCBI Conserved Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)).

**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 (4) at LipoScience, Inc. (Raleigh, NC).

**Generation of Abca8b^−/− mice**
Sperm was obtained from Abca8b<sup>−/−</sup> mice (Abca8b<sup>tm1a(EUCOMM)Wtsi</sup>, 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-TTCATGTGTCATTGACACTTG, Rv-GGATCGGCATCCATTTTCTC; hHPRT1: Fw-TGACACTGGGAAAAACAAATGCA, Rv-GGTCCTTTTCACCAGCAAGCT; hTBP: Fw-TGCACAGGAGCCAAGAGTGA, Rv-CACATCACAGCTCCCCACCA; mRPL37: Fw-GGAGTGCAGGCTAAGAGAC, Rv-TCTGAATCTGCAGTACATCTC; mHPRT: Fw-AGTGGTGGATACAGGCCAGAC, Rv-CGTGATTCAAATCCCTGAAGT; mAbca8a: Fw-CGTGGCCCTATTGGCAAGA, Rv-CAGGTCCACATCAGGCGATG; mAbca8b: Fw-ATAAGTGTGCGCCAACAAACT, Rv-TGACAGGCGTGACCTATCA; mAbca1: Fw-TCCGAGCCAATGTCCCTTC, Rv-GCGCTCAAACCTTACGAAGGC). Relative quantification 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 (5).

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 Na$_2$HPO$_4$, 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 (6), 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.

**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 5x10^8 pfu of adeno-AP (alkaline phosphatase) to inactivate Kupffer cells (7), and tail vein injections were performed as previously described (8). 1x10^9 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 (9). 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% CO₂ 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, resuspended, and 2 million cells per mouse were injected intra-peritoneally into individually housed recipient mice that had been injected 6 hours before with 1x10⁹ 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 (9). 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.

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 (6).

**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 (10) to adjust for the
small sample size. The statistical analyses were performed with the MIXED
procedure in SAS 9.4.

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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*<sup>−/−</sup> 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 ($2^{(\Delta\DeltaCT)}$). 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.