CHAPTER 4

The contribution of Abcg5/Abcg8-independent pathways to hepatobiliary cholesterol secretion in mice

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Chapter 4

Summary

The ABC half-transporters ABCG5 and ABCG8 have been proposed to heterodimerize into a functional complex that mediates secretion of plant sterols and cholesterol by hepatocytes into bile and their apical efflux from enterocytes, thus limiting sterol accumulation in the body. We have addressed the putatively rate-controlling role of Abcg5/Abcg8 in hepatobiliary cholesterol excretion in mice. Despite similar bile salt (BS) excretion rates, total sterol and phospholipid (PL) output were reduced by 82 % and 35 %, respectively, in chow-fed Abcg5−/− mice compared to wild-type mice. Upon infusion with the hydrophilic bile salt TUDCA, similar relative increases in bile flow, BS output, PL output and total sterol output were observed in wild-type, Abcg5+/− and Abcg5−/− mice. Maximal cholesterol and PL output rates in Abcg5−/− mice were 15 % and 69 %, respectively, of wild-type values. The ratio cholesterol:PL remained similar during TUDCA infusion, i.e., 0.13 in wild-type and 0.027 in Abcg5−/− mice. In Abcg5+/− mice, this ratio changed from values similar to that in Abcg5−/− mice under basal conditions to that of wild-type mice upon TUDCA infusion. Infusion of increasing amounts of the hydrophobic bile salt TDCA increased cholesterol excretion 3.0- and 2.4-fold in wild-type and Abcg5−/− mice, respectively, but rapidly induced cholestasis in Abcg5−/− mice. Treatment with the LXR agonist T0901317 (0.015 % w/w in the diet, 7 days) increased the maximal sterol excretion capacity in wild-type mice but not in Abcg5−/− mice. In a separate study, LXRα−/− mice were fed chow containing 1 % w/w cholesterol. As expected, hepatic expression of Abcg5 and Abcg8 was strongly induced (5-fold and 4-fold, respectively) in wild-type but not in LXRα−/− mice. Surprisingly, hepatobiliary cholesterol excretion was increased to the same extent, i.e., 2.2-fold in wild-type mice and 2.0-fold in LXRα−/− mice, upon cholesterol-feeding. Our data confirm that Abcg5, probably as part of the Abcg5/Abcg8 heterodimer, is strongly involved in the control of hepatobiliary cholesterol secretion. However, BS infusions in Abcg5−/− mice and feeding of a high cholesterol-diet to LXRα−/− mice demonstrate that Abcg5/Abcg8 heterodimer-independent, inducible routes exist that contribute to total hepatobiliary cholesterol output.
Introduction

Mutations in the ATP-binding-cassette (ABC) half-transporters ABCG5 and ABCG8 cause sitosterolemia, which is characterized by the accumulation of plant sterols in the body. It has been proposed that ABCG5 and ABCG8, which are highly expressed in the liver and the small intestine, heterodimerize into a functional complex. Mutations in either one of the genes cause the biochemical hallmarks of the disease in humans as well as in mouse models. The daily intake of plant sterols, like sitosterol and campesterol, from a "Western-type" diet is in the same order of magnitude as that of cholesterol. However, only trace amount of plant sterols are absorbed in healthy subjects. Abcg5/Abcg8 mediates efflux of plant sterols from enterocytes back into the intestinal lumen and their excretion into bile, thus limiting their accumulation in the body. Expression of the Abcg5 and Abcg8 genes is controlled by the liver X-receptor (LXRα/NR1H3) and possibly by the liver-receptor-homologue 1 (LRH-1). LXR is activated by oxysterols and hence is considered a cholesterol sensor. Overexpression of Abcg5/Abcg8 in transgenic mice or via activation of LXR with synthetic ligands has been demonstrated to result in reduced intestinal cholesterol absorption and strongly increased hepatobiliary cholesterol excretion, while deletion of the genes, in general, has the opposite effects. It is therefore generally assumed that in the liver Abcg5/Abcg8 also mediates transport of cholesterol into bile. Indeed, treatment with synthetic LXR agonists resulted in induction of Abcg5 and Abcg8 gene expression in mice and, in parallel, increased hepatobiliary cholesterol excretion independent from phospholipid excretion. Data on biliary lipid composition have been reported so far in mice lacking Abcg5, Abcg8 or both. We have shown that gallbladder cholesterol concentrations are reduced by 50% in Abcg5/−/− mice compared to wild-type mice. Yu et al. reported a more dramatic decrease of gallbladder cholesterol concentrations (-90%) in Abcg5/−/−/Abcg8/−/− double-knockout mice. So far, data on actual biliary excretion rates are only available from Abcg8/−/− mice: total sterol secretion was found to be reduced by approximately 70% in comparison to wild-type mice. Surprisingly, sitosterol excretion was comparable to that in wild-type mice.

Several potential explanations exist for the apparent discrepancies between results obtained in the different animal models used in different laboratories. These include methodological aspects related to the bile collection procedure as well as the putative existence of Abcg5/g8-independent excretion pathways. In this study, we have used two strategies to address this issue, i.e., quantification of biliary output rates during bile salt infusion experiments in LXR-agonist-treated Abcg5/−/− mice and feeding high-cholesterol diet to LXRα/−/− mice. The data from these studies provide evidence to suggest that, under certain metabolic conditions, Abcg5/Abcg8-independent routes significantly contribute to total hepatobiliary cholesterol output in mice.
Materials and Methods

Animals and diets
Mice homozygous (\textit{Abcg5}^{-/-}) and heterozygous (\textit{Abcg5}^{+/+}) for the disruption of the \textit{Abcg5} gene and their wild-type littermates were used. Animals were housed in temperature-controlled rooms (21°C) with 12 hours light cycling and received standard mouse chow (Arie Blok, Woerden, The Netherlands) and water \textit{ad libitum}. The diet contained 0.017% (w/w) cholesterol and 0.045% (w/w) plant sterols.

\textit{Lxr}^{\alpha^{-/-}} mice, generated by Deltagen, Inc. (Redwood City, USA) using standard gene-targeting methods, were kindly provided by Tularik Inc. (South San Francisco, USA). In short, a 42 bp fragment corresponding to a segment of exon 2 was replaced by a beta-galactosidase cDNA and a phosphoglycerate kinase promoter-driven neomycin resistance cassette. The remaining procedure followed that described for the \textit{Abcg5}^{-/-} mice. Mice were genotyped via PCR using allele-specific primers (wild-type: GTTTCTCTCCCCTATCTATC-TAGGGAGAC; CACCCATTCTCCGTGTTCTCTTTG; knockout: GGGCCAGCTCATT-CCTCCACTCAT). Mice homozygous (\textit{Lxr}^{\alpha^{-/-}}) and heterozygous (\textit{Lxr}^{\alpha^{+/+}}) for the disruption of the \textit{Lxr}^{\alpha} gene and their wild-type littermates received either standard mouse chow or chow diet containing 1% cholesterol (wt/wt; Arie Blok, Woerden, The Netherlands) for two weeks. Male mice of 2-4 months were used. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Experiments in \textit{Abcg5}^{-/-} mice
\textit{Abcg5}^{-/-} and wild-type mice were fed either standard laboratory chow or chow supplemented with the synthetic LXR-agonist T0901317 (0.015%, w/w; Cayman Chemicals, Ann Arbor, MI, USA) for 7 days. \textit{Abcg5}^{+/+} mice received standard chow only. Female mice of 3-6 months were used.

Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and Diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder. After two basal bile samples of 15 minutes, mice were continuously infused with tauroursodeoxycholate (TUDCA; Calbiochem/Merck Biosciences, Darmstadt, Germany) or taurodeoxycholate (TDCA; Calbiochem/Merck Biosciences) in PBS via the jugular vein. Infusion rates were increased in a stepwise manner: 150, 300, 450, 600 nmol/min and 25, 50, 75, 100 nmol/min for TUDCA and TDCA, respectively. During bile collection, body temperature was stabilized using an humidified incubator. At the end of the collection period, animals were killed by cardiac puncture. Livers were excised and weighed.

Biochemical analysis and hepatic lipid contents was studied in an independent experiment. 5-7 months old male \textit{Abcg5}^{-/-}, \textit{Abcg5}^{+/+} and wild-type mice were fed standard laboratory chow. Mice were anesthetized with isofluorane and killed by cardiac puncture. Blood was collected in EDTA-containing tubes. Livers were excised and weighed.
Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses and RNA isolation.

**Experiments in Lxra^{+/+} mice**

Lxra^{+/+}, Lxra^{+/} and Lxra^{−/−} mice were anaesthetized by intraperitoneal injection with Hypnorm and Diazepam as described above. Bile was collected by cannulation of the gallbladder for 30 minutes. Body temperature was stabilized using an humidified incubator. Subsequently, animals were killed by cardiac puncture and livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses and RNA isolation.

**Analytical procedures**

Biliary bile salt concentrations were measured enzymatically. Biliary phospholipid and sterol concentrations in the Abcg5 experiments were determined as described previously; no difference was made between cholesterol and plant sterols. In Lxra mice, phospholipids and cholesterol in bile were determined as described by Böttcher et al. and Gamble et al., respectively, after extraction according to Bligh and Dyer. The same extraction method was applied for hepatic lipids, after which commercially available kits were used for the determination of unesterified and total cholesterol (Wako, Neuss, Germany), and for triglycerides (Roche, Mannheim, Germany). Pooled plasma samples from all animals of one group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) as described previously.

**RNA isolation and PCR procedures**

Total RNA was extracted from frozen tissues with TriReagent (Sigma, St. Louis, MO, USA) and quantified photometrically. cDNA synthesis was performed using recombinant M-MLV reverse transcriptase (10 U/µl), the appropriate buffer, dNTPs (500 µM), random nonamers (1 µM), RNase inhibitor (2 U/µl; all from Sigma) and total RNA (50 ng/µl). The reaction mix was incubated for 10 minutes at 25°C for primer annealing, 60 minutes at 37°C for synthesis and 5 minutes at 94°C to denature the RT enzyme. Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector as previously described. Primers were obtained from Invitrogen (Carlsbad, USA). Fluorogenic probes, labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes used in these studies have been described elsewhere (Srebp1a, Srebp1c, Srebp2, Lxra, Srb1, Acat1, Acat2, Hmgr, Cyp7a1, Cyp27, Abca1, Abcg5, Abcg8, Mdr2, Bsep, 18S rRNA; beta actin, Ldlr, with the exception of Abcg1, Abcg2, Npc111, 36b4 and Cyclophilin (see Table I for further information). All data of the Abcg5 experiment were subsequently normalized to the median of beta-actin, 36b4, 18S rRNA, and cyclophilin as described by Vandesompele et al. In the Lxra experiments, beta-actin alone was used for normalization.
Table I: Primer sequences used in mRNA quantification by real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcg1</td>
<td>NM_009593</td>
<td>forward CAAGACCTTTTGAAAGGATCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse GCCAGAATATTCATGAGGAGCCAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>probe CCCATGATGCACCCATCTCCTCC</td>
</tr>
<tr>
<td>Abcg2</td>
<td>AF140218</td>
<td>forward AATCAGGGCATCGAATCTGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse CAGGTAGGCAATTGAGGAAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>probe CAAGCCAGGGCCACATCTTCC</td>
</tr>
<tr>
<td>Npc1l1</td>
<td>NM_207242</td>
<td>forward GAGAGCCAAAGATGCTACTTTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse CCCGGGAAGTTGTCATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>probe ACTCCAGCAAACACGCACTG</td>
</tr>
<tr>
<td>36b4</td>
<td>XM_356471</td>
<td>forward GCTTCATTGTGGGAGCAGACA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse CATGGTGTCTTGCAATTGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>probe TCCAAGCAGATGCAGATCG</td>
</tr>
<tr>
<td>cyclophilin</td>
<td>XM_356256</td>
<td>forward CAGATCGAGGGATCGATTGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse TCACACCTTGAGACCCTATTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>probe CTCCTCCACATTGGAGACAGAGATGCA</td>
</tr>
</tbody>
</table>

All probes are labeled with FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamine) at the 5’- and 3’-end, respectively.

Statistics
Statistical analyses were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, USA). Differences between genotypes were evaluated using the Mann-Whitney-U-test. A p-value smaller than 0.05 was considered statistically significant.

Results

Plasma and hepatic lipid composition in Abcg5−/− mice
We have recently reported that Abcg5−/− mice have elevated plasma triglyceride levels compared to wild-type mice, whereas plasma cholesterol concentrations (measured by gas chromatography) were decreased. To further investigate plasma lipid composition, lipoproteins were fractionated by FPLC and both triglycerides and total sterols (cholesterol + plant sterols) were measured enzymatically (Fig. I). Total sterol content was virtually identical in the two genotypes. The increase in plasma triglycerides was exclusively found in the VLDL-sized fraction; peak height was 2.5 times in the Abcg5−/− mice compared to wild-types.

As previously reported, liver weight was slightly increased in Abcg5−/− mice compared to wild-type littermates (Table II). This was not due to steatosis, because triglyceride concentrations were unaffected. The concentrations of phospholipids as well as that of total sterols and unesterified sterols were almost identical between all groups. Sterol ester concentrations in Abcg5−/− mice were reduced by 45% compared to wild-type and heterozygotes. It should be
noted that sterol concentrations were measured enzymatically and consisted of both cholesterol and plant sterols. We have previously shown that plant sterols comprise up to 42% of total sterols in livers of Abcg5−/− mice while plant sterols were present in low amounts in livers of wild-type mice.5

**Figure 1:** Distribution of sterols (above) and triglycerides (below) in plasma lipoprotein fractions of wild-type and Abcg5−/− mice.

Blood was collected via cardiac puncture and pooled before FPLC analysis. Analysis was performed as described in MATERIALS AND METHODS.

**Table II:** Liver parameters of male Abcg5+/+, Abcg5+/− and Abcg5−/− mice on chow diet.

<table>
<thead>
<tr>
<th></th>
<th>Abcg5+/+</th>
<th>Abcg5+/−</th>
<th>Abcg5−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio liver weight/body weight</td>
<td>0.049 ± 0.002</td>
<td>0.049 ± 0.004</td>
<td>0.058 ± 0.002*</td>
</tr>
<tr>
<td>Total sterols</td>
<td>5.14 ± 0.64</td>
<td>5.52 ± 0.72</td>
<td>5.24 ± 0.43</td>
</tr>
<tr>
<td>Sterol ester</td>
<td>0.77 ± 0.29</td>
<td>0.79 ± 0.50</td>
<td>0.43 ± 0.19*</td>
</tr>
<tr>
<td>Unesterified sterols</td>
<td>4.37 ± 0.53</td>
<td>4.73 ± 0.26</td>
<td>4.80 ± 0.44</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>19.2 ± 15.4</td>
<td>11.7 ± 9.8</td>
<td>8.9 ± 3.5</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>34.8 ± 1.4</td>
<td>36.0 ± 0.4</td>
<td>36.8 ± 2.1</td>
</tr>
</tbody>
</table>

Male Abcg5+/−, Abcg5+/+ and littermate control mice, 5-7 months old, were fed standard laboratory chow. Mice were anesthetized with isoflurane and killed by cardiac puncture. Livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses. Lipids were extracted and analysed as described in MATERIALS AND METHODS. No difference was made between cholesterol and other sterols. * indicates significant difference from wild-type group (Mann-Whitney-U-test, p<0.05). n =5-7 per group.
Table III: Hepatic mRNA expression levels in male Abcg5+/+, Abcg5+/- and Abcg5-/- mice on chow diet measured by real-time RT-PCR.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Abcg5+/+</th>
<th>Abcg5+/-</th>
<th>Abcg5-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srebp1a</td>
<td>1.00 ± 0.11</td>
<td>1.11 ± 0.11</td>
<td>1.06 ± 0.12</td>
</tr>
<tr>
<td>Srebp1c</td>
<td>1.00 ± 0.41</td>
<td>1.08 ± 0.29</td>
<td>0.95 ± 0.42</td>
</tr>
<tr>
<td>Srebp2</td>
<td>1.00 ± 0.14</td>
<td>1.08 ± 0.35</td>
<td>0.78 ± 0.17</td>
</tr>
<tr>
<td>Lxra</td>
<td>1.00 ± 0.08</td>
<td>0.99 ± 0.12</td>
<td>1.05 ± 0.08</td>
</tr>
<tr>
<td>Ldlr</td>
<td>1.00 ± 0.11</td>
<td>1.18 ± 0.24</td>
<td>0.78 ± 0.28</td>
</tr>
<tr>
<td>Srb1</td>
<td>1.00 ± 0.20</td>
<td>1.06 ± 0.19</td>
<td>0.98 ± 0.10</td>
</tr>
<tr>
<td>Hmgcr</td>
<td>1.00 ± 0.35</td>
<td>1.36 ± 0.56</td>
<td>0.50 ± 0.23*</td>
</tr>
<tr>
<td>Acat1</td>
<td>1.00 ± 0.11</td>
<td>0.94 ± 0.25</td>
<td>1.19 ± 0.20</td>
</tr>
<tr>
<td>Acat2</td>
<td>1.00 ± 0.18</td>
<td>1.24 ± 0.28</td>
<td>0.89 ± 0.32</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>1.00 ± 0.59</td>
<td>0.85 ± 0.66</td>
<td>1.82 ± 0.59*</td>
</tr>
<tr>
<td>Cyp27</td>
<td>1.00 ± 0.13</td>
<td>1.24 ± 0.14</td>
<td>1.22 ± 0.22</td>
</tr>
<tr>
<td>Abcg8</td>
<td>1.00 ± 0.19</td>
<td>1.41 ± 0.44</td>
<td>1.26 ± 0.37</td>
</tr>
<tr>
<td>Abca1</td>
<td>1.00 ± 0.12</td>
<td>1.01 ± 0.09</td>
<td>1.24 ± 0.11*</td>
</tr>
<tr>
<td>Abcg1</td>
<td>1.00 ± 0.36</td>
<td>0.98 ± 0.21</td>
<td>1.04 ± 0.13</td>
</tr>
<tr>
<td>Abcg2</td>
<td>1.00 ± 0.29</td>
<td>0.96 ± 0.19</td>
<td>0.98 ± 0.20</td>
</tr>
<tr>
<td>Npc1l1</td>
<td>1.00 ± 0.13</td>
<td>0.97 ± 0.60</td>
<td>1.41 ± 0.26*</td>
</tr>
<tr>
<td>Bsep</td>
<td>1.00 ± 0.18</td>
<td>1.00 ± 0.13</td>
<td>0.99 ± 0.05</td>
</tr>
</tbody>
</table>

Male Abcg5+/+, Abcg5+/- and littermate control mice, 5-7 months old, were fed standard laboratory chow. Mice were anesthetized with isoflurane and killed by cardiac puncture. Liver pieces were snap frozen in liquid nitrogen and stored at -80°C. mRNA extraction, cDNA synthesis and TaqMan PCR analysis was done as described in MATERIALS AND METHODS. All data were normalized to the median of beta-actin, 36b4, 18S rRNA, and cyclophilin as described by Vandesompele et al.23 n = 5-8 per group. * indicates significant difference from wild-type group (Mann-Whitney-U-test, p<0.05).

Hepatic gene expression
In male mice, hepatic gene expression was determined by real-time RT-PCR (Table III). The mRNA levels of regulatory genes (SREBP1a, 1c, 2; LXRa) were identical in all groups. Expression of hepatic cholesterol uptake systems, i.e., Ldlr and Srb1, also did not differ between groups. On the other hand, expression of hepatic cholesterol metabolism genes was different in Abcg5-/- mice compared to wild-type and Abcg5+/- mice: the expression of Hmgcr, rate-controlling for cholesterol synthesis was reduced by 50% in Abcg5-/- mice. Expression of Cyp7a1, encoding the enzyme responsible for the majority of bile salt synthesis, was upregu-
lated by 82% in Abcg5+/− mice. Expression levels of Acat1 and Acat2, involved in cholesteryl ester formation, were not impaired in Abcg5+/− and Abcg5−/− mice compared to wild-type controls.

Gene expression of a wide spectrum of transporters potentially involved in cholesterol transport was screened: only the expression of Abca1 (+24%) and Npc1l1 (+41%) did show significant increases in Abcg5−/− mice compared to their wild-type littermates. Expression of Abcg1, Abcg2, Abcg8 and Bsep was identical in all three genotypes. As anticipated, Abcg5 mRNA was virtually absent (5% mRNA remaining compared to wild-type) in Abcg5−/− mice (Figure 2A). Surprisingly, Abcg5 expression in heterozygous mice was 76% of wild-type values. No effect on the expression of the phospholipid flippase Mdr2 (Abcb4) was noticed (Figure 2B).

![Graph A: Abcg5 mRNA expression](https://example.com/grapha.png)

![Graph B: Mdr2 mRNA expression](https://example.com/graphb.png)

![Graph C: Sterol output](https://example.com/graphc.png)

![Graph D: Phospholipid output](https://example.com/graphd.png)

**Figure 2:** Hepatic Abcg5 and Mdr2 mRNA expression levels (above) and hepatobiliary sterol and phospholipid output rates (below) of Abcg5+/+, Abcg5+/- and Abcg5−/− mice.

A and B: Liver pieces were snap frozen in liquid nitrogen. mRNA extraction, cDNA synthesis and TaqMan PCR analysis was done as described in MATERIALS AND METHODS. All data were normalized to the median of beta-actin, 36b4, 18S rRNA, and cyclophilin as described by Vandesompele *et al.*23 * indicates significant difference from wild-type group (Mann-Whitney-U-test, p<0.05).

C and D: Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and Diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder for 15 minutes. Biliary phospholipid and sterol concentrations were determined enzymatically as described previously16 no difference was made between cholesterol and plant sterols. * indicates significant difference from wild-type group (Mann-Whitney-U-test, p<0.05).
Biliary sterol and phospholipid secretion rates are decreased in *Abcg5*<sup>−/−</sup> mice

*Abcg8*<sup>−/−</sup> mice have been reported to have decreased hepatobiliary cholesterol and phospholipid secretion rates, while for *Abcg5*<sup>−/−</sup> mice only gallbladder concentration data are available. Therefore, hepatic bile was collected from *Abcg5*<sup>+/+</sup>, *Abcg5*<sup>+/−</sup> and *Abcg5*<sup>−/−</sup> mice for 15 minutes to determine basal biliary lipid output rates. *Abcg5* knockout mice presented with a significantly increased bile flow compared to heterozygotes and wild-type littermates (8.9 ± 2.3 in *Abcg5*<sup>−/−</sup> mice vs. 6.6 ± 1.5 and 5.0 ± 2.9 µl/min/100 g body weight in *Abcg5*<sup>+/+</sup> and *Abcg5*<sup>+/−</sup> mice, respectively). Hepatobiliary sterol (page 91, Fig. 2C) and phospholipid excretion rates (Fig. 2D) were significantly decreased in both heterozygous and homozygous knockout mice compared to wild-type controls. Bile salt output rates were unaffected, *i.e.*, 325 ± 121, 309 ± 198 and 332 ± 164 nmol/min/100 g body weight in *Abcg5*<sup>+/+</sup>, *Abcg5*<sup>+/−</sup> and *Abcg5*<sup>−/−</sup> mice, respectively.

Figure 3: Hepatobiliary phospholipid and sterol output rates and the sterol:phospholipid ratio under basal conditions and during infusion with TUDCA in *Abcg5*<sup>+/−</sup>, *Abcg5*<sup>+/−</sup> and *Abcg5*<sup>−/−</sup> mice.

Phospholipids and sterols were measured enzymatically as described previously, no difference was made between cholesterol and plant sterols. * indicates significant difference between basal level and maximal level during TUDCA infusion (Mann-Whitney-U-test, p<0.05).
Biliary lipid excretion increases upon infusion with TUDCA in Abcg5\(^{-/-}\), Abcg5\(^{+/+}\) and wild-type mice

Systemic infusion of hydrophilic bile salts increases bile flow and facilitates hepatobiliary lipid secretion in wild-type mice.\(^{24}\) To investigate if this forced flow could restore impaired phospholipid- and sterol secretion in Abcg5\(^{5/-}\) mice, we infused Abcg5\(^{5/+}\), Abcg5\(^{5/-}\) and Abcg5\(^{5/-}\) mice with increasing concentrations of tauroursodeoxycholate (TUDCA). Bile flow was increased in Abcg5\(^{5/-}\) mice compared to wild-type and heterozygote mice already under basal conditions. This effect was even more pronounced at higher infusion rates (data not shown). Bile salt excretion rates were indistinguishable between genotypes (data not shown).

As depicted in Figure 3, phospholipid excretion increased upon TUDCA infusion in all genotypes (+140 %, +280 % and +150 % in Abcg5\(^{5/+}\), Abcg5\(^{5/-}\) and Abcg5\(^{5/-}\) mice, respectively). In parallel, hepatobiliary sterol excretion also increased in all strains (+230 %, +700 %, and +120 % Abcg5\(^{5/+}\), Abcg5\(^{5/-}\) and Abcg5\(^{5/-}\) mice, respectively). Noteworthy, the initially low sterol excretion rate in heterozygous Abcg5 mice recovered upon infusion of TUDCA to reach wild-type-levels. Taken together, the sterol:phospholipid-ratios in Abcg5\(^{5/-}\) mice and in Abcg5\(^{5/-}\) mice were significantly lower than in wild-type mice on the basal level. Upon infusion with TUDCA, the ratio normalized in Abcg5\(^{5/-}\) mice but not in Abcg5\(^{5/-}\) mice.

Figure 4: Bile flow and hepatobiliary bile salt, phospholipid and sterol output rates during infusion with increasing amounts of TDCA in Abcg5\(^{5/-}\) and wild-type mice. TDCA was infused via the jugular vein at rates of 25, 50, 75 and 100 nmol/min. Measurements were done as described in MATERIALS AND METHODS. The arrow points at the appearance of red-colored bile in Abcg5\(^{5/-}\) mice.
Biliary lipid excretion increases upon infusion with TDCA in both Abcg5<sup>−/−</sup> and wild-type mice

To determine if a more hydrophobic bile would restore hepatobiliary cholesterol excretion in Abcg5<sup>−/−</sup> mice, Abcg5<sup>−/−</sup> mice and littermate controls were infused with increasing amounts of the hydrophobic bile salt taurodeoxycholate (TDCA; page 93, Figure 4). At infusion rates of up to 75 nmol/min, bile flow and hepatobiliary bile salt output were indistinguishable between the two groups. At 100 nmol TDCA/min, the highest infusion rate tested, bile of the Abcg5 knockout mice turned red and bile flow dramatically decreased. Therefore, we calculated the maximal capacity of hepatobiliary lipid secretion at the second highest rate (75 nmol/min).

Both phospholipid excretion curves and sterol excretion curves were lower in the Abcg5<sup>−/−</sup> mice compared to wild-type controls. However, both phospholipid (3.5-times) and sterol (2.4-times) excretion increased in Abcg5<sup>−/−</sup> mice upon infusion with TDCA. This increase was not statistically different from that in wild-type mice (2.1-times for phospholipids and 3.5-times for sterols).

The Abcg5-independent sterol secretion is LXR-independent

LXR is involved in the regulation of cholesterol homeostasis at various levels. To unravel if the remaining sterol excretion capacity in Abcg5<sup>−/−</sup> mice is LXR-dependent, wild-type and Abcg5<sup>−/−</sup> mice were fed the LXR agonist T0901317 for two weeks. Similar to results obtained before, LXR activation led to the presence of large, triglyceride-rich HDL particles in Abcg5<sup>−/−</sup> mice (data not shown).<sup>21</sup> As also reported before, treatment with T0901317 yielded an increased hepatobiliary sterol secretion (+210 %) and a reduced phospholipid secretion (-35 %), resulting in a relative sterol hypersecretion in wild-type mice.<sup>21</sup> In Abcg5<sup>−/−</sup> mice, however, no increase in sterol secretion was observed. In contrast, a slight drop of the already low sterol secretion rate occurred (from 1.1 to 0.7 nmol/min/100 g BW).

Upon infusion with TUDCA, phospholipid secretion rates more than doubled in all groups. The maximal excretory rate for sterols went up 4 times in wild-type mice treated with the LXR agonist compared to non-treated wild-type mice. However, in Abcg5<sup>−/−</sup> mice no difference was observed between LXR-treated and non-treated mice. Figure 5 visualizes the effect of LXR activation in Abcg5<sup>−/−</sup> mice and wild-type littermates. The sterol:bile salt ratio is increased in wild-type mice upon treatment with T0901317, whereas the lines for Abcg5<sup>−/−</sup> mice are similar to each other and significantly lower than the former.
**Figure 5:** The effect of LXR activation in Abcg5<sup>-/-</sup> mice and wild-type littermates during TUDCA infusion on hepatobiliary cholesterol output.

TUDCA was infused via the jugular vein in a stepwise manner as described in MATERIALS AND METHODS: 150, 300, 450, 600 nmol/min. Sterols were measured enzymatically as described previously;<sup>16</sup> no difference was made between cholesterol and plant sterols.
Increased hepatobiliary cholesterol excretion independent of Abcg5 and Abcg8

To test whether the reported increase in hepatobiliary cholesterol excretion upon cholesterol feeding is LXR- (and Abcg5-) dependent, LXRα−/−, LXRα+/− and wild-type control mice were fed a diet containing 1% cholesterol to increase delivery of cholesterol to the liver (Figure 6). Consequently, hepatic expression of Abcg5 and Abcg8 was increased in control mice and heterozygotes on high-cholesterol diet. In LXRα−/− mice on high cholesterol diet, Abcg5 and Abcg8 expression did not differ from chow-fed animals. Bile flow, biliary bile salt and phospholipid excretion rates did not differ between the groups. Surprisingly, hepatobiliary cholesterol excretion was increased in all mice on high cholesterol diet, regardless of genotype (Figure 6).

**Figure 6:** Hepatic Abcg5 and Abcg8 mRNA expression levels (right) and hepatobiliary cholesterol and phospholipid output rates (left) of LXRα+/+, LXRα+/− and LXRα−/− mice fed chow (open bars) or high-cholesterol diet (filled bars).

A and B: Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and Diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder for 30 minutes. Biliary sterol and phospholipid concentrations were determined as described in MATERIALS AND METHODS. * indicates significant difference between chow-fed and high-cholesterol groups (Mann-Whitney-U-test, p<0.05).

C and D: Liver pieces were snap frozen in liquid nitrogen. mRNA extraction, cDNA synthesis and TaqMan PCR analysis was done as described in MATERIALS AND METHODS. All data were normalized to beta-actin. * indicates significant difference between chow-fed and high-cholesterol groups (Mann-Whitney-U-test, p<0.05).
Discussion

The mechanism by which cholesterol is excreted from the hepatocyte into the bile is still an unsolved problem in lipid biochemistry. The recent discovery of the ABC-half transporters Abcg5 and Abcg8 as important players herein\textsuperscript{1,2} has shed some light on the involvement of transporter proteins in this process, but substantial parts have remained in the dark. Knocking out the genes encoding Abcg5, Abcg8 or both in mice dramatically reduces biliary cholesterol concentrations\textsuperscript{5,6,14}, which was taken to indicate that Abcg5 and Abcg8 function as a heterodimer in the excretion process. In this study we wanted to address the extent of rate-control by Abcg5 in hepatobiliary (chole)sterol secretion. For this purpose, actual hepatobiliary sterol secretion rates were determined under basal conditions and during bile salt infusions in Abcg5-deficient mice with and without treatment with the synthetic LXR agonist T0901317. In addition, LXRα-deficient mice fed a high-cholesterol diet were studied.

Both hepatobiliary phospholipid and sterol secretion were strongly impaired under basal, non-stimulated conditions in Abcg5\textsuperscript{+/-} and Abcg5\textsuperscript{-/-} mice. Hepatic gene expression of Abcg5 was reduced by only 24% in the heterozygotes, whereas that of Mdr2 (Abcb4) was identical in all groups. The relatively small reduction of Abcg5 expression in heterozygotes already almost mimicked the effect of complete absence of Abcg5 on sterol secretion. Therefore, it is reasonable to conclude that under these conditions Abcg5 expression is limiting for a large fraction of hepatobiliary sterol secretion and for a part, either directly or indirectly, also for phospholipid secretion. This high degree of control was partly overcome when bile salt secretion was stimulated by infusion of the hydrophilic bile salt TUDCA. Under these conditions, both sterol and phospholipid secretion rates approximated wild-type levels in the heterozygotes, resulting in a normalization of the sterol:phospholipid ratio in bile. This indicates that a high bile salt flux creates a situation in which biliary sterol excretion becomes (partly) independent of Abcg5 expression levels. Under the same conditions, biliary sterol- and phospholipid secretion in Abcg5\textsuperscript{-/-} mice did increase substantially, but remained low when compared to Abcg5\textsuperscript{+/-} and Abcg5\textsuperscript{-/-} mice. It is tempting to speculate that this increase reflects an Abcg5/Abcg8-independent, but bile salt-dependent part of biliary cholesterol secretion that contributes approximately 20% of total under chow-fed conditions.

Infusion of the hydrophobic bile salt TDCA restored cholesterol secretion, but not phospholipid secretion, in mice lacking Mdr2 (Abcb4).\textsuperscript{24} TDCA infusions at low concentrations increased phospholipid and sterol secretion in Abcg5\textsuperscript{-/-} mice at the same relative rate as observed in wild-type mice, however, at a much lower absolute level. At higher concentration, bile turned red and mice became cholestatic. It is likely that this is caused by the detergent effect of TDCA on canalicular membranes in the absence of sufficient amounts of cholesterol and phospholipids.

In a series of classical papers, Yousef and colleagues studied the effects of bile salts on biliary lipid composition in rats.\textsuperscript{25-28} Upon infusion of hydrophobic bile salts, typically phospholipid
secretion declined first, followed by decreases in bile flow, bile salt output and cholesterol output. Concomitantly with the decline in phospholipid output, phospholipid composition changed from mainly phosphatidylcholine to more phosphatidylethanolamine and sphingomyelins, which was attributed to partial solubilization of the canalicular membrane. Our data from TDCA-infused Abcg5/−/− mice, however, differ in the kinetics of the process reported by Yousef et al.: in Abcg5/−/− mice, the maximum secretory rate for phospholipids and bile salts as well as the maximal bile flow rate were reached earlier than that of sterols. This may indicate that in the outer leaflet of the canalicular membrane, sufficient sterols were present—even in the absence of Abcg5— which could be "dissolved" by hydrophobic micelles. Obviously, this model would argue against a role of Abcg5/Abcg8 as a flippase and would favor a liftase mode of action as proposed by Small. However, further studies are necessary to rule out that the late increase in sterol excretion in the Abcg5/−/− mice is caused by hepatic micro-bleedings which could theoretically provide erythrocyte membranes as a source for the sterols measured in bile. A recent study has demonstrated that the expression of Abcg5, Abcg8, Abca1 and other genes involved in the regulation of cholesterol transport is increased by the LXR agonist T0901317. LXR activation dramatically increased sterol excretion in wild-type mice on basal levels and upon infusion with TUDCA. However, no additional effect of LXR activation was observed in Abcg5/−/− mice. The remaining, Abcg5-independent sterol secretion is obviously independent from LXR-activated systems. We have recently reported that cholesterol concentrations in the gallbladder of Abcg5/−/− mice are reduced by 50% compared to wild-type mice. In this study, we found a much stronger reduction of biliary cholesterol output rates, as measured after cannulation through the gallbladder. This discrepancy between low cholesterol output rates upon cannulation and downstream concentrations in the gallbladder may be caused by processes in the gallbladder itself. It can be speculated that gallbladder epithelial cells might be able to deliver cholesterol to the bile. This issue clearly needs further investigation. To further substantiate the hypothesis that Abcg5-independent cholesterol secretion mechanism exist, we examined a model in which the expression of this transporter remains unchanged upon loading of the liver with dietary cholesterol, i.e., the LXRα−/− mouse on a high cholesterol diet. Wild-type mice react with an up-regulation of Abcg5/Abcg8 expression when challenged with a high cholesterol diet. This response is mediated via LXR and, consequently, abolished in LXRα−/− mice. Nevertheless, both wild-type and LXRα−/− mice showed a significant increase in hepatobiliary cholesterol excretion, independent of the Abcg5/Abcg8 expression level. This could either mean that the Abcg5/Abcg8 heterodimer is not rate-controlling under these conditions or that other routes compensate for the missing excretory system under this particular stress. Recently, Kosters et al. reported that, across various mouse models, a strong correlation exists between biliary cholesterol excretion and hepatic Abcg5/g8 expression. An exception to this rule was the diosgenin-treated mouse.
Diosgenin is a plant sterol-like compound known to induce hypersecretion of cholesterol by so far unidentified mechanisms. Absence of LXRα in combination with a high dietary cholesterol intake seems to add another model in which Abcg5/Abcg8 expression does not correlate with cholesterol excretion rates. We therefore favor the hypothesis that (an) LXR- and Abcg5/Abcg8-independent route(s) might come into play in specific situations.

Taken together, our studies support the notion that Abcg5/Abcg8 has rate-controlling function for the majority of hepatobiliary cholesterol transport in mice. However, a considerable fraction of cholesterol reaches the bile via an Abcg5/Abcg8-independent route. Furthermore, our data indicate that cholesterol excretion can be greatly increased without changes in Abcg5/Abcg8 expression levels. In other words, changes in hepatic Abcg5/Abcg8 expression alone do not always predict changes in the actual metabolic flux.

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References


