Mouse models of cholestatic liver disease: PFIC revisited

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Abcg5/8 independent biliary cholesterol excretion in Atp8b1-deficient mice

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ABSTRACT

BACKGROUND & AIMS: ATP8B1 is a phosphatidylserine flippase in the canalicular membrane; patients with mutations in ATP8B1 develop severe chronic (PFIC1) or periodic (BRIC1) cholestatic liver disease. We have observed that Atp8b1 deficiency leads to enhanced biliary cholesterol excretion. It has been established that biliary cholesterol excretion depends on transport by the heterodimer Abcg5/Abcg8. We hypothesized that the increased cholesterol output was due to enhanced extraction from the altered canalicular membrane rather than to higher Abcg5/Abcg8 activity. We therefore studied the relation between Abcg5/Abcg8 expression and biliary cholesterol excretion in mice lacking Atp8b1, Abcg8 or both.

METHODS: Bile formation was studied in LXR agonist-fed wild-type mice as well as mice lacking Atp8b1, Abcg8, or both (GF mice) upon infusion of taurocholate. Bile samples were analyzed for cholesterol, bile salt, phospholipids and ecto-enzyme content.

RESULTS: LXR agonist increased Abcg5/8 expression and this was accompanied by increased biliary cholesterol output in both wild-type and Atp8b1<sup>G308V/G308V</sup> mice. However, Atp8b1<sup>G308V/G308V</sup> mice maintained higher cholesterol output. While in Abcg8<sup>−/−</sup> mice biliary cholesterol output was severely reduced, GF mice displayed high biliary cholesterol output which was comparable to wild-type. Bile of both Atp8b1<sup>G308V/G308V</sup> and GF mice displayed elevated levels of phosphatidylserine and sphingomyelin, indicating membrane stress.

CONCLUSIONS: Our data demonstrate that the increased biliary cholesterol excretion in Atp8b1-deficient mice is independent of Abcg5/8 activity. This implicates that Atp8b1 deficiency leads to a decrease in the detergent resistance and subsequent nonspecific extraction of cholesterol from the canalicular membrane by bile salts.
INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is a severe form of inherited cholestasis. Three types of PFIC, type 1-3, are known and are caused by mutations in respectively \( \text{ATP8B1, ABCB11, and ABCB4} \) (for review see ref \(^1\)). All these genes encode proteins necessary for proper bile formation: \( \text{ABCB11} \) (or BSEP) mediates the hepatobiliary transport of bile salts. \( \text{ABCB4} \) (or MDR3) translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane, while \( \text{ATP8B1} \) (or FIC1) translocates phosphatidylserine from the outer to the inner leaflet of this membrane.\(^2\) Many PFIC patients develop severe liver failure and need to undergo liver transplantation.

Apart from PFIC type 1, mutations in \( \text{ATP8B1} \) also cause benign recurrent intrahepatic cholestasis (BRIC1).\(^3,4\) While PFIC1 manifests as a chronic cholestasis which progresses to endstage liver disease, BRIC1 is characterized by bouts of cholestasis leaving no overt liver damage. PFIC1 was first described in the Amish population.\(^5\) The most common mutation in this population is a conserved glycine to valine substitution at amino acid 308 (G308V), which leads to the severe form of the disease. ATP8B1 is expressed in the apical membrane of many epithelial cells, including the canalicular membrane of hepatocytes.\(^6,7\)

To study the effect of Atp8b1 deficiency, Pawlikowska et al.\(^8\) produced mice bearing the G308V mutation, designated \( \text{Atp8b1}^{G308V/G308V} \) mice. This mutation leads to near complete absence of Atp8b1 protein.\(^9\) These animals have elevated serum bile salt levels, which was initially contributed to increased intestinal bile salt uptake. However, we have recently shown that intestinal bile salt uptake is not enhanced in these mice.\(^10\)

In contrast to PFIC1 patients, Atp8b1-deficient mice only displayed a mild cholestasis.\(^9\) In the isolated perfused liver, biliary output of taurodeoxycholate (TDC) was \( >50\% \) reduced in Atp8b1-deficient mice compared to wild-type. Importantly, upon infusion with taurocholate, these mice had an increased biliary output of canalicular ecto-enzymes and cholesterol. Furthermore, phosphatidylserine (PS) was recovered from bile of Atp8b1-deficient animals, indicating that Atp8b1 is necessary to restrict PS to the inner leaflet of the canalicular membrane. Indeed, we and others have
demonstrated that ATP8B1 is a flippase for PS, translocating PS from the outer to the inner leaflet of the bilayer.\textsuperscript{2,11} All our observations suggested that the canalicular membrane is unstable when Atp8b1 is not active and is sensitive to extraction of cholesterol and other membrane constituents by hydrophobic bile salts.

Biliary cholesterol excretion is driven by the two half ABC transporters, Abcg5 and Abcg8, which form an active heterodimer.\textsuperscript{12} Mice lacking either Abcg8 or Abcg5 have an 80% reduced biliary cholesterol output\textsuperscript{13,14}, indicating that Abcg5/8 function is essential for the largest part of biliary cholesterol excretion. The increase in biliary cholesterol excretion in Atp8b1-deficient mice raises the question whether this involves enhanced Abcg5/8 activity or aspecific extraction of cholesterol from the membrane. To study this, we produced mice deficient in both Atp8b1 and Abcg8. These Atp8b1\textsuperscript{G308V/G308V}/Abcg8\textsuperscript{-/-} double transgenic mice will further be described as GF mice. Our data show that enhanced biliary cholesterol excretion in Atp8b1-deficient mice is not dependent on Abcg5/8 activity. This indicates that the increase in biliary cholesterol output in these mice is caused by direct extraction of cholesterol from the canalicular membrane by hydrophobic bile salts. Our study also provides new fundamental insights in canalicular membrane function in general, and of intramembraneous cholesterol translocation in particular.

MATERIALS & METHODS

**ANIMALS:** All experiments were performed with age-matched male mice at 2-6 months of age. Wild-type, Atp8b1\textsuperscript{G308V/G308V}, Abcg8\textsuperscript{-/-} and Atp8b1\textsuperscript{G308V/G308V}/Abcg8\textsuperscript{-/-} (GF) mice used in the experiments were all of C57Bl/6 background and were bred in a pathogen free environment on a 12h light-dark cycle at the animal facility of the Academic Medical Center. Mice were fed AM-II chow (Arie Blok Diervoeders, Woerden, The Netherlands). In experiments with administration of cholate or the LXR agonist T0901317, the animals received a purified semi-synthetic diet (K4068.02, Arie Blok, Woerden, The Netherlands) with or without 0.5% cholate (Merck, Darmstadt, Germany) or 0.025% (w/w) LXR agonist T0901317 (Cayman Chemicals, Ann Arbor, USA). Food and water were supplied ad libitum. All animal experiments were approved
by the institutional animal care and use committee (IUCAC) of the Academic Medical Center.

**IN VIVO BILE SALT INFUSIONS:** The gallbladder and jugular vein were cannulated. Mice were placed on a thermostatted heating pad and body temperature was kept at 37°C. Prior to bile salt infusion, mice were depleted of endogenous bile salts by bile collection without infusion. At t = 90 min, taurocholate (TC) was infused via the jugular vein. Infusion rates were increased every 30 min with 400 nmol/min*100g (400-800-1200-1600 nmol/min*100g). Bile samples were collected every 10 minutes.

**HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY:** Phospholipids extracted from bile samples were dissolved in chloroform/methanol (1:2) and run on silica gel 60 plates (Merck, Darmstadt, Germany). Spot densities were quantified via densitometric scanning using Quantity One-4.2.3 software (BioRad, Veenendaal, the Netherlands).

**DETERMINATION OF mRNA LEVELS:** Total RNA was isolated using the Trizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Purified RNA was treated with RQ1 RNase-free DNase (1 units/2 μg of total RNA, Promega, Madison, WI, USA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the protocols supplied by the manufacturers. Gene expression analysis was performed on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) by using the Bio-Rad iQ SYBR Green Supermix. PCR primers (supplemental table) were designed on the basis of Primer Express 1.7 software with the manufacturer’s default settings (Applied Biosystems, Foster City, CA, USA) or found by consulting the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/citation.html). All primers were validated for identical efficiencies. The measured mRNA level was normalized for total mRNA content by dividing though the levels of 36B4 (acidic ribosomal phosphoprotein P0). Lack of changes in 36B4 expression was checked by comparison with the other housekeeping genes: Hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophilin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**PROTEIN ANALYSIS:** Abcg5 and Atp1a1 protein levels (the latter as a loading control) were determined by western blot in total liver homogenates exactly as described in
Klett et al.\textsuperscript{14} using rabbit anti-ABCG5 (raised at the Academic Medical Center) and rabbit anti-ATP1A1 in a 1:1000 dilution. Homogenates were prepared by cutting liver tissue into small pieces and homogenization in ice cold lysis buffer (5 mM Tris-pH7.5, 250 mM sucrose with protease inhibitors) by 10 strokes in a dounce homogenizer.

**Bile Salts, Phospholipids, and Cholesterol Assays:** Bile salt, choline-containing phospholipids, and cholesterol were determined enzymatically.\textsuperscript{17} CD13 activity was determined using L-alanine-ß-naphtylamide-HBr (ICN, Aurora, OH, USA) as substrate.\textsuperscript{18} All measurements were done on a Novostar analyzer (BMG Labtech GmbH, Offenburg, Germany).

**Statistics:** All data are given as means ± SD. Significance was tested by use of the One-Way ANOVA with Bonferroni’s correction for multiple testing. In case of the RT-PCR experiments, a Student’s t-test was performed.

**Results**

**LXR-Dependent Abcg5/8 Expression in Mice Lacking Atp8b1**

We have previously shown that biliary cholesterol excretion is significantly increased in mice lacking Atp8b1 (Atp8b1\textsuperscript{G308V/G308V} mice). To elucidate the mechanism of this enhanced excretion we studied the expression level of Abcg5 and Abcg8 and their induction by Lxr. Figure 1a shows the normalized mRNA levels in control and Atp8b1\textsuperscript{G308V/G308V} mice after treatment with LXR agonist. Figure 1b gives the corresponding data for Abcg5 protein as determined by western blotting. Although Fig. 1a suggests that Abcg5 mRNA is induced stronger in Atp8b1\textsuperscript{G308V/G308V} mice than in wild-type mice, this does not translate into higher protein levels. In fact, Abcg5 protein tends to be lower in Atp8b1\textsuperscript{G308V/G308V} mice compared to wild-type after treatment with the agonist but this was not significant. We also checked this at the functional level by measuring biliary cholesterol excretion in both strains of mice after administration of diet in the absence or presence of T0901317. Figure 1b shows that our original observation of increased biliary cholesterol excretion in Atp8b1-deficient mice could be replicated in mice against a different genetic background (C57Bl/6). Upon
Abcg5/8 dependent cholesterol excretion in Atp8b1 deficient mice

administration of T0901317 cholesterol excretion was increased to the same extent in both strains. Strikingly, under both feeding conditions the elevated levels of biliary cholesterol in the Atp8b1<sup>G308V/G308V</sup> mice compared to the levels found in wild-type mice remained, suggesting that this increase is not dependent on the level of Abcg5/8 expression. Because we have observed that these mice have slight but significant increases in serum bile salt levels, we investigated whether Abcg5/g8 induction by bile salt was different in wild-type vs. Atp8b1<sup>G308V/G308V</sup> mice. To this end we fed both strains with lab chow,
purified diet or purified diet supplemented with 0.5% cholate and we measured the hepatic expression of Abcg5 and Abcg8. In wild-type mice cholate feeding induced the expression of both halftransporters (Fig. 2a), in line with what has been published before.\textsuperscript{19} However, in \textit{Atp8b1}\textsuperscript{G308V/G308V} mice this induction was completely blunted. Figure 2b gives the Abcg5 protein levels in liver membranes from the same animals. This figure demonstrates that induction of Abcg5 also occurs at the protein level and confirms the lack of induction in \textit{Atp8b1}\textsuperscript{G308V/G308V} mice. Hence, the increased biliary cholesterol excretion in these animals cannot be explained by (bile salt induced) up-regulation of Abcg5/8 levels; in fact expression does not change with a cholate challenge.

It has been shown before that biliary cholesterol excretion is nearly completely dependent on Abcg5/8 activity and that biliary cholesterol excretion is reduced by 80% in \textit{Abcg8}\textsuperscript{−/−} mice, as well as in \textit{Abcg5/8} double knockout mice\textsuperscript{13,20,21}. In order to further delineate the mechanism of enhanced cholesterol excretion in \textit{Atp8b1}\textsuperscript{G308V/G308V} mice, we produced a double transgenic strain which bears the mutant \textit{Atp8b1} gene as well as the disruption of the \textit{Abcg8} gene. We designated these homozygous double transgenic mice, GF mice.

Table 1 shows the basal serum values for ASAT, ALAT and bilirubin for all four animal groups. Serum bile salt levels are significantly increased in the \textit{Atp8b1}\textsuperscript{G308V/G308V} mice (as previously reported), but these levels were even substantially higher in the GF mice. It is important to notice that serum bile salt levels in these \textit{Atp8b1}\textsuperscript{G308V/G308V} mice on a C57Bl/6 background are considerably higher than the levels we earlier reported in the same genotype on a 129Sv background. On both types of genetic background the serum bile salt levels proved to be highly variable, however. As expected, cholesterol excretion into bile was strongly depressed in \textit{Abcg8}\textsuperscript{−/−} animals. Liver weight of \textit{Atp8b1}\textsuperscript{G308V/G308V} animals was significantly higher than that of wild-type animals and this tended to be similar in the GF mice.
**FIGURE 2. Induction of Abcg5/8 expression by chronic cholate feeding.**

Wild-type and Atp8b1<sup>G308V/G308V</sup> mice were fed the indicated diet for one week. Subsequently, livers were harvested and processed for western blotting and quantitative RT-PCR. Panel a shows mRNA levels for Abcg5 and Abcg8. Expression was normalized to housekeeping genes as indicated in Materials and Methods and is given as averages ± S.D. (n=4). Panel b shows a representative western blot for Abcg5. The figure displays duplicate lanes with liver homogenates from two independent mice treated with the same indicated experimental condition. The plasma membrane protein Atp8a1 was taken as a loading control. Staining intensity on western blots was subsequently quantified and is given as averages ± S.D. (n = 4). Significant differences are indicated as: *P < .005, cholate vs corresponding control; #P < .01, Atp8b1<sup>G308V/G308V</sup> vs. wild-type under the same condition. Solid bars: wild-type; open bars: Atp8b1<sup>G308V/G308V</sup>. 

A

B

Panel a shows mRNA levels for Abcg5 and Abcg8. Expression was normalized to housekeeping genes as indicated in Materials and Methods and is given as averages ± S.D. (n=4). Panel b shows a representative western blot for Abcg5. The figure displays duplicate lanes with liver homogenates from two independent mice treated with the same indicated experimental condition. The plasma membrane protein Atp8a1 was taken as a loading control. Staining intensity on western blots was subsequently quantified and is given as averages ± S.D. (n = 4). Significant differences are indicated as: *P < .005, cholate vs corresponding control; #P < .01, Atp8b1<sup>G308V/G308V</sup> vs. wild-type under the same condition. Solid bars: wild-type; open bars: Atp8b1<sup>G308V/G308V</sup>. 

FIGURE 2. Induction of Abcg5/8 expression by chronic cholate feeding.
### Table 1. Plasma and bile parameters of wild-type, Atp8b1<sup>G308V/G308V</sup>, Abcg8<sup>−/−</sup> and GF mice.

Hepatic bile was collected during the first 15 minutes after cannulation of the gallbladder. Body weight is given as absolute values and liver weight is given as percentage of body weight. ASAT: aspartate aminotransferase; ALAT: alanine aminotransferase. Values are given as mean ± SD. *P < .05 GF vs. all groups, †P < .05 Abcg8<sup>−/−</sup> vs. wild-type, ‡P < .05 Atp8b1<sup>G308V/G308V</sup> vs. wild-type.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type&lt;sub&gt;(n = 5)&lt;/sub&gt;</th>
<th>Atp8b1&lt;sup&gt;G308V/G308V&lt;/sup&gt;&lt;br&gt;(n = 5)</th>
<th>Abcg8&lt;sup&gt;−/−&lt;/sup&gt;&lt;br&gt;(n = 4)</th>
<th>Atp8b1&lt;sup&gt;G308V/G308V&lt;/sup&gt;&lt;br&gt;&lt;sup&gt;†&lt;/sup&gt;Atp8b1&lt;sup&gt;G308V/G308V&lt;/sup&gt; (n = 4)</th>
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<tr>
<td>Plasma</td>
<td></td>
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<tr>
<td>AST (U/L)</td>
<td>194.4 ± 165.0</td>
<td>244.0 ± 195.8</td>
<td>79.0 ± 30.4</td>
<td>261.0 ± 36.90</td>
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<tr>
<td>ALT (U/L)</td>
<td>26.4 ± 10.4</td>
<td>50.4 ± 20.1</td>
<td>31.0 ± 17.1</td>
<td>91.0 ± 69.8</td>
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<tr>
<td>Bilirubin (μmol/L)</td>
<td>3.2 ± 2.0</td>
<td>8.0 ± 6.3</td>
<td>1.0 ± 2.0</td>
<td>8.0 ± 4.6</td>
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<tr>
<td>Bile salt (total μmol/L)</td>
<td>2.16 ± 0.57</td>
<td>30.6 ± 38.0</td>
<td>1.67 ± 0.67</td>
<td>100.6 ± 40.34&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Bile flow (μL/min·100 g)</td>
<td>9.55 ± 2.60</td>
<td>12.8 ± 5.20</td>
<td>9.7 ± 3.3</td>
<td>13.9 ± 4.76</td>
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<td>Bile salt (nmol/min·100 g)</td>
<td>502 ± 254</td>
<td>855.1 ± 410.7</td>
<td>362.2 ± 193.3</td>
<td>628.4 ± 261.1</td>
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<td>Phospholipids (nmol/min·100 g)</td>
<td>56.9 ± 24.8</td>
<td>76.1 ± 21.8</td>
<td>30.1 ± 11.6</td>
<td>82.1 ± 32.3</td>
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<tr>
<td>Cholesterol (μL/min·100 g)</td>
<td>2.2 ± 2.6</td>
<td>5.1 ± 2.7</td>
<td>0.12 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6 ± 1.8</td>
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<tr>
<td>Body weight</td>
<td>25.3 ± 2.00</td>
<td>29.0 ± 0.70</td>
<td>32.0 ± 2.0</td>
<td>31.8 ± 1.70</td>
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<tr>
<td>Liver weight (% of body weight)</td>
<td>4.6 ± 0.5</td>
<td>6.6 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.0 ± 0.6</td>
<td>6.3 ± 1.5</td>
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**SERUM BILE SALT SPECIES**

We have previously shown that Atp8b1<sup>G308V/G308V</sup> mice have increased levels of tri- and tetrahydroxylated bile salts. We therefore checked whether the single and double mutant mice have similar bile salt compositions. Fig. 3 shows that the serum levels of most bile salt species are increased in animals lacking Atp8b1 and this tended to be higher in GF mice than in Atp8b1<sup>G308V/G308V</sup> mice. It is clear, however, that in animals with high plasma bile salt levels and Atp8b1 deficiency the absolute and relative levels of tetra- and pentahydroxylated bile salts are increased. Hence, the relative serum bile salt composition is similar in Atp8b1<sup>G308V/G308V</sup> and GF mice.
Abcg5/8 dependent cholesterol excretion in Atp8b1 deficient mice

**FIGURE 3. Serum bile salt species.**
Serum bile salt species were determined in peripheral serum by HPLC-MS. Panel a shows values for the bile salts presenting trace amounts (up to 6 μmol/L), panel b shows values for the more abundant bile salts. pentahydroxyl: pentahydroxyl bile salt; tetrahydroxyl: tetrahydroxyl bile salt; muricholate: CA; cholate: UDCA: ursodeoxycholate; di-OH: chenodeoxycholate/deoxycholate. The values represent means ± S.D.. Solid bars: wild-type (n = 5), open bars: Atp8b1G308V/G308V (n = 5), dark shaded bars: Abcg8−/− (n = 3), light shaded bars: GF (n = 3). Significance was tested using one-way ANOVA and Bonferroni’s correction for multiple testing. *P < .05 GF vs. all other groups, #P < .05 Atp8b1G308V/G308V vs. Abcg8−/−.

**BILIARY FLOW, BILE SALT AND ChoLESTEROL EXCRETION IN SINGLE AND DOUBLE MUTANT MICE**

Fig. 4 depicts bile flow (a) and bile salt excretion (b) upon bile depletion and subsequent infusion of taurocholate. Only in Abcg8−/− animals bile flow drops during the third step of infusion, indicating hepatic stress and onset of cholestasis. Figure 5a shows the cholesterol output related to the bile salt output for wild-type and Atp8b1G308V/G308V mice for each bile sample during the infusion phase. As we have reported previously, Atp8b1G308V/G308V mice secrete significantly higher amounts of cholesterol into bile than wild-type animals. Figure 5b shows the same correlation for Abcg8−/− and GF mice. While cholesterol excretion is dramatically reduced in Abcg8−/− mice, GF mice still secrete substantial amounts of cholesterol (comparable to wild-type) although excretion was lower than in Atp8b1G308V/G308V mice. These data clearly indicate that the increased cholesterol output in the absence of Atp8b1 is independent of Abcg5/8 function. To ensure that the biliary cholesterol we measured was cholesterol and not other (plant) sterols, we determined the sterol species in bile by use of GC-MS. We did not find other sterols than cholesterol (data not shown).
CHAPTER 2

FIGURE 4. Bile flow and biliary bile salt output upon bile salt infusion in various genotypes.
Intravenous taurocholate infusion was performed in the indicated strains of mice. Bile flow (panel a) and bile salt output (panel b) are plotted in time. Depletion phase, 0-90 minutes; at t=90 min taurocholate was infused at increasing rates (see Materials and Methods). Values represent means ± S.D. from 4 to 6 animals for each strain. ■, wild-type; □, Atp8b1\textsuperscript{G308V/G308V}; ▲, Abcg8\textsuperscript{−/−}; △, GF.

Figure 5c shows that phospholipid output in all groups has a similar dependence on the bile salt output. Figure 5d shows the output of the ecto-enzyme CD13. In both Atp8b1\textsuperscript{G308V/G308V} and GF mice there was enhanced excretion of this ecto-enzyme into bile, whereas this is not the case for the wild-type and Abcg8\textsuperscript{−/−} animals. This also confirms that the increased sensitivity of the canalicular membrane towards bile salt-mediated extraction is maintained in GF mice.
**Abcg5/8 dependent cholesterol excretion in Atp8b1 deficient mice**

**FIGURE 5.** Bile salt-dependent output of cholesterol, phospholipids, and aminopeptidase N/Cd13 in mice lacking Atp8b1, Abcg8 or both proteins.

Bile samples from the experiment displayed in Figure 4 were analyzed for the content of bile salt, cholesterol, choline-containing phospholipids, and aminopeptidase N/Cd13 activity. The data for all individual bile samples were plotted against the corresponding bile salt excretion rates. Panel A: bile salt-dependent cholesterol (CH) excretion in wild-type and Atp8b1<sup>G308V/G308V</sup> mice. Panel B: bile salt-dependent cholesterol excretion in Abcg8<sup>−/−</sup> and GF mice. Panel C: bile salt-dependent excretion of choline-containing phospholipid (PL) in wild-type and Atp8b1<sup>G308V/G308V</sup> mice. Panel D: bile salt-dependent excretion of choline-containing phospholipid (PL) in Abcg8<sup>−/−</sup> and GF mice. Panel E: bile salt-dependent excretion of aminopeptidase N/Cd13 in wild-type and Atp8b1<sup>G308V/G308V</sup> mice. Panel F: bile salt-dependent excretion of aminopeptidase N/Cd13 in Abcg8<sup>−/−</sup> and GF mice. Values represent measurements from 4 to 6 animals for each strain.

**PHOSPHOLIPID SPECIES IN BILE DURING DEPLETION AND INFUSION PHASE**

Bile samples taken during the bile salt infusion phase were analyzed for phospholipid species by means of HPTLC analysis. The values are given as a percentage of total phospholipid. Figure 6 shows that PS shows up in bile of both strains of animals lacking the Atp8b1 protein. Mice that do express Atp8b1 have undetectable levels of PS. Similarly, SM levels were increased in both strains lacking Atp8b1. A significant decrease in biliary PC can be seen in bile from animals lacking Atp8b1, which can be
explained by the fact that the values are presented as percentage of total and not as absolute values.

![Phospholipid species in bile during taurocholate infusion phase.](image)

**FIGURE 6.** Phospholipid species in bile during taurocholate infusion phase. Bile samples obtained in the experiment of Figure 5 at t = 160 min were analyzed for phospholipid composition by high-performance thin-layer chromatography. PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; L-PC, lysophosphatidylcholine. Values are given as mean percentage of total ± S.D.. Significance was tested using one-way ANOVA and Bonferroni correction for multiple testing. *P < .05 for Atp8b1<sup>G308V/G308V</sup> and GF vs. wild-type and Abcg8<sup>−/−</sup>. Solid bars: wild-type (n = 3), open bars: Atp8b1<sup>G308V/G308V</sup> (n = 3), dark shaded bars: Abcg8<sup>−/−</sup> (n = 3), light shaded bars: GF (n = 3).

**EXPRESSION LEVELS OF OTHER GENES INVOLVED IN BILE FORMATION**

Finally, we have measured the expression levels of several other genes relevant for bile formation (Figure 7). As expected, no expression of Abcg8 was observed in the corresponding knockouts and, as reported earlier for the single knockout, these strains have reduced Abcg5 expression. Expression of Mrp2 and Mrp3 were significantly increased in the Atp8b1<sup>G308V/G308V</sup> mice although variation was very high. We have also semi-quantitatively determined the protein levels of Abcb11 and Mrp2 by western blotting and found no significant differences among the four strains of mice (data not shown). In line with our previous report we observed no difference in Abcb11 protein levels among the different mouse strains<sup>9, 14</sup>. We also did not find a significant difference in Mrp2 protein levels among any of the four strains. Hence, the observed
difference in mRNA levels was not big enough to translate into significantly different protein levels. Interestingly, the expression of Fxr was significantly reduced in both strains lacking Abcg8 expression.

**DISCUSSION**

In this study we present data to substantiate our hypothesis that Atp8b1 is required to render the canalicular membrane resistant towards lipid extraction by bile salts. Atp8b1 is a P-type ATPase that flips PS from the outer to the inner leaflet of the canalicular membrane. This function reduces the relative PS content of the canalicular outer leaflet and, as a consequence, increases the relative SM content of this leaflet, thereby making it more rigid. As we have previously reported, absence of Atp8b1 leads to enhanced biliary output of PS, cholesterol and ectoenzymes. Upon an intravenous infusion of taurocholate, Atp8b1<sup>G308V/G308V</sup> mice show an approximate doubling in biliary cholesterol excretion compared to wild-type mice. It is well established that biliary cholesterol excretion is highly dependent on the activity of the ABC transporter couple Abcg5/8. To investigate whether the increased cholesterol excretion in Atp8b1-deficient mice depends on activity of the cholesterol transporter...
Abcg5/Abcg8, we created a double mutant mouse for Atp8b1 and Abcg8. The absence of Abcg8 in the double transgenic GF mice did not prevent the increased cholesterol excretion, caused by Atp8b1 deficiency. In fact, in absolute terms the difference in cholesterol excretion between Atp8b1<sup>G308V/G308V</sup> and wild-type mice was similar to that between Abcg8<sup>−/−</sup> and GF mice (about 3 nmol/min.100gr at a bile salt excretion rate of 1000 nmol/min.100gr). These observations prove that the observed increase in biliary cholesterol excretion is fully Abcg5/Abcg8-independent.

Other studies have reported that under normal conditions a small fraction of biliary cholesterol excretion in the mouse is independent of Abcg5/8 function. Plösch et al.<sup>20</sup> showed that in Abcg5<sup>−/−</sup> mice cholesterol excretion is reduced by 82% suggesting that only about 18% of the excretion occurs in an Abcg5/8-independent manner. Since Abcg5 and Abcg8 are halftransporters and only function as a heterodimer<sup>12</sup>, the Abcg5<sup>−/−</sup> mouse will have a very similar phenotype as the Abcg8<sup>−/−</sup> mouse. Studies by Kosters et al.<sup>21</sup> also provide proof for an Abcg5/Abcg8-independent fraction of biliary cholesterol excretion. In Abcg8<sup>−/−</sup> mice cholesterol excretion was decreased by 75%. Importantly, this percentage was independent of the type of bile salt that was infused. Thus, even infusion of the hydrophobic TDC did not elicit appreciable levels of cholesterol excretion.

Our findings provide a dramatic insight in the function of the canalicular membrane. Apparently, substantial amounts of cholesterol can be secreted in the absence of a cholesterol translocation machinery in case the asymmetry of the canalicular membrane is compromised. From this it can be inferred that spontaneous flipping of cholesterol from the inner to the outer leaflet is sufficient to drive a cholesterol excretion rate of at least 4 nmol/min.100gr., which was the maximal cholesterol excretion rate observed in the GF mice. For human standards this is a low rate but in rodents this is in fact higher than the cholesterol excretion rate observed in wild-type mice under conditions of endogenous bile formation (see table 1). Under these conditions Abcg5/8 function is apparently not required for sufficient supply of cholesterol to the outer leaflet of the membrane. Nevertheless, table 1 also shows that in the single Abcg8<sup>−/−</sup> mouse cholesterol excretion is negligible. Hence, when the canalicular membrane is asymmetric and contains little/no PS in the outer leaflet,
cholesterol excretion nearly completely depends on Abcg5/8 function. Conversely, when this asymmetry is (partly) lost, cholesterol can be fairly easily extracted from the membrane and does not require translocation by Abcg5/8. It may be suggested from these observations that biliary cholesterol excretion does not primarily depend on translocation but rather depends on the removal of the molecule out of the membrane. Thus, the function of Abcg5/8 as a "liftase", liberating cholesterol from the membrane may be more important for actual excretion than its translocator function, as was already hypothesized by Small.\(^{23}\) Our data show that the asymmetry of the canalicular membrane is crucial for these particular characteristics: in the presence of Atp8b1, which flips PS from the outer to the inner leaflet, the outer membrane leaflet is apparently so rigid that cholesterol is buried in the membrane and not available for bile salt-mediated extraction. This will be mainly due to the high relative content of SM in this condition (see for more elaborate discussion: ref \(^{1}\)). In the absence of Atp8b1, the outer leaflet will contain considerable amounts of PS which fluidizes this leaflet and allows bile salt-mediated extraction of cholesterol and other membrane constituents.

We can not exclude the possibility that in the absence of Atp8b1 another compensatory system for cholesterol excretion is induced. However, as long as no evidence for the existence of such system exists, we hypothesize that the increased cholesterol excretion into bile is caused by enhanced leaching of cholesterol from the more fluid canalicular membrane of these mice. This hypothesis is supported by the fact that the enhanced cholesterol excretion only occurs during excretion of more hydrophobic bile salts, such as taurocholate and taurodeoxycholate and was not observed during infusion of tauroursodeoxycholate.\(^{9}\)

Our data clearly show that the enhanced cholesterol excretion in Atp8b1 deficient mice cannot be caused by an upregulation of Abcg5/8 expression. We observed that the mRNA level of Abcg5 and Abcg8 is equal in wild-type and Atp8b1-deficient mice. Moreover, regulation of Abcg5/8 expression by Lxr was intact in these mice because it could be induced to the same extent as in wild-type mice by the administration of the LXR-agonist T0901317. This upregulation was accompanied by an expected increase in biliary cholesterol excretion in both strains of mice. Interestingly, the induction of
Abcg5/8 expression by chronic cholate administration in wild-type mice was completely blunted in Atp8b1-deficient mice. The cause of this is unclear but may have its background in the mild cholestasis that prevails in these animals. In accordance with the study of Paulusma et al.\(^9\), PS and SM end up in bile of Atp8b1-deficient mice when they are stressed with bile salts. This phenomenon is purely depending on the absence of the Atp8b1 protein and not a more general phenomenon of bile salt-induced cholestasis. As previously reported by Kosters et al.\(^{21}\) the Abcg8\(^{−/−}\) animals become cholestatic with the higher levels of taurocholate infusion (fig. 4a). However, even under this condition PS and SM are not detectable in the bile of these animals. It was shown that the onset of cholestasis upon high bile salt infusion is associated with a depletion of cholesterol from the canalicular membrane. A reduced cholesterol content of the membrane could cause impaired activity of Abcb11 or withdrawal of Abcb11 from the membrane, but this remains to be demonstrated. It is striking, however, that the taurocholate infusion induces acute cholestasis in the Abcg8\(^{−/−}\) mice but not in the GF mice. This could indicate that depletion of cholesterol from the membrane does not take place in these double knockout mice, which would be striking as the cholesterol excretion in these animals is in fact higher than in the single knockouts. A possible explanation for this apparent paradox could be that spontaneous flip-flop of cholesterol to the outer leaflet is slower in a rigid membrane (rich in SM and poor in outer leaflet PS as occurs in the presence of Atp8b1) than in a fluid leaflet (rich in outer leaflet PS as occurs in the absence of Atp8b1). It is, however, not possible to ascertain the asymmetric distribution in canalicular membranes \textit{in vivo}. Therefore, this hypothetical mechanism must be tested by \textit{in vitro} studies.

How our data can be translated into a disease mechanism for PFIC1 remains to be elucidated. However, they do demonstrate that changes in membrane asymmetry that occur in the absence of ATPB1 lead to nonspecific and disproportionate leaching of lipids, including cholesterol, from the membrane. This may very well affect proper function of the bile salt export pump (BSEP), with cholestasis as a consequence. Such a mechanism would explain how cholestasis can develop in the context of normal BSEP levels and a normal localization of the protein. It would also explain why bile diversion improves bile formation in these patients, as diversion leads to a shift of the bile salt
Abcg5/8 dependent cholesterol excretion in Atp8b1 deficient mice

composition towards more primary bile salts and less secondary bile salts. The latter will reduce nonspecific leaching of cholesterol and other lipids from the membrane and improve BSEP activity.

In conclusion, increased biliary cholesterol output in Atp8b1-deficient mice does not depend on the presence of Abcg5/8, and probably represents direct extraction of cholesterol from the canalicular membrane. These data provide a fundamental insight in the function of the canalicular membrane and provide a basis for one of the mechanisms of several forms of cholestasis in which the rigidity of this membrane may be compromised.
REFERENCE LIST


