Mouse models of cholestatic liver disease: PFIC revisited

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Citation for published version (APA):
Hepatic cytochrome p450 deficiency in mouse models for intrahepatic cholestasis predispose to bile salt induced cholestasis

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Submitted to the journal of hepatology
CHAPTER 5

ABSTRACT
Progressive familial intrahepatic cholestasis (PFIC) types 1 and 3 are severe cholestatic liver diseases caused by deficiency of ABTB8B1 and ABCB4, respectively. Mouse models for PFIC 1 and 3 display mild phenotypes when compared to corresponding human patients. Mice, unlike human, have the ability to detoxify hydrophobic bile salts by (re)hydroxylation and as a consequence have a more hydrophilic, less toxic bile salt pool. The enzymes responsible for this very efficient detoxification belong to the family of the cytochrome P450 system. In the present study we have generated mouse models for PFIC1 and PFIC3 which have impaired bile salt synthesis and are unable to hydroxylate secondary bile salts. A humanized bile salt pool was instilled directly from weaning by cholic acid supplementation of the diet. These double transgenic mouse models were obtained by backcrossing Atp8b1\textsuperscript{G308V/G308V} and Abcb4\textsuperscript{−/−} mice with Hrn mice, the latter having a liver-specific disruption of the cytochrome P450 reductase gene and therefore impaired P450 activity. Compared to Atp8b1\textsuperscript{G308V/G308V} and Abcb4\textsuperscript{−/−} single transgenes, cholic acid fed Atp8b1\textsuperscript{G308V/G308V}/Hrn and Abcb4\textsuperscript{−/−}/Hrn double transgenic mice displayed strongly enhanced plasma bilirubin and bile salt levels. Atp8b1\textsuperscript{G308V/G308V}/Hrn mice had a strongly reduced bile flow and displayed mild liver damage. The plasma bile salt pool was more hydrophobic compared to that of Atp8b1\textsuperscript{G308V/G308V} mice. Compared to Abcb4\textsuperscript{−/−} mice, bile flow in Abcb4\textsuperscript{−/−}/Hrn mice was elevated and coincided with enhanced bile duct proliferation and strongly aggravated liver damage. The biliary bile salt pool was more hydrophobic compared to that in Abcb4\textsuperscript{−/−} mice. Conclusion: the bile salt pool composition is a critical determinant in the initiation and progression of cholestasis and inducer of liver pathology in PFIC1 and PFIC3.
INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) type 1 and 3 are defined on basis of mutations in genes encoding two phospholipid transporting proteins that are essential in the formation of primary bile, i.e. ATP8B1 (PFIC1) and ABCB4 (PFIC3) \(^1\). PFIC1 patients present in the first years of life with jaundice and pruritis and suffer from progressive liver disease culminating in cirrhosis \(^2\). Young patients usually show a failure to thrive. Liver histology reveals bridging fibrosis but no bile duct proliferation. PFIC1 patients can develop extrahepatic symptoms, including hearing loss and diarrhea even after liver transplantation \(^3\). ATP8B1 is a flippase that translocates phosphatidylserine from the exoplasmic to the cytosolic leaflet of biological membranes \(^4,5\). We and others have previously shown that ATP8B1 deficiency leads to loss of the normal phospholipid asymmetry of the canalicular membrane. As a result the canalicular membrane becomes more sensitive to extraction of cholesterol by hydrophobic bile salts, which impairs the activity of the bile salt export pump (ABCB11) and, as a consequence, causes cholestasis \(^6\). PFIC3 patients suffer from a chronic and progressive cholestasis with high serum γ-glutamyltransferase levels; liver histology reveals fibrosis, which progresses into cirrhosis with portal inflammation and strong bile duct proliferation. In addition, milder mutations in ABCB4 have been shown to predispose to intrahepatic cholestasis of pregnancy, cholelithiasis, adult biliary cirrhosis, primary sclerosing cholangitis, and drug- and cytokine-induced cholestatic injury \(^7\). ABCB4 is a floppase that translocates phosphatidylcholine from the cytosolic to the exoplasmic leaflet of the canalicular membrane \(^8,9\). Phosphatidylcholine in the exoplasmic leaflet of the apical membrane can be extracted by bile salts micelles to form mixed micelles, thus reducing the detergent effect of bile salts \(^9\). Consequently, ABCB4 deficiency results in bile that is cytotoxic to the cells lining the biliary tree. For both PFIC1 and PFIC3 mouse models have been generated i.e. Atp8b1\(^{G308V/G308V}\) and Abcb4\(^{-/-}\) mice \(^10-12\). In contrast to PFIC1 patients, Atp8b1\(^{G308V/G308V}\) mice develop very little liver pathology and are only mildly cholestatic \(^10,13\). Similar to patients, Atp8b1\(^{G308V/G308V}\) mice the canalicular content has a coarsely granular appearance upon EM that is reminiscent of Byler’s bile \(^6\); In addition, these mice also suffer from hearing
loss caused by a progressive degeneration of stereocilia and cochlear hair cells.\textsuperscript{14} Abcb4\textsuperscript{-/-} mice display mild fibrosis that does not progress into cirrhosis; mice do display cholangiopathy i.e. periductal inflammation and bile duct proliferation. Still, liver failure does not occur during the life span of these animals.\textsuperscript{11,15,16}

Overall, these mouse models display much less severe phenotypes compared to those of human patients.\textsuperscript{11,12} A likely explanation for this discrepancy may be the difference in bile salt pool composition. The cytotoxic effect of bile depends on the hydrophobicity of the bile salt pool, which is different in mice and men. In contrast to men, mice are capable of rehydroxylating toxic, dihydroxy bile salts. Therefore, the bile salt pool of mice mainly consists of trihydroxy bile salts while the human bile salt pool contains more dihydroxy bile salts, including secondary bile salts that are formed in the gut by bacterial dehydroxylation of bile salts.

Bile salt synthesis and hydroxylation are mediated by the cytochrome P450 monoxygenase system in hepatocytes.\textsuperscript{19} All cytochrome P450s receive an electron from a single donor, a reaction mediated by the cytochrome P450 oxidoreductase (Cpr).\textsuperscript{20,21} Whole body deletion of cytochrome P450 oxidoreductase inactivates all cytochrome P450s and is embryonic lethal.\textsuperscript{22} Henderson et al. have generated a hepatocyte-specific cytochrome P450 oxidoreductase knockout mouse, also termed hepatic reductase null (Hrn) mouse.\textsuperscript{21} In this model, bile salt synthesis and (re)hydroxylation are reduced by 95%. Endogenous bile salt output in Hrn mice was 3-fold reduced compared to wild type mice, which is likely due to strongly reduced bile salt synthesis. In contrast to wild type mice of which the bile salt pool consists of 90% trihydroxy and 10% dihydroxy bile salts, the bile salt pool of Hrn mice contains approximately 55% trihydroxy and 45% dihydroxy bile salts after feeding these mice a cholic acid-supplemented diet.\textsuperscript{23}

We hypothesized that backcrossing Hrn mice with the existing mouse models for PFIC1 and PFIC3 would render mouse models that predispose to cholestatic liver disease upon instillation of a humanized bile salt pool.
MATERIALS AND METHODS

ANIMALS: C57bl/6 Cprlox/lox + CreALB (Hrn) animals were bred with C57bl/6 Atp8b1G308V/G308V mutant mice or FVB Abcb4<sup>−/−</sup> to obtain C57bl/6 Atp8b1G308V/G308V Cprlox/lox + Cre<sup>ALB</sup> (further designated as Atp8b1G308V/G308V<sup>−/−</sup>/Hrn) and C57bl/6/FVB Abcb4<sup>−/−</sup>/Cprlox/lox + Cre<sup>ALB</sup> (further designated as Abcb4<sup>−/−</sup>/Hrn), respectively. Littermates and WT mice (Harlan) were included as controls. The animals were kept in a pathogen-free environment on a controlled 12 hr light-dark regime in the animal facility of the Academic Medical Center Amsterdam. Male mice were fed a standard purified semi-synthetic diet (K4068.02; Arie Blok diervoeders, Woerden, The Netherlands) either or not supplemented with 0.03% cholic acid (CA) and water ad libitum starting from weaning. Abcb4<sup>−/−</sup>/Hrn were fed for 8 weeks, whereas Atp8b1G308V/G308V<sup>−/−</sup>/Hrn mice were fed a control diet for 4 weeks followed by a 0.03% CA-supplemented diet for an additional 4 weeks. Blood was taken every two weeks. After the feeding period, bile was collected after which the animals were sacrificed and blood and livers were collected. All animal experiments were approved by the institutional animal care and use committee of the Academic Medical Center.

BILE COLLECTION: In order to obtain bile, mice were anesthetized and placed on a thermostatted heating pad to remain body temperature. The common bile duct was closed with a ligature and the gallbladder canulated. Bile was collected in fractions of 10 or 15 minutes. At the end of the experiment blood and liver were harvested.

Plasma biochemistry:
Plasma biochemistry was performed by the routine laboratory of clinical chemistry of the AMC.

BILE SALT, PHOSPHOLIPID AND CHOLESTEROL ASSAYS: Biliary bile salts, choline-containing phospholipids, and cholesterol were determined enzymatically using a Novostar analyzer (BMG Labtech GmbH, Offenburg, Germany) as described earlier <sup>6</sup>. Hepatic bile salts were extracted after homogenizing in water using acetonitrile and dissolved in 25% methanol. Bile salt species were determined by reverse phase HPLC using a nano quantity analyte detector (NQAD) QT-500 (Quant technologies, Blaine, USA). In brief, bile was injected after dilution with water. 100 µl was applied to a
Hypersil C18, 3 µm, 15 cm HPLC column (Thermo Scientific, Breda, The Netherlands). The starting eluent consisted of 6.8 mM ammoniumformate (pH 3.9), followed by several steps of linear gradients to different concentrations of acetonitrile (ACN) (Biosolve, Valkenswaard, The Netherlands): 1 min 27% ACN, 13 min 42% ACN, 19 min 42% ACN, 20 min 60% ACN, 26 min 66% ACN, 27 min 80% ACN, 29 min 80% ACN and 30 min 0% ACN. Detection was performed using a NQAD QT-500. Quantification of the different bile salt species was performed by using a calibration curve for all different bile salt species.

**Quantitative PCR Analysis of Gene Expression:** Liver tissue was snap frozen and stored at -80°C. RNA was isolated using Trizol reagent (Invitrogen, Bleiswijk, The Netherlands). cDNA synthesis was transcribed using locked oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, Bleiswijk, The Netherlands). Quantitative PCR was performed using a Lightcycler 2.0 with the Fast Start DNA MasterPlus SYBR Green I kit (Roche, Woerden, The Netherlands). Expression levels were normalized for cyclophylin/hypoxanthine-guanine phosphoribosyltransferase.

**Immunohistochemistry:** Livers were fixed in 4% paraformaldehyde solution and embedded in paraffin wax. 7-µm Sections were stained with sirius red (collagen deposition), CK19 (bile duct proliferation) or Ki67 (cell proliferation). Oil red O (lipids) stainings were performed on frozen sections.

**Liver Triglycerides:** Liver triglycerides were extracted by a chloroform/methanol extraction method adapted from Srivastava et al. and measured using a Trig/GB-kit (Roche, Woerden, The Netherlands).

**Liver Hydroxyprolines:** Liver hydroxyprolines were extracted and measured according to van Westrhenen et al. After the extraction the amino acids (hydroxyprolines) were measured using HPLC and fluorescent detection.

**Statistical Analysis:** All data are given as means ± SEM or indicated else. Significance was tested by use of the Two-Way ANOVA with Bonferroni’s correction for multiple testing considering statistical significance when p<0.05.
RESULTS

We generated double transgenic mouse models for PFIC1 and PFIC3 by crossing Atp8b1<sup>G308V/G308V</sup> and Abcb4<sup>−/−</sup>, respectively, with hepatic reductase null (Hrn) mice. On top of deficiency for the respective transport proteins (ATP8B1 or ABCB4), both mouse models are for ~95% impaired in the synthesis of primary bile salts and the rehydroxylation of secondary bile salts (Hrn). The characteristics of the single transgenic Hrn mice with regard to bile formation have been described before. In the double transgenic mice we instilled a humanized bile salt pool by feeding 0.03% cholic acid (CA)-supplemented diets directly from weaning.

**AT WEANING ATP8B1<sup>G308V/G308V</sup>/HRN MICE ARE EXTREMELY SENSITIVE TO BILE SALT FEEDING**

When Atp8b1<sup>G308V/G308V</sup>/Hrn mice were fed a 0.03% CA-supplemented diet directly from weaning, they died within a week after the start of the diet. This precluded further analysis of bile salt feeding at young age. We therefore shifted to a protocol in which the animals were first fed a control diet from weaning for 4 weeks followed by a 0.03% CA-supplemented diet for another 4 weeks. Using this protocol the mice survived and all further experiments with these mice were done in this fashion.

**CA-FED ATP8B1<sup>G308V/G308V</sup>/HRN MICE ARE CHOLESTATIC AND DISPLAY MILD LIVER DAMAGE**

Mice fed control diets displayed normal weight gain, plasma biochemistry and bile flow (data not shown). In contrast to wild-type (WT), Hrn and Atp8b1<sup>G308V/G308V</sup> mice, Atp8b1<sup>G308V/G308V</sup>/Hrn mice failed to gain, and even lost weight after the start of the
**FIGURE 1. Characteristics of Atp8b1^{G308V/G308V}/Hrn**. Mice were fed semisynthetic control diet for 4 weeks starting from weaning followed by 0.03% CA supplemented diet for 4 weeks. Panel A displays the bodyweight gain (n=3-8), panel B represents plasma bilirubin (n=4-11) and panel C plasma AST (n=5-13). In panel D total plasma bile salt concentration (n=3-7) is plotted. Data are shown as mean ± SEM. *p<0.05; **p<0.001; ***p<0.0001.
New mouse models for PFIC predispose to cholestasis

Experimental diet at week 4 (Fig. 1A). After 4 weeks of CA feeding, Atp8b1G308V/G308V/Hrn mice displayed strongly enhanced plasma bilirubin levels compared to Atp8b1G308V/G308V, WT and Hrn controls (Fig. 1B). Plasma aspartate aminotransferase (AST) (Fig. 1C) and alanine transaminase (ALT) and alkaline phosphatase (AP) (data not shown) were mildly elevated. Plasma bile salt levels were strongly elevated compared to Atp8b1G308V/G308V mice (Fig. 1D). Bile flow was strongly (~2-fold) reduced compared to controls while bile flow in Atp8b1G308V/G308V mice was intermediate to controls and double transgenes (Fig. 3A). Biliary bile salt output, on the other hand, was ~3-fold higher in Atp8b1G308V/G308V/Hrn compared to the other groups (Fig. 3B). Due to >15% bodyweight-loss in the Atp8b1G308V/G308V/Hrn, the experiment had to be terminated 4 weeks after the start of the experimental diet. These data indicate that Atp8b1G308V/G308V/Hrn mice develop a much more severe cholestatic phenotype compared to Atp8b1G308V/G308V mice, whereas hepatic damage is comparable.

CA-FED ABCB4−/−/HRN ARE CHOLESTATIC AND DEVELOP SEVERE LIVER DAMAGE

Abcb4−/−/Hrn mice showed normal growth on both control (data not shown) and CA-supplemented diet when compared to Abcb4−/−, WT and Hrn control mice (Fig. 2A). In contrast to Abcb4−/− mice, plasma bilirubin levels of Abcb4−/−/Hrn mice were mildly increased already at weaning and further increased after 8 weeks of CA-supplemented diet (Fig. 2B). Plasma AST (Fig. 2C), ALT and AP (data not shown) and plasma bile salt levels (Fig. 2D) were strongly elevated in Abcb4−/−/Hrn, but not in Abcb4−/− mice. Bile flow and biliary bile salt output were approximately 2-fold increased compared to Abcb4−/−, WT and Hrn mice (Fig. 3C and 3D, respectively). These data indicate that cholestasis and hepatic damage are aggravated in Abcb4−/−/Hrn double transgenes compared to Abcb4−/− single transgenes.
FIGURE 2. Characteristics of Abcb4<sup>−/−</sup>/Hrn<sup>−/−</sup>. Mice were fed control diet or 0.03% CA supplemented diet for 8 weeks starting from weaning. Panel A displays the bodyweight gain (n=3-8), panel B shows plasma bilirubin (n=4-16) and panel C plasma AST (n=4-17). In panel D total plasma bile salt concentration (n=3-8) is plotted. Data are shown as mean ± SEM. *p<0.05; **p<0.001; ***p<0.0001.
New mouse models for PFIC predispose to cholestasis

**FIGURE 3. Bile flow and biliary bile salt output.** At the end of the bile salt feeding period, gallbladders were canulated and bile was sampled for 45 minutes. From the samples, bile flow was calculated and bile salts were measured. (A) Bile flow and (B) biliary bile salt output in wild-type (n=9), Hrn (n=6), Atp8b1<sup>G308V/G308V</sup> (n=3) and Atp8b1<sup>G308V/G308V</sup>/Hrn mice (n=7). (C) Bile flow and (D) biliary bile salt output in wild-type (n=4), Hrn (n=4 for flow), Abcb4<sup>−/−</sup> (n=7) and Abcb4<sup>−/−</sup>/Hrn (n=4) mice. Data are shown as mean ± SEM.

**PLASMA AND BILIARY BILE SALT SPECIES IN CA-FED ATP8B1<sup>G308V/G308V</sup>/HRN AND ABCB4<sup>−/−</sup>/HRN MICE**

We determined plasma- and biliary bile salt species at the end of the bile salt feeding period. Plasma bile salts in Atp8b1<sup>G308V/G308V</sup>/Hrn mice predominantly consisted of TC (~90% of total) and TßMC (~7% of total; Table 1 and Suppl. fig. 1A). Low concentrations of the secondary bile salt TDC (~0.4% of total) were detected. In Atp8b1<sup>G308V/G308V</sup> mice, plasma TC and TßMC made up ~70% and 20% of total bile salts, respectively. Biliary bile salts in Atp8b1<sup>G308V/G308V</sup>/Hrn mice predominantly consisted of TC (~90% of total)
Table 1  Plasma bile salt species in 0.03% cholic acid fed mice.
Plasma bile salt species of cholic acid fed mice were determined by HPLC analyses and are expressed as percentage of total bile salt. For clarity, total plasma bile salt concentrations are indicated. Hydrophobicity indices were calculated using the individual hydrophobicity indices as determined by Heuman [33]. Data are given as mean ± SD. *p = 0.0016 for Atp8b1<sup>G308V/G308V</sup> versus Atp8b1<sup>G308V/G308V</sup>/Hrn and *p = 0.49 for Abcb4<sup>−/−</sup> versus Abcb4<sup>−/−</sup>/Hrn using students t-test.; n.d., not detectable.

<table>
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<th>Species ( % of total )</th>
<th>WT (n=3)</th>
<th>Hrn (n=10)</th>
<th>Atp8b1&lt;sup&gt;G308V/G308V&lt;/sup&gt; (n=4)</th>
<th>Atp8b1&lt;sup&gt;G308V/G308V&lt;/sup&gt;/Hrn (n=6)</th>
<th>Abcb4&lt;sup&gt;−/−&lt;/sup&gt; (n=8)</th>
<th>Abcb4&lt;sup&gt;−/−&lt;/sup&gt;/Hrn (n=5)</th>
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<tr>
<td>Plasma total bile salts (μM)</td>
<td>4.1 ± 3.5</td>
<td>3.9 ± 3.0</td>
<td>187.8 ± 156.7</td>
<td>764.7 ± 698.5</td>
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<td>0.7 ± 0.6</td>
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<td>11.9 ± 16.9</td>
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<td>22.8 ± 3.3</td>
<td>12.3 ± 12.7</td>
<td>20.2 ± 7.7</td>
<td>8.7 ± 9.3</td>
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<td>Tauroursodeoxycholate</td>
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<td>0.3 ± 0.5</td>
<td>1.1 ± 2.2</td>
<td>0.4 ± 0.7</td>
<td>1.7 ± 2.4</td>
<td>4.0 ± 8.8</td>
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<td>Taurocholate</td>
<td>71.3 ± 5.7</td>
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<td>47.8 ± 36.1</td>
<td>81.9 ± 19.6</td>
<td>46.9 ± 19.8</td>
<td>76.4 ± 19.7</td>
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<td>1.2 ± 2.7</td>
<td>n.d.</td>
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<td>O-Muricholate</td>
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<td>n.d.</td>
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<td>1.2 ± 2.8</td>
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<td>13.6 ± 19.6</td>
<td>5.1 ± 8.9</td>
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<td>α-Muricholate</td>
<td>n.d.</td>
<td>0.7 ± 2.3</td>
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<td>β-Muricholate</td>
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<td>n.d.</td>
<td>5.5 ± 7.2</td>
<td>0.2 ± 0.2</td>
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<td>n.d.</td>
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<td>Cholate</td>
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<td>n.d.</td>
<td>n.d.</td>
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<td>16.5 ± 10.9</td>
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<td>-0.13 ± 0.22</td>
<td>-0.20 ± 0.01</td>
<td>-0.06 ± 0.05&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.03 ± 0.13</td>
<td>-0.06 ± 0.26&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>WT (n=8)</td>
<td>Hrn (n=9)</td>
<td>Atp8b1&lt;sup&gt;0/0&lt;/sup&gt;/Hrn (n=3)</td>
<td>Atp8b1&lt;sup&gt;0/0&lt;/sup&gt;/Hrn/Abcb4&lt;sup&gt;−/−&lt;/sup&gt; (n=6)</td>
<td>Abcb4&lt;sup&gt;−/−&lt;/sup&gt; (n=4)</td>
<td>Abcb4&lt;sup&gt;−/−&lt;/sup&gt;/Hrn (n=5)</td>
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<td><strong>Bile total bile salts (mM)</strong></td>
<td>84.2 ± 55.6</td>
<td>148.8 ± 73.8</td>
<td>114.3 ± 145.6</td>
<td>107.0 ± 92.3</td>
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<td>Species (% of total)</td>
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<td>Tauro-α-muricholate</td>
<td>2.3 ± 0.6</td>
<td>4.4 ± 2.9</td>
<td>n.d.</td>
<td>1.0 ± 2.3</td>
<td>0.9 ± 1.0</td>
<td>1.1 ± 0.5</td>
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<td>Tauro-β-muricholate</td>
<td>17.5 ± 4.2</td>
<td>4.4 ± 4.8</td>
<td>32.3 ± 14.9</td>
<td>27.4 ± 26.9</td>
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<td>Taurodeoxycholate</td>
<td>n.d.</td>
<td>7.1 ± 11.1</td>
<td>0.3 ± 0.6</td>
<td>4.2 ± 9.9</td>
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<td>3.6 ± 2.0</td>
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<td><strong>Hydrophobicity index</strong></td>
<td>-0.11 ± 0.07</td>
<td>0.01 ± 0.11</td>
<td>-0.25 ± 0.12</td>
<td>-0.20 ± 0.17&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-0.16 ± 0.05</td>
<td>-0.01 ± 0.01&lt;sup&gt;**&lt;/sup&gt;</td>
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*Table 2 Biliary bile salt species in 0.03% cholic acid fed mice.*

Biliary bile salt species of cholic acid fed mice were determined by HPLC analyses and are expressed as percentage of total bile salt. For clarity, total biliary bile salt concentrations are indicated. Hydrophobicity indices were calculated using the individual hydrophobicity indices as determined by Heuman [33]. Data are given as mean ± SD. *p = 0.63 for Atp8b1<sup>0/0</sup>/Hrn versus Atp8b1<sup>0/0</sup>/Hrn/Abcb4<sup>−/−</sup> and *p = 0.0003 for Abcb4<sup>−/−</sup>/Hrn using students t-test; n.d., not detectable.
and TßMC, (~9% of total) while in Atp8b1<sup>G308V/G308V</sup> mice TC and TßMC made up ~55% and ~40% of total biliary bile salts, respectively (Table 2 and Suppl. fig 1B). Compared to the single transgenes, the bile salt hydrophobicity indices of plasma, but not of bile, were elevated in the double transgenes indicating that the bile salt pool was more hydrophobic (tables 1 and 2). The plasma bile salt pool of Abcb4<sup>/</sup>/Hrn mice consisted predominantly of TC (~90% of total) and TßMC (~7%) of total (Table 1 and Suppl. fig. 1C). Plasma bile salt species in Abcb4<sup>/</sup> single knockout mice were similar to those of WT and Hrn control animals, with predominantly TC (~60%) and TßMC (~23%). Biliary bile salts in Abcb4<sup>/</sup>/Hrn mice predominantly consisted of TC (~90%) and low concentrations of TDC (~2.5%; Table 2 and Suppl. fig. 1D), while in Abcb4<sup>/</sup> mice TC and TßMC made up ~75% and ~20% of total biliary bile salts, respectively. Biliary hydrophobicity indices, but not plasma hydrophobicity indices, were elevated in the double transgenes indicative of a more hydrophobic biliary bile salt pool (tables 1 and 2).

**LIVER HISTOLOGY IN CA-FED ATP8B1<sup>G308V/G308V</sup>/HRN AND ABCB4<sup>/</sup>/HRN MICE**

Sirius red staining (collagen deposition) of liver showed no fibrosis in Atp8b1<sup>G308V/G308V</sup>/Hrn mice nor in Atp8b1<sup>G308V/G308V</sup> (Fig. 4A), which was confirmed by quantification of hepatic hydroxyproline levels (Fig. 5A). Bile duct proliferation, evidenced by CK19 staining, was slightly increased in Atp8b1<sup>G308V/G308V</sup>/Hrn mice compared to controls (Fig. 4B). No hepatocyte proliferation was observed, as shown by Ki67 immunostaining (Fig. 4C). Since Hrn mice have a fatty liver<sup>21</sup>, stainings for oil red O were done (Fig. 4D) and quantified by measuring liver triglyceride (TG) content. Compared to control-fed Hrn mice, liver triglyceride content was significantly reduced in Atp8b1<sup>G308V/G308V</sup>/Hrn mice, and was even further reduced when mice were fed CA-supplemented diets (Fig. 5C).
New mouse models for PFIC predispose to cholestasis

A

WT  | Hrn
---|---

\[ \text{Atp8b1}^{G308V/G308V} \]  |  \[ \text{Abcb4}^{-/-} \]

\[ \text{Atp8b1}^{G308V/G308V} / \text{Hrn} \]  |  \[ \text{Abcb4}^{-/-} / \text{Hrn} \]
New mouse models for PFIC predispose to cholestasis

C

WT

Hm

Atpb1\textsuperscript{G308V/G308V}

Atpb1\textsuperscript{G308V/G308V}/Hm

Abcb4\textsuperscript{-/-}

Abcb4\textsuperscript{-/-}/Hrn

5
D

Control  0.03%CA

WT

WT

Hrn

Hrn

Atp8b1^{G308V/G308V}

Atp8b1^{G308V/G308V}

Atp8b1^{G308V/G308V}/Hrn

Atp8b1^{G308V/G308V}/Hrn
New mouse models for PFIC predispose to cholestasis

E

<table>
<thead>
<tr>
<th>Control</th>
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<tr>
<td>WT</td>
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<tr>
<td>Hrn</td>
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<tr>
<td>Abcb4^{-/-}</td>
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FIGURE 4 Histology of the liver. Liver pathology of mice fed 0.03% CA supplemented diets (panels A,B,C; genotype as indicated). Livers were stained for fibrosis with picrosirius red (PSR) (A), for bile duct proliferation with CK19 (B), for cell proliferation with Ki67 (C) and for neutral lipid with Oil red O (D, Atp8b1<sup>G308V/G308V</sup> and E, Abcb4<sup>−/−</sup>). In panels D and E the animals received the indicated diet. Genotypes and antigens are depicted in the pictures.

Sirius red staining of livers of Abcb4<sup>−/−</sup> livers indicated portal fibrosis which was aggravated in livers of Abcb4<sup>−/−</sup>/Hrn mice, where portal-bridging fibrosis was observed (Fig. 4A). Quantification of liver hydroxyprolines confirmed that the fibrosis in CA-fed

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

FIGURE 5. Hydroxyprolines and liver triglycerides. After the feeding period mice were sacrificed and livers were collected. Hydroxyprolines were extracted and measured using HPLC. (A) liver hydroxyprolines from WT, Hrn, Atp8b1<sup>G308V/G308V</sup> and Atp8b1<sup>G308V/G308V</sup>/Hrn mice (n=3-9). (B) liver hydroxyprolines from WT, Hrn, Abcb4<sup>−/−</sup> and Abcb4<sup>−/−</sup>/Hrn mice (n=3-10). Liver triglycerides (TGs) were extracted using a chloroform/methanol extraction method. (C) liver TGs from WT, Hrn, Atp8b1<sup>G308V/G308V</sup> and Atp8b1<sup>G308V/G308V</sup>/Hrn mice (n=4-8). (D) liver TGs from WT; Hrn; Abcb4<sup>−/−</sup> and Abcb4<sup>−/−</sup>/Hrn mice (n=6). Data are shown as mean ± SEM.

Abcb4<sup>−/−</sup>/Hrn mice was more severe than in Abcb4<sup>−/−</sup> mice, and was already present in control-fed Abcb4<sup>−/−</sup>/Hrn mice to a similar level as in CA-fed Abcb4<sup>−/−</sup> mice (Fig. 5B).
Cytokeratin 19 (CK19) staining was much more prominent in \textit{Abcb4}^{-/-}/Hrn mice compared to \textit{Abcb4}^{-/-} mice, indicating bile duct proliferation (Fig. 4B). In addition, hepatocyte proliferation (by Ki67) was strongly increased throughout the whole liver in \textit{Abcb4}^{-/-}/Hrn mice (Fig. 4C). Both \textit{Ck19} and \textit{Ki67} mRNA levels were significantly elevated in \textit{Abcb4}^{-/-}/Hrn livers compared to controls (not shown). Compared to \textit{Hrn} mice, liver triglycerides were reduced in control-fed \textit{Abcb4}^{-/-}/Hrn mice and even further declined when mice were fed a CA-supplemented diet (Fig. 5D).

**DISCUSSION**

In this study we have characterized novel mouse models for PFIC1 and PFIC3 disease, \textit{Atp8b1}^{G308V/G308V}/Hrn and \textit{Abcb4}^{-/-}/Hrn double transgenic mice in which we have instilled a human bile salt pool by feeding 0.03% CA-supplemented diets. Our data indicate that the cholestatic phenotypes of the double transgenes are aggravated compared to those of the single transgenes. As expected, the more hydrophobic plasma and biliary bile salt pool are responsible for these aggravated phenotypes. For instance, \textit{Atp8b1}^{G308V/G308V}/Hrn mice displayed failure to thrive and accumulated higher concentrations of bilirubin and bile salts compared to \textit{Atp8b1}^{G308V/G308V} single mutants. Still, liver pathology was very mild, which may be due to the short experimental period (i.e. 4 weeks). Nevertheless, the lack of hepatic damage demonstrates that the cholestatic component in the PFIC1 model is a much more important one than in the PFIC3 model, which is characterized by severe damage and cell proliferation. Biliary bile salt output in \textit{Atp8b1}^{G308V/G308V}/Hrn mice was 3-fold increased compared to the single transgene. It is unclear why biliary bile salt output was strongly enhanced in these animals, but the main cause for this may be the strongly increased bile salt pool. We have previously already described that in single \textit{Atp8b1}^{G308V/G308V} mice there is a strong increase in the bile salt pool and this is most likely even stronger in the double knockouts. Indeed, the area under the curve in Fig. 3B, which is an indicative measure for bile salt pool size is at least 2 fold higher in \textit{Atp8b1}^{G308V/G308V}/Hrn compared to \textit{Atp8b1}^{G308V/G308V} single mutant mice.
Similarly, \textit{Abcb4} \textsuperscript{+/−}/\textit{Hrn} double transgenic mice developed a more severe phenotype than \textit{Abcb4} \textsuperscript{+/−} single transgenic mice. Liver pathology was much more severe compared to pathology in \textit{Abcb4} \textsuperscript{+/−} mice, evidenced by portal-bridging fibrosis and extensive hepatocyte and bile duct proliferation. \textit{Abcb4} \textsuperscript{+/−}/\textit{Hrn} mice have a strongly increased bile flow, which may be explained by the bile duct proliferation in these animals. We have previously already determined that bile salt-independent bile flow is increased in the single \textit{Abcb4} \textsuperscript{+/−}.\textsuperscript{26} Obviously, the more hydrophobic bile salt pool causes more hepatocyte and cholangiocyte damage, which induces massive fibrosis and hepatocyte and cholangiocyte proliferation.

Because of the more hydrophobic biliary bile salt pool, we had expected \textit{Abcb4} \textsuperscript{+/−}/\textit{Hrn} mice to develop HCC quickly. \textit{Abcb4} \textsuperscript{+/−} mice have shown to be a model for inflammation-induced hepatocarcinogenesis\textsuperscript{27}; regurgitation of bile salts into the portal tracts induces portal inflammation\textsuperscript{15}, which initiates the gradual development of hepatocellular carcinoma\textsuperscript{11,27}. We could, however, not detect any tumors, not even after feeding mice up to one year with CA-supplemented diets (C. Kunne, unpublished observation). A possible explanation may be the difference in genetic background. Previously, hepatic tumors were observed after 6-18 months of chow feeding in \textit{Abcb4} \textsuperscript{+/−} mice in a 129/OLA background\textsuperscript{27}. In another study, in which \textit{Abcb4} \textsuperscript{+/−} mice in an FVB/N background were fed CA-supplemented diets directly from weaning for 22 weeks, no liver tumors were detected, despite more pronounced portal inflammation\textsuperscript{11,27}. In a recent publication Barashi et al.\textsuperscript{28} confirmed that significant development of HCC in \textit{Abcb4} \textsuperscript{+/−} mice against FVB/N background only occurs after about 16 months. Apparently, there also is little predisposition towards bile salt-induced hepatocarcinogenesis in mice in a mixed FVB/C57BL/6 background. It should be noted that hepatocellular carcinoma has not been reported in PFIC3 patients, but this may be due to the fact that these patients are usually transplanted at young age.

When \textit{Atp8b1}\textsuperscript{G308V/G308V}/\textit{Hrn} mice were fed a 0.03% CA-supplemented diet directly from weaning (3 week old mice), the mice died within a few days. We have previously shown that feeding \textit{Hrn} mice 0.1% CA was not toxic and restored biliary salt output to that of control mice\textsuperscript{29}. \textit{Atp8b1}\textsuperscript{G308V/G308V} mice can also tolerate 0.1% CA or even 0.5% CA\textsuperscript{6,30}, whereas \textit{Abcb4} \textsuperscript{+/−} mice are very sensitive to bile salt feeding\textsuperscript{31}. Hence, we
decided to supplement the diets with 0.03% CA, which obviously was toxic to \textit{Atp8b1}^{G308V/G308V}/\textit{Hrn} mice during weaning, but not when started four weeks later. A possible explanation may be the combination of ATP8B1 deficiency, a more hydrophobic biliary bile salt pool upon CA feeding and simultaneous low \textit{Abcb4} expression. Naive 3 week old wild type mice have a relatively hydrophilic bile salt pool (i.e. \textasciitilde95% TC and T\textbeta MC) but a \textasciitilde10-fold lower \textit{Abcb4} expression compared to 8 week old mice (Suppl. fig. 2); The low \textit{Abcb4} levels apparently guarantee sufficient biliary phosphatidylcholine excretion to protect the canicular membrane from luminal bile salt insult. However, when fed a CA diet, the mice will have a more hydrophobic bile salt pool, which in combination with low \textit{Abcb4} expression and deficiency of ATP8B1 renders the canicular membrane more sensitive to cholestasis. This may be the cause of death during weaning in CA-fed double transgenes. Lethality was overcome when mice were included 4 weeks post-weaning (i.e. 7 week old mice).

Our data indicate that the bile salt pool of the double transgenic mice is more hydrophobic compared to the single transgenes, and is an essential determinant of the severity of the phenotypes. Interestingly, the plasma and biliary hydrophobicity indices indicated that in \textit{Atp8b1}^{G308V/G308V}/\textit{Hrn} mice the plasma, but not biliary bile pool was more hydrophobic compared to the single transgenes, while this was the reverse in \textit{Abcb4}^{+/−}/\textit{Hrn} mice (tables 1 and 2). It must be stressed that calculation of the hydrophobicity index of the plasma bile salts in single \textit{Abcb4}^{+/−} mice (table 1) is prone to potentially large errors because these mice are not cholestatic and their plasma bile salt levels are exceedingly low. In the double transgenic \textit{Abcb4}^{+/−}/\textit{Hrn} mice bile salts are strongly elevated and the hydrophobicity index is comparable to \textit{Atp8b1}^{G308V/G308V}/\textit{Hrn} and single \textit{Hrn} mice.

The more hydrophobic biliary bile salt pool in \textit{Abcb4}^{+/−}/\textit{Hrn} is likely to contribute to the aggravated liver damage observed in these animals whereas the more hydrophobic plasma pool in \textit{Atp8b1}^{G308V/G308V}/\textit{Hrn} mice may aggravate the cholestasis in these mice. Still, a comparison with the patient phenotypes is difficult. The bile salt pool of humans mainly consists of dihydroxy bile salts (chenodeoxycholate and deoxycholate) and about 30% trihydroxy bile salts (cholate). This contrasts our CA-fed double transgenes, in which \textasciitilde90% of plasma and biliary bile salts are taurocholate and muricholate. We
had expected a higher percentage of dihydroxy bile salts in the double transgenes. A possible explanation for near absence of dihydroxy bile salts in the double transgenes may be that hepatic bile salt retention is enhanced due to cholestasis, which in combination with the 5% residual cytochrome P450 activity renders rehydroxylation capacity sufficient to partially detoxify most dihydroxy bile salts (Table 1 and 2). Indeed, CA-feeding of single transgenic Hrn mice resulted in a mole fraction dihydroxy bile salts of ~ 20% (TCDC + TDC), whereas in the double transgenes CA-supplementation of the diet resulted in a mole fraction dihydroxy bile salts of maximal 5% (table 2). Another essential difference between mice and men with regard to bile salt pool hydrophobicity is the nature of bile salt conjugation. In men, approximately 50% of bile salts are conjugated with glycine whereas in mice bile salts are exclusively conjugated with taurine. Glycine-conjugated bile salts are more cytotoxic than taurine-conjugated bile salts, especially under mildly acidic conditions, which may occur in cholestasis.

The hydrophobic bile salt pool in CA-fed double transgenes apparently ameliorated liver steatosis. We have recently shown that bile salt feeding of Hrn mice, which predispose to liver steatosis, ameliorates the steatosis (Kunne et al. submitted). In the present study, liver triglyceride content was already strongly reduced in control-fed double transgenes compared to Hrn mice and was further reduced in the CA-fed condition. It shows that accumulation of bile salts in hepatocytes of these cholestatic mice even further reduces hepatic lipid accumulation.

In conclusion, we have characterized new mouse models for PFIC1 and PFIC3 disease with a more hydrophobic bile salt pool composition. Mice developed pathology that better resembled the pathology observed in patients. We conclude that the bile salt pool composition is a critical determinant in the initiation and progression of cholestasis and inducer of liver pathology in PFIC1 and PFIC3.

ACKNOWLEDGEMENTS

We thank Dr. Johanna Verheij for helpful discussions. This work was supported by a grant from the Dutch Digestive Disease Foundation (WO 08-63 to CCP).
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SUPPL. FIG. 1 Bile salt species in plasma and bile.
Plasma (A, C) and biliary (B, D) bile salt species were determined at the end of the feeding period. Bile salt species were measured using HPLC. Panel A and B: wild-type (n=2 and n=4), Hrn (n=5), Atp8b1G308V/G308V (n=5 and n=3), Atp8b1G308V/G308V/Hrn (n=7 and n=6). Panel C and D: wild-type (n=2 and n=4), Hrn (n=5 and n=4), Abcb4-/- (n=8 and n=4), Abcb4-/-/Hrn (n=5). Data are shown as mean ± SEM. Bile salt species are indicated from left to right with increasing hydrophobicity.

SUPPL. FIG. 2 Hepatic Abcb4 expression. Livers from 3 and 8 week old chow fed WT animals were collected and snapfrozen. mRNA expression for Abcb4 was determined and expression levels were normalized for cyclophylin/hypoxanthine guanine phosphoribosyltransferase.