Chapter 7

Bile duct proliferation associated with bile salt-induced hypercholeresis in Mdr2 P-glycoprotein-deficient mice

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ABSTRACT

Background/Aims: Bile flow consists of bile salt-dependent bile flow (BSDF), generated by canalicular secretion of bile salts, and bile salt-independent flow (BSIF), probably of combined canalicular and ductular origin. Bile salt transport proteins have been identified in cholangiocytes, suggesting a role in control of BSDF and/or in control of bile salt synthesis through cholehepatic shunting.

Methods: We studied effects of bile duct proliferation under non-cholestatic conditions in Mdr2 P-glycoprotein (Abcb4)-deficient (Mdr2(-/-)) mice. BSDF and BSIF were determined in wild-type and Mdr2(-/-) mice during infusion of step-wise increasing dosages of tauroursodeoxycholate (TUDC). Cholate synthesis rate was determined by [2H₄]-cholate dilution. Results were related to expression of transport proteins in liver and intestine.

Results: During TUDC infusion, BSDF was increased by ~50% and BSIF by ~100% in Mdr2(-/-) mice compared to controls. Cholate synthesis rate was unaffected in Mdr2(-/-) mice. Hepatic expression of the apical sodium-dependent bile salt transporter (Asbt), its truncated form (tAbst) and the multidrug resistance-related protein (Mrp3) were up-regulated in Mdr2(-/-) mice.

Conclusions: Bile duct proliferation in Mdr2(-/-) mice enhances cholehepatic shunting of bile salts, which is associated with a disproportionally high bile flow but does not affect bile salt synthesis.
INTRODUCTION

Bile flow is generated via canalicular and ductular processes and is composed of bile salt-dependent (BSDF) and bile salt-independent flow (BSIF)\(^1\). Active transport of bile salts by hepatocytes into bile canaliculi is responsible for the bile salt-dependent canalicular bile flow\(^2\). Bile salt-independent generation of bile is considered to result from net transport into bile of organic solutes (e.g., glutathione) and inorganic electrolytes by hepatocytes or cholangiocytes, followed by osmotic movement of water, putatively mediated by aquaporins\(^3;4\).

Several studies support the concept that cholangiocytes interact with biliary bile salts. Cholangiocytes are able to conjugate bile salts to taurine or glycine and bile salts increase proliferation of cholangiocytes in vitro\(^5;6\). In vivo, bile salt feeding in rats induces cholangiocyte proliferation and an increases secretin-induced, i.e., bile salt independent, ductular bile flow\(^7;8\). Cholangiocytes have also been suggested to influence the BSDF, via their role in the so-called “cholehepatic shunt pathway”, originally proposed to explain the hypercholeresis observed after administration of certain (unconjugated) bile salt species\(^9;10\). According to this concept, cholangiocytes absorb unconjugated bile salts after their protonation by passive diffusion. Subsequently, these bile salts return to the liver to be resecreted into the bile, thereby promoting additional movement of water. The apical sodium-dependent bile salt transporter (Asbt/ Slc10a2) is, in addition to the terminal ileum, present at the apical membrane of cholangiocytes lining the large hepatic bile ducts in rats and facilitates uptake of conjugated bile salt species\(^11;12\). Bile salt efflux from the basolateral membrane of cholangiocytes may involve a truncated isoform of Asbt (t-Asbt) and/or the multidrug-resistance protein 3 (Mrp3/ Abcc3)\(^13;15\).

Cholehepatic circulation could play a role in feedback repression of de novo bile salt synthesis and control expression of hepatocytic transport systems for bile salts, i.e., Ntcp and Bsep, via activation of the farnesoid X receptor (FXR; NR1H4)\(^16;17\). Especially during conditions associated with bile duct proliferation such as extrahepatic cholestasis, cholehepatic circulation of bile salts could thus protect the liver from further bile salt toxicity by limiting intracellular accumulation.

Bile duct proliferation has been shown to enhance de novo secretin-stimulated bile flow in rat models and humans\(^8;18;19;20\). It is not known whether bile duct proliferation is associated with changes in BSDF and/or bile salt synthesis. Therefore, we studied whether bile duct proliferation without cholestasis is associated with altered BSDF, BSIF and/or bile salt synthesis. As a model for bile duct proliferation without cholestasis we made use of Mdr2 P-glycoprotein (or Abcb4) -deficient (Mdr2\(^{-/-}\)) mice that are unable to secrete phospholipids into bile and develop progressive bile duct proliferation in the absence of obstructive cholestasis\(^21;22\).

MATERIALS AND METHODS

Animals

Mice homozygous for disruption of the multidrug resistance gene-2 (Mdr2\(^{-/-}\)) and control (Mdr2\(^{+/+}\)) mice of the same FVB background were obtained from
the Animal Facility of the AMC, Amsterdam. Mice were ~4 months old, were housed in a light- and temperature-controlled facility and fed standard lab-chow. Experiments were approved by the ethical committee on animal testing.

**Experimental procedures**

Gallbladders of anaesthetized mice were cannulated for bile collection to analyze biliary bile salt composition and output rate, before and during intravenously infusion of tauroursodeoxycholate (TUDC) as described23. Bile flow was determined gravimetrically. Determination of [$^2$H$_4$]-cholate kinetics was performed in six male Mdr2$^{+/+}$ and six male Mdr2$^{-/-}$ mice as reported previously24. At the end of the experiment, liver and intestine were removed. The intestine was divided in three segments: proximal, medial and distal. The proximal part includes the duodenum and proximal jejunum, the medial part consists of jejunum and the distal part includes the terminal ileum. Samples were frozen in liquid nitrogen and stored at -80°C for membrane and RNA isolation.

**Bile salt analyses**

Plasma samples were prepared for bile salt enrichments by gas chromatography mass spectrometry (GC-MS) as described24. Biliary bile salt composition was determined by capillary gas chromatography as described25. Biliary bile salt concentrations were determined by an enzymatic fluorimetric assay26.

**Steady state mRNA levels in liver and intestine**

Total RNA was isolated using TRizol (GIBCO BRL, Grand Island, NY). Reverse transcription was performed with random primers and real time quantitative PCR was performed as described previously27. Primer and probe sequences have been described for β-actin, Fxr, Asbt, truncated Abst and Ibabp28, for Bsep29, and for Mrp2 and Mrp330, except for Cfr (sense: 5’-CCGTTGACAACATGGAACAC-3; antisense: 5’-AGAAGCAGCCACCTCAACCA-3; probe:5’-TCTCCATAAAGGCTTTACTGCTAGTGCTGATTG-3’; accession number: NM_021050). Probes were labelled with a reporter (6-carboxy-fluorescein) and a quenching dye (6-carboxy-tetramethyl-rhodamine). Fluorescence was measured by an ABI Prism 7700 Sequence Detector v. 1.6 software (Perkin-Elmer Corp., Foster City, California). β-actin was used as internal control. The mRNA levels of -actin and Asbt in intestine were determined by semi-quantitative RT-PCR using the following primers; β-actin (sense: AACACCCAGCCATGTACG; antisense: ATGTCACGCACGATTTCCC), Asbt (sense primer: GCTTCTGTGGACTTGGCCAT; antisense primer: TGGAGCAAGTGGTCATGCTA). Relative intensity of the bands was determined using a the ImageMaster VDS system (Pharmacia, Upsalla, Sweden).

**Immunohistochemistry**

To localize the Asbt protein in liver and intestine, 4 µm sections were cut of these tissues and fixed with acetone. The first antibody, anti-Asbt (guinea pig anti-Asbt in 1% BSA/PBS)31 was incubated and washed with PBS. Endogenous peroxidase was inhibited using 30% methanol, 0.3% H$_2$O$_2$ and detection was done with peroxidase-linked rabbit anti-guinea pig-Ig (Dako A/S, Glostrup, Denmark).
with an amplification step using goat anti-rabbit-Ig (Dako A/S). 3-Amino-9-ethylcarbozole (Sigma, St. Louis, MO) was used as a substrate and tissue was counterstained with haematoxylin.

**Western blotting**
Liver homogenates and total membranes were isolated as described by Wolters et al.\(^{32}\). Intestinal brush border membranes were isolated as described by Schmitz et al.\(^{33}\). Protein concentrations were determined according to Lowry et al.\(^{34}\). Hepatic total membranes (30 µg protein), intestinal homogenates and intestinal brush border membranes (2.5 µg of protein) were separated using 4-15% Tris-HCL ready gradient gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose (Amersham, Little Chalfont, UK), using a tankblotting system (Bio-Rad laboratories, Hercules, CA). Mrp3 and Asbt protein content were determined using goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and polyclonal anti-rat Asbt antibody\(^{31}\), respectively. Ibabp protein content was determined using anti-murine Ibabp antibody\(^{35}\). Immunecomplexes were detected using horseradish peroxidase - conjugated antibodies, and ECL detection (Amersham, Little Chalfont, UK).

**Calculations**

**Isotope dilution technique**
The isotope dilution technique was performed as described by Hulzebos et al.\(^{24}\). Enrichment was defined as the increase of M\(_4\)-CA/M\(_0\)-CA relative to baseline measurements after administration of [\(^2\)H\(_4\)]-CA and expressed as the natural logarithm of atom % excess (ln APE)\(^{36}\). The fractional turnover rate (FTR) and pool size of CA were calculated from the decay of ln APE. The FTR (day\(^{-1}\)) equals the slope of the regression line. The pool size (µmol·100g\(^{-1}\)) is determined according to the formula: (D·b·100)/e\(^{a}\)·D, where D is the administered amount of label, b is the isotopic purity, and “a” is the intercept on the y-axis of the ln APE curve. Cholate synthesis rate (µmol·100g\(^{-1}\)·day\(^{-1}\)) was determined by multiplying pool size and FTR.

**Cycling time**
Cholate cycling time was estimated by dividing mean cholate pool size by mean biliary secretion rate of cholate. The fraction of cholate lost per enterohepatic cycle was calculated by dividing fractional cholate synthesis rate by cholate cycling frequency and expressed as percentage of total cholate pool size\(^{25}\).

**Statistical analysis**
Results are presented as means ± standard deviation for the number of animals indicated. Differences between groups were determined by Student’s t-test or Mann Whitney exact 2-tailed U-test. Level of significance for all statistical analyses was p < 0.05. Analysis was performed using SPSS 10.0 (SPSS, Chicago, IL, USA).
RESULTS

Bile salt independent flow and bile salt dependent flow in control and Mdr2\(^{(-/-)}\) mice
To examine the consequence of bile duct proliferation on bile flow, the gallbladders of Mdr2\(^{(-/-)}\) and control mice were cannulated and the bile salt pool was depleted to exclude potential effects of differences in bile salt pool composition. Analysis of biliary bile salt composition revealed relatively decreased proportions of cholate and increased proportions of β-muricholate in Mdr2\(^{(-/-)}\) mice compared to control mice (Table 1), in accordance with previously published results\(^{23}\). Total biliary bile salt secretion tended to be higher in Mdr2\(^{(-/-)}\) mice than in controls (p = 0.06), but cholate secretion rates were similar (Table 1). TUDC was infused intravenously in step-wise increasing dosages. Analysis of the relationship between bile flow and biliary bile salt output (Figure 1) revealed a ~50% higher choleretic activity of TUDC in Mdr2\(^{(-/-)}\) mice compared to controls (7.2 ± 1.6 µL/µmol vs. 4.7 ± 0.9 µL/µmol, Mdr2\(^{(-/-)}\) vs. control mice; p < 0.05). The BSIF, i.e., the theoretical value of the bile flow in the absence of bile salt output was 2-fold increase in Mdr2\(^{(-/-)}\) mice (10.8 ± 1.7 µL-min\(^{-1}\).100g\(^{-1}\) vs. 5.4 ± 1.1 µL-min\(^{-1}\).100g\(^{-1}\), Mdr2\(^{(-/-)}\) vs. control mice; p < 0.05).

Table 1. Biliary bile salt output rates and bile salt composition (% total) in chow-fed male wild-type and Mdr2\(^{(-/-)}\) mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild-type</th>
<th>Mdr2(^{(-/-)})</th>
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</thead>
<tbody>
<tr>
<td>Bile salt output (nmol-min(^{-1}).100g body wt(^{-1}))</td>
<td>309 ± 88</td>
<td>394 ± 88</td>
</tr>
<tr>
<td>Cholate output (nmol-min(^{-1}).100g body wt(^{-1}))</td>
<td>130 ± 37</td>
<td>109 ± 24</td>
</tr>
<tr>
<td>Cholate composition (% total)</td>
<td>42.2 ± 5.4</td>
<td>27.7 ± 4.3*</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>2.0 ± 0.6</td>
<td>0.6 ± 0.4*</td>
</tr>
<tr>
<td>α-Muricholate</td>
<td>3.8 ± 0.7</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>β-Muricholate</td>
<td>31.3 ± 4.5</td>
<td>47.3 ± 0.7*</td>
</tr>
<tr>
<td>ω-Muricholate</td>
<td>14.0 ± 1.5</td>
<td>13.4 ± 4.3</td>
</tr>
<tr>
<td>Lithocholate</td>
<td>5.4 ± 0.4</td>
<td>8.6 ± 0.2*</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>1.3 ± 0.1</td>
<td>0.8 ± 0.1*</td>
</tr>
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Total bile salt and cholate biliary output rates determined in Mdr2\(^{(-/-)}\) and Mdr2\(^{(+/-)}\) mice. The gallbladders of the mice were cannulated and bile was collected for 30 minutes. Biliary bile salts represent >90% of all bile salt species. Values are expressed as means ± SD (n = 3 per group). *Significant difference between wild-type and Mdr2\(^{(-/-)}\) mice, p < 0.05.

Effects of Mdr2-Pgp deficiency on expression of hepatic bile salt transport proteins
Hepatic mRNA levels of genes encoding transporters considered to be involved in bile salt transport in cholangiocytes, i.e., Asbt, and tAsbt were clearly increased in Mdr2\(^{(-/-)}\) mice (Figure 2A). Moreover, hepatic mRNA levels of Mrp3, putatively involved in bile salt efflux from the cholangiocyte was also markedly increased in Mdr2\(^{(-/-)}\) mice. Yet, Mrp3 is also expressed in hepatocytes and these results do not allow to differentiate hepatocytes and cholangiocytes. Levels of Fxr mRNA were increased in Mdr2-P-glycoprotein deficient mice. Expression of hepatocytic Mrp2
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Figure 1. Relation between bile flow and biliary bile salt output for Mdr2(-/-) (closed circles) and control (open circles) mice. After cannulation of the gallbladder and depletion of the bile salt pool, TUDC in phosphate-buffered saline was infused intravenously in a step-wise increasing dose as described previously. Data represent means ± standard deviation of 4 animals per group. The slope of the line yields the bile salt-dependent flow (BSDF), i.e., the choleretic activity of TUDC in µL/µmol. The mean values for the apparent choleretic activity were 4.7 ± 0.9 and 7.2 ± 1.6 µL/µmol for control and Mdr2(-/-) mice, respectively (p < 0.05). The bile salt-independent bile flow (BSIF), obtained by extrapolation to the Y-axis was also significantly increased in Mdr2(-/-) mice (5.2 ± 1.1 µL.min⁻¹.100g⁻¹ vs. 10.9 ± 0.3 µL.min⁻¹.100g⁻¹, Mdr2(-/-) vs. control mice; p < 0.05).

(Abcc2), crucially involved in hepatobiliary transport of glutathione and identified as an FXR-target gene, was ~ 50% higher in Mdr2(-/-) mice. On the other hand, expression of Shp, and Bsep (Abcc11) were not significantly affected. Expression of Cftr (Abcc7) encoding for a chloride transport protein present in cholangiocytes, but not on hepatocytes, was 6-fold induced in livers of Mdr2(-/-) mice Asbt and Mrp3 protein levels were increased in livers of Mdr2(-/-) mice when compared to those of controls (Figure 2B). Immunohistochemistry on frozen sections of the liver and intestines of Mdr2(-/-) and control mice showed clear Asbt staining of the apical membrane of cholangiocytes (data not shown) and enterocytes (Figure 3), respectively.

In vivo kinetics of [2H₄]-Cholate. We investigated whether bile duct proliferation affects in cholate kinetics in the Mdr2(-/-) and Mdr2(+/-) mice. Deuterated [²H₄]-cholate disappeared from plasma at the same rate in Mdr2(-/-) and wild-type mice (Figure 4). The fractional turnover rate of cholate (Figure 5A) was similar in both groups of mice (0.4 ± 0.1 day⁻¹ vs. 0.5 ± 0.1 day⁻¹, Mdr2(-/-) mice vs. wild-type, NS). Pool sizes of cholate (Figure 5B), estimated from the Y-intercept of the In APE
The calculated cholate synthesis rate (Figure 5C) did not differ between both strains (9.9 ± 2.9 µmol·100g⁻¹·day⁻¹ vs. 7.3 ± 1.6 µmol·100g⁻¹·day⁻¹, Mdr2(-/-) vs. wild-type mice; NS). Consequently, the calculated cholate synthesis rate (Figure 5C) did not differ between both strains (9.9 ± 2.9 µmol·100g⁻¹·day⁻¹ vs. 7.3 ± 1.6 µmol·100g⁻¹·day⁻¹, Mdr2(-/-) vs. wild-type mice; NS).

**Effects of Mdr2 deficiency on intestinal protein levels of Asbt and Ibabp.**

Asbt mRNA levels were clearly most abundant in the distal parts of the small intestine (Figure 6A) and no differences were observed between Mdr2(-/-) and wild-type mice. In accordance with mRNA expression patterns, Asbt protein was mainly present in the distal part of the intestine of Mdr2(-/-) and wild-type mice (Figure 6B). Ibabp protein expression was also confined to the distal

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**Figure 2.** (A) Effects of Mdr2-Pgp deficiency on steady-state mRNA levels of genes involved in hepatocytic and cholangiolytic bile salt transport in Mdr2(+/-) and Mdr2(-/-) mice. Total mRNA was isolated from Mdr2(+/-) mice (white bars) and Mdr2(-/-) mice (black bars), transcribed into cDNA, and subjected to real-time PCR. Data represent hepatic mRNA levels of Fxr, Shp, Bsep, Mrp2, Mrp3, Asbt, Cft3, and tAsbt, respectively. All data were standardized for β-actin. Expression in wild-type mice was set to 1.00. The asterisks indicate significant difference, p < 0.05. Values are expressed as means ± standard deviation of RNA isolated from 4 animals in each group. (B) Effects of Mdr2-Pgp deficiency on hepatic protein levels of Asbt and Mrp3. Hepatic Asbt and Mrp3 protein levels were increased in Mdr2(-/-) mice when compared to wild-type mice. Data are shown as typical examples of two independent preparations of each group isolated from 4 animals in each group.

[^2H_4]-CA vs. time plot, were also similar for Mdr2(+/-) and Mdr2(-/-) mice (18.7 ± 2.1 µmol.100g⁻¹ vs. 16.9 ± 2.2 µmol.100g⁻¹, Mdr2(-/-) vs. wild-type mice; NS)
Figure 3. Immunohistochemical staining for Asbt on frozen sections of terminal small intestine from control (A) and Mdr2(-/-) (B) mice. The arrow heads indicate the apical staining by anti-Asbt antibodies (magnification 40x). No clear differences were observed in the intensities of staining between Mdr2(-/-) mice and control mice. No signal was obtained when primary or secondary antibodies were omitted from the incubation medium.

Figure 4. Decay of intravenously administered [3H4]-cholate in wild-type (open circles) and Mdr2(-/-) (closed circles) mice. A solution of with 210 µg [3H4]-cholate, 0.5% NaHCO3 and phosphate-buffered saline (PBS) was intravenously injected into six Mdr2(+/+) and six Mdr2(-/-) mice. Blood samples were collected and plasma was analyzed for the increase of M4-CA/M0-CA relative to baseline measurements and expressed as the natural logarithm of atom % excess (ln APE) enrichment of cholate. Deuterated [3H4]-cholate disappeared from plasma at the same rate in Mdr2(-/-) and wild-type mice. Data are expressed as means ± standard deviation of n = 3-6 mice per time point.
Figure 5: Effects of Mdr2- P glycoprotein-deficiency on fractional turnover rate (A), pool size (B) and synthesis rate (C) of cholate. Derived from $[^{2}H_{4}]$-cholate isotope enrichment measurements in plasma of wild-type and Mdr2$^{(-/-)}$ mice (n=6 per group), fractional turnover rate, pool sizes and synthesis rates of cholate were calculated in wild-type (white bars) and Mdr2$^{(-/-)}$ (black bars) mice. The fractional turnover rate of cholate, calculated from the slope of the linear regression curve, was similar in both groups of mice (A). Pool sizes of cholate (B), estimated from the Y-intercept of the ln APE $[^{2}H_{4}]$-CA vs. time plot, were also similar for Mdr2$^{(+/+)}$ and Mdr2$^{(-/-)}$ mice. The cholate synthesis rate (C), calculated by multiplying pool size and FTR did not differ. Data represent means ± SD.

Figure 6: Expression of Asbt mRNA, Asbt protein and Ibabp protein levels in proximal (P), medial (M) and distal (D) segments of the intestines of Mdr2$^{(+/+)}$ and Mdr2$^{(-/-)}$ mice. Asbt mRNA (A) and protein levels (B) increased towards the distal end of the small intestine and no clear differences were observed between Mdr2$^{(-/-)}$ and wild-type mice. Ibabp protein expression (Fig. 6B) was also confined to the distal terminal ileum in both groups of mice. Samples shown are representative for 3-4 individual intestinal preparations.
terminal ileum in both groups of mice. No significant differences existed upon semiquantitative analysis (average of 3–4 mice per group) in intestinal Asbt or Ibabp expression between $Mdr2^{(+/-)}$ and $Mdr2^{(-/-)}$ mice.

**DISCUSSION**

In the present study we investigated whether bile duct proliferation without cholestasis affects BSDF, BSIF and/or bile salt synthesis in mice. The results show that bile duct proliferation in (non-cholestatic) $Mdr2$ P-glycoprotein-deficient mice is not only associated with a higher BSIF but also with an increased BSDF. The increased BSIF and BSDF coincided with an increased hepatic expression of Asbt, tAasbt, and Mrp3, i.e., bile salt transporters putatively involved in cholehepatic shunting of bile salts.

We made use of a mouse model of bile duct proliferation without obstructive cholestasis. $Mdr2$ P-glycoprotein-deficiency in mice leads to the formation of phospholipid-free bile, resulting in exposure of the bile ducts to the detergent actions of bile salts leading to proliferation. $Mdr2^{(-/-)}$ mice thus represent a “spontaneous” model of bile duct proliferation unlike other experimental models, i.e., bile duct ligation, bile salt feeding, or partial hepatectomy. Previously, the increased bile flow in $Mdr2^{(-/-)}$ mice has been attributed to an increase in BSIF. Accordingly, BSIF in the $Mdr2^{(-/-)}$ mice in the present study was twice as high as in controls. An increase in BSIF is also seen in other experimental models of bile duct proliferation and is characterized by increased basal and secretin-stimulated bile flow. This increment of BSIF in the $Mdr2^{(-/-)}$ mice coincided with a decreased glutathione secretion, as previously reported by Smit et al. Biliary glutathione secretion occurs almost exclusively via the canalicular organic anion transporter Mrp2 and is an important contributor to BSIF in rodents. Since Mrp2 mRNA expression was increased in $Mdr2^{(-/-)}$ mice, reduced synthesis or enhanced turnover probably underlie decreased glutathione secretion. Secretion of other organic anions in combination with other mechanisms, e.g., increased net ductular secretion, might therefore contribute to a larger extent to enhanced BSIF in $Mdr2^{(-/-)}$ mice. Upregulation of Cftr expresion, a gene involved in biliary chloride secretion at the apical membrane of the cholangiocyte, could also contribute to enhanced bile flow in $Mdr2^{(-/-)}$ mice. Indeed, an increase in biliary chloride secretion in $Mdr2^{(-/-)}$ mice has been reported. Although theoretically, another localization of Cftr protein than at the apical membrane or at a subapical domain of cholangiocytes is possible, this has not been described so far.

Cholehepatic shunting will be associated with an increased amount of fluid per mole of biliary bile salt, i.e., an increased BSDF or choleretic activity, as a result of the amplified osmotic activity per molecule. The amount of fluid generated per mole of biliary TUDC was higher in $Mdr2^{(-/-)}$ mice than in wild-type mice, i.e., 7.2 µL/µmol in the $Mdr2^{(-/-)}$ mice and 4.7 µL/µmol in wild-type mice. Although this finding is consistent with TUDC-induced hypercholeresis, it obviously does not provide definitive proof for the presence of cholehepatic shunting of TUDC. Bile duct-specific delivery of Asbt inhibitors or $Mdr2/Asbt$ double knock-out mice would be suitable models to address this hypothesis. Yet, the combination of increased expression of Asbt, tAasbt, and Mrp3 with the increased choleretic activity does
strongly support the existence of cholehepatic shunting. Absence of mixed micelle formation, as occurs in Mdr2\(^{(-/-)}\) mice, is not a major determinant of the choleretic activity of bile salt. The choleretic activity of conjugates of dihydroxy bile salts, such as TUDC, is comparable to that of tauroursodeoxycholate (TUC), a conjugated trihydroxy bile salt that does not induce biliary lipid secretion\(^{39}\). In another model of bile duct proliferation, i.e., bile duct ligation, infusion of TUDC and of taurohyodeoxycholate (THDC) induced hypercholeresis to a greater extent than in normal rats, suggesting a ductal origin for enhanced bile flow by these bile salts\(^{40}\). For UDC, used as a therapeutic agent in various liver diseases, indications for cholehepatic shunting have been reported previously\(^{41};^{42}\).

Data on some hepatocellular transporters in Mdr2\(^{(-/-)}\) mice, i.e., Ntcp, have been previously published\(^{43}\). Yet, to the best of our knowledge, this study is the first to describe bile salt-induced hypercholeresis in relation to induced Asbt, tAsbt, and Mrp3 expression. Since Mdr2\(^{(-/-)}\) mice develop marked bile duct proliferation, the increased protein and mRNA levels of transport proteins in their livers are most likely be attributed to increased numbers of cholangiocytes. Yet, it can not be excluded that the amount of protein or mRNA per cholangiocyte has changed. Literature data on other bile duct proliferation models are not conclusive. In bile duct-ligated rats, the content of Asbt protein in liver increases while Asbt mRNA expression in isolated cholangiocytes remains unchanged, suggesting an increased number of cholangiocytes with an unaffected amount of protein per cell\(^{44}\). In contrast, bile salt feeding in rats stimulates proliferation of cholangiocytes associated with overexpression of the Asbt gene in isolated cholangiocytes\(^{45}\). Cholehepatic circulation of bile salts could be involved in feedback repression of de novo bile salts synthesis and serve to protect the liver from further bile salt toxicity in conditions with extrahepatic cholestasis\(^{11}\). To evaluate the physiological

![Figure 7: Estimated fluxes and kinetics of the enterohepatic circulation of cholate in Mdr2\(^{(+/-)}\) and Mdr2\(^{(-/-)}\) mice. The values represent calculations based on the mean values for biliary cholate secretion, cholate pool sizes and synthesis rates. Mdr2 Pgp-deficiency associated with bile duct proliferation and increased expression of bile salt transport proteins enhances cholehepatic shunting (represented by the spring), increases cycling time (CT) of cholate and increases the calculated percentage of cholate lost per enterohepatic cycle.](image)
Bile duct proliferation enhances bile flow

consequences of Mdr2 P-glycoprotein-deficiency on the synthesis rate of bile salts, cholate kinetics were compared in the Mdr2+/+ and Mdr2−/− mice, using a stable isotope procedure24. As demonstrated previously, the contribution of cholate to the bile salt pool is reduced in Mdr2−/− mice, but its biliary secretion rate is unaffected23. Upregulation of bile salt transporters mainly confined to the biliary epithelium did not affect synthesis rate of cholate nor its pool size. Bile salts mediate feedback regulation at the level of cholesterol 7α-hydroxylase (Cyp7A1), which encodes the rate-limiting enzyme in the neutral bile salt synthetic pathway via the farnesoid receptor (FXR)17. Cholate synthesis was not repressed in Mdr2−/− mice. This is in accordance with earlier findings that Cyp7A1 expression at the mRNA level is not affected in livers of Mdr2−/− mice 25.

Based on the data provided in this study it is possible to estimate the cycling time (CT) of cholate, i.e., the time it takes the cholate pool to circulate a single time. Cycling time for cholate was found to be ~2 h and ~3 h in control and Mdr2−/− mice, respectively (Figure 7). The cholate pool thus cycled ~11 versus ~8 per day in control and Mdr2−/− mice, respectively. The calculated fraction of cholate lost per enterohepatic cycle was increased by ~50% (~4 versus ~6 % for control and Mdr2−/− mice, respectively). Thus, in Mdr2−/− mice cholate tends to complete its enterohepatic cycle at a slower rate and to partially escape intestinal reabsorption. Yet, Mdr2 Pgp-deficiency did not affect mRNA and/or protein expression of ileal bile salt transporters considered to facilitate intestinal bile salt reabsorption, i.e., the apical sodium-dependent bile salt transporter (Asbt; Slc10a2) and the ileal bile acid-binding protein (Ibabp). It is tempting to speculate that an increased cholehepatic shunting in Mdr2−/− mice contributes to a slower enterohepatic circulation.

In conclusion, bile duct proliferation in Mdr2 Pgp-deficiency results in an increased choleretic activity of biliary bile salts, which is associated with increased hepatic expression of Asbt, tAsbt, and Mrp3, suggestive for Asbt/tAsbt/Mrp3-mediated cholehepatic circulation. In spite of indications for cholehepatic shunting in Mdr2 P-glycoprotein-deficiency, cholate synthesis was unaffected. Our results support the concept that bile duct proliferation can be a protective response against toxic stimuli by enhancing bile flow. Rather than previously thought, this protective response may not remain confined to stimulation of BSIF, but may also involve an enhanced BSDF through cholehepatic shunting.

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