General discussion
Alterations in bile acid (BA) metabolism have been linked to metabolic diseases, including obesity, type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) (1). In general, elevated plasma BA concentrations are frequently present in patients with T2DM and NAFLD (1), while levels of 12α-hydroxylated BAs (cholic acid, CA + deoxycholic acid, DCA) appear to be positively associated with insulin resistance (2). Besides, signaling pathways that are induced upon binding of BAs to BA receptors such as FXR (NR1H4) and TGR5 (GPBAR1), are involved in the pathophysiology of metabolic diseases due to their regulation of lipid, glucose and energy homeostasis (Chapter 2). Therefore, BA profile might serve as an attractive candidate for the diagnosis or identification of treatment targets of metabolic diseases. Interestingly, the classical anti-diabetic drug metformin has been recently found to improve hyperglycemia in part via a BAs- intestinal FXR- glucagonlike peptide-1 (GLP-1) pathway (3). The exact role of BA and BA signaling pathways in the etiology of metabolic diseases, however, remains to be further delineated, but in vivo studies and clinical trials do show promising results of FXR agonists in T2DM and NAFLD (4–8). Making use of genetically-modified mouse models, the underlying mechanisms for BA-based therapies are gradually being uncovered. However, fundamental differences in BA metabolism between humans and mice complicate the extrapolation of observations in mouse models to the human situation. Since the responsible enzyme for rodent-specific muricholic acids (MCAs) formation has been recently identified, i.e., CYP2C70 (cytochrome P450, family 2, subfamily c, polypeptide 70) (9), work in this dissertation aims to generate and characterize Cyp2c70-deficient mice with a human-like BA composition to gain better understanding of the role of BAs and BA-based treatment in metabolic and liver diseases.

Bile acid homeostasis in Cyp2c70<sup>−/−</sup> mice

Enzymes of the cytochrome P450 (CYP) superfamily function as monooxygenases and are involved in metabolism of steroids, fatty acids, BAs as well as in the clearance of xenobiotics (10). A number of CYPs take part in BA synthesis. For example, CYP7A1 (cholesterol 7α-hydroxylase) is known as the rate-limiting enzyme for BA synthesis while CYP8B1 (sterol 12α-hydroxylase) is the determinant of 12α-hydroxylated BA production (11). In 2016, CYP2C70 was identified as the responsible enzyme for rodent-specific MCAs formation (9). Specifically, CYP2C70 mediates 6β-hydroxylation on chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) to generate αMCAs and βMCA, respectively. In 2017, CYP3A11, a known BA 6β-hydroxylase, was found not to be essential for MCAs synthesis in mice (12). In 2019, CYP2C70 was reported to also possess 7-epimerization activity (13). A two-step reaction CDCA- αMCA-βMCA is identified as the major pathway for βMCA synthesis in mice (13), in which CDCA is converted into βMCA via αMCA as an intermediate metabolite. Hepatic Cyp2c70-knockout mice (Cyp2c70<sup>ako</sup>) were generated and displayed a hydrophobic human-like BA pool, with a strong reduction of MCAs (13). The hydrophobicity index increased from ~0.3 in WT (wild type) mice to ~0 in Cyp2c70<sup>ako</sup> mice, with still ~18% of MCAs present in the bile. Although plasma transaminases were 5-fold higher in Cyp2c70<sup>ako</sup> mice, liver histology did not reveal major differences between Cyp2c70<sup>ako</sup> and WT mice. In addition, bile formation was maintained in...
Cyp2c70 \textit{ako} mice (13). In 2020, full body Cyp2c70-knockout mice (Cyp2c70\textsuperscript{-/-}) were produced by us (Chapter 3) and other groups (14,15). Similar phenotypes were observed due to the deletion of Cyp2c70 by all these groups. The MCA precursors CDCA and UDCA are enriched while MCAs are absent and the hydrophobicity index of biliary bile is ~0.3 in Cyp2c70\textsuperscript{-/-} mice, comparable to the hydrophobicity of BAs in human bile. Moreover, BA synthesis is suppressed, as reflected by inhibition of gene expression of Cyp7a1 and Cyp8b1 and reduction of fecal BA secretion. In line with a strong inhibition of Cyp8b1, CA synthesis and CA pool size are significantly decreased in both male and female Cyp2c70\textsuperscript{-/-} mice. This leads to an unanswered question, how is BA synthesis regulated in Cyp2c70\textsuperscript{-/-} mice, the modulation of Cyp8b1 in particular? Surprisingly, despite high abundances of the potent endogenous FXR agonist CDCA, we (Chapter 3 and 4) as well as others do not observe a clear induction of FXR target genes in the liver and only a slight induction of FXR target genes in ileum (14,15). Alternatively, Honda A, et al. (15) hypothesized that unconjugated CDCA induced hepatic inflammation and cytokine production, which subsequently could inhibit expression of Cyp7a1 and Cyp8b1. Therefore, multifaceted mechanisms including intestinal FXR activation and hepatic cytokine induction might be involved in the regulation of BA synthesis in Cyp2c70\textsuperscript{-/-} mice. Moreover, other nuclear receptors such as hepatocyte nuclear factor 4α (HNF-4α) and peroxisome proliferator-activated receptor α (PPARα) (11) and hormones including thyroid hormone (11), estrogen (16) and insulin (17) have been reported to mediate the regulation of BA synthesis, which might be of great interest for the future studies.

The data presented in Chapter 3 show a normal bile flow in both male and female Cyp2c70\textsuperscript{-/-} mice compared to WT controls, indicating absence of cholestasis. Due to the hydrophobic BAs, biliary phospholipid and cholesterol secretion are slightly increased in Cyp2c70\textsuperscript{-/-} mice. Total BA pool size is reduced by 30% in female Cyp2c70\textsuperscript{-/-} mice only in our study while Honda A, et al. (15) found a similar reduction in both male and female Cyp2c70\textsuperscript{-/-} mice. More importantly, plasma BA levels were increased and ileum BA contents were decreased in Cyp2c70\textsuperscript{-/-} mice (14,15). How would that impact the liver histology and intestinal fat absorption will be discussed in the following sections.

**Lipid metabolism in Cyp2c70\textsuperscript{-/-} mice**

BAs play a central role in lipid metabolism. With manipulation of BA homeostasis, data in Chapter 3 display that Cyp2c70\textsuperscript{-/-} mice have slightly higher hepatic cholesterol contents. Intriguingly, gene expression of Npc1l1, encoding a reported cholesterol transporter in jejunum, is significantly down-regulated, while plasma plant sterols, surrogates for intestinal cholesterol absorption, show a 50% reduction in Cyp2c70\textsuperscript{-/-} mice, suggesting a possible suppression of cholesterol absorption (14,15). However, kinetic studies with stable isotopically labeled-cholesterol tracers do not support a reduction of cholesterol absorption in Cyp2c70\textsuperscript{-/-} mice under a chow diet condition. Cholesterol synthesis rates, determined by isotopically labeled-acetate using mass isotopomer distribution analysis (MIDA), also show no differences between Cyp2c70\textsuperscript{-/-} and WT mice. In line, unchanged serum surrogate markers for
cholesterol biosynthesis (lathosterol and desmosterol) (15) also indicates that cholesterol synthesis is not altered in chow-fed Cyp2c70−/− mice compared to WT controls. Conceivably, inhibition of BA synthesis contributes to the increased cholesterol contents in livers of Cyp2c70−/− mice. In plasma, total cholesterol levels are increased in Cyp2c70−/− mice compared to their WT controls, mainly due to the higher levels of LDL-c (low-density lipoprotein cholesterol). The clearance of plasma LCL-c is mainly carried out by the liver via LDL receptor (LDLR), which has been shown to be decreased by CDCA treatment in human hepatocytes (18). In line, both our study and others (14) suggest a lower expression of hepatic LDLR, which possibly explains the higher LDL-c levels in plasma of Cyp2c70−/− mice. Sterol regulatory element binding protein 2 (SREBP2) (19) and proprotein convertase subtilisin kexin 9 (PCSK9) (20) are two major mediators of LDLR regulation, follow-up studies on which might shed more light on the mechanisms of decreased LDLR in livers of Cyp2c70−/− mice.

Regarding the fatty acid and triglyceride metabolism, Cyp2c70−/− mice have comparable plasma triglyceride levels but lower hepatic triglyceride contents compared to their WT controls. As shown in Chapter 3 of this thesis, de novo lipogenesis is only marginally inhibited in female Cyp2c70−/− mice while pre-existing fat contents show a bigger reduction. Pre-existing fat contents in the liver are the balance between hepatic lipid uptake, fatty acid oxidation and (very low-density lipoprotein) VLDL production. VLDL production remains unaltered between Cyp2c70−/− and WT mice (data not shown), while hepatic lipid uptake and fatty acid oxidation have not been measured in the current study. Unconjugated BAs including UDCA and DCA can inhibit hepatic fatty acid uptake in a fatty acid transport protein 5 (FATP5)-dependent manner (21). Cyp2c70−/− mice have increased abundances of UDCA, but decreased abundances of DCA, which might counteract regarding the effects on hepatic fatty acid uptake. Future studies will be required to elucidate how hepatic lipid uptake is modulated by BAs in Cyp2c70−/− mice. Although CDCA was reported to activate human PPARα promoter and likely enhance fatty acid oxidation in mitochondria, the murine PPARα promoter was not affected by CDCA (22). Therefore, suppression of de novo lipogenesis might partly contribute to the lower hepatic triglyceride contents in Cyp2c70−/− mice. Other steps including hepatic lipid uptake and fatty acid oxidation await further investigation.

Under a western-type diet (WTD), both hepatic cholesterol and triglyceride contents are strongly reduced in Cyp2c70−/− mice, in particular in female Cyp2c70−/− mice. Data presented in Chapter 4 show elevated plasma LDL-c levels in female Cyp2c70−/− mice while plasma triglycerides and total cholesterol levels remain similar between Cyp2c70−/− and WT mice. Gene expression of lipogenic pathway Lxrα-Srebp1c-Acc1/Fasn/Scd1 is down-regulated in Cyp2c70−/− mice, (partly) contributing to the lower fat contents in the liver. Genes involved in fatty acid oxidation including Ppara, Cpt1a, Acox1 are surprisingly also down-regulated in livers of Cyp2c70−/− mice. In addition, our data in Chapter 4 do not support roles of increased energy burning in brown adipose tissue or browning of white adipose tissue in Cyp2c70−/− mice. Unexpectedly, both cholesterol and fatty acid absorption are reduced in Cyp2c70−/− mice as measured by balance method and plant sterols (as surrogate markers). In line, fecal loss of
neutral sterols and fatty acids are increased in Cyp2c70−/− mice. Of note, the absorption of lipids involves lipolysis by pancreatic enzymes, micelle formation as well as intestinal uptake either mediated by transporters or through passive diffusion. Within the enterocytes, free fatty acids or cholesterol will be utilized for reconstitution of triglycerides and cholesterol esters. Chylomicrons will be assembled and secreted into lymph, which cycle to the circulation, reaching to the adipose tissues and liver (23). A BODIPY-labeled palmitic acid gavage experiment in Chapter 4 demonstrates a markedly reduced fatty acid uptake by the intestine of both male and female Cyp2c70−/− mice. Because palmitic acid is a saturated long-chain fatty acid, enzymatic lipolysis is not involved in its intestinal absorption. Moreover, protein level of CD36, a major fatty acid transporter in ileum, is not affected between Cyp2c70−/− and WT mice, suggesting that impaired lipid absorption in Cyp2c70−/− mice is CD36-independent. Therefore, other steps such as an impairment of micelle formation and/or passive entry of fatty acids into enterocytes might explain the observed effects. Unaffected bile flow and biliary BA secretion in Cyp2c70−/− mice do not suggest a shortage of intestinal BA content, which obviously play a crucial role in micelle formation (24). However, ratio of 12α/non 12α-hydroxylated BAs is evidently decreased in Cyp2c70−/− mice, and is strongly correlated with intestinal fat absorption and hepatic fat accumulation, demonstrating that reduction of 12α-hydroxylated BAs most likely explains the impaired intestinal fat absorption, possibly interrupting the micelle formation or passive intestinal lipid uptake. Cyp2c cluster-deficient mice showed a similar amelioration of hepatic fat contents and impairment of fat absorption (25). More importantly, low-dose TCA (taurine conjugated-cholic acid) treatment normalized the fat absorption, supporting the crucial role of 12α-hydroxylated BAs in intestinal fat absorption.

In line with much lower hepatic fat contents in livers of female Cyp2c70−/− mice compared to male Cyp2c70−/− mice, expression of Cyp8b1 and ratio of 12α/non 12α-hydroxylated BAs also show a stronger reduction in female Cyp2c70−/− mice. Differential modulation of Cyp8b1 between genders is most likely estrogen-related since estrogen down-regulates Cyp8a1 expression in mice (16,26). Intriguingly, lipid droplets in enterocytes are in a comparable low level in both male and female Cyp2c70−/− mice in the labeled-palmitic acid experiment. Delayed lipid absorption in distal intestine might compensate for some lipid absorption in male Cyp2c70−/− mice, which needs to be confirmed in future studies. Taken together, our data demonstrate that 12α-hydroxylated BAs represent a promising target for the treatment of NAFLD.

**Cholangiopathic features in Cyp2c70−/− mice**

Cholangiopathies refer to diseases that affect the hepatic biliary system. Cholangiopathies can be caused by genetic, viral or environmental factors (27). BA-induced injury is present in livers of Cyp2c70−/− mice, predominantly in the periductal area (Chapter 3). Young Cyp2c70−/− mice at 3 weeks of age show a normal liver histology despite elevated plasma levels of AST (aspartate aminotransferase), ALT (alanine aminotransferase) and BAs. Adult Cyp2c70−/− mice have bigger livers, increased plasma levels of AST and ALT and higher expression of cytokines in the liver.
In addition, liver histology shows proliferation of cholangiocytes and fibrosis in adult Cyp2c70−/− mice. Intriguingly, Honda A, et al. (15) observed more liver damage in male Cyp2c70−/− mice, while we detected more liver damage in female Cyp2c70−/− mice. Mice from both groups have the same background (C57BL/6J) and similar age (~12 weeks old), which thus do not explain the differences in liver phenotypes. Serum concentration of CDCA is much higher in female Cyp2c70−/− mice compared to male Cyp2c70−/− mice in our study, which might contribute to a worse liver phenotype in female Cyp2c70−/− mice, while CDCA level is comparable between male and female Cyp2c70−/− mice in the study of Honda A, et al. (15). Whether the housing or diet conditions might contribute to the observed variations needs further exploration since mice from Honda A, et al. (15) were kept under specified pathogen-free conditions while our mice were conventionally housed. When mice grow older in our study, up to 33 weeks, cholangiopathy develops progressively in female Cyp2c70−/− mice with bridging fibrosis but is improved in male Cyp2c70−/− mice. Liver pathology in Cyp2c70−/− mice shows a more progressive development course in female mice, the mechanism for which requires further investigation. Estrogen is known to induce hepatotoxicity (26), which might make female mice more susceptible to BA-induced injury. Data presented in Chapter 4 demonstrate that 12 weeks of WTD does not worsen the cholangiopathy in both male and female Cyp2c70−/− mice compared to the regular chow diet-fed cohorts. Plasma albumin levels are quite stable and comparable between Cyp2c70−/− and WT mice, suggesting that Cyp2c70−/− mice maintain considerable liver function.

Several mechanisms have been proposed to be involved in BA-induced liver injury (28,29). On one hand, BAs can directly damage cell membranes through their detergent actions. On the other hand, the accumulation of BAs may promote the generation of reactive oxygen species and cause mitochondrial dysfunction, endoplasmic reticulum (ER) stress, DNA damage or cellular senescence and, eventually, induce apoptosis or necrosis of liver cells including hepatocytes, cholangiocytes and endothelial cells. Moreover, increased activity of inflammatory and fibrogenic pathways may amplify the liver insult and trigger cholangiocyte proliferation. Our data suggest a potential role of ER stress (Chapter 3) and cellular senescence (Chapter 6) in the development of cholangiopathy in female Cyp2c70−/− mice. Inflammation markers are elevated in livers of Cyp2c70−/− mice, but F4/80 staining does not indicate an increased number of macrophages. Hepatic macrophages consist of tissue-resident Kupffer cells and an infiltrating group of bone marrow-derived macrophages. The role of macrophages in cholestatic liver injury remains controversial (28). Infiltration of neutrophils rather than macrophages correlated best with liver injury in cholestasis (30,31). Recently, liver-gut crosstalk has been proposed to contribute to cholestatic liver diseases by promoting intestinal permeability and endotoxin flux into the liver (32). We observed an increased intestinal permeability in Cyp2c70−/− mice in Chapter 3, but the endotoxin concentrations in portal plasma were undetectable. Moreover, serum LPS (lipopolysaccharide) and TNFα levels were not increased in Cyp2c70−/− mice compared to their WT controls in the study of Honda A, et al. (15).
Gut microbiome in Cyp2c70⁻/⁻ mice

Gut bacteria are known to mediate the conversion of primary BAs to secondary BAs mainly through deconjugation and dehydroxylation reactions (33). On the other hand, BAs exert direct anti-microbial effects through interrupting membrane integrity leading to cell death or indirect anti-microbial effects through FXR-stimulating mucosal defense mechanisms (34). Given the interactions between BAs and the gut microbiome, it is not surprising to see that cecal gut microbiome communities are considerably affected by Cyp2c70-deficiency both on a chow diet (Chapter 3) as well as on a WTD (Chapter 4). When fed a chow diet, gut microbiome changes in Cyp2c70⁻/⁻ mice show certain similarities with microbiome alterations observed in PBC patients (35), with lower abundances of Akkermansia, Rikenella and Christensenellaceae, while Prevotella and Veillonella are enriched. It suggests that cholangiopathies might share similar BA-gut flora changes, which might be involved in the disease development and/or progression. When fed a WTD, Lachnospiraceae are dominant in caecum contents of both Cyp2c70⁻/⁻ and WT mice, accounting for over 40% of total reads. In line with the inhibition of fat absorption and improvement of hepatic steatosis in Cyp2c70⁻/⁻ mice, a “beneficial” bacterial profile is observed, with lower abundance of Desulfovibrioaceae and higher abundances of Bacteroidales_S24-7_group and Erysipelotrichaceae, which have been reported to be related to improvement of diet-induced obesity (36,37). More importantly, data in Chapter 4 show a strong association at the biliary 12α/ non 12α-hydroxylated BAs ratio with intestinal fatty acid absorption as well as with the abundance of Erysipelotrichaceae. It will be interesting to work further on 12α-hydroxylated BAs and Erysipelotrichaceae in intestinal lipid absorption. In addition, genera involved in short-chain fatty acid (SCFA) production, such as Clostridiales_vadinBB60 and Erysipelotrichaceae, are increased in Cyp2c70⁻/⁻ mice. However, SCFAs are not measured in Chapter 4 and their potential roles in lipid metabolism and cholangiopathy in Cyp2c70⁻/⁻ mice awaits further studies.

Pharmacological response in Cyp2c70⁻/⁻ mice

The human-like BA profile and cholangiopathic phenotypes make Cyp2c70⁻/⁻ mice a useful model to investigate the potential therapeutic approaches for cholangiopathies. To date, there are limited pharmacological options for cholangiopathies, other than liver transplantation (38). UDCA and OCA (obeticholic acid) are the FDA-approved first- and second-line treatment options for PBC patients, respectively, while there are no approved mediations for PSC patients (38). The potential new therapies including PPAR agonists and non-steroidal FXR agonists are in clinical trials for the treatment of PBC while UDCA and FXR agonists are under investigation for PSC (27). Furthermore, accumulating data address the important role of cellular senescence in cholangiopathies (39–41), which supports the potential application of senolytics as therapeutic options for PBC and PSC. Fisetin is a natural senolytic, which has shown beneficial effects in progeroid mice (42) as well as in mdr2⁻/⁻ mice (43) that show cholangiopathy due to defective secretion of phospholipids into the bile. In this thesis, it was
first explored whether UDCA could improve the cholangiopathy in female *Cyp2c70*<sup>−/−</sup> mice (Chapter 3). 7 weeks of UDCA treatment starting at 5 weeks of age strongly reduces plasma total BA levels. The BA pool at UDCA-treated *Cyp2c70*<sup>−/−</sup> mice is enriched with ~60% of (T)UDCA. Consequently, hydrophobicity index of biliary BAs is decreased from +0.3 in *Cyp2c70*<sup>−/−</sup> mice to -0.15 in UDCA-treated *Cyp2c70*<sup>−/−</sup> mice. UDCA treatment restores the elevated plasma transaminases as well as inflammation, proliferation of cholangiocytes and fibrosis in livers of female *Cyp2c70*<sup>−/−</sup> mice. More importantly, expression of ER stress markers and gene sets involved in extracellular matrix (re)organization and senescence are normalized by UDCA treatment, demonstrating the multifaceted effects of UDCA in cholangiopathy. However, expression of *Bsep*, *Mrp2* and *Mrp3* is unaltered upon UDCA treatment (data not shown) and effects on bile flow have not been explored. Whether UDCA exerts choleretic action in *Cyp2c70*<sup>−/−</sup> mice requires formal confirmation, but it would be expected based on other studies (44,45).

Briefly, OCA is a CDCA-derivative and a strong FXR activator. It is believed to decrease accumulation of toxic BAs in the liver, resulting in amelioration of inflammation and fibrosis via inhibition of BA synthesis and stimulation of bile export (46,47). Next we treated both male and female *Cyp2c70*<sup>−/−</sup> mice with 10 mg/kg OCA for 4 weeks starting at an age when fibrosis is already present in *Cyp2c70*<sup>−/−</sup> mice (Chapter 5). We use ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) to assess the hydrophobicity of OCA and its conjugates. Interestingly, (T)OCA itself is as hydrophobic as (T)LCA, making the biliary BA composition even more hydrophobic in OCA-fed *Cyp2c70*<sup>−/−</sup> mice. Although OCA significantly inhibits BA synthesis in *Cyp2c70*<sup>−/−</sup> mice and is detected in plasma in a concentration that is comparable to those observed in OCA-treated individuals, liver phenotypes including inflammation, proliferation of cholangiocytes and fibrosis are not improved. Expression of genes involved in senescence (*P16<sup>INK4A</sup>, *P21*) or BA transport (*Ntcp, Bsep*) is not altered either. It has been reported that activated hepatic stellate cells in fibrosis showed limited response to OCA due to an enhanced FXR SUMOylation (48). Whether this post transcriptional modulation of FXR activation is present in livers of *Cyp2c70*<sup>−/−</sup> mice with established fibrosis, which might lead to a low response to OCA treatment, needs to be evaluated. In addition, 3-year of OCA treatment has been associated with improvement or stabilization of fibrosis in PBC patients compared to baseline (49). Whether long term OCA treatment or higher dose of OCA treatment will improve cholangiopathy in *Cyp2c70*<sup>−/−</sup> mice also requires further investigation.

Senescent cholangiocytes might contribute to chronic biliary diseases by secreting pro-inflammatory senescent associated secretory phenotypes (SASPs), thereby negatively modulating their microenvironment (50). Subsequently, cellular senescence serves as a therapeutic target for cholangiopathies. The potential beneficial effects of the senolytic fisetin on cholangiopathy are explored in female *Cyp2c70*<sup>−/−</sup> mice in Chapter 6. Multiple senescence gene sets strongly suggest up-regulation of senescence pathways in livers of female *Cyp2c70*<sup>−/−</sup> mice. However, fisetin treatment does not appear to remove the senescent cells. Instead, it
reduces SASP expression possibly by modulating the function of senescent cells. Fisetin therefore seems to exert mainly senomorphic effects in livers of Cyp2c70−/− mice. In addition, fisetin exerts a marked inhibition of proliferative pathways in the liver, G1/S phase in particular. But fisetin does not alleviate cholangiopathy in Cyp2c70−/− mice as it did in Mdr2−/− mice. Of note, Mdr2−/− mice have a hydrophilic BA pool with high abundances of MCAs, while Cyp2c70−/− mice are mainly enriched with hydrophobic CDCAs. Fisetin barely reduced the GCDCA-induced senescent cholangiocytes in vitro (40). Therefore, it is likely that fisetin has limited effects in CDCA-induced senescence. Indeed, out data suggest senomorphic effects of fisetin in livers of female Cyp2c70−/− mice, which might not be strong enough to restore cholangiopathy. Alternatively, we cannot rule out the possibility that senescence does not play an essential role in the development of cholangiopathy in female Cyp2c70−/− mice. To confirm the important role of cellular senescence and to elucidate the potential beneficial effects of senescence removal in cholangiopathies, genetic mouse models, such as Cyp2c70 x P16-3MR mice, might represent a promising strategy. P16-3MR (trimodality reporter) fusion protein contains functional domains of Renilla luciferase (LUC), monomeric red fluorescent protein (mRFP), and a truncated herpes simplex virus (HSV)-1 thymidine kinase (tTK) under the control of a senescence-sensitive p16INK4a promoter (51). LUC and mRFP allow the detection of 3MR-expressing cells by luminescence and fluorescence, while HSV-TK allows their selectively killing by ganciclovir (GCV) (51). Therefore, Cyp2c70 X p16-3MR mice can be employed to track senescent cells in the development of cholangiopathy in live animals and to identify senescent cell types as well as to selectively remove senescent cells.

**Limitations of Cyp2c70−/− mice as a human-like mouse model**

Of note, except for the major differences in murine MCAs, there are some other differences regarding BA metabolism between humans and mice (Table 1). 1) BA conjugation is catalyzed by BA CoA : amino acid N-acyltransferase (BAAT). Mouse BAAT only utilizes taurine as a substrate, while human BAAT can conjugate BAs with both taurine and glycine (52). It appears that the ratio of glycine to taurine-conjugated BAs has only limited functional consequences (11). For example, conjugation with either glycine or taurine causes little changes in the critical micellar concentration (CMC) (53), suggesting that conjugation of BAs with glycine or taurine has little effect on fat absorption. In addition, the type of conjugation (glycine or taurine) has little impact on BA hydrophobicity (54) and on the activation of human FXR (55). 2) CYP2A12 (a 7-alpha-hydroxylase) is responsible for the 7α-rehydroxylation of DCA and LCA (lithocholic acid) in livers of mice (9). Such rehydroxylation does not occur in humans. Due to the conversion of DCA back into CA in the liver, the BA pool in mice has relatively low levels of DCA compared to humans. 3) The BA synthesis rate in mice is ~5-fold higher than that in humans (56) and bile flow is ~20-fold greater in mice when normalized for body weight (57). Despite devoid of MCAs, Cyp2c70−/− mice have comparable bile flow and biliary BA secretion compared to WT mice, which does not support the contribution of MCAs to the increased bile flow in WT mice. 4) The differences of gut microbiota between humans and mice could impact the synthesis of secondary BAs and subsequently BA composition (33).
Table 1. Major differences of BA homeostasis between humans and mice.

<table>
<thead>
<tr>
<th></th>
<th>HUMANS</th>
<th>MICE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major primary BAs</td>
<td>CA, CDCA</td>
<td>CA, α-MCA, β-MCA</td>
</tr>
<tr>
<td>Conjugation</td>
<td>Glycine: taurine ≈ 3:1</td>
<td>Only taurine</td>
</tr>
<tr>
<td>Hydrophobicity index</td>
<td>~+0.3</td>
<td>~-0.3</td>
</tr>
<tr>
<td>Re-hydroxylation of secondary BAs in the liver</td>
<td>No</td>
<td>Yes, at C-7α position</td>
</tr>
<tr>
<td>BA synthesis</td>
<td>~ 20 μmol/kg BW/day</td>
<td>~ 100 μmol/kg BW/day</td>
</tr>
<tr>
<td>Bile flow</td>
<td>3.6 μl/min/kg BW</td>
<td>69 μl/min/kg BW</td>
</tr>
<tr>
<td>Gut microbiome</td>
<td>Largely different</td>
<td></td>
</tr>
</tbody>
</table>

BA, bile acid; BW, body weight; MCA, muricholic acid.

Therefore, Cyp2a12 deficiency and fecal transplants of human microbiota into germ-free Cyp2c70−/− mice might make it an even more human-like mouse model. Cyp2a12/Cyp2c70 double knockout (DKO) mice actually have been generated and showed accumulation of DCA and LCA in both plasma and bile compared to Cyp2c70−/− mice (15). The hydrophobicity of biliary BAs in DKO mice was further increased (~+0.5). Intriguingly, liver damage was attenuated in male DKO mice as evidenced by lower liver weight, lower plasma ALT activity and suppression of expression of inflammatory markers (Il-1β, Tnfa, Ccl2, Tgfβ1), while liver damage was not exacerbated in female DKO compared to Cyp2c70−/− mice, respectively (15). The underlying mechanisms for the improvement in DKO mice remain unclear. The role of individual BA species on the development of liver damage might be of great interest. In addition, baseline liver damage in adult Cyp2c70−/− mice might raise concerns when using this model for studies on BAs and metabolic diseases. For this purpose, an improved humanized mouse model is needed and our study suggest that UDCA treatment represents a promising strategy.

Defense mechanisms against BA-induced injury

High concentration of hydrophobic BAs might be toxic to the liver and cause liver disease, as evidenced in Cyp2c70−/− mice. Therefore, BA synthesis and circulation are highly regulated by negative feedback loops mediated by the BA sensor FXR in both liver and intestine (5 8). In addition, the formation of mixed micelles represents an important cytoprotective action against BA exposure in the biliary tree (59). Biliary phospholipids and cholesterol are secreted via MDR2 and 3 (Multidrug resistance protein 2 and 3) and ABCG5 and 8 (ATP-binding cassette sub-family G member 5 and 8), respectively, in a ratio of BA : phospholipid : cholesterol = ~50:10:1 in mice (60) and they form mixed micelles in bile, limiting the toxicity of BAs (Figure 1). Mdr2 (Abcb4)-knockout mice develop progressive cholangitis and fibrosis due to an absence of biliary phospholipid secretion and a strong reduction of biliary cholesterol secretion, which is well-established, i.e., progressive familial intrahepatic cholestasis type 3 (PFIC3) (61,62). Furthermore, BA-induced cytotoxicity is highly pH-dependent (63) because cell entry of BAs is increased by decreasing pH (64). The so-called biliary bicarbonate umbrella
subsequently provides an additional way of protection against hydrophobic BAs entering the cells and BA-induced injury in bile ducts (65). Multiple transporters and channels are involved in the formation of the biliary bicarbonate umbrella and some differences between species are present (Figure 1). The Na+ independent Cl−/HCO3− exchanger, anion exchanger 2 (AE2, SLC4A2) mediates biliary HCO3− secretion in both humans and mice, while an alternative Na+/HCO3− cotransporter (NBC1, Slc4a4) is only present in mouse cholangiocytes, but not in humans (66). Both in vitro and in vivo studies have indicated that defects of the biliary bicarbonate umbrella may cause the chronic cholangiopathies (63,67).

The above mentioned defense mechanisms against BA-induced injury might serve as therapeutical targets for the cholangiopathies in future studies. For example, PPARα agonists were reported to stimulate biliary phospholipid secretion (68) and potentially protect against cholangiopathy. Furthermore, NorUDCA (24-norursodeoxycholic acid) has been reported to be effective in the activation of the cholehepatic shunt and stimulation of bicarbonate secretion, representing a promising candidate for the treatment of cholangiopathies (69). Moreover, ASBT (apical sodium-dependent bile acid transporter) inhibitors, which suppress BA absorption and interrupt the enterohepatic circulation (70), might reduce BA accumulation in the liver and subsequently ameliorate BA-induced liver damage. Like ASBT inhibitors, BA sequestrants that bind to BAs in the intestine and inhibit their (re)absorption have potential to reduce BA pool size and alleviate liver injury.

Figure 1. Defense mechanisms against BA-induced injury.
BAs, bile acids; chol, cholesterol; PL, phospholipids; AE2, anion exchanger 2; cAMP, cyclic adenosine monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; TMEM16A, Transmembrane member 16A, also known as Anoctamin-1, ANO1; NBC1, sodium bicarbonate cotransporter isoform 1; BSEP, ATP-dependent bile salt excretory pump; NTCP, Na+ taurocholate co-transporting peptide; ABCG5 and 8, ATP-binding cassette sub-family G member 5 and 8; MDR2 and 3, multidrug resistance protein 2 and 3; OATP, organic anion transport polypeptide. (Created with Biorender)
Conclusion and perspective

To conclude, this thesis describes the generation of a mouse model with a human-like BA composition (i.e., Cyp2c70−/− mice) and its impact on lipid metabolism and liver pathophysiology. A strong reduction of ratio 12α/non 12α-hydroxylated BAs is associated with decreased intestinal fat absorption in Cyp2c70−/− mice, supporting a crucial role of 12α-hydroxylated BAs in fat absorption. 12α-hydroxylated BAs might serve as therapeutic targets for NAFLD, which is worth further exploration.

With a hydrophobic BA pool, cholangiopathy and fibrosis are manifested in Cyp2c70−/− mice, which raises concerns when using this model to study BA metabolism and signaling in human diseases. Our finding suggests that UDCA treatment can restore the cholangiopathy in Cyp2c70−/− mice, which represents a promising strategy to improve this human-like mouse model. Follow-up studies of treating mice with UDCA during pregnancy or in neonatal period should be considered. Alternatively, we employ this model for investigating the potential pharmacotherapies on cholangiopathy. So far, only UDCA treatment shows beneficial effects in Cyp2c70−/− mice under our experimental settings. Both OCA and fisetin have limited effects on fibrosis of Cyp2c70−/− mice although we cannot rule out any long-term benefits. In order to explore the potential therapeutic approaches, mechanistic research will be required regarding the BA-induced injury in cholangiopathies. On the other hand, female predominance of Cyp8b1 inhibition and liver damage are observed in Cyp2c70−/− mice, which needs further investigation, especially on the potential roles of estrogen and estrogen receptors.
General discussion

References


3. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1); liver regeneration and neuronal differentiation. Proc Natl Acad Sci U S A. 2003 Feb 4;100(3):928–33.


43. Alsurailh M, O’Hara SP, Woodrum JE, Pirius NE, LaRusso NF. Genetic or pharmacological


58. Choudhuri S, Claussen CD. MOLECULAR REGULATION OF BILE ACID HOMEOSTASIS. Drug Metab Dispos. 2021 Oct 22;


64. Amelsberg A, Schteingart CD, Ton-Nu HT, Hofmann AF. Carrier-mediated jejunal...


