Cholangiopathy and Biliary Fibrosis in Cyp2c70-Deficient Mice Are Fully Reversed by Ursodeoxycholic Acid

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SUMMARY

We characterized Cyp2c70-deficient mice, possessing a human-like bile acid composition. While both sexes display transient neonatal cholestasis, only female mice develop considerable pathologic features with age. Treatment with the hydrophilic bile acid ursodeoxycholic acid reverses liver pathology in female Cyp2c70-deficient mice.

BACKGROUND AND AIMS: Bile acids (BAs) aid intestinal fat absorption and exert systemic actions by receptor-mediated signaling. BA receptors have been identified as drug targets for liver diseases. Yet, differences in BA metabolism between humans and mice hamper translation of pre-clinical outcomes. Cyp2c70-ablation in mice prevents synthesis of mouse/rat-specific muricholic acids (MCAs), but potential (patho)physiological consequences of their absence are unknown. We therefore assessed age- and gender-dependent effects of Cyp2c70-deficiency in mice.

METHODS: The consequences of Cyp2c70-deficiency were assessed in male and female mice at different ages.

RESULTS: Cyp2c70−/− mice were devoid of MCAs and showed high abundances of chenodeoxycholic and lithocholic acids. Cyp2c70-deficiency profoundly impacted microbiome composition. Bile flow and biliary BA secretion were normal in Cyp2c70−/− mice of both sexes. Yet, the pathophysiological consequences of Cyp2c70-deficiency differed considerably between sexes. Three-week-old female Cyp2c70−/− mice showed high plasma BAs and transaminases, which spontaneously decreased thereafter to near-normal levels. Only mild ductular reactions were observed in male Cyp2c70−/− mice up to 8 months of age. In female Cyp2c70−/− mice, plasma BAs and transaminases remained substantially elevated with age, gut barrier function was impaired and bridging fibrosis was observed at advanced age. Addition of 0.1% ursodeoxycholic acid to the diet fully normalized hepatic and intestinal functions in female Cyp2c70−/− mice.

CONCLUSION: Cyp2c70−/− mice show transient neonatal cholestasis and develop cholangiopathic features that progress to bridging fibrosis in females only. These consequences of Cyp2c70-deficiency are restored by treatment with UDCA, indicating a role of BA hydrophobicity in disease development. (Cell Mol Gastroenterol Hepatol 2021;11:1045–1069; https://doi.org/10.1016/j.jcmgh.2020.12.004)

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plasma BAs, while some liver diseases that develop at more transient period of neonatal cholestasis with elevated parameters in this study because human infants often experience a ohepatic circulation). Age and sex were included as vari-
liver and intestine (ie, the organs that constitute the enter-
composition may differentially affect BA-mediated signaling or (pharmacological) interventions that impact BA pool
MCAs in mice, the intrinsic potency of the circulating BA
leads to a complete absence of MCAs in mice.6,7

Given the highly divergent physicochemical properties of the various BA species, differences in BA metabolism between animal species obviously complicate translational research on the role of BAs in disease development. Accumulation of BAs contributes to liver damage in obstructive cholestasis, progressive familial intrahepatic cholestasis, primary sclerosing cholangitis, and primary biliary cholangitis (PBC).10,9 Human diseases involving BA-induced liver damage are difficult to mimic in mouse models, because of the aforementioned high abundance of MCAs in the murine BA pool.3 These trihydroxylated BAs are very hydrophilic,10 have a high critical micellar concentration, and possess poor lipid-solubilizing properties. Like ursodeoxycholic acid (UDCA), which is used in treatment of cholestatic liver diseases like PBC, MCAs do not cause cell damage and can even reduce cytotoxic effects of more hydrophobic BAs.11 The BA receptors FXR and TGR5 have been identified as bona fide drug targets for the treatment of metabolic and cholestatic liver diseases, including nonalcoholic fatty liver disease12,13 and PBC.14 Furthermore, these receptors play important roles in BA, glucose, lipid, and cholesterol metabol-
If, TGR5, however, differs substantially between BA species. CDCA is the most potent endogenous FXR agonist,16 while MCAs act oppositely and actually inhibit FXR activation.17 In general, more hydrophobic BAs are also more potent agonists of TGR5.18 Hence, owing to the efficient conversion of CDCA into MCAs in mice, the intrinsic potency of the circulating BA pool to activate these receptors differs substantially between mice and humans. Furthermore, metabolic conditions or (pharmacological) interventions that impact BA pool composition may differentially affect BA-mediated signaling pathways in mice and men.5

The recent discovery that CYP2C70 catalyzes the conversion of CDCA into MCAs in mice4–7 paved the way for the generation of mouse models with a more human-like BA profile to study BA-related liver diseases, as well as the impact of BAs and pharmacological agents that target their signaling pathways on metabolic diseases.9,19 However, the (patho)physiological consequences of the absence of MCAs in mice are still unexplored. Therefore, we assessed the effects of the human-like BA pool in Cyp2c70-deficient mice on liver and intestine (ie, the organs that constitute the enter-
transient period of neonatal cholestasis with elevated plasma BAs, while some liver diseases that develop at more advanced ages display a sex bias, such as PBC10 and nonalcoholic fatty liver disease.21 We found that male Cyp2c70-deficient mice displayed transient liver dysfunction at weaning, which gradually improved with age, while liver pathology worsened and actually progressed to bridging fibrosis in female Cyp2c70-deficient mice at advanced age. Intriguingly, aberrations in liver function in Cyp2c70-defi-
cient mice were fully restored by increasing the hydrophilicity of the BA pool by treatment with UDCA.

Results
Generation of Cyp2c70-Deficient Mice
Targeted inactivation of Cyp2c70 yielded 3 mouse lines with mutations resulting in the introduction of an early stop codon in exon 1 (Figure 1A). Adult mice from all 3 Cyp2c70+/− lines displayed similar biliary BA compositions with a complete absence of MCAs (Figure 1B). Biliary BA profiles of Cyp2c70+/− mice were only performed for mice from the breeding lines containing the 11 nucleotide deletion or the 2 nucleotide insertion in the Cyp2c70 gene and were very similar to their wild-type (WT) littermates. The breeding line with an 11 nucleotide deletion was used for further characterization of the (patho)physiological consequences of this human-like BA pool composition in mice. Absence of CYP2C70 protein in the livers of Cyp2c70−/− mice was confirmed by targeted proteomics (Figure 1C). Cyp2c70−/− mice were born in the expected Mendelian ratio (Figure 1D), but some Cyp2c70-deficient mice died shortly after weaning when pups were weaned at 3 weeks of age. This could be prevented by postponing weaning until the age of 4 weeks.

Cyp2c70−/− Mice Show Features of Neonatal Cholestasis
To evaluate potential early effects of Cyp2c70 deficiency, we sacrificed mice at the age of 3 weeks and examined plasma and hepatic parameters as well as liver histology. Body weights did not differ between WT and Cyp2c70−/− mice (Figure 2A), but liver sizes were increased in male as well as female mice lacking Cyp2c70 at this age (Figure 2B). Plasma transaminases were strongly elevated in young Cyp2c70−/− mice of both sexes (Figure 2C), as were total plasma BA levels (Figure 2D, inserts). Cyp2c70−/− mice of both sexes showed high abundances of (tauro-)CDCA in plasma (Figure 2D). Likely due to ingestion of exogenous BAs via milk from their heterozygous mothers or consumption of feces from mother and littermates, low levels of MCAs could be detected in these young Cyp2c70−/− mice. Hepatic gene expression analysis revealed that expression of Fxr was reduced in male and female Cyp2c70−/− mice, whereas expression of its target genes Shp (Nr0b2) and Bsep (Abcb11) was not significantly altered (Figure 2E). Nonetheless, robust decreases in hepatic expression of Cyp7a1 and Cyp8b1 indicate reduced BA synthesis in the Cyp2c70−/− animals (Figure 2E). A profibrotic gene expression pattern with increased collagen type 1a1 (Col1a1) and tissue inhibitor of metalloproteinase (Timp1) was evident (Figure 2E), but histology revealed only mild collagen deposition in the livers of Cyp2c70−/− mice at this age (Figure 2F). Modest cholangiocyte proliferation appeared to be present, as evidenced by slightly increased Krt19 gene expression and modestly higher numbers CK19-positive cells in the livers of
Inflammation pathways were activated. Taken together, several characteristics of “neonatal cholestasis” were evident in 3-week-old Cyp2c70−/− mice, with no overt differences between male and female mice.
Characterization of BA Profiles in 12-Week-Old Cyp2c70–/– Mice

Body weight and food intake were not affected by absence of CYP2C70 in 12-week-old, young adult male and female mice (Table 1). These individually housed Cyp2c70–/– mice were completely devoid of α, β, and ω MCAs (Figure 3A and B). Instead, substantial amounts of CDCA were present. In contrast to their WT littermates,
(tauro-)LCA was clearly present in Cyp2c70−/− mice. Owing to the inability to convert UDCA into βMCA, the amounts of (tauro-)UDCA in the BA pool were increased upon Cyp2c70 deficiency (Figures 1B and 3A). Female WT mice had a more hydrophilic BA pool compared with WT male mice (Figure 3C). As a result of the altered abundance of BA species, the hydrophobicity index of biliary BAs was substantially increased in male as well as in female mice lacking Cyp2c70 (Figure 3C). Interestingly, owing to the stronger increase in the relative abundance of hydrophobic BA species in female Cyp2c70−/− mice compared with WT, the hydrophobicity index of biliary BAs did not differ between male and female mice lacking Cyp2c70. Compared with WT, total plasma BA levels were elevated in 12-week-old Cyp2c70−/− mice of both sexes, but the increase was more pronounced in female mice (Figure 3D). Yet, plasma BA levels in the 12-week-old Cyp2c70−/− mice were considerably lower than at 3 weeks of age. In Cyp2c70−/− mice of both sexes, the relative abundances of secondary BAs were reduced compared with WT, whereas Cyp2c70 deficiency did not affect the fraction of unconjugated BAs in the blood (Figure 3E and F).

### Altered Bile Composition and BA Synthesis in Young adult Cyp2c70−/− Mice

Bile formation and biliary lipid secretion is driven by biliary BA secretion. Gallbladder cannulations demonstrated that bile flow was unaffected in Cyp2c70−/− mice, showing that these mice were not cholestatic by definition (Figure 4A). Biliary BA secretion was also not impacted by Cyp2c70 ablation (Figure 4B), although it was higher in female mice compared with male mice. However, in line with the greater potential of the hydrophobic BAs to promote biliary lipid secretion (Figure 4C and D), the ratios of phospholipids and cholesterol to BAs were markedly increased in Cyp2c70−/− mice (Figure 4E and F).

Compared with male mice, female mice excreted more BAs with the feces. Cyp2c70 deficiency tended to reduce fecal BA excretion in female mice but not in male mice (Figure 5A). Because fecal BA loss is compensated by BA synthesis, these data indicate that BA synthesis is higher in female mice compared with male mice and reduced only in female mice upon Cyp2c70 deficiency. Indeed, hepatic Cyp7a1, Cyp8b1, Cyp27a1, and Cyp7b1 were more strongly reduced in female mice than in male mice in absence of Cyp2c70 (Figure 5B and C). Ileal expression of Fgf15, the intestine-derived regulator of hepatic BA synthesis, was not altered in either sex.

To quantify the impact of Cyp2c70 deficiency on BA metabolism in detail, cholic acid (CA) kinetics were studied by isotope dilution after an intravenous bolus of [24,13C]-CA (Figure 5D–I). WT female mice had larger CA and total BA pool sizes compared with WT male mice (Figure 5F and G). In male mice, CA pool size was reduced, whereas total BA pool size was not significantly impacted by Cyp2c70 deficiency (Figure 5F and G). The fractional turnover rate of CA tended to be lower, whereas CA synthesis was reduced by 40% in male Cyp2c70−/− mice (Figure 5H and I). In female Cyp2c70−/− mice, CA pool size was reduced by 70% compared with WT mice, whereas total BA pool size was decreased by 40% (Figure 5F and G). The fractional turnover of CA was strongly decreased in female Cyp2c70−/− mice compared with WT (Figure 5H), and it was synthesized at only ~10% of the rate in age-matched WT female mice (Figure 5I). Collectively, the impact of Cyp2c70 deficiency on BA metabolism was clearly more pronounced in female mice than in male mice.

### Table 1. Characteristics of 12-Week-Old Cyp2c70−/− Mice

| Characteristic                  | Male mice          | Female mice         |
|--------------------------------|--------------------|---------------------|
|                                | WT (n = 12)        | 2c70−/− (n = 12)    | WT (n = 7)         | 2c70−/− (n = 10) |
| Body weight, g                 | 25.2 (24.5–25.6)   | 25.3 (24.4–26.3)    | 21.4 (20.7–23.4)   | 22.1 (21.8–22.4) |
| Food intake, g/d               | 4.4 (4.2–4.5)      | 4.8 (4.3–4.9)       | 4.0 (3.7–4.3)      | 3.7 (3.6–3.9)    |
| Plasma                         |                    |                     |                    |                   |
| Total cholesterol, mmol/L      | 2.4 (2.3–2.5)      | 2.4 (2.2–2.5)       | 1.9 (1.8–2.3)      | 2.3 (2.2–2.5)    |
| Free cholesterol, mmol/L       | 0.73 (0.69–0.75)   | 0.80 (0.74–0.83)    | 0.67 (0.63–0.70)   | 0.66 (0.59–0.78) |
| Cholesterol ester, mmol/L      | 1.6 (1.6–1.8)      | 1.6 (1.5–1.7)       | 1.3 (1.2–1.5)      | 1.6 (1.5–1.8)    |
| Triglycerides, mmol/L          | 0.54 (0.39–0.69)   | 0.48 (0.29–0.72)    | 0.34 (0.29–0.51)   | 0.34 (0.26–0.50) |
| Free fatty acids, mmol/L       | 0.41 (0.36–1.2)    | 0.41 (0.31–0.59)    | 0.67 (0.32–0.81)   | 0.61 (0.44–0.65) |
| Liver                          |                    |                     |                    |                   |
| Total cholesterol, µmol/g      | 5.9 (5.5–6.1)      | 6.6 (6.4–6.8)       | 5.2 (4.7–5.5)      | 5.6 (5.2–6.0)    |
| Free cholesterol, µmol/g       | 4.9 (4.7–5.0)      | 5.3 (5.2–5.7)       | 4.1 (3.8–4.2)      | 4.5 (4.3–4.9)    |
| Cholesterol ester, µmol/g      | 0.95 (0.93–1.08)   | 1.24 (1.18–1.47)    | 1.1 (1.0–1.4)      | 1.0 (0.8–1.3)    |
| Triglycerides, µmol/g          | 18.6 (16.3–20.5)   | 15.9 (12.7–18.5)    | 12.7 (10.2–18.3)   | 6.5 (5.3–7.4)    |
| Phospholipids, µmol/g          | 29 (28–30)         | 29 (27–32)          | 29.6 (27.6–31.3)   | 28.7 (27.2–30.9) |

**NOTE.** Values are median (interquartile range).

*WT, wild-type; 2c70−/−, Cyp2c70−/−.*

*P < .05 vs WT of same gender.

*P < .01 vs WT of same gender using the Mann-Whitney U test.*
Because BA synthesis contributes significantly to whole body cholesterol turnover and BAs are important for fat absorption, plasma and hepatic lipids were analyzed (Table 1). Cyp2c70−/− mice of both genders displayed increased low-density lipoprotein (LDL) cholesterol levels compared with WT mice (Figure 6A and B). This was likely attributable to posttranscriptional downregulation of hepatic LDL receptor (LDLR) (Figure 6C and D), while cholesterol synthesis and intestinal fractional cholesterol absorption were unaffected in Cyp2c70−/− mice despite downregulation of Npc1l1 in the small intestine (Figure 6E-G). Hepatic cholesterol content was slightly

Figure 3. Young adult Cyp2c70-deficient mice possess a hydrophobic BA pool with substantial amounts of CDCA and LCA. Cyp2c70−/− mice and WT littermates were fed a standard rodent diet until the age of 12 weeks. BAs were quantified in (A) bile and (B) plasma. (C) Hydrophobicity index of biliary BAs. (D) Total plasma BA levels. (E) Percent secondary BAs in plasma. (F) Ratio unconjugated to conjugated BAs in plasma. *P < .05 between groups (n = 4–12 mice/group).
higher in Cyp2c70−/− mice compared with WT, whereas triglycerides were lower (Table 1). Hepatic messenger RNA (mRNA) expression levels of genes involved in fatty acid synthesis suggested that de novo lipogenesis (DNL) was decreased in female Cyp2c70−/− mice (Figure 7A). However, direct quantification of DNL by measuring incorporation of [1-13C]-acetate into fatty acid molecules revealed that the lower hepatic lipid content was primarily due to a reduction of old, preexisting fat (Figure 7B).

Mild Portal Fibrosis and Proliferation of Cholangiocytes in Young Adult Cyp2c70−/− Mice

Although young adult Cyp2c70−/− mice were not cholestatic, a distinctive hepatic phenotype was evident. The hydrophobic BA pool was associated with mild ductular reactions in 12-week-old mice (Figure 8A). Some portal fibrosis and proliferation of cholangiocytes was observed. In agreement with these observations, mRNA expression of Col1a1, Col1a2, and Timp1 were increased in Cyp2c70−/− mice (Figure 8B). Liver weights were higher in female Cyp2c70−/− mice, and plasma transaminases were moderately elevated compared with WT littermates in both sexes (Figure 8C and D), indicating the presence of some liver damage in Cyp2c70−/− mice. Intestinal barrier function was clearly impaired in female mice lacking Cyp2c70. This effect was less obvious in male Cyp2c70-deficient mice due to high variability (Figure 8E). Furthermore, spleens were enlarged in Cyp2c70−/− mice (Figure 8F). Immune cells in a group of female mice were analyzed to investigate whether Cyp2c70 deficiency was associated with systemic inflammation. Circulating white blood cells were higher in Cyp2c70-deficient animals compared with control animals (Figure 9A). However, flow cytometry did not reveal altered relative abundances of specific immune cell subsets in blood (Figure 9B). In the liver, non–Kupffer cell macrophages were present in increased numbers in mice lacking Cyp2c70 (Figure 9C). Furthermore, CD8+ T cells appeared to be enriched, while CD4+ T cells tended to be reduced in livers of Cyp2c70−/− mice. Despite impaired intestinal barrier function in female Cyp2c70−/− mice, endotoxin concentrations in portal plasma were below the detection limit (0.15 EU/mL) in these animals.

Cyp2c70 deficiency Is Associated With Marked Changes in Bacterial Colonization of the Gut

Because intestinal microbiota may modulate metabolic and immune functions and BAs can modify microbiome composition,24 bacterial colonization of the cecum was...
Figure 5. The impact of Cyp2c70 deficiency on BA metabolism is more pronounced in female mice. Parameters of BA metabolism were assessed in 12-week-old male and female Cyp2c70<sup>−/−</sup> mice and WT littermates. (A) Fecal BA excretion. Messenger RNA expression in liver and intestine of (B) male and (C) female mice. Plasma enrichment of labeled cholic acid following administration of 400 μg [24-<sup>13</sup>C] cholic acid in (D) male and (E) female mice. (F) Cholic acid pool size. (G) Total BA pool size. (H) CA turnover rate. (I) CA synthesis rate. *P < .05 between groups (panels A–C, n = 6–10 mice/group; panels D–I, n = 5–6 mice/group). APE, atom percent excess; FTR, fractional turnover rate.
Figure 6. Cyp2c70-deficient mice have increased plasma LDL cholesterol levels. Parameters of lipoprotein metabolism were analyzed in 12-week-old Cyp2c70−/− mice and WT littermates. (A) Cholesterol distribution after lipoprotein fractionation of plasma samples by fast protein liquid chromatography (n = 7–12 mice/group). (B) Plasma apolipoprotein B (APOB) protein levels measured using targeted proteomics (n = 4–5 female mice/group). (C) Hepatic Ldlr mRNA expression levels (n = 7–12 mice/group). (D) Hepatic LDLR protein expression in male mice quantified by Western blot (n = 12 mice/group). (E) Fractional cholesterol synthesis determined by mass isotopomer distribution analysis following administration of [1-13C]acetate to drinking water of the mice for 3 days (n = 7–11 mice/group). (F) Npc1l1 mRNA expression in the small intestine of female mice (n = 7–10 mice/group). (G) Fractional cholesterol absorption determined using orally and intravenously administered stable isotopically labeled cholesterol tracers, as detailed in the Materials and Methods section (n = 7–10 mice/group). *P < .05 between groups. HDL, high-density lipoprotein.
analyzed in a cohort of male mice. The more hydrophobic BA composition was associated with distinct alterations in the bacterial species present within the gut of Cyp2c70−/− mice. Principal coordinates analysis clearly separated Cyp2c70−/− from WT mice and hierarchical clustering grouped the mice by genotype (Figure 10A and B). A total of 46 genera showed differential abundance in Cyp2c70−/− mice compared with WT (Figure 11A). A number of bacterial species known to produce short-chain fatty acids, including Roseburia and Butyricicoccus, were more abundant in Cyp2c70−/− mice. Other species, including Akkermansia, Rikenella, and Christensenellaceae, were less abundant in the gut of Cyp2c70−/− mice, whereas Prevotella and Veillonella were more prominent in Cyp2c70−/− mice compared with control mice (Figure 11A and B). Several species belonging to the Ruminococcaceae and Lachnospiraceae, reported to exert BA modifying activity, were also more abundant in Cyp2c70−/− mice (see Figure 11A and B).

Marked Preponderance of Liver Disease in Aged Female Cyp2c70−/− Mice

Because plasma BA, alanine aminotransferase, and aspartate aminotransferase levels in Cyp2c70−/− mice were
Figure 8. Mild ductular reactions in young adult Cyp2c70-deficient mice. Histology and parameters of liver function were assessed in 12-week-old Cyp2c70<sup>−/−</sup> mice and WT littermates. (A) Representative images of liver sections from male and female mice stained with hematoxylin and eosin, Sirius red, or an anti-CK19 antibody (black bars represent 200 μm, arrowheads indicate collagen deposition). (B) Hepatic mRNA expression of genes involved in fibrosis and inflammation. (C) Liver weights. (D) Plasma transaminase levels. (E) Plasma fluorescence 45 minutes after administration of FITC-Dextran. (F) Spleen weights. *P < .05 between groups (panels A–D and F, n = 7–10 mice/group; panel E, n = 5–7 mice/group).
lower at 12 weeks compared with 3 weeks of age, yet still elevated compared with WT animals, we next questioned what would happen to these parameters beyond the young adult state. Therefore, cohorts of mice were aged until 32–34 weeks. Body weights of WT and Cyp2c70−/− mice were similar in both genders (Figure 12A). However, remarkable differences between sexes became apparent at this advanced age. Male Cyp2c70−/− mice showed a normal liver and spleen size and only mildly elevated plasma transaminases, and plasma BA levels comparable to those in WT mice (Figure 12B–F). Conversely, female Cyp2c70−/− mice had enlarged livers and spleens as well as substantially higher plasma transaminases (Figure 12B–D). Plasma BAs in female Cyp2c70−/− mice were increased compared with WT control mice and considerably higher than in male Cyp2c70−/− mice (Figure 12E and F). Plasma of male Cyp2c70−/− mice contained ~2.5 μmol/L (T)CDCA. However, plasma concentrations of this potent endogenous FXR agonist reached ~100 μmol/L in female Cyp2c70−/− mice, while high levels of (T)LCA were present as well. Yet, increased hepatic FXR signaling was not evident, as expression of Fxr and its target genes Shp and Bsep was not increased in either of the genders (Figure 13A). Expression of Cyp8b1 was reduced by ~40% in male Cyp2c70−/− mice compared with WT control mice, but was almost completely absent in female mice lacking Cyp2c70 (Figure 13A), indicating very low CA synthesis in these animals. The expression of genes involved in fibrogenesis showed striking differences between male and female Cyp2c70−/− mice. In old male Cyp2c70−/− mice, Col1a1, Col1a2, and Timp1 were only moderately higher than in age- and sex-matched control mice, whereas expression of these genes was increased to a much greater extent in female mice upon Cyp2c70 deficiency (Figure 13B). Expression of the cholangiocyte marker Krt19, encoding CK19, showed a similar pattern (Figure 13B). In line with the mRNA data, livers of male Cyp2c70−/− mice showed near normal liver histology, whereas livers of female Cyp2c70-deficient mice displayed bridging fibrosis and had strongly increased numbers of CK19-positive cells (Figure 13C). Inflammation markers were mildly increased in both genders in absence of Cyp2c70 (Figure 13B). Together, these results clearly demonstrate that the development of liver disease in Cyp2c70−/− mice is sex-dependent, with female mice being substantially more affected than male mice.

**Reversal of Liver Disease Associated With Cyp2c70 Deficiency by UDCA**

Liver disease and compromised intestinal barrier function in Cyp2c70−/− mice may be related to the more hydrophobic composition of the circulating BA pool, but could also result from other, yet unknown, functions of CYP2C70. Therefore, we added 0.1% UDCA to the diet of a cohort of 5-
week-old female Cyp2c70−/− mice until they reached the age of 12 weeks. UDCA accumulated in the BA pool of the treated mice and accounted for ~60% of circulating BAs at the end of treatment in WT and Cyp2c70−/− mice (Figure 14A). Consequently, the BA pool in the UDCA-treated Cyp2c70−/− mice became substantially more hydrophilic (Figure 14B). Body weights were not impacted by UDCA treatment (data not shown). Liver sizes of UDCA-treated Cyp2c70−/− mice were similar to those of WT animals (Figure 14C), and hepatocyte damage was reversed, as evidenced by the complete normalization of plasma transaminases and liver histology (Figure 14D and E). Ductular reactions and portal fibrosis were no longer observed when Cyp2c70−/− mice were treated with UDCA (Figure 14E) and expression levels of genes associated with fibrogenesis and inflammation were normalized (Figure 14F). In addition, CK19-positive cells in the liver were reduced to normal numbers upon treatment (Figure 14E, lower panels), indicating that UDCA corrected cholangiocyte proliferation in Cyp2c70−/− mice. To obtain more insight into the changes in the processes underlying the liver pathology in Cyp2c70−/− mice and its reversal by UDCA, transcriptome analysis was performed by RNA microarray. Principal component analysis separated the WT mice from the nontreated Cyp2c70−/− mice, whereas UDCA-treated Cyp2c70−/− mice essentially overlapped with WT mice, indicating that little residual differences in hepatic gene expression were left in UDCA-treated Cyp2c70−/− mice (Figure 15A). Hierarchical clustering, based on genes that were differentially expressed between groups (false discovery rate [FDR] < 5% and a fold-change ≥ 1.5), clearly separated the nontreated Cyp2c70−/− mice from the other groups (Figure 15B). Interestingly, UDCA treatment essentially normalized gene expression patterns in Cyp2c70−/− mice. Gene set enrichment analysis revealed that pathways involved in extracellular matrix (re)organization were most upregulated in nontreated Cyp2c70−/− mice compared with WT control mice, whereas peroxisomal pathways and fatty acid metabolism were among the most downregulated pathways (Figure 16A). When Cyp2c70−/− mice were treated with UDCA, pathways involved in extracellular matrix (re)organization were no longer among the most altered pathways compared with WT mice (data not shown) and strongly downregulated compared with untreated Cyp2c70−/− mice (Figure 16B). UDCA functions as a chaperone and can relieve endoplasmic reticulum (ER) stress.26 Therefore, we explored whether

Figure 10. Altered BA composition in Cyp2c70-deficient mice impacts bacterial colonization of the gut. Bacterial DNA was isolated from cecal contents of 12-week-old male mice and analyzed by 16S sequencing. (A) To compare the overall microbial communities between mice, the unweighted UniFrac distance was calculated based on the taxonomic tree at the genus level using the Phyloseq package for R, and principal coordinates analysis was performed. (B) Hierarchical clustering of the mice based on the unweighted UniFrac distance of bacteria present in the cecum of male Cyp2c70−/− mice and WT littermates.
Amelioration of ER stress was involved in the restoration of normal liver physiology in Cyp2c70−/− mice by UDCA. Female Cyp2c70−/− mice indeed displayed signs of increased ER stress. Hepatic mRNA expression of Ddit3 (Chop) showed a tendency toward an increase, and expressions of Grp78 (Bip) and Dnajc3 (Hsp40) were significantly increased in the Cyp2c70−/− mice compared with WT animals fed a control diet (Figure 17A). Hepatic protein levels of binding immunoglobulin protein (BIP) were also higher in the Cyp2c70−/− mice compared with control mice (Figure 17B and C). UDCA treatment rescued the increased expression of these ER stress markers in Cyp2c70−/− mice (Figure 17A–C), suggesting that reduction of ER stress is involved in the reversal of liver pathology in Cyp2c70−/− mice. In addition to the normalization of liver physiology by UDCA, spleen size and white blood cell counts returned to normal upon...
treatment (Figure 18A and B). The impaired intestinal barrier function observed in Cyp2c70–/– mice was corrected by UDCA treatment as well (Figure 18C). Taken together, these results indicate that UDCA treatment effectively restores liver and intestinal dysfunction in Cyp2c70–/– mice.

**Discussion**

In the present study, we assessed the (patho)physiological consequences of Cyp2c70-deletion in mice in an age- and gender-dependent manner. We show that absence of CYP2C70 leads to a hydrophobic BA pool containing substantial amounts of CDCA and LCA throughout development. Cyp2c70–/– mice of both sexes display features of transient "neonatal" cholestasis. Interestingly, the phenotype spontaneously improves into adulthood in male Cyp2c70–/– mice, while female Cyp2c70–/– mice develop a clear cholangiopathy that progresses to bridging fibrosis at advanced age. The pathologic features in female Cyp2c70–/– mice could, however, be fully reversed by treatment with UDCA.

The differences in BA composition between humans and mice result in marked disparity in physicochemical...
This obviously complicates translation of preclinical observations, eg, data concerning effects of pharmacological FXR modulation, to the human situation. “Humanization” of BA metabolism in mice by knocking out \textit{Cyp2c70} can facilitate translation of murine data while preserving the benefits of the mouse as a preclinical model, such as the possibilities for selective genetic modifications. Hence, \textit{Cyp2c70}\textsuperscript{−/−} mice represent an
Figure 14. UDCA reverses cholangiopathy in female Cyp2c70–/– deficient mice. Female Cyp2c70–/– mice and WT littermates were fed a diet with 0.1% UDCA or a control diet from 5 to 12 weeks of age. BA composition was measured and parameters related to liver function were assessed at the age of 12 weeks. (A) BA profiles in plasma. (B) Hydrophobicity index of biliary BAs. (C) Liver weights. (D) Plasma transaminases. (E) Representative images of liver sections stained with hematoxylin and eosin, Sirius red, or an anti-CK19 antibody (black bars represent 200 μm, arrow heads indicate collagen deposition). (F) Hepatic mRNA expression levels of genes involved in fibrogenesis and inflammation. *P < .05 between groups (n = 5–6 mice/group). CTRL, control.
Figure 15. UDCA normalizes hepatic gene expression patterns in female Cyp2c70-deficient mice. (A) Principal component analysis of hepatic mRNA microarray data obtained from 12-week-old female Cyp2c70^{-/-} mice and WT littermates treated with and without 0.1% UDCA in their diet from 5 to 12 weeks of age. (B) Hierarchical clustering of mice based on genes that were differentially expressed between groups using an FDR cutoff of 5% and a fold-change $>1.5$ ($n = 5-6$ mice/group).

interesting model to study BA-related liver diseases and could also be employed to explore the impact of BAs and pharmacological manipulations of BA signaling pathways on metabolism. Not surprisingly, therefore, several research groups have recently generated mice that specifically lack Cyp2c70.

The BA pool of Cyp2c70^{-/-} mice contained considerable amounts of hydrophobic CDCA and LCA and therefore had a much higher cytotoxic potential than the BA pool in WT mice. Oval cell proliferation has indeed been reported in livers of Cyp2c cluster null mice and Cyp2c70^{-/-} mice, but the (patho)physiological consequences of the more hydrophobic BA pool in Cyp2c70^{-/-} mice have remained ill defined. In the current study, we demonstrate that Cyp2c70^{-/-} mice of both sexes display transient "neonatal" cholestasis. Intriguingly, marked differences between genders became apparent when age-dependent consequences of Cyp2c70 deficiency were assessed. Female, but not male, Cyp2c70^{-/-} mice displayed progression of liver disease and bridging fibrosis at more advanced ages. Furthermore, female Cyp2c70^{-/-} mice had a markedly reduced total BA pool size compared with WT control mice, whereas the pool size was not impacted by Cyp2c70-deletion in male mice. Hence, the overall impact of Cyp2c70-deletion was more pronounced in female mice compared with male mice in our studies. Honda et al recently reported ductular reactions in male Cyp2c70^{-/-} mice, whereas female mice showed a more variable phenotype. In contrast to our study, these authors only studied mice at young adult age and did not assess fibrosis development. The reason for the more pronounced phenotype in adult female Cyp2c70^{-/-} mice in our studies remains elusive, but the minimal phenotypic differences between male and female Cyp2c70^{-/-} mice at 3 weeks of age suggest involvement of sex hormones in the development of liver pathology in female Cyp2c70^{-/-} mice at more advanced ages. Interestingly, estrogen was shown to repress the expression of Cyp8b1 in bile-diverted rats. Indeed, the ratio of 12a-hydroxylated to non-12a-hydroxylated BAs was lower in Cyp2c70-deficient female mice compared with male mice. However, the differences in 12a-hydroxylation between male mice and female mice only appeared upon Cyp2c70-ablation (Figure 1B) despite similar hepatic expression levels of Cyp2c70 in both sexes in C57BL/6 mice.

The BA pool in Cyp2c70^{-/-} mice clearly comprises more potent FXR agonists than the pool of WT mice. Yet, in line with our previous findings as well as with data reported by others, this did not translate into evidently increased hepatic FXR activation. In the ileum, we did observe increased expression of multiple FXR target genes in Cyp2c70^{-/-} mice, but expression of Fgf15, important in control of hepatic BA synthesis, was hardly affected. Nevertheless, mRNA expression levels of key enzymes in the BA synthesis pathways were clearly reduced in Cyp2c70^{-/-} mice. Taken together, the impact of the more human-like BA composition on the regulation of BA synthesis appears to be complex and requires more investigation.

In apparent contrast to the dogma that hydrophobic BAs facilitate intestinal lipid absorption more efficiently than hydrophilic ones, we did not observe increased fractional cholesterol absorption in Cyp2c70^{-/-} mice compared with WT control mice. Decreased expression of the cholesterol uptake transporter Npc1l1 in the proximal small intestine...
may have counteracted the effects of the hydrophobic BA pool in Cyp2c70−/− mice. Others recently reported reduced levels of plant sterols, which are used as surrogate markers of cholesterol absorption, in plasma of Cyp2c70−/− mice compared with control mice, suggesting decreased cholesterol absorption. This was, however, not apparent from our direct measurements of fractional cholesterol absorption.

BAs are known to impact the gut microbiome. Because the bactericidal properties differ between BA species, we
hypothesized that the altered composition of the BA pool in Cyp2c70−/− would affect the microbial community in the gut. Indeed, differences in bacterial colonization of the intestinal tract were observed between Cyp2c70−/− mice and their WT littermates. Genera of Akkermansia, Rikenella, and Christensenellaceae were less abundant, while Prevotella and Veillonella were more abundant in Cyp2c70−/− mice compared with control mice. Interestingly, the abundances of these genera were recently reported to be changed in similar directions in the gut of PBC patients compared with healthy control mice.33 The bacterial signature in Cyp2c70−/− mice thus appears to show certain similarities with the alterations in gut colonization observed in PBC patients.33 Akkermansia muciniphila contributes to maintenance of barrier integrity in the gut.34 Although 16S DNA sequencing does not allow quantification of individual bacterial species, it is tempting to speculate that the strong reduction of the abundance of species belonging to the genera Akkermansia may have contributed to the increased intestinal permeability observed in Cyp2c70−/− mice.

Although the physicochemical properties of the BA pool in Cyp2c70−/− mice are considerably more similar to humans than those of the pool in WT mice, certain differences in BA metabolism between Cyp2c70−/− mice and humans do remain. Cyp2c70−/− mice are able to rehydroxylate DCA and therefore still have a slightly more hydrophilic BA pool than humans. While this manuscript was in preparation, CYP2A12 was identified as the enzyme responsible for the conversion of DCA into CA in mouse livers by 7α-rehydroxylation.6 Another difference between Cyp2c70−/− mice and humans concerns the conjugation of BAs in the liver. Although BAs are predominantly conjugated to taurine in human neonates,35 glycine conjugation predominates in humans >1 year old. Glycine-conjugated BAs are only slightly more hydrophobic than their taurine-conjugated counterparts.10 Despite these differences, the hydrophobicity index of biliary BAs in Cyp2c70−/− mice (∼+0.2) is more comparable to that of humans (∼+0.3) than to that of WT mice (∼−0.3). The physicochemical characteristics of the BA pool in Cyp2c70−/− mice thus closely resemble those of the human BA pool. Cyp2c70−/− mice therefore represent a valuable model to study BA-related liver diseases and metabolic actions of BAs in vivo. The cholangiopathy that is observed in female mice upon “humanization” of the BA pool obviously represents a point of concern for their application in metabolism-oriented preclinical studies.19 Interestingly, these phenotypic distortions could be fully restored by adding 0.1% UDCA to the diet. Thus, our data indicate that altered composition of the BA pool indeed caused the cholangiopathy rather than other, yet unknown, functions of CYP2C70. Consequently, it would be interesting to investigate to what extend variations in BA composition between human subjects affect their risk of developing liver disease (eg, whether people with a high relative abundance of hydrophobic CDCA are at increased risk). The mechanism by which UDCA restores liver physiology in Cyp2c70−/− mice might involve reduction of ER stress, as UDCA is known to ameliorate ER stress by acting as a chemical chaperone.26 Indeed, UDCA treatment alleviated the signs of ER stress in the livers of female Cyp2c70−/− mice. It is likely that elevated LDL cholesterol levels in Cyp2c70−/− mice are related to an ER stress-induced decrease of LDLR protein levels (eg, due to increased occupancy of the chaperone GRP94 by misfolded proteins), resulting in a reduction of its inhibitory interaction with intracellular PCSK9 and, hence, in reduced protection of LDLR from PCSK9-induced degradation.36 In line with this hypothesis, LDL cholesterol was reduced to normal levels upon UDCA treatment. UDCA was provided from 5 to 12 weeks of age in our experiments. It would be interesting to study whether UDCA would also be able to fully prevent the aging-associated liver pathology in female Cyp2c70−/− mice by extending treatment beyond 12 weeks of age.

Taken together, chow-fed Cyp2c70−/− mice develop liver pathology in a sex-specific manner. Multiple characteristics of the development of the hepatic phenotype in Cyp2c70−/− mice are similar to those in humans with PBC, including female preponderance of liver disease and beneficial effects.
of UDCA on liver function. Cyp2c70–/– mice may thus serve as a model for PBC and be used to study other BA-related liver pathologies such as progressive familiar intrahepatic cholestasis. For instance, crossbreeding of Cyp2c70–/– mice with mice lacking the bile salt export pump (BSEP) could be envisioned to serve as a preclinical model for progressive familiar intrahepatic cholestasis. Besides, modulation of CYP8B1 activity in Cyp2c70–/– mice could be employed to assess the impact of BA pool composition on insulin resistance and energy homeostasis. Furthermore, Cyp2c70–/– mice are anticipated to be very instrumental for the study of the versatile interactions between BAs, the gut microbiome and host metabolism. Such future studies may contribute to delineation of the underlying reasons for the observed marked differences between male and female Cyp2c70–/– mice. Elucidation of these mechanisms fell beyond the scope of the current study, but clarification of the interactions between sex hormones and BA metabolism as well as of potential sex differences in the interactions between BAs and the intestinal microbiome in these mice will be subject of our future studies.

Materials and Methods

Animals

Cyp2c70 knockout mice were generated using CRISPR/Cas9-technology. Zygotes were isolated from a female C57BL/6J mouse 1 day after fertilization and injected with a mRNA encoding the Cas9 endonuclease as well as a sgRNA (5’-CCCACCTCTTATCAATTGT-3’) directed against the Cyp2c70 gene. The zygotes were then transplanted into the infundibulum of a pseudo pregnant B6CBF1/J mouse. The targeted region of the Cyp2c70 gene was sequenced in the offspring and mosaic mice were crossed with WT C57BL/6J mice to obtain heterozygous founders.

Animals were housed under climate-controlled conditions (21°C) with a 12-hour light/dark day/night-cycle. Animals had ad libitum access to a standard rodent diet (RM-1; Special Diet Services, Essex, United Kingdom) during the experiments. When indicated, 0.1% UDCA (w/w) (Sigma-Aldrich, St Louis, MO) was mixed into the food. Bile cannulations were performed in dedicated cohorts of 12-week-old mice. These mice were anesthetized by intraperitoneal injection of Hypnorn (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg) prior to ligation of the common bile duct and cannulation of the gallbladder. Directly after cannulation, mice were placed in a humidified incubator to maintain body temperature. Bile that was secreted during the first 5 minutes was discarded to prevent collection of, more concentrated, gallbladder bile. Next, hepatic bile was collected continuously for 30 minutes. Bile production was determined gravimetrically, whereas concentrations of BAs, phospholipids and cholesterol in the bile were determined as described subsequently. Animal experiments were performed in accordance with the Dutch law and were approved by the Dutch Central Committee for Animal Experiments and the Animal Welfare Body of the University of Groningen.

Targeted Proteomics

Protein levels of CYP2C70 and APOB were quantified by targeted proteomics using an isotopically labeled peptide (TDSSLISR; Thermo Fisher Scientific, Rockford, IL) for CYP2C70 and a labeled concatamer-derived peptide (QSFDSLVK; PolyQuant GmbH, Bad Abbach, Germany) for apolipoprotein B as standards.

Western Blot

Total protein was isolated from liver homogenates (10%, w/w) and quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein (~20 μg) were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for 1 hour and subsequently incubated with anti-LDLR (PAB8804, 1:1000; Abnova, Taipei, Taiwan), anti-BIP (3177S, 1:1000; Cell Signaling Technology, Danvers, MA), anti-tubulin (2144S, 1:1000; Cell Signaling Technology) or anti-GAPDH (1:2000, CB1001; Millipore, Burlington, MA) overnight at 4°C. Horseradish peroxidase–conjugated secondary antibodies were then added to detect the proteins of interest. Antibody binding was visualized using SuperSignal West Femto substrate (Thermo Fisher Scientific, Waltham, MA), and signal quantification was carried out using the freely available ImageJ software (v1.53a, National Institutes of Health, Bethesda, MD).

Plasma Parameters

Plasma triglycerides, free fatty acids, total cholesterol, and free cholesterol were measured using commercially available kits (DiaSys Diagnostic Systems, Holzheim, Germany; Roche Diagnostics, Basel, Switzerland). Plasma lipoproteins were separated by fast protein liquid chromatography using a system containing a PU-4180 pump with a linear degasser and UV-4075 UV/VIS detectors (Jasco, Tokyo, Japan), as described. Plasma (25 μL) was diluted with phosphate-buffered saline (PBS) (pH 7.4) in a 1:1 ratio before being loaded onto the column (Superose 6 Increase 10/300 GL; GE Healthcare, Hoevelaken, the Netherlands). Lipoproteins were then separated using PBS (pH 7.4, flow rate of 0.31 mL/min) as eluent. Total cholesterol concentrations were quantified using a colorimetric reagent (11489232; Roche Diagnostics) that was added in line at a rate of 0.1 mL/min using an additional PU-4080i infusion pump (Jasco). Data acquisition and analysis were performed using ChromNav software (version 1.0; Jasco). Plasma transaminases were analyzed using a Cobas 6000 analyzer with standard reagents (Roche Diagnostics). Endotoxin was measured using Endosafe limulus amoebocyte lysate cartridges for the NeXgen-PTS (Charles River, Leiden, the Netherlands) after a dilution of 30× in limulus amoebocyte lysate water.

Measurement of BAs, Neutral Sterols, and Biliary Phospholipids

BAs in plasma and bile were measured by ultra high-performance liquid chromatography tandem mass
spectrumometry,5 while fecal BAs were quantified by gas-liquid chromatography.5 Biliary cholesterol concentrations were determined by gas-liquid chromatography as described elsewhere.37 Biliary phospholipids were determined as described.39

Real-Time Quantitative Polymerase Chain Reaction
Real-time quantitative polymerase chain reaction was performed on reverse transcribed RNA using either TaqMan primer-probe combinations (Applied Biosystems, Foster City, CA) or SYBR green mastermix (Roche Diagnostics). Data were normalized to cyclophilin as a housekeeping gene.

Microarray Analysis
Transcriptome analysis of livers obtained from female Cyp2c70−/− mice and WT littermates, fed a diet with or without 0.1% UDCA from 5 to 12 weeks of age, was performed using Mouse Gene 2.0ST arrays (Affymetrix; Thermo Fisher Scientific) as described.40 The MADMAX pipeline was used for processing microarray data. After normalization, differentially expressed genes were extracted using IBMT statistics with the FDR cutoff set at 5%. Gene set enrichment analyses were performed using the C2 canonical KEGG pathways. A heat map of differentially expressed genes was generated following processing of array data with Bioconductor and normalization of gene expression using robust multichip averaging.45 Differential gene expression between groups was assessed using limma with the FDR set at 5% (and a fold-change cutoff of 1.5). Hierarchical clustering of differentially expressed genes was carried out using the hopach package with the cosine distance metric. The raw data were deposited in the GEO database (GSE138779).

Histology
Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin, picrosirius red or cytokeratin-19 (CK19, ab52625; Abcam, Cambridge, United Kingdom) according to standard protocols. Images were obtained using a Hamamatsu NanoZoomer (Hamamatsu Photonics, Almere, the Netherlands).

Flow Cytometry
EDTA blood samples from mice were collected and after red blood cell lysis incubated with Fc Block and labeled with conjugated antibodies. Immune cells were isolated from mouse liver by digestion for 30 minutes at 37°C with collagenase D, and subsequent centrifugation with 35% Percoll. Cells were treated with Zombie UV to discriminate live and dead cells. Cells from liver and blood were incubated with Fc Block and labeled with conjugated antibodies: TCRβ (BUV395), CD4 (BUV737), CD8α (BV510), CD3 (BV785), CD11b (FITC), F4/80 (PE-CY5), B220 (PE), CD45 (PE-Texas Red), CD19 (APC), TCRγδ (APC-CY7). For myeloid cell staining, an additional panel was used with the following antibodies: CD45.2 (BUV737), B220 (BV421), CD11b (BV605), Ly6C (BV785), CD115 (FITC), CLEC4F (PE-CY7), F4/80 (PE-CY5), CCR2 (PE), CD19, CD3, NK1.1 (PE-Texas Red), MHCI (AF700), CD11c (APC-CY7). The Abcam Antibody Coupling Kit (ab102903) was used to couple the CLEC4F antibody to PE-CY7. Flow cytometry analysis was performed on a BD LSR Fortessa X-20 (Becton Dickinson, Franklin Lakes, New Jersey). Results were acquired with the Diva software (v7.0, Becton Dickinson) and analyzed using FlowJo software (v10.6.1, Tree Star, Ashland, OR).

Microbiota Analysis
Composition of the microbiota was analyzed by sequencing of 16S ribosomal DNA, isolated from cecal contents of male mice sacrificed at the age of 12 weeks, on an Illumina HiSeq platform (Novogene, Hong Kong, China), essentially as described.49 Operational taxonomic units abundance (97% similarity) information was normalized using a standard number of sequences corresponding to the sample with the least reads (<60,000). Subsequent analyses were performed based the normalized data. Taxonomic differences between the groups were determined at the genus level after removal of unidentified and very low-abundant genera, leaving 115 genera for analysis. To compare the overall microbial communities between mice, the unweighted UniFrac distance was calculated based on the taxonomic tree at the genus level using the Phyloseq package for R software (v3.6.1, R Foundation for Statistical Computing, Vienna, Austria) and visualized using principal coordinates analysis. Significance of differences between groups was assessed by the nonparametric Wilcoxon test. The P value was adjusted for multiple comparisons using the Benjamini-Hochberg procedure with the FDR set at 5%.

Measurement of Metabolic Fluxes Using Stable Isotopes
Fractional cholesterol absorption was measured following intravenous administration of cholesterol-D3 and oral administration of cholesteryl-D3.53 DNL and cholesterol synthesis rates were measured following addition of [1-13C]acetate to the drinking water of the mice and calculated as described.52 CA kinetics were determined following intravenous administration of 400 μg [24-13C]-CA, essentially as described previously.53 Total BA pool size was calculated by dividing CA pool size by the fractional abundance of CA in the total BA pool.

Hepatic Lipid Measurements
Livers were homogenized (15%, w/w) in PBS and lipids were extracted according to Bligh and Dyer.54 Cholesterol and triglycerides were subsequently measured using commercially available reagents (DiaSys Diagnostic Systems and Roche Diagnostics), whereas phospholipids were quantified as described.59
Statistics

Data in graphs are presented as bar graphs with SEM, Tukey box-and-whisker plots or line graphs with median and interquartile range. Statistical analyses between 2 groups were performed by Mann-Whitney U nonparametric comparisons (GraphPad Software, San Diego, CA), whereas the Kruskal-Wallis H test followed by Conover post hoc analysis (Brightstat) was used for multiple group comparisons. Differences were considered statistically significant when P values were <.05.

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Conflicts of Interest
The authors disclose no conflicts.

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