ORIGINAL RESEARCH

Sex Dimorphic Effects of Bile Acid Metabolism in Liver Cancer in Mice

Rachel M. Golonka,[1] Beng San Yeoh,[1] Piu Saha,[1] Yuan Tian,[2] John Y. L. Chiang,[3] Andrew D. Patterson,[2] Andrew T. Gewirtz,[4] Bina Joe,[1] and Matam Vijay-Kumar[1]

[1]UT Microbiome Consortium, Department of Physiology and Pharmacology, University of Toledo College of Medicine and Life Sciences, Toledo, Ohio; [2]Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, Pennsylvania; [3]Department of Integrative Medical Sciences, Northeast Ohio Medical University, Rootstown, Ohio; and [4]Center for Inflammation, Immunity & Infection, Institute for Biomedical Sciences, Georgia State University, Atlanta, Georgia

SUMMARY

Our study found bile acids to be sex-dimorphic metabolites in a HCC mouse model. We suggest bile acids as potential therapeutic targets and biomarkers for HCC cases. This study supports for advancements in sex-personalized therapies to treat liver cancer.

BACKGROUND & AIMS: Hepatocellular carcinoma (HCC) is a male-dominant disease, but targeted sex hormone therapies have not been successful. Bile acids are a potential liver carcinogen and are biomolecules with hormone-like effects. A few studies highlight their potential sex dimorphism in physiology and disease. We hypothesized that bile acids could be a potential molecular signature that explains sex disparity in HCC.

METHODS & RESULTS: We used the farnesoid X receptor knockout (FxrKO) mouse model to study bile acid-dependent HCC. Temporal tracking of circulating bile acids determined more than 80% of FxrKO females developed spontaneous cholemia (ie, serum total bile acids $\geq 40\ \mu$mol/L) as early as 8 weeks old. Opposingly, FxrKO males were highly resistant to cholemia, with ∼23% incidence even when 26 weeks old. However, FxrKO males demonstrated higher levels of deoxycholate than females. Compared with males, FxrKO females had more severe cholestatic liver injury and further aberrancies in bile acid metabolism. Yet, FxrKO females expressed more detoxification transcripts and had greater renal excretion of bile acids. Intervention with CYP7A1 (rate limiting enzyme for bile acid biosynthesis) deficiency or taurine supplementation either completely or partially normalized bile acid levels and liver injury in FxrKO females. Despite higher cholemia prevalence in FxrKO females, their tumor burden was less compared with FxrKO males. An exception to this sex-dimorphic pattern was found in a subset of male and female FxrKO mice born with congenital cholemia due to portosystemic shunt, where both sexes had comparable robust HCC.

CONCLUSIONS: Our study highlights bile acids as sex-dimorphic metabolites in HCC except in the case of portosystemic shunt. *(Cell Mol Gastroenterol Hepatol 2024;17:719–735;* https://doi.org/10.1016/j.jcmgh.2024.01.011*)*

Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer, and recent statistics estimate it to be the third leading cause of cancer mortality worldwide.[1] A sequence of liver injury, chronic inflammation, fibrosis, and cirrhosis normally precedes HCC. However, the mechanism(s) behind HCC pathogenesis is complex and varies depending on its risk factor(s). For instance, it is well-known that aflatoxins drive liver carcinogenesis by the formation of mutagenic DNA adducts and induction of oxidative stress.[2,3] Moreover, hepatitis B virus (HBV)-mediated HCC is determined by the viral regulatory protein HBx, which disrupts the p53-dependent tumor suppressive pathway and promotes telomerase expression in cancer cells.[4] Unlike the aforementioned risk factors, another HCC determinant is sex disparity, but the driving mechanism(s) is not well-established.

Epidemiologic surveillance with sex-specific rates demonstrates a male predominance in HCC. For every 1 female patient, there is an average ratio of 2–4 male patients with HCC, and mortality rates are at least 2-fold greater in the male population.[1] Previous research has investigated chromatin-modifying proto-oncogenes and sex hormones as possible mechanisms behind sex disparity in HCC. Several Y-

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linked proto-oncogenes, including testis specific protein Y-encoded and RNA-binding motif gene on Y chromosome, have been identified as positive HCC biomarkers.[5,6] Alongside, the X-chromosome gene TSPY homologue X, known to promote proteasomal degradation of the HBx oncoprotein, was found to be significantly down-regulated in HCC tumors.[7] These findings have been positively pursued for diagnostic and prognostic applications in HCC cases. Investigation with targetable sex hormones and their receptors has suggested male-dominant androgens to be pathologic by accelerating cell proliferation, whereas female-dominant estrogens protected from HCC by inducing cell apoptosis and anti-tumor immune responses.[8–10] However, high variability of estrogen receptor expression and failed survival benefits of anti-androgen agents have questioned hormonal therapy for HCC.[11,12] Accordingly, the objective of this study was to identify molecular signature(s) that could explain sex disparity and be a relevant therapeutic target in HCC.

The liver is responsible for the detoxification of xenobiotics/endobiotics via redox and conjugation reactions that make the biotic less toxic and water soluble for excretion.[13] Bile acids are one such metabolite that undergo hydroxylation, glucuronidation, and sulfation reactions to prevent their accumulation toward toxic liver injury levels.[14,15] Bile acids are detergent-like metabolites produced in the liver that aid in the assimilation of dietary lipids. Sex differences in bile acid metabolism are sparsely noted, but a few reports conducted in hamsters and rats suggest that females have increased expression for enzymes in bile acid detoxification at physiological conditions.[16,17] Bile acid levels are also regulated by the farnesoid X receptor (FXR), a major bile acid sensor, where its activation leads to tissue-specific suppression of bile acid biosynthesis.[18] In cases where bile acid metabolism is dysregulated, hepatic diseases such as cholestasis and HCC can result from the pro-inflammatory effects of bile acids.[19–23] A well-established example is reported with FXR knockout (FxrKO) mice that develop cholemia (ie, elevated bile acids) and spontaneous HCC at 16 months of age.[24,25] Interestingly, the majority of reports with FxrKO mice have focused only on males,[26–30] probably because of the convention that liver pathologies, such as HCC, are outwardly more severe in males than females.

We generated the hypothesis that sex differences in bile acid metabolism could drive sex disparity in HCC. Using FxrKO mice as a bile acid-dependent HCC model, we found females to have a higher prevalence of spontaneous cholemia (serum total bile acids ≥40 μmol/L) at both precancerous and cancerous time points. Comparatively, the male counterparts were more resistant to spontaneous cholemia irrespective of age. Despite the toxic accumulation of bile acids in FxrKO females, these mice had lower tumor burden than FxrKO males, suggesting an inverse relationship between spontaneous cholemia and HCC severity. This pattern might be attributed to the increased expression of bile acid detoxification transcripts and renal excretion of bile acids in FxrKO females. Noteworthy, we stumbled on an exception to the sex dimorphism paradigm where a subset of male and female FxrKO mice had congenital cholemia due to a developmental vascular defect called portosystemic shunt (PSS). Both male and female FxrKO mice with congenital cholemia exhibited equally robust HCC, negating the sex dimorphism effects we originally observed. Overall, our study highlights bile acids as targetable, sex-dimorphic metabolites with therapeutic potential in HCC.

Results

Cholemia and Cholestatic Liver Injury Are Sexually Dimorphic in FxrKO Mice

FXR is a nuclear receptor predominantly localized in the liver and ileum. On activation, FXR inhibits bile acid biosynthesis in a tissue-specific manner.[18] Hepatic FXR induces the expression of small heterodimer partner (SHP), which inhibits hepatocyte nuclear factor 4α (HNF4α); HNF4α normally positively regulates 2 bile acid biosynthetic genes, cholesterol 7α-hydroxylase (Cyp7a1) and Cyp8b1. In the ileum, FXR induces fibroblast growth factor 15 (FGF15), and FGF15 promotes hepatic FGFR4-ERK1/2 signaling that suppresses Cyp7a1 and Cyp8b1 expression. FxrKO mice have high bile acids and hypercholesterolemia at an early age, and they develop HCC later in life.[24,25,31] Reports with the FxrKO mouse model have mostly focused on males because of the convention that liver pathologies such as HCC are outwardly more severe in males than females. In this study, we investigated for potential sex-specific alterations in bile acid metabolism that could explain HCC disparity in males vs females.

Our first objective was to determine a possible sex disparity in bile acid levels at the precancerous age in FxrKO mice. As an initial reference, normal levels of serum total bile acids (TBA) in wild-type (WT) mice are between 1–5 μmol/L. Temporal tracking depicted surprisingly higher serum TBA levels in FxrKO females than males (mean TBA, females, 23.5 μmol/L vs males, 11.6 μmol/L) when 6 weeks of age (Figure 1A). TBA levels continued to rapidly increase in FxrKO females as they aged, whereas males were resistant to spontaneous increases in TBA levels (Figure 1A). When aged to 26 weeks (ie, 6 months old), 12 of 14 female FxrKO mice reached cholemia levels (TBA ≥40 μmol/L), but only 4 of 17 male FxrKO mice surpassed the cholemia threshold, which gave this cohort an approximate cholemia incidence of 85% in females and 23% in males (Figure 1A and B). The cholemia benchmark was determined on the basis of reports with intrahepatic cholestasis of pregnancy that fetal complications occurred when TBA levels were ≥40 μmol/L.[32] Serum metabolomic analysis suggested some variation in individual bile acids between male and female FxrKO mice (Figure 2A–D). One notable difference was FxrKO males had a significant increase in deoxycholic acid compared with their female counterparts (P = .0024). As expected, both sexes of FxrKO mice had hypercholesterolemia when compared with WT mice; however, FxrKO females had lower serum total cholesterol than their male counterparts (Figure 1C). These results demonstrate spontaneous cholemia is most prevalent in female FxrKO mice.

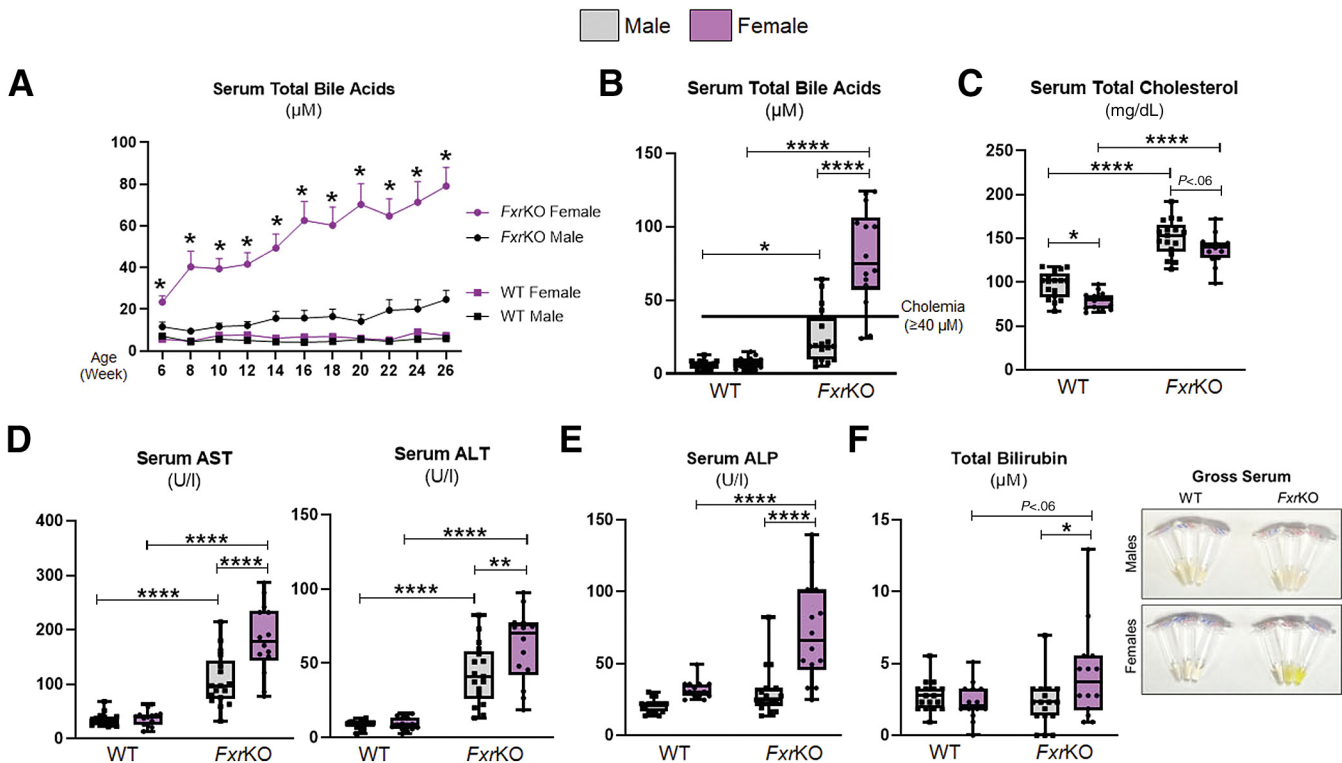Because bile acids can be hepatotoxic agents, we next measured clinical parameters of cholestatic liver injury

Figure 1. Female *Fxr*KO mice are more susceptible to cholemia and cholestatic liver injury. (*A*) Male and female WT and *Fxr*KO mice were bled every 2 weeks from 6–26 weeks of age and analyzed for serum total bile acid levels. Serum levels of (*B*) total bile acids, (*C*) total cholesterol, (*D*) AST and ALT, (*E*) ALP, and (*F*) total bilirubin and gross serum (depicting hyper-bilirubinemia from the yellow fluorescent jaundice) were analyzed when WT and *Fxr*KO mice were 26 weeks of age. Sample sizes: WT (male, N = 17; female, N = 14) and *Fxr*KO (male, N = 17; female, N=14). Results are expressed as means ± SEM. *P < .05, **P < .01, ***P < .001, ****P < .0001.

when *Fxr*KO mice were 26 weeks old. Compared with WT mice, both sexes of *Fxr*KO mice had transaminitis, ie, elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (Figure 1*D*). In accord with the sex disparity in serum TBA levels, *Fxr*KO females had a higher degree of elevation in AST (P < .0001) and ALT (P = .01) compared with males (Figure 1*D*). Impressively, the cholestasis-specific marker alkaline phosphatase (ALP) was significantly increased only in *Fxr*KO females (P < .0001), whereas ALP levels in *Fxr*KO males were comparable with WT mice (Figure 1*E*). Visualization of the serum color showed most (albeit not all) *Fxr*KO females also had jaundice with high serum bilirubin (Figure 1*F*). Altogether, our profound findings demonstrate female *Fxr*KO mice to have cholemia predominance and increased cholestatic liver injury.

Female FxrKO Mice Have Exacerbated Bile Acid Dysmetabolism

To discern the possible reason behind cholemia dominance in female *Fxr*KO mice, a panel of bile acid metabolism genes were quantitated at sites of FXR signaling, ie, liver and ileum. When analyzing for the major bile acid biosynthesis genes (Figure 3*A*), the hepatic gene *Cyp7a1*, which encodes

the rate limiting enzyme, was equally up-regulated in both sexes of *Fxr*KO mice compared with WT mice. Opposingly, female but not male WT and *Fxr*KO mice had a depression in *Cyp8b1* levels (P = .001), suggesting females might produce less cholic acid. Correspondingly, *Fxr*KO females had the most significant down-regulation of *Cyp27a1* and *Cyp7b1* transcripts (P < .0001), the latter of which is related to chenodeoxycholic acid production. Measuring the expression levels of bile acid regulators (Figure 3*B*) demonstrated *Fxr*KO females, but not males, to have repression in *Hnf4α* and *Shp*, two *Cyp7a1* regulators. Similarly, the hepatic membrane receptor complex that mediates ileum FXR-FGF15 signaling, ie, fibroblast growth factor receptor 4 (*Fgfr4*) and β-Klotho (*Klb*), were significantly suppressed in *Fxr*KO females. Yet, there was an equal down-regulation of ileal *Fgf15* seen in male and female *Fxr*KO mice (Figure 4*A*). These data suggest that part of the reason for cholemia dominance in female *Fxr*KO mice is the greater lack of negative feedback regulation of bile acid biosynthesis.

We next asked whether there could be a compromise in enterohepatic bile acid circulation to explain the sex disparity in cholemia. Indeed, *Fxr*KO females had lessened expression of hepatic sodium-dependent taurocholate co-transport peptide (NTCP encoded by *Slc10a1*) and sodium-independent organic anion transport peptide
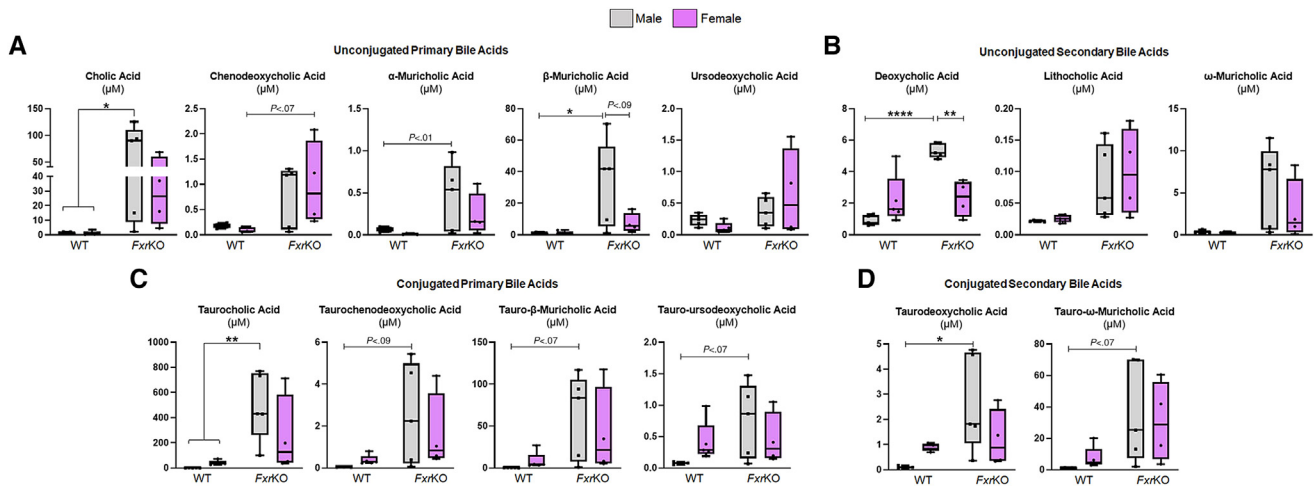
Figure 2. *Fxr*KO male mice have greater levels of deoxycholic acid and β-muricholic acid in circulation. (*A*–*D*) Serum samples were collected from 16-week-old male and female WT and *Fxr*KO mice and sent for bile acid profiling via metabolomics. (*A*) Unconjugated primary bile acids (cholic acid, chenodeoxycholic acid, α-muricholic acid, β-muricholic acid, and ursodeoxycholic acid), (*B*) unconjugated secondary bile acids (deoxycholic acid, lithocholic acid, and ω-muricholic acid), (*C*) conjugated primary bile acids (taurocholic acid, taurochenodeoxycholic acid, tauro-β-muricholic acid, and tauro-ursodeoxycholic acid), and (*D*) conjugated secondary bile acids (taurodeoxycholic acid and tauro-ω-muricholic acid). Sample sizes: WT (male, N = 5; female, N = 5) and *Fxr*KO (male, N = 5; female, N = 4). Results are expressed as means $\pm$ SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

(OATP1B2 encoded by *Slco1b2*) for bile acid uptake into hepatocytes (Figure 3*C*). We also found that the expression of bile salt exporter pump (BSEP) (encoded by *Abcb11*) was further diminished in *Fxr*KO females ($P < .0001$), and only the female groups had a suppression of multidrug resistant protein 2 (encoded by *Abcb4*) (Figure 3*D*). Interestingly, male and female *Fxr*KO mice had equal down-regulation in transcripts related to bile acid reabsorption in the ileum, ie, apical sodium-dependent bile salt transporter (*Asbt*) (Figure 4*B*), fatty acid binding protein 6 (*Fabp6*) (Figure 4*C*), and sinusoidal bile acid efflux transporters, organic solute transporter α and β (*Ostα* and *Ostβ*) (Figure 4*D* and *E*). These data suggest that lowered bile acid secretion into the biliary tree could be another reason for cholemia dominance in female *Fxr*KO mice.

Female FxrKO Mice Exhibit Increased Expression for Bile Acid Detoxification

Several studies highlight the notion that females have better bile acid detoxification because of elevated xenobiotics-processing CYP450 hydroxylating enzymes and sulfotransferases.[16,17,33,34] When analyzing bile acid hydroxylating genes (Figure 3*E*), both sexes of *Fxr*KO mice had increased *Cyp3a11* expression compared with WT mice, but the levels were substantially higher in *Fxr*KO females ($P < .0001$). Moreover, *Fxr*KO females had greater *Cyp2b10* expression than *Fxr*KO males ($P = .0005$). Of note, *Cyp2b10* expression in WT females was elevated, even more than *Fxr*KO mice, compared with WT males. When analyzing sulfotransferase transcripts (Figure 3*F*), *Fxr*KO males had a greater expression in the male-dominant *Sult2a8* compared with WT males, whereas both female groups had reduced mRNA levels. Comparatively, when measuring the female-dominant *Sult2a1*, WT females had around 500-fold

increase, and *Fxr*KO females had around 1500-fold increase in mRNA expression than their respective male groups. Of note, analysis of amidation (*Baat*, *Slc27a5,* or *Bacs*), glucuronidation (*Ugt2b1*, *2b5*, *3a1*), and glucoside genes (*Gba2*) were decreased in *Fxr*KO females (Figure 5*A*–*C*). Hydroxylation and sulfonation detoxify bile acids by promoting their renal excretion, and indeed *Fxr*KO females had the highest urinary TBA levels ($P < .0001$) (Figure 3*G*). These data emphasize cholemia dominance in female *Fxr*KO mice is associated with an up-regulation in bile acid detoxification and excretion.

Deletion of CYP7A1 Normalizes TBA Levels and Liver Injury in Female FxrKO Mice

Our next goal was to find a relevant intervention that can restore bile acid metabolism in *Fxr*KO mice with emphasis to females. We attempted cholestyramine intervention that sequesters luminal bile acids, enhances their fecal excretion, and stimulates bile acid biosynthesis.[35] Introducing dietary cholestyramine to *Fxr*KO females at time points before (Figure 6*A*–*C*) or after (Figure 6*D*–*F*) cholemia did not lower TBA or liver injury. Similarly, we attempted treating with the HMG-CoA reductase inhibitor atorvastatin to reduce substrate availability (ie, cholesterol) for bile acid biosynthesis, but *Fxr*KO females still retained spontaneous cholemia and liver injury (Figure 7*A*–*H*). Because the gut microbiota is responsible for generating secondary bile acids, we also administered broad-spectrum antibiotics orally to *Fxr*KO females with no success to reduce TBA and liver injury (Figure 8*A*–*C*). All of these data indicate and confirm that bile acid metabolism is dysregulated in *Fxr*KO mice.

For a more direct and genetic approach, we crossed *Fxr*KO mice with *Cyp7a1*-deficient (*Cyp7a1*KO) mice to
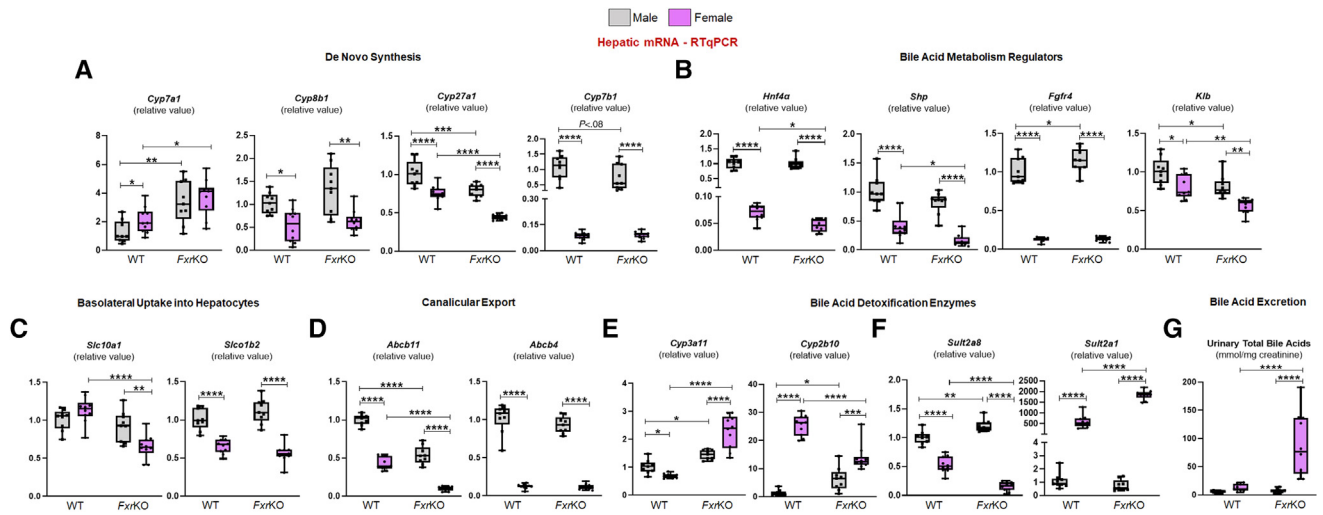
Figure 3. Enhanced bile acid dysmetabolism and detoxification could explain the cholemia disparity in female *Fxr*KO mice. (*A–F*) Liver samples from 16-week-old male and female WT and *Fxr*KO mice were analyzed by qRT-PCR) for transcripts related to bile acid de novo synthesis, regulators, transporters, and detoxification. Relative expressions of genes for bile acid biosynthesis (*A*) *Cyp7a1*, *Cyp8b1*, *Cyp27a1*, and *Cyp7b1*; bile acid regulators (*B*) *Hnf4α*, *Shp*, *Fgfr4*, and *Klb*; bile acid uptake transporters (*C*) *Slc10a1* and *Slco1b2*; bile acid canicular exporters (*D*) *Abcb11* and *Abcb4*; bile acid hydroxylation (*E*) *Cyp3a11* and *Cyp2b10*; and bile acid sulfonation (*F*) *Sult2a8* and *Sult2a1*. 36b4 was used as internal control for qRT-PCR. (*G*) Urinary total bile acid levels (normalized to creatinine) in 16-week-old male and female WT and *Fxr*KO mice. Sample sizes: WT (male, N = 9; female, N = 10) and *Fxr*KO (male, N = 9; female, N = 10). Results are expressed as means ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

generate double knockouts (DKO) (Figure 9*A*). *Cyp7a1*KO mice are reported to have only half the bile acid biosynthesis rate typically found in WT mice.[36] Impressively, 8- and 16-week-old male ($P = .1658$) and female ($P < .0001$) DKO mice had normalized TBA levels to that seen in their sex-matched WT counterparts (Figure 9*B* and *E*), possibly due in part to their lowered cholesterol levels (Figure 9*C* and *F*). It must be emphasized that this intervention was most remarkable in the females because the TBA shift was from an average of 107.3 μmol/L in *Fxr*KO to 2.7 μmol/L in DKO mice compared with the TBA reduction from 12.2 μmol/L to 5.6 μmol/L in male *Fxr*KO and DKO mice, respectively. Even though TBA was normalized, serum ALT levels in DKO males were higher than in *Fxr*KO males ($P = .0121$), and DKO males had hyperbilirubinemia (Figure 9*D* and *H*). Comparatively, DKO females had significantly reduced ALT levels to that found in WT females and were protected from jaundice (Figure 9*G* and *H*). These results demonstrate that female *Fxr*KO mice better responded to the intervention of *Cyp7a1* deficiency with restoration toward low TBA and ALT levels.
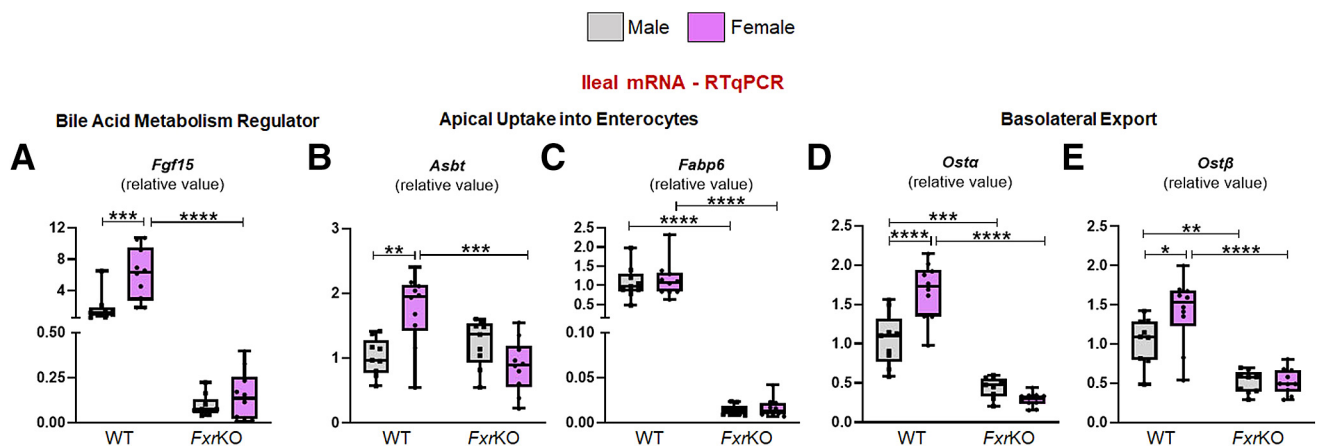

Figure 4. *Fxr*KO mice, irrespective of sex, have equal suppression of ileal transcripts related to bile acid regulation and reabsorption. (*A–E*) Ileal samples from 16-week-old male and female WT and *Fxr*KO mice were analyzed by qRT-PCR for transcripts related to bile acid metabolism. Relative expressions of (*A*) *Fgf15*, (*B*) *Asbt*, (*C*) *Fabp6*, (*D*) *Ostα*, and (*E*) *Ostβ*. 36b4 was used as internal control for qRT-PCR. Sample sizes: WT (male, N = 9; female, N = 10) and *Fxr*KO (male, N = 9; female, N = 10). Results are expressed as means ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.
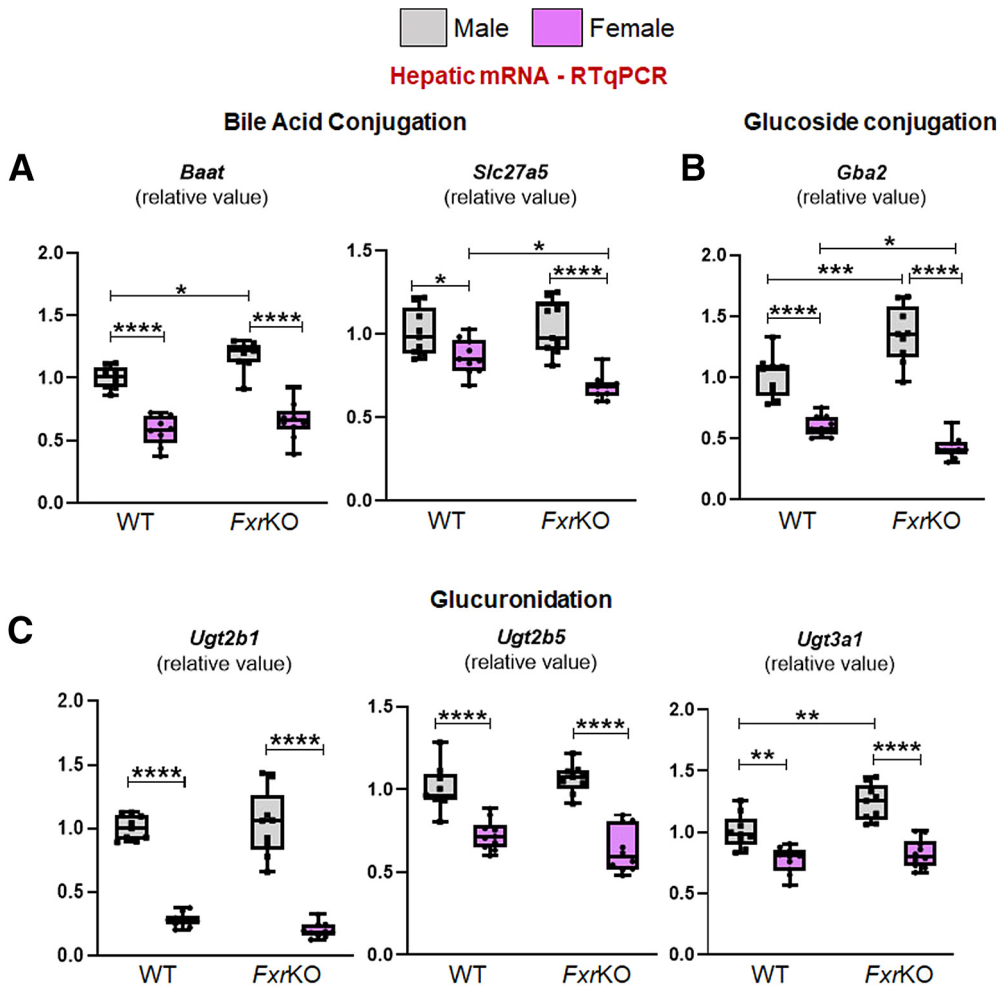
Figure 5. Female mice have lowered hepatic expression of glucuronidation and glucoside genes. (*A–C*) Liver samples from 16-week-old male and female WT and *Fxr*KO mice were analyzed by qRT-PCR for transcripts related to bile acid conjugation (*A*) *Baat* and *Slc27a5*; glucoside formation (*B*) *Gba2*; and glucuronidation (*C*) *Ugt2b1*, *Ugt2b5*, and *Ugt3a1*. 36b4 was used as internal control for qRT-PCR. Sample sizes: WT (male, N = 9; female, N = 10) and *Fxr*KO (male, N = 9; female, N = 10). Results are expressed as means ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

Cholemia in Male FxrKO Mice Is Accelerated by Taurine Supplementation

Taurine is a sulfur-containing non-proteinaceous amino acid that can conjugate with bile acids, which increases their solubility and biliary excretion.[37] Bile acid conjugation enzyme BAAT (bile acid-CoA: amino acid N-acyltransferase) deficiency is known to cause hypercholanemia in mice.[38] We found that female WT and *Fxr*KO mice had significantly lowered *Baat* mRNA expression (Figure 5A). We hypothesized that taurine supplementation (Figure 10A) could serve as a clinically relevant approach to prevent cholemia and liver injury in *Fxr*KO mice. Taurine feeding to *Fxr*KO females resulted in a modest reduction in serum TBA and ALT levels that was associated with increased urinary TBA excretion but no changes in serum cholesterol (Figure 10E–H). Unexpectedly, we observed *Fxr*KO males to have an acceleration in spontaneous cholemia where 7 of 8 males fed taurine reached cholemia by 18 weeks of age compared with the 3 of 8 control *Fxr*KO males (Figure 10B). The rapid cholemia development after taurine also resulted in a significant increase in serum ALT levels for *Fxr*KO males (Figure 10D) but no changes in cholesterol levels (Figure 10C) or urinary TBA levels (data not shown). Overall, taurine seems to have sex-dependent effects on cholemia and liver injury where males exhibit more pathologic indices.

Sex Heterogeneity in Cholemia Is Inversely Associated With HCC Severity

Our next objective was to determine whether sex-dependent spontaneous cholemia at the precancerous age reflects HCC severity in *Fxr*KO mice when 16 months of age. Our initial thought was *Fxr*KO females could have worse HCC because of their high TBA, and they had altered mRNA expression of the oncogene *Myc* and cell cycle regulators at the precancerous stage (Figure 11A–D). At the cancer time point, male and female *Fxr*KO mice who never developed cholemia (TBA <40 μmol/L) had the least HCC severity, whereas tumor burden increased in both sexes for *Fxr*KO mice with spontaneous cholemia (TBA ≥40 μmol/L) (Figure 12A). There were no apparent differences in HCC severity when comparing male and female *Fxr*KO mice without cholemia. However, the few *Fxr*KO males that became cholemic had aggravated tumor burden compared with *Fxr*KO females with prevalent cholemia (Figure 12A). Both male and female *Fxr*KO mice had equivalent intensity of hepatomegaly and jaundice (Figure 12B and C), but females still dominated with the highest levels of TBA ($P = .0015$), ALT ($P = .0192$), and ALP ($P < .0001$) (Figure 12D). When measuring the widely used serologic biomarker for HCC, α-fetoprotein (AFP), *Fxr*KO males had sustainably high
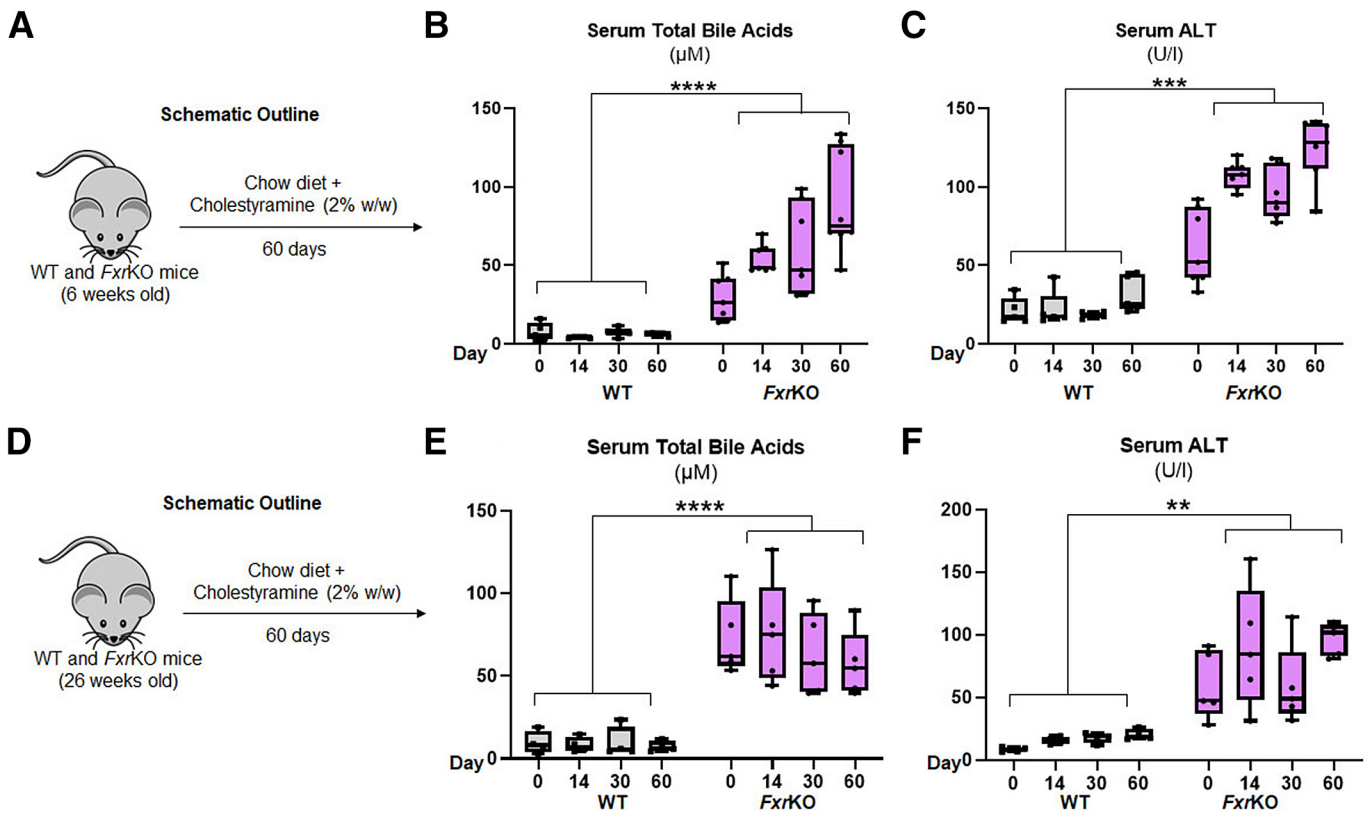
Figure 6. Cholestyramine does not lower bile acids or reduce liver injury in female *Fxr*KO mice. (*A*–*C*) Grain-based chow diet (LabDiet 5001) was supplemented with cholestyramine (2% w/w, Sigma-Aldrich) and given to 6-week-old female WT (N = 5) and *Fxr*KO (N = 7) mice for 60 days. (*A*) Schematic outline, (*B*) serum total bile acids, and (*C*) serum ALT. (*D*–*F*) Grain-based chow diet (LabDiet 5001) was supplemented with cholestyramine (2% w/w, Sigma-Aldrich) and given to 26-week-old female WT (N = 4) and *Fxr*KO (N = 5) mice for 60 days. (*D*) Schematic outline, (*E*) serum total bile acids, and (*F*) serum ALT. Results are expressed as means ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

levels, whereas *Fxr*KO females barely had an elevation compared with their WT counterparts (Figure 12*E*). Pearson correlation demonstrated that TBA levels in *Fxr*KO males had a positive correlation with ALT, ALP, and AFP (Figure 12*F*). Alternately, TBA levels in *Fxr*KO females had a modest positive relationship with serum ALT but negative correlations with ALP and AFP (Figure 12*G*). Urinary TBA levels were over 10-fold more in *Fxr*KO females compared with *Fxr*KO males (Figure 12*H*), suggesting females have more efficient detoxification. Collectively, the summation of all data to this point underscores female *Fxr*KO mice are more susceptible to spontaneous cholemia but are substantially protected from HCC severity because of potential adaptions for better bile acid detoxification.

Portosystemic Shunt Is an Exception to the Sex-dependent Effects of Cholemia on HCC

We stumbled on an unexpected exception to the sex disparity that has been highlighted. When screening *Fxr*KO mice at the pre-weaning age (16–18 days old), we found a subset of males (13.8%) and females (10.1%) exhibited congenital cholemia with mostly similar TBA levels (Figure 13*A*). Essentially, these *Fxr*KO mice are born with

high TBA concentrations, and they remain elevated throughout their lifespan (Figure 13*B*). Recently, we reported the presence of congenital cholemia in BL6 WT mice who had hypocholesterolemia (serum total cholesterol <75 mg/dL),[39] but surprisingly, *Fxr*KO mice with congenital cholemia still retained high cholesterol levels above 100 mg/dL (Figure 13*C*). We previously denoted the reason for congenital cholemia in WT mice was from PSS, which is an abnormal blood vessel architecture that "shunts" blood returning from the intestine directly to the heart, thus bypassing the liver.[40] To assess whether *Fxr*KO mice with congenital cholemia also have PSS, we first injected 4 kDa fluorescein isothiocyanate (FITC)–dextran into the portal vein and imaged the livers by an in vivo imaging system (IVIS). In male and female *Fxr*KO mice with congenital cholemia, we observed FITC–dextran was not retained in the liver and rather entered the inferior vena cava (Figure 13*D*), which supported our hypothesis that these mice have PSS. To better confirm anatomically, resin casting was performed, and we could visualize the shunt architecture in male and female *Fxr*KO mice with congenital cholemia (Figure 13*E*). Last, we aged the mice to the HCC time point and found that male and female *Fxr*KO mice with congenital cholemia/PSS had equally aggravated tumor
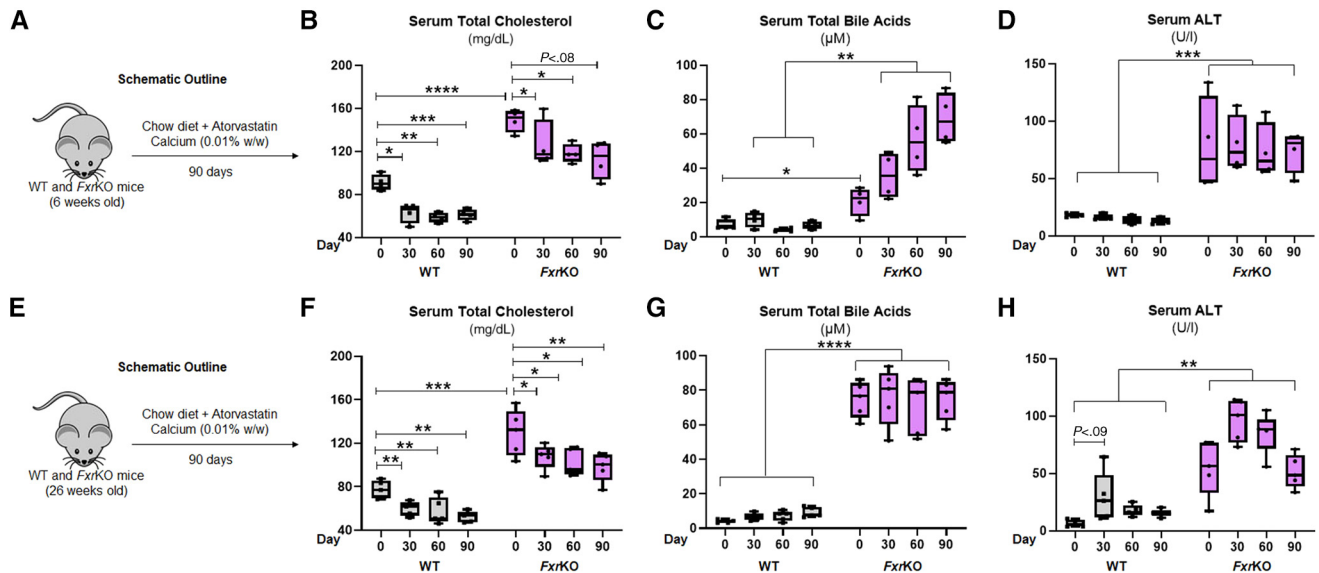
Figure 7. Atorvastatin does not lower bile acids or liver injury during either delayed onset or established cholemia in female *Fxr*KO mice. (*A–D*) Grain-based chow diet (LabDiet 5001) was supplemented with atorvastatin calcium (0.01% w/w, MilliporeSigma) and given to 6-week-old female WT (N = 4) and *Fxr*KO (N = 4) mice for 90 days. (*A*) Schematic outline, (*B*) serum total cholesterol, (*C*) serum total bile acids, and (*D*) serum ALT. (*E–H*) Grain-based chow diet (LabDiet 5001) was supplemented with atorvastatin calcium (0.01% w/w, MilliporeSigma) and given to 26-week-old female WT (N = 5) and *Fxr*KO (N = 5) mice for 90 days. (*E*) Schematic outline, (*F*) serum total cholesterol, (*G*) serum total bile acids, and (*H*) serum ALT. Results are expressed as means ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

burden and jaundice (Figure 13*F*). The tumor burden in mice with congenital cholemia/PSS was more severe than the prior results of spontaneous, delayed onset cholemia in *Fxr*KO mice without PSS (Figure 12). Congenital cholemia in *Fxr*KO mice also accelerated mortality in several mice toward 12 months of age instead of the 16-month cancer time point (data not shown). Overall, PSS is an exception to the sex-dependent effects of cholemia on HCC pathogenesis in *Fxr*KO mice.

Discussion

Sex dimorphism is an unavoidable feature in a plethora of diseases including HCC, and it can dictate susceptibility, severity, outcomes, and response to therapy. Data mining and case studies of HCC specimens have identified several Y-linked proto-oncogenes[41] and possible epigenetic modifications such as histone methylation[42] to discern sex differences. Sex-biased molecular signatures in HCC have focused on sex hormones, ie, androgen and estrogen. Much
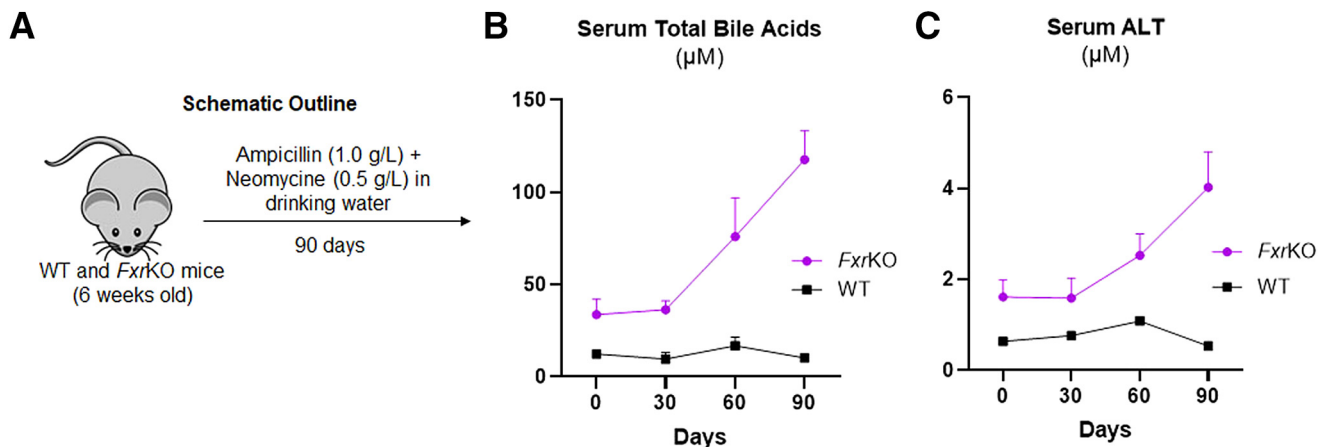

Figure 8. Antibiotics do not inhibit delayed onset cholemia or liver injury in female *Fxr*KO mice. (*A–C*) Broad-spectrum antibiotics (ampicillin [1.0 g/L] + neomycin [0.5 g/L]; Sigma-Aldrich) were given to 6-week-old female WT (N = 4) and *Fxr*KO (N = 4) mice in the drinking water for 90 days. (*A*) Schematic outline, (*B*) serum total bile acids, and (*C*) serum ALT. Results are expressed as means ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.
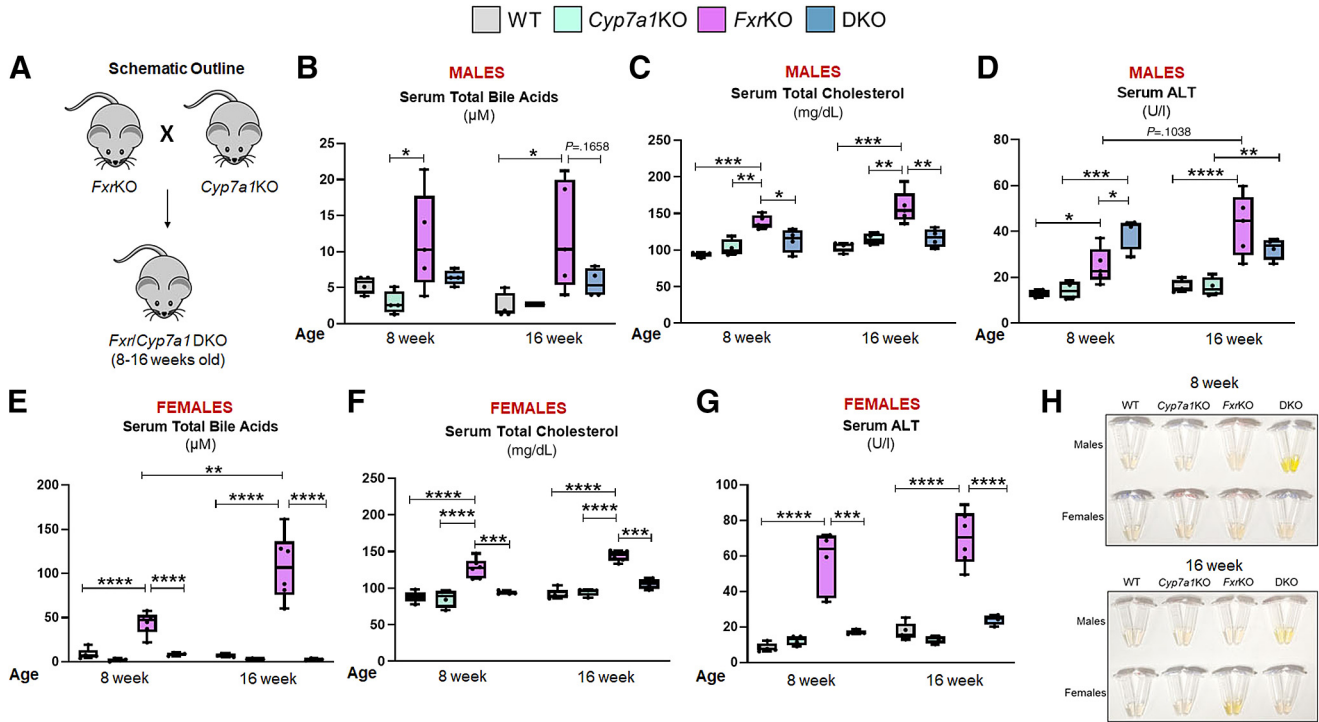
Figure 9. Bile acids and liver injury are normalized in female *Fxr*KO mice on CYP7A1 deletion. (*A–H*) *Fxr*KO mice were crossed with *Cyp7a1*KO mice to generate DKO and were analyzed for cholemia and liver injury markers when 8 and 16 weeks old. (*A*) Schematic outline. Serum (*B*) total bile acids, (*C*) total cholesterol, and (*D*) ALT levels in the male mice. Serum (*E*) total bile acids, (*F*) total cholesterol, and (*G*) ALT levels in the female mice. (*H*) Gross serum (depicting hyperbilirubinemia from the yellow fluorescent jaundice). Sample sizes: WT (male, N = 4; female, N = 5), *Cyp7a1*KO (male, N = 4; female, N = 4), *Fxr*KO (male, N = 5; female, N = 6), and DKO (male, N = 4; female, N = 4). Results are expressed as means ± SEM.*P < .05, **P < .01, ***P < .001, ****P < .0001.
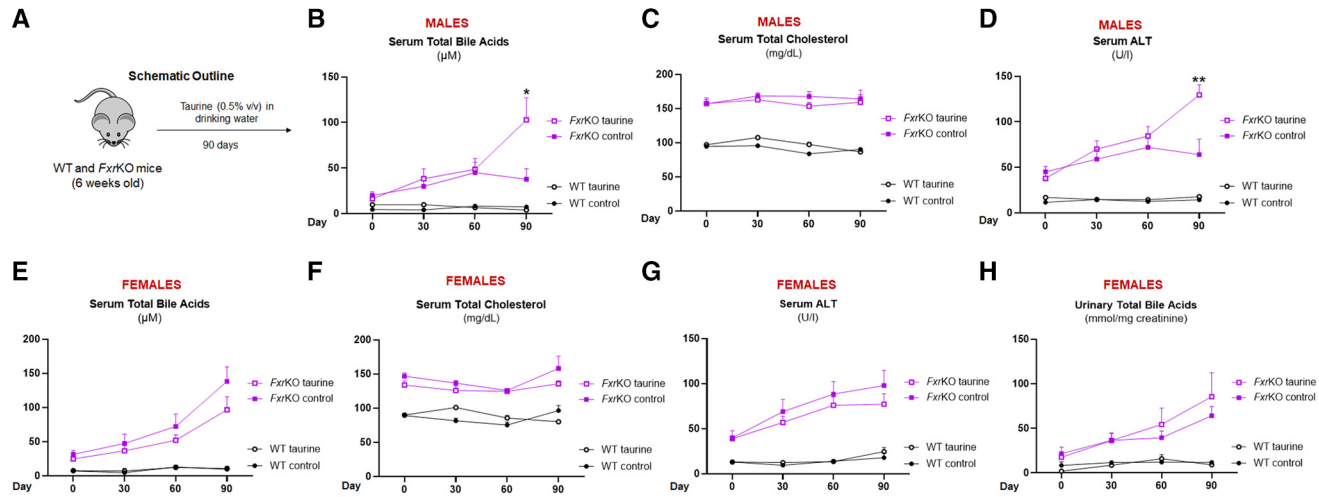

Figure 10. Taurine supplementation lessens cholemia development in female *Fxr*KO mice but accelerates cholemia in male *Fxr*KO mice. (*A–H*) Drinking water containing 0.5% v/v of taurine (Sigma-Aldrich) was given to 6-week-old WT and *Fxr*KO mice for 90 days, and mice were tracked for changes in cholemia and liver injury markers. Serum (*B*) total bile acids, (*C*) total cholesterol, and (*D*) ALT levels in the male mice. Serum (*E*) total bile acids, (*F*) total cholesterol, and (*G*) ALT levels in the female mice. (*F*) Urinary total bile acid levels (normalized to creatinine) in taurine-fed female WT and *Fxr*KO mice. Sample sizes: control WT (male, N = 5; female, N = 5), taurine WT (male, N = 5; female, N = 5), control *Fxr*KO (male, N = 7; female, N = 7), and taurine *Fxr*KO (male, N = 8; female, N=8). Results are expressed as means ± SEM. *P < .05, **P < .01, ***P < .001, ****P < .0001.
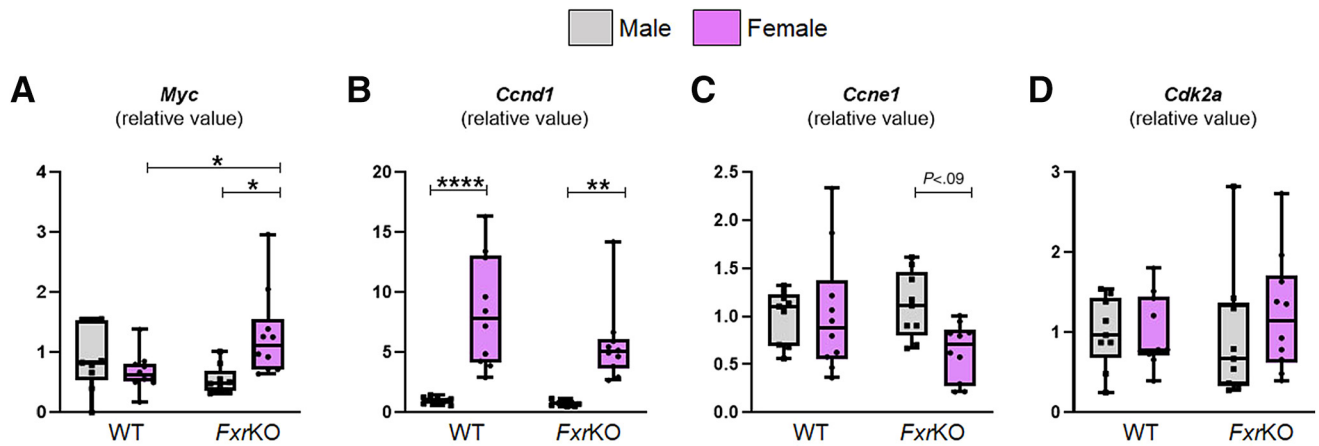
Figure 11. Female *Fxr*KO mice have increased hepatic expression of *Myc* and *Ccnd1*, whereas *Ccne1* and *Cdk2a* are comparable. (*A–D*) Liver samples from 16-week-old male and female WT and *Fxr*KO mice were analyzed by qRT-PCR for transcripts related to oncogenes and cell cycle regulators. (*A*) *Myc*, (*B*) *Ccnd1*, (*C*) *Ccne1*, and (*D*) *Cdk2a*. 36b4 was used as internal control for qRT-PCR. Sample sizes: WT (male, N = 9; female, N = 10) and *Fxr*KO (male, N = 9; female, N = 10). Results are expressed as means ± SEM. *P < .05, **P < .01, ***P < .001, ****P < .0001.

research over the last 20 years provides the concept that androgen is pro-carcinogenic by accelerating cell proliferation via androgen receptor signaling, whereas estrogen is tumor suppressive by inducing cell apoptosis and immune responses.[43] Of note, a novel estrogen-related gene prognostic signature was recently discovered to predict patient survival and immunotherapy response of HCC patients.[44] Overall, these markers have provided diagnostic and
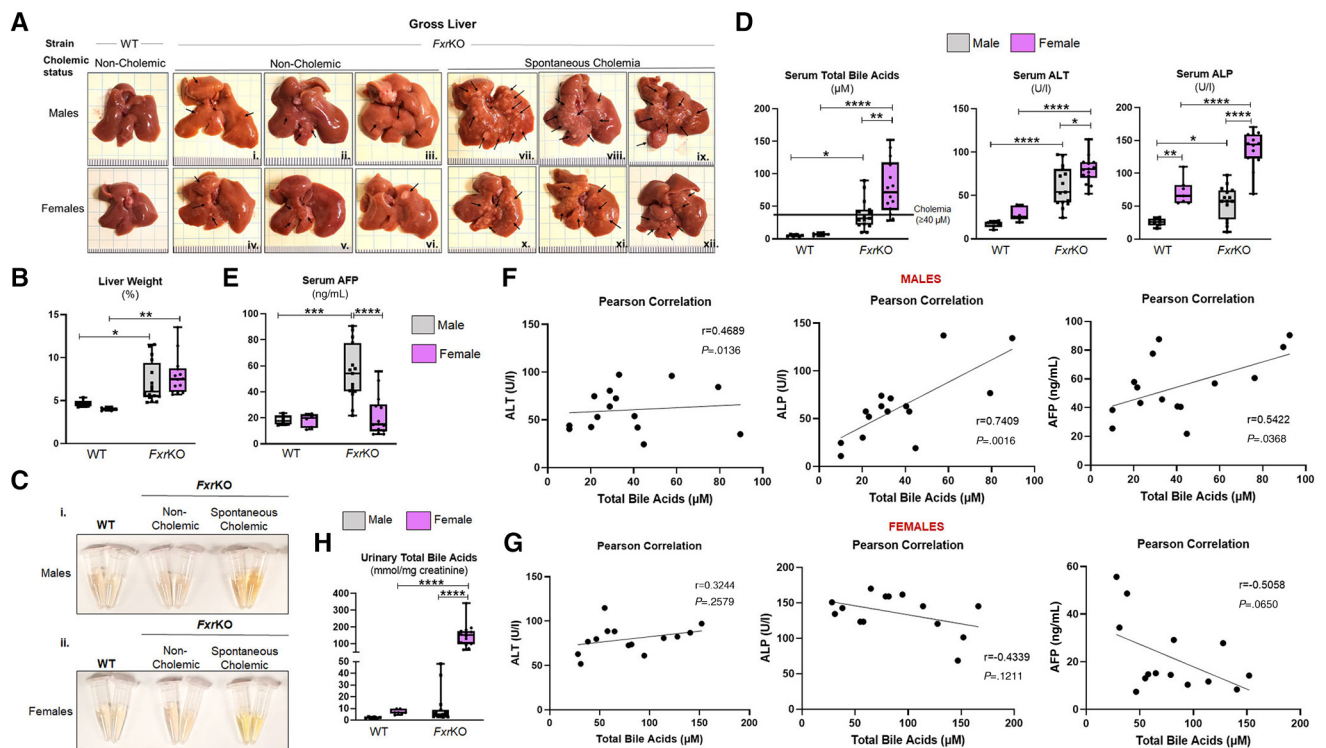

Figure 12. Heterogeneity in cholestasis and HCC is positively associated with bile acids in male *Fxr*KO mice. (*A–H*) Male and female WT and *Fxr*KO mice were aged until 16 months and assessed for liver cancer. (*A*) Gross livers, (*B*) % liver weight, (*C*) gross serum (depicting hyperbilirubinemia from the yellow fluorescent jaundice), serum (*D*) total bile acids, ALT, ALP, and (*E*) AFP levels. Pearson correlations for (*F*) male *Fxr*KO mice comparing serum total bile acids and ALT, ALP, and AFP. Pearson correlations for (*G*) female *Fxr*KO mice comparing serum total bile acids and ALT, ALP, and AFP. (*H*) Urinary total bile acid levels (normalized to creatinine) in WT and *Fxr*KO mice. Sample sizes: WT (male, N = 6; female, N = 6) and *Fxr*KO (male, N = 15; female, N = 14). Results are expressed as means ± SEM. *P < .05, **P < .01, ***P < .001, ****P < .0001.
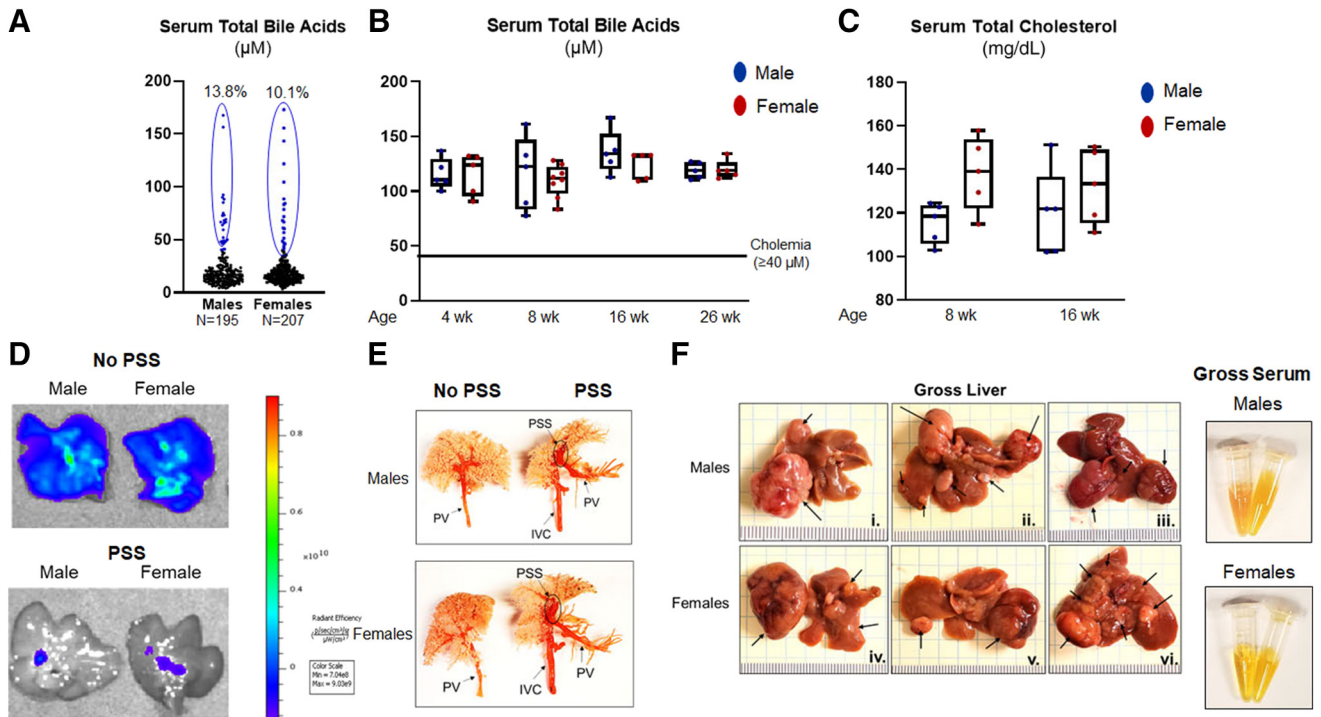
Figure 13. Congenital cholemia due to portosystemic shunt is an exception to the sex dimorphism and HCC severity relationship in *Fxr*KO mice. (*A*) Pre-weaned male (N = 195) and female (N = 207) *Fxr*KO mice (16–18 days old) were bled, and serum total bile acid levels were measured to identify congenital cholemia. (*B*) Male (n = 5) and female (n = 5) *Fxr*KO with congenital cholemia were tracked for their bile acid levels from 4–26 weeks of age. (*C*) Male (n = 5) and female (n = 5) *Fxr*KO with congenital cholemia were tracked for their total cholesterol levels at 8 and 16 weeks of age. (*D* and *E*) Two methods were used to assess PSS in *Fxr*KO mice. (*D*) 4 kDa FITC–dextran (Sigma-Aldrich, 0.136 mg/g body weight) was injected into the portal vein, and then the liver was extracted and imaged via IVIS at excitation 490 nm and emission 520 nm. (*E*) Mercox II resin (Ladd Research) was injected into the portal vein with 30-gauge, 0.5-inch needle at necropsy/dissection. Resin cast was immersed in 15% KOH overnight, rinsed with water, and followed by visual inspection. (*F*) Male (n = 5) and female (n = 5) *Fxr*KO mice with congenital cholemia were aged to 16 months and assessed for liver cancer. Gross liver (3 representative images per sex) and serum (depicting hyperbilirubinemia from the yellow fluorescent jaundice). Results are expressed as means ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

prognosis potential and suggest that genetic and/or hormonal mechanisms could explain male predominance in HCC.

Translational research has focused on targeting sex hormones for HCC treatment. On a therapeutic level, inhibiting androgen receptor signaling has not yielded clinical benefits, but new approaches such as proteolysis-targeting chimeras are being studied.[12] Feminization via estrogen administration in mouse and zebrafish HCC models has demonstrated regressed tumor progression.[9,10] However, there is concern in exogenous estrogenic exposure to humans. In the Liver Cancer Pooling Project, exogenous estrogenic exposures such as oral contraceptives were not associated with increased risk for HCC,[45] but there was 62% increased risk for intrahepatic cholangiocarcinoma, the second most common type of liver cancer.[46] Moreover, a recent report demonstrated that loss of hepatocyte-specific histone deacetylase 3 in female mice reduced FOXA1/2 and promoted HCC development in an estrogen-dependent manner.[47] Collectively, the literature supports the drive for our current study to discover new sex-based, molecular targets that are clinically relevant for HCC patients.

Substantial evidence emphasizes bile acids as a liver oncogenic metabolite,[23] and new findings continue to accumulate.[48,49] In accord, odds ratio by multivariable conditional logistic regression models demonstrated individuals with high circulating TBA to have at least 2-fold increase in HCC risk.[50] Moreover, several reported molecular signatures and computational models based on bile acid metabolism-related genes were found to effectively predict the prognosis of HCC patients and their immunotherapeutic response.[50–54] A few available reports hint the possibility that bile acid metabolism could explain sex divergences in liver disease pathologies. One 2007 study highlights a female-specific resistance in hepatotoxic and cholestasis mouse models due to their better bile acid detoxification.[55] Two consecutive publications in 2012 and 2017 also suggested that male-specific liver adenomas in mice were associated with changes in xenobiotic-processing cytochrome p450 (*Cyp*) genes, including those for bile acid detoxification.[56,57] As such, we hypothesized that physiological sex deviations in bile acid metabolism could explain the pathology differences observed between males and females with HCC.

Using the *Fxr*KO mouse model, we determined that bile acids are a sex-dimorphic molecule in HCC. Our findings demonstrate that *Fxr*KO females have greater incidence and severity of cholemia but have lower tumor burden when compared with males. This is an important observation because the majority of reports using the *Fxr*KO mouse model for liver cancer investigation did not compare males vs females. The literature demonstrates only males were used, or sometimes the sex was not specified.[26–30] The first 2 publications using the *Fxr*KO mouse model did analyze HCC in both males and females but did not highlight potential factors underlying the difference in tumor burden between the sexes.[24,25] Of note, one study that investigated HBV-induced hepatocarcinogenesis with both sexes did observe *Fxr*KO females to have worsened HCC than males,[58] which emphasizes that etiology can influence sex-dependent effects in HCC. As such, other HCC models such as diethylnitrosamine,[21,59,60] high-fat and high-cholesterol,[61] alcohol,[62] and circadian dysfunction (jet lag),[63,64] which are noted with pathologic changes in bile acid pathways, should be better investigated for possible sex variations. Regardless, our study herein supports the future exploration for possible polymorphisms in bile acid genes, including FXR, that may explain sex dimorphism of HCC in humans.

Physiological sex differences in bile acid metabolism are sparsely noted in the literature, where the first line of evidence from the late 1970s uncovered heterogeneity in bile acid sulfonation with significantly increased activity in female hamsters and rats.[16,17] Additional studies in hamsters and rats found females to have a higher rate of bile acid hydroxylation,[33,34] which is another method of bile acid detoxification along with sulfonation. We believe that our observation of lessened hepatocarcinogenesis in cholemic *Fxr*KO female mice could be due to adaptations that promote bile acid detoxification. The up-regulated expressions of *Cyp3a11*, *Cyp2b10*, and the female-dominate *Sult2a1* and the stark increase of urinary TBA excretion in *Fxr*KO females support the possible concept of better bile acid detoxification. Notably, a recent analysis from the Gene Expression Omnibus found the CYP3A4 gene (human ortholog of *Cyp3a11*) to be down-regulated in male HCC patients.[65] A prior study highlights a female-specific resistance in hepatotoxic and cholestasis mouse models due to their better bile acid detoxification[55]; however, we observed increased cholestatic liver injury for cholemic *Fxr*KO females. We believe this could be partially attributed to their lowered hepatic *Cyp7b1* expression, a male-dominate gene, where this compromise would increase oxidized steroid intermediates that can induce liver injury.[66] Furthermore, it is possible that dysregulation in *Cyp7b1* could result in high levels of 27-hydroxycholesterol and thus cause abnormalities in estrogen-mediated gene expression[67] that may change HCC risk.

Our study provides the groundwork for follow-up studies to expand on the possible mechanisms behind the sex-specific role of bile acids in HCC. One of the future steps includes performing unbiased gene expression analysis such as RNA sequencing. Our quantitative reverse transcription-polymerase chain reaction (qRT-PCR) results demonstrate

that *Fxr*KO females, rather than males, have increased *Myc* expression and changes in cell cycle regulation at the precancerous age. Interestingly, *Fxr*KO males are reported to have elevated *Myc* expression at the cancerous time point,[26] which could explain their worsened HCC. Besides oncogenes such as *Myc*, other known mechanisms for how bile acids promote HCC development, eg, activating inflammasome,[19] inducing an immunosuppressive environment,[48,68] and altering the gut microbiota[69] should be further researched for possible sex variations. Another mechanistic approach includes studying how individual bile acids activate inflammatory and/or oncogenic pathways. On the other hand, some individual bile acids such as ursodeoxycholic acid are noted to reduce transforming growth factor-β activity and promote anti-tumor immune responses.[70] Metabolomic analyses in our study delineated *Fxr*KO males had significantly higher deoxycholic acid. This secondary bile acid is known to provoke the malignant behavior of senescence in hepatic stellate cells via induction of interleukin 8 and transforming growth factor-β.[71,72] It would be important to next test whether blocking secondary bile acid production via a bile salt hydrolase inhibitor could be of therapeutic relevance in the *Fxr*KO mouse model.

Growing evidence for sex-dependent variability in treatment responses has encouraged for sex-specific therapies as a new line of personalized medicine, including for liver diseases.[73] There are current approaches to use anti-tumorigenic FGF19 (human ortholog of *Fgf15*) and other FXR agonists to protect against HCC,[74–76] but whether these interventions have sex-dependent effects is unknown. Of note, a recent study found that *Fgf15* overexpression in healthy male mice can cause a phenotypical switch toward a female pattern of drug metabolizing enzymes.[77] Administering FGF15/19 could be thought as one therapeutic idea for *Fxr*KO mice, but studies demonstrate FGF15/FGFR4 signaling to have pro-HCC effects.[78,79] An alternative intervention is to introduce a xenobiotic-responsive receptor agonist that targets constitutive androstane receptor (CAR). However, although the CAR-specific agonist ligand 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) can strongly enrich xenobiotic and drug metabolism better in female mice, TCPOBOP exposure up-regulates hepatic oncogenes in males.[80] Furthermore, another CAR activator called phenobarbital is well-documented to be a liver tumor promoter.[81]

In this study we sought to therapeutically intervene by *Cyp7a1* deficiency or taurine supplementation instead. Interestingly, our intervention studies of *Cyp7a1* deletion and taurine supplementation had opposing sex-dependent effects in *Fxr*KO mice. These treatments either completely or partially alleviated cholemia and liver injury in *Fxr*KO females, but males had an unexpected acceleration in spontaneous cholemia and worsened liver injury. It is plausible that taurine-mediated detoxification in *Fxr*KO males lowered TBA below physiological levels, and that inadvertently triggered more production. Alongside, taurine restoration may have caused a bloom in sulfidogenic bacteria[38] that could contribute to the unexpected increase in cholemia. As such, further studies are required to test other

possible therapies. One option is targeting the pregnane X receptor (PXR). However, there is controversial knowledge about the role of PXR in cell cycle regulation,[81] which must be kept in mind when interpreting results of such an intervention.

Intriguingly, we did find a sex-independent etiologic factor of PSS in male and female *Fxr*KO mice that resulted in congenital cholemia and comparable aggravation in HCC pathogenesis. This observation matches our recent publication that a subset of male and female C57BL/6 mice have PSS and were equally prone to HCC only when fed a compositionally defined diet (alias purified diet).[39] In essence, mice with PSS are experimental outliers and should be statistically accounted for appropriate sample sizing. Regardless, the continual exposure of high TBA levels from birth is probably why *Fxr*KO mice with congenital cholemia have the worst HCC compared with *Fxr*KO mice with delayed onset, spontaneous cholemia. It would be interesting to determine whether TBA levels in congenital cholemic *Fxr*KO mice are resistant to interventions such as lactulose, which is used to prevent hepatic encephalopathy for liver cirrhosis patients who surgically undergo transjugular intrahepatic portosystemic shunt.[82]

Overall, our study newly identifies bile acids as sex-dimorphic metabolites in HCC with the exception of PSS cases. This study advocates for the rigorous incorporation of sex as a biological variable and bile acids to be a possible screening biomarker for HCC. The continual evaluation of sex-dependent markers in HCC will help to determine personalized medicines, including for those undergoing or completed gender-affirming hormone therapy. Moving forward, future studies should expand on the possible mechanisms behind the sex-specific role of bile acids in HCC pathogenesis and their clinical implications.

Materials and Methods
Mice

*Fxr*KO mice on C57BL/6J background[31] were a gift from Dr Frank J. Gonzalez (National Cancer Institute, NIH). Homozygous *Fxr*KO and cholesterol 7α-hydroxylase-deficient (*Cyp7a1*KO, C57BL/6J background[83]) mice were crossed to generate the heterozygous F1 generation, and continual breeding resulted in DKO mice. In our studies, second to fourth generation male and female offspring were used. The above mice were bred with C57BL/6J WT mice in our colony to generate their WT littermates. All mice were bred and maintained under specific pathogen-free conditions at the University of Toledo. Housing was in cages (N = 5 mice/cage) containing corncob bedding (Bed-O-Cob, The Andersons Co) and nestlets (cat# CABFM00088, Ancare) at 23°C under a 12-hour light/dark phase cycle. Mice were fed ad libitum (LabDiet 5020 for breeders and LabDiet 5001 for weaned mice) and given unrestricted access to regular, non-acidified drinking water. Custom diets were generated and supplied by Cincinnati Lab Supply (Certified LabDiet Dealer). Using an alpha of 0.05 (two-sided) and a power of 0.80, we calculated 5–7 mice per genotype (WT, *Cyp7a1*KO, *Fxr*KO, and DKO) and per intervention as the minimum

number necessary to get statistically significant data. All animal studies were conducted per institutional animal care and use committee (IACUC) approvals obtained from University of Toledo.

Serum and Urine Bile Acid Tracking

Submandibular blood was collected from male and female WT and *Fxr*KO mice using a BD microtainer (Becton, Dickinson). Hemolysis-free sera were obtained after centrifugation (10,000 rpm for 10 minutes at 4°C) and stored at –80°C until further analysis for serum TBA. Mice were bled biweekly from 6–26 weeks of age. Urine was also collected for tracking TBA excretion. Same approaches were applied for another study with male and female WT, *Cyp7a1*KO, *Fxr*KO, and *Fxr/Cyp7a1* DKO mice from 8 and 16 weeks of age. In another independent study, pre-weaned *Fxr*KO mice (16–18 days old) were bled, and TBA levels were measured to identify congenital cholemia and then tracked from 4–26 weeks of age. The cholemia threshold (pathologic TBA levels) was determined on the basis of reports in intrahepatic cholestasis of pregnancy where fetal complications occurred when TBA levels were ≥ 40 μmol/L.[32]

Total Bile Acid Quantification

TBA in hemolysis-free sera and urine were measured using a TBA assay kit (enzyme cycling method; Diazyme Laboratories) according to the manufacturer's protocol. Urinary TBA was normalized to creatinine measured by the creatinine kit (Randox).

Bile Acid Quantitation by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

Bile acid levels were measured using a published protocol[84] with minor modification. Targeted analysis of bile acids in serum was performed using a Vanquish UHPLC system coupled with a TSQ Quantis Triple Quadrupole mass spectrometer (Thermo Fisher Scientific) with an ACQUITY C8 BEH UPLC column (2.1 ×100 mm, 1.7 μm) (Waters). Sera (50 μL) were extracted with 150 μL ice-cold methanol containing 0.5 μmol/L deuterated internal standards. After 20 minutes of incubation at –20°C, the samples were centrifuged (12,000g, 4°C, and 10 minutes), 150 μL of supernatants was transferred to autosampler vials. Bile acids were detected by multiple reaction monitoring and selected ion monitoring modes. The results were quantified by comparing integrated peak areas against standard curves with Skyline (MacCoss Lab Software).

PSS Detection

Two methods were used to assess PSS in *Fxr*KO mice with congenital cholemia. First, we injected Mercox II resin (Ladd Research) into the portal vein with a 30-gauge, 0.5-inch needle at necropsy/dissection. Resin cast was immersed in 15% KOH overnight, rinsed with water, and followed by visual inspection. Second, 4 kDa FITC–dextran

(Sigma-Aldrich, 0.136 mg/g body weight) was injected into the portal vein, and then the liver was extracted and imaged via IVIS at excitation 490 nm and emission 520 nm.

Intervention With Dietary Cholestyramine

Cholestyramine is a large cationic exchange resin polymer that sequesters bile acids and enhances their fecal excretion.[35] Grain-based chow diet (LabDiet 5001) was supplemented with cholestyramine (2% w/w, Sigma-Aldrich) and given to female WT and *Fxr*KO mice starting either at 6 weeks of age (before cholemia) or at 26 weeks of age (after cholemia). Mice were fed cholestyramine for 60 days and tracked for changes in serum TBA levels and liver injury markers.

Atorvastatin Supplemented Diet Intervention

Atorvastatin, an inhibitor of HMG-CoA reductase, lowers cholesterol levels. LabDiet 5001 was supplemented with atorvastatin calcium (0.01% w/w, MilliporeSigma) and fed to female WT and *Fxr*KO mice starting either at 6 weeks of age (before cholemia) or at 26 weeks of age (after cholemia). Mice were fed atorvastatin diet for 90 days and tracked for changes in serum TBA levels and liver injury markers.

Antibiotics Administration for Gut Microbiota Ablation

Broad-spectrum antibiotics (ampicillin [1.0 g/L] + neomycin [0.5 g/L]; Sigma-Aldrich) were given to 6-week-old female WT and *Fxr*KO mice in the drinking water, as described previously,[39] for 90 days. Antibiotics were changed once weekly. Mice were tracked for changes in serum TBA levels and liver injury markers.

Taurine Treatment

Taurine is a sulfur-containing, non-proteinaceous amino acid that can conjugate with bile acids, which increases their excretion rate.[37] Drinking water containing 0.5% v/v of taurine (Sigma-Aldrich) was given to 6-week-old female WT and *Fxr*KO mice for 90 days, and mice were tracked for changes in serum TBA levels and liver injury markers.

Liver Cancer Study

*Fxr*KO mice develop HCC at 15–16 months of age, and this happens spontaneously without the introduction of a hepatocarcinogen or dietary intervention.[24,25] Accordingly, male and female WT and *Fxr*KO mice were aged until 16 months and assessed for spontaneous HCC.

Euthanasia With Tissue and Serum Collection

At the end of each bile acid tracking and intervention studies, male and female WT and *Fxr*KO mice underwent 5-hour fasting, were euthanized via CO_2 asphyxiation, and their blood was collected using a BD microtainer (Becton, Dickinson) via cardiac puncture. Hemolysis-free sera were obtained after centrifugation (10,000 rpm for 10 minutes at 4°C) and stored at −80°C until further analysis of cholestatic injury and liver cancer markers as described below. For the bile acid tracking study, livers from 16-week-old WT and *Fxr*KO mice were processed for qRT-PCR. For the 16-month liver cancer study, livers were collected for organ weight and gross images.

Serum Lipids and Transaminases Measurement

Serum total cholesterol, AST, ALT, ALP, and total bilirubin were measured by using biochemical kits from Randox Laboratories (Kearneysville, WV).

Enzyme-linked Immunosorbent Assay

Serum AFP was measured using the Duoset ELISA kit (R&D Systems; Minneapolis, MN) according to the manufacturer's protocol.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Liver samples from 16-week-old male and female WT and *Fxr*KO mice were analyzed for the relative expression of genes encoding for bile acid de novo synthesis, conjugation, regulators, transporters, and detoxification. Moreover, select oncogenes and cell cycle regulator genes were analyzed. RNA was extracted from liver tissue using TRI reagent (Sigma-Aldrich) as per the manufacturer's protocol. The cDNA was synthesized from 800 ng of purified RNA using the qScript cDNA Synthesis Kit (Quanta BioSciences). QRT-PCR was performed using the Step One Plus Real-Time PCR System (Applied Biosystems) in a 10 μL reaction mixture containing cDNA, SYBR Green Master Mix (Quanta BioSciences), and mouse-specific oligonucleotides (Supplementary Table 1). The *36B4* gene was used as the housekeeping gene. The data were expressed as a relative fold-change in comparison with the WT male as control.

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance between 2 groups was calculated using unpaired, two-tailed Student *t* test. Data from more than 2 groups were compared using a one-way analysis of variance, followed by Tukey's multiple comparison test (to compare the mean of each column with the mean of every other column). Pearson correlation was used to establish the association of 2 variables. $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$ were considered statistically significant. All statistical analyses were performed with the GraphPad Prism 9.0 program (GraphPad).

Supplementary Material

Note: To access the supplementary material accompanying this article, go to the full text version at http://doi.org/10.1016/j.jcmgh.2024.01.011.

References

1. Rumgay H, Arnold M, Ferlay J, et al. Global burden of primary liver cancer in 2020 and predictions to 2040. J Hepatol 2022;77:1598–1606.

2. Cao W, Yu P, Yang K, et al. Aflatoxin B1: metabolism, toxicology, and its involvement in oxidative stress and cancer development. Toxicol Mech Methods 2022; 32:395–419.

3. Coskun E, Jaruga P, Vartanian V, et al. Aflatoxin-guanine DNA adducts and oxidatively induced DNA damage in aflatoxin-treated mice in vivo as measured by liquid chromatography-tandem mass spectrometry with isotope dilution. Chem Res Toxicol 2019;32:80–89.

4. Chidambaranathan-Reghupaty S, Fisher PB, Sarkar D. Hepatocellular carcinoma (HCC): epidemiology, etiology and molecular classification. Adv Cancer Res 2021; 149:1–61.

5. Li S, Mo C, Huang S, et al. Over-expressed testis-specific protein Y-encoded 1 as a novel biomarker for male hepatocellular carcinoma. PLoS One 2014;9: e89219.

6. Tsuei DJ, Hsu HC, Lee PH, et al. RBMY, a male germ cell-specific RNA-binding protein, activated in human liver cancers and transforms rodent fibroblasts. Oncogene 2004;23:5815–5822.

7. Kido T, Lo RC, Li Y, et al. The potential contributions of a Y-located protooncogene and its X homologue in sexual dimorphisms in hepatocellular carcinoma. Hum Pathol 2014;45:1847–1858.

8. Nakatani T, Roy G, Fujimoto N, et al. Sex hormone dependency of diethylnitrosamine-induced liver tumors in mice and chemoprevention by leuprorelin. Jpn J Cancer Res 2001;92:249–256.

9. Naugler WE, Sakurai T, Kim S, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007;317:121–124.

10. Li Y, Li H, Spitsbergen JM, et al. Males develop faster and more severe hepatocellular carcinoma than females in kras(V12) transgenic zebrafish. Sci Rep 2017; 7:41280.

11. Sukocheva OA. Estrogen, estrogen receptors, and hepatocellular carcinoma: are we there yet? World J Gastroenterol 2018;24:1–4.

12. Zhang H, Spencer K, Burley SK, et al. Toward improving androgen receptor-targeted therapies in male-dominant hepatocellular carcinoma. Drug Discov Today 2021; 26:1539–1546.

13. Marin JJG, Serrano MA, Monte MJ, et al. Role of genetic variations in the hepatic handling of drugs. Int J Mol Sci 2020;21.

14. Camilleri M. Bile acid detergency: permeability, inflammation, and effects of sulfation. Am J Physiol Gastrointest Liver Physiol 2022;322:G480–G488.

15. Kastrinou Lampou V, Poller B, Huth F, et al. Novel insights into bile acid detoxification via CYP, UGT and SULT enzymes. Toxicol In Vitro 2023;87:105533.

16. Barnes S, Burhol PG, Zander R, et al. Enzymatic sulfation of glycochenodeoxycholic acid by tissue fractions from adult hamsters. J Lipid Res 1979;20:952–959.

17. Hammerman KJ, Chen LJ, Fernandez-Corugedo A, et al. Sex differences in hepatic sulfation of taurolithocholate in the rat. Gastroenterology 1978;75:1021–1025.

18. Kong B, Wang L, Chiang JY, et al. Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. Hepatology 2012;56:1034–1043.

19. Chen W, Ding M, Ji L, et al. Bile acids promote the development of HCC by activating inflammasome. Hepatol Commun 2023;7.

20. Ohtaki Y, Hida T, Hiramatsu K, et al. Deoxycholic acid as an endogenous risk factor for hepatocarcinogenesis and effects of gomisin A, a lignan component of Schizandra fruits. Anticancer Res 1996;16:751–755.

21. Sun L, Beggs K, Borude P, et al. Bile acids promote diethylnitrosamine-induced hepatocellular carcinoma via increased inflammatory signaling. Am J Physiol Gastrointest Liver Physiol 2016;311:G91–G104.

22. Yamada S, Takashina Y, Watanabe M, et al. Bile acid metabolism regulated by the gut microbiota promotes non-alcoholic steatohepatitis-associated hepatocellular carcinoma in mice. Oncotarget 2018;9: 9925–9939.

23. Li T, Apte U. Bile acid metabolism and signaling in cholestasis, inflammation, and cancer. Adv Pharmacol 2015;74:263–302.

24. Kim I, Morimura K, Shah Y, et al. Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice. Carcinogenesis 2007;28:940–946.

25. Yang F, Huang X, Yi T, et al. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 2007;67:863–867.

26. Takahashi S, Tanaka N, Fukami T, et al. Role of farnesoid X receptor and bile acids in hepatic tumor development. Hepatol Commun 2018;2:1567–1582.

27. Degirolamo C, Modica S, Vacca M, et al. Prevention of spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice by intestinal-specific farnesoid X receptor reactivation. Hepatology 2015;61:161–170.

28. Wang YD, Chen WD, Li C, et al. Farnesoid X receptor antagonizes JNK signaling pathway in liver carcinogenesis by activating SOD3. Mol Endocrinol 2015;29:322–331.

29. Anakk S, Bhosale M, Schmidt VA, et al. Bile acids activate YAP to promote liver carcinogenesis. Cell Rep 2013; 5:1060–1069.

30. Wolfe A, Thomas A, Edwards G, et al. Increased activation of the Wnt/beta-catenin pathway in spontaneous hepatocellular carcinoma observed in farnesoid X receptor knockout mice. J Pharmacol Exp Ther 2011; 338:12–21.

31. Sinal CJ, Tohkin M, Miyata M, et al. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell 2000;102:731–744.

32. Glantz A, Marschall HU, Mattsson LA. Intrahepatic cholestasis of pregnancy: relationships between bile acid levels and fetal complication rates. Hepatology 2004; 40:467–474.

33. Carlson SE, Mitchell AD, Goldfarb S. Sex-related differences in diurnal activities and development of hepatic microsomal 3-hydroxy-3-methylglutaryl coenzyme A

reductase and cholesterol 7alpha-hydroxylase. Biochim Biophys Acta 1978;531:115–124.

34. Kuroki S, Muramoto S, Kuramoto T, et al. Sex differences in gallbladder bile acid composition and hepatic steroid 12 alpha-hydroxylase activity in hamsters. J Lipid Res 1983;24:1543–1549.

35. Riaz S, John S. Cholestyramine resin. Treasure Island. FL: StatPearls, 2022.

36. Schwarz M, Russell DW, Dietschy JM, et al. Alternate pathways of bile acid synthesis in the cholesterol 7alpha-hydroxylase knockout mouse are not upregulated by either cholesterol or cholestyramine feeding. J Lipid Res 2001;42:1594–1603.

37. Bellentani S, Pecorari M, Cordoma P, et al. Taurine increases bile acid pool size and reduces bile saturation index in the hamster. J Lipid Res 1987;28:1021–1027.

38. Alrehaili BD, Lee M, Takahashi S, et al. Bile acid conjugation deficiency causes hypercholanemia, hyperphagia, islet dysfunction, and gut dysbiosis in mice. Hepatol Commun 2022;6:2765–2780.

39. Yeoh BS, Saha P, Golonka RM, et al. Enterohepatic shunt-driven cholemia predisposes to liver cancer. Gastroenterology 2022;163:1658–1671 e16.

40. Franchi-Abella S, Gonzales E, Ackermann O, et al. Congenital portosystemic shunts: diagnosis and treatment. Abdom Radiol (NY) 2018;43:2023–2036.

41. Heydari R, Jangravi Z, Maleknia S, et al. Y chromosome is moving out of sex determination shadow. Cell Biosci 2022;12:4.

42. Chlamydas S, Markouli M, Strepkos D, et al. Epigenetic mechanisms regulate sex-specific bias in disease manifestations. J Mol Med (Berl) 2022;100:1111–1123.

43. Zhang L, Wu J, Wu Q, et al. Sex steroid axes in determining male predominance in hepatocellular carcinoma. Cancer Lett 2023;555:216037.

44. Gao B, Wang Y, Li C, et al. Estrogen-related genes influence immune cell infiltration and immunotherapy response in hepatocellular carcinoma. Front Immunol 2023;14:1114717.

45. McGlynn KA, Sahasrabuddhe VV, Campbell PT, et al. Reproductive factors, exogenous hormone use and risk of hepatocellular carcinoma among US women: results from the Liver Cancer Pooling Project. Br J Cancer 2015;112:1266–1272.

46. Petrick JL, McMenamin UC, Zhang X, et al. Exogenous hormone use, reproductive factors and risk of intrahepatic cholangiocarcinoma among women: results from cohort studies in the Liver Cancer Pooling Project and the UK Biobank. Br J Cancer 2020;123:316–324.

47. Xu Y, Zhu Y, Wu Z, et al. Hepatocyte-specific HDAC3 ablation promotes hepatocellular carcinoma in females by suppressing Foxa1/2. BMC Cancer 2023;23:906.

48. Sun R, Zhang Z, Bao R, et al. Loss of SIRT5 promotes bile acid-induced immunosuppressive microenvironment and hepatocarcinogenesis. J Hepatol 2022;77:453–466.

49. Wang L, Luo Q, Zeng S, et al. Disordered farnesoid X receptor signaling is associated with liver carcinogenesis in Abcb11-deficient mice. J Pathol 2021;255:412–424.

50. Stepien M, Lopez-Nogueroles M, Lahoz A, et al. Pre-diagnostic alterations in circulating bile acid profiles in the development of hepatocellular carcinoma. Int J Cancer 2022;150:1255–1268.

51. Shi Q, Yuan X, Xue C, et al. Establishment and validation of a novel risk score for hepatocellular carcinoma based on bile acid and bile salt metabolism-related genes. Int J Mol Sci 2023:24.

52. Zhang W, Zhang Y, Wan Y, et al. A bile acid-related prognostic signature in hepatocellular carcinoma. Sci Rep 2022;12:22355.

53. Cui H, Lian J, Xu B, et al. Identification of a bile acid and bile salt metabolism-related lncRNA signature for predicting prognosis and treatment response in hepatocellular carcinoma. Sci Rep 2023;13:19512.

54. Wang G, Guan J, Yang Q, et al. Development of a bile acid-related gene signature for predicting survival in patients with hepatocellular carcinoma. Comput Math Methods Med 2022;2022:9076175.

55. Uppal H, Saini SP, Moschetta A, et al. Activation of LXRs prevents bile acid toxicity and cholestasis in female mice. Hepatology 2007;45:422–432.

56. Bojkowska K, Aloisio F, Cassano M, et al. Liver-specific ablation of Kruppel-associated box-associated protein 1 in mice leads to male-predominant hepatosteatosis and development of liver adenoma. Hepatology 2012;56:1279–1290.

57. Cassano M, Offner S, Planet E, et al. Polyphenic trait promotes liver cancer in a model of epigenetic instability in mice. Hepatology 2017;66:235–251.

58. Niu Y, Xu M, Slagle BL, et al. Farnesoid X receptor ablation sensitizes mice to hepatitis B virus X protein-induced hepatocarcinogenesis. Hepatology 2017;65:893–906.

59. Cameron RG, Imaida K, Tsuda H, et al. Promotive effects of steroids and bile acids on hepatocarcinogenesis initiated by diethylnitrosamine. Cancer Res 1982;42:2426–2428.

60. Xing L, Zhang Y, Li S, et al. A dual coverage monitoring of the bile acids profile in the liver-gut axis throughout the whole inflammation-cancer transformation progressive: reveal hepatocellular carcinoma pathogenesis. Int J Mol Sci 2023:24.

61. Chun HJ, Shim YJ, Kwon YH. Cholic acid supplementation accelerates the progression of nonalcoholic fatty liver disease to the procarcinogenic state in mice fed a high-fat and high-cholesterol diet. J Nutr Biochem 2022;100:108869.

62. Chen W, Zhang Q, Ding M, et al. Alcohol triggered bile acid disequilibrium by suppressing BSEP to sustain hepatocellular carcinoma progression. Chem Biol Interact 2022;356:109847.

63. Kettner NM, Voicu H, Finegold MJ, et al. Circadian homeostasis of liver metabolism suppresses hepatocarcinogenesis. Cancer Cell 2016;30:909–924.

64. Padilla J, Osman NM, Bissig-Choisat B, et al. Circadian dysfunction induces NAFLD-related human liver cancer in a mouse model. J Hepatol 2023.

65. Wu Y, Yao N, Feng Y, et al. Identification and characterization of sexual dimorphism-linked gene expression profile in hepatocellular carcinoma. Oncol Rep 2019;42:937–952.

66. Setchell KD, Schwarz M, O'Connell NC, et al. Identification of a new inborn error in bile acid synthesis: mutation of the oxysterol 7alpha-hydroxylase gene causes severe neonatal liver disease. J Clin Invest 1998; 102:1690–1703.

67. Umetani M, Domoto H, Gormley AK, et al. 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. Nat Med 2007;13:1185–1192.

68. Xia JK, Tang N, Wu XY, et al. Deregulated bile acids may drive hepatocellular carcinoma metastasis by inducing an immunosuppressive microenvironment. Front Oncol 2022;12:1033145.

69. Shen R, Ke L, Li Q, et al. Abnormal bile acid-microbiota crosstalk promotes the development of hepatocellular carcinoma. Hepatol Int 2022;16:396–411.

70. Shen Y, Lu C, Song Z, et al. Ursodeoxycholic acid reduces antitumor immunosuppression by inducing CHIP-mediated TGF-beta degradation. Nat Commun 2022; 13:3419.

71. Nguyen PT, Kanno K, Pham QT, et al. Senescent hepatic stellate cells caused by deoxycholic acid modulates malignant behavior of hepatocellular carcinoma. J Cancer Res Clin Oncol 2020;146:3255–3268.

72. Yoshimoto S, Loo TM, Atarashi K, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature 2013;499:97–101.

73. Durazzo M, Belci P, Collo A, et al. Gender specific medicine in liver diseases: a point of view. World J Gastroenterol 2014;20:2127–2135.

74. Cariello M, Peres C, Zerlotin R, et al. Long-term administration of nuclear bile acid receptor FXR agonist prevents spontaneous hepatocarcinogenesis in Abcb4(-/-) mice. Sci Rep 2017;7:11203.

75. Gadaleta RM, Scialpi N, Peres C, et al. Suppression of hepatic bile acid synthesis by a non-tumorigenic FGF19 analogue protects mice from fibrosis and hepatocarcinogenesis. Sci Rep 2018;8:17210.

76. Shi L, Zhao T, Huang L, et al. Engineered FGF19(DeltaKLB) protects against intrahepatic cholestatic liver injury in ANIT-induced and Mdr2-/- mice model. BMC Biotechnol 2023;23:43.

77. Rizzolo D, Kong B, Piekos S, et al. Effects of overexpression of fibroblast growth factor 15/19 on hepatic drug metabolizing enzymes. Drug Metab Dispos 2022; 50:468–477.

78. Cui G, Martin RC, Jin H, et al. Up-regulation of FGF15/19 signaling promotes hepatocellular carcinoma in the background of fatty liver. J Exp Clin Cancer Res 2018; 37:136.

79. Lin BC, Desnoyers LR. FGF19 and cancer. Adv Exp Med Biol 2012;728:183–194.

80. Lodato NJ, Melia T, Rampersaud A, et al. Sex-differential responses of tumor promotion-associated genes and dysregulation of novel long noncoding RNAs in constitutive androstane receptor-activated mouse liver. Toxicol Sci 2017;159:25–41.

81. Yoshinari K, Shizu R. Distinct roles of the sister nuclear receptors PXR and CAR in liver cancer development. Drug Metab Dispos 2022;50:1019–1026.

82. de Wit K, Schaapman JJ, Nevens F, et al. Prevention of hepatic encephalopathy by administration of rifaximin and lactulose in patients with liver cirrhosis undergoing placement of a transjugular intrahepatic portosystemic shunt (TIPS): a multicentre randomised, double blind, placebo controlled trial (PEARL trial). BMJ Open Gastroenterol 2020;7:e000531.

83. Ferrell JM, Boehme S, Li F, et al. Cholesterol 7alpha-hydroxylase-deficient mice are protected from high-fat/high-cholesterol diet-induced metabolic disorders. J Lipid Res 2016;57:1144–1154.

84. Tian Y, Cai J, Allman EL, et al. Quantitative analysis of bile acid with UHPLC-MS/MS. Methods Mol Biol 2021; 2194:291–300.

Correspondence
Address correspondence to: Matam Vijay-Kumar, PhD, Department of Physiology & Pharmacology, University of Toledo College of Medicine and Life Sciences, Toledo, Ohio 43614. e-mail: MatamVijay.Kumar@UToledo.edu.