



Bile acids contribute to the development of non-alcoholic steatohepatitis in mice

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Background & Aims: Through FXR and TGR5 signaling, bile acids (BAs) modulate lipid and glucose metabolism, inflammation and fibrosis. Hence, BAs returning to the liver after enteric secretion, modification and reabsorption may contribute to the pathogenesis of non-alcoholic steatohepatitis (NASH). Herein, we characterized the enterohepatic profile and signaling of BAs in preclinical models of NASH, and explored the consequences of experimental manipulation of BA composition.

Methods: We used high-fat diet (HFD)-fed *foz/foz* and high-fructose western diet-fed C57BL/6J mice, and compared them to their respective controls. Mice received a diet supplemented with deoxycholic acid (DCA) to modulate BA composition.

Results: Compared to controls, mice with NASH had lower concentrations of BAs in their portal blood and bile, while systemic BA concentrations were not significantly altered. Notably, the concentrations of secondary BAs, and especially of DCA, and the ratio of secondary to primary BAs were strikingly lower in bile and portal blood of mice with NASH. Hence, portal blood was poor in FXR and TGR5 ligands, and conferred poor anti-inflammatory protection in mice with NASH. Enhanced primary BAs synthesis and conversion of secondary to primary BAs in NASH livers contributed to the depletion in secondary BAs. Dietary DCA supplementation in HFD-fed *foz/foz* mice restored the BA concentrations in portal blood, increased TGR5 and FXR signaling, improved the dysmetabolic status, protected from steatosis and hepatocellular ballooning, and reduced macrophage infiltration.

Conclusions: BA composition in the enterohepatic cycle, but not in systemic circulation, is profoundly altered in preclinical models of NASH, with specific depletion in secondary BAs. Dietary correction of the BA profile protected from NASH, supporting a role for enterohepatic BAs in the pathogenesis of NASH.

Lay summary: This study clearly demonstrates that the alterations of enterohepatic bile acids significantly contribute to the development of non-alcoholic steatohepatitis in relevant preclinical models. Indeed, experimental modulation of bile acid composition restored perturbed FXR and TGR5 signaling and prevented non-alcoholic steatohepatitis and associated metabolic disorders.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a progressive disease ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Steatosis is described as the presence of lipid droplets in more than 5% of hepatocytes, while superimposed lobular inflammation and hepatocyte ballooning with variable fibrosis define NASH.¹ NAFLD is considered as the hepatic manifestation of the metabolic syndrome and strongly associates with obesity, insulin resistance and type 2 diabetes.¹ In line with the rising obesity rates, NAFLD is becoming increasingly common worldwide, with a global prevalence of 25% in the adult population.¹

Despite this, the pathogenesis of NAFLD is still not fully understood and efficient pharmacological treatments are lacking.

Primary bile acids (BAs) are synthesized from cholesterol in hepatocytes. After conjugation with taurine or glycine, they are secreted through the bile in the intestine where they enhance the digestion and the absorption of lipids and liposoluble vitamins. In the gut, bacterial enzymes can deconjugate and dehydroxylate primary BAs to form secondary BAs.² Most of the BAs are reabsorbed and brought back to the liver by the portal flow. A small proportion escapes this enterohepatic cycle and is excreted in feces or enters the systemic circulation.³

Primary and secondary BAs interact with receptors such as the nuclear farnesoid X receptor (FXR) and the membrane Takeda G-protein coupled receptor 5 (TGR5) with specific affinities. Primary chenodeoxycholic acid (CDCA) activates, while tauro α - and tauro β -muricholic acids (T α - and T β -MCA) inhibit FXR.⁴ Secondary lithocholic and deoxycholic acids (LCA and DCA) have high affinity for and activate TGR5.⁴ The two

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receptors are expressed on cells along, but also outside, the enterohepatic cycle. Notably, BA-induced FXR activation in enterocytes stimulates the production of FGF19 (or FGF15 the mouse ortholog), a key component of enterohepatic FXR signaling.⁵ FXR orchestrates BA metabolism,^{5,6} while both FXR and TGR5 regulate lipid and glucose homeostasis,^{7–11} energy expenditure,^{12–14} inflammation^{14–17} and fibrosis.^{18,19} Thereby, any modification of BA pool size and composition would modulate TGR5 and FXR signaling and potentially impact on NAFLD pathogenesis, making BAs attractive candidates for therapeutic development.

Literature reports that explore the association between BA composition and NAFLD are discordant.^{20–28} Proper interpretation of available data is difficult and speculative because the sampling of BAs in humans is often restricted to systemic blood and feces, as sampling of enterohepatic BAs is challenging. BAs in feces and systemic blood have escaped the enterohepatic cycle and thereby do not reflect the BA cycling between the liver and the gut. Hence, the question about alterations of enterohepatic BA pool and their impact on FXR and TGR5 signaling remains elusive in patients with NASH.

With this study, we aimed to evaluate the composition, metabolism and signaling of BAs in the enterohepatic compartment of two validated mouse models of NASH, that recapitulate the metabolic context and the histological hepatic hallmarks of human NASH. We found low BA concentrations and important changes in BA composition of bile and portal blood that resulted in a reduction of BA signaling through FXR and TGR5 in mice with NASH. Dietary DCA supplementation to restore FXR and TGR5 signaling significantly protected from the development of key histological NASH features. Taken together, our data support the contribution of a shift in BA composition to the pathogenesis of NASH in relevant preclinical models, highlighting the therapeutic potential of BA manipulation.

Materials and methods

Animals and diets

Six-week-old male *foz/foz* (*Alms1*^{-/-}) and wild-type (WT, *Alms1*^{+/+}) mice on a NOD.B10 background,^{29,30} co-housed in the same cage were fed a high-fat diet (HFD, ResearchDiets D12492) for 12 weeks. As described,³¹ HFD-fed *foz/foz* mice (*n* = 6) were used as a model of obesity, insulin resistance and NASH (NAFLD activity score [NAS] ≥ 7) while HFD-fed WT mice (NAS ≤ 2, Fig. S1) served as healthy controls (*n* = 5–6/group).

Eight-week-old male C57BL/6J mice were fed a normal diet (ND, SAFE diets A03) or a western diet containing 0.5% cholesterol (WD, ResearchDiets D05011404) and 30% fructose in drinking water for 20 weeks (WDF). After 20 weeks, C57BL/6J mice fed a WDF have NASH (NAS ≥ 7) while C57BL/6J mice fed a ND have no liver disease (NAS ≤ 1, *n* = 10/group, Fig. S2).

For modulation of the BA composition, *foz/foz* mice were randomized into 3 groups based on their body weight and glycemia and received a HFD or a HFD supplemented with 0.03% or 0.1% (w/w) DCA for 12 weeks, and were compared to WT mice fed a HFD for 12 weeks (*n* = 6–7/group).

Body weight, glycemia and food intake were measured weekly. For sacrifice, mice were fasted for 12 hours then refed for 4 hours (to ensure synchronization for intestinal bile secretion) and anesthetized with ketamine-xylazine. Portal and systemic blood were collected in heparin-coated tubes and plasma stored at -80°C. Bile, liver and distal ileum were harvested, weighed,

snap frozen in liquid nitrogen and stored at -80°C or fixed in 4% formalin.

Animal care was provided in accordance with the guidelines for humane care for laboratory animals as per the European regulations and data reported in conformity with ARRIVE guidelines. The study protocol was approved by the university ethics committee for the use of experimental animals under the reference 2016/UCL/MED/016 and 2020/UCL/MD/018.

BA analyses

BAs were extracted from bile, portal and systemic plasma by precipitation with iced methanol.³² The BA species were quantified by high-performance liquid chromatography (UFLC-XR device, Shimadzu) coupled to tandem mass spectrometry (QTRAP5500 hybrid system, equipped with a Turbo VTM ion source, Sciex) using 5 deuterated BAs (d4-cholic acid [CA], d4-glycocholid acid, d4-taurocholic acid, d4-CDCA, d4-glycochenodeoxycholic acid) as internal standards. CA, CDCA, ursodeoxycholic acid, αMCA and βMCA are mouse primary BAs. DCA, LCA, ωMCA and hyodeoxycholic acid are mouse secondary BAs.

TGR5 ligand activity

HEK293T (ATCC CRL-3216) were cultured in DMEM containing 10% FBS and 1% Penicillin/Streptomycin. At 80% confluence in a 96 well plate, cells were transfected with 20 ng of pCMV-SPORT6 human TGR5 (Harvard Medical School MGC:40597), 40 ng of pGL4.29 (CRE-luciferase, Promega) and 5 ng of pGL4.73 (SV40-Renilla, Promega) using Lipofectamine 2000. Twenty-four hours later, cells were incubated with FBS-free medium or FBS-free medium containing tauro-lithocholic acid (TLCA) 10 μM, or portal plasma (20% or 40%) for 3 hours. Then, cells were lysed and assayed according to the Dual-Luciferase Reporter Assay System (Promega E1910). Firefly and renilla luminescences were quantified using a GloMax 20/20 Luminometer. The firefly luciferase signal was normalized to the renilla luciferase signal as an internal control of the transfection rate. The signal is TGR5-dependent as no signal was detected in cells transfected with pGL4.29 and pGL4.73 only, without pCMV-SPORT6 human TGR5 (Fig. S3).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8. Data are presented as mean ± standard deviation and graphed as individual dots as per the ARRIVE guidelines. Outliers were removed based on Grubbs' test. Normality was assessed using the Shapiro-Wilk test. When comparing 2 groups, an unpaired 2-tailed *t* test or Mann-Whitney test were used to calculate significance. When comparing more than 2 groups, one-way or two-way ANOVA followed by *post hoc* Bonferroni correction or Kruskal-Wallis followed by Dunn's multiple comparisons test was used to calculate significance.

For further details regarding the materials and methods used, please refer to the CTAT table and [supplementary information](#).

Results

In HFD-fed *foz/foz* mice with NASH, enterohepatic BA composition is altered

As previously described,^{31,33} *foz/foz* mice fed a HFD for 12 weeks are obese, severely insulin resistant and have developed the histological hepatic features of human NASH (NAS ≥ 7). Their co-

housed WT littermates fed the same HFD for the same duration do not have metabolic or liver disease (NAS ≤ 2 , Fig. S1).

We first quantified and profiled BAs in systemic blood, as commonly sampled in humans. Total BA concentration (Fig. 1A) and proportions of unconjugated and conjugated BAs (Fig. 1B) were similar between *foz/foz* and WT mice. The concentrations of individual BA species in systemic blood were not significantly different between the 2 groups (Fig. 1C), although the concentrations of CA and DCA tended to be respectively higher and lower in *foz/foz* mice. The only significant difference was the higher proportion of primary BAs in *foz/foz* compared to WT mice (Fig. S4A); hence there was a lower ratio of secondary to primary BAs in the systemic blood in *foz/foz* mice (Fig. 1D).

As the systemic BA composition was not significantly altered, we focused on the enterohepatic cycle and profiled BA species in bile and portal blood. In bile, the total BA concentration was similar between *foz/foz* and WT mice (Fig. 2A) and was composed exclusively of tauro-conjugated BAs (Fig. 2B). The proportion of primary BAs was higher in *foz/foz* than in WT mice (Fig. S4B), mainly due to a 2-fold higher concentration of CA (Fig. 2C). Accordingly, the proportion of secondary BAs was lower in *foz/foz* compared to WT mice (Fig. S4B), owed to a lower DCA concentration (Fig. 2C). Hence, the ratio of secondary to primary BAs was 3-fold lower in bile of *foz/foz* than of WT mice (Fig. 2D).

In portal blood, the total BA concentration was significantly lower in *foz/foz* than in WT mice (Fig. 2E), with similar proportions of conjugated BAs (Fig. 2F) but with a global reduction

in the concentration of all primary and secondary BA species (although not significant for CA and α MCA, Fig. 2G). The proportion of secondary BAs was lower in the portal blood of *foz/foz* than WT mice (Fig. S4C) and, consequently, the ratio of secondary to primary BAs was reduced in the portal blood of *foz/foz* mice (Fig. 2H).

Altogether, these data suggest that BA composition is significantly altered in the enterohepatic circulation of *foz/foz* mice with NASH, with a depletion in secondary BAs in bile and portal blood. Interestingly, among all BAs considered, DCA is the only BA whose concentration is significantly different both in the bile and portal blood. By contrast, there is no significant difference in systemic BA profile, indicating that the analysis of BAs in systemic blood is not representative of the enterohepatic BA composition.

In mice with NASH, hepatic FXR signaling is reduced and BA synthesis through the classical pathway increased

Since FXR regulates BA synthesis and transport, we investigated FXR signaling in the ileum and the liver. When BAs activate FXR in enterocytes, FGF15 is produced, released in portal blood and stimulates the hepatic FGFR4- β Klotho complex, thereby inhibiting hepatic BA synthesis.⁵ In accordance with similar amounts of biliary BAs secreted by the liver (Fig. S5A-C), the ileal expression of FXR target genes, small heterodimer partner (SHP) and FGF15, as well as the FGF15 portal concentration were similar between *foz/foz* and WT mice (Fig. 3A-B). The similar ileal gene expression of BA transporters, modulated by FXR activa-

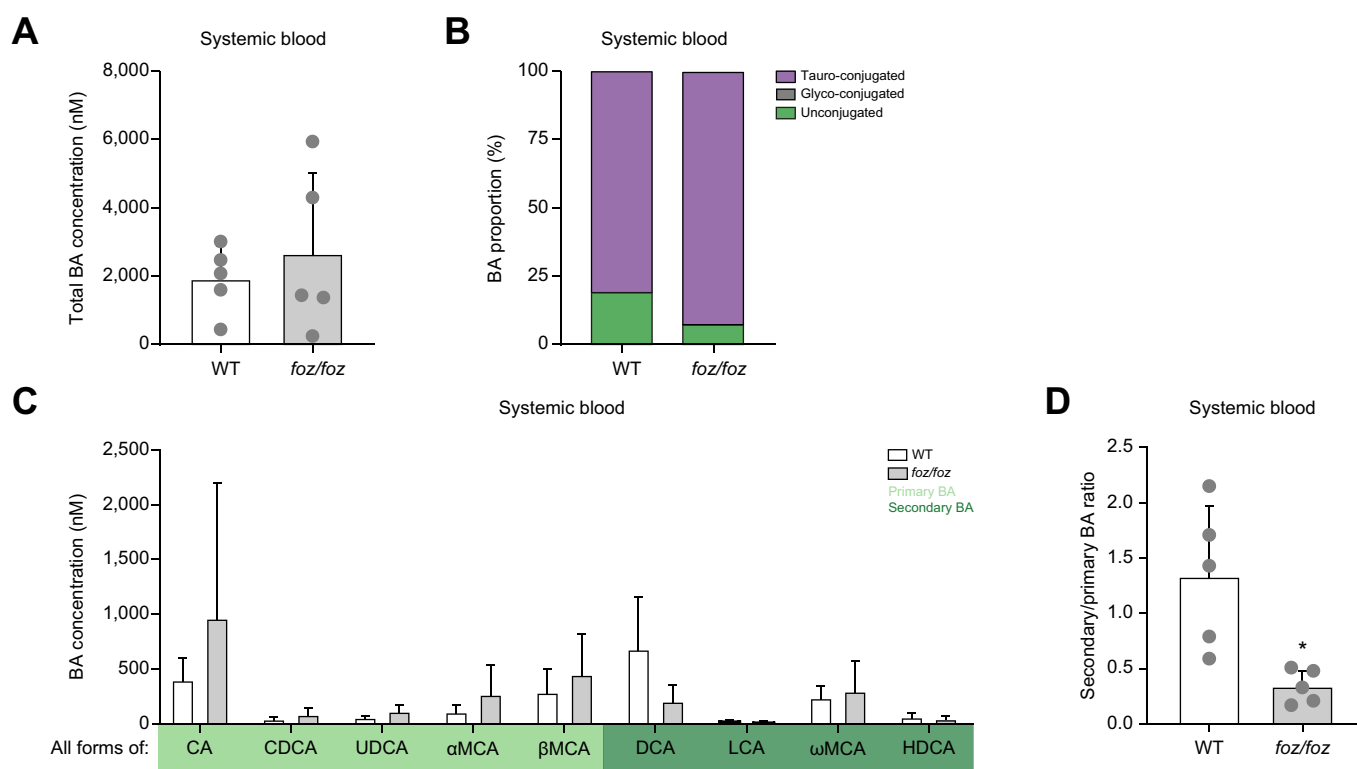


Fig. 1. HFD-fed *foz/foz* mice with NASH do not have an altered systemic BA profile. (A) Total BA concentrations, (B) proportions of conjugated and unconjugated BAs and (C) individual BA concentrations (sum of conjugated and unconjugated BA) and (D) ratio of secondary to primary BAs in systemic plasma of HFD-fed *foz/foz* and WT mice ($n = 5$ /group). Mean $\pm$ standard deviation. Unpaired 2-tailed t test: * $p < 0.05$. BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; HFD, high-fat diet; LCA, lithocholic acid; α MCA, α -muricholic acid; β MCA, β -muricholic acid; ω MCA, ω -muricholic acid; NASH, non-alcoholic steatohepatitis; UDCA, ursodeoxycholic acid; WT, wild-type.

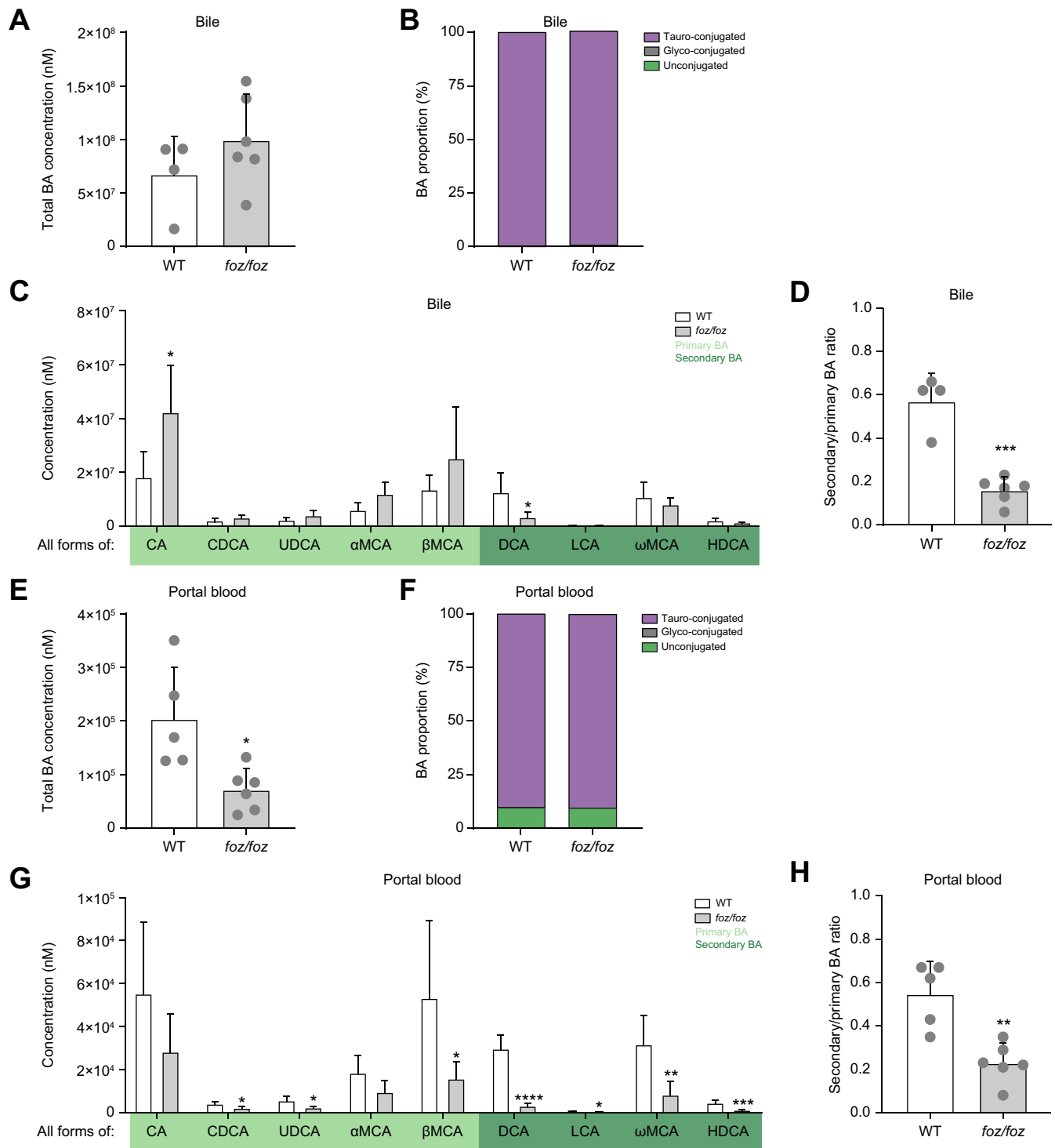


Fig. 2. HFD-fed *foz/foz* mice with NASH have an altered enterohepatic BA composition. Total BA concentrations, proportions of conjugated and unconjugated BAs, individual BA concentrations (sum of conjugated and unconjugated BA) and ratio of secondary to primary BAs in bile (A-D) and portal plasma (E-H) of HFD-fed *foz/foz* and WT mice ($n = 4-6$ /group). Mean $\pm$ standard deviation. Unpaired 2-tailed t test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$. BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HDCA, hydoxycholic acid; HFD, high-fat diet; LCA, lithocholic acid; α MCA, α -muricholic acid; β MCA, β -muricholic acid; ω MCA, ω -muricholic acid; NASH, non-alcoholic steatohepatitis; UDCA, ursodeoxycholic acid; WT, wild-type.

tion,³ and similar fecal BA loss in *foz/foz* and WT mice indicates that intestinal BA reabsorption was not altered (Fig. 3C-D). In hepatocytes, BAs also activate FXR to inhibit BA synthesis in a SHP-dependent manner.⁶ Compared to WT mice, lower hepatic

SHP expression in *foz/foz* mice supported a reduction in hepatic FXR signaling (Fig. 3E), in accordance with lower total BA concentration in portal blood (Fig. 2E). The expression of CYP7A1 and CYP8B1, key enzymes of the classical pathway for BA

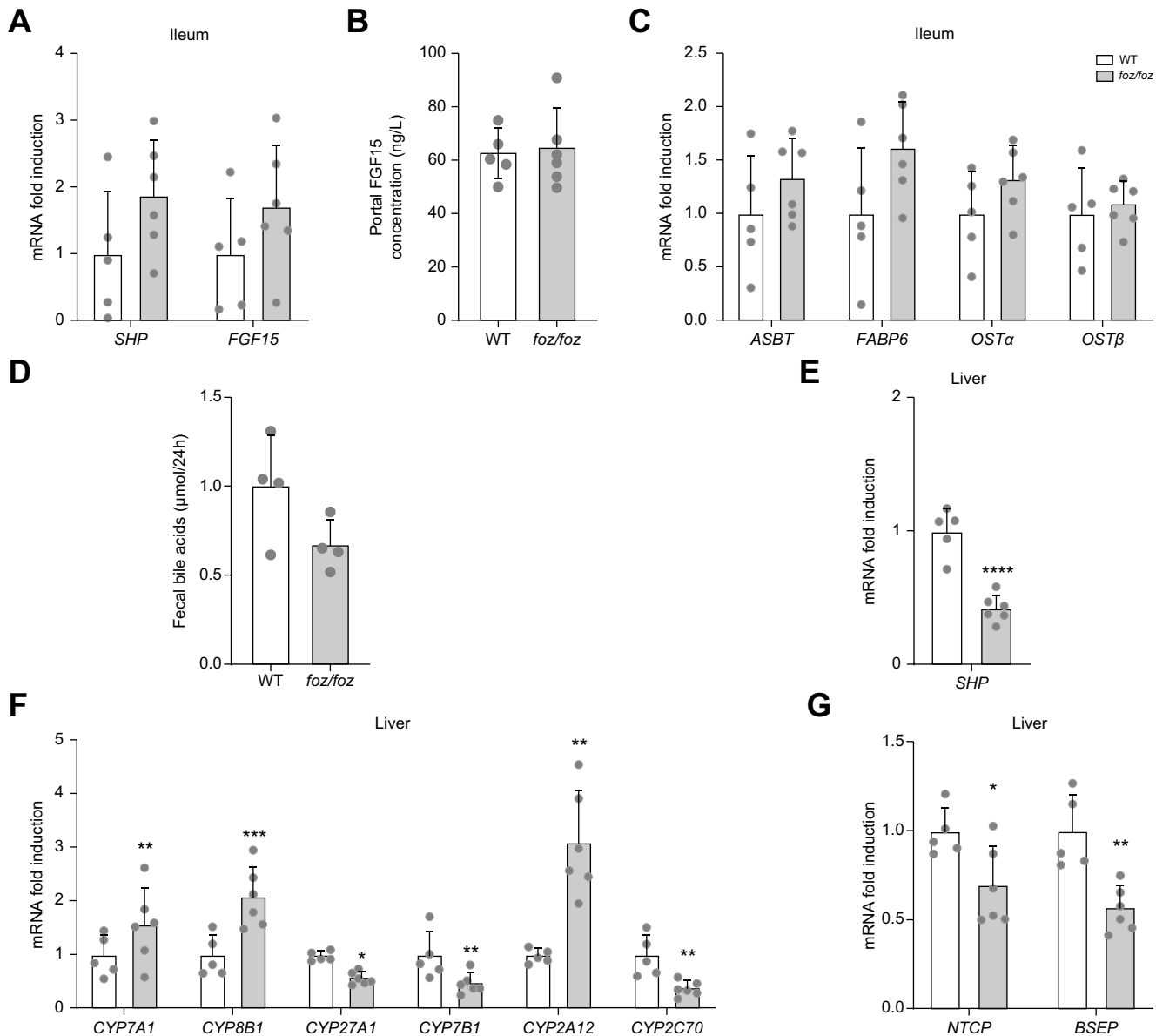


Fig. 3. In HFD-fed *foz/foz* mice with NASH, hepatic FXR signaling is reduced and BA synthesis through the classical pathway increased. (A) Ileal expression of genes involved in BA metabolism, (B) concentration of FGF15 in the portal plasma and (C) ileal gene expression of BA transporters in HFD-fed *foz/foz* and WT mice ($n = 5-6$ /group). (D) Total fecal BA loss per day ($n = 4$ /group). Gene expression of (E) SHP, enzymes involved in BA (F) metabolism and (G) transport in the liver of HFD-fed *foz/foz* and WT mice ($n = 5-6$ /group). Mean $\pm$ standard deviation. Unpaired 2-tailed t test: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. BA, bile acid; HFD, high-fat diet; NASH, non-alcoholic steatohepatitis; WT, wild-type.

synthesis, was accordingly upregulated and that of CYP27A1 and CYP7B1, key enzymes of the alternative pathway, downregulated in *foz/foz* livers (Fig. 3F). Altogether, this supports a low FXR stimulation and an increased BA synthesis through the classical pathway in *foz/foz* mice. Interestingly, CYP2A12 and CYP2A22 were induced in the liver of *foz/foz* mice (Fig. 3F & Fig. S6A). In the mouse liver, CYP2A12 (and likely CYP2A22 which is highly homologous) is the BA 7α -hydroxylase that transforms secondary DCA and LCA back to primary CA and CDCA, respectively.³⁴ In addition, CYP2C70 (that forms α - and β -MCA^{35,36}), the Na⁺-taurocholate cotransporting polypeptide (NTCP) and the bile salt export pump (BSEP) were downregulated in *foz/foz* livers (Fig. 3F-G).

Thus, FXR signaling is reduced in the liver of mice with NASH, but not in the ileum, and as expected associates with increased hepatic BA synthesis.

TGR5 activation is reduced in mice with NASH

Because DCA and LCA – considered natural TGR5 agonists – were 8-fold less concentrated in the portal blood of *foz/foz* compared to WT mice (Fig. 4A), we questioned the amount of TGR5 ligands in portal blood by using a TGR5 activation reporter assay. The *foz/foz* portal plasma was indeed less effective in activating TGR5 than the WT portal plasma (Fig. 4B). TLCA was used as a positive control and the TGR5 dependency was proven, as no signal was detected in cells not transfected with the TGR5 plasmid (Fig. S3).

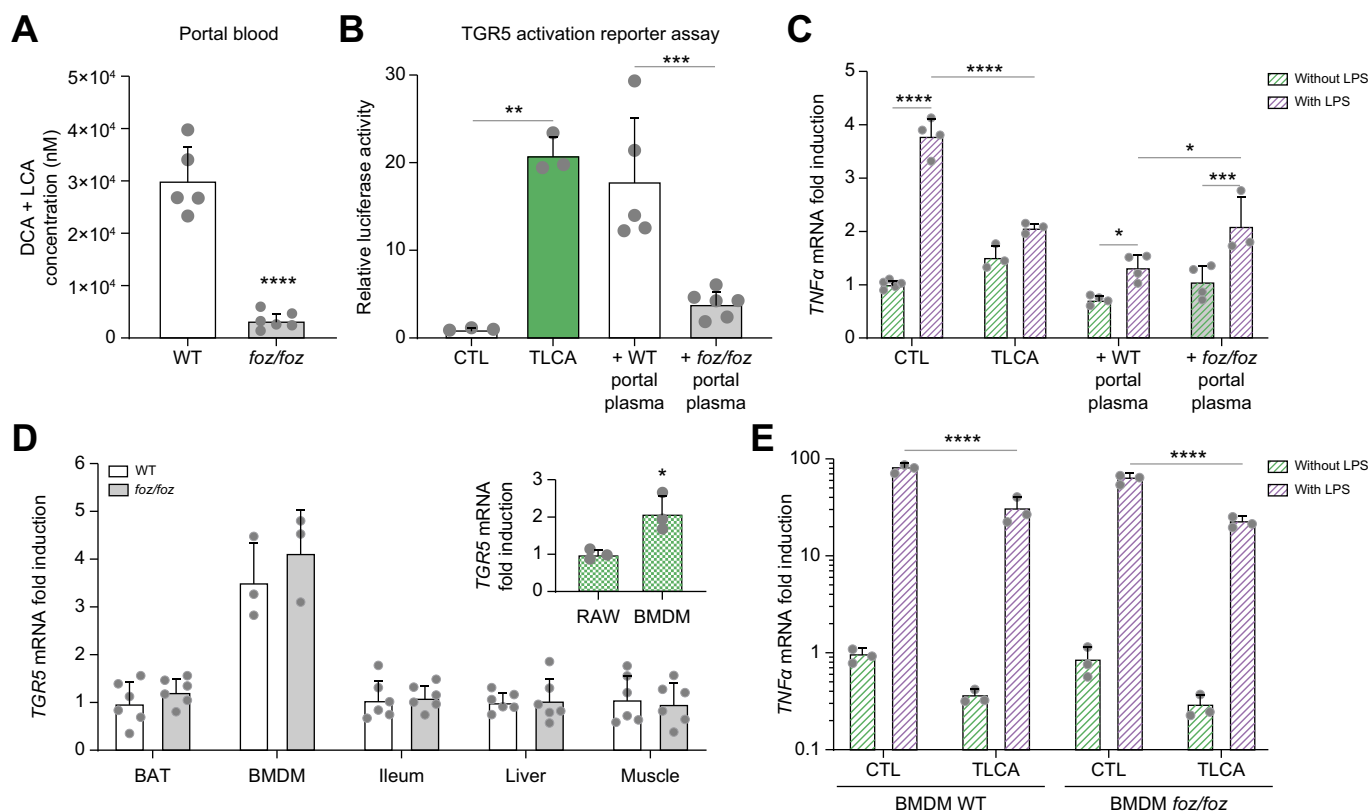


Fig. 4. TGR5 activation is reduced in HFD-fed *foz/foz* mice with NASH. (A) Total concentration of LCA and DCA in portal plasma of HFD-fed *foz/foz* and WT mice ($n = 5-6$ /group). (B) TGR5 activation by medium containing TLCA (used as a positive control), WT or *foz/foz* portal plasma in the cell reporter assay. (C) TNF α gene expression in RAW264.7 macrophages stimulated or not with LPS and treated with medium (CTL) containing TLCA, WT or *foz/foz* portal plasma ($n = 3-4$ /group). (D) TGR5 gene expression in brown adipose tissue (BAT), BMDMs, distal ileum, liver and quadriceps of WT and *foz/foz* mice ($n = 3-6$ /group), normalized to TGR5 gene expression in BAT of WT mice. (E) TNF α gene expression in BMDMs of WT and *foz/foz* mice stimulated or not with LPS and treated with medium (CTL) containing TLCA, normalized to TNF α gene expression in untreated BMDMs WT. For each condition, TNF α was significantly upregulated by LPS. Mean $\pm$ standard deviation. Unpaired 2-tailed *t* test for (A) and for comparison between RAW264.7 and BMDM in (D), One-way ANOVA followed by *post hoc* Bonferroni correction for (C), (D) and (E). * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. BMDM, bone marrow-derived macrophage; DCA, deoxycholic acid; HFD, high-fat diet; LCA, lithocholic acid; LPS, lipopolysaccharide; NASH, non-alcoholic steatohepatitis; TLCA, tauro-lithocholic acid; WT, wild-type.

As TGR5 activation is known to reduce the pro-inflammatory cytokine response in macrophages,^{15,17} we investigated whether a deficit in TGR5 agonists in portal blood promotes inflammation in *foz/foz* mice. In RAW264.7 macrophages, the lipopolysaccharide (LPS)-induced upregulation of tumor necrosis factor α (TNF α) was significantly blunted when cells were exposed to TLCA or to portal plasma of *foz/foz* or WT mice (Fig. 4C). However, this reduction was of a lesser magnitude when macrophages were exposed to portal plasma of *foz/foz* mice (Fig. 4C). Thus, compared to WT, the *foz/foz* portal plasma is less effective in reducing LPS-induced expression of pro-inflammatory cytokines, in line with higher macrophage activation in the liver of *foz/foz* mice (Fig. S1F).

Of note, TGR5 was similarly expressed in brown adipose tissue (BAT), bone-marrow derived macrophages (BMDMs), ileum, liver and muscle of *foz/foz* and WT mice (Fig. 4D). Also, the *foz/foz* genotype did not alter TGR5 functionality as LPS similarly enhanced TNF α gene expression and TLCA blunted it to the same extent in BMDMs from both genotypes (Fig. 4E). As *foz/foz* mice carry a mutation in the *Alms1* gene involved in primary cilium function³⁰ and as TGR5 might be found on primary cilia of cholangiocytes,³⁷ we verified the hepatic bile flow, the absence

of ductular reaction and the gene expression of the apical sodium-dependent BA transporter (ASBT) in the liver of these mice: all the parameters were similar to WT mice (Fig. S5A-F), ruling out that *foz/foz* mice are ill equipped for TGR5 signaling.

In conclusion, a lower concentration of circulating TGR5 agonists in the portal blood of mice with NASH dampens TGR5 activation.

Alterations in BA signaling are not model specific

To determine whether BA alterations are model specific, we compared C57BL/6J mice fed a western and high-fructose diet (WDF) for 20 weeks to mice fed a ND. WDF-fed mice became obese and insulin resistant (Fig. S2A-C). Glucose tolerance was globally maintained by hyperinsulinemia (Fig. S2B-C). Similarly to *foz/foz* mice,³¹ WDF-fed mice had a higher liver weight and lower liver density (a surrogate for liver steatosis) and recapitulated all histological features of NASH, resulting in a NAS >7 , whereas ND-fed mice had normal livers (Fig. S2D-G).

Reminiscent of the changes in *foz/foz* livers, FXR was less activated in the liver of WDF-fed mice, as shown by a down-regulation of SHP expression (Fig. 5A). Accordingly, the gene expression of *CYP7A1* was upregulated, and that of *CYP8B1* and

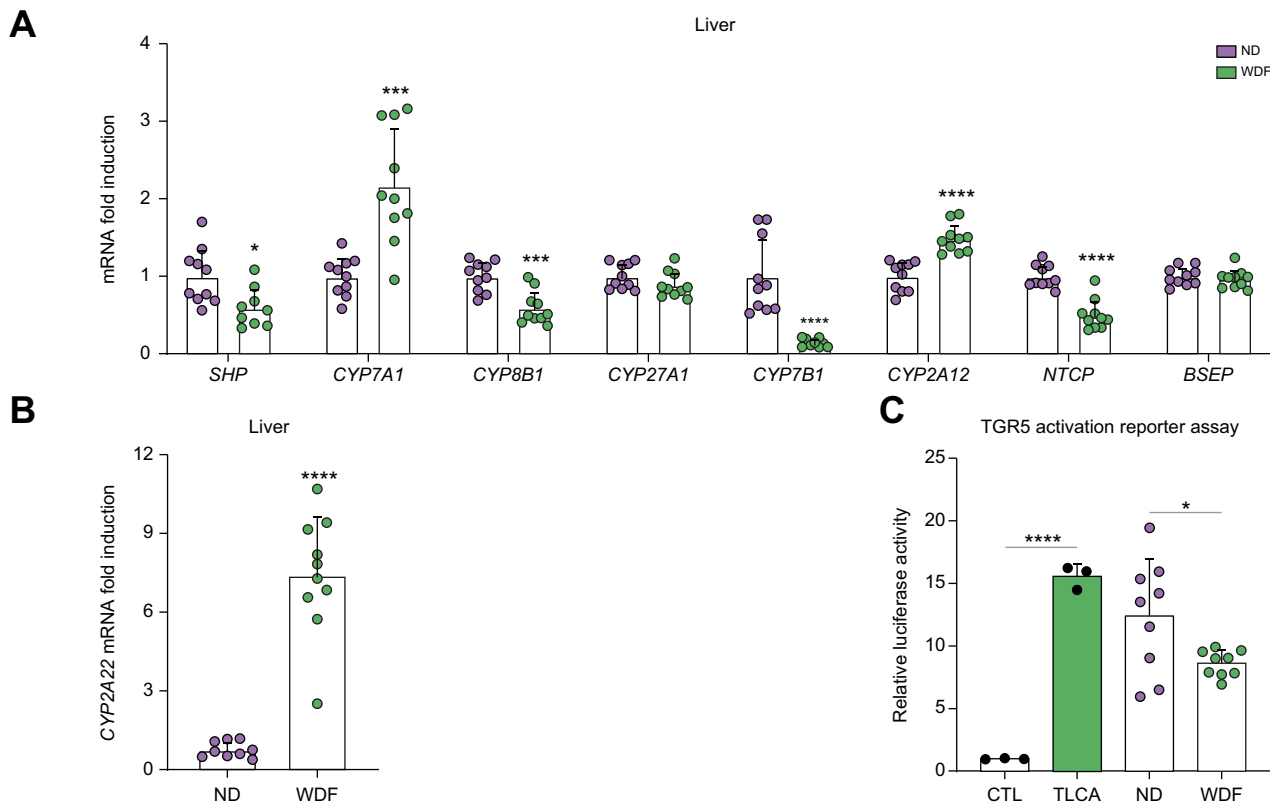


Fig. 5. Alterations in FXR and TGR5 signaling are not model specific. (A-B) Hepatic expression of genes involved in BA metabolism and transport. (C) TGR5 activation by medium (CTL) containing TLCA, portal plasma of C57BL/6J fed a ND or a WDF for 20 weeks (n = 9-10/group) in the cell reporter assay. Mean $\pm$ standard deviation. Unpaired 2-tailed *t* test for (A) and (B) and One-way ANOVA followed by *post hoc* Bonferroni correction for (C): **p* < 0.05; ****p* < 0.001 and *****p* < 0.0001. BA, bile acid; ND, normal diet; TLCA, tauro-lithocholic acid; WDF, western high-fructose diet.

CYP7B1 downregulated in the liver of WDF- compared to ND-fed mice (Fig. 5A). Notably, CYP2A12 and CYP2A22, converting secondary into primary BAs, were also upregulated in the liver of WDF-fed mice (Fig. 5A-B). Strikingly, TGR5 was also less activated by the portal plasma of WDF- than ND-fed mice (Fig. 5C).

Altogether, these results confirm that FXR and TGR5 signaling are reduced in another model of NASH and that the alterations are independent of the genetic background and of the *Alms1* mutation.

Modulation of BA composition and recovery of FXR and TGR5 signaling prevent obesity, insulin resistance and NASH

Then, we hypothesized that the altered portal BA composition that compromises FXR and TGR5 signaling contributes to NASH pathogenesis. To test this, we modulated BAs in *foz/foz* mice to increase FXR and TGR5 activation and study the effect on glucose metabolism and liver pathology. Since DCA concentration was consistently low in *foz/foz* bile and portal blood (Fig. 2C&G), we supplemented the HFD with 0.03% and 0.1% of DCA for 12 weeks and compared them with *foz/foz* mice fed a HFD without supplementation.

The supplementation with 0.1%, but not 0.03% DCA, increased total BA concentrations in the portal blood of *foz/foz* mice (Fig. 6A). Feeding *foz/foz* mice 0.03% DCA failed to significantly increase portal DCA but increased CA (Fig. 6B). The high expression of CYP2A12 and CYP2A22, which convert

DCA to CA, in the liver of *foz/foz* mice (Fig. 6C and Fig. S7A) could explain the increased CA concentration (Fig. 6B). By contrast, 0.1% DCA increased DCA and CA portal concentrations in *foz/foz* mice (Fig. 6B). In accordance, FXR was activated, as shown by the induction of SHP and the downregulation of CYP7A1 and CYP8B1 in the liver of *foz/foz* mice treated with 0.1% DCA (Fig. 6D). As expected, because of the higher gut BA concentration, FXR was also activated in enterocytes, as shown by the upregulation of SHP and FGF15 in *foz/foz* mice treated with 0.1% DCA (Fig. S7B). In addition, while slightly (or not) modified by 0.03% DCA supplementation, the ratio of secondary to primary BAs, the concentration of DCA and LCA considered as natural TGR5 agonists, and the TGR5 ligand activity were strikingly increased in the portal blood of *foz/foz* mice supplemented with 0.1% DCA (Fig. 6E-G). Thus, supplementation with the secondary BA DCA modulated BA profile and in consequence, restored FXR and TGR5 activation in *foz/foz* mice.

We then investigated the impact of the BA modulation on NASH and associated metabolic features. Feeding the *foz/foz* mice a HFD supplemented with 0.03% DCA did not alter body weight gain, fat mass, glucose intolerance, fasting hyperglycemia and hyperinsulinemia (Fig. 7A-C & Fig. S7C-G). By contrast, DCA 0.1% supplementation significantly reduced body weight gain and fat mass in HFD-fed *foz/foz* mice, in spite of slightly increased food intake compared to untreated *foz/foz* mice (Fig. 7A & Fig. S7C-E). Glucose intolerance, fasting glycemia and

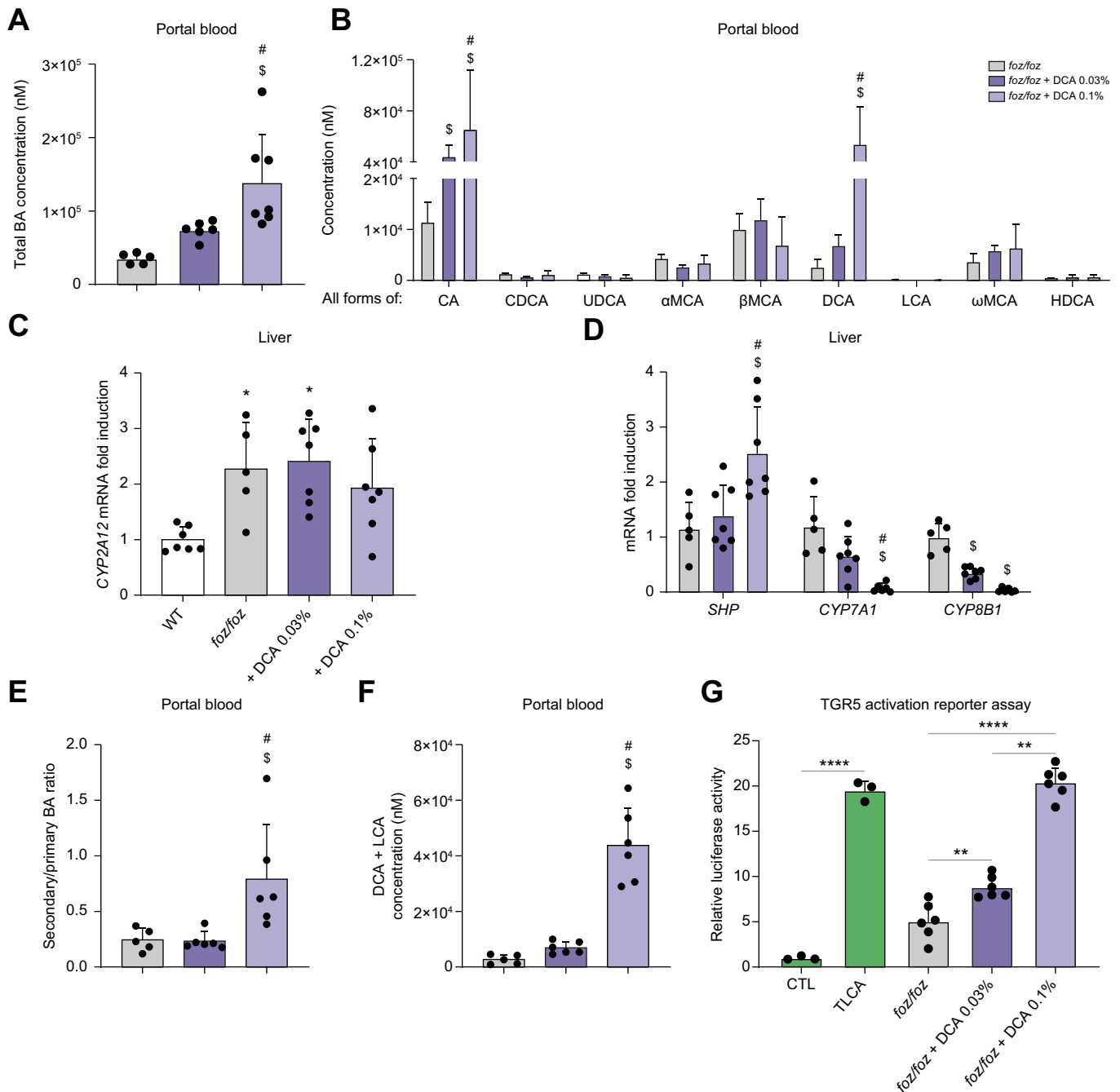


Fig. 6. Dietary 0.1% DCA modulates BA composition and restores FXR and TGR5 signaling in HFD-fed *foz/foz* mice. (A) Total BA and (B) individual BA concentrations (sum of conjugated and unconjugated BA) in portal plasma of HFD-fed *foz/foz*, *foz/foz* + DCA 0.03% or *foz/foz* + DCA 0.1% mice ($n = 5/\text{group}$). Hepatic gene expression of (C) CYP2A12, (D) SHP and enzymes involved in BA synthesis. (E) Ratio of secondary to primary BAs and (F) total concentration of LCA and DCA in portal plasma ($n = 5-6/\text{group}$) and (G) TGR5 activation by medium (CTL) containing TLCA, portal plasma of *foz/foz*, *foz/foz* + DCA 0.03% or *foz/foz* + DCA 0.1% mice ($n = 6/\text{group}$) in the cell reporter assay. Mean $\pm$ standard deviation. One-way ANOVA followed by *post hoc* Bonferroni correction. Statistical significance ($p < 0.05$) is represented by * when compared to WT; \$ when compared to *foz/foz* and # when compared to *foz/foz* + DCA 0.03% for (A) to (F) and by ** $p < 0.01$ and **** $p < 0.0001$ for (G). BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; HFD, high-fat diet; LCA, lithocholic acid; αMCA, α-muricholic acid; βMCA, β-muricholic acid; ωMCA, ω-muricholic acid; TLCA, tauro-lithocholic acid; UDCA, ursodeoxycholic acid; WT, wild-type.

insulinemia were also reduced by DCA 0.1% (Fig. 7B-C & Fig. S7F-G).

Low 0.03% DCA supplementation partly reduced steatosis and corrected liver density but did not significantly change the severity of liver disease, as shown by a marginal reduction in the

histological NAS (Fig. 7D-G). Accordingly, serum transaminase levels were unchanged compared to HFD-fed *foz/foz* mice (Fig. 7H & Fig. S7H). By contrast, 0.1% DCA reduced the liver weight of HFD-fed *foz/foz* mice and protected them from hepatic steatosis and hepatocellular ballooning (Fig. 7D-G). Despite a

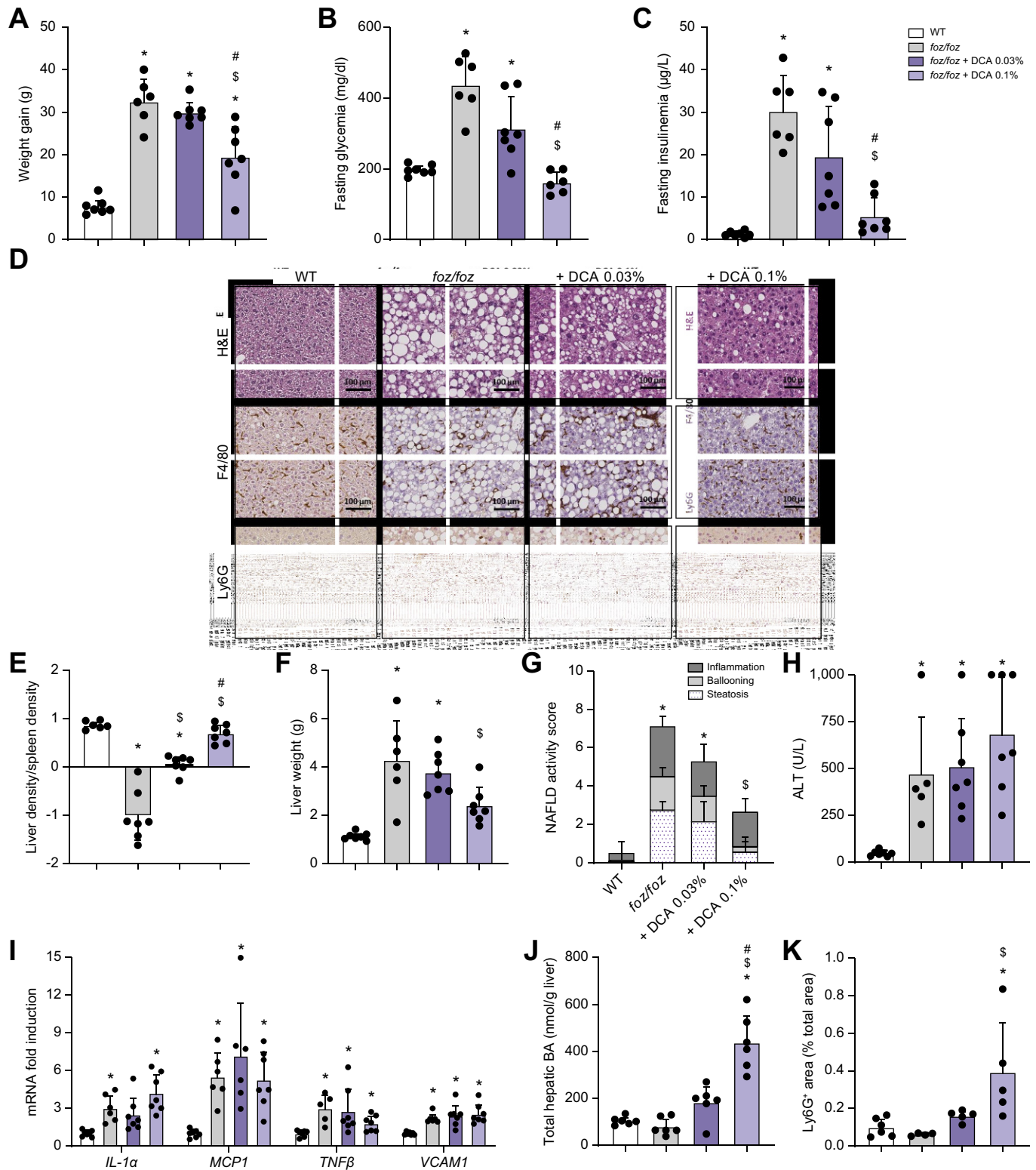


Fig. 7. Restoration of the FXR and TGR5 signaling prevents obesity, insulin resistance and NASH in HFD-fed *foz/foz* mice. (A) Body weight gain, (B) fasting glycemia and (C) insulinemia of WT, *foz/foz*, *foz/foz* + DCA 0.03% and *foz/foz* + DCA 0.1% mice (n = 6-7/group). (D) Representative H&E, F4/80 and Ly6G staining of liver sections (bar size: 100 μm). (E) Liver density, (F) weight and (G) NAS, (H) plasmatic ALT levels, (I) hepatic expression of pro-inflammatory genes, (J) hepatic concentration of total BA and (K) quantification of neutrophils in the liver of WT, *foz/foz*, *foz/foz* + DCA 0.03% and *foz/foz* + DCA 0.1% mice (n = 4-7/group). Mean ± standard deviation. One-way ANOVA followed by *post hoc* Bonferroni correction for (A-F) and (H-K), and Kruskal-Wallis followed by Dunn's multiple comparisons test for (G). Statistical significance ($p < 0.05$) is represented by * when compared to WT; \$ when compared to *foz/foz* and # when compared to *foz/foz* + DCA 0.03%. ALT, alanine aminotransferase; BA, bile acid; DCA, deoxycholic acid; HFD, high-fat diet; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; WT, wild-type.

reduction in the number of macrophage aggregates (Fig. 7D, F4/80 staining), the histological inflammation score, hepatic *Il1 β* (interleukin-1 β), *Mcp1* (monocyte chemoattractant protein-1), *Tnfx* and *Vcam1* (vascular cell adhesion molecule 1) mRNA and transaminase levels were unchanged by 0.1% DCA (Fig. 7G-I & Fig. S7H). In the light of high hepatic BA concentration, neutrophilic infiltration and increased number of apoptotic hepatocytes and mitotic figures (Fig. 7D&J-K, Ly6G staining), we suspected a BA-induced hepatotoxicity.³⁸ Owing to the pronounced effect on steatosis and ballooning, the treatment significantly decreased NAS, such that only 1 out of the 7 *foz/foz* mice treated with 0.1% DCA presented NASH vs. 6 out of the 6 untreated *foz/foz* mice (Fig. 7G).

Discussion

In this study, we characterized the enterohepatic BA profile, metabolism and signaling in different mouse models of NASH. Our data showed that BA composition was profoundly altered in bile and portal blood of mice with NASH. Indeed, concentrations of total BAs as well as of secondary BAs were lower in mice with NASH compared to controls that do not have liver disease. Hence, BA perturbations resulted in a reduction of BA signaling through FXR and TGR5, with consequences on BA, glucose and lipid metabolism, as well as on the inflammatory response. In addition, we demonstrated the contribution of BAs to NASH pathogenesis, as correcting BA composition restored FXR and TGR5 signaling and prevented the development of NASH and associated metabolic disorders.

Herein, we used validated models of NASH in which, contrary to numerous other mouse models,^{39–41} mice exhibit the histological hepatic features of human NASH in a dysmetabolic context.²⁹ By comparing hyperphagic *foz/foz* mice and their WT littermates fed the same HFD, we isolated the effects due to dysmetabolism and liver disease from those directly influenced by food composition. By comparing C57BL/6J mice fed a WDF and C57BL/6J fed a ND, we validated that the alterations were independent of the genetic background and the *Alms1* mutation. Strikingly, all the perturbations observed in HFD-fed *foz/foz* mice were validated in WDF-fed C57BL/6J mice.

Mice with NASH have less total BAs in their portal blood, but not in their bile. The concentrations of BAs arriving to the liver are lower in mice with NASH and, consequently, FXR is less activated, SHP downregulated and CYP7A1 upregulated. Thus, increased primary BA production through the classical synthesis compensates for low hepatic exposure to BAs, but completely changes the balance between primary and secondary BAs secreted by the liver. Our data in preclinical models are similar to reports of upregulated CYP7A1 expression and increased BA synthesis in livers of NASH vs. no-NASH patients,^{23,25,27} which indicate reduced FXR signaling.

The low concentration of total BAs in the portal blood of mice with NASH could be caused by reduced intestinal BA reabsorption. Conjugated BAs are mostly actively reabsorbed by the ASBT into the enterocytes, while unconjugated secondary BAs are mostly passively reabsorbed through diffusion across the intestinal epithelium.³ Logically, loss of ASBT-driven absorption drastically increases BA fecal loss and hepatic synthesis and, consequently, changes the BA pool composition.⁴² However, BA reabsorption was similar in HFD-fed *foz/foz* and WT mice, invalidating this hypothesis.

The most striking and constant modification observed in mice with NASH is the low concentration of secondary BAs in all compartments. A defective production of secondary BAs by the gut microbiota might be an explanation. Conjugated BAs can be deconjugated by the bile salt hydrolase activity widely distributed in the commensal bacteria of the small intestine and colon.⁴³ Then, unconjugated primary BAs, under the operation of enzymes produced by anaerobic bacteria, may undergo transformation into 7 α -dehydroxylated secondary BAs.² Thereby, a reduced 7 α -dehydroxylase activity could explain the lower concentration of secondary BAs. Gut microbiota tightly control BA metabolism, as in germ-free and antibiotic-treated mice, the BA pool is depleted in secondary BAs.⁴⁴ However, in our experiment, HFD-fed WT and *foz/foz* littermates were co-housed, an experimental setting that levels out microbial differences.⁴⁵ In addition, their ratios of conjugated vs. unconjugated BAs in portal blood, as well as their levels of intestinal FXR activation (directly linked to active BA reabsorption) were similar and invalidate massive perturbation in BA gut deconjugation. By contrast, the BA 7 α -hydroxylase CYP2A12 and CYP2A22, expressed in murine hepatocytes, are substantially upregulated in the liver of HFD-fed *foz/foz* and WDF-fed C57BL/6J mice compared to their respective controls. Back transformation of secondary into primary BAs likely explains the shift in BA proportions. In support of this, in mice receiving a 0.03% DCA-supplemented diet, the constant DCA dietary supply does not increase the DCA concentration but significantly increases CA, even though *de novo* synthesis through CYP7A1 is repressed. With supraphysiological supply (DCA 0.1%), there is a rise in DCA concentration, but accompanied by a major increase in primary CA concentration despite the silencing of the enzymatic machinery for *de novo* synthesis. The hypothesis that upregulated CYP2A12 contributes to decreased secondary BA concentrations in mice with NASH is reinforced by a recent study reporting that *Cyp2a12* knockout mice had higher proportion of secondary BAs and lower proportion of primary BAs in their BA pool than WT controls.³⁴ In addition, Honda *et al.* suggested that CYP2A22, highly homologous to CYP2A12, could also hydroxylate secondary BAs at the 7 α position.³⁴

Insulin resistance, a risk factor and key player in NAFLD pathogenesis, also influences the metabolism of BAs. Haeusler *et al.* showed that proper insulin signaling inhibits FoxO1, an activator of the sterol 12 α -hydroxylase CYP8B1, and reduces the synthesis of 12 α -hydroxylated CA.⁴⁶ In addition, Legry *et al.* reported that BA alterations were correlated with insulin resistance rather than NASH.²⁷ Herein, we showed that CYP8B1 expression and the biliary concentration of CA were higher in insulin-resistant HFD-fed *foz/foz* mice than in their controls. On the contrary, CYP8B1 was downregulated in the liver of WDF-fed C57BL/6J mice. This could be explained by the less pronounced insulin resistance in WDF-fed C57BL/6J than in HFD-fed *foz/foz* mice. Although treatment with DCA 0.1% improved insulin resistance in HFD-fed *foz/foz* mice and downregulated CYP8B1 expression, the concentration of CA was increased (most likely by a 7 α -hydroxylation of DCA in the liver of mice with NASH).

Conversely, BAs fine tune insulin responsiveness. Indeed, FXR and TGR5 regulate glucose and lipid metabolism as well as inflammation, so that pharmacological agonists are proposed for the treatment of obesity, type 2 diabetes and NASH.^{4,47} Here, it is conceivable that low FXR and TGR5 signaling, as a consequence of the altered BA composition in mice with NASH could worsen insulin resistance. Supporting this, we showed that the

restoration of BA composition increases FXR and TGR5 signaling, prevents hyperglycemia, intrahepatic lipid accumulation and insulin resistance in HFD-fed *foz/foz* mice.

As DCA concentrations were notably lower in mice with NASH, we supplemented their HFD with DCA 0.03% or 0.1%. When administered for 1 week to C57BL/6J mice, these concentrations have been shown to significantly increase secondary BAs without raising total BA concentrations or inducing side effects or toxicity.^{48,49} In HFD-fed *foz/foz* mice, DCA 0.03% was not sufficient to correct low secondary BA concentrations and to reset TGR5 and FXR signaling, likely because of the induction of hepatic 7 α -rehydroxylation of DCA to CA. Accordingly, DCA 0.03% did not change liver pathology and associated metabolic parameters in HFD-fed *foz/foz* mice. By contrast, DCA 0.1%, supposedly because the level of DCA overwhelms the enzymatic activities of CYP2A12 and CYP2A22, augmented secondary BA concentrations and, as a result, TGR5 and FXR activation in HFD-fed *foz/foz* mice. Ensuing from these changes, glucose tolerance, insulin resistance and obesity improved and at liver histology, steatosis and ballooned hepatocytes vanished. Also, macrophage aggregates were far less numerous, supportive of decreased lipotoxicity. By contrast, these livers remain inflamed, although with a switch from focal monocyte-macrophage aggregates in HFD-fed *foz/foz* mice to a more diffuse polymorphonuclear neutrophil infiltration in those supplemented with DCA 0.1%. In line with a significant (5-fold) increase in liver total BA concentration in mice that received 0.1% DCA and with persistent elevation in transaminases, we suspect BA-induced toxicity concomitant to NASH improvement. Given the positive anti-metabolic syndrome and anti-

NASH effects of DCA, it will be of great interest to test, as a proof of principle, the therapeutic effects of strategies that restore the BA profile without increasing the BA pool size in NASH.

One observation of importance regarding translational studies is that the enterohepatic cycle sees the main changes in BAs. Hence, as shown here and by others,⁵⁰ the alterations are not recapitulated in systemic blood usually sampled in human studies. First, BAs are at least 100-fold less concentrated in the systemic than in the portal blood and, second, the systemic and the portal BA profiles are not similar. Indeed, while the concentrations of nearly all BA species were changed in portal blood of mice with NASH, no corresponding alterations were observed in systemic blood. In addition, the fecal BA excretion is similar in mice with or without NASH. Hence, in the clinic, sampling of systemic blood or feces could provide misleading information with regard to the BA pool and its influence on metabolism and inflammation in the enterohepatic hub. This likely also explains the discordant reports among BA studies in patients with NASH.²⁸

Targeting BA receptors through FXR, TGR5 or dual agonists led to promising results regarding NASH treatment, but this was at the cost of secondary effects, such as decreased HDLc, increased cholesterol, LDLc, pruritus and gallbladder volume.⁴⁷ This study clearly demonstrates that enterohepatic BA alterations significantly contribute to the development of NASH in relevant pre-clinical models. In the light of our data, understanding how the enterohepatic BA pool, metabolism and signaling are changed in patients could be the key to developing an effective treatment without side effects and therefore should be fully explored.

Abbreviations

ASBT, apical sodium-dependent BA transporter; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; CYP2A12, bile acid 7 α -hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP7A1, cholesterol 7 α -hydroxylase; CYP7B1, oxysterol 7 α -hydroxylase; CYP8B1, sterol 12 α -hydroxylase; DCA, deoxycholic acid; FABP6, fatty acid binding protein 6; FGF15, fibroblast growth factor 15; FGFR4, fibroblast growth factor receptor 4; FXR, Farnesoid X receptor; GLP-1, glucagon-like peptide-1; HFD, high-fat diet; LCA, lithocholic acid; LPS, lipopolysaccharide; α MCA, α -muricholic acid; β MCA, β -muricholic acid; ω MCA, ω -muricholic acid; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; ND, normal diet; OGTT, oral glucose tolerance test; OST, organic solute transporter; SHP, small heterodimer protein; TGR5, Takeda G-protein coupled receptor 5; TLCA, tauro-lithocholic acid; TNF α , tumor necrosis factor α ; WDF, western and high-fructose diet; WT, wild-type.

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Conflict of interest

The authors declare that they have no conflict of interest in relation to this work to disclose.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

JG and IAL conceived and designed the study. JG performed the *in vivo* experiments, prepared the material, performed biochemical, histological

and gene expression analyses. AT managed the LC-MS/MS analyses. JG and LAC optimized and performed the *in vitro* experiments. MN acquired and analyzed micro-CT data. CS provided help with histological analyses. JG, BS, LBB, AT and IAL critically reviewed and analyzed the data. JG and IAL wrote the manuscript. All authors read and revised the manuscript.

Data availability statement

All data that support the findings in this study can be found within this article. Nevertheless, raw data on bile acid analysis are available from the corresponding author upon a reasonable request.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2021.100387>.

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Author names in bold designate shared co-first authorship

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