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BASIC SCIENCE ARTICLE

Induction of fecal cholesterol excretion is not effective for the treatment of hyperbilirubinemia in Gunn rats

Maaïke Blankestijn¹, Ivo P. van de Peppel¹, Ales Dvorak², Nikola Capkova², Libor Vitek², Johan W. Jonker¹ and Henkjan J. Verkade¹

BACKGROUND: Unconjugated hyperbilirubinemia, a feature of neonatal jaundice or Crigler–Najjar syndrome, can lead to neurotoxicity and even death. We previously demonstrated that unconjugated bilirubin (UCB) can be eliminated via transintestinal excretion in Gunn rats, a model of unconjugated hyperbilirubinemia, and that this is stimulated by enhancing fecal fatty acid excretion. Since transintestinal excretion also occurs for cholesterol (TICE), we hypothesized that increasing fecal cholesterol excretion and/or TICE could also enhance fecal UCB disposal and subsequently lower plasma UCB concentrations.

METHODS: To determine whether increasing fecal cholesterol excretion could ameliorate unconjugated hyperbilirubinemia, we treated hyperbilirubinemic Gunn rats with ezetimibe (EZE), an intestinal cholesterol absorption inhibitor, and/or a liver X receptor (LXR) and farnesoid X receptor (FXR) agonist (T0901317 (T09) and obeticholic acid (OCA), respectively), known to stimulate TICE.

RESULTS: We found that EZE treatment alone or in combination with T09 or OCA increased fecal cholesterol disposal but did not lower plasma UCB levels.

CONCLUSIONS: These findings do not support a link between the regulation of transintestinal excretion of cholesterol and bilirubin. Furthermore, induction of fecal cholesterol excretion is not a potential therapy for unconjugated hyperbilirubinemia.

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IMPACT:

- Increasing fecal cholesterol excretion is not effective to treat unconjugated hyperbilirubinemia.
- This is the first time a potential relation between transintestinal excretion of cholesterol and unconjugated bilirubin is investigated.
- Transintestinal excretion of cholesterol and unconjugated bilirubin do not seem to be quantitatively linked.
- Unlike intestinal fatty acids, cholesterol cannot “capture” unconjugated bilirubin to increase its excretion.
- These results add to our understanding of ways to improve and factors regulating unconjugated bilirubin disposal in hyperbilirubinemic conditions.

INTRODUCTION

Unconjugated hyperbilirubinemia, such as present in neonatal jaundice or Crigler–Najjar syndrome, can lead to bilirubin-induced neurotoxicity, kernicterus, and even to death.¹ In the liver, hydrophobic unconjugated bilirubin (UCB) is conjugated by bilirubin UDP glucuronic acid (UGT1A1) to form the more water-soluble bilirubin monoglucuronoside and bilirubin diglucuronoside, facilitating excretion into the bile. UGT1A1 is not only present in the liver but also highly expressed in the human intestine and was found to contribute to conjugation of UCB.² Despite its hydrophobic character, UCB is still partially excreted into the bile during unconjugated hyperbilirubinemia.^{3,4} In Gunn rats, an animal model for Crigler–Najjar disease type 1, around 2–15% of intestinal UCB originates from biliary disposal, while 85–98% is derived from transintestinal UCB excretion.⁴ This makes transintestinal bilirubin excretion the major route of UCB disposal in Gunn rats.^{4–6}

Transintestinal excretion also occurs for cholesterol (TICE) and can be stimulated by activation of several nuclear receptors, including peroxisome proliferator-activated receptor delta, liver X receptor (LXR), and farnesoid X receptor (FXR).^{7–12} Both LXR and FXR are involved in regulating hepatic and intestinal cholesterol metabolism. The ATP-binding cassette sub-family (ABC)G5/G8 heterodimer is expressed on the canalicular membrane of hepatocytes and the apical membrane of enterocytes and regulated by LXR and FXR, where it facilitates the efflux of sterols. The intestinal ABCG5/G8 transporter is at least partially responsible for TICE.^{8,13} The Niemann–Pick C1-like 1 (NPC1L1) protein regulates intestinal cholesterol absorption and inhibition of NPC1L1 by ezetimibe (EZE) induces fecal neutral sterol (FNS) output (cholesterol and its bacterial metabolites) in mice and rats.^{9,11,14} Van de Peppel et al. showed that, under physiological conditions in mice, cholesterol excreted via TICE is largely reabsorbed.¹⁵ Decreasing intestinal cholesterol (re)absorption,

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either directly via an inhibition of NPC1L1 or indirectly via reducing the bile acid pool, resulted in a profound increase in FNS output beyond biliary and dietary input.¹⁵ The underlying mechanism of transintestinal excretion of UCB and that of cholesterol are not fully understood.

Earlier studies showed that plasma UCB decreased by administration of a high-fat diet (HFD) and/or the lipase inhibitor orlistat in Gunn rats.^{5,16–18} The increase in fecal fat excretion was correlated to the decrease in plasma UCB levels.^{16,17} It was demonstrated in a kinetic study using radiolabeled bilirubin that the decrease in UCB upon orlistat was due to an increase in transintestinal excretion of UCB.¹⁶ In addition, feeding a HFD to mice enhanced TICE, resulting in increased FNS excretion.¹⁹ It has been hypothesized that the increase in fecal UCB and subsequent decrease in plasma UCB levels upon higher intestinal fat concentrations is the result of UCB “capturing” by fatty acids, meaning that the reabsorption of UCB is decreased upon its association with non-absorbed fat in the intestinal lumen.^{5,20} In the present study, we tested whether selectively increasing FNS excretion, a different class of lipid, also exerts hypobilirubinemic effects in Gunn rats, similar to what was found by increasing fecal fatty acid excretion. We applied three manipulations to enhance FNS excretion: (1) activation of LXR by T09, a synthetic LXR activator, (2) activation of FXR by obeticholic acid (OCA), and/or (3) inhibition of intestinal cholesterol absorption using EZE. These different approaches allowed further differentiation into what effects were mediated through merely enhancing intestinal cholesterol concentrations (inhibition of cholesterol absorption) and effects specific to stimulating TICE (FXR and LXR stimulation). Insight into novel ways to lower plasma UCB could provide new possibilities to treat patients with unconjugated hyperbilirubinemia.

METHODS

Animals

Gunn rats (Gunn-UGT1A1j/BluHsdRrrc) were obtained from the Rat Resource & Research Center (Columbia, MO) and bred in our animal facility in the University Medical Center Groningen (Groningen, The Netherlands). Animals were individually housed in a temperature- and light-controlled facility (12-h dark/light rhythm) and had ad libitum access to laboratory chow (RM3, Special Diets Services, Essex, UK) and normal drinking water during the experiments. Animal experiments were performed with the approval of the local Ethics Committee for Animal Experiments of the University of Groningen. Experiments were performed in accordance with relevant guidelines and regulations, including laboratory and biosafety.

Materials

The synthetic LXR ligand T09 was obtained from Cayman Chemical (Ann Arbor, MI, USA). The semi-synthetic bile acid OCA (INT-747; 6 α -ethyl-chenodeoxycholic acid) is a potent and selective FXR agonist and was purchased from MedChem Express (South Brunswick, NJ, USA). The cholesterol absorption inhibitor EZE was obtained as Ezetrol (Merck Sharp & Dohme, Haarlem, The Netherlands). EZE and OCA were prepared for oral administration using the oral suspension solution “ORA-Plus” (Perrigo, Allegan, MI, USA).

Experimental design

LXR agonist T09. For studying the effects of administration of T09, male Gunn rats were first fed with normal chow, and 24-h fecal outputs were collected. Subsequently, the rats were randomly divided over four experimental groups ($n = 7$ per group) and received either chow diet (control group) or chow diet supplemented with EZE (0.005% w/w), T09 (0.015% w/w), or a combination of these two compounds (T09+EZE) for 14 days.

Body weights (BWs) were measured two times per week. Feces were collected and food intake was measured during the last 24 h of treatment. At day 14, rats were anesthetized using isoflurane, and the bile duct was cannulated for 20 min. Bile flow was determined gravimetrically (1 g = 1 mL bile secretion). Subsequently, blood was collected via cardiac puncture, protected against light, and stored under argon at -80°C . The liver was harvested and snap-frozen in liquid nitrogen. The small intestine was flushed with ice-cold phosphate-buffered saline (PBS) containing a protease inhibitor (cOmplete, Roche, Mannheim, Germany), snap-frozen, and stored at -80°C . Rats were terminated via decapitation under isoflurane anesthesia.

FXR agonist OCA. Rats were randomly divided to receive vehicle (ORA-Plus suspension, control group, $n = 4$), EZE ($n = 3$), OCA ($n = 4$) or OCA+EZE ($n = 5$) by oral gavage for 14 days. EZE was administered at a dose of 5 mg/kg/day and OCA at 10 mg/kg/day. A maximal administration volume of 5 mL per kg was applied. Feces were collected, and food intake was measured during the last 24 h of treatment. At day 14, the bile duct was cannulated for 20 min under isoflurane anesthesia within 6 h after the last dose, followed by blood collection via cardiac puncture. The liver was harvested and snap-frozen in liquid nitrogen. The small intestine was flushed with ice-cold PBS containing a protease inhibitor (cOmplete, Roche, Mannheim, Germany), snap-frozen, and stored at -80°C . Rats were terminated by decapitation under isoflurane anesthesia.

Analytical methods

Dietary NS and FNS and bile acids. Feces were freeze-dried, followed by mechanical homogenization. FNS and dietary NS and bile acids were extracted from 50 mg of fecal or dietary samples by 2 h of heating (80°C) with a mixture of 1 M sodium hydroxide and methanol (1:3). Specific extraction of NS was performed by petroleum ether (2 times 2 mL) and derivatized with BSTFA–pyridine–TMCS mixture (5:5:0.1). Extraction of fecal bile acids was performed using Sep-Pak C-18 columns (Waters Corporation, Milford, MA, USA), and samples were methylated with methanol/acetyl chloride (20:1) and subsequently derivatized with BSTFA–pyridine–TMCS (5:5:0.1). Dietary NS and FNS and bile acids were then both analyzed by gas chromatography (GC) as described.²¹

Plasma total bilirubin (TB) and haptoglobin. Terminal blood samples were collected and kept in EDTA-coated collecting tubes (MiniCollect, Greiner Bio-One, Kremsmünster, Austria) on ice in the dark. After centrifugation, plasma was stored in light-protecting tubes (Eppendorf, Hamburg, Germany) under argon at -80°C upon analysis. Plasma TB and free haptoglobin were analyzed on a Roche/Hitachi Cobas 501 Analyzer (Hitachi, Tokyo, Japan) using, respectively, the Bilirubin Total Gen 3 Kit and Tina-quant Haptoglobin ver.2 Kit (Roche Diagnostics, Rotkreuz, Switzerland).

Bilirubin determination in feces and bile. Fecal samples were put into the pre-weighted centrifugation tubes; then internal standard [mesobilirubin (MBR)] was added (10 μL of 5 μM MBR per sample). Then samples were extracted with concomitant protein precipitation by 2 mL of basic methanol (0.3% butylated hydroxytoluene, 0.1% ascorbic acid, and 0.5% ammonium acetate). The mixture was vigorously shaken and vortexed, and the suspension was centrifuged (3000 $\times g/5$ min). One mL of supernatant was collected into micro-tubes, and the solution was centrifuged again (16,000 $\times g/30$ min) for elimination of impurities before analytical measurement.

Bile samples were prepared similarly. Five μL of bile was pipetted into micro-tubes and 10 μL of MBR was added (5 μM); the extraction was performed with 1 mL of basic methanol. Samples were vigorously shaken and vortexed and the mixture was centrifuged (16,000 $\times g/30$ min).

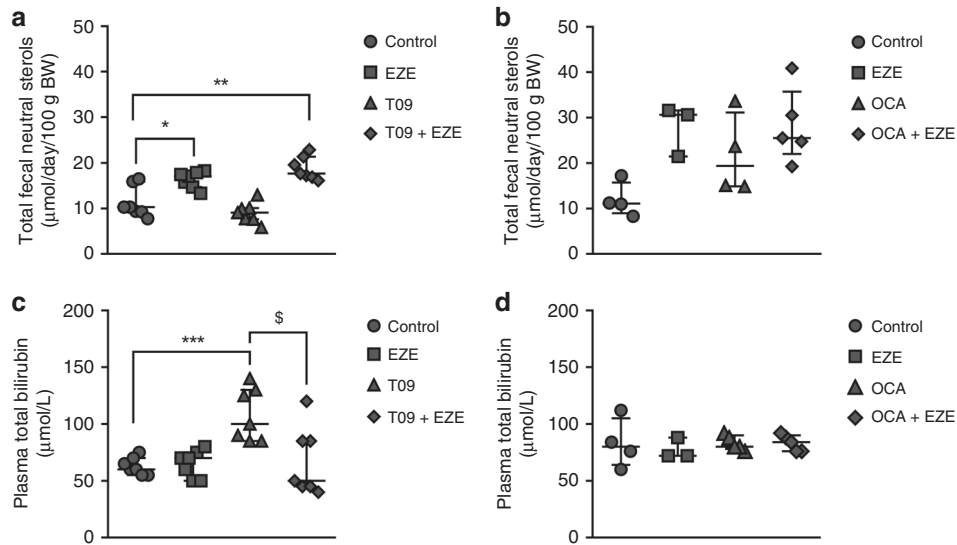


Fig. 1 The effect of stimulation of FNS on plasma levels of bilirubin in hyperbilirubinemic rats. Total FNS excretion after 2 weeks of **a** LXR or **b** FXR stimulation. Plasma TB after 2 weeks of **c** LXR or **d** FXR stimulation. **a + c**: $n = 7$; **b + d**, $n = 3-5$.

After final centrifugation steps, 100 μL of supernatant from bile as well as fecal samples was pipetted into glass vials with the inert insert (suitable for liquid chromatography tandem mass spectrometry (LC-MS) analysis), and 2 μL was directly injected to LC-MS apparatus.

LC-MS/MS analysis was performed using a high-performance liquid chromatography (Dionex Ultimate 3000, Dionex Softron GmbH, Germany) equipped with a Poroshell 120 EC-C18 column (2.1 μm , 3.0×100 mm; Agilent, CA, USA). For a gradient elution, the phase was prepared by mixing 1 mM of NH_4F (Honeywell, International Inc., Morris Plains, NJ, USA) in water and methanol (Biosolve Chimie SARL, France). The analytes were detected by mass spectrometer (TSQ Quantum Access Max with HESI-II probe, Thermo Fisher Scientific, Inc., USA) operating in a positive SRM mode: bilirubin [585.3 \rightarrow 299.1 (20 V); 585.3 \rightarrow 271.2 (18 V)]; MBR [589.3 \rightarrow 301.1 (20 V); 589.3 \rightarrow 273.2 (44 V)].

Samples remaining after extraction of feces were lyophilized overnight and freeze-dried tubes with dry matter were weighed by analytical scales. The empty tube weight (before analysis) was subtracted from the weight of the lyophilized tube.

Integrated areas of bilirubin were related to MBR areas, and ratios were normalized to dry weight of feces or volume of bile, respectively. The concentration was calculated according calibration curves measured in relevant matrices.

Hepatic and biliary lipids. Livers were crushed and homogenized in liquid nitrogen. A 15% liver homogenate was made with PBS to extract hepatic lipids using the Bligh and Dyer method.²² Hepatic triglycerides (TGs) and free and total cholesterol were measured using commercially available kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany, respectively). For analysis of biliary lipids, 15 μL of bile was used for extraction using the Bligh and Dyer method. Biliary cholesterol was derivatized using pyridine-acetic anhydride (1:1) for analysis by GC as previously described.²¹

Gene expression analysis. Isolation of total RNA from the liver and duodenum was performed using TRI-reagent (Sigma, St. Louis, MO, USA). RNA was quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA), and 1 μg RNA was used to create cDNA. Analysis of real-time quantitative polymerase chain reaction was performed on QuantStudio 7 Flex machine (Applied Biosystems, Thermo Fisher Scientific, Darmstadt, Germany). Gene

expression levels were normalized to *cyclophilin* for liver and *36b4* for intestine.

Plasma lipids. Plasma total cholesterol, triglycerides, and non-esterified fatty acids were spectrophotometrically analyzed using commercially available kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany).

Bile acid composition of bile and plasma. Biliary and plasma bile acid species were determined using LC-MS as described previously.¹⁵ Biliary hydrophobicity was calculated using Heuman values.²³ Total bile acid concentrations were calculated as the sum of the individual bile acid species. Total bile acid secretion was calculated using biliary bile acid concentrations multiplied by the bile flow and corrected for BW.

Statistics

For all statistical analyses, GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used. Unless stated otherwise, graphs are presented as a scatter dot plot representing individual values with a median \pm interquartile range. Statistical significance was tested by a non-parametric one-way analysis of variance (Kruskal-Wallis) test, followed by Mann-Whitney *U* tests to assess differences between the experimental groups. Differences before and after treatment were assessed using Wilcoxon signed-rank test. Statistical significance compared to control relative untreated groups is indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistical significance between the T09 and T09+EZE groups is indicated as § $p < 0.05$ and §§ $p < 0.01$. Owing to low animal numbers, no statistical significance was determined for the OCA experiment.

RESULTS

The effect of EZE, LXR, and FXR activation on FNS excretion and plasma bilirubin in Gunn rats

To assess the effect of stimulated FNS excretion on plasma bilirubin levels in Gunn rats, we used three approaches previously shown to increase FNS excretion in mice: inhibition of cholesterol absorption using EZE^{9,10,14,15} and pharmacological activation of LXR and FXR using T09 and OCA, respectively.^{7,8,11,24,25} In line with murine studies, EZE treatment, either administered via the diet or via oral gavage, increased FNS excretion by about twofold to threefold (Fig. 1a, b). In contrast to studies in mice, T09

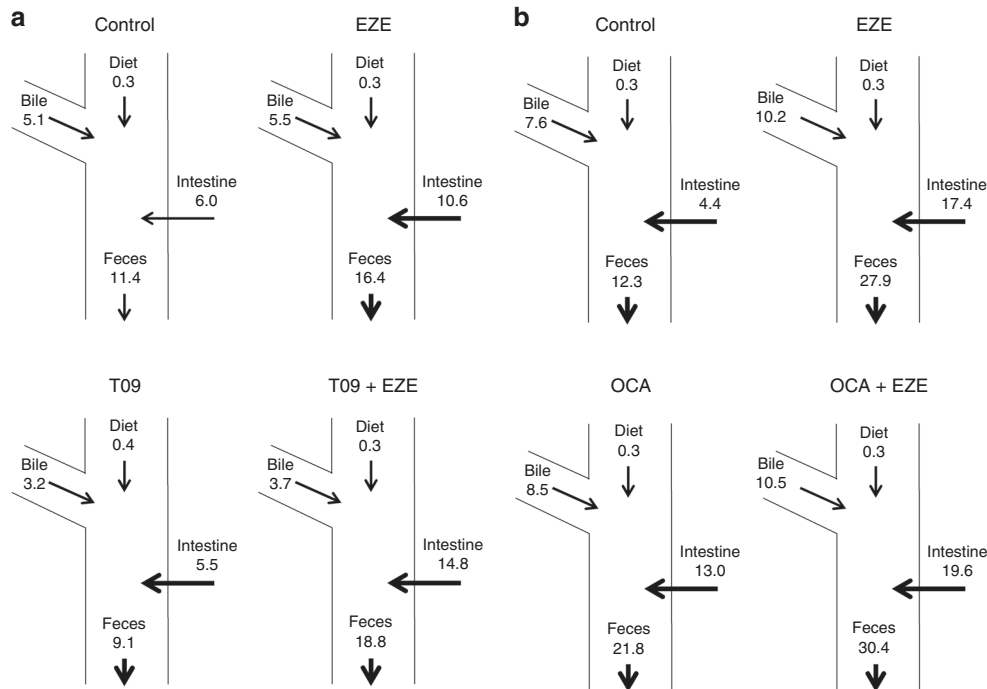


Fig. 2 Schematic representation of the calculated cholesterol fluxes. Net intestinal cholesterol balance was calculated by subtraction of mean dietary cholesterol intake and biliary cholesterol secretion from the FNS excretion for **a** LXR, $n = 7$, and **b** FXR activation, $n = 3-5$. Treatments in **a** were mixed in the diet, and treatments in **b** were administered through oral gavage. Numbers are represented as mean ($\mu\text{mol/day}/100\text{ g}$ body weight).

administration by itself did not stimulate FNS excretion in Gunn rats (Fig. 1a). Moreover, T09 even lowered FNS excretion compared to baseline, while FNS excretion remained unchanged in untreated controls (Supplementary Fig. S1A, B). Combined EZE and T09 treatment increased FNS excretion to a similar extent as EZE alone (Fig. 1a).^{9,26}

While OCA treatment showed a trend towards increased FNS, co-administration of OCA and EZE did not further increase FNS excretion as compared to EZE treatment alone (Fig. 1b). To assess whether the increase in FNS excretion affected hyperbilirubinaemia, we determined plasma TB levels. Since Gunn rats lack the conjugating activity of UGT1A1, plasma TB levels are equal to plasma UCB levels. Figure 1c shows that EZE did not lower plasma TB levels in Gunn rats. T09 increased plasma TB levels in Gunn rats, and this was partially prevented by co-administration of EZE (Fig. 1c). Treatment with T09 with or without EZE resulted in decreased free haptoglobin levels, an indication of increased intravascular hemolysis,²⁷ compared to control Gunn rats (Supplementary Fig. S2). Plasma TB levels were unaffected by administration of OCA, either alone or in combination with EZE (Fig. 1d). Taken together, these results demonstrate that plasma levels of bilirubin are not lowered by stimulation of FNS excretion in Gunn rats.

The effect of LXR and FXR activation on intestinal cholesterol fluxes in Gunn rats

The increase in FNS by EZE, T09, and OCA in previous studies has been explained by its effects on intestinal cholesterol fluxes.^{8,24,28} While T09 in our study did not affect total FNS excretion, this does not exclude the possibility of changes in intestinal cholesterol fluxes. To investigate this, we measured dietary cholesterol intake and biliary cholesterol secretion and subtracted these measurements from FNS excretion to obtain the net intestinal cholesterol balance (Fig. 2). In untreated Gunn rats, the intestinal cholesterol balance indicated a net cholesterol excretion into the intestine ($+6.0\ \mu\text{mol/day}/100\text{ g}$ BW) and was increased about twofold by

EZE treatment (Fig. 2a). These findings were similar in heterozygous Gunn rats indicating that the observed effects of EZE are independent of UGT1A1^{29,30} (Supplementary Fig. S3).

While T09 administration alone did not change the net intestinal cholesterol balance, the combination of T09 with EZE increased net intestinal cholesterol excretion into the intestine more than EZE alone (14.8 vs. 10.6 $\mu\text{mol/day}/100\text{ g}$ BW, Fig. 2a). This change was associated with a slight decrease in biliary cholesterol secretion, observed in both the T09-treated groups (Fig. 2a).

When EZE was administered through oral gavage instead of via the diet, net intestinal cholesterol excretion increased even further, to about 17.4 $\mu\text{mol/day}/100\text{ g}$ BW compared to 10.6 $\mu\text{mol/day}/100\text{ g}$ BW with dietary EZE (Fig. 2a, b). OCA alone increased net intestinal cholesterol excretion threefold. Previous reports indicated that OCA reduces cholesterol absorption in mice.²⁴ In our study, the addition of OCA to EZE treatment did not result in a major additive effect on the intestinal cholesterol balance compared to EZE alone (17.4 vs. 19.6 $\mu\text{mol/day}/100\text{ g}$ BW, Fig. 2b). Taken together, our results show that an increase in net intestinal cholesterol excretion does not result in lowering of plasma TB levels.

The effect of EZE, LXR, and FXR activation on biliary and fecal bilirubin secretion

Previous studies in rats have demonstrated that plasma UCB levels can be lowered through stimulation of fecal bilirubin excretion.^{5,16} We assessed whether EZE treatment or activation of LXR and FXR affected biliary and fecal UCB secretion rates. EZE resulted in a lower biliary UCB secretion rate both alone and combined with T09 (Fig. 3a). T09 by itself did not significantly alter biliary UCB secretion (Fig. 3a). Fecal UCB excretion rates were not altered by dietary administration of EZE; however, T09 alone or combined with EZE significantly increased fecal UCB excretion (Fig. 3b). No differences were observed in biliary and fecal UCB excretion after oral administration of EZE, OCA, or

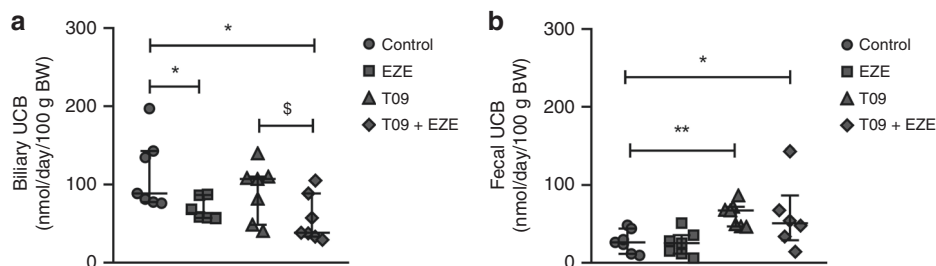


Fig. 3 The effect of T09 and EZE on UCB in bile and feces in Gunn rats. Secretion rates of UCB after administration of EZE, T09, or co-administration in **a** bile, $n = 6-7$, and **b** feces, $n = 7$.

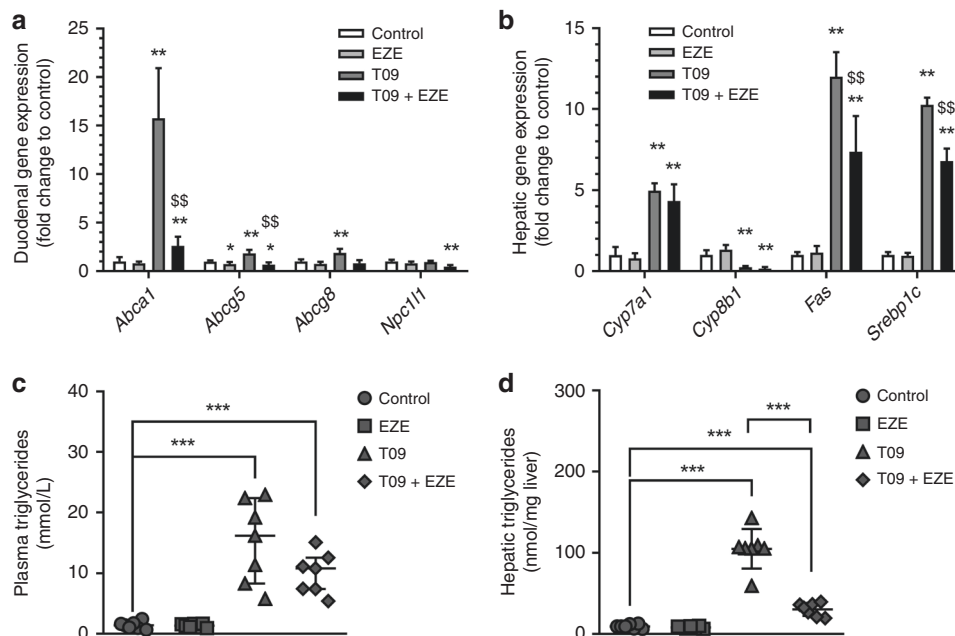


Fig. 4 The effect of T09 and EZE treatment on LXR target gene expression and triglyceride levels. Gene expression of target genes of LXR in the **a** duodenum and **b** liver, $n = 5$. **c** Triglyceride concentration in plasma after administration of EZE, T09, or combination treatment and **d** hepatic triglyceride content after administration of EZE, T09, or combination treatment, $n = 7$.

OCA+EZE (data not shown). These results show that, while T09 increased fecal UCB levels, this was not accompanied by a decrease in plasma UCB concentrations.

Validation of LXR activation by T09 in Gunn rats

In mice, the effect of LXR on FNS excretion are (at least partially) mediated via induction of ABCG5/8-mediated cholesterol efflux.^{8,13,31} Since we did not observe an increase in FNS excretion upon T09 treatment in Gunn rats (Fig. 1a), we wanted to exclude the possibility that LXR was not activated by T09 in our experiment. To determine the activation of LXR by T09, we analyzed duodenal mRNA expression of LXR target genes involved in cholesterol metabolism (Fig. 4a). In Gunn rats, T09 increased the expression of LXR target genes *Abcg5/8* and *Abca1* (Fig. 4a). *Npc11* gene expression was unchanged upon T09 treatment alone but lower, compared to untreated controls, when combined with EZE. Figure 4b depicts hepatic target genes of LXR involved in bile acid synthesis and lipogenesis. T09, with or without co-administration of EZE, increased the expression of cholesterol 7 α -hydroxylase (*Cyp7a1*), the rate-limiting enzyme in bile acid synthesis, while it decreased the expression of microsomal sterol 12 α hydroxylase (*Cyp8b1*), responsible for the synthesis of cholic acid (Fig. 4b). Administration of EZE alone did not affect the expression of these hepatic genes. The changes in gene expression levels of *Cyp7a1* and *Cyp8b1* by T09 were accompanied by a percentual decrease

of taurocholic acid (TCA) in the bile (Supplementary Fig. S4A). Treatment with T09 increased the hepatic mRNA expression of sterol-regulatory element-binding protein 1C (*Srebp-1c*) and fatty acid synthase (*Fas*), genes involved in lipogenesis. In line with an increase in lipogenesis, T09 highly increased both plasma and hepatic TG levels (Fig. 4c, d), similar to previous observations in mice,³² and were partly attenuated by EZE. Supplementary Fig. 5 shows that T09-induced increase in plasma TG is positively correlated to the increase in plasma TB in Gunn rats. Conversely, plasma and hepatic total cholesterol levels were lowered upon T09 treatment with or without EZE co-treatment (Supplementary Fig. S6A, B). Finally, T09 treatment also increased liver weight and bile flow (Supplementary Table S1). These data demonstrate that, while T09 did not affect FNS excretion in Gunn rats, it did induce other known LXR-mediated effects on lipid, cholesterol, and bile acid homeostasis without affecting plasma TB levels.

Validation of FXR activation by OCA in Gunn rats

In mice, OCA treatment activates intestinal and hepatic FXR, thereby reducing the hepatic expression of *Cyp7a1* and *Cyp8b1*, resulting in decreased bile acid synthesis and a subsequent reduction in size and hydrophobicity of the bile acid pool.¹¹ To determine whether OCA treatment resulted in similar effects in Gunn rats, we measured the expression of hepatic FXR target genes and the biliary bile acid concentration and composition.

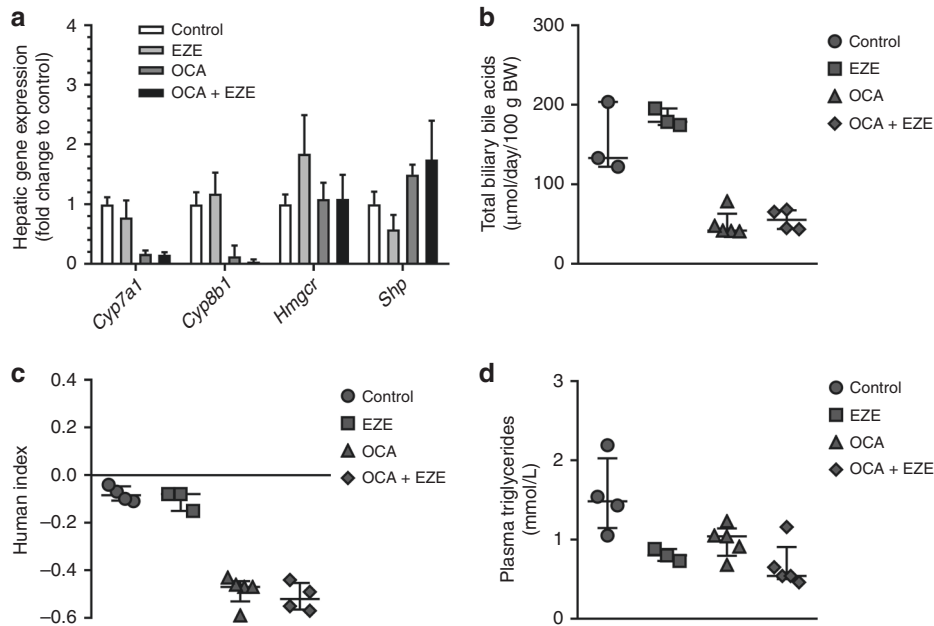


Fig. 5 The effect of OCA and EZE treatment on FXR target genes and bile acids in Gunn rats. **a** Hepatic gene expression of FXR target genes after administration of EZE, OCA, or co-administration. **b** Total biliary bile acid levels corrected for bile flow after FXR activation. **c** Hydrophobicity index of the bile. **d** Plasma triglyceride concentration after administration of EZE, OCA, or combination treatment, $n = 3-5$.

OCA treatment, either alone or with EZE, reduced hepatic *Cyp7a1* and *Cyp8b1* mRNA levels compared to untreated controls (Fig. 5a). EZE alone did not significantly alter *Cyp7a1* or *Cyp8b1* gene expression. OCA treatment decreased total biliary bile acid secretion by about 75%, either with or without co-administration of EZE (Fig. 5b). This was associated with a similar decrease in plasma and fecal bile acids (Supplementary Fig. S7). OCA reduced biliary hydrophobicity compared to controls, which was unaffected by EZE co-administration (Fig. 5c). The decrease in hydrophobicity resulted from a fractional decrease of TCA and increase of tauro- α - and tauro- β -muricholic acids (Supplementary Fig. S4B). In contrast to LXR agonist treatment, activation of FXR has been associated with a reduction of plasma TGs (reviewed in ref. 33). In Gunn rats, both EZE and OCA treatment, irrespective of EZE co-treatment, decreased plasma TG concentration compared to controls (Fig. 5d). Plasma and hepatic cholesterol levels were unaffected upon OCA and/or EZE treatment (Supplementary Fig. S6C, D). These results show that OCA treatment results in similar FXR-mediated effects on bile acid and cholesterol metabolism in Gunn rats compared to mice but did not affect plasma TB levels.

DISCUSSION

In this study, we tested the hypothesis that stimulation of FNS excretion lowers plasma TB in hyperbilirubinemic Gunn rats. To increase FNS excretion, we inhibited intestinal cholesterol absorption using EZE and stimulated TICE via LXR or FXR activation. We show that increasing FNS excretion by inhibition of intestinal cholesterol absorption did not lower plasma TB. We conclude that neither stimulation of FNS excretion nor LXR or FXR stimulation exert hypobilirubinemic effects in Gunn rats.

We previously demonstrated that increased fecal fat excretion, either via a HFD or orlistat, is associated with decreased plasma UCB in Gunn rats.^{5,17,18} Here we tested whether induction of fecal excretion of another lipid, cholesterol, could also exert hypobilirubinemic effects. In addition, a transintestinal excretion route is present for both UCB and cholesterol.^{4,13,16} Therefore, we hypothesized that treatments that stimulate TICE could potentially affect

transintestinal UCB excretion. Most of the induced FNS excretion in our present experiment originates from (non-reabsorbed) transintestinally excreted cholesterol. Our results provide two important new insights: (1) the transintestinal excretion pathways for cholesterol and for UCB are not quantitatively linked and (2) the intestinal "capture" hypothesis only relates to non-absorbed TGs/fatty acids and not to cholesterol.

While LXR activation did not lower plasma bilirubin levels in Gunn rats, fecal UCB excretion was increased (Fig. 3b). However, it should be realized that fecal UCB excretion only accounts for an estimated ~50% of TB turnover.³⁴ Since our analyses do not provide a quantitative estimation of UCB secretion, (disposal and degradation, into urobilinoids and other compounds), we cannot definitively determine whether the increase in fecal UCB was due to (1) increased transintestinal UCB secretion, (2) decreased transintestinal UCB reabsorption, or (3) decreased intraluminal (microbial) UCB degradation. Based on biliary UCB secretion rates, however, it can be excluded that the T09-induced fecal UCB excretion is due to increased biliary excretion (Fig. 3a).

Despite clear activation of LXR by T09 in Gunn rats, as evidenced from upregulation of known target genes (e.g., *Abca1*, *Abcg5*, and *Abcg8*) and increased lipogenesis, we did not observe an effect on FNS excretion, in contrast to previous observations in mice.^{7,8} Intestinal ABCG5/8 stimulation has been implicated in the increase of TICE upon T09 treatment in mice.⁷ Van de Peppel et al. showed that, under physiological conditions, cholesterol excreted via TICE is largely reabsorbed by the intestine in an NPC1L1-dependent process.¹⁵ In mice, activation of LXR decreases *Npc1l1* expression, which could result in decreased cholesterol (re) absorption, thereby increasing FNS excretion independent of an effect on ABCG5/8.³⁵ In our study, *Npc1l1* gene expression in Gunn rats was unaffected upon T09 treatment, while *Abcg5/8* expression was increased. Potential differences between mice and rats in regulation of these transporters are illustrated by a study by Kawase et al., showing that a 2% cholesterol-enriched diet decreased intestinal mRNA and protein levels of NPC1L1 in mice, but not in rats, while increasing ABCG5/8 only in rats.³⁶ These observations suggest that the lack of an effect of T09 on FNS excretion in (Gunn) rats is potentially due to a species-specific

effect on intestinal *Npc1l1* expression and subsequent cholesterol (re)absorption. Interestingly, combined T09 and EZE treatment increased net intestinal cholesterol excretion more than EZE treatment alone (Fig. 2a), suggesting that T09 does increase TICE, but under the conditions of unaffected NPC1L1 function or expression, this is reabsorbed and does not contribute to FNS excretion.

The second approach to study whether higher FNS excretion could lower plasma bilirubin was by activating FXR using OCA. The underlying mechanism by which OCA increases FNS excretion in mice has been suggested to be mediated by a smaller and more hydrophilic bile acid pool.¹¹ Hydrophilic muricholic bile acids inhibit intestinal cholesterol absorption, thereby promoting FNS excretion. In our current study, we showed that Gunn rats treated with OCA with or without EZE also had lower biliary bile acid concentrations and a more hydrophilic profile (Fig. 5b, c). While we did not measure intestinal cholesterol absorption directly, intestinal cholesterol balance data demonstrated net intestinal excretion upon OCA treatment (Fig. 2b). Co-administration of OCA with EZE slightly increased net intestinal cholesterol excretion further than either treatment alone, suggesting that, while most of the effects of OCA are also likely mediated through decreased cholesterol absorption in Gunn rats, a small part of the effects could be due to direct stimulation of TICE.

Surprisingly, administration of T09 led to an increase in plasma bilirubin, and this was partially prevented by EZE co-treatment. Our T09-treated Gunn rats displayed severely increased plasma TG levels, a well-known effect of LXR activation, resulting in an optical yellow-colored and turbid appearance. Therefore, hypertriglyceridemia could have potentially interfered with the plasma bilirubin measurements, resulting in higher values. However, it has also been demonstrated that plasma TG levels >12 mmol/L increase hemolysis, possibly due to increased membrane instability of erythrocytes.³⁷ Bilirubin is a degradation product of heme metabolism and can thus be further increased upon hemolysis in our T09-treated Gunn rats.³⁸ We observed a strong, positive correlation between plasma TG and bilirubin in Gunn rats treated with T09 with or without EZE (Supplementary Fig. S5). The presence of increased hemolysis due to hypertriglyceridemia upon T09 treatment is supported by decreased plasma-free haptoglobin concentrations, a marker for intravascular hemolysis,²⁷ in both T09-treated groups (Supplementary Fig. S2). However, decreased haptoglobin levels are not conclusive of hemolysis, and we did not have whole-blood samples to perform other red blood cell analyses to determine whether hemolysis indeed had been induced. Taken together, the present study demonstrates that neither induction of FNS excretion nor LXR or FXR activation results in a hypobilirubinemic effect in Gunn rats, in contrast to induction of fecal fat excretion.^{5,17} This shows that clinically relevant “capturing” of bilirubin can be realized only by fatty acids and not by cholesterol. These data also demonstrate that there is no quantitative link between TICE or FNS excretion and transintestinal UCB excretion. Therefore, increasing FNS excretion is not a potential target to treat unconjugated hyperbilirubinemia. Of further interest, we found that T09 did not increase FNS excretion in Gunn rats but did induce intestinal *Abcg5/8* expression without affecting *Npc1l1* expression. Based on this observation, we conclude that the increased expression of *Abcg5/8* per se upon T09 treatment is not critical for the induction of FNS excretion observed in mice but perhaps rather the reduced expression of *Npc1l1*.^{32,33} This underlines important species differences in (intestinal) lipid homeostasis, and caution is warranted in choosing what model to study.

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AUTHOR CONTRIBUTIONS

M.B. designed and performed the experiments, analyzed and interpreted data, and wrote the manuscript. I.P.v.d.P. interpreted data and wrote the manuscript. A.D. and N.C. analyzed data and reviewed the manuscript. L.V. interpreted data and reviewed the manuscript. J.W.J. and H.J.V. designed the experiments, interpreted data, and wrote the manuscript.

ADDITIONAL INFORMATION

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