

# Dysregulation of bile acid homeostasis in parenteral nutrition mouse model

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**Zhan L, Yang I, Kong B, Shen J, Gorczyca L, Memon N, Buckley BT, Guo GL.** Dysregulation of bile acid homeostasis in parenteral nutrition mouse model. *Am J Physiol Gastrointest Liver Physiol* 310: G93–G102, 2016. First published November 12, 2015; doi:10.1152/ajpgi.00252.2015.—Long-term parenteral nutrition (PN) administration can lead to PN-associated liver diseases (PNALD). Although multiple risk factors have been identified for PNALD, to date, the roles of bile acids (BAs) and the pathways involved in BA homeostasis in the development and progression of PNALD are still unclear. We have established a mouse PN model with IV infusion of PN solution containing soybean oil-based lipid emulsion (SOLE). Our results showed that PN altered the expression of genes involved in a variety of liver functions at the mRNA levels. PN increased liver gene expression of *Cyp7a1* and markedly decreased that of *Cyp8b1*, *Cyp7b1*, *Bsep*, and *Shp*. CYP7A1 and CYP8B1 are important for synthesizing the total amount of BAs and regulating the hydrophobicity of BAs, respectively. Consistently, both the levels and the percentages of primary BAs as well as total non-12 $\alpha$ -OH BAs increased significantly in the serum of PN mice compared with saline controls, whereas liver BA profiles were largely similar. The expression of several key liver-X receptor- $\alpha$  (LXR $\alpha$ ) target genes involved in lipid synthesis was also increased in PN mouse livers. Retinoid acid-related orphan receptor- $\alpha$  (ROR $\alpha$ ) has been shown to induce the expression of *Cyp8b1* and *Cyp7b1*, as well as to suppress LXR $\alpha$  function. Western blot showed significantly reduced nuclear migration of ROR $\alpha$  protein in PN mouse livers. This study shows that continuous PN infusion with SOLE in mice leads to dysregulation of BA homeostasis. Alterations of liver ROR $\alpha$  signaling in PN mice may be one of the mechanisms implicated in the pathogenesis of PNALD.

parenteral nutrition; liver; bile acid; *Cyp7a1*; *Cyp8b1*; retinoid acid-related orphan receptor- $\alpha$

LONG-TERM PARENTERAL NUTRITION (PN) infusion has been shown to be associated with a spectrum of hepatobiliary disorders, including cholestasis, steatosis, fibrosis, and end-stage liver complication, cirrhosis, collectively known as PN-associated liver diseases (PNALD) (8). Among these disorders, steatosis, cholestasis, and cholelithiasis (i.e., gallbladder sludge/stones) are most common (8). PN-associated steatosis occurs mostly in adults and is generally benign (8). PN-associated cholestasis occurs predominantly in term and preterm infants (14). Given the immature livers and intestines, infants with PNALD can rapidly develop devastating liver diseases if they are not able to be weaned off of PN in a timely fashion. There are no effective preventative or therapeutic approaches to PNALD other than discontinuation of PN and advancement to full enteral food

intake, which is challenging and often impossible in patients dependent on PN because of their poor intestinal function (24).

PNALD is multifactorial in etiology, with many associated risk factors including lack of enteral feeding (i.e., nutrition obtained by mouth), inflammation, infection, micronutrient deficiencies, contaminants in PN products, and the composition and/or source of macronutrients such as intravenous fat emulsions (19). A significant amount of research has been conducted on soybean oil-based lipid emulsion (SOLE). The presence of plant phytosterols, large amounts of proinflammatory  $\omega$ -6 polyunsaturated fatty acids (PUFAs), and the lack of anti-inflammatory  $\omega$ -3 PUFAs in SOLE have all been implicated in the pathogenesis of PNALD (17). In recent years, newer generations of lipid emulsion, including the fish oil-based lipid emulsion, which mainly contains  $\omega$ -3 PUFAs, and SMOFlipid (a mixture of soybean oil, medium-chain triglycerides, olive oil, and fish oil) have been shown to be beneficial to improve or even reverse the PNALD in both pediatric and adult patients (19).

Intra- or extrahepatic bile acid (BA) accumulation in the liver leads to cholestatic liver disorders. Farnesoid X receptor (FXR) is a BA sensor, which plays an essential role in maintaining enterohepatic BA homeostasis (23). To date, changes in liver and intestine FXR function as well as BA signaling pathways within PNALD are still not fully understood. It has been shown that Toll-like receptor 4-mediated immune responses were critically involved in PN-induced liver injury when PN mice were pretreated with dextran sulfate sodium (DSS) (2). DSS is a toxic chemical agent that could induce intestinal damage to mimic human clinical conditions. The same group also showed that plant sterols contained in SOLE, particularly stigmaterols, were associated with cholestatic liver injury by inhibiting FXR function in PN mouse livers (3). However, studies using PN piglet models do not support the idea that phytosterols are associated with suppression of hepatic FXR function and pathogenesis of PNALD (17). Therefore, a comprehensive understanding of the BA homeostasis and FXR function under PN is urgently needed.

To test our hypothesis that PN infusion will disrupt BA homeostasis, especially enterohepatic circulation of BAs, we have established a valid mouse PN model. We characterized the expression profiles of liver genes critically involved in BA homeostasis, as well as the serum and liver BA profiles for the PN mice. The results of this study will aid in determining contributing factors involved in the development and management of PNALD in the future.

## MATERIALS AND METHODS

**Animals and surgery.** All animal protocols and procedures were approved by the Institutional Animal Care and Use Committee (IA-

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CUC) of Rutgers University. Unless otherwise specified, all mice used for the PN study were maintained in the pathogen-free animal facilities in the Comparative Medicine Resources at the Nelson Animal Facility at Rutgers University, under a standard 12-h:12-h light/dark cycle (6 AM/6 PM) with temperature- and humidity-controlled conditions. Mice were fed ad libitum with standard mouse chow and autoclaved tap water before initiation of PN infusion. Wild-type (WT) C57BL/6J mice were obtained from the Jackson Laboratory. Male mice, between 8 to 20 wk of age, were used for the study.

For jugular vein catheterization, mice were anesthetized via intraperitoneal injection with 80 mg/kg ketamine and 10 mg/kg xylazine, followed by placement of a central venous catheter (polyurethane tubing, 1F in O.D.) (SAI Infusion Technologies) into the right jugular vein. A minimal amount of blood was carefully withdrawn to verify the catheter patency. Afterward, the saline solution in the catheter was replaced by heparin/glycerol catheter lock solution (SAI Infusion Technologies). The proximal end of the catheter was then tunneled subcutaneously, exited between the shoulder blades, and properly secured. On the next day, a fully recovered surgical mouse was placed in a plastic harness (SAI Infusion Technologies), and the catheter was connected to an infusion pump (Harvard Apparatus) through the extension lines (SAI Infusion Technologies). Catheterized mice were then kept on intravenous infusion of normal saline (NS, 0.9%) at an initial rate of 6 ml/day and had free access to food and water. The next day, mice in the PN group (hereafter referred to as PN mice) started to receive intravenous PN infusion prepared by CAPS and still had free access to water but not food. Saline control mice (hereafter referred to as saline mice) continued to receive saline infusion and have free access to food and water. One day after, the infusion rates for both saline and PN mice were increased to 8 ml/day and kept as 8 ml/day throughout the study period with the PN solution providing an adequate caloric intake of 10.62 kcal/day (Table 1). This regimen was based on the report that a graded infusion period is necessary for the mice to adapt to the continuous infusion of fluid and nutrients (16). All saline and PN mice were housed individually in metabolic cages to prevent PN mice from coprophagia. Because metabolic cages were maintained in a nonsterile environment, mice used in this study were allowed at least 1 wk to acclimatize. After 8 days of PN infusion, mice were killed, and tissues were harvested for analysis.

Table 1. Components of PN per 100 ml

Component	Amount	Unit
Concentrated amino acids*	4.000	g
Dextrose	25.500	g
Lipid†	3.000	g
Sodium phosphate	1.340	mM
Potassium chloride	1.600	mEq
Sodium chloride	3.200	mEq
Potassium acetate	12.000	mEq
Magnesium sulfate	0.800	mEq
Calcium gluconate	1.320	mEq
Multitrace-5 concentrate§	0.100	ml
Heparin	500.000	U
Multivitamin‡	2.000	ml
Protein content (amino acid)	4.000	g
Nitrogen content	0.632	g
Nonprotein calorie	116.700	kcal
Carbohydrate calorie	86.700	kcal
Lipid calorie	30.000	kcal
Protein calorie	16.000	kcal
Total calorie	132.700	kcal

\*Clinisol 15%, obtained from Baxter International (Deerfield, IL). †Intralipid 20%, obtained from Baxter International. ‡Inluvite Adult, obtained from Baxter International. §Multitrace-5 concentrate, contains zinc, copper, manganese, chromium, and selenium, obtained from American Regent (Shirley, NY). PN, parenteral nutrition.

**Serum biochemical analysis.** Commercially available testing kits were used to measure serum levels of biomarkers, including alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) (Pointe Scientific), and total serum BAs (TSBA) (Diazyme Laboratories). Procedures were scaled up or down based on the manufacturer's instructions.

**RNA isolation, qRT-PCR, and microarray analysis.** Total RNA was isolated using TRI reagent (Sigma) according to the manufacturer's instructions. Liver gene expression at the mRNA level was analyzed by qRT-PCR. After rigorous analysis of gene expression in saline and PN mice, RNA samples ( $n = 3$ ) obtained from the livers from saline and PN mice were pooled, respectively, for microarray analysis (Microarray-PN/Saline). Liver mRNA microarray analysis was determined using Mouse Gene 2.0 ST Array system manufactured by Affymetrix. Microarray data were analyzed using the Affymetrix Power Tools (<http://www.affymetrix.com>). Data retrieved from the microarray analysis were further validated by qRT-PCR analysis. All the primer sequences for qRT-PCR are listed in Table 2. The raw and processed data files for microarray analysis have been deposited in NCBI Gene Expression Omnibus (1) and are accessible through GEO Series accession number GSE71286 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ylmficimlhexpcj&acc=GSE71286>).

**Organic extraction and Ultra Performance Liquid Chromatography/mass spectrometry profiling of serum and liver BAs.** Serum and liver total BAs were extracted as previously described (21). Final BA extracts were introduced to the Thermo Finnigan Ultra Performance Liquid Chromatography (UPLC) system (Thermo Fisher Scientific) coupled with a Thermo Finnigan LTQ XL Ion Trap Mass Spectrometer (Thermo Fisher Scientific). An Electrospray (ESI)/ITMS was operated in multiple mass spectrometry (MS)/MS and Selective Reaction Monitoring modes for simultaneous determination of 23 BAs including: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), tauroolithocholic acid (TLCA), taurooursodeoxycholic acid (TUDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA),  $\beta$ -muricholic acid ( $\beta$ -MCA),  $\alpha$ -muricholic acid ( $\alpha$ -MCA),  $\omega$ -muricholic acid ( $\omega$ -MCA), tauro- $\beta$ -muricholic acid (T- $\beta$ -MCA), tauro- $\alpha$ -muricholic acid (T- $\alpha$ -MCA), tauro- $\omega$ -muricholic acid (T- $\omega$ -MCA), hyodeoxycholic acid (HDCA), taurohyodeoxycholic acid (THDCA), and glycohyodeoxycholic acid (GHDCA).

For BA standards, CA, GCA, TCA, DCA, GDCA, TDCA, CDCA, GCDCA, TCDCA, LCA, TLCA, UDCA, and TUDCA were purchased from Sigma-Aldrich; GLCA,  $\beta$ -MCA,  $\alpha$ -MCA,  $\omega$ -MCA, T- $\beta$ -MCA, T- $\alpha$ -MCA, T- $\omega$ -MCA, HDCA, THDCA, and GHDCA were purchased from Steraloids. For internal standards, chenodeoxycholic-2,2,4,4-d<sub>4</sub> acid (<sup>2</sup>H<sub>4</sub>-CDCA) and glycochenodeoxycholic-2,2,4,4-d<sub>4</sub> acid (<sup>2</sup>H<sub>4</sub>-GCDCA) were purchased from C/D/N Isotopes.

**Western blot.** Nuclear, cytoplasmic, and total protein extraction and Western blot analysis were performed as previously described (10). Antibodies against lamin a/c (Developmental Studies Hybridoma Bank) and  $\beta$ -actin (Santa Cruz Biotechnology) were used as the loading controls for nuclear and total proteins, respectively. Antibody for retinoid acid-related orphan receptor- $\alpha$  (ROR $\alpha$ ) was purchased from BioLegend.

**Oil red O staining.** The optimal cutting temperature compound-embedded frozen livers were sectioned at 8  $\mu$ M and stained with Oil Red O with a standard protocol (6).

**Statistical analysis.** Data are presented as mean  $\pm$  SE. Student's *t*-test was used to compare the data obtained from the saline and PN groups.  $P < 0.05$  was considered statistically significant. The numbers of animals used for each experiment are shown in the legends of each figure and table.

Table 2. List of qPCR primers

Gene	Forward Primer Sequence (5'–3')	Reverse Primer Sequence (5'–3')
<i>Abcd2</i>	CACAGCGTGCACCTCTAC	AGGACATCTTTCCAGTCCA
<i>Abcg5</i>	TGGATCCAACACCTCTATGCTAAA	GGCAGGTTTTCTCGATGAAGTG
<i>Acc</i>	TGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
<i><math>\beta</math>-actin</i>	GGGTGACATCAAAGAGAAGC	CTCGTTGCCAATAGTGATGAC
<i>Bsep</i>	CTGCCAAGGATGCTAATGCA	CGATGGCTACCCCTTGTCTTCT
<i>Cd36</i>	GATGACGTGGCAAAGAACAG	TCCTCGGGGTCCTGAGTTAT
<i>Cyp2b10</i>	GACTTTGGGATGGGAAAGAG	CCAAACACAATGGAGCAGAT
<i>Cyp27a1</i>	GCCTCACCTATGGGATCTTCA	TCAAAGCTGAGCGCAGATG
<i>Cyp7a1</i>	AACAACCTGCCAGTACTAGATAGC	GTGTAGAGTGAAGTCCCTCTTAGC
<i>Cyp7b1</i>	CAGCTATGTTCTGGGCAATG	TGGGATGATGCTGGAGTATG
<i>Cyp8b1</i>	AGTACACATGGACCCGACATC	GGGTGCCATCCGGGTTGAG
<i>Fas</i>	GCTGCGAACTTCAGGAAAT	AGAGACGTGTACTCTGGACTT
<i>Fgf15</i>	GCCATCAAGGACGTCAGCA	CTTCCTCCGAGTAGCGAATCAG
<i>Fxr</i>	TCCGGACATTCAACCATCAC	TCACTGCACATCCAGATCTC
<i>Lepr-b</i>	GCATGCAGAATCAGTGATTTTGG	CAAGCTGTATCGACACTGATTCTTC
<i>Lpl</i>	AGGACCCCTGAAGACAC	GGCACCCAACTCTCATA
<i>Mrp3</i>	AGAGCTGGGCTCCAAGTTCT	TGGTGTCTCAGGTAAACAGGTAGCA
<i>Pgc-1<math>\alpha</math></i>	CGGAAATCATATCCAACCGAG	TGAGGACCGCTAGCAAGTTTG
<i>Marco</i>	GCACTGCTGCTGATTCAAGTTC	AGTTGCTCCTGGCTGGTATG
<i>Nocturnin</i>	ACCAGCCAGACATACTGTGC	CTTGGGGAACCGTGCCT
<i>Nurr77</i>	AGCTTGGGTGTGATGTTCC	AATGCGATTCTGCAGTCTT
<i>Scd1</i>	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAGATTTCTGCAAACC
<i>Shp</i>	CGATCCTCTTCAACCCAGATG	AGGGCTCCAAGACTTCACACA
<i>Srebp-1c</i>	GGAGCCATGGATTGCACATT	GTTTCCAGAGAGGAGCCAG

## RESULTS

PN mice had decreased body and liver weight. Upon infusion system setup, average body weight (BW) for saline and PN mice was 21.28 and 21.22 g, respectively (Table 3). The initial infusion rate for PN mice was set to 7 ml/day, which was adapted from a previous study and provided a calorie intake of 8.4 kcal/day (2). After 8 days of PN infusion, mice consistently exhibited an average BW loss of around 20% (data not shown). Therefore, the lipid content in the PN solution was increased from 2 g per 100 ml to 3 g per 100 ml. The infusion rate was also increased from 7 ml/day to 8 ml/day for mice with an initial BW around 22 g (Table 3). After administration of the modified PN for 8 days (6 ml/day for 1 day, 8 ml/day afterwards), average BW change for PN mice was  $-2.10$  g ( $0.06$  to  $-3.44$  g, Table 3), whereas the average BW change for saline mice was  $-0.06$  g ( $1.28$  to  $-2.02$  g, Table 3). PN mice had around 10% BW loss (Table 3). This trend of BW change is similar to the trend reported previously (2). In addition to BW loss, final liver weight (LW) and the ratio of LW to BW also decreased in PN mice (Table 3). Upon animal euthanasia, the gallbladders of PN mice were substantially smaller than those found in saline mice (data not shown).

PN mice had altered serum biochemical parameters. Serum levels of ALT were similar between saline and PN mice (Fig. 1A). However, PN mice had significantly increased serum levels of AST (Fig. 1B) and TSBA (Fig. 1D) and significantly

decreased serum levels of ALP (Fig. 1C) compared with saline controls. Decreased ALP levels and empty gallbladders observed in PN mice indicate that PN infusion was associated with diminished bile flow from the hepatocytes into the biliary tract. This suggests that PN mice may have an increased risk of developing intrahepatic cholestatic complications after long-term PN infusion, especially when other risk factors, such as intestinal inflammation coupled with lipopolysaccharide infiltration, are presented.

PN altered hepatic gene expression for BA homeostasis and lipid synthesis. Substantial changes in the expression of genes involved in BA homeostasis and lipid metabolism were detected in the livers of PN mice. Relative mRNA levels of *Cyp7a1*, which encodes the rate-limiting enzyme in BA synthesis, showed a significant increase in the livers of PN mice compared with saline mice (around 3-fold,  $P < 0.005$ ) (Fig. 2A). Relative mRNA levels of *Cyp27a1*, *Fxr*, bile salt export pump (*Bsep*), and small heterodimer partner (*Shp*) decreased significantly in PN mice ( $P < 0.05$ ,  $0.05$ ,  $0.0005$ ,  $0.05$ , respectively) (Fig. 2A). Relative mRNA levels of *Cyp7b1* and multidrug-related protein 3 (*Mrp3*) decreased  $>70\%$  ( $P < 0.005$  for both), whereas relative mRNA levels of *Cyp8b1* decreased  $>90\%$  ( $P < 0.005$ ) (Fig. 2A). CYP8B1 is critical in determining BA hydrophobicity, as it functions to generate CA, a more water-soluble BA. Relative mRNA levels of genes involved in cholesterol and lipid metabolism were also quantified (Fig.

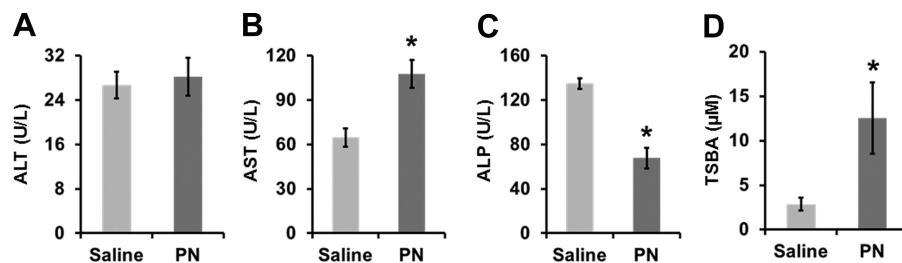
Table 3. Summary of body and liver weight for PN and saline mice

Group	Initial BW, g	Final BW, g	BW Change, g	BW Change	LW, g	LW/BW Ratio
Saline*	21.28 ( $\pm 0.51$ )	21.22 ( $\pm 0.55$ )	$-0.06$ ( $\pm 0.50$ )	$-0.17$ ( $\pm 2.36\%$ )	$1.11 \pm 0.08$	$0.052 \pm 0.003$
PN	21.22 ( $\pm 0.53$ )	19.12 ( $\pm 0.67$ )	$-2.10$ ( $\pm 0.34$ )	$-10.03$ ( $\pm 1.70\%$ )	$0.75 \pm 0.10$	$0.038 \pm 0.004$

Values are means  $\pm$  SE. \* $P < 0.05$ , PN group compared with saline group ( $n = 6$  to  $8$ ), Student's  $t$ -test, for all the categories in this table except for initial body weight (BW). Initial BW was recorded upon infusion setup; final BW and liver weight (LW) were recorded upon animal euthanasia (PN mice had gone through 8 days of PN infusion; corresponding saline controls were killed at the same time).



Fig. 1. Parenteral nutrition (PN) mice had altered serum biochemistry. Serum levels of alanine transaminase (ALT) (A), aspartate transaminase (AST) (B), alkaline phosphatase (ALP) (C), and total serum bile acids (BA) (TSBA) (D) in saline and PN mice ( $n = 6$  to 8). Data were expressed as means  $\pm$  SE. \* $P < 0.05$ , compared with saline group, Student's  $t$ -test.



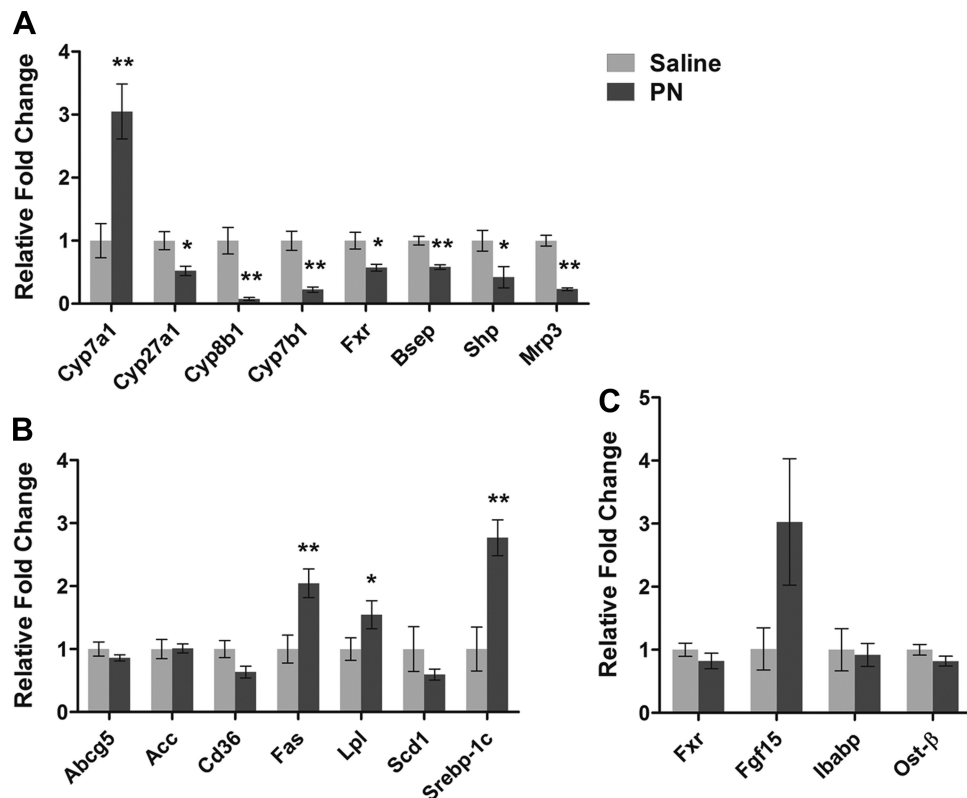
2B). Relative mRNA levels of *Cd36*, which encodes a long-chain fatty acid uptake transporter, decreased 50% ( $P < 0.05$ ), whereas relative mRNA levels of fatty acid synthase (*Fas*), lipoprotein lipase (*Lpl*), and sterol regulatory element-binding protein-1c (*Srebp-1c*) increased significantly in the livers of PN mice ( $P < 0.005$ , 0.05, 0.005, respectively) (Fig. 2B).

In the distal ileum, whereas the relative mRNA levels of *Fxr*, ileum BA-binding protein (*Ibabb*), and organic solute transporter- $\beta$  (*Ost- $\beta$* ) were similar between saline and PN mice, the relative mRNA levels of fibroblast growth factor 15 (*Fgf15*) increased in four out of seven of the PN mice (Fig. 2C,  $P > 0.05$ ). Nevertheless, no correlation between the mRNA levels of ileal *Fgf15* and liver *Cyp7a1* was found when the data from individual mice were examined.

**PN altered serum BA profiles but not liver BA profiles.** Among all the 23 BAs tested, serum and liver concentrations of GDCA, LCA, and GUDCA in both saline and PN samples were below the UPLC-MS detection limits. The analyzed serum concentration for each of the other 20 BAs in the saline ( $n = 5$ ) and PN mice ( $n = 6$ ) is plotted in Fig. 3. For most BAs, levels of glycol conjugates were the lowest, whereas tauroconjugates were much higher compared with the unconjugated

forms. Calculated total serum concentrations of the 20 BAs increased fivefold in PN mice compared with saline mice (mean  $\pm$  SE, ng/ml: saline mice,  $1,601.2 \pm 417.9$ ; PN mice,  $8,112.4 \pm 605.0$ ). Compared with saline mice, serum levels of TCDCA,  $\beta$ -MCA, T- $\beta$ -MCA, T- $\alpha$ -MCA,  $\omega$ -MCA, T- $\omega$ -MCA, TLCA, and TUDCA increased significantly in PN mice ( $P < 0.05$ ), whereas serum levels of GLCA in PN mice decreased dramatically below the detection limit (Fig. 3). Among these, levels of TCDCA, T- $\beta$ -MCA, T- $\alpha$ -MCA, and TLCA increased more than 10-fold in PN mice. Levels of CDCA increased threefold in PN mice although without significance ( $P = 0.09$ ). Levels of TCA, GCA,  $\alpha$ -MCA, and THDCA also increased dramatically ( $P < 0.07$ ) in PN mice (Fig. 3). The total serum concentration and percentage of each BA species (unconjugated BA and its conjugates), total primary BAs, and total secondary BAs, as well as total 12 $\alpha$ -OH BAs (CA, DCA, and their conjugates) and total non-12 $\alpha$ -OH BAs (CDCA, MCA, LCA, HDCA, UDCA, and their conjugates) in the total BA pool are summarized in Table 4. The percentage of total CDCA increased more than threefold, and the percentage of total  $\alpha/\beta$ -MCA increased almost twofold in PN mice. The percentage of total primary and secondary BAs was similar in saline

Fig. 2. PN altered the expression of genes involved in BA homeostasis and lipid metabolism. qRT-PCR analysis of relative mRNA levels of genes in the livers for BA homeostasis (A) and lipid metabolism (B) and genes in the distal ileums (C) of saline and PN mice ( $n = 6$  to 8) are shown. Gene expression data were expressed as means  $\pm$  SE. Relative mRNA levels were first normalized to mouse  $\beta$ -actin, and then relative fold changes were normalized to saline group. \* $P < 0.05$ , \*\* $P < 0.005$ , compared with saline group, Student's  $t$ -test.



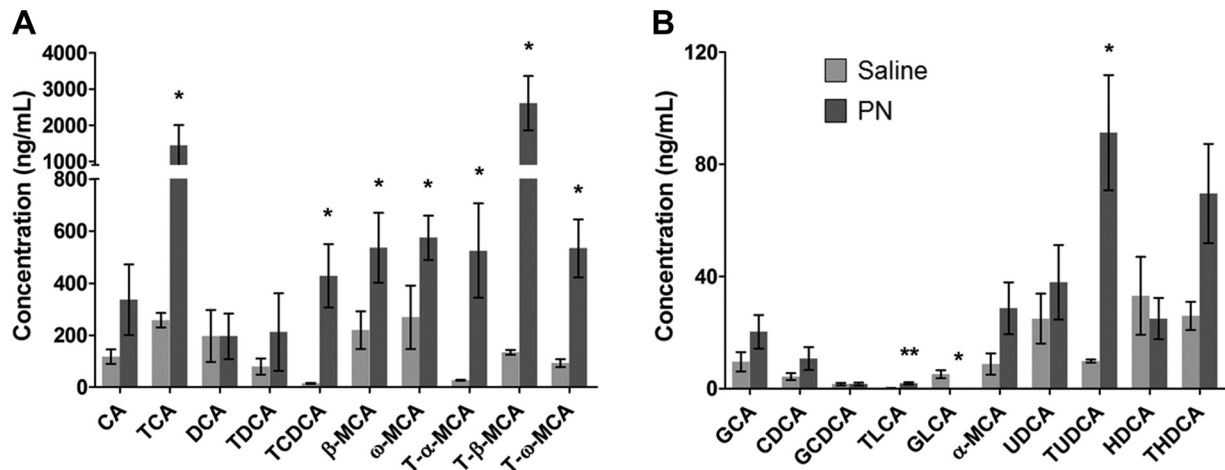


Fig. 3. PN altered serum BA profiles in mice. Quantified serum concentrations (ng/ml of serum sample) of the 20 BAs by Ultra Performance Liquid Chromatography mass spectrometry (UPLC/MS) for saline ( $n = 5$ ) and PN mice ( $n = 6$ ). Full names of the 20 BAs are described in MATERIALS AND METHODS. Data were presented as means  $\pm$  SE. \* $P < 0.05$ , \*\* $P < 0.005$ , compared with saline group, Student's  $t$ -test. For A and B, the scales on the y-axis are different for better clarification.

mice (mean: 56.93% vs. 43.07%), whereas, in PN mice, the percentage of total primary BAs was 3.1-fold compared with the secondary BAs (mean: 75.75% vs. 24.25%). In addition, the percentage of total 12 $\alpha$ -OH BAs and non-12 $\alpha$ -OH BAs was also similar in saline mice (mean: 44.45% vs. 55.55%), whereas the percentage of total non-12 $\alpha$ -OH BAs was 2.8-fold of that of total 12 $\alpha$ -OH BAs in PN mice (mean: 73.73% vs. 26.27%).

In contrast, levels of total BAs and individual BAs were overall similar in the livers of PN and saline mice. Levels of TCDCA, T- $\beta$ -MCA, T- $\alpha$ -MCA, and TUDCA increased significantly in the livers of PN mice, whereas the fold changes were substantially smaller compared with the changes detected in serum samples (Fig. 4). Nevertheless, compared with unconjugated BAs, these tauro-conjugated BAs are not toxic to the liver (9). Consistent with these relatively normal liver BA profiles, hematoxylin and eosin staining showed relatively normal liver histology of PN mice (data not shown) despite

altered gene expression and slightly altered liver biochemistry parameters.

*PN altered the expression of genes in many biological pathways.* To determine the changes of gene expression in pathways altered by PN, other than the previously mentioned BA and lipid pathways, a microarray was performed. Genes with fold change  $\geq 4$  (upregulated) or  $\leq -4$  (downregulated) obtained from the microarray analysis, with their full names and the corresponding fold changes, are presented in Table 5. qRT-PCR was performed to validate the microarray results (Fig. 5). Both upregulated and downregulated genes were tested. Among these, the relative mRNA levels of ATP-binding cassette, subfamily d, member 2 (*Abcd2*), *Nocturnin*, and macrophage receptor with collagenous structure (*Marco*) increased significantly in PN mice compared with saline controls (2.2-, 5.0-, and 6.8-fold,  $P < 0.0005$ , 0.05, 0.05, for *Abcd2*, *Nocturnin*, and *Marco*, respectively). Relative mRNA levels of *Cyb2b10* decreased more than 20-fold in PN mice ( $P < 0.005$ ).

Table 4. Summary of serum BAs in PN and saline mice

BAs	Concentration, ng/ml			Percentage	
	Saline	PN	Fold	Saline	PN
Total CA	384.071	1802.12	4.69	29.55%	22.03%
Total CDCA	20.557	440.13*	21.41	1.46%	6.36%
Total $\alpha/\beta$ -MCA	409.629	4137.79*	10.10	27.38%	53.44%
Total DCA	276.050	407.79	1.48	14.90%	4.24%
Total LCA	5.234	1.89*	0.36	0.37%	0.03%
Total $\omega$ -MCA	361.724	1108.15*	3.06	21.42%	17.14%
Total HDCA	59.056	94.53	1.60	3.95%	1.30%
Total UDCA	34.828	129.12*	3.71	2.43%	1.83%
Total primary BAs	793.700	5939.91*	7.48	56.93%	75.47%
Total secondary BAs	736.892	1741.48*	2.36	43.07%	24.53%
Total unconjugated BAs	873.506	1744.56	2.00	49.84%	25.21%
Total conjugated BAs	657.087	5936.83*	9.04	50.16%	74.79%
Total 12 $\alpha$ -OH BA	660.121	2209.91	3.35	44.45%	26.27%
Total non-12 $\alpha$ -OH BA	870.472	5471.48*	6.29	55.55%	73.73%
Total BAs	1530.592	7681.39*	5.02		

Data for concentration and percentage were expressed as mean for each category of bile acids (BAs) in saline or PN mice. Total BAs from Ultra Performance Liquid Chromatography mass spectrometry (UPLC/MS) analysis for saline ( $n = 5$ ) and PN mice ( $n = 6$ ). Full names of the 20 BAs are described in MATERIALS AND METHODS. Fold was calculated by dividing the averaged total serum concentration of BAs in each category detected in PN mice by that in saline mice. \* $P < 0.05$ , compared with saline group, Student's  $t$ -test.

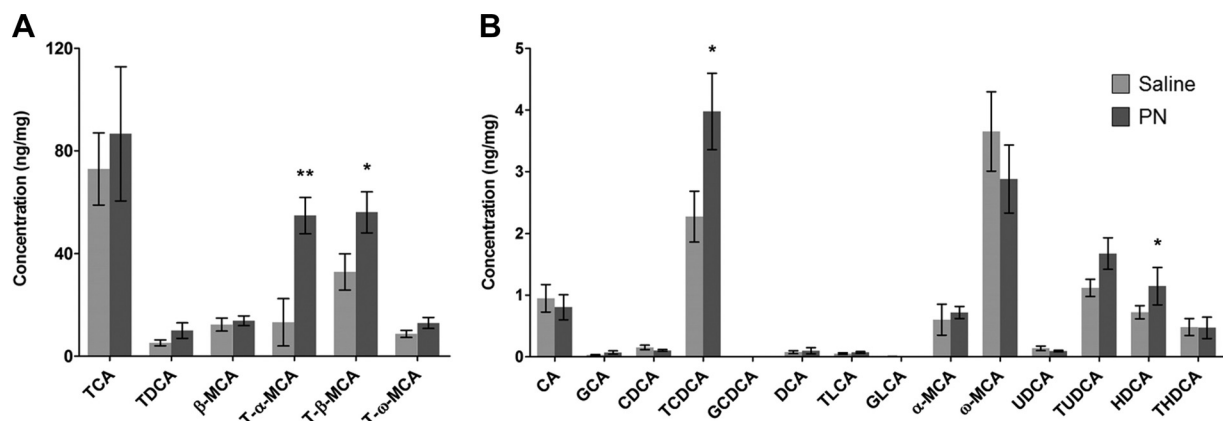


Fig. 4. Liver BA profiles were similar between PN and saline mice. Quantified liver concentrations (ng/mg of liver tissue) of the 20 BAs by UPLC/MS for saline ( $n = 6$ ) and PN mice ( $n = 6$ ). Full names of the 20 BAs are described in MATERIALS AND METHODS. Data were presented as means  $\pm$  SE. \* $P < 0.05$ , \*\* $P < 0.005$ , compared with saline group, Student's  $t$ -test. For A and B, the scales on the y-axis are different for better clarification.

However, for the other genes tested, the means of fold changes were  $<2$  and without significance (Fig. 5).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was used to further categorize the altered genes found in the microarray analysis (Table 6). Many genes, which are related to immune and inflammatory responses, were up-regulated, including genes involved in cytokine-cytokine receptor interaction (19 out of 28), Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway (12 out of 15), and natural killer cell-mediated cytotoxicity (11 out of 15). Most genes in the category of fat digestion and absorption as well as biosynthesis of unsaturated fatty acids were upregulated (10 out of 11). On the contrary, the majority of the genes involved in metabolism of endobiotics and xenobiotics were downregulated (13 out of 19 in drug metabolism, cytochrome P450; 10 out of 14 in retinol metabolism; 8 out of 12 in arachidonic acid metabolism; all the 8 in glutathione metabolism; and all the 6 in fatty acid metabolism).

**PN altered liver ROR $\alpha$  signaling.** Previous studies have shown that ROR $\alpha$  plays a key role in regulating the gene

expression of *Cyp8b1* and *Cyp7b1* in mouse liver (5, 13, 18). A detailed comparison of our microarray data (Microarray-PN/Saline) with previously published microarray data, obtained from ROR $\alpha$  knockout mice (Microarray-ROR $\alpha^{sg/sg}/WT$ ) (5), revealed similar trends of alterations for a number of genes in the two datasets (Table 7). A few genes with different trends were also identified. As shown in Table 7, the mRNA expression of ROR $\alpha$  also slightly decreased in the livers of PN mice. As a nuclear receptor, ROR $\alpha$  protein level, especially nuclear localization of ROR $\alpha$ , is more important in determining its physiological function. Western blot analysis showed that nuclear levels of ROR $\alpha$  protein decreased significantly in the livers of PN mice, whereas total ROR $\alpha$  protein levels actually increased (Fig. 6).

**PN mice had increased hepatic lipid accumulation.** Consistent with increased mRNA expression of lipid synthesis genes (Fig. 2), Oil Red *O* staining showed that PN mouse livers had significantly increased levels of lipid staining (Fig. 7), but not reaching the levels of steatosis.

Table 5. Top upregulated and downregulated liver genes from Microarray-PN/Saline

Gene	Fold Change	Direction	Full Transcript Name
<i>Sult1e1</i>	17.17	Up	Sulfotransferase family 1E, member 1, mRNA
<i>Fmo3</i>	7.68	Up	Flavin-containing monooxygenase 3, mRNA
<i>Lepr</i>	7.05	Up	Leptin receptor, transcript variant 2, mRNA
<i>Abcd2</i>	5.97	Up	ATP-binding cassette, subfamily D (ALD), member 2, mRNA
<i>Cyp17a1</i>	5.28	Up	Cytochrome P450, family 17, subfamily a, polypeptide 1, mRNA
<i>Nr4a1</i>	5.16	Up	Nuclear receptor subfamily 4, group A, member 1, mRNA
<i>Marco</i>	5.15	Up	Macrophage receptor with collagenous structure, mRNA
<i>Egr1</i>	5.1	Up	Early growth response 1, mRNA
<i>Ccrn4l</i>	5.02	Up	NOCTURNIN (Nocturnin)
<i>Btg2</i>	4.64	Up	B-cell translocation gene 2, antiproliferative, mRNA
<i>Cyp4a12a</i>	9.17	Down	Cytochrome P450, family 4, subfamily a, polypeptide 12a, mRNA
<i>Ces2</i>	8.37	Down	Carboxylesterase 2, mRNA
<i>Cyp2b10</i>	6.72	Down	Cyp2b10-like pseudogene, mRNA sequence
<i>Orm3</i>	6.61	Down	Orosomucoid 3, mRNA
<i>Hist1 h2bg</i>	6.28	Down	Histone cluster 1, H2bg, mRNA
<i>Clec2 h</i>	6.12	Down	C-type lectin domain family 2, member h, mRNA
<i>Orm2</i>	6.07	Down	Orosomucoid 2, mRNA
<i>Cyp4a12b</i>	4.87	Down	Highly similar to CYTOCHROME P450 4A8
<i>Cml5</i>	4.85	Down	Camello-like 5, mRNA
<i>Selenbp2</i>	4.49	Down	Selenium binding protein 2, mRNA
<i>Cyp2c55</i>	4.28	Down	Cytochrome P450, family 2, subfamily c, polypeptide 55, mRNA

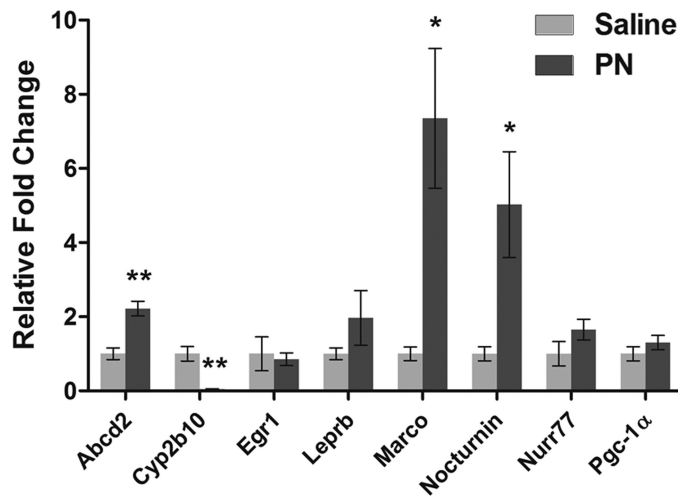


Fig. 5. Validation of microarray analysis (Microarray-PN/Saline). qRT-PCR validation of selected genes retrieved from Microarray-PN/Saline (genes with full names are shown in Table 5, *Nur77* is for *Nr4a1*). Hepatic gene expression data are expressed as means  $\pm$  SE. Samples from the same mice were used as in Fig. 2. Relative mRNA levels were first normalized to  $\beta$ -actin, and then relative fold changes were normalized to saline mice. \* $P < 0.05$ , \*\* $P < 0.005$ , compared with saline group, Student's *t*-test.

## DISCUSSION

The PN regimen, based on a previous publication (2), was modified to maintain sufficient calorie intake for adult mice to prevent excessive BW loss. One interesting change observed in this model was increased extent of LW loss in the PN mice.

Table 6. KEGG pathway analysis for Microarray-PN/Saline

KEGG Pathway	List	Up	Down	Gene Set
Cytokine-cytokine receptor interaction	28	19	9	243
Neuroactive ligand-receptor interaction	16	11	5	309
Jak-STAT signaling pathway	15	12	3	146
Natural killer cell-mediated cytotoxicity	15	11	4	132
Osteoclast differentiation	11	10	1	107
<i>Staphylococcus aureus</i> infection	11	9	2	48
Glycolysis/Gluconeogenesis	9	6	3	58
p53 signaling pathway	9	7	2	65
Inositol phosphate metabolism	7	7	0	55
Fat digestion and absorption	6	6	0	41
Biosynthesis of unsaturated fatty acids	5	4	1	24
Steroid biosynthesis	4	4	0	18
Drug metabolism-cytochrome P450	19	6	13	74
Retinol metabolism	14	4	10	64
Steroid hormone biosynthesis	13	5	8	46
Arachidonic acid metabolism	12	4	8	82
Complement and coagulation cascades	11	4	7	70
Glutathione metabolism	8	0	8	53
Pentose and glucuronate interconversions	7	1	6	22
Fatty acid metabolism	6	0	6	45
Circadian rhythm-mammal	4	1	3	21
Bile secretion	16	9	7	65
PPAR signaling pathway	15	8	7	74

Total number of genes retrieved from Microarray-PN/Saline for each Kyoto Encyclopedia of Genes and Genomes (KEGG) category is listed in the list column. Total number of genes for each category in the mouse genome in the database for DAVID is listed in the gene set column. PPAR, peroxisome proliferator-activated receptor.

Table 7. Comparison of microarray analysis

Functional Category	Gene	Microarray-PN/Saline		Microarray-ROR $\alpha^{sg/sg}$ /WT	
		Fold Change	Direction	Fold Change	Direction
Cytochrome P450	<i>Cyp2b9</i>	9.40	Up	7.2	Up
	<i>Cyp8b1</i>	3.56	Down	1.8	Down
	<i>Cyp7b1</i>	2.22	Down	4.2	Down
	<b><i>Cyp2b10</i></b>	<b>6.72</b>	<b>Down</b>	<b>1.2</b>	<b>Up</b>
	<b><i>Cyp4a10</i></b>	<b>2.74</b>	<b>Down</b>	<b>3.3</b>	<b>Up</b>
Steroid	<b><i>Hmgcr</i></b>	<b>2.61</b>	<b>Up</b>	<b>1.1</b>	<b>Down</b>
	<b><i>Hsd17b7</i></b>	<b>1.68</b>	<b>Up</b>	<b>1.3</b>	<b>Down</b>
	<i>Hsd3b4</i>	2.45	Down	1.9	Down
	<i>Hsd3b5</i>	2.39	Down	2.8	Down
Lipid and fatty acid	<i>Scd2</i>	2.11	Up	1.0	Up
	<i>Elovl6</i>	1.66	Up	1.3	Up
	<i>Elovl3</i>	3.01	Down	2.2	Down
Cell signaling	<b><i>Igf1bp1</i></b>	<b>2.88</b>	<b>Up</b>	<b>1.1</b>	<b>Down</b>
Transport	<i>Abcd2</i>	5.97	Up	1.3	Up
	<i>Apoa4</i>	3.87	Up	1.4	Up
Carbohydrate	<i>Ppp1r3c</i>	3.55	Down	1.7	Down
Circadian rhythm	<i>Rora</i>	1.60	Down	2.6	Down
	<b><i>Rorc</i></b>	<b>1.61</b>	<b>Down</b>	<b>1.2</b>	<b>Up</b>
	<i>Ccm4l</i>	5.02	Up	1.7	Up
Miscellaneous	<i>Keg1</i>	2.22	Down	1.6	Down
	<i>Lpin2</i>	2.57	Up	1.4	Up
	<i>Selenbp2</i>	4.49	Down	3.1	Down
	<b><i>Ccnb1</i></b>	<b>2.22</b>	<b>Up</b>	<b>1.3</b>	<b>Down</b>

Selected genes with the corresponding fold changes and directions of change were retrieved from Microarray-PN/Saline and Microarray-ROR $\alpha^{sg/sg}$ /WT (5). The functional categories of these genes were obtained from the previous microarray study (5). The genes with different directions of changes in the 2 microarray datasets are in bold font. ROR $\alpha$ , retinoid acid-related orphan receptor- $\alpha$ .

Future studies will be needed to uncover the underlying mechanisms.

Similar to the previous study (2), serum ALT levels were not increased in the PN mice, whereas serum AST levels increased significantly in our model but were still in the normal range. TSBA levels increased significantly in the PN mice. Interestingly, serum ALP levels decreased significantly in the PN mice. It has been shown that FGF15 is essential for gallbladder refill in mice (4). Without normal food intake, the lack of enteral stimulus in PN mice will lead to impaired BA release into the GI tract. This could further impair enterohepatic FGF15 signaling, causing diminished gallbladder refilling from the liver and subsequently less biliary BA exposure, leading to less biliary injury indicated by decreased serum ALP levels. In addition, recent studies also showed that elevated levels of ALP in patients with intestinal failure and PNALD can be caused by metabolic bone diseases (MBD), rather than liver diseases (11). From the KEGG pathway analysis, we did see that 10 out of 11 genes in the category of osteoclast differentiation were upregulated in the Microarray-PN/Saline (Table 6). These findings from the microarray analysis can potentially provide novel insights into the association of long-term PN-induced liver dysfunction to the development of MBD, for which, the underlying mechanisms are still poorly understood (11).

The lack of enterohepatic FGF15 signaling could also be responsible for the upregulation of *Cyp7a1* gene expression seen in the PN mice. It has been shown that WT mice with bile duct ligation had a threefold increase in *Cyp7a1* expression,



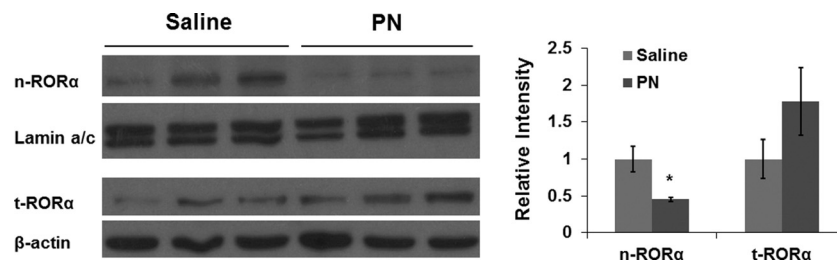


Fig. 6. PN decreased liver nuclear protein levels of retinoid acid-related orphan receptor- $\alpha$  (ROR $\alpha$ ). *Left*: Western blot analysis of ROR $\alpha$  protein in saline and PN mouse livers (n-ROR $\alpha$ , nuclear fraction of ROR $\alpha$ ; t-ROR $\alpha$ , total cellular ROR $\alpha$ ). Lamin a/c and  $\beta$ -actin were used as the loading controls for nuclear and total protein, respectively. Each band represents 1 single mouse sample in the indicated treatment group. *Right*: relative band density was determined using the ImageJ software. Data are presented as means  $\pm$  SE. The relative intensity of ROR $\alpha$  for each mouse sample was first normalized to the corresponding loading control, and then the results from PN mice were normalized to the saline mice. Results from saline mice were set as arbitrary 1. \* $P < 0.05$ , compared with saline group, Student's  $t$ -test.

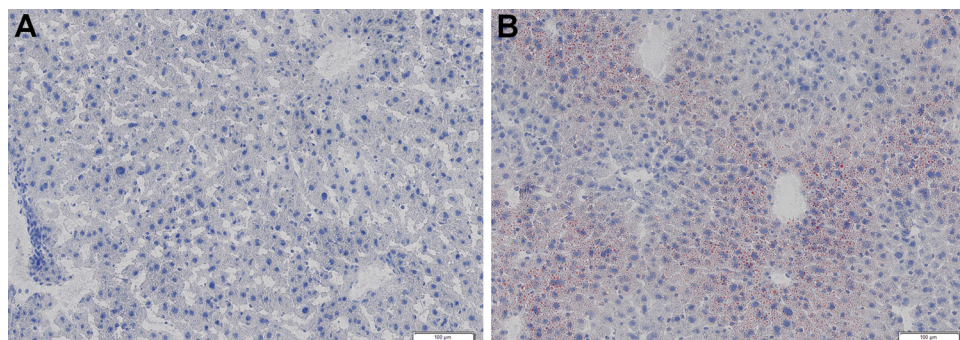
whereas the expression of small heterodimer partner (*Shp*) was not changed (4). The increased mRNA levels of *Cyp7a1* in PN mice may lead to the accumulation of cholesterol metabolites in the liver, which could, in turn, activate liver-X receptor- $\alpha$  (LXR $\alpha$ ) and induce the expression of LXR $\alpha$  target genes. Indeed, we have observed upregulation of LXR $\alpha$  target genes in PN mouse livers, including *Lpl*, *Srebp-1c*, and *Fas* (Fig. 2B). Long-term LXR $\alpha$  activation coupled with upregulation of its target genes could be responsible for PN-induced liver steatosis in adults although PN mice tend to lose BW.

Similar to prior findings from PN piglets (17), we also detected decreased gene expression of *Fxr*, *Bsep*, and *Mrp3* in the livers of PN mice. A previous report did show reduced expression of *Fxr* and *Bsep* in DSS-pretreated PN mice (PN/DSS); however, the expression of these genes in PN-only mice was not reported (2, 3). Consistent with the downregulation of *Fxr* and FXR target genes (*Bsep*, *Cd36*), we also detected a reduced gene expression of *Shp* in the livers of PN mice. Although a previous study has shown that intestinal FXR and FGF15 play a major role in suppressing *Cyp7a1* gene expression (7), the downregulation of *Shp* could also contribute to the upregulation of *Cyp7a1*. Interestingly, similar to the findings in PN piglets, ileum *Fgf15* gene expression also increased in the distal ileums of PN mice. Nevertheless, plasma FGF19 level in PN piglets actually decreased, measured by ELISA (17). Because of the lack of a good ELISA antibody for FGF15 protein, the data for portal FGF15 levels are not provided. Nevertheless, similar trends of decreased gene expression in the adult mice and preterm piglets suggest common underlying mechanisms in different species, which are most likely PN dependent. Future studies will be needed to uncover the underlying mechanisms, especially the downregulation of *Fxr* gene expression

and FXR signaling. The increase of *Cyp7a1* and decrease of *Fxr*, *Bsep*, *Shp*, and *Mrp3* gene expression, taken together with the lack of hepatic bile flow into the gallbladder, could lead to BA accumulation in hepatocytes, causing the elevation of TSBA in PN mice. BA accumulation in hepatocytes of PN mice could lead to cholestasis after long-term PN, especially with the presence of additional risk factors, such as catheter-related infections or intestinal tract inflammation. These risk factors could magnify liver stress and cause severe liver damage in a relatively short time, as detected in PN/DSS mice (2).

Results from serum BA profiling by UPLC-MS were consistent with gene expression results. PN mice exhibited a threefold increase of *Cyp7a1* gene expression as well as an increase in serum levels of the total and individual BAs. CYP8B1 determines the ratio of 12 $\alpha$ -OH BAs vs. non-12 $\alpha$ -OH BAs. As expected, the lack of intestinal FXR-FGF15 signaling and the decreased mRNA expression of *Cyp8b1* in PN mice could lead to a dramatic increase of both the levels and percentages of serum TCDCA, T- $\beta$ -MCA, and T- $\alpha$ -MCA in PN mice (Fig. 3, Table 4). Although serum levels of CA, TCA, DCA, and TDCA also increased in PN mice, their percentage in the total serum BA pool actually decreased. As a result, the percentage of total non-12 $\alpha$ -OH BAs was much higher in PN mice (Table 4). The decrease in the percentage of total secondary BAs in PN mice could also be caused by decreased bile flow from the liver into the intestinal tract, leading to a proportional decrease of secondary BA formation in the gut. Although the levels of unconjugated secondary BAs in saline and PN mice were similar (Fig. 3), the levels of conjugated secondary BAs were much higher in PN mice, except for GLCA (undetectable). Because fecal excretion is mainly responsible for the direct loss of secondary BAs, the increased

Fig. 7. PN increased hepatic lipid deposition in mice. Representative low-throughput images from Oil Red O staining of frozen mouse liver sections from saline (A) and PN (B) mice. Scale bar = 100  $\mu$ m.





levels of conjugated secondary BAs in PN mice could be caused by the decreased fecal loss of BAs (9). Studies in germ-free mice suggest that T- $\beta$ -MCA and T- $\alpha$ -MCA are FXR antagonists (15). Indeed, the most significant changes detected in the PN mice were the increased levels of T- $\beta$ -MCA and T- $\alpha$ -MCA in both mouse serum and liver extracts, which could explain the downregulation of FXR signaling in the PN mice, even without DSS pretreatment. Of note, liver TCDCA also increased significantly, but the concentration was much lower compared with T- $\beta$ -MCA or T- $\alpha$ -MCA.

ROR $\alpha$  has been shown to play critical roles in regulating the expression of many genes involved in phase I and phase II metabolism (5). The expression patterns of many genes involved in cellular metabolism in the liver are also regulated by the fast-feed circadian rhythm (20, 22). It has been shown that the expression of ROR $\alpha$  exhibited an oscillatory pattern consistent with the liver circadian rhythm (5). Under continuous PN infusion, it is very likely that the normal fast-feed circadian rhythm, maintained in saline control mice, is disrupted in PN mice. It has also been shown that glucagon/protein kinase A (PKA) signaling could phosphorylate and stabilize ROR $\alpha$  protein upon fasting to induce *Cyp8b1* gene expression and diurnal rhythm (13). The continuous supply of dextrose in the PN solution could potentially lead to a downregulation of PKA signaling and therefore cause a suppression of ROR $\alpha$  target genes, such as *Cyp7b1* and *Cyp8a1*. Furthermore, many indirect target genes were also altered in the PN mice (Table 7) because of the suppression of ROR $\alpha$  by the continuous PN infusion, as exhibited by significantly decreased nuclear protein levels of ROR $\alpha$  in PN mouse livers. Previous studies have also shown that ROR $\alpha$  and LXR $\alpha$  could mutually suppress the function of each other (18). Therefore, the dramatic downregulation of ROR $\alpha$  signaling could also lead to the upregulation of LXR $\alpha$  target genes as well as the subsequent lipid accumulation in the livers of PN mice. It has been shown that ROR $\alpha$  and ROR $\gamma$  could redundantly regulate the expression of many genes involved in phase I and phase II metabolism (5). However, nuclear protein levels of ROR $\gamma$  by Western blot did not show any correlation (data not shown) with the alterations of the gene expression of ROR $\gamma$  targets.

It is also important to note that the expression of several genes listed in Table 7 showed opposite trends of alterations (*Cyp2b10*, *Cyp4a10*, and *Ccna1*, etc.). These changes could be mediated by additional transcriptional factors altered by PN infusion. For example, the expression of *Cyp2b10* has been shown to be directly regulated by both constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (12). In this case, the potential alterations of CAR and/or PXR signaling could explain the dramatic decrease of *Cyp2b10* expression in PN mice.

In summary, our studies showed altered hepatic gene expression, altered serum/liver BA profiles, and increased hepatic lipid accumulation in mice after 8 days of PN infusion. We provided mechanistic evidence of altered ROR $\alpha$  signaling in PN mice. We further showed additional alterations of gene expression in various functional pathways in the livers of PN mice. These novel results will provide critical insights for future studies of PN-induced cholestasis (BA homeostasis), steatosis (LXR $\alpha$  and lipid metabolism), MBDs, etc., when PN infusion is combined with genetic, pharmacological, or toxicological manipulations in mice or other animal models.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

L.Z. and G.L.G. conception and design of research; L.Z., I.Y., J.S., and L.G. performed experiments; L.Z., I.Y., and B.T.B. analyzed data; L.Z., B.K., N.M., and G.L.G. interpreted results of experiments; L.Z. prepared figures; L.Z. drafted manuscript; L.Z., I.Y., B.K., L.G., N.M., and G.L.G. edited and revised manuscript; L.Z., B.K., and G.L.G. approved final version of manuscript.

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