Neurotensin contributes to pediatric intestinal failure-associated liver disease via regulating intestinal bile acids uptake

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Abstract

Although the pathogenesis of intestinal failure (IF)-associated liver disease (IFALD) is uncertain, IF-associated cholestasis mediated by the combination of intestinal injury and parenteral nutrition (PN) can lead to disturbed hepatocyte bile acids (BA) homeostasis and cause liver damages. We here show that neurotensin (NT; also known as NTS) concentrations were lower compared to healthy matched controls. Patients with cholestasis [56.1 ng/L (9.7–154.7) vs. 210.4 ng/L (134–400.4), p < .001] had lower serum NT concentrations than others. In patients' ileum, the levels of NT mRNA were positively correlated with the apical sodium dependent bile acid transporter (ASBT) mRNA levels. In mice and in cultured intestinal cells, NT treatments stimulated the expression of ASBT and led to increase BA uptake via NT receptors (NTR1 and NTR3; also known as NTSR1 and NTSR3). In conclusion, these findings directly link NT with BA homeostasis, which provide an insight into the complex mechanisms mediating the development of liver disease in pediatric patients with IF.

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1. Introduction

Pediatric intestinal failure (IF) is often caused by short bowel syndrome (SBS) or gastrointestinal motility disorders, which is characterized by insufficient bowel function to maintain hydration and nutrient absorption for growth and development. IF-associated liver disease (IFALD) that is serious complication and the leading cause of morbidity and mortality in pediatric IF patients. However, the mechanisms underlying the development of IFALD are poorly understood. To understand the involved mechanisms, we performed population based cross-sectional study on neurotensin (NT) in relation to bile acids (BA) homeostasis in pediatric IF patients and investigated the roles of NT on BA uptake in vitro and in vivo.
dyshomeostasis, leading to liver injury. To this end, we determined the serum concentrations of NT, biochemical liver function tests and serum BA levels, and analyzed the relationship between them. We also investigated the roles of NT on BA uptake and mechanisms involved in vitro and in vivo.

2. Materials and methods

2.1. Reagents

NTR1 antibody (Cat. No. sc-374492) was from Santa Cruz Biotechnology (Dallas, TX), Anti-PPARα (phospho S12) antibody (Cat. No. ab3484), PPARα (ab8934) and β-Actin (ab8226) antibodies were obtained from Abcam (NT, HK). NTR3 (Cat. No. 12369-1-AP) and PPARα (Cat. No. 15540-1-AP) antibodies were obtained from Proteintech (Rosemont, IL). Neurotensin (Cat. No. N5266), SR 48692 (Cat. No. SML0278), Sodium taurocholate-2, 4, 4-d4 (TCA-D4, Cat. No. 900036) and Anti-ASBT antibody (Cat. No. HPA004795) were from Sigma-Aldrich (St. Louis, MO), SYBR-Green Universal Master Mix kit (Cat. No. 4385610) and a High Capacity cDNA Reverse Transcription kit (Cat. No. 4368814) were from Applied Biosystems (Foster City, CA). Dual Luciferase Assay Kit (Cat. No. E2920) was from Promega (Madison, WI). Lipofectamine® RNAiMAX (Cat. No. 11668019) and Lipofectamine™ 2000 (Cat. No. 11668027) transfection reagents and TRizol reagent (Cat. No. 15596026) were from Life Technologies (Foster, CA). The siRNAs were synthesized by GenePharma (Shanghai, China). The sequences for siRNAs were described as previously and listed in Supplementary Materials and Methods.

2.2. Patients

A total of forty patients at median age 6.0 months (IQR 3.25–26.9) enrolled in the study (Supplementary Table 1). Causes of IFL included short bowel syndrome (necrotizing enterocolitis (NEC): n = 8, small bowel atresia: n = 5, and mid-gut volvulus: n = 3) and intestinal dysmotility disorders (chronic intestinal pseudo-obstruction (CIPO): n = 20 and extensive aganglionosis of hirschsprung’s disease: n = 4). In total, 32 patients preserved ileocecal valve and ileum. 21 patients were on PN and 19 had weaned off PN 51 days (28–78) earlier, after 1.9 months (0.8–12) on PN. The PN energy comprised 51% (47–55) of glucose and 32% (28–36) of fat. PN fat was given as soy oil-based emulsion [1.5 g/kg/day (1.10–1.78)] and combined with fish oil-based emulsion [1.0 g/kg/day] in four patients.

A total of 40 blood samples from patients were analyzed in this study. 16 blood samples from healthy day-surgery patients with matched age without gastrointestinal diseases used as controls. The

Table 1

| Variable | Healthy control (n = 16) | All patients (n = 40) | Patients without cholestasis (n = 19) | Patients with cholestasis (n = 21) | p value | Correlation with NT |
|-----------------|-------------------------|------------------------|---------------------------------------|-----------------------------------|---------|-------------------|
| Serum Neurotensin, NT (ng/L) | 282.5 (167.2–531.3) | 139.3 (48.2–259.7) | 210.4 (134–400.4) | 56.1 (9.7–154.7) | <0.001 | <0.001 |

Liver enzymes

| Plasma alkaline phosphatase, ALP (U/L) | 25 (5.2–45.2) | 274 (202.5–351.5) | 260.5 (212.5–361.5) | 285 (183–331.7) | <0.01 | 0.18 |
| Plasma alanine aminotransferase, ALT (U/L) | 18.6 (15.3–27) | 45 (30.5–87.5) | 45 (31–87) | 45 (32.5–82) | <0.01 | 0.41 |
| Plasma aspartate aminotransferase, AST (U/L) | 31 (23–50) | 59 (46.5–100.5) | 59 (49–91.7) | 57 (46.5–114.5) | <0.01 | 0.21 |

Markers of cholestasis

| Plasma total bilirubin (μmol/L) | 7.2 (3.5–6) | 13.7 (9.45–23.4) | 10.7 (10.2–17.4) | 13.7 (9.25–33.4) | 0.02 | 0.09 |
| Plasma conjugated bilirubin (μmol/L) | 2.7 (2.5–4) | 6 (0.25–11.65) | 2.5 (0.2–4) | 11.7 (10.3–20.5) | 0.89 | 0.007 |

Serum lipids

| Serum HDL cholesterol (mmol/L) | 0.72 (0.62–0.8) | 0.62 (0.54–0.7) | 0.32 (0.24–0.47) | 0.41 (0.34–0.59) | 0.06 | 0.065 |
| Serum LDL cholesterol (mmol/L) | 1.48 (1.22–1.84) | 1.66 (1.38–2.08) | 1.69 (1.42–2.1) | 1.39 (1.21–1.86) | 0.03 | 0.092 |
| Serum total cholesterol, TC (mmol/L) | 2.1 (1.63–2.41) | 2.32 (1.89–2.66) | 2.38 (1.82–2.74) | 2.02 (1.79–2.45) | 0.04 | 0.25 |
| Serum triglycerides, TG (mmol/L) | 0.75 (0.54–1.06) | 0.83 (0.64–1.36) | 0.89 (0.68–1.45) | 0.68 (0.54–1.15) | 0.02 | 0.045 |
| Plasma glucose (mmol/L) | 3.53 (3.06–4.32) | 4.6 (4.1–5.3) | 5.1 (4.5–6.0) | 4.2 (3.4–4.6) | 0.14 | 0.002 |

Data are median (range). A comparison between patients with and without cholestasis using Fisher’s exact test or Mann Whitney U test.
serum samples were prepared centrifugation after blood collection and stored at −80 °C until analyzed. The blood samples were analyzed for levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin and conjugated bilirubin, total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C), low density lipoprotein, cholesterol (LDL-C), neurotensin (NT) and bile acids (BA).

A total of 40 liver specimens were obtained from pediatric IF patients who underwent surgery. 10 normal adjacent non-tumour tissues, taken from hepatoblastoma patients, were used as liver controls. A piece of the liver samples were snap-frozen in liquid nitrogen and stored at −80 °C for analyzing the concentration of hepatic bile acids and levels of related genes. The remained liver biopsies were routinely stained with hematoxylin and eosin (HE), Periodic-acid-Schiff and Masson trichrome assay. Liver cholestasis, steatosis and fibrosis were analyzed by two researchers and a pathologist, blinded to clinical data, until consensus was reached. The cut-off for the diagnosis of cholestasis was set at serum direct bilirubin (DB) > 2 mg/dl [8]. Liver cholestasis was graded (0–3) as intracellular, canalicular and ductular cholestasis (0 = absent, 1 = minimal, 2 = marked, 3 = prominent) [9]. Steatosis was determined and graded as described previously [10]. The liver fibrosis was assessed by Metavir fibrosis stage [11].

Surgical samples of ileum (n = 20) from IF patients were used to performed quantitative real-time polymerase chain reaction (qRT-PCR), histology and immunohistochemistry (IHC). All patients’ guardians provided written informed consent. This study was approved by the Faculty of Medicine’s Ethics Committee of Xiu Hua hospital School of Medicine, Shanghai Jiao Tong University, Shanghai, China. All methods in this study were carried out in accordance with the relevant guidelines.

### Table 2

| Bile acids (nM) | HC (n = 16) | Patients (n = 40) | P value<sup>a</sup> (HC vs. Patients) | Spearman ρ (Correlation with NT) | P value<sup>a</sup> (Patients without Cholestasis vs. Patients with Cholestasis) |
|----------------|------------|----------------|-------------------------------------|---------------------------------|--------------------------------------------------|
| CA             | 338.2 ± 651.3 | 1428.3 ± 2868.0 | 166.8 ± 333.7 | 2107.6 ± 3376.8 | 0.140 | 0.040 | −0.012 |
| o-MCA          | 17.6 ± 20.3 | 53.3 ± 110.1 | 5.9 ± 4.3 | 73.3 ± 126.4 | 0.221 | 0.102 | −0.286 |
| CDCA           | 251.1 ± 466.6 | 708.1 ± 1246.7 | 151.2 ± 251.0 | 1007.9 ± 1457.6 | 0.161 | 0.036 | 0.322 |
| HCA            | 45.9 ± 70.7 | 30.1 ± 51.1 | 5.6 ± 7.0 | 23.1 ± 58.7 | 0.390 | 0.052 | 0.129 |
| 7DHCA          | 103.9 ± 312.7 | 90.9 ± 253.6 | 9.1 ± 23.3 | 138.2 ± 311.2 | 0.895 | 0.184 | −0.283 |
| 3DHCA          | 8.0 ± 10.9 | 28.1 ± 73.5 | 5.4 ± 6.3 | 38.9 ± 87.8 | 0.284 | 0.198 | 0.076 |
| Total BA       | 718.9 ± 1037.0 | 2301.4 ± 4144.9 | 338.1 ± 566.7 | 3538.6 ± 4823.1 | 0.139 | 0.026 | 0.327 |

*Comparison between patients with and without cholestasis using Fisher’s exact test or Mann Whitney U test.

2.3. Histology and immunohistochemistry (IHC)

Histological examination was stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was performed using the method of diaminobenzidine (DAB) chromogen. Briefly, paraffin-embedded tissues were deparaffinized using xylol and descending concentrations of ethanol. Tissue buffer (pH 6.0 or PH 8.0) was used for antigen retrieval. Endogenous peroxidases were removed by 0.3% H2O2 and then blocked using 10% bovine serum albumin (BSA). Primary antibodies were applied in an optimal concentration overnight in a wet chamber (NTR1, dilution, 1: 25; NTR3, dilution, 1: 50; Phospho-PPARα, dilution, 1: 100; ASBT, 1: 100). Antibody binding was visualized by a liquid DAB Substrate Chromogen System (Dako, Glostrup, Denmark). The IHC images analysis was used software Image Pro Plus (Media Cybernetics) 10 fields/sample.

2.4. Biochemical measurements and enzyme linked immunosorbent assay (ELISA)

The human blood samples were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin and conjugated bilirubin by using routine hospital laboratory methods. Serum total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) were determined enzymatically. The plasma biochemistries for all animals were analyzed with a CHEMUX-180 multiple analyzer (Sysmex Corporation, Japan) as previously described [12]. For ELISA analysis, the human IL-6 Platinum ELISA (BMS213, eBioscience, Waltham, MA) Kit, human TNF-α Alpha Platinum ELISA (BMS223, eBioscience, Waltham, MA) Kit and human...
Neurotensin ELISA Kit (CSB-E09144h, Cusabio, Wuhan, China) were used in this study according to protocols of manufactures.

2.5. Bile acids measurements

Bile acids measurements were performed with a Waters ACQUITY ultra performance liquid chromatography coupled with Waters Xevo TQ-S triple quadrupole mass spectrometry according to the previously reported method [13, 14]. Data acquisition and bile acids quantification were performed using the MassLynx 4.1 software (Waters). The methods were detailed in Supplementary Materials and Methods.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

The trusses samples from human and mice were homogenized using MagNA Lyser Instrument and MagNA Lyser Green Beads (Manassas, VA, USA). Total RNA was extracted with Trizol according to the protocol of the manufacture (Invitrogen, Foster, CA). cDNA synthesized from 1 μg of total RNA with a High Capacity cDNA Reverse Transcription kit. A SYBR-Green Universal Master Mix kit was employed to detect the levels of the genes. The primers were listed in Supplementary Materials and Methods.

2.7. Western blotting

For Western blot, the equal amounts of proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in 5% nonfat dry milk, 0.2% Tween-20 at room temperature (RT) for 30 min, the membranes were incubated overnight at 4 °C with primary antibodies. Antibodies for NTR1 (dilution, 1: 50), NTR3 (dilution, 1: 100), Phospho-PPARα, (dilution, 1: 100), PPARα (1: 200), ASBT (1: 100) and β-Actin (dilution, 1: 500) were analyzed. The membranes were washed with PBS (containing 0.1% Tween) and incubated with horseradish-peroxidase conjugated detected the antigen-antibody complexes using an ECL Plus chemiluminescence reagent kit (Pierce, Rockford, IL, USA).

2.8. Cell culture and treatments

The human intestinal epithelial cells Caco2 and rat intestinal epithelial cells IEC-6 were purchased from American Type Culture Collection (Manassas, VA), and was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Reverse transfection was performed using RNAiMAX (for siRNA) or Lipofectamine 2000 (for plasmid) transfection reagents according to the protocol of manufactures. Final siRNA concentrations were used at 200 (NTR3), 100 (PPARα) or 200 (NTR1) nM. Cells were treated after 72 h (siRNA) or 48 h (plasmid) after transfection. For combined treatment of TCA with NT, cells were pre-treated with NT (2.5 μM) or NT at various dosages for 30 min followed by TCA (0.1 mM) for 1 h. For analysis of sodium Taurocholate-2, 2, 4, 4-D4 (TCA-D4) in vitro, siRNAs against NTR1, NTR3 or PPARα were transfected into Caco-2 cells firstly. After 72 h, the cells were pre-treated with or without NT (2.5 μM) for 30 min and followed by TCA-D4 (0.1 mM) for 2 h. The cells washed with cold PBS twice and collected for further bile acid analysis.

Fig. 2. The expression of NT and its receptors in intestinal of mice and human. (A) Quantification of Nt, Ntr1, Ntr2 and Ntr3 in the mouse proximal (pro), middle (mid), distal (dis) small bowel and colon. (B) Total RNA was isolated from human ileum (n = 4) and RT-PCR performed using specific primers targeting human NTR1, 2 and 3.
2.9. Animal experiments

All procedures were approved by the Shanghai jiao tong University School of Medicine affiliated Xin Hua hospital Animal Care and Use Committee. Mice were maintained with a 14 h light/10 h dark cycle and provided with food and water ad libitum. For intestinal transporting bile acids studies, 34 male C57BL/6 mice (6-week-old) were divided into four groups: controls (n = 10), TDA-D4 (n = 8), TDA-D4 + SR 48692 (n = 8), and TDA-D4 + SR 48692 + NT group. Mice were injected with SR 48692 (2.5 mg/kg body weight, i.p.). Thirty min after the injection, mice were given 0.5 mL TCA-D4 (200 μg/mL) by oral gavage twice a day and then human euthanasia. TDA-D4 + NT group: Mice were injected with NT (4000 nmol/kg body weight, i.p.) [7]. Thirty minutes after the injection, mice were given 0.5 mL TCA-D4 (200 μg/mL) by oral gavage twice a day and then human euthanasia. TCA-D4 group: Mice were injected with saline and were given 0.5 mL TCA-D4 (200 μg/mL) by oral gavage twice a day. Controls group: Mice injected with saline and were given 0.5 mL saline by oral gavage twice a day. After euthanasia, the liver, intestinal, blood and feces were collected and further analysis.

2.10. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells and intestinal samples using Trizol (Invitrogen, Foster, CA) according to the manufacturer’s instructions. RT-PCR analysis was performed using cDNA synthesized from 1 μg of total RNA. β-actin was used as the internal control. The primers were synthesized by Invitrogen (Shanghai, China). The primers sequences were described as previously and listed in Supplementary Materials and Methods. The PCR products were analyzed on a 2% agarose gel.

Fig. 3. The expression of NT in relation to ASBT levels in the ileum of IF patients. (A) Quantification of mRNAs of NT, NTR1, NTR2, NTR3, PPARα, SLC10A2 in the ileum of patients. (B) The NT mRNA correlated with SLC10A2 mRNA. (C) The PPARα mRNA correlated with SLC10A2 mRNA. (D) Representative images of IHC for NTR1, NTR3, p-PPARα and ASBT in the ileum of patients. (E) Western Blotting analysis for proteins PPARα, p-PPARα and ASBT in the ileum of patients. Scale bar = 50 μm.
2.11. Reporter analysis

To construct reporter vectors carrying promoters SLC10A2, we synthesized the fragments containing the promoters for human SLC10A2 (–1688 to +408) and cloned them into the psciCHECK2 luciferase vector (Promega, Madison, WI). Transient transfection of Caco-2 cells with PPARα siRNAs, promoters of SLC10A2 was carried out with Lipofectamine 2000. After 72 h, cells were harvested and washed with PBS. Cell lysates were assayed for luciferase enzyme activities as described by the dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA). GW7647 (2 μM) treated for 2 h to stimulate the PPARα. Luciferase activities were normalized to Renilla luciferase activity to correct for differences in transfection efficiency and cell numbers. All transfection studies were repeated at least three times.

2.12. Statistical analysis

The statistics are presented as frequencies and percentages, medians IQR or as mean ± SD. The Kolmogorov-Smirnov test was used to assess distributions. Mann Whitney U test, Fisher exact test or one-way ANOVA were used to compare differences between groups. Correlations were tested by Spearman rank correlation test. The level of statistical significance was set at 0.05.

3. Results

3.1. Neurotensin (NT) decreases in IF patients

In IF patients, serum neurotensin (NT) concentration was significantly lower \( n = 40, 139.3 \text{ ng/L} (48.2.2–259.7) \), \( p < .001 \) compared to controls \( n = 16, 282.5 \text{ ng/L} (167.2.2–531.3) \) (Fig. 1A and Table 1). Overall, 52% (21/40) of patients had cholestasis (Supplementary Table 1). Patients with cholestasis had about four folds lower serum NT concentrations in relation to patients without cholestasis (Fig. 1B and Table 1). As shown in Table 1, serum NT levels were inversely correlated with the levels of plasma alkaline phosphatase (ALP) \( r = −0.29, p = .03 \) and plasma alanine aminotransferase (ALT) \( r = −0.23, p = .07 \) (Table 1).

3.2. Altered NT is associated with BA dyshomeostasis

It showed that the serum BA (2812.3 ± 2371.3 vs. 7859.4 ± 8559.9, nmol/L, \( p = .038 \)) and liver (431 ± 255 vs. 884 ± 231, nmol/mg, \( p = .03 \)) elevated significantly in patients with cholestasis, related to patients without cholestasis (Table 2 and Supplementary Table 2). In addition, the BA compositions of liver and serum were markedly altered in patients with cholestasis, exhibiting a significant increase in the proportion of the primary BA (Including cholic acid (CA) and chenodeoxycholic acid (CDCA)) (Table 2 and Supplemental Table 2). In line with BA increased in patients with cholestasis, the classic bile acid synthesized enzyme cholesterol 7α-hydroxylase (CYP7A1) expression increased significantly in patients with cholestasis when compared to those without cholestasis (Supplementary Fig. 1). Moreover, the patients with cholestasis had higher degree of liver damages and portal fibrosis when compared to those without cholestasis (Supplementary Fig. 1).

As shown in Table 2, serum NT levels negatively correlated with plasma BA concentrations (\( r = −0.294, p = .028 \)) (Table 2). The BA profiles analysis indicated that plasma primary bile acid CA \( r = −0.332, p = .012 \) and CDCA \( r = −0.352, p = .008 \) inversely correlated with NT levels (Table 2). In addition, it observed that patients with cholestasis had increased fecal taurocholic acid (TCA) \( (7.2 ± 12.6 \text{ vs. } 191.9 ± 254.9 \text{ nmol/mg}, p = .017) \) compared to the ones without cholestasis (Supplementary Table 3).

3.3. NT levels in relation to ileal ASBT expression

As shown in Fig. 2A, mucosa from mouse proximal (pro), middle (mid), distal (dis) small bowel and colon expressed Nt and its receptors (Ntr1, Ntr2 and Ntr 3). The expression of Nt mRNA was higher in the mice small intestinal when compared to the colon. The mRNAs of ntr1 and ntr2 decreased from the proximal small bowel to the colon. In contrast, Ntr3 mRNA increasingly expressed from the proximal small bowel to the colon (Fig. 2A). In patients, the RT-PCR analysis showed that NTR1, NTR3 but not NTR2 strongly expressed in the ileum (Fig. 2B). The qRT-PCR indicated that IF patients with cholestasis \( n = 9 \) had lower mRNAs of NT, NTR1, NTR3, PPARα and SLC10A2 (ASBT) in ileum, compared to the ones without cholestasis \( n = 7 \) (Fig. 3A). Correlated analysis showed that NT mRNA levels positively correlated with levels of SLC10A2 \( r = 0.47, p = .06 \) (Fig. 3B). Moreover, the PPARα mRNA levels significantly correlated with SLC10A2 mRNA expression \( r = 0.49, p = .05 \) (Fig. 3C). In line with changes in these mRNAs, the proteins for NTR1, NTR3, phosphorylated–PPARα (p–PPARα) and ASBT were decreased in ileum of IF patients with cholestasis related to the ones without cholestasis (Fig. 3D and E).

3.4. NT and BA treatments increase expression of ASBT

RT-PCR analysis showed that NTR1, NTR3 expressed in the human intestinal epithelial cells Caco2 (Fig. 4A). In contrast, the rat intestinal epithelial cells IEC6 expressed all Nt receptors (Ntr1, 2 and 3) (Fig. 4B). Treatment of Caco2 with different dose of NT (0–5 μM) or different concentration of sodium taurocholate (TCA) (0–400 μM) led to an increase in expression of ASBT (Fig. 4C and D). Moreover, the NT or TCA administration also increased the phosphorylation of PPARα in Caco2 cells (Fig. 4C and D).

![Fig. 4. NT or TCA treatment increased expression ASBT and phosphorylation of PPARα in vitro.](image-url)
3.5. NT treatment promotes BA absorption via its receptors in vitro

Caco2 cells transfected with either human NTR1 siRNA or human NTR3 siRNA for 72 h abrogated expression of NTR1 or NTR3. As shown in Fig. 5, NTR1 or NTR3 knockdown prevented NT- or TCA-induced expression of p-PPARα and ASBT (Fig. 5A). Similarly, NT- or TCA-induced p-PPARα and ASBT in IEC6 cells were evidently suppressed by either Ntr1 or Ntr3 knockdown (Fig. 5B). Furthermore, it was also found that PPARα knockdown reduced expression of ASBT in presence of NT or TCA (Fig. 6A). To determine roles NT in intestinal BA absorption in vitro, Caco2 cells transfected with NTR1, NTR3, PPARα or control siRNAs for 72 h were pretreated with NT (2.5 μM) for 30 min followed by TCA-D4 (0.1 mM) for 2 h, and cellular contents of TCA-D4 were determined. As shown in Fig. 6B, the NT treatment significantly increased Caco2 cells absorbed TCA-D4 related to controls (77.9 ± 4.2 vs. 129.5 ± 0.6 nmol/mg protein, p < .01) (Fig. 6B). NTR1 or PPARα knockdown successfully abrogated the NT-increased TCA-D4 absorption in Caco2 cells (Fig. 6B).

To investigate whether a transcriptional mechanism exists that PPARα regulates ASBT, Caco2 cells transfected with SLC10A2 promoter-luciferase vectors and PPARα siRNA or control for 72 h were pretreated with NT (2.5 μM) or PPARα agonist GW 7647 (2 μM) for 30 min. As shown in Fig. 6C, Caco2 cells treatment with NT resulted in a 2.5-fold induction of ASBT promoter activity (Fig. 6C). The PPARα knockdown significantly suppressed activity of ASBT promoter (Fig. 6C). In contrast, addition of PPARα agonist GW 7647 led to an increase in ASBT promoter activity. Consistently, ASBT mRNA was reduced after PPARα knockdown (Fig. 6D).

3.6. NT administration stimulates BA uptake in mice

To further determine the roles of NT in regulation of BA uptake in vivo, we pretreated C57BL/6 mice with NT (4000 nmol/kg, i.p.), SR 48692 (a selective nonpeptide NTR1 antagonist, 2.5 mg/kg, i.p.) or saline, and given 0.5 mL TCA-D4 (200 μg/mL) by oral gavage. As shown in Table 3, the blood levels of TCA-D4 in NT-treated mice significantly compared to saline administered mice (85.29 ± 30.61 vs. 40.80 ± 10.55, nM, p = .015) (Table 3). NT treatment also enhanced the return of TCA-D4 from the intestine to the liver by increased the contents of liver TCA-D4 (62.45 ± 37.9 vs. 22.86 ± 7.41, nmol/mg, p = .012) (Table 3 and Supplementary Table 4). In agreement with the TCA-D4 increased in blood and liver, the NT treatment reduced the fecal TCA-D4 and TCA-D4 metabolites (TCA-D4, CA-D4, TCA-D4, CA-D4, TCA-D4) loss (Table 3). The NTR1 antagonist SR 48692 treatment inhibited intestinal TCA-D4 uptake and increased fecal TCA-D4 loss (Table 3).

Using qRT-PCR analysis, we showed that mice with TCA-D4 administration increased expression of ileal Nt, NT receptors (ntr1, ntr 2 and ntr 3) and ASBT (Slc10a2) mRNAs (Supplementary Fig. 2). The NT treatment also increased expression of Slc10a2, while SR 48692 treatment inhibited Slc10a2 expression in ileum (Supplementary Fig. 2). Consistent with this analysis, the Western blotting and Immunohistochemistry (IHC) revealed that the expression of the ASBT protein was reduced in ileum of SR 48692-treated mice, and was increased in NT-treated mice, compared to mice with saline treatment (Fig. 7A and Supplementary Fig. 3). In addition, the levels of p-PPARα in the ileum were induced by NT administration but suppressed by SR 48692 treatment (Fig. 7A and Supplementary Fig. 3). Interestingly, we observed that SR
48692 treatment increased the expression of BA synthesized enzymes Cyp7a1 and cyp8b1 related to the TCA-D4-treated mice (Supplementary Fig.7A).

4. Discussion

In this study, we firstly indicated that over half of the pediatric IF patients exhibited liver cholestasis and was coupled with liver injury. Secondly, the cholestasis in patients was tightly associated with the levels of NT. Thirdly, NT promotes BA uptake in mice and in cultured intestinal cells via increasing expression of ASBT.

In infants, the predominant histological feature of IFALD is cholestasis [15, 16]. Indeed, we here found that 52% (21/40) of pediatric IF patients had liver cholestasis. The IF patients with cholestasis led to BA dyshomeostasis featured by high levels of primary BA in blood and liver. The primary BA increased in IF patients with cholestasis might be attributed to dysfunction of BA enterohepatic circulation. It is previously reported that fibroblast growth factors 19 (FGF) mediates BA homeostasis through a negative feedback way during BA enterohepatic circulation [17]. In the enterocyte, BA reclaimed by terminal ileum can increase FGF19 gene expression via activation of farnesoid X receptor (FXR) [18]. After releasing into circulation, FGF19 reaches the liver and inhibits hepatic bile acids synthesis through suppression of CYP7A1 [19]. In our previous study, we showed that serum FGF19 concentrations were markedly decreased in IF patients compared to healthy controls [20]. Amnika and colleagues recently showed that loss of ileum led to reduce FGF19 production in patients with IF [9]. Thus, BA uptake by terminal ileum is essential to BA homeostasis by promoting the FGF19 production.

Neurotensin (NT), a 13-amino acid peptide predominantly mainly located in neuronal synaptic vesicles of hypothalamus and in neuroendocrine cells of the small bowel, participates in enteric digestive processes, gut motility and intestinal inflammation through three known NT receptors (NTR1, 2 and 3) [21, 22]. In animals and human, ingestion of fat was the strongest stimulus for NT release in the intestinal [23, 24]. Moreover, the recent studies indicate that that NT might involve in hepatic BA output and BA enterohepatic circulation [25, 26]. In the present study, IF patients with cholestasis had about four folds lower serum NT concentration than those without cholestasis. In addition, the serum NT concentration is inversely related to serum BA contents, suggested that NT may regulate the BA homeostasis in IF patients. Indeed, NT treatment increased BA absorption in mice and in cultured intestinal cells via enhancing expression of ASBT. NTR1 and NTR3 have been demonstrated to heterodimerize on the human intestinal epithelial Caco2 cells surface and to broaden the response range for NT signaling [27]. In vitro, NT treatment increased BA uptake in Caco2 cells, and siRNA knockdown of either NTR1 or NTR3 reduced NT-mediated BA absorption. Consistent with a role for NT in BA uptake in vitro, treatment with NTR1 inhibitor SR 48692 significantly attenuated intestinal BA absorption in vivo. Collectively, these results indicate that NT promotes intestinal BA uptake through NTR1 or NTR3. The nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) has been played an important role in the fatty acid metabolism and BA metabolisms [28–30]. As transcription factors, PPARα regulates many target genes, such as carnitine palmityltransferase 1A (CPT1A) and peroxisomal acyl-coenzyme oxidase 1 (ACOX1) [31]. In this study, we indicated that NT treatment could increase the activation of PPARα by increased levels of phosphorylated PPARα (p- PPARα). Mechanistic studies showed that PPARα could bind to DR-1 motifs (DR-1 motifs were previously identified as positive response elements for PPARα [32]) of ASBT promoter and increased its transcription.

There are some limitations of our study, including small sample size and various distributions of the patients. Further prospective longitudinal studies on serum NT, liver histology and hepatic expression of key regulators of BA metabolism are needed to assess the diagnostic value of NT. To better manage IFALD, more studies are required to investigate the relationship between serum NT levels and duration of PN as well as relationship between NT levels and remaining small bowel length. Taken together, our study showed that altered NT directly linked with development of IFALD. NT increase intestinal BA uptake via binding NTR1 and NTR3 to activate PPARα (Fig. 7B).

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**Table 3**

| Bile acids          | TCA-D4 | NT + TCA-D4 | SR + TCA-D4 | P value* |
|---------------------|--------|-------------|-------------|----------|
| Serum TCA-D4 (nM)   | 40.806 | ± 10.552    | 85.290 ± 30.614 | 28.837 ± 6.569 | 0.015 | 0.003 |
| Liver TCA-D4 (nM/mg)| 22.865 | ± 7.416     | 62.453 ± 37.920 | 14.357 ± 7.122 | 0.012 | 0.035 |
| Fecal TCA-D4 (nM/mg)| 0.382  | ± 0.403     | 0.134 ± 0.134 | 1.065 ± 0.669 | 0.119 | 0.027 |
| Fecal CA-D4 (nM/mg)| 4.702  | ± 1.881     | 2.605 ± 2.605 | 13.725 ± 7.164 | 0.022 | 0.004 |

Data are means ± SD.

* One-way ANOVA were used to compare differences between groups.
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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Author contributions

Yongtao Xiao and Wei Cai accomplished the study concept and design, acquisition of data, analysis and interpretation of data, obtained funding and drafting of the manuscript; Yongtao Xiao, Weihui Yan, Kejun Zhou and Ying Lu performed most of the experiments. Ying Lu gave the administrative, technical, or material support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.08.006.

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