Differential and organ-specific functions of organic solute transporter α and β in experimental cholestasis

Authors
Sandra M.W. van de Wiel, Begoña Porteiro, Saskia C. Belt, Esther W.M. Vogels, Isabelle Bolt, Jacqueline L.M. Vermeulen, D. Rudi de Waart, Joanne Verheij, Vanesa Muncan, Ronald P.J. Oude Elferink, Stan F.J. van de Graaf

Correspondence
k.f.vandegraaf@amsterdamumc.nl (S.F.J. van de Graaf).

Graphical abstract

Highlights
- This manuscript describes the first mouse model of OSTβ deficiency.
- Ostβ−/− mice are viable and fertile, but show increased length and weight of the small intestine, blunted villi and deeper crypts.
- Ostβ deficiency leads to an altered microbiome compared to both wild-type and Ostα−/− mice.
- Cholestasis led to lower survival and worse body weight loss, but an improved liver phenotype, in Ostβ−/− mice compared to Ostα−/− mice.

Lay summary
Organic solute transporter (OST) subunits OSTα and OSTβ together facilitate the efflux of conjugated bile acids into the portal circulation. Ostα knockout mice have longer and thicker small intestines and are largely protected against experimental cholestatic liver injury. Herein, we generated and characterized Ostβ knockout mice for the first time. Ostα and Ostβ knockout mice shared a similar phenotype under normal conditions. However, in cholestasis, Ostβ knockout mice had a worsened overall phenotype which indicates a separate and specific role of OSTβ, possibly as an interacting partner of other intestinal proteins.

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Differential and organ-specific functions of organic solute transporter α and β in experimental cholestasis

Sandra M.W. van de Wiel,1,2 Begoña Porteiro,1,2,3 Saskia C. Belt,1,2 Esther W.M. Vogels,1,2 Isabelle Bolt,1,2 Jacqueline L.M. Vermeulen,1,2 D. Rudi de Waart,1,2 Joanne Verheij,2,4 Vanesa Muncan,1,2,5 Ronald P.J. Oude Elferink,1,2,5 Stan F.J. van de Graaf1,2,5,*

1Tygat Institute for Liver and Intestinal Research, Amsterdam UMC, University of Amsterdam, the Netherlands; 2Amsterdam Gastroenterology Endocrinology Metabolism, Amsterdam, the Netherlands; 3Amsterdam Gastroenterology Endocrinology Metabolism, Amsterdam, the Netherlands; 4Department of Pathology, Amsterdam UMC, University of Amsterdam, the Netherlands; 5Department of Gastroenterology and Hepatology, Amsterdam UMC, University of Amsterdam, the Netherlands

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Introduction

Bile acids facilitate the intestinal digestion and absorption of fats and fat-soluble vitamins. Bile acids are synthesized in hepatocytes from cholesterol via several enzymatic steps that form the primary bile acids cholic acid and chenodeoxycholic acid. The first and rate-limiting step of this cascade is mediated by CYP7A1. Bile acids are subsequently conjugated with amino acids glycine and taurine to form glycocholic acid, taurocholic acid, glycinecholodeoxycholic acid and taurochenodeoxycholic acid.1 A portion of primary bile acids are converted into the secondary bile acids deoxycholic acid, lithocholic acid and ursodeoxycholic acid by gut bacteria in the intestine.1 Compared to humans, mice have a more hydrophilic bile acid composition as they can also synthesize (α-, β- or ω-) muricholic acid from chenodeoxycholic acid.

Tight regulation of bile acid homeostasis prevents intracellular accumulation of toxic bile acids, which can disrupt membranes, and lead to generation of reactive oxygen species and initiation of apoptosis.2 The nuclear farnesoid X receptor (FXR) plays a central role in regulating several genes involved in the enterohepatic circulation of bile acids. Intestinal FXR increases gene expression of fibroblast growth factor (FGF)19, the human homolog of mouse FGF15, upon binding by bile acids.3 FGF15/19 is released by the enterocyte into the portal circulation and binds to the FGF receptor 4 (FGFR4)-β-Klotho complex on hepatocytes, which triggers several pathways including the suppression of the

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* Corresponding author. Address: Meibergdreef 69-71, 1105 BK Amsterdam, the Netherlands; Tel.: 020-5668832, fax: 020-5669190.
E-mail address: k.f.vandegraaf@amsterdamumc.nl (S.F.J. van de Graaf).
rate-limiting enzyme in bile acid synthesis, CYP7A.\textsuperscript{3} In addition, activation of FXR also protects against bile acid overload in both enterocytes and hepatocytes. This is achieved by inhibiting bile acid influx via downregulation of the apical sodium-dependent bile acid transporter (ASBT) and the hepatic uptake transporter sodium taurocholate cotransporting polypeptide and stimulating export of bile acid by upregulation of efflux transporters, such as the bile salt export pump and the organic solute transporter $\alpha$-$\beta$ (OST-$\alpha$-OST-$\beta$).\textsuperscript{4}

OST-$\alpha$-OST-$\beta$ transports conjugated bile acids across the basolateral membrane of enterocytes into the portal circulation.\textsuperscript{5,7} This transporter is a heterodimer that consists of 2 distinct subunits; $\alpha$ and $\beta$,\textsuperscript{8} encoded by 2 different genes, SLC51A and SLC51B, located on separate chromosomes. The $\alpha$-subunit consists of 340 amino acids with 7 transmembrane domains, while the beta-subunit only has 128 amino acids and includes 1 transmembrane domain.\textsuperscript{9} Heterodimerization of the 2 subunits leads to increased stability of the proteins and is necessary for plasma membrane trafficking and transport activity.\textsuperscript{9}

OST-$\alpha$-OST-$\beta$ functions in cellular efflux of both conjugated bile acids and steroid hormones, independently of the sodium gradient.\textsuperscript{9} Moreover, in vitro studies show that OST-$\alpha$-OST-$\beta$ is able to mediate both cellular efflux and influx, dependent on the concentration gradient of the substrate and extracellular pH.\textsuperscript{7} Highest expression levels of OST-$\alpha$-OST-$\beta$ are detected in the distal part of the ileum. However, OST-$\alpha$-OST-$\beta$ also shows expression in other tissues involved in bile acid homeostasis, such as the kidney and liver, and tissues involved in steroid hormone homeostasis.\textsuperscript{10} Of note, OST-$\alpha$ and OST-$\beta$ are expressed with highly varying protein ratios and their transcriptional regulation is poorly correlated.\textsuperscript{11} The relevance of this is not yet known.

To elucidate the physiological role and pathophysiological implications of OST-$\alpha$ deficiency, Ost-$\alpha$-/- mice have previously been generated.\textsuperscript{10-14} Knockout of the Ost-$\alpha$ gene leads to complete loss of the OST-$\alpha$ protein, strongly reduced OST-$\beta$,\textsuperscript{10,11,13} and results in impaired intestinal bile acid absorption and bile acid accumulation in enterocytes.\textsuperscript{11} Compared to control mice, Ost-$\alpha$-/- mice display an ameliorated liver phenotype upon bile duct ligation (BDL), and this has been attributed to increased urinary bile acid excretion.\textsuperscript{14} Bile acid accumulation and associated histological changes in the intestine are prevented in Ost-$\alpha$-/- mice that also lack Asbt while Fxr depletion did not resolve the phenotype of Ost-$\alpha$-/- mice. While mutations in the Asbt gene are known to cause bile acid malabsorption in humans,\textsuperscript{15} genetic defects in Asbt do not account for all hereditary cases of bile acid malabsorption.\textsuperscript{16} In 2019, 2 brothers were identified with a frameshift mutation in the OST-$\beta$/SLC51B gene causing impaired bile acid transport activity.\textsuperscript{17} These patients had diarrhea, fat-soluble vitamin deficiencies and features of cholestasis, including moderately increased levels of the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT).\textsuperscript{17} Due to the limited availability of biospecimens from these 2 patients, little is known about the consequence of OST-$\beta$ deficiency in humans. Recently, the first OST-$\alpha$-deficient patient was identified; this patient had diarrhea and cholestasis,\textsuperscript{18} which is not observed in Ost-$\alpha$-/- mice.\textsuperscript{11,14} The OST-$\alpha$-OST-$\beta$ complex has an overall topology similar to the heteromeric structure of G-protein coupled receptors associated to receptor activity-modifying proteins,\textsuperscript{19} where OST-$\alpha$ adopts a 7-pass transmembrane structure, and OST-$\beta$ is a transmembrane protein that crosses the membrane once. OST-$\beta$ expression is necessary for glycosylation and trafficking of OST-$\alpha$ to the plasma membrane as well as for functional bile acid transport,\textsuperscript{5,9,20} but whether its function is restricted to this chaperone function is unknown. Therefore, an OST-$\beta$ knockout mouse model was generated to study the role of OST-$\beta$ and to analyze whether deficiency of Ost-$\beta$ in mice affects cholestatic liver injury.

**Materials and methods**

For further details regarding the materials and methods used, please refer to the CITAT table and supplementary information.

**Animals**

Ost-$\beta$-/- mice were generated in C57BL/6J mice by precise targeted deletion via CRISPR/Cas9, which resulted in a large deletion in exon 3 of the Ost-$\beta$ (Slc51b) gene. To this end, 2 single-guide RNA (sgRNA) target sequences in the Ost-$\beta$ gene were selected and inserted in a pDR274 gRNA cas9-guide plasmid. The sgRNA were synthesized in vitro, purified and microinjected together with Cas9 mRNA into 1-cell stage wild-type embryos. These mice were backcrossed once to wild-type mice and resulting Ost-$\beta$-/- animals were crossed to create Ost-$\beta$-/- and wild-type littermates for analysis. Sequencing was performed to confirm the exact genotypes of the mutated Ost-$\beta$ gene and to analyze whether mutations occurred in potential off-target genes, which was not the case. Ost-$\alpha$-/- mice were generated by Rao et al.\textsuperscript{13} and purchased from the Jackson Laboratory. Male and female Ost-$\alpha$-/-, Ost-$\beta$-/- and control wild-type C57BL/6J mice (Janvier Labs) were housed under a 12 h light/dark cycle and bred in the Animal Research Institute Amsterdam. Mice were fed with normal chow diet and given ab libitum access to water. The study design, animal care and handling were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands).

**Cholestatic mice models**

Wild-type and Ost-$\beta$-/- female and male adult mice (littermates) 8–12 weeks of age were subjected to a common BDL as previously described.\textsuperscript{21} All surviving mice (both males and females) were sacrificed at day 5 because of animal welfare regulations (body weight loss >15%). A second cohort of male mice, including wild-type, Ost-$\alpha$-/- and Ost-$\beta$-/- adult (age 20–30 weeks) mice, were sacrificed 2 days after BDL. In a third cohort of mice, cholestasis was induced by supplementing the chow diet (D12450B1, Open Source Diets, USA) with 0.1% 3,5-diethoxycarbonyl-1,4-dihiydroxocollidine (DDC, Sigma) during 8 days.\textsuperscript{22} In indicated experiments, DDC diet was initiated 2 weeks after administration via the tail vein of 2x10$^{12}$ adeno-associated virus serotype 8 (AAV8) particles/kg encoding codon optimized mouse Ost-$\beta$ (Vectorbuilder). All mice were sacrificed under anesthesia and blood, bile and tissues were collected as described in the supplementary information.

**Statistical analysis**

Data are provided as mean ±SD with individual points shown in dots. Differences between groups were analyzed using a one-way ANOVA test, and Dunnett’s test to compare with the wild-type littermates or Sidak’s multiple comparisons test. Differences in survival were assessed using a log-rank test. Statistical significance was considered at $p <0.05(*)$. Graphs were generated using GraphPad Prism software (version 8.0.2; GraphPad Software Inc.).
Differences in microbiota α diversity were tested using ANOVA. Permanova was used to test compositional differences in terms of Bray-Curtis dissimilarity and Weighted Unifrac distances. Differential abundance of taxa was tested using DESeq2.23

Results

Generation of OSTβ knockout mice
To study the role of OSTβ in mice, targeted deletion was performed using CRISPR/Cas9, resulting in a 190 base pair deletion in exon 3 of the Ostβ gene (Fig. 1A). Ostα and Ostβ mRNA were not expressed in Ostx-/- and Ostβ-/- mice, respectively (Fig. 1B). Western blotting confirmed the complete absence of OSTα and OSTβ protein in Ostx-/- and Ostβ-/- mice, respectively (Fig. 1C). In line with previous Ostx-/- studies, we found that Ostβ-/- mice lack the OSTα protein and have strongly reduced OSTβ protein expression, while Ostβ-/- mice lack both the OSTβ protein as well as the OSTα protein. Consistent with the western blot, immunohistochemistry showed protein expression of OSTβ on the basolateral membrane of ileal enterocytes in wild-type mice, while this signal was absent in Ostβ-/- mice (Fig. 1D).

Phenotype of Ostx-/- and Ostβ-/- mice
Both Ostx-/- and Ostβ-/- mice are viable and showed no obvious change in appearance and growth. Crossing heterozygous Ostβ-/- mice produced a Mendelian distribution of wild-type and knockout genotypes. In contrast to the OSTβ-deficient patients, Ostβ-/- mice showed no signs of diarrhea. Only a trend towards a modestly increased plasma level of the liver enzymes ALT (p = 0.073) and alkaline phosphatase (ALP; p = 0.075) was detected and AST levels were unchanged (Fig. 2A). Ostx-/- mice showed no significant change in body weight at 4 and 8 weeks after birth in both females and males. Likewise, Ostβ-/- mice did not demonstrate altered body weight except for 8-week-old females that showed a modest reduction in body weight compared to wild-type littersmates (Fig. 2B). The length and weight of the small intestine were significantly and similarly increased in the Ostx-/- and Ostβ-/- mice in both 4- and 8-week-old mice (Fig. 2C,D). The weight per length had a tendency to increase in the Ostx-/- and Ostβ-/- mice that were 4 weeks of age, and was significantly increased in 8-week-old male mice and female Ostβ-/- mice (Fig. S1A). Liver weight and kidney weight were not changed in the Ostx-/- and Ostβ-/- mice (Fig. S1B,C). The length, weight and weight per length of the colon were not altered in Ostx-/- and Ostβ-/- mice (Fig. S1D-F). The small intestine phenotype was preserved in older Ostβ-/- mice (32-37 weeks) (Fig. S1G).

Altered ileal histology in Ostx-/- and Ostβ-/- mice
Analysis of the ileum showed an altered histology in Ostx-/- and Ostβ-/- male and female mice (Fig. 3A, Fig. S2A). While the ileum of wild-type mice comprises normal-appearing long, thin villi, Ostx-/- and Ostβ-/- mice exhibit blunted villi and elongated crypt depth. This altered ileal histology is similar between Ostx-/- and Ostβ-/- mice. Quantification of villus height showed a 25% reduction in 4-week-old female and a (not significant) 21% reduction in male Ostβ-/- mice compared to wild-type littermates (female mice: Ostβ-/- 121.7 μm ± 24.45 vs. wild-type 163.4 μm ± 25.20) (male mice: Ostβ-/- 115.5 μm ± 17.66 vs. wild-type 146.8 μm ± 19.30)
Additionally, crypt depth was significantly increased at both 4 weeks of age by 57% and 38% in female and male Ostβ-/- mice respectively, and 8 weeks of age by 83% and 64% in female and male Ostβ-/- mice respectively (Fig. 3C). As a result of the increased crypt depth and decreased villus height, the ratio was significantly decreased in Ostα-/- and Ostβ-/- mice (Fig. S2B). The top of the ileal villi of Ostα-/- and Ostβ-/- mice have increased numbers of mucus-filled vacuoles (Fig. 3D). Furthermore, intestinal proliferation was determined using phosphohistone H3 staining and demonstrated a more widespread distribution along the villi in both Ostα-/- and Ostβ-/- mice compared to wild-type mice probably due to the increased crypt depth (Fig. 3E). Other parts of the small intestine, the duodenum and jejunum, were not histologically altered.

Altered expression of differentiation markers in enterocytes of Ostα-/- and Ostβ-/- mice
Caudal type homeobox 2 (CDX2) induces transcription of several genes implicated in intestinal differentiation and epithelial cell maturation.24-26 Ostβ-/- mice showed a significant decrease in

| A | Plasma ALT | | Plasma ALP | | Plasma AST |
|   | Units/L | Units/L | Units/L |
| --- | --- | --- | --- |
| WT | | | |
| Ostβ-/- | | | |

| B | Males (4 wk) | Females (4 wk) | Females (8 wk) | Males (8 wk) |
| --- | --- | --- | --- | --- |
| Body weight (g) | | | | |
| WT | | | | |
| Ostα-/- | | | | |
| Ostβ-/- | | | | |

| C | Length small intestine (cm) |
| --- | --- |
| WT | | |
| Ostα-/- | | |
| Ostβ-/- | | |

| D | Weight small intestine (mg/g BW) |
| --- | --- |
| WT | | |
| Ostα-/- | | |
| Ostβ-/- | | |

Fig. 2. The phenotype of Ostα-/- and Ostβ-/- mice compared with wild-type littermates at 4 and 8 weeks of age in females and males. (A) Plasma ALT, ALP and AST levels of 8–12-week-old male mice. (B) Body weight, (C) length of small intestine and (D) weight of small intestine of 4- or 8-week-old male and female mice. Data are expressed as the mean ± SD with individual points shown in dots (n = 7–13 mice per group). Statistical analysis was done using a one-way ANOVA test and Dunnett’s test to compare with wild-type littermates. *Indicates p values of <0.05. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; Ostα, organic solute transporter alpha; Ostβ, organic solute transporter beta; wk, week.

μm ± 12.20) (Fig. 3B). Additionally, crypt depth was significantly increased at both 4 weeks of age by 57% and 38% in female and male Ostβ-/- mice respectively, and 8 weeks of age by 83% and 64% in female and male Ostβ-/- mice respectively (Fig. 3C). As a result of the increased crypt depth and decreased villus height, the ratio was significantly decreased in Ostα-/- and Ostβ-/- mice (Fig. S2B). The top of the ileal villi of Ostα-/- and Ostβ-/- mice have increased numbers of mucus-filled vacuoles (Fig. 3D). Furthermore, intestinal proliferation was determined using phosphohistone H3 staining and demonstrated a more widespread distribution along the villi in both Ostα-/- and Ostβ-/- mice compared to wild-type mice probably due to the increased crypt depth (Fig. 3E). Other parts of the small intestine, the duodenum and jejunum, were not histologically altered.

Altered expression of differentiation markers in enterocytes of Ostα-/- and Ostβ-/- mice
Caudal type homeobox 2 (CDX2) induces transcription of several genes implicated in intestinal differentiation and epithelial cell maturation.24-26 Ostβ-/- mice showed a significant decrease in
expression of Cdx2 in the ileum in males at 4 and 8 weeks of age (41% and 50% reduction, respectively), and a similar decreased trend is seen in 4-week-old Ostβ−/− females (−31%; p = 0.15) (Fig. 4A, Fig. S3A). Ostα−/− and Ostβ−/− mice showed no change in mRNA expression of Muc2, a marker for goblet cells, and Lysozyme, a marker for Paneth cells (Fig. S3E,F). However, 4-week-old female and male Ostβ−/− mice had a significantly decreased expression of sucrase-isomaltase (Sis; 65% and 63% reduction,
Bile salt-related gene expression changes in ileal enterocytes of Ostx<sup>-/-</sup> and Ostβ<sup>-/-</sup> mice

Gene expression levels of Fabp6, Fgf15, Mrp3 and Asbt were measured to assess possible adaptations related to bile acid transport. Fabp6 (encoding IBABP) and Mrp3 mRNA levels were not increased in Ostx<sup>-/-</sup> and Ostβ<sup>-/-</sup> mice (Fig. 4A,B). In contrast, Fgf15 levels increased 3.8-fold and 2.9-fold in Ostx<sup>-/-</sup> females and Ostβ<sup>-/-</sup> males at 4 weeks of age. Furthermore, Fgf15 levels tended to increase 3.2-fold (p = 0.056) in 4-week-old female Ostβ<sup>-/-</sup> mice and 2.4-fold (p = 0.026) in 4-week-old Ostx<sup>-/-</sup> males (Fig. 4C). Both Ostx<sup>-/-</sup> and Ostβ<sup>-/-</sup> mice that were 8 weeks of age did not show increased Fgf15 levels (Fig. 4D). Furthermore, Ostx<sup>-/-</sup> and Ostβ<sup>-/-</sup> mice show decreased expression of the apical bile acid uptake transporter Asbt at both ages, which could serve as a protective mechanism against bile acid overload (Fig. 4D, Fig. S5C). Organoids were cultured from ileal stem cells of the Ostx<sup>-/-</sup>, Ostβ<sup>-/-</sup> and wild-type mice to investigate whether the altered ileal morphology and gene expression is due to cell-intrinsic factors (Fig. S5A,B). Both Ostx<sup>-/-</sup>, Ostβ<sup>-/-</sup> and wild-type organoids grew in the same manner regarding their size and number of buds (Fig. S5A). Furthermore, expression levels of Fgf15 and Ibabp are similar in Ostx<sup>-/-</sup>, Ostβ<sup>-/-</sup> organoids and wild-type organoids (Fig. 5D,E).

Bile acid concentration and composition in circulation and excretory systems

Next, we investigated the effect of Ostβ deficiency on concentrations and composition in the circulation and excretory systems.

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Fig. 4. Altered expression of differentiation markers in Ostx<sup>-/-</sup> and Ostβ<sup>-/-</sup> mice. Ileal mRNA expression of 4- and 8-week-old Ostx<sup>-/-</sup>, Ostβ<sup>-/-</sup> and wild-type males of (A) Cdx2, (B) Sis, (C) Fgf15 and (D) Asbt. Data are normalized using the geometric mean of CyclophilinB and Rpl4. Data are shown as the mean ±SD with individual points shown in dots (n = 4-7). Statistical analysis was performed using a one-way ANOVA test, and Dunnett’s test to compare with wild-type littermates. *Indicates p values of <0.05. Asbt, apical sodium-dependent bile acid transporter; Cdx2, caudal type homeobox 2; Fgf15, fibroblast growth factor 15; Ostx, organic solute transporter alpha; Ostβ, organic solute transporter beta; Sis, sucrase-isomaltase; wk, week; WT, wild-type.
systems. Ostαβ−/− and Ostβ−/− mice showed unaltered bile acid concentration and composition in bile or plasma. Furthermore, no increased bile acid excretion in urine or feces was observed and the bile acid hydrophobicity index of bile was unchanged (Fig. S6).

**Decreased β diversity in Ostβ−/− microbiome**

We evaluated bacterial α and β diversity in Ostβ−/−, Ostαβ−/− mice and their wild-type littermates. We found no significant differences in terms of α diversity (a metric of microbial richness) analyzed in 3 different ways (Fig. S7A). In contrast, β diversity
showed significant differences in bacterial composition between groups as shown in the principal coordinates analysis plots (PERMANOVA p = 0.001, R2 = 0.19; Fig. S7B). Weighted UniFrac analysis, which takes the relatedness of the microbes into account, did not show significant differences, indicating the microbiota are more similar at higher taxonomic ranks.

Comparing abundances of taxa in Ostβ-/ mice with both wild-type mice and Ostx-/ mice shows a decrease of Lactobacillus, various Lachnospiraceae and Candidatus_Saccharimonas and increase of Bifidobacteria and Faecalibaculum (Fig. S7C).

**Ostβ-/** mice show lower survival rates while displaying hepatoprotective effects during BDL-induced liver injury

While OSTβ- and OSTx-deficient patients show features of cholestatic liver injury,7,12,18 OSTx-deficient mice display attenuated liver disease upon induction of cholestasis by ligation of the common bile duct.14 Therefore, we wondered whether challenging Ostβ-/ mice by inducing cholestasis would affect liver injury. To this end, we performed experiments with 2 distinct cholestasis models; common BDL and a 0.1% DDC-containing diet. Both models revealed an unexpected phenotype specifically in Ostβ-/ mice. First, 18 Ostβ-/ mice (8 male) and 20 wild-type littersmates (10 male; 8-10 weeks of age) were subjected to common BDL (Fig. 5A). While all wild-type mice survived, 40% of the Ostβ-/ mice died within 5 days (Fig. 5B; p = 0.02 log-rank test). No difference in body weight loss was observed in surviving Ostβ-/ mice compared to wild-type littersmates (Fig. S8A). Remarkably, livers of surviving Ostβ-/ mice were completely devoid of necrotic areas, which covered 15-20% of the area in wild-type mice (Fig. 5C and Fig. S8B). A clear reduction was observed in bile acid levels (62%), plasma bilirubin (42%) and in cholesterol (38%) levels in the surviving Ostβ-/ mice, while plasma ALT, ALP and AST levels were unchanged (Fig. 5D,E and Fig. S9A,B). Tauroursodeoxycholic acid levels are increased in Ostβ-/ mice compared to wild-type littersmates (Fig. 5F).

In general, expression levels of markers of hepatic inflammation (Mcp1; p = 0.0163), fibrosis (Timp1; p = 0.093), TGFβ (p = 0.17) and proliferation (Afp; p = 0.14), but not Cyp7a1 and Il6 tended towards being reduced in surviving Ostβ-/ mice compared to wild-type mice after BDL (Fig. 5G, Fig. S8D). The high mortality upon BDL in Ostβ-/ mice was confirmed in a second experiment with a group of 3 Ostβ-/ mice (male, 20-30 weeks old). In this experiment we also included wild-type littersmates and Ostx-/ mice (Fig. 5H). On day 3, animals were sacrificed due to severe symptoms of distress, including hunched posture and lethargy, specifically presented by

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**Fig. 6. Challenging adult wild-type, Ostx-/ and Ostβ-/ mice by inducing cholestasis using a DDC diet.** (A) Body weight change in Ostx-/ and WT (right panel) and body weight change in Ostx-/ and WT littersmates (left panel) after 8 days with DDC diet (n = 6-9 per group). (B) Hepatic mRNA expression of Asbt, Fgf15 and hepatic mRNA expression of Cyp7a1 (n = 6-9 per group). Data are normalized using the geometric mean of CyclophilinB-Hprt (ileum) and Tbp-Hprt (liver). Adult Ostx-/ mice were injected via the tail vein with an AAV8 vector encoding mouse Ostβ and then cholestasis was induced using a DDC diet. (C) Body weight change in Ostβ-/ WT/HET (left panel) and Ostβ-/ and Ostβ-/ AAV8 (right panel) after 12 days of a DDC diet (n = 8-10 mice per group). (D) Hepatic mRNA expression of Slc51β; data are normalized using the geometric mean of CyclophilinB-Hprt. (E) Small intestine length and (F) liver weight of adult Ostβ+/HET mice, Ostβ-/ mice and Ostβ+/ AAV8 were measured (n = 8-10 mice per group). Statistical analysis was performed using a one-way ANOVA test, and Dunnett’s test to compare with the wild-type littersmates. *p values of <0.05 were considered statistically significant. AAV8, adeno-associated virus serotype 8. AAV, adeno-associated virus; Asbt, apical sodium-dependent bile acid transporter; Cyp7a1, Cytochrome P450 Family 7 Subfamily A Member 1; DDC diet, 3,5-dihydroxy-4-carboxyl-1,4-dihydrocollidine diet; Fgf15, fibroblast growth factor 15; HET, heterozygous; HOM, homozygous; Ostx, organic solute transporter alpha; Ostβ, organic solute transporter beta; Slc51β, solute carrier family 51, beta subunit; wk, week.
the \( \text{Ost}^- \) mice. Furthermore, the contents of the stomach and the intestines were dark colored and were located throughout the small intestine while the small intestine of both \( \text{Ost}^+ \) mice and wild-type mice showed a normal color and contained less alimentary matter (Fig. S1 and Fig. S8). Remarkably, the cagеs of \( \text{Ost}^- \) mice contained considerably less feces compared to the cagеs of \( \text{Ost}^+ \) mice (data not shown). In line with results of the first BDL experiment, examination of the liver suggested a protective effect in both \( \text{Ost}^- \) and \( \text{Ost}^+ \) mice with respect to liver damage due to BDL, with obvious pre-necrotic areas in the wild-type animals (Fig. 5J).

**Ost\(^+\) mice show lower body weight gain while displaying hepatoprotective effects when challenged with a DDC diet**

After 8 days on a DDC diet, \( \text{Ost}^- \) mice showed marked body weight loss compared to wild-type and \( \text{Ost}^+ \) mice (Fig. 6A). In contrast, \( \text{Mcp-1} \) levels were significantly lower in \( \text{Ost}^- \) mice when compared with wild-type littermates and a significant reduction was found in AST in \( \text{Ost}^- \) and \( \text{Ost}^+ \) mice when compared with wild-type mice (Fig. S9A,B) while plasma bilirubin, ALP and ALT levels as well as \( \alpha\)-Sma, \( \text{Col1a1} \) and \( \text{Afp} \) expression remained unchanged (Fig. S9B). Intestinal \( \text{Abst} \) expression was decreased and \( \text{Fgf15} \) expression increased in \( \text{Ost}^- \) and \( \text{Ost}^+ \) mice also under these cholestatic conditions (Fig. 6B). The discrepancy between overall health status and (selected) markers for liver damage mimics the BDL phenotype and suggests that, in \( \text{Ost}^- \) mice, an extrahepatic phenotype is unmasked under cholestatic conditions which is distinct from \( \text{Ost}^- \) mice. The increased weight loss in \( \text{Ost}^+ \) mice was confirmed in a second DDC-induced cholestasis experiment where we tested the role of hepatic \( \text{Ost}^- \) (Fig. 6C). To this end, we included a group that received AAV8 encoding mouse \( \text{OST}^\beta \)-Sma mice. The increased weight loss in \( \text{Ost}^- \) mice was more severe in \( \text{Ost}^+ \) mice than wild-type mice (Fig. 6C). Body weight differences across the entire experiment are calculated as area under the curve (%/day) and were -86.07 ± 5.25 in mice expressing endogenous \( \text{Ost}^- \) and -129.6 ± 10.9 and -127.0 ± 9.34 in DDC-fed \( \text{Ost}^- \) mice (respectively mock injected or treated with \( \text{Ost}^- \)-AAV8) (Fig. 6C). Hepatic expression of \( \text{Ost}^- \) was confirmed in the latter group (Fig. 6D). Also, cholestatic \( \text{Ost}^- \) mice displayed elongated small intestines (irrespective of restored hepatic \( \text{Ost}^- \) expression) (Fig. 6E), while no difference in liver weight was present (Fig. 6F). This indicates that the increased body weight loss seen in cholestatic \( \text{Ost}^- \) mice likely has an extrahepatic origin.

### Discussion

Here, we generated \( \text{Ost}^- \)-deficient mice and show that disruption of \( \text{Ost}^- \) results in profound ileal morphological changes. When unchallenged, no major differences are observed between \( \text{Ost}^- \) and \( \text{Ost}^+ \) mice and \( \text{Ost}^+ \) mice are phenocopying \( \text{Ost}^- \) mice. Our results are mostly in line with previous \( \text{Ost}^- \) studies, suggesting that \( \text{Ost}^- \) and \( \text{Ost}^+ \) function in the same manner in bile acid homeostasis under normal conditions. However, under cholestatic conditions, \( \text{Ost}^- \) mice have a worsened phenotype, a significant lower survival rate and lower body weight compared to both wild-type and \( \text{Ost}^- \) mice. This phenotype is independent of hepatic \( \text{Ost}^- \) expression status. As the contents of the intestine and stomach of \( \text{Ost}^- \) mice were dark colored, while \( \text{Ost}^- \) mice were indistinguishable from wild-type littermates, an intestinal origin of this phenotype is likely. Furthermore, these data indicate that there might be a difference between the function of \( \text{Ost}^- \) and \( \text{Ost}^- \).

The \( \text{Ost}^- \)-deficiency phenotype under cholestatic conditions does not relate to liver damage since the lower survival rates of \( \text{Ost}^- \) mice do not seem to correlate with histology and markers of bile acid-induced liver injury. The \( \text{Ost}^- \) knockout mice even showed some level of protection against liver injury during cholestasis by BDL, similar to \( \text{Ost}^- \) mice. The discrepancy in content in the colon vs. the stomach of cholestatic \( \text{Ost}^- \) mice may point to an intestinal motility phenotype. Several papers suggest a link between cholestasis and/or altered bile acid signaling and alterations in intestinal transit via 3 possible mechanisms.29–32

First, the endogenous opioid system has been demonstrated to be activated in cholestatic conditions in mice, leading to decreased intestinal transit. Therefore, induction of cholestasis may reveal or enhance an intestinal mobility phenotype in \( \text{Ost}^- \) mice. Second, activation of TGR5, the GPCR for bile acids, is essential for peristalsis and gastric emptying, possibly via induction of glucagon-like peptide-1 secretion.30,31 \( \text{Ost}^- \) mice may have reduced TGR5 activation as the bile acid pool is likely reduced due to chronically elevated \( \text{Fgf15} \) expression. Third, NGM282, an \( \text{FGF15/19} \) mimetic has prokinetic activity itself.32 Chronic overexpression of \( \text{FGF15} \), as seen in \( \text{Ost}^- \) and \( \text{Ost}^- \) mice may lead to desensitization of \( \text{Fgfr/Klb} \), just as chronic \( \text{FGF23} \) overexpression desensitizes this receptor complex.33 A rapid reduction in \( \text{FGF15} \), as would occur during cholestasis may then lower intestinal motility to pathologically relevant levels. Although such mechanisms could contribute to the intestinal phenotype of \( \text{Ost}^- \) mice in cholestatic conditions, it remains unclear why this phenotype is not exposed in \( \text{Ost}^- \) mice, which are largely indistinguishable with regard to bile acid homeostasis. This suggests that \( \text{Ost}^- \) might have another function besides forming a bile acid efflux transporter upon heterodimerization with \( \text{Ost}^- \). Early after the cloning of \( \text{Ost}^- \) it was postulated that \( \text{Ost}^- \) may function as a chaperone or regulatory subunit for other proteins as the topology of \( \text{Ost}^- \) is similar to that of \( \text{G} \)-protein coupled receptors associated to receptor activity-modifying proteins.34 This may also explain why the regulation of gene expression of these 2 subunits is so different.3 For example, hepatic upregulation of \( \text{Ost}^- \) expression is much higher than that of \( \text{Ost}^- \) in patients with primary biliary cholangitis and in obstructive cholestasis.38 Finally, the modest but evident differences in microbial composition may lead to or reflect differences in intestinal function. \( \text{Ost}^- \) mice were more sensitive to the DDC diet than wild-type or \( \text{Ost}^- \) mice. \( \text{Ost}^- \) mice lost significantly more body weight which may be related to the altered microbiota as this could lower the efficiency of energy harvest.37 This would also explain why the effect is independent of hepatic \( \text{Ost}^- \) expression.

Our \( \text{Ost}^- \) model made it possible to compare the consequence of \( \text{Ost}^- \) deficiency and \( \text{Ost}^- \) deficiency in mice but also to compare this to the few individuals described to date with SLC5A1 or SLC51B deficiency. In contrast to \( \text{Abst}^- \) mice which show a similar malabsorptive phenotype as patients with an \( \text{Abst} \) mutation,15,38 \( \text{Ost}^- \) mice do not reflect all characteristics of the 2 \( \text{Ost}^- \)-deficient patients. The \( \text{Ost}^- \)-deficient brothers suffer from congenital diarrhea and features of cholestasis, whereas \( \text{Ost}^- \) and \( \text{Ost}^- \) mice do not. Furthermore, the \( \text{Ost}^- \)-deficient
patient who was recently identified showed symptoms similar to the OSTβ-deficient brothers, albeit with more severe signs of cholestasis. While expression of Ostx and Ostβ is high in human livers, it is marginal in mouse livers under normal circumstances. This may explain why OSTα- and OSTβ-deficient patients experience liver histological changes and elevated liver enzymes ALT, AST and GGT, while there is only a trend towards a modest increase in ALT and ALP in the Ostβ−/− mice. Protective mechanisms are initiated in mice with OSTα or OSTβ deficiency to reduce the bile acid load, which likely explains the ameliorated phenotype in older mice, although the elongated small intestine remains present in aged Ostβ−/− mice. In addition, mice have a different gut microbiome composition and enzymatic bile acid (re)hydroxylation repertoire leading to a distinct bile acid composition and conjugation. The mouse bile acid pool is less hydrophobic and toxic which may dampen liver damage and is also much reduced in OSTα- and OSTβ-deficient mice, lowering the level of diarrhea despite the severely affected ileal morphology.

Gene expression of ileal Fgf15 was increased, inversely correlated with Asbt expression in the ileum and Cyp7a1 in the liver, implying accumulation of bile acids in the enteroocyte and dampening of bile acid synthesis. Surprisingly, gene expression of Fabp6 was not elevated, however, conflicting results on gene expression of Fabp6 have been observed in Ostx−/− mice before. Short-term inhibition of OSTα-OSTβ in vivo leads to increased FXR activation in enterocytes and it was previously demonstrated that the increase in Fgf15 expression in Ostx−/− mice is due to FXR activation. Recent evidence indicates that the ileal histological changes in Ostx−/− are secondary to enterocyte injury caused by bile acid accumulation, since disruption of Asbt in Ostx−/− mice restores the intestinal phenotype completely. Even though expression of Asbt is partly down-regulated, Ostx−/− and Ostβ−/− mice are not able to fully restore the ileal morphology, suggesting that bile acid accumulation in enterocytes is still present. Furthermore, expression of ileal Mrp3 is not increased, supporting the evidence that MRP3 does not have a major role in conjugated bile acid transport. Finally, Ostx−/− and Ostβ−/− organoids do not show an altered phenotype, suggesting that bile acids cause the altered phenotype in the ileum.

In conclusion, OSTα-OSTβ is an important heterodimeric bile acid transporter. Knockout of either Ostx or Ostβ results in a severe ileal phenotype that is in line with previous Ostx knockout studies. During cholestasis, knockout of either Ostx or Ostβ seems to ameliorate liver damage. However, unlike in Ostx−/− mice, these beneficial effects are paralleled by an intestinal motility phenotype in Ostβ−/− mice, potentially contributing to a significantly lower survival rate and higher body weight loss. This is the first evidence that the role of OSTβ differs from OSTα and suggests that OSTβ might also have an additional, unidentified, intestinal function.

Abbreviations
AAV8, adeno-associated virus serotype 8; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ASBT, apical sodium-dependent bile acid transporter; AST, aspartate aminotransferase; BDL, bile duct ligation; CDX2, caudal type homeobox 2; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FGF, fibroblast growth factor; FXR, farnesoid X receptor; OST, organic solute transporter.

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Authors’ contributions
SMWvdW, BP, SCB: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; EWMV, IB, JLMV, DRdW, JV: technical support; acquisition of data; analysis and interpretation of data; VM, RPJOE, SFJvdG: study concept and design, study supervision, analysis and interpretation of data; critical revision of the manuscript.

Data availability statement
All raw data are available upon request to the corresponding author.

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Author names in bold designate shared co-first authorship

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