Reduced ileal expression of OSTα-OSTβ in non-obese gallstone disease

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Abstract Cholelithiasis is a multifactorial process, and several mechanisms have been postulated. A decreased expression of the ileal apical sodium-dependent bile acid transporter (ASBT) and of the cytosolic ileal lipid binding protein (ILBP) was recently described in female non-obese patients. The role of the recently identified organic solute transporters α and β (OSTα, OSTβ) in gallstone pathogenesis remains unclear. Therefore, we performed analysis of OSTα-OSTβ in gallstone patients according to body weight. Ileal mucosal biopsies were collected during routine colonoscopy from female gallstone carriers (n = 19) and controls (n = 34). OSTα-OSTβ mRNA expression was measured using the LightCycler sequence detection system; protein was analyzed by immunohistochemistry and Western blot. The mRNA expression of OSTα-OSTβ was significantly reduced (OSTα: 3.3-fold, $P = 0.006$; OSTβ: 2.6-fold, $P = 0.03$) in normal-weight but not overweight gallstone carriers compared with controls. OSTα-OSTβ protein levels also showed a reduction by 40–67%. The expression of OSTα-OSTβ correlated positively with ASBT ($r = 0.65, 0.58$, respectively), ILBP ($r = 0.77, 0.67$), and the farnesoid X receptor ($r = 0.58, 0.50$). Fibroblast growth factor-19 showed a 2.8-fold reduction ($P = 0.06$), and liver receptor homolog-1 showed a 2-fold reduction ($P = 0.04$) in non-obese patients. In conclusion, an impaired function of all three ileal bile acid transporters may lead to low ileal bile acid reabsorption and an altered bile acid pool composition and therefore may contribute to the formation of gallstones in non-obese patients.—Renner, O., S. Harsch, A. Strohmeyer, S. Schimmel, and E. F. Stange. **Reduced ileal expression of OSTα-OSTβ in non-obese gallstone disease.** ***J. Lipid Res.*** **2008.** 49: **2045–2054.**

Supplementary key words gallstones • intestine • organic solute transporters α and β • farnesoid X receptor • fibroblast growth factor-19 • liver receptor homolog-1

Bile acids are powerful detergents and play a critical role in multiple biological processes. They are synthesized in the liver, stored in the gallbladder, and released postprandially into the small intestine, where they are crucial for the absorption of lipophilic nutrients (1). The reuptake of bile acids from the terminal ileum is an important step in bile acid homeostasis and a major factor determining bile acid pool size (2). The major mechanism for ileal bile acid uptake is the active transport by the apical sodium-dependent bile acid transporter (ASBT), located in the brush border (3, 4). The subsequent intracellular transport of bile acids is mediated by the cytosolic ileal lipid binding protein (ILBP), which shuttles bile acids to the basolateral membrane (5, 6). Responsible for secretion of bile acids into the portal circulation are the basolateral organic solute transporters α and β (OSTα, OSTβ) (7).

In a recent study, we showed that female gallstone carriers exhibit a decreased ileal expression of ASBT and ILBP and their most relevant transcription factors, farnesoid X receptor (FXR) and hepatic nuclear factor 1 α (HNF1α) (8). This decrease was observed on both the mRNA and protein levels and may explain bile lithogenicity by an intestinal bile acid loss. However, compatible with differences in cholesterol handling in lean and obese subjects (9), this effect was weight specific and observed only in normal-weight subjects (8). Since then, the bile acid transporter on the basolateral side of the enterocyte has been identified as OSTα-OSTβ and it has been possible to complete an assessment of transporter-mediated mechanisms of transepithelial bile acid flux in gallstone disease. Wang et al. (10) identified a novel type of organic solute and steroid transporter in the liver of the little skate *Raja erinacea,* an evolutionarily ancient vertebrate. This transporter could also be described in mammals, including human and mouse. Human OSTα is a 340 amino acid protein, with a predicted 7-transmembrane-domain structure, whereas OSTβ is a putative 128 amino acid protein, single-transmembrane-domain ancillary polypeptide (11). OST-mediated basolateral transport requires the coexpression

Abbreviations: ASBT, apical sodium-dependent bile acid transporter; FGF-19, fibroblast growth factor-19; FXR, farnesoid X receptor; HNF1α, hepatic nuclear factor 1 α; ILBP, ileal lipid binding protein; LRH-1, liver receptor homolog-1; OSTα, organic solute transporter α; QRT-PCR, quantitative RT-PCR; RAR/RXR, retinoic acid receptor/retinoid X receptor; SHP, short heteromeric partner.

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of the two distinct gene products OSTα and OSTβ to deliver different bile acids, estron 3-sulfate, and prostaglandin E2, to the plasma membrane (7, 10, 11). The OSTα subunit is converted to a mature N-glycosylated form that is relevant for the movement through the Golgi apparatus. Alternatively, the OSTβ subunit may function as a chaperone to promote the α-subunit to the cell surface (10). Using indirect immunofluorescence microscopy, the subcellular localization of the two human proteins to the basolateral membrane of epithelial cells was demonstrated (12). This heteromeric transporter is colocalized at relatively high levels in the kidney, liver, and intestine of mouse, rat, and human species, the same tissues that express ASBT, indicating that OSTα-OSTβ is a major basolateral transporter mediating the final step of the reabsorption of steroid-derived molecules (7, 11, 12). Very recently, the major importance of these basolateral transporters for intestinal bile acid export and homeostasis was confirmed in the OSTα knockout mouse (13).

For the transcriptional regulation of OSTα-OSTβ gene expression, two major factors, liver receptor homolog-1 (LRH-1) and FXR, appear to be essential (14, 15). Following reabsorption, the bile acids modulate their own hepatic de novo synthesis via inhibition of cholesterol 7α-hydroxylase (CYP7A1) (16). The transcription factor FXR, after binding bile acids, induces the ileal expression of fibroblast growth factor-19 (FGF-19). Secreted into the portal circulation, FGF-19 acts as a signal on the liver and represses CYP7A1 expression and bile acid synthesis (16, 17).

In the present study, we asked whether ileal OSTα and OSTβ are also affected in gallstone disease and whether they are linked to FXR, FGF-19, or LRH-1.

MATERIALS AND METHODS

Subjects

Biopsies were sampled from a total of 53 individuals undergoing routine colonoscopy for various clinical indications. The presence or absence of gallstones was determined by abdominal ultrasound. Thirty-four subjects were gallstone-free controls and nineteen were gallstone carriers. None of the individuals had symptomatic gallstone disease or displayed any macroscopic or histological signs of inflammation in the ileum. To exclude gender differences, only females were studied. Subjects included in this study had *a*) normal serum lipid values and no history of taking lipid-lowering drugs or drugs interfering with bile acid uptake, *b*) no known medical conditions affecting lipid metabolism, *c*) normal liver function and no signs of hemolysis or other conditions associated with pigment stones, *d*) no intestinal surgery, and *e*) no clinical indication of impaired nutritional status. As in our previous study (8), subjects were stratified into a group with normal body weight [body mass index (BMI) ≤25 kg/m^2] and a group of overweight subjects (BMI >25 kg/m^2), with a mean BMI of 30 kg/m^2. Several relevant characteristics of patients and controls, including age and serum lipid as well as bilirubin levels, did not differ significantly and are summarized in **Table 1**. Informed consent was obtained from all subjects and the study was approved by the ethics committee of the University of Tübingen.

Isolation of total RNA and real-time quantitative RT-PCR

Total RNA and protein were isolated from biopsy specimens using the TRIzol extraction procedure (Invitrogen) based on the single-step method described by Chomczynski and Sacchi (18). The integrity, quality, and quantity of RNA were analyzed by gel electrophoresis and absorption measurement. Subsequently, 400 ng of total RNA was reverse transcribed to cDNA using the avian myeloblastosis virus reverse transcriptase system (AMV; Promega) and random hexamers according to the manufacturer's recommendation.

The quantitative RT-PCR (QRT-PCR) was performed in duplicate with the LightCycler sequence detection system (Roche Diagnostics). Primer sequences for amplification of human OSTα (sense: 5′-TGTTGGGCCCTTTCCAATAC-3′; antisense: 5′-GGCTCCCATGTTCTGCTCAC-3′) and human OSTβ (sense: 5′-CAGGCAAGCAGAAAAGAAAAGATG-3′; antisense: 5′-CCGGAAGGAAAACTGACA- 3′) were used as described by Seward et al. (11). QRT-PCR was performed as follows: 95°C for 5 min, 40 cycles at 95°C for 10 s, followed by 60°C for 5 s, then 72°C for 9 s (OSTα) and 58°C for 5 s, then 72°C for 7 s (OSTβ). At the end of the PCR, a dissociation curve was set from 57°C to 95°C. Primers used for amplification of human FXR, ASBT, ILBP, FGF-19, and LRH-1 are listed in **Table 2**. The quantity of any given transcript was calculated using the second derivative maximum method according to the manufacturer's instructions. Single-stranded cDNA from human biopsies corresponding to 10 ng of RNA or gene-specific plasmids as controls served as a template with specific oligonucleotide primer pairs. The measurement of mRNA copy numbers allows the direct comparison of expression levels between different gene products.

Plasmid constructs

OSTα and OSTβ plasmids were constructed from human cDNA, with primers described above (11). Plasmids were produced with the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Four microliters of PCR product (OSTα: 221 base pairs and OSTβ: 187 base pairs) was subcloned into the *Eco*RI sites of a 2.1 TOPO TA cloning vector. Transforma-

TABLE 1. Characteristics of study participants

	All Women		Normal-weight Women		Overweight Women	
Variables	Controls	Gallstone Carriers	Controls	Gallstone Carriers	Controls	Gallstone Carriers
Number	34	19	22	7	12	12
Age (years)	59 ± 2	64 ± 3	61 ± 3	65 ± 5	56 ± 4	63 ± 3
BMI (kg/m^2)	24.9 ± 0.9	27.8 ± 1.0	22.1 ± 0.5	23.9 ± 0.5	30.0 ± 1.4	30.1 ± 1.0
Triglyceride (mg/dl) (1–200)[a]	121 ± 7	123 ± 11	109 ± 7	125 ± 16	144 ± 13	122 ± 16
Cholesterol (mg/dl) (140–240)[a]	205 ± 6	197 ± 8	198 ± 7	212 ± 9	217 ± 9	188 ± 10
Bilirubin (mg/dl) (0.2–1.4)[a]	0.53 ± 0.04	0.67 ± 1.00	0.55 ± 0.06	0.93 ± 0.19	0.51 ± 0.06	0.51 ± 0.08

BMI, body mass index. Values are given as means ± SEM.
[a] Normal range.

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TABLE 2. Primer sequences used for QRT-PCR

Fragment	Primer	Sequence	Product Size (base pairs)
Exon 2/3	FXR F	5′ - ATCAAAGGGGATGAGCTGTG - 3′	213
	FXR R	5′ - TCCCATCTCTTTGCATTTCC - 3′	
Exon 4/5	ASBT F	5′ - TATTTCCTGTGGCGGGTTAC - 3′	182
	ASBT R	5′ - GATGAGCGGGAAGGTGAATA - 3′	
Exon 3/4	ILBP F	5′ - ACTTCACTTGGTCCCAGCAC - 3′	184
	ILBP R	5′ - TTGTCACCCACGATCTCTGA - 3′	
Exon 3	FGF-19 F	5′ - CATCGATGGGGAACTCACTT - 3′	209
	FGF-19 R	5′ - GCAAATGGTCCCTGGAAGTA - 3′	
Exon 1/3	LRH-1 F	5′ - GGGTACCATTATGGGCTCCT - 3′	227
	LRH-1 R	5′ - GGCCCAAACTTATTCCTTCC - 3′	

ASBT, apical sodium-dependent bile acid transporter; FGF-19, fibroblast growth factor-19; FXR, farnesoid X receptor; HNF1α, hepatic nuclear factor 1 α; ILBP, ileal lipid binding protein; LRH-1, liver receptor homolog-1; QRT-PCR, quantitative RT-PCR.

tion was performed with 2 μl of the ligation onset to TOP 10 cells (*Escherichia coli*). Transformed bacteria were spread to agar plates appended with X-Gal and ampicillin. The plates with bacteria were incubated overnight at 37°C. White colonies were picked and the positive clones were verified by DNA sequencing.

Preparation of membrane-enriched tissue fractions

For protein analysis, a separate method with cell membrane enrichment of the OSTα-OSTβ protein was required. Ileal mucosal biopsy specimens were homogenized (1 mg/10 μl) in ice-cold homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1% Triton X-100, 1 mM PMSF, 10 mM dipotassium magnesium salt (EDTA), and protease inhibitor cocktail (P8340; Sigma). Sucrose was added to a final concentration of 250 mmol/l, and the homogenate was centrifuged at 10,000 *g* for 5 min at 4°C. The supernatant was recovered and further centrifuged at 100,000 *g* for 30 min at 4°C to obtain a total membrane fraction. The pellet was resuspended in the buffer containing 10 mM Tris-HCl (pH 7.4), 125 mM sucrose, 2 mM PMSF, 0.68 mM EDTA, and protease inhibitor cocktail (P8340, Sigma).

Western blot analysis

Antibodies used for the detection of human OSTα and OSTβ proteins were the kind gift of Professor N. Ballatori (University of Rochester). Protein concentration was determined using a commercial kit (Bio-Rad). Laemmli sample buffer (1:2; Bio-Rad) was added to 20 μg protein. Protein samples were heated at 37°C for 20 min, mixed every 5 min, and then run on 4–20% ready Tris-HCl gels (Bio-Rad). After transfer to polyvinylidene difluoride membranes, nonspecific bindings were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20 overnight at 4°C. The blots were subsequently incubated with a dilution of primary antibody against OSTα (1:1,000) and OSTβ (1:300) for 2 h at room temperature. Afterwards, membranes were washed five times for 5 min, then probed with a secondary antibody, anti-rabbit IgG HRP-linked $F(ab')^2$ (1:3,000; Santa Cruz Biotechnology) and exposed to a chemiluminescent reagent (SuperSignal*T* West Dura; Pierce). An immunoreactive band was obtained with the predicted size of 40 kDa representing OSTα monomer, whereas OSTβ staining was detected at 19 kDa. Bands were photographed, and immunoquantitation was accomplished by densitometric analysis using the software AIDA (Raytest). To account for variability in the amounts of enterocytes in biopsy specimens, villin contents of all samples were also determined. Therefore, membranes were incubated with a primary antibody against human villin (1:2,000; Chemicon International), followed by incubation with a secondary peroxidase-conjugated anti-mouse IgG antibody (1:10,000; Pierce). Protein staining was obtained at the predicted size of 95 kDa.

Immunhistochemistry

Formalin-fixed biopsies were embedded in paraffin and cut into 3 μm sections. The immunohistochemical localization of OSTα and OSTβ was performed using the EnVision technique (EnVision™ Detection Kit; DAKO) according to the manufacturer's instructions. Sample slides were incubated with anti-OSTα (1:500) and -OSTβ (1:100) rabbit antibodies, followed by 30 min incubation with an HRP-labeled polymer secondary antibody. To stop the reaction, sections were rinsed with water and counterstained with hematoxylin. The human OSTα-OSTβ antibodies used for this procedure were the same as for the Western blot analysis.

Statistics

Data are presented as means ± SEM. Differences between groups were determined using the Mann-Whitney test. Variables were correlated with Spearman's Rank test. A P value of <0.05 was considered statistically significant.

RESULTS

Ileal OSTα and OSTβ mRNA expression

Overall, there was no significant difference in OSTα or OSTβ expression between gallstone carriers and controls in the whole study population (**Fig. 1A, B**). When divided into subgroups according to weight, again no significant differences were observed in the overweight subgroup. However, a significant reduction in mRNA expression was found in the normal-weight gallstone-carrier group compared with both normal-weight controls as well as overweight gallstone carriers. OSTα mRNA transcript levels were decreased 3.3-fold ($P = 0.006$) in the normal-weight gallstone-carrier group compared with normal-weight controls (Fig. 1A). Additionally, the considerable difference of 2.6-fold ($P = 0.03$) reduction in OSTβ mRNA expression was noted between normal-weight gallstone patients and controls (Fig. 1B). These differences were not observed in the overweight group. Moreover, OSTα transcript levels of all study participants were positively correlated with OSTβ transcripts (correlation coefficient, $r = 0.76$, $P < 0.0001$) (Fig. 1C). Finally, the expression of villin, an

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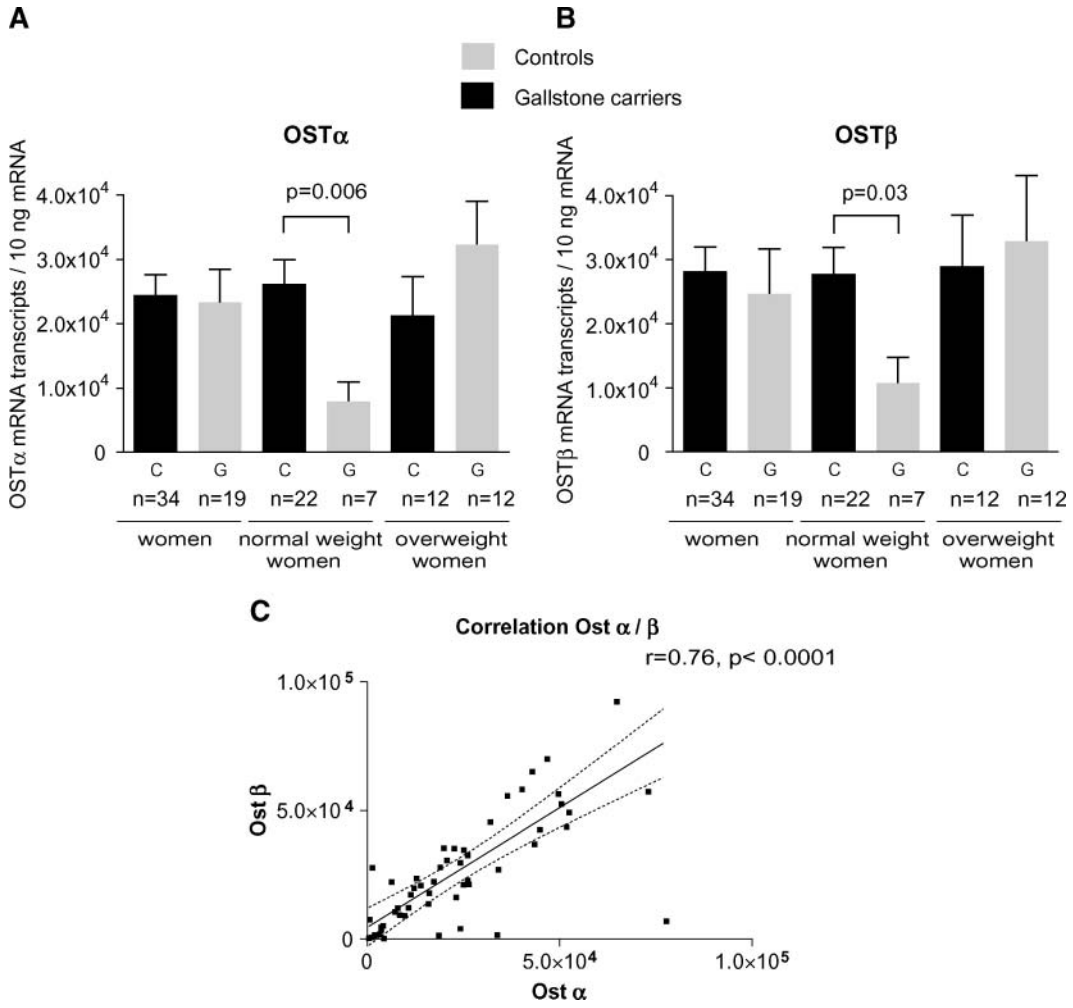

Fig. 1. Organic solute transporters α and β (OSTα-OSTβ) mRNA expression in human ileal mucosal biopsies of women. Quantitative analysis of OSTα mRNA expression (A), and OSTβ mRNA expression (B) is given as copy numbers. All data are presented as means ± SEM. C: Correlation of ileal OSTα and OSTβ mRNA ($r = 0.76$, $P < 0.0001$), n = 53.

epithelial housekeeping gene, exhibited no significant differences between the groups (data not shown).

Ileal OSTα-OSTβ localization and protein levels

To examine the cellular localization of OSTα and OSTβ proteins, immunostaining was performed in ileal biopsies. **Figure 2A–D** shows representative photomicrographs of OSTα and OSTβ in a gallstone carrier and a control. In line with previous investigations (7, 12), OSTα and OSTβ immunostaining was positive in ileal tissue, and most of the OSTα and OSTβ protein was localized at the basolateral side of the ileal enterocytes. An additional staining of OSTα-OSTβ was detectable in the cytoplasmic compartments, possibly plasma membrane-targeted proteins. The staining of cytoplasm and apical membrane was weaker in the gallstone carriers than in the corresponding control samples.

Figure 2E represents the Western blot analysis of OSTα-OSTβ protein expression in the normal-weight subgroup. When protein content was quantified using densitometric analysis, consistent with mRNA levels, the mean of OSTα protein levels was 3.1-fold lower in the normal-weight gallstone-carrier group than in corresponding controls. The expression of the OSTβ subunit also showed a clear reduction in the normal-weight gallstone group compared with controls (1.7-fold). Finally, the protein contents were normalized to villin in all samples (Fig. 2E).

A positive correlation between mRNA expression and protein levels was obtained for both transporter subunits ($r = 0.77$ for OSTα and $r = 0.89$ for OSTβ). These data provide an important indication that human ileal OSTα and OSTβ are transcriptionally regulated.

Correlation of OSTα and OSTβ mRNA with ASBT, ILBP, and the transcriptional regulator FXR

Because FXR is known to be an important transcriptional regulator of OSTα and OSTβ, a correlation analysis of these factors was performed. Indeed, OSTα correlated significantly in a positive manner with FXR ($r = 0.58$, $P < 0.0001$) (**Fig. 3A**). Moreover, the correlation coefficient for OSTβ and FXR was similar ($r = 0.50$, $P < 0.0002$) (Fig. 3B). As previously described, the apical bile acid transporter ASBT and the cytosolic transporter ILBP exhibit a reduced expression in the female normal-weight gallstone subgroup and are positively correlated with each other

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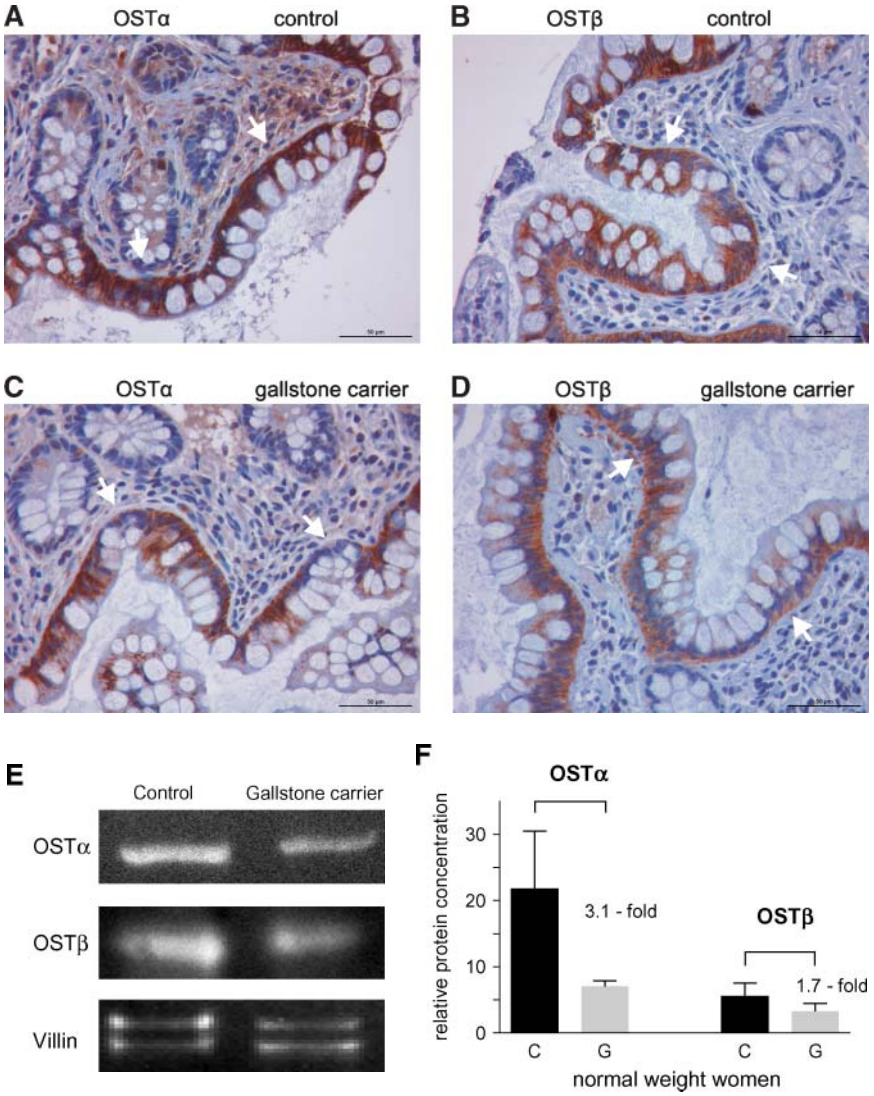

Fig. 2. Protein levels and localization of OSTα-OSTβ in human ileal biopsies of female gallstone carriers and controls. Representative immunostaining (A–D) of OSTα-OSTβ in paraffin-embedded ileal tissue. Arrows represent localization of OSTα or OSTβ in the epithelium. Representative Western blot of a female normal-weight gallstone carrier and corresponding control (E). Densitometric analysis of OSTα-OSTβ protein expression in normal-weight group (n = 3) (F). C, controls; G, gallstone carriers. All data are presented as means ± SEM. Protein levels were normalized to villin.

(8). To look for a possible regulatory link between OSTα-OSTβ and ASBT or ILBP, the correlation coefficients were determined. This analysis demonstrated a close and positive correlation of mRNA levels of the OSTα subunit to ASBT ($r = 0.65$, $P < 0.0001$) (Fig. 3C) and to ILBP ($r = 0.77$, $P < 0.0001$) (Fig. 3E). The subunit OSTβ also correlated positively with ASBT ($r = 0.58$, $P < 0.0001$) (Fig. 3D) and with ILBP ($r = 0.67$, $P < 0.0001$) (Fig. 3F).

Ileal FGF-19 and LRH-1 mRNA expression

To investigate the regulatory link between intestine and liver, we also measured ileal mRNA expression of FGF-19 in the normal-weight group and correlated it with the OSTα-OSTβ and FXR data. FGF-19 showed an mRNA expression pattern similar to that of the ileal bile acid transporters (ASBT, ILBP, OSTα-OSTβ) and the transcriptional factor, FXR. As shown in **Fig. 4A**, FGF-19 exhibited a considerable (2.8-fold) mRNA reduction ($P = 0.06$) in the gallstone-carrier group. Interestingly, the expression data of FGF-19 showed no correlation with that of OSTα-OSTβ mRNA ($r = 0.24$, $r = 0.25$, respectively) or FXR mRNA ($r = 0.1$).

Finally, we examined the expression of the nuclear receptor LRH-1. A significant (2-fold) reduction of mRNA transcript levels ($P = 0.04$) was detected in gallstone carriers compared with controls (Fig. 4B). However, the correlation coefficient of LRH-1 mRNA with OSTα was low ($r = 0.17$), similar to that of OSTβ ($r = 0.13$) and FXR ($r = 0.25$).

DISCUSSION

The present study demonstrates reduced ileal mRNA and protein expression of basolateral ileal bile acid ex-

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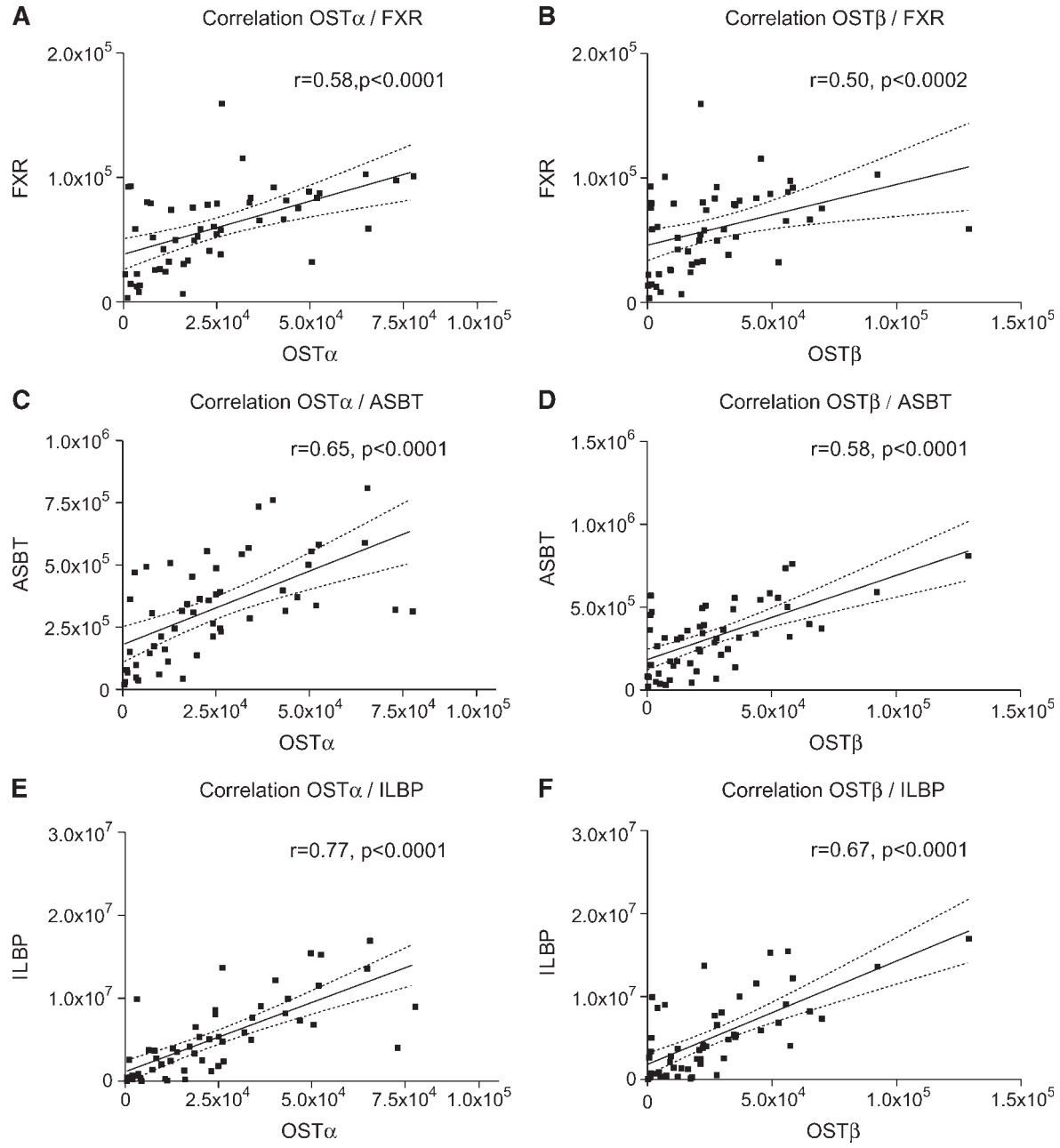

Fig. 3. Correlation data of ileal OSTα-OSTβ mRNA expression from controls and gallstone carriers (n = 53). Determination of the correlation coefficient between OSTα mRNA expression and the farnesoid X receptor (FXR) ($r = 0.58$, $P < 0.0001$) (A) and OSTβ expression with FXR ($r = 0.50$, $P < 0.0002$) (B). Correlation of OSTα expression with ileal apical sodium-dependent bile acid transporter (ASBT) ($r = 0.65$, $P < 0.0001$) (C) and OSTβ expression levels with ASBT ($r = 0.58$, $P < 0.0001$) (D). Correlation of OSTα transcript levels with cytosolic ileal lipid binding protein (ILBP) ($r = 0.77$, $P < 0.0001$) (E) and OSTβ mRNA expression with ILBP ($r = 0.67$, $P < 0.0001$) (F).

porter protein OSTα-OSTβ in non-obese gallstone disease. Importantly, OSTα and OSTβ mRNA expression was positively correlated with the regulatory nuclear receptor FXR, with the ileal expression of the apical bile acid transporter ASBT, as well as with the cytosolic bile acid transporter protein ILBP. Furthermore the intestinal growth hormone FGF-19 and the transcriptional factor LRH-1 were also considerably reduced in non-obese gallstone carriers, although unrelated to OSTα and OSTβ mRNA expression. Together with our previous observations of diminished apical and cytosolic bile acid transporters ASBT and ILBP (8), the present investigation completes the scenario of three impaired bile acid transporters and, probably, transepithelial bile acid flux in non-obese gallstone disease.

Disorders of bile acid metabolism in cholesterol gallstone disease have been described extensively, but some aspects are still controversial. There is consensus that in most gallstone patients, the proportion of deoxycholate in bile is increased (19, 20) and the bile acid pool size is diminished (19, 21). In experimental animals, deoxycholate is a strong inhibitor of bile acid synthesis (22), compatible with a low rate of bile acid formation in some gallstone patients (23).

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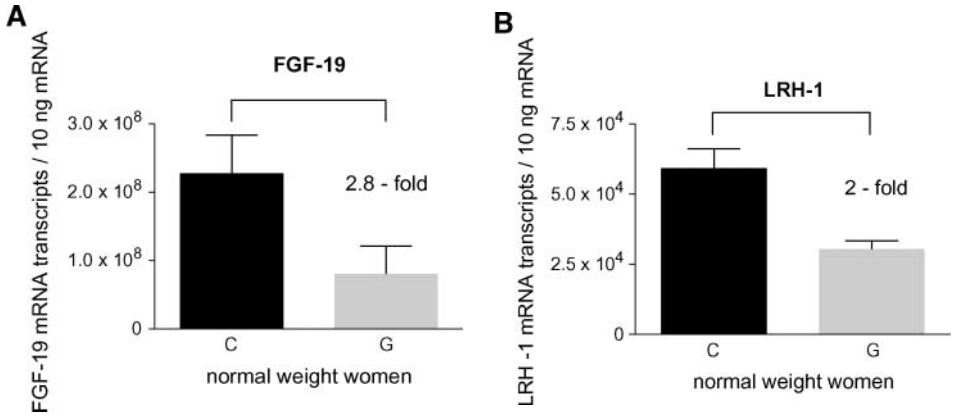

Fig. 4. Fibroblast growth factor-19 (FGF-19) and liver receptor homolog-1 (LRH-1) mRNA expression in human ileal mucosal biopsies of normal-weight women. Quantitative analysis of FGF-19 mRNA expression (A) and LRH-1 mRNA expression (B) is given as copy numbers. All data are presented as means ± SEM. (controls: n = 20, gallstone carriers: n = 6). $P = 0.06$ for FGF-19 and $P = 0.04$ for LRH-1.

On the other hand, the effect of deoxycholate in humans is controversial (24, 25) and most studies in gallstone patients report an increase in both bile acid turnover (19, 26) and synthesis (27, 28). Increased biliary deoxycholate levels, bile acid turnover, and synthesis may all occur as a response to an increased ileal loss of bile acids. Alternatively, higher levels of biliary deoxycholate may be due to increased amounts of Gram-positive anaerobic bacteria and increased activity of 7α-dehydroxylase in the cecum of patients with cholesterol gallstones versus controls, but this change in flora may actually be a consequence of primary ileal bile acid loss (29).

As mentioned above, we have recently described an impaired expression of the epithelial transporters ASBT and ILBP in normal-weight but not overweight gallstone disease (8). In the present study, we have complemented the basolateral transporters OSTα and OSTβ, which were also diminished both on the mRNA and the protein level. The combined impaired expression of all three transporters may lead to a compromised ileal reuptake of bile acids and loss toward the colon, which may also explain the increased level of biliary deoxycholate in gallstone disease. As discussed above, our own and many previous studies in the field suggest that the distal small intestine may play a major role in lithogenesis (28, 30), at least in female normal-weight gallstone carriers. It is possible that some of the discrepancies in the literature may be explained by the weight heterogeneity of the gallstone groups, because metabolic handling of cholesterol and bile acids is different between normal-weight and overweight individuals (8, 9).

Interestingly, a similar mechanism was observed in hypertriglyceridemia, a state often associated with gallstones. The elegant study by Duane et al. (31) explains impaired intestinal bile acid absorption in patients with hypertriglyceridemia as a consequence of diminished expression of ASBT protein. Compatible with these findings, it has been suggested that the enhanced activity of microsomal triglyceride transfer protein in the liver is a response to the increased fecal loss of bile acids (28). On the other hand, in the present study, triglyceride levels were similar in the different groups, suggesting that low ASBT activity alone may not necessarily lead to an increase in serum lipids.

Increased hepatic bile acid synthesis is a well-recognized mechanism to compensate for interruption of the enterohepatic circulation of bile acids (32). Principally, the increased bile acid synthesis observed in some studies on gallstone patients (27, 28) would be consistent with the diminished bile acid carrier expression and consequent bile acid malabsorption described in our prior investigation (8). Notably, in $ASBT^{-/-}$ mice, ileal expression of FGF-15, the rodent ortholog of human FGF-19, was reduced and the activity of CYP7A1 in the liver was increased (13, 33). By contrast, in $OST\alpha^{-/-}$ mice, the expression of bile acid-activated FGF-15 was increased, followed by depressed CYP7A1 activity (13). Applied to our human study, the diminished ASBT expression may lead to the low enterocyte bile acid levels, explaining the reduced FGF-19 expression.

The expression of OSTα and OSTβ was closely correlated. A similar positive correlation coefficient between mRNA levels of the α- and β-subunits ($r = 0.93$) was previously described for the ileal expression in the mouse (34). To gain further insight into the mechanism behind these findings, the key transcription factor of OSTα and OSTβ, FXR, was investigated. FXR, the bile acid nuclear receptor, is a molecular link between bile acid synthesis, transport, and detoxification (15, 34–36). In wild-type animals, but not in $FXR^{-/-}$ animals, cholic acid markedly induces the expression of OSTα-OSTβ mRNA, indicating an FXR-dependent regulation (37). Moreover, mouse OSTα and -β promoters harbor both FXR and LRH-1 elements, which mediate positive and negative feedback regulation, respectively. The positive regulatory pathway appears to be dominant (34). In addition, an intestinal LRH-1 deficiency in mouse was associated with lower OSTα-OSTβ mRNA levels, whereby the expression of ILBP and FGF-15, the rodent ortholog of FGF-19, were significantly reduced (14). Although the low LRH-1 expression in gallstone patients would be compatible with this finding, the lack of a close correlation calls into question the importance of this transcription factor for OSTα-OSTβ regulation in man. In cultured Huh7-cells, hu-

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man OSTα-OSTβ expression is induced, due to bile acids, through ligand-dependent transactivation of both OST genes by FXR. Two functional FXR binding motifs have been identified in the human OSTα gene and one in the OSTβ gene (15). Accordingly, targeted mutation of these elements led to a reduced inducibility of both OST promoters by FXR. Therefore, we also analyzed the connection between OSTα and OSTβ and the transcriptional regulator FXR in our study population with regard to gallstone pathogenesis. The positive correlation coefficient between OSTα-OSTβ and FXR confirmed this relationship. The fact that both OST genes are regulated by FXR and OSTα-OSTβ expression is diminished in ileal tissue of female normal-weight gallstone carriers could be the consequence of a reduced activation through the transcriptional regulator FXR.

In addition to the transcriptional regulation of OSTα-OSTβ, FXR directly induces the expression of the human ILBP gene (38). The correlative association between OSTα-OSTβ and ILBP may well be explained by a coordinated regulation of both transporters through FXR. In contrast, the regulation of ASBT, which also shows a positive correlation with OSTα-OSTβ, is much more complicated. Multiple steps have been found in the bile acid-mediated feedback regulation of ASBT. In mice, bile acids bind to the FXR receptor, leading to an upregulation of short heteromeric partner (SHP) expression and to repression of LRH-1-dependent activation of the ASBT gene (39). Bile acid responsiveness of the human ASBT gene is mediated by an FXR-bile acid complex-dependent activation of SHP and, subsequently, inhibition of retinoic acid receptor/retinoid X receptor (RAR/RXR) (40). Recently, a further study by Duane, Xiong, and Wolvers (41) demonstrated the bile acid-dependent activation of the human ASBT promoter via an AP-1 response element in the 5′-untranslated region, which is consistent with our findings of a positive feedback regulation of bile acid absorption in animal models (42). In addition, three HNF1α binding sites, all responsible for transcriptional induction, were identified in the promoter region of the ASBT gene (43). Thus, HNF1α seems to be of major importance for the regulation of ASBT expression (44). Our prior work indicated that ileal expression of HNF1α was significantly reduced in gallstone carriers and correlated positively with FXR levels (8). Furthermore, an HNF1α binding site was identified in the promoter region of FXR, indicating that HNF1α may also act as a transcriptional activator of FXR (44).

Taken together, several scenarios appear possible to explain the changes in three bile acid carriers. First, it is pos-

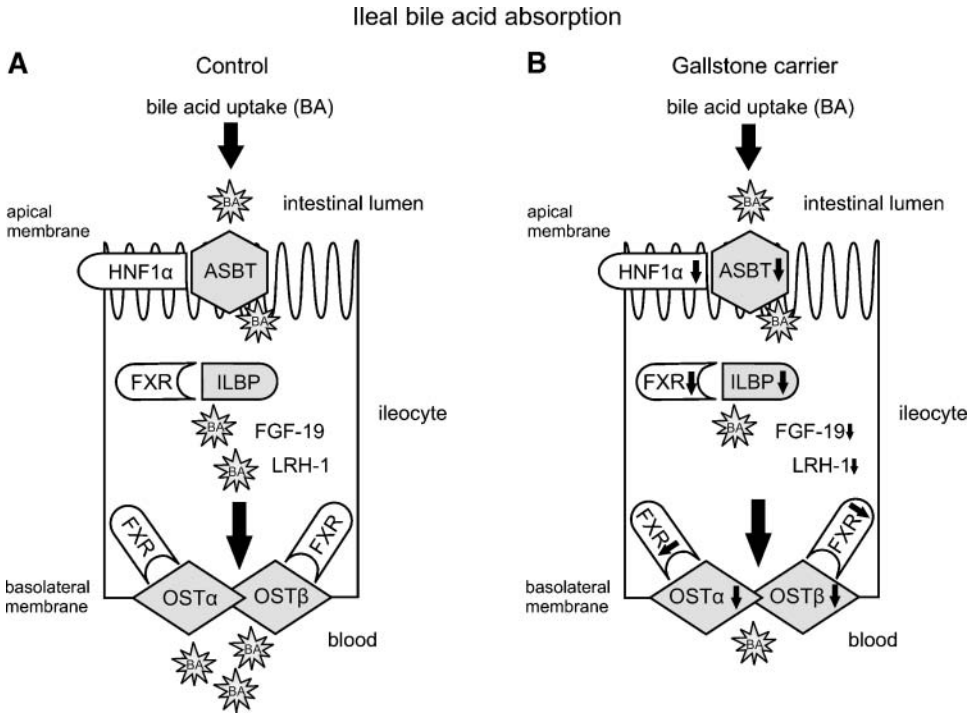

Fig. 5. Schematic model of intestinal bile acid absorption in normal-weight women. A: The initial step of the ileal bile acid absorption is mediated by ASBT, which is located at the apical membrane of the ileocyte. The cytosolic bile acid transporter ILBP shuttles bile acids to the basolateral membrane. OSTα-OSTβ exports bile acids from the basolateral side of the ileocyte into the portal circulation. Several transcription factors are directly involved in this absorption process, e.g., HNF1α, which has three responsive elements in the ASBT promoter, and FXR as activator of ILBP and OSTα-OSTβ gene expression. LRH-1 is known to be involved in bile acid and cholesterol homeostasis. FGF-19 is expressed in the intestine, activated by bile acids, and modulates as a systemic signal the hepatic bile acid synthesis. B: The defective intestinal bile acid absorption in female non-obese gallstone carriers is shown schematically, where the expression of all three bile acid transporters (ASBT, ILBP, and OSTα-OSTβ), as well as the transcription factors FXR, HNF1α, LRH-1 and the growth factor FGF-19 are diminished compared with female normal-weight controls.

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sible that in some gallstone patients, a genetic defect in ASBT expression leads to impaired epithelial bile acid uptake and diminished activation and expression of FXR. As a consequence, the expression of FXR-controlled ILBP as well as OSTα and OSTβ would be reduced. Second, it cannot be excluded that the low FXR expression is a primary and independent defect in some patients with gallstone disease. Alternatively, it may be hypothesized that HNF1α or other relevant transcription factors such as RAR/RXR (40) are major determinants of the reduced transepithelial bile acid flux in normal-weight gallstone disease. Notably, these explanations for the current findings are not mutually exclusive.

In summary, in addition to low ASBT and ILBP (8), mRNA expression and protein levels of the heteromeric bile acid transporter OSTα-OSTβ are diminished in the ileum of female non-obese gallstone carriers compared with controls, as depicted in **Fig. 5**. Ileal mRNA expression of FGF-19 and LRH-1 exhibits a similarly reduced pattern. A primary reduced ileal expression of ASBT or of the transcriptional factors FXR or HNF1α (8) might play a crucial role in gallstone formation in normal-weight women.

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