Transgenic Overexpression of *Abcb11* Enhances Biliary Bile Salt Outputs, But Does Not Affect Cholesterol Cholelithogenesis in Mice

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Abstract

Background—Cholesterol gallstone disease is a complex genetic trait and induced by multiple but as yet unknown genes. A major *Lith* gene, *Lith1* was first identified on chromosome 2 in gallstone-susceptible C57L mice compared with resistant AKR mice. *Abcb11*, encoding the canalicular bile salt export pump in the hepatocyte, co-localizes with the *Lith1* QTL region and its hepatic expression is significantly higher in C57L mice than in AKR mice.

Material and methods—To investigate whether *Abcb11* influences cholesterol gallstone formation, we created an *Abcb11* transgenic strain on the AKR genetic background and fed these mice with a lithogenic diet for 56 days.

Result—We excluded functionally relevant polymorphisms of the *Abcb11* gene and its promoter region between C57L and AKR mice. Overexpression of *Abcb11* significantly promoted biliary bile salt secretion and increased circulating bile salt pool size and bile salt-dependent bile flow rate. However, biliary cholesterol and phospholipid secretion, as well as gallbladder size and contractility were comparable in transgenic and wild-type mice. At 56 days on the lithogenic diet, cholesterol saturation indexes of gallbladder biles and gallstone prevalence rates were essentially similar in these two groups of mice.

Conclusion—Overexpression of *Abcb11* augments biliary bile salt secretion, but does not affect cholelithogenesis in mice.

Keywords

bile; bile flow; bile salt; biliary secretion; cholesterol saturation index; crystallization; liquid crystals; microscopy; *Lith* gene

INTRODUCTION

Cholesterol cholelithiasis is one of the most prevalent digestive diseases, resulting in a considerable amount of financial and social burden worldwide [1,2]. Because prevalence of gallstones is rising due to the worldwide obesity epidemic with insulin resistance, a key feature of the metabolic syndrome, it is imperative to investigate cholelithogenesis and find

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potential ways to prevent the formation of gallstones. Bile formation depends mainly on the sequential action of several membrane lipid transport proteins that are located on the canalicular membrane of the hepatocyte [1–4]. The hepatocyte possesses an ensemble of export pumps belonging to the ATP-binding cassette (ABC) family of membrane transporters that enable the transport of biliary lipids against their concentration gradients into bile. Alterations in the expression or function of these proteins may affect the lipid compositions of bile and contribute to cholesterol gallstone formation [2–4]. Five major genes encoding ABC transporters involved mainly in bile formation have been cloned and characterized. A major progress in the search for the canalicular lipid transporter comes from the discovery that *ABCB4* encodes a phosphatidylcholine transmembrane transporter and there is a lack in hepatic secretion of biliary phosphatidylcholine in *Abcb4*$^{-/-}$ mice [5,6]. Targeted disruption of *Abcb4* induces spontaneous formation of cholesterol gallstones in mice [7], and mutations of *ABCB4* result in gallstone formation in humans [8,9]. Moreover, the biochemical and histological characteristics of *Abcb4*$^{-/-}$ mice closely resemble the corresponding phenotypes of patients with progressive familial intrahepatic cholestasis (PFIC type 3) [10]. Mutations in the genes encoding human ABCG5 and ABCG8 transporters cause sitosterolemia [11,12]. By using transgene techniques, it has been found that overexpression of the human *ABCG5/G8* genes in mice markedly alters cholesterol transport in the liver and small intestine: biliary cholesterol secretion and saturation are increased, but fractional absorption of dietary cholesterol is decreased [13]. Furthermore, *Abcg5/g8* are mapped to murine chromosome 17 and are candidate genes for the gallstone susceptibility locus *Lith9* in the (I/LN×PERA)F$_2$ cross [14]. Furthermore, genome-wide association and family studies have identified the common ABCG8 polymorphism p.D19H as a susceptibility gene for gallstone disease in humans [15,16]. *Abcc2* is mapped to chromosome 19 in the mouse and promotes biliary secretion of dianionic xeno- and endobiotics, including glutathione conjugates, bilirubin diglucuronides and bile salts conjugated with sulfate or glucuronide. Thus, ABCC2 may be responsible for the generation of bile salt-independent bile flow, apparently through glutathione secretion. Also, it has been proposed that *Abcc2* is a candidate gene for the *Lith2* locus [17,18]. The *Abcb11* gene encodes a protein called bile salt export pump that transports bile salts from the hepatocyte into bile [19,20] and maps to mouse chromosome 2. In particular, the first quantitative trait locus (QTL) analysis of cholesterol gallstones was carried out between inbred strains of gallstone-susceptible C57L and resistant AKR mice [21]. The results from these studies [22,23] showed that *Abcb11* co-localized with the *Lith1* QTL and its expression in the liver is significantly higher in C57L mice than in AKR mice. Therefore, it has been proposed that *Abcb11* is a creedal candidate gene for *Lith1* in the C57L strain [22]. In the present study, we created a new *Abcb11* transgenic strain (*Abcb11.Tg*) in the gallstone-resistant AKR genetic background to investigate whether overexpression of the *Abcb11* gene in the liver influences cholesterol cholelithogenesis.

MATERIALS AND METHODS

Genetically modified mice and diets

Generation of bacterial artificial chromosome (BAC) transgenic mice with overexpression of the *Abcb11* gene in a 129S1/SvImJ background has been reported in details in a published paper [24]. Mice were genotyped as described [24], and Southern blot analysis and semiquantitative PCR analysis indicated that these transgenic mice possess two additional intact *Abcb11* gene copies. The 129S1 strain shows intermediate susceptibility to gallstone formation in response to the lithogenic diet [25]. It has been suggested that *Abcb11* is one of the strongest candidate genes underlying *Lith1* in C57L mice, since it co-localizes with the *Lith1* QTL [17,22]. To explore whether overexpression of *Abcb11* in the liver contributes to gallstone formation, we created a new *Abcb11* transgenic strain (*Abcb11.Tg*) on the

gallstone-resistant AKR background by marker-assisted backcrossing [26] of the *Abcb11*-BAC transgenic strain with the 129S1 background on the AKR background for five generations (N5). These breeding regimen resulted in *Abcb11.Tg* mice that carry at least 97% AKR genome and ~3% 129S1 genome. Non-transgenic littermates with the same genetic background were used as controls.

Male mice, at 8 to 10 weeks of age, were fed normal mouse chow (Harlan Teklad Laboratory Animal Diets, Madison, WI), or a lithogenic diet containing 1% cholesterol, 0.5% cholic acid and 15% butter fat for 56 days [27]. Mice were maintained in a temperature-controlled room ($22\pm1°C$) with a 12-hour day cycle (6 AM-6 PM), and were provided free access to water. All procedures were in accordance with current National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Harvard University.

Microscopic studies of gallbladder biles and gallstones

After cholecystectomy, gallbladder volume was measured by weighing (n=20 per group). Fresh gallbladder bile was examined for mucin gel, solid and liquid crystals, and gallstones, which were defined according to previously established criteria [27]. Pooled gallbladder biles were frozen and stored at $-20°C$ for further lipid analyses.

Hepatic secretion of biliary lipids

After cholecystectomy, the common bile duct was cannulated and hepatic bile was collected by gravity in additional groups of mice (n=5 per group). The first hour collection of hepatic biles was used to study biliary lipid outputs [28]. To determine bile salt-dependent and bile salt-independent bile flow rates, as well as bile salt pool sizes, 8-hour biliary "washout" studies were carried out [28]. During surgery and hepatic bile collection, mouse body temperature was maintained at $37\pm0.5°C$ with a heating lamp and monitored with a thermometer.

Lipid analyses

Total and individual bile salt concentrations were measured by reverse-phase HPLC [29]. Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett [30]. Bile cholesterol, as well as cholesterol content in gallstones and the lithogenic diet were determined by HPLC [27]. Cholesterol saturation indexes (CSI) in pooled gallbladder biles were calculated from the critical tables [31]. Hydrophobicity indexes of bile salts in hepatic biles were calculated according to Heuman's method [32]. Fecal neutral steroids were saponified, extracted and measured by HPLC [33].

Quantitative real-time PCR assays

Total RNA was extracted from fresh liver tissues (n=4 per group) using RNeasy Midi (Qiagen, Valencia, CA). Reverse-transcription reactions were performed using the SuperScript II First-strand Synthesis System (Invitrogen, Carlsbad, CA) with 5 µg of total RNA and random hexamers to generate cDNA. Primers and probes for mouse *Abca1, Abcb4, Abcg5, Abcg8, Cyp7a1, Hmgcr, Npc1l1,* and *Srb1* genes have been described elsewhere [34,35]. Real-time PCR assays for all samples were performed in triplicate. To obtain a normalized target level, all values were divided by *Gapdh* as the invariant control.

Determination of activities of hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase

Fresh liver samples were collected from nonfasted mice (n=5 per group) on chow or fed the lithogenic diet for 56 days. To minimize diurnal variations of hepatic enzyme activities, all procedures were performed between 9:00 and 10:00 AM. Microsomal activities of HMG-

CoA reductase were determined by measuring the conversion rate of $[^{14}C]$HMG-CoA to $[^{14}C]$mevalonic acid and with $[^3H]$mevalonolactone as internal standard [36]. Hepatic activities of cholesterol 7α-hydroxylase were determined by HPLC as described elsewhere [36].

Gallbladder contraction study

To explore whether gallbladder emptying was altered during the lithogenic diet feeding, we measured gallbladder contraction function in response to exogenously administered cholecystokinin (CCK) (17 nmol/kg body weight) or phosphate buffered saline (PBS) solution as a control in *Abcb11.Tg* and wild-type mice (n=5 per group) on chow (day 0) and at 56 days on the lithogenic diet [35]. Gallbladder contractile function was determined by comparing bile flow rates and bile salt concentrations in the collected hepatic bile samples before and after the IV administration of CCK-8 [35].

Measurement of intestinal cholesterol absorption

(i) Fecal dual-isotope ratio method. Nonfasted and non-anesthetized mice (n=5 per group) were given by gavage an intragastric bolus of 1 μCi of $[^{14}C]$cholesterol and 2 μCi of $[^3H]$sitostanol mixed with 150 μl of medium-chain triglyceride. To calculate the percent cholesterol absorption, the ratios of the two radiolabels in the fecal extract from the 4-day pooled feces and the dosing mixture were assayed, respectively [33,37]. (ii) Plasma dual-isotope ratio method. Additional groups of chow-fed mice (n=5 per group) were injected intravenously with 100 μl of Intralipid containing 2.5 μCi of $[^3H]$cholesterol. Immediately, each animal was given by gavage an intragastric bolus of 150 μl of medium-chain triglyceride containing 1 μCi of $[^{14}C]$cholesterol. The ratio of the two radiolabels in the plasma sample taken on the third day was used for calculating the percent cholesterol absorption [33,37].

Cholesterol balance analysis

Mice housed in individual metabolic cages with wire mesh bottoms were allowed to adapt to the environment for 2 weeks. When body weight, food ingestion, and fecal excretion were constant, indicating an apparent metabolic steady state, food intake was measured and feces were collected daily for the balance study. The mice (n=5 per group) were fed the lithogenic diet for 7 days. Then, cholecystectomy was performed and the common bile duct was cannulated and hepatic bile was collected for the first hour of biliary secretion. Percent cholesterol absorption was calculated as described previously [33].

Comparative sequencing of mouse *Abcb11* cDNA and promoter sequences

cDNA from strains AKR and C57L was obtained by reverse-transcription-PCR (Superscript, Gibco, Eggenstein, Germany) from RNA isolated from liver, using Ultraspec reagent (Biotecx, TX). All *Abcb11* exons were amplified with 20 ng DNA, 100 nM primers (MWG, Ebersberg, Germany), 20 mM TRIS/HCl, pH 8.55, 1.5 mM $MgCl_2$, 1.6 mM $(NH_4)_2SO_4$, 50 μM dNTPs, 0.4 U *Taq* polymerase (Peqlab, Erlangen, Germany) in 20 μl reaction volume; 95°C/5 min, 95°C/1 min, −55°C/30 sec, −72°C 1 min (35 cycles), and 72°C/7 min).

Promoter sequence (−1,785 bp) from the strain C57L (GenBank Accession No. AF303740) was generated by Genome Walking (Clontech, Heidelberg, Germany), using gene specific primers 5′-ttgaagtgacccttgtgacatcctg-3′ and 5′-cttctgctcagaactctgtgcacac-3′. Additional promoter sequence (−5,485 bp) was obtained by direct sequencing of BAC clone RP23-291P1 [24] (*Trans NIH Mouse Initiative*). The open reading frame of the *Abcb11* cDNA (4,866 bp) and promoter sequences (5,485 bp) were compared between strains AKR

and C57L by direct sequencing of PCR products [38]. Primer sequences are available upon request.

Potential transcription factor bindings sites were identified using MatInspector 2.2 based on the Transfac Database 4.0 (Genomatix Software, München, Germany). Dual luciferase reporter gene assays using vectors pGL3b and pRL-TK (Promega, Madison, WI) and cotransfection with expression vectors hFXR and hRXR (kindly provided by Dr. David Mangelsdorf, Dallas, TX) were carried out as described [39].

Statistical methods

All data are expressed as means±SD. Statistically significant differences among groups of mice were assessed by Student's *t*-test or Chi-square test. By linear regression analyses, parameters significantly associated with bile flow rates and bile salt secretion rates were further assessed by a stepwise multiple regression analysis to identify the independence of the association. Analyses were performed with *SuperANOVA* software. Statistical significance was defined as a two-tailed probability of less than 0.05.

RESULTS

Effects of *Abcb11* overexpression on biliary secretion

As shown in Table 1, mean bile flow rates following acute (first hour) interruption of the enterohepatic circulation are significantly higher in *Abcb11.Tg* mice than in wild-type mice on chow or fed the lithogenic diet. On chow, hepatic outputs of biliary cholesterol and phospholipid were essentially similar between *Abcb11.Tg* and wild-type mice. Compared with the chow diet, feeding the lithogenic diet significantly increased secretion rates of biliary cholesterol and phospholipids; however, no significant differences in these secretion rates existed between *Abcb11.Tg* and wild-type mice. In contrast, bile salt outputs were significantly higher in *Abcb11.Tg* mice than in wild-type mice, regardless of whether chow or the lithogenic diet was fed. Furthermore, we used biliary "washout" techniques to measure the circulating bile salt pool sizes, which were significantly larger in *Abcb11.Tg* mice than in wild-type mice. Moreover, the total bile salt pool sizes, i.e., the circulating bile salt pool size plus the bile salt pool in the gallbladder, were still markedly larger in *Abcb11.Tg* mice than in wild-type mice. Furthermore, we found that under basal physiological conditions (on chow, day 0), the relative mRNA levels for the hepatic lipid transporters *Abcb4*, *Abcg5*, and *Abcg8* were essentially similar in both groups of mice. In the lithogenic state, there was a 4-fold increase (P<0.05) in gene expression levels of all these transporters (data not shown). However, no significant statistical differences in their relative mRNA levels were found between *Abcb11.Tg* and wild-type mice.

Figure 1 displays the relationships of bile flow to bile salt secretion rates from the "washout" data demonstrating combined hepatic bile data in both strains on chow and fed the lithogenic diet. The slope of the lines represents the relationships between bile flow and bile salt output that defines bile salt-dependent bile flow. Its intercept with the ordinate axis estimates the bile salt-independent bile flow. When the data were analyzed with a conventional regression line, the slopes were significantly different. They were 33±3 μl (bile H$_2$O)/μmol bile salts for *Abcb11.Tg* mice, being significantly greater (P<0.001) than those (16±2 μl/μmol) for wild-type mice. However, the extrapolated *y*-intercepts of the regressions displayed similar values between *Abcb11.Tg* (120±15μl/h/100g) and wild-type mice (146±10μl/h/100g), indicating that the bile salt-independent bile flow was essentially similar in these two groups of mice.

Consistent with previous studies [27,28], analysis of individual bile salts by HPLC revealed that all were taurine conjugated. On chow, *Abcb11.Tg* and wild-type mice displayed

identical distributions of bile salt species in hepatic biles, with taurocholate and tauro-β-muricholate being the predominant bile salts and others being present in smaller concentrations (Table 1). In the lithogenic state, taurocholate was significantly increased, but tauro-β-muricholate was significantly decreased, most contributable to cholic acid in the lithogenic diet. In addition, both taurochenodeoxycholate and taurodeoxycholate were significantly increased. However, tauro-ω-muricholate and tauroursodeoxycholate remained unchanged. Again, no differences in the distributions of bile salt species were found in both strains of mice. Of note, the changes of bile salt compositions in gallbladder biles (data not shown) were similar to those in hepatic biles (Table 1).

Effects of *Abcb11* overexpression on gallstone formation

On chow, the gallbladder wall was thin and transparent. Macroscopic and light microscopic examination of gallbladder biles showed no evidence of mucin gel, solid and liquid crystals, or gallstones. At 56 days on the lithogenic diet, gallstone prevalence rates were essentially similar in these two strains of mice, with 25% of *Abcb11.Tg* mice and 20% of wild-type mice forming gallstones. The cholesterol extracted from these stones constituted >99% of stone weight. Moreover, gallstone diameters were similar between *Abcb11.Tg* mice (0.32±0.14 mm) and wild-type mice (0.24±0.13 mm). It should be emphasized that 75% of *Abcb11.Tg* mice and 80% of wild-type mice were gallstone free; however, most of these mice contained a thin layer of mucin gel, as well as some solid and liquid crystals.

Lipid compositions of pooled gallbladder and individual hepatic biles

Table 2 shows biliary lipid compositions of pooled gallbladder biles (n=20 per group). On chow, *Abcb11.Tg* mice displayed slightly higher mole percent cholesterol (=2.18%) and cholesterol saturation index (CSI=0.45) compared with wild-type mice (mole percent cholesterol=1.73% and CSI=0.35, respectively). However, at 56 days on the lithogenic diet, gallbladder biles became supersaturated with cholesterol, as well as mole percent cholesterol and CSIs reached essentially similar values between two strains of mice. Furthermore, the biles of *Abcb11.Tg* mice contained identical mole percent phospholipid and bile salts compared with wild-type mice.

Table 2 also lists the biliary lipid compositions of individual hepatic biles from *Abcb11.Tg* and wild-type mice during the first hour of interrupted enterohepatic circulation while on chow and on the lithogenic diet for 56 days. As discussed elsewhere [40], if these biles were at equilibrium, they would be composed of liquid crystals and saturated micelles but never solid crystals. We occasionally detected sparse numbers of small liquid crystals in fresh hepatic bile samples under microscopic examination (×800 magnification), and when observed over several days, these biles never formed solid cholesterol monohydrate crystals. This is in complete agreement with equilibrium two-phase nature of region E [40], as shown in Figure 2. Moreover, the relative lipid compositions of pooled gallbladder biles in both groups of mice were located in region C of the central three-phase zone in which biles are composed of cholesterol monohydrate crystals, liquid crystals and saturated micelles at equilibrium [40].

Gallbladder volumes and emptying

On chow, gallbladder volumes were similar between *Abcb11.Tg* mice (15±7 μl) and wild-type mice (13±6 μl). In the lithogenic state, gallbladder sizes (25±9 μl) were enlarged in these mice while cholesterol crystallization and gallstones were developed. Figure 3 shows gallbladder emptying function as indicated by changes in bile flow rates (top panels) and biliary bile salt outputs (bottom panels) stimulated by IV injection of CCK-8 (as shown by arrows) in mice on chow or fed the lithogenic diet. As shown in Figure 3A (on chow, day 0), because of gallbladder emptying, bile flow rates and biliary bile salt outputs were increased

sharply and significantly by exogenously administered CCK-8 and these parameters were comparable in *Abcb11.Tg* mice vs. wild-type mice. In the lithogenic state (Figure 3B), bile flow rates and biliary bile salt outputs were increased slightly by CCK-8 in both strains of mice. These alterations suggest that gallbladder contractile function is impaired partially in both strains of mice at 56 days on the lithogenic diet compared with those on the chow diet. Again, gallbladder contractility in response to CCK-8 was comparable in *Abcb11.Tg* mice vs. wild-type mice. As expected, PBS administration (data not shown) did not influence gallbladder sizes in either group.

Hepatic cholesterol and bile salt synthesis

Of special note is that mRNA levels and enzymatic activities of hepatic HMG-CoA reductase were comparable in *Abcb11.Tg* mice vs. wild-type mice, regardless of whether (Figure 4A) chow or (Figure 4B) the lithogenic diet is fed. Noticeably, the lithogenic diet significantly reduced expression levels and activities of hepatic HMG-CoA reductase in these mice. Furthermore, on chow (Figure 4C), expression levels and enzymatic activities of cholesterol 7α-hydroxylase in the liver were significantly lower in *Abcb11.Tg* mice than in wild-type mice. Feeding the lithogenic diet reduced enzymatic activities and gene expression levels for hepatic bile salt synthesis in both groups of mice (Figure 4D). However, *Abcb11.Tg* mice still showed significantly lower gene expression levels and enzymatic activities of hepatic cholesterol 7α-hydroxylase compared with those in wild-type mice.

Effects of *Abcb11* overexpression on intestinal cholesterol absorption

On chow, cholesterol absorption efficiency was significantly higher in *Abcb11.Tg* mice than in wild-type mice as measured by two independent techniques, the plasma (Figure 5A) and the fecal dual-isotope ratio methods (Figure 5B). Because high dietary cholesterol exerts the effect of radioisotope dilution on the specific activity of cholesterol in the upper small intestine, we used cholesterol balance analysis to determine cholesterol absorption efficiency in mice challenged with the lithogenic diet. Cholesterol absorption efficiency measured by the mass balance method was significantly increased to 35±4% in wild-type mice mice and to 47±4% in *Abcb11.Tg* mice (P<0.01, compared with wild-type mice) in challenge to the lithogenic diet, because of the dietary cholic acid [33]. These results suggest that, regardless of whether chow or the lithogenic diet was fed, increased hepatic outputs of biliary bile salts and larger circulating bile salt pool sizes in *Abcb11.Tg* mice greatly enhance intestinal cholesterol absorption, mostly because these alterations resulted in more cholesterol dissolved in micelles within the small intestinal lumen.

Of note is that relative mRNA levels of the intestinal *Npc1l1* gene in chow-fed *Abcb11.Tg* mice were 3-fold higher (P<0.05) than those in wild-type mice. In contrast, no significant differences in expression levels of *Abca1, Abcg5*, *Abcg8*, and *Srb1* in the small intestine were found between two groups of mice (data not shown).

Comparative sequencing of the mouse *Abcb11* in strains AKR and C57L and reporter gene assays

Sequencing of cDNAs, representing all 28 *Abcb11* exons, and the 5′-flanking regions (see *Methods*) revealed no differences between strains AKR and C57L, except for a C/T-transition at position −3,757 in the strain C57L. This transition is localized in an Lx8 repeat element, which represents a long interspersed repeat (LINE) 1 fragment that might have regulatory functions [41,42]. Albeit no potential transcription factor binding sites were identified in the Lx8 repeat element, we performed additional luciferase assays. We cloned the sequences of both mouse strains (−4,050/−3,561 bp) in front of the *Abcb11* minimal promoter (−376/+64 bp). This led to a significant (P<0.01) reduction of promoter activity

from 0.32±0.05 to 0.17±0.01 RLU, but no differences were observed between constructs harboring sequences from strains C57L and AKR.

DISCUSSION

Cholesterol cholelithiasis, similar to most common diseases such as atherosclerosis and obesity, is a complex trait and does not qualify readily to simple Mendelian analysis for single-gene traits [3,4]. A powerful genetic technique, QTL analysis provides a crucial method for identifying, locating and estimating the lithogenic effects of genes that underlie complex traits in inbred mice challenged to the lithogenic diet. The mouse model of cholesterol gallstones is an important experimental system for identifying pathophysiologically relevant *Lith* QTL as well as finding candidate *Lith* genes and the biochemical and physiological pathways for this complex disease. A genome-wide search was first applied to the AKR and C57L inbred strains for genetic analysis of cholesterol gallstones [21]. After AKR, C57L and (AKR×C57L)F_1 mice were fed with the lithogenic diet for 56 days, it was found that cholesterol gallstone formation is a dominant trait because the F_1 mice have gallstones of similar prevalence and weight to the C57L parental strain [27]. The first gallstone QTL locus was mapped to chromosome 2 and named *Lith1* [17,21,22]. Subsequently, the second locus *Lith2* was localized on chromosome 19 [17,18,21]. Each QTL has been confirmed to have a strong, independent impact on gallstone risk as shown by two congenic strains: one with the C57L allele at the *Lith1* locus, and the other with the C57L allele at the *Lith2* locus introgressed into the AKR background [17].

Since the *Lith1* locus was identified, the cholesterol gallstone phenotypes in C57L and AKR strains and their F_1 progeny were systematically characterized. Compared with AKR mice, C57L mice display higher hepatic secretion of biliary cholesterol and bile salts, bile salt-dependent bile flow, and CSIs, as well as larger gallbladders, greater accumulation of gallbladder mucin gel, and gallbladder hypomotility [27,28]. Also, C57L mice show significantly higher hepatic HMG-CoA reductase activities for cholesterol biosynthesis and relatively lower activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase for bile salt synthesis than AKR mice [33].

The *Lith1* QTL that was identified in previous studies [21] showed peak linkage to markers *D2Mit11* (71.7 Mb) and *D2Mit66* (84.7 Mb). Subsequently, *Lith1* was confirmed using additional backcrosses with congenic mice and refined to the interval between *D2Mit182* (68.8 Mb) and *D2Mit11* (71.6 Mb) [17,43]. However, this region still encompasses many genes, some of which are related to lipid metabolism and transport. For example, the *Lrp2* gene encoding the LDL-receptor-related protein 2, previously known as megalin (*Gp330*), a member of the LDL receptor gene family, maps to the *Lith1* region. LRP2 is expressed in gallbladder epithelium and regulated by bile acids [44]; however, it is not expressed in the hepatocyte and appears not to have major effects on biliary lipid secretion as well as hepatic lipid and lipoprotein metabolism. Another candidate for *Lith1* is *Abcb11*, a gene encoding an ABC transporter, previously known as bile salt export pump (*Bsep*) or sister to P-glycoprotein (*Spgp*) [19,20]. This protein is expressed on the canalicular membrane of hepatocytes and transports bile salts into the canalicular bile [19,20,23]. Indeed, compared with AKR mice, C57L mice display significantly higher expression levels of *Abcb11* in the hepatocyte as well as hypersecretion of biliary bile salts and cholesterol [22,23,28].

In the present study, we observed that overexpression of *Abcb11* promotes hepatic secretion of biliary bile salts and increases circulating bile salt pool sizes as well as bile salt-dependent bile flow rates. However, mice overexpressing the *Abcb11* gene show essentially similar secretion rates of biliary cholesterol compared with AKR mice, and the *Abcb11* overexpression does not influence cholesterol cholelithogenesis in mice challenged to the

lithogenic diet. Henkel et al. [45] developed TTR-*Abcb11* transgenic mice in the FVB/NJ genetic background and reported that cholesterol gallstone prevalence was increased in mice overexpressing *Abcb11* upon feeding the lithogenic diet. It has been found [24] that overexpression of the *Abcb11* gene in TTR-*Abcb11* transgenic mice increases biliary secretion of not only bile salts, but also cholesterol and phospholipids. Of note is that TTR-*Abcb11* transgenic mice were created in the FVB/NJ background. Since FVB/NJ mice are a gallstone-susceptible inbred strain, it could not be excluded that rapid gallstone formation is conferred by other *Lith* genes. In the present study, we developed a new *Abcb11* transgenic strain in the gallstone-resistant AKR genetic background, demonstrating that the murine strain background markedly influences the phenotype of transgenic mice overexpressing *Abcb11*. We observed that compared with wild-type mice, *Abcb11.Tg* mice secrete an essentially similar amount of cholesterol and a larger amount of bile salts in relation to phospholipid, even in the lithogenic state. One possible explanation is that the lack of lithogenic effects in the present study is due to the increased bile flow, which decreases the relative concentration of bile salts in the canalicular space. Apparently, the increased bile salt secretion could just compensate for this effect, resulting in an essentially identical canalicular bile salt concentration in *Abcb11.Tg* vs. wild-type mice. Therefore, our results indicate that the *Abcb11* overexpression does neither induce relative nor absolute biliary cholesterol hypersecretion for the formation of lithogenic hepatic bile. As a result, no differences in gallstone formation are found in these two groups of mice. Since the phenotypes of gallbladder and hepatic biles in *Abcb11.Tg* mice mimic the gallstone-resistant AKR strain, these observations do not support the notion that the *Abcb11* gene underlies *Lith1*. Furthermore, we found that neither polymorphisms in the *Abcb11* gene nor functionally relevant promoter variants exist between strains C57L and AKR. In addition, Schafmayer et al. did not find evidence of association of the *ABCB11* gene to gallstone susceptibility in a Caucasian population, suggesting that systematic fine mapping of the *Lith1* region is required to identify the causative genetic variants for gallstones [46,47].

Using biliary "washout" techniques, we measured the circulating bile salt pool sizes and observed that the pool sizes are significantly enlarged in mice overexpressing *Abcb11*. This is mainly contributed to increased hepatic secretion of biliary bile salts in the transgenic mice. Canalicular bile formation has been traditionally divided into two components: bile salt-dependent flow, defined as the slope of the line relating canalicular bile flow to bile salt output, and bile salt-independent flow, attributed to the active secretion of inorganic electrolytes and other solutes, and defined as the extrapolated *y*-intercept of this line. Our results reveal that bile salt-dependent flow is significantly greater in *Abcb11.Tg* mice than in wild-type mice, mainly due to biliary bile salt hypersecretion induced by overexpression of the hepatic *Abcb11* gene. In contrast, bile salt-independent flow is similar in these two groups of mice. Moreover, because augmented biliary bile salt outputs enhance micellar cholesterol solubilization, intestinal cholesterol absorption efficiency is significantly increased in mice overexpressing *Abcb11*. This result is consistent with the striking induction of *Npc1l1* expression in the intestine of *Abcb11.Tg* mice.

In our phenotypic study of the rate-limiting enzymes for bile salt syntheses, we observed that compared with wild-type mice, *Abcb11.Tg* mice display significantly lower mRNA levels and enzymatic activities of cholesterol 7α-hydroxylase. It probably relates to larger circulating bile salt pool size in the transgenic mice, which could result in increased enterohepatic return of bile salts to the liver. Also, intestinal FXR stimulated by enlarged circulating bile salt pool size could increase expression of fibroblast growth factor-19 (FGF-19), a secreted growth factor that signals through the FGFR4 cell-surface receptor tyrosine kinase. In turn, FGF-19 might reduce expression of cholesterol 7α-hydroxylase in the liver through c-Jun N-terminal kinase (JNK) or extracellular signal-regulated kinase 1/2 (Erk1/2)-dependent pathways in *Abcb11.Tg* mice [48,49]. Furthermore, mRNA levels and

enzymatic activities of cholesterol 7α-hydroxylase are reduced in all mice in response to the lithogenic diet, mostly due to the presence of 0.5% cholic acid in the diet. In contrast, no differences in mRNA levels and enzymatic activities of HMG-CoA reductase are found between *Abcb11.Tg* and wild-type mice, consistent with identical hepatic secretion of biliary cholesterol in these mice.

In summary, the pathophysiological phenotypes of the *Abcb11* gene as inferred from comprehensive biliary lipid secretion and gallstone studies in *Abcb11.Tg* mice suggest that an increased hepatic secretion of biliary bile salts is not a major factor for the development of supersaturated bile and the formation of cholesterol gallstones in mice. The present results support the notion that the *Abcb11* overexpression enhances hepatic secretion of biliary bile salts, enlarges the circulating bile salt pool size, and induces higher bile salt-dependent bile flow. Our work offers a critical clue for positional cloning of *Lith1*, as well as a basic framework for further investigating how individual *Lith* genes promote biliary cholesterol secretion and enhance cholesterol cholelithogenesis in mice. Furthermore, because of the homology of human and mouse chromosomes, it is possible to identify human *Lith* genes from mouse studies.

Acknowledgments

Abbreviations used in this paper

ABC	ATP-binding cassette (transporter)
BAC	bacterial artificial chromosome
CCK-8	sulfated cholecystokinin octapeptide
CSI	cholesterol saturation index
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
QTL	quantitativetrait locus

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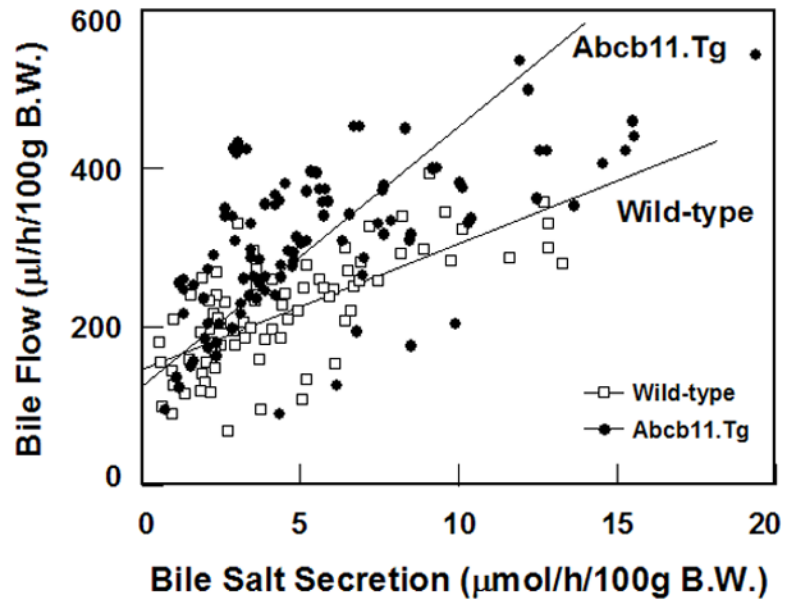
Figure 1.
Bile flow as a function of bile salt output in mice before (on chow, day 0) and at 56 days on the lithogenic diet. Each point represents bile flow and bile salt output measurements in the same sample during 8-hour periods of biliary "washout". Bile salt-dependent bile flow in *Abcb11.Tg* mice is significantly (P<0.001) greater than that in wild-type mice. Equations for canalicular bile salt-dependent flow are $y=120(\pm15)+33(\pm3)x$ (r=0.66; P<0.001) for *Abcb11.Tg* mice and $y=146(\pm10)+16(\pm2)x$ (r=0.70; P<0.001) for wild-type mice. Upon extrapolation of the regression lines to the ordinate, the *y*-intercepts are similar in these two groups of mice, suggesting that bile salt-independent flow is similar between *Abcb11.Tg* and wild-type mice.
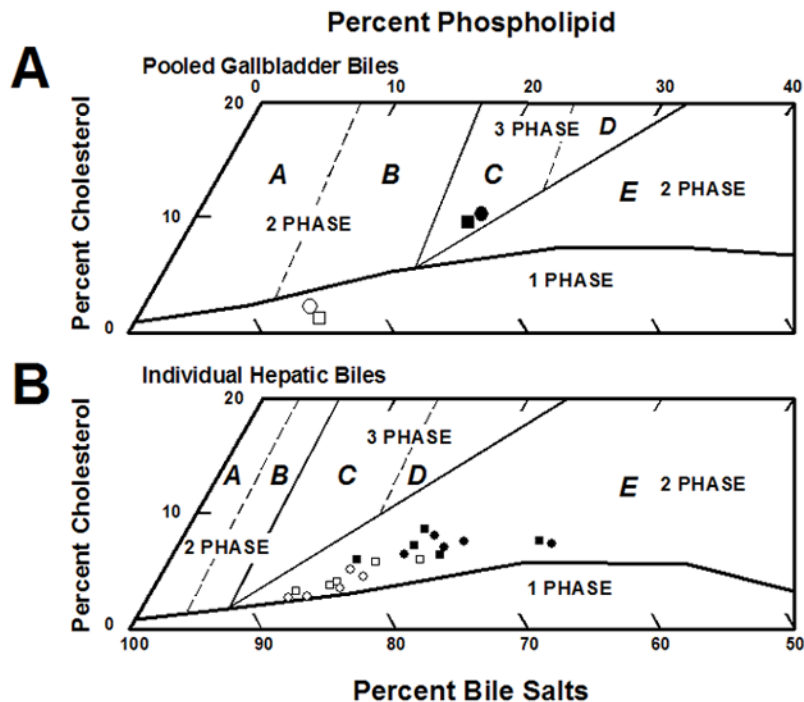
Figure 2.
(**A**) Relative lipid compositions of pooled gallbladder biles (n=20 per group) plotted on a condensed phase diagram according to average total lipid concentration (10.0 g/dl) of biles (see Table 2). One-phase micellar zone is enclosed by solid curved line. Above the micellar zone, two solid and two dashed lines divide the phase diagram into regions A to E with different crystallization sequences [40]. Relative lipid compositions of pooled biles from chow-fed *Abcb11.Tg* and wild-type mice are located in one-phase micellar zone. Upon challenge to the lithogenic diet, lipid compositions of pooled gallbladder biles from these mice plot in central three-phase zone, where at equilibrium biles would be composed of cholesterol-saturated micelles, solid cholesterol crystals, and liquid crystals. (**B**) Lipid compositions of individual hepatic biles (n=5 per group) are plotted on a condensed phase diagram for average total lipid compositions (2.5 g/dl) of biles (see Table 2). This system exhibits the same physical states at equilibrium as for gallbladder biles, but all crystallization pathways are shifted to the left with decreases in total lipid concentration [40]. These changes generate a new condensed phase diagram with smaller one-phase micellar zone and enlarged region E. Relative lipid compositions of all hepatic biles locate in region E, where at theoretical equilibrium biles would be composed of liquid crystals and saturated micelles but not solid cholesterol crystals. In this work, fresh hepatic biles of all mice are generally clear microscopically, suggesting their nonequilibrium nature. Symbols (white for the chow diet; black for the lithogenic diet): ○/● *Abcb11.Tg* mice; □/▪ wild-type mice.
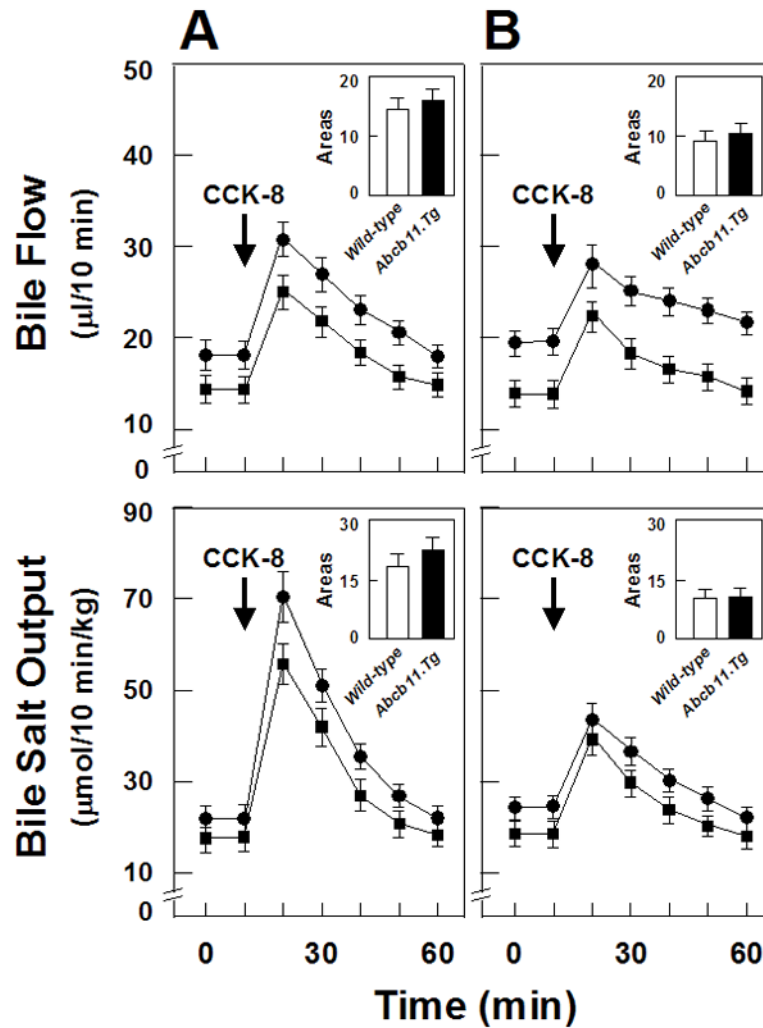
Figure 3.
Gallbladder emptying function as shown by changes in bile flow rates and biliary bile salt outputs stimulated by IV injection of CCK-8 in *Abcb11.Tg* and wild-type mice (**A**) on chow or (**B**) fed the lithogenic diet. Because of gallbladder emptying, bile flow rates and biliary bile salt outputs are increased sharply and significantly by exogenously administered CCK-8 (as indicated by the arrows). Although gallbladder contractility is impaired partially in the lithogenic state compared with the chow diet, no differences in these parameters are found in both groups of mice. Symbol ● represents *Abcb11.Tg* mice and ▪ wild-type mice.
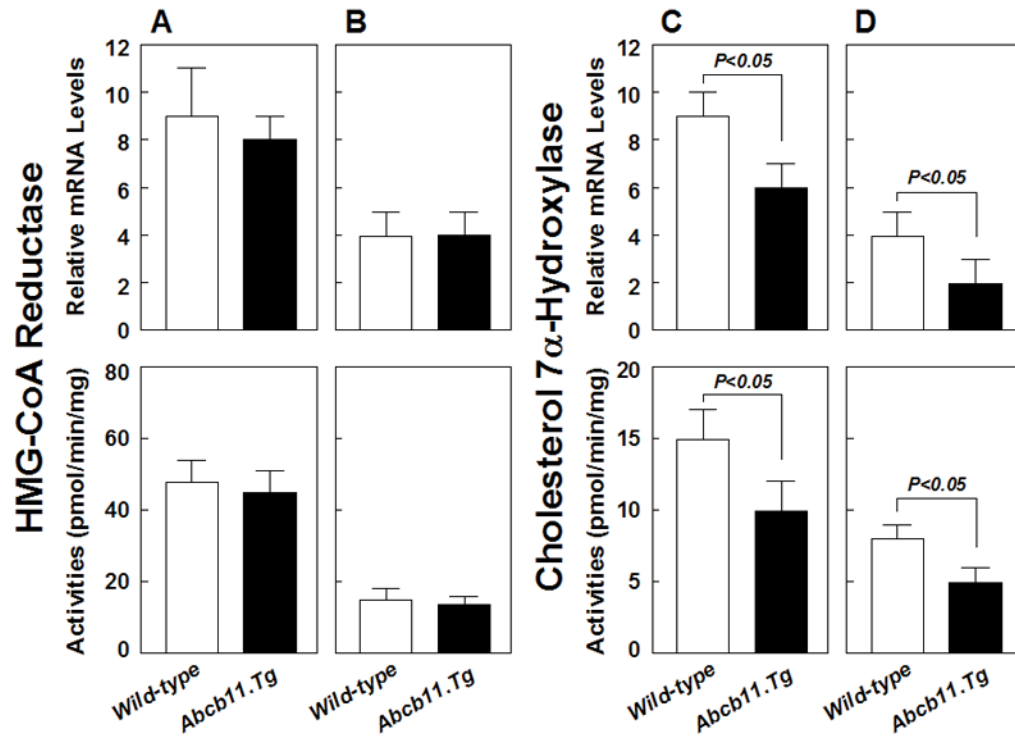
Figure 4.
Gene expression levels and enzymatic activities of HMG-CoA reductase and cholesterol 7α-hydroxylase in livers of *Abcb11.Tg* and wild-type mice (n=5 per group). mRNA levels and activities of HMG-CoA reductase are essentially similar in these mice, regardless of whether (**A**) the chow or (**B**) the lithogenic diet is fed. Obviously, mRNA levels and activities of HMG-CoA reductase are significantly (P<0.01) reduced under high dietary cholesterol loads. Compared with (**C**) the chow diet, (**D**) feeding the lithogenic diet significantly reduces mRNA levels and enzymatic activities of cholesterol 7α-hydroxylase in all mice. Of note is that mRNA levels and enzymatic activities are significantly lower in *Abcb11.Tg* mice than in wild-type mice.

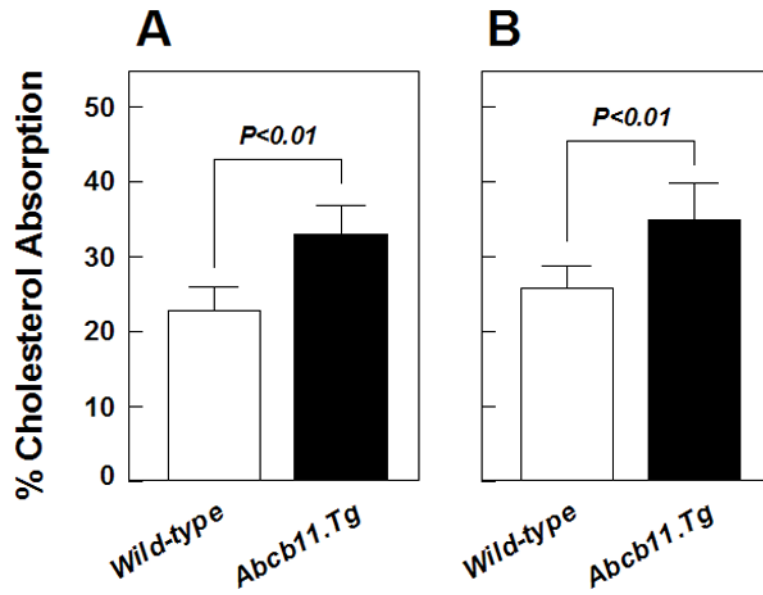**Figure 5.**
Effects of *Abcb11* overexpression on intestinal cholesterol absorption. Cholesterol absorption efficiency was determined by (**A**) the plasma and (**B**) the fecal dual-isotope ratio methods in chow-fed mice (n=5 per group). As measured by the plasma and the fecal dual-isotope ratio methods, intestinal cholesterol absorption is significantly increased in *Abcb11.Tg* mice than in wild-type mice. No differences in percent cholesterol absorption are found by two independent methods.

Table 1

Bile flow rates, biliary lipid outputs, as well as bile salt pool sizes and species

Parameter	Wild-type	*Abcb11.Tg*
Chow diet		
Bile flow (μl/min/kg)	49.7±2.7	72.2±10.8[A]
Biliary output		
Cholesterol (μmol/h/kg)	6.0±1.3	6.1±1.1
Phospholipid (μmol/h/kg)	17.0±2.4	20.5±1.9
Bile salt (μmol/h/kg)	105.3±13.4	134.5±15.1[A]
Circulating bile salt pool size (μmol)	5.6±0.5	6.1±0.7
Total bile salt pool size (μmol)	9.9	10.8
Bile salt species		
Taurocholate	49.7±6.7	51.4±8.0
Tauro-β-muricholate	42.2±5.6	43.6±7.2
Taurochenodeoxycholate	0.6±0.3	0.5±0.2
Tauro-ω-muricholate	1.4±0.6	1.3±0.7
Tauroursodeoxycholate	2.6±1.6	2.1±0.6
Taurodeoxycholate	3.4±1.3	1.1±0.5
Hydrophobicity index	−0.33±0.05	−0.35±0.06
Lithogenic diet		
Bile flow (μl/min/kg)	56.9±7.0	75.7±12.4[B]
Biliary output		
Cholesterol (μmol/h/kg)	9.5±1.1[A]	10.2±3.3[C]
Phospholipid (μmol/h/kg)	35.7±11.6[A]	45.0±15.6[C]
Bile salt (μmol/h/kg)	115.0±20.1	152.9±28.8[B]
Circulating bile salt pool size (μmol)	5.3±0.8	7.0±1.2[B]
Total bile salt pool size (μmol)	11.4	14.1
Bile salt species		
Taurocholate	74.3±7.3[A]	69.6±4.0[C]
Tauro-β-muricholate	2.9±0.2[A]	3.1±1.5[C]
Taurochenodeoxycholate	11.1±3.4[A]	13.4±0.9[C]
Tauro-ω-muricholate	1.7±0.4	2.6±1.9
Tauroursodeoxycholate	3.2±1.0	2.4±0.7
Taurodeoxycholate	6.9±3.3[A]	9.0±1.3[C]
Hydrophobicity index	0.04±0.03[A]	0.06±0.02[C]

Values represent mean±SD of five mice per group. All mice were fed normal rodent chow containing trace (<0.02%) amounts of cholesterol at day 0, or the lithogenic diet containing 1% cholesterol, 0.5% cholic acid and 15% butter fat for 56 days.

[A]P<0.05, compared with wild-type mice on the chow diet;

[B]P<0.05, compared with wild-type mice on the lithogenic diet;

^{C}P<0.05, compared with *Abcb11.Tg* mice on the chow diet.

Table 2

Biliary lipid compositions of pooled gallbladder and individual hepatic biles[A]

Mouse	Mole%Ch[B]	Mole%PL	Mole%BS	PL/(PL+BS)	[TL] (g/dl)	CSI[C]
Chow diet						
Pooled gallbladder biles						
Wild-type	1.73	13.28	85.00	0.14	10.14	0.35
Abcb11.Tg	2.18	12.77	85.04	0.13	10.76	0.45
Individual hepatic biles						
Wild-type	4.75±0.96	14.19±3.45	81.06±4.38	0.15±0.04	2.33±0.28	1.23±0.10
Abcb11.Tg	4.05±0.72	12.80±2.02	83.15±2.43	0.13±0.02	1.98±0.30	1.19±0.16
Lithogenic diet						
Pooled gallbladder biles						
Wild-type	9.23	22.08	68.69	0.24	13.83	1.20
Abcb11.Tg	9.71	21.75	68.54	0.24	14.80	1.26
Individual hepatic biles						
Wild-type	6.76±1.05	20.94±5.09	72.31±5.77	0.22±0.06	2.55±0.47	1.29±0.16
Abcb11.Tg	7.10±0.68	21.35±4.22	71.55±4.53	0.23±0.05	2.57±0.29	1.33±0.15

[A] Values were measured from pooled gallbladder biles (n=20 per group) and individual hepatic biles (n=5 per group).

[B] Abbreviations: Ch, cholesterol; PL, phospholipid; BS, bile salt; [TL], total lipid concentration; CSI, cholesterol saturation index.

[C] The CSI values of pooled gallbladder and six hepatic biles were calculated from the critical tables [31].