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Increased expression of *LXRα*, *ABCG5*, *ABCG8*, and *SR-BI* in the liver from normolipidemic, nonobese Chinese gallstone patients

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Abstract **Cholesterol supersaturation of bile is one prerequisite for gallstone formation. In the present study of Chinese patients with gallstones, we investigated whether this phenomenon was correlated with the hepatic expression of genes participating in the metabolism of cholesterol and bile acids. Twenty-two nonobese, normolipidemic patients (female-male, 11:11) with gallstones were investigated with 13 age- and body mass index-matched gallstone-free controls (female-male, 10:3). The bile from the gallstone patients had higher cholesterol saturation than that from the controls. The mRNA levels of *ABCG5*, *ABCG8*, and liver X receptor α (*LXRα*) in the gallstone patients were increased by 51, 59, and 102%, respectively, and significantly correlated with the molar percentage of biliary cholesterol and cholesterol saturation index (CSI). The mRNA and protein levels of the hepatic scavenger receptor class B type I (SR-BI) were increased, and a significant correlation was found between the protein levels and the CSI. No differences were recorded between the two groups concerning the hepatic synthesis of cholesterol, bile acids, and esterification of cholesterol. Our results suggest that the upregulation of *ABCG5/ABCG8* in gallstone patients, possibly mediated by increased *LXRα*, may contribute to the cholesterol supersaturation of bile. Our data are consistent with the possibility that increased amounts of biliary cholesterol may originate from plasma HDL cholesterol by enhanced transfer via SR-BI.**—Jiang, Z-Y., P. Parini, G. Eggertsen, M. A. Davis. H. Hu, G-J. Suo, S-D. Zhang, L. L. Rudel, T-Q. Han, and C. Einarsson. **Increased expression of *LXRα*, *ABCG5*, *ABCG8*, and *SR-BI* in the liver from normolipidemic, nonobese Chinese gallstone patients.** ***J. Lipid Res.*** **2008.** 49: **464–472.**

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Cholesterol gallstone disease is common in both industrialized and developing countries (1, 2). In 1995, the Chinese National Survey reported that gallstone disease accounted for nearly 10% of all diagnoses of patients hospitalized in surgical clinics, and the majority of their gallstones were composed of cholesterol (2).

Gallstone disease can be viewed as the terminal outcome of different metabolic disorders caused by diverse genetic and environmental factors. It is a multifactorial disease, and the causes of gallstones are heterogeneous and mostly intrahepatic. The critical element for gallstone formation is supersaturation of bile with cholesterol (3).

Early studies attempted to define enzymatic defects in the liver contributing to the cholesterol supersaturation of bile. The activity of 3-hydroxy-3-methylglutaryl coenzyme A

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Abbreviations: apoA-I, apolipoprotein A-I; CSI, cholesterol saturation index; CYP7A1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; GS, gallstone patients; GSF, gallstone-free controls; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HNF4A, hepatocyte nuclear factor 4α; LXRα, liver X receptor α; MTTP, microsomal triglyceride transfer protein; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; SR-BI, scavenger receptor class B type I; SREBP, sterol-regulatory element binding protein.

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reductase (HMGCR; the rate-limiting enzyme for de novo synthesis of cholesterol) was shown to be increased in gallstone patients (4). Conversely, the activities of cholesterol 7α-hydroxylase (CYP7A1; the rate-limiting enzymes for bile acid synthesis) and ACAT (the enzyme catalyzing cholesterol esterification) were both decreased in gallstone patients (4, 5). However, larger studies of different populations could not confirm these results (6, 7). Recently, in a study in Chilean Hispanics and Mapuche Indians, a surrogate marker of bile acid synthesis was measured in the plasma of gallstone patients that suggested increased CYP7A1 activity (8).

The identification and characterization of the ATP binding cassette (ABC) transporters for cholesterol, bile acids, and phospholipids has brought new insights to our understanding of gallstone disease. ABCG5 and ABCG8 appear to function as a heterodimer for the secretion of cholesterol into the bile canaliculus (9). As shown previously in mice (10, 11), both the Abcg5 and Abcg8 genes are targets for the liver X receptor α (LXRα) and could be induced by LXRα agonists. ABCB11 (12) (also known as BSEP, for bile salt export pump) is a major bile acid transporter, and ABCB4 (also known as MDR3, for multiple drug-resistant transporter 3) is a phospholipid transporter. In animal models, either overexpression of Abcg5/Abcg8 (9) or depletion of Abcb11 (13) or Abcb4 (14) modifies biliary lipid secretion and, in some cases, leads to the supersaturation of bile with cholesterol. As with most of the studies performed in mice, the role of ABC transporters in the pathogenesis of cholesterol gallstones is not well understood in humans.

In this study, we attempted to identify some of the molecular defects in hepatic cholesterol and bile acid metabolism involved in the pathogenesis of cholesterol gallstone disease. We studied a group of normolipidemic, nonobese Chinese patients who had neither diabetes mellitus nor signs of insulin resistance. Our present results suggest that in these humans, supersaturation of bile is associated with an increased expression of *ABCG5/ABCG8* and *LXRα*. We also observed an increased expression of scavenger receptor class B type I (SR-BI), which mediates the hepatic uptake of cholesterol from HDL (15) that is directed to biliary cholesterol secretion.

MATERIALS AND METHODS

Subjects

Twenty-two Chinese patients (11 females and 11 males) with cholesterol gallstone disease (GS) and 13 Chinese gallstone-free patients (GSF; 10 females and 3 males) were included in this study. The GS underwent open or laparoscopic cholecystectomy. Gallbladder size was evaluated by ultrasonography, and gallbladder function was assessed by analysis of total biliary lipids. Cholesterol gallstones were confirmed by visual inspection of the typical cut-surface of gallstones or, when necessary, by enzymatic cholesterol analysis. The GSF included nine patients with gallbladder polyps (seven females and two males) undergoing cholecystectomy and four patients treated with liver resection because of right hepatic hemangioma (three females and one male). No gallstones were found in any of these controls after resection of the gallbladder, nor were cholesterol crystals found in bile by polarized light microscopy. The pathological examination of the polyps showed an inflammatory nature. None of the patients had any other disorders affecting hepatic, gastrointestinal, renal, and endocrine functions (i.e., either diabetes mellitus or signs of insulin resistance). The patients were not subjected to lipid-lowering treatment. Before enrollment in the study, informed consent to participate in the study and to the collection of a liver biopsy was obtained. The study protocol was approved by the ethics committees at both the Ruijin Hospital, Medical School of Shanghai Jiaotong University, and the Karolinska University Hospital at Huddinge.

Procedure for sample collection

Patients were fasted overnight before surgery, which was performed between 9 and 10 AM. After opening the abdomen, or after the application of pneumoperitoneum, a wedge biopsy of ~0.5–1.0 g was taken from the right lobe of the liver, snap-frozen in liquid nitrogen, and stored at −70°C. Criteria for a functioning gallbladder consisted of *i*) the presence of dark concentrated bile in the gallbladder and *ii*) no evidence of impacted stones in the neck of the cystic duct at operation. After clamping the cystic duct, bile from the gallbladder was obtained by aspiration. All of the cholecystectomies were performed without any complications. Participation in the study did not result in prolonged hospitalization, and no serious adverse events were reported.

Analysis of plasma lipids

Plasma total cholesterol, triglycerides, HDL cholesterol, apolipoprotein A-I (apoA-I), and apoB were analyzed with an automated bioanalyzer (Roche Hitachi Modular P800) and corrected by dilution with the addition of EDTA (2%). LDL cholesterol in plasma was calculated according to Friedewald's equation. Lipoproteins were separated by size-exclusion chromatography as described previously (16).

Analysis of biliary lipids and bile acid composition

Biliary cholesterol, total bile acids, and phospholipids in gallbladder bile were measured as described previously (17). The cholesterol saturation index (CSI) was calculated using Carey's critical table (18).

Analysis of liver lipids

Crude liver homogenates were prepared as described previously (7) and extracted in chloroform-methanol (2:1, v/v). Hepatic cholesterol concentrations were then assayed by gas chromatography-mass spectrometry (7). Unesterified lathosterol was determined by isotope dilution-mass spectrometry using a deuterium-labeled internal standard (19). Hepatic triglycerides were determined in liver lipid extracts by colorimetric enzymatic methods (TG Roche/Hitachi, Roche Diagnostic GmbH, Mannheim, Germany). Protein content was determined according to Lowry's method.

Assay of microsomal ACAT1 and ACAT2 activity

Total ACAT enzymatic activity was determined in hepatic microsomes, including a 30 min preincubation with a cholesterol-saturated solution of β-hydroxypropyl cyclodextrin before addition of the [^{14}C]oleoyl-CoA, as described (20). In a parallel incubation, pyripyropene A, a specific ACAT2 inhibitor, was included in the preincubation and reaction mixtures at a con-

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centration of 5 μmol/l to separately identify ACAT1 and ACAT2 activities (20).

Relative RNA expression level measurements

Hepatic total RNA was extracted with Trizol® (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA (Omniscript; Qiagen, Inc., Valencia, CA). Real-time quantitative PCR assays were performed in triplicate using SYBR-Green (MedProbe, Oslo, Norway). Primers (primer sequences are available on request) were designed using Primer Express 2.0, all with sequences crossing exon-exon boundaries. Data were calculated by the delta-C_t method, expressed in arbitrary units, and were normalized by the signals obtained from the same cDNA for cyclophilin A. The fold change for each mRNA expression level in the GS was expressed relative to the obtained value in the GSF, the mean value of which was arbitrarily set at 1.

Western blot analysis

Twenty micrograms of liver membranes from each patient sample was separated on a 10% SDS-PAGE gel and then transferred onto nitrocellulose membranes (Invitrogen). After blocking in 5% nonfat dry milk in PBST (PBS with 0.05% Tween-20), the nitrocellulose membranes were incubated overnight at 4°C with rabbit anti-SR-BI (1:3,000; Abcam, Ltd., Cambridge, UK) in 5% nonfat milk powder in PBST. After washing with PBST, donkey anti-rabbit IgG $F(ab')_2$ antibodies were added (1:50,000; Amersham Bioscience AB, Uppsala, Sweden). The signals were detected using the SuperSignal chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL) and a Fuji BAS 1800 analyzer (Fuji Photo Film Co.) and quantified by Image Gauge software (Science Lab 98, version 3.12; Fuji Photo Film Co.). After cleaning the membranes by stripping, they were further blotted with rabbit anti-β-actin (1:3,000, Abcam) as a loading control. Data are expressed as arbitrary units and normalized to β-actin expression.

Statistics

Data are reported as means ± SEM. Student's *t*-test was used to compare the differences of variables between GS and GSF (Statistica 7 .0 software; StatSoft, Inc., Tulsa, OK). Variables were correlated with Spearman's rank test. Statistical significance was set at $P < 0.05$.

RESULTS

Clinic characteristics and plasma lipids

Demographic data for GS and GSF are shown in **Table 1**. No significant differences in age and body mass index were observed. No differences in plasma lipids between GS and GSF were present (Table 1). Neither were there any significant differences in the distribution of cholesterol or triglycerides in the lipoprotein classes.

TABLE 1. Age, body mass index, and plasma lipids of GS and GSF

Variable	GS (n = 22)	GSF (n = 13)
Age (years)	45.0 ± 2.6	44.1 ± 2.9
Body mass index (kg/m^2)	23.3 ± 0.6	21.9 ± 0.9
Cholesterol (mmol/l)	4.21 ± 0.28	4.16 ± 0.38
Triglyceride (mmol/l)	1.66 ± 0.16	1.78 ± 0.26
HDL (mmol/l)	1.09 ± 0.08	1.02 ± 0.09
LDL (mmol/l)	2.41 ± 0.20	2.33 ± 0.26
Apolipoprotein A-I (g/l)	1.18 ± 0.06	1.10 ± 0.09
Apolipoprotein B (g/l)	0.75 ± 0.04	0.74 ± 0.07

GS, gallstone patients; GSF, gallstone-free controls.

Biliary lipid composition and hepatic lipid content

Analysis of gallbladder bile was not possible for eight patients because of technical problems during the surgical procedure. Furthermore, for ethical reasons, bile was not collected from four GSF who underwent surgery for the removal of hepatic hemangioma. In the samples analyzed, a significantly greater molar percentage of cholesterol was present in the bile of the GS compared with the GSF (**Fig. 1A**), as well as a significantly greater cholesterol saturation index (GS vs. GSF, 1.04 ± 0.08 vs. 0.71 ± 0.05; $P < 0.01$). Neither the total bile acids nor the phospholipids in bile differed between the groups (Fig. 1A). No differences were found in total biliary lipids (GS vs. GSF, 13.4 ± 1.26 vs. 13.5 ± 1.60 g/dl; P = NS), possibly because we ensured that all of the GS had normal gallbladder function. The bile acid composition of the available gallbladder bile samples is shown in Fig. 1B. In both groups, cholic acid and chenodeoxycholic acid constituted 70–80% of the total bile acids, without any differences between groups. Unexpectedly, in GSF, a significantly greater percentage of deoxycholic acid was found.

In the liver tissue, GS tended to have higher free cholesterol and lower cholesteryl ester concentrations than GSF, but the differences were not statistically significant (Fig. 1C). Similarly, no significant differences were observed for hepatic triglyceride (Fig. 1D) and lathosterol (Fig. 1E) concentrations between GS and GSF.

ABCG5 and ABCG8 correlate with biliary cholesterol

ABCG5 and *ABCG8* mRNAs were significantly increased in GS compared with GSF [+51% for *ABCG5* ($P < 0.01$) and +59% for *ABCG8* ($P < 0.01$)] (**Fig. 2A**), and their mRNA levels correlated very well ($r = 0.89$, $P < 0.05$) (Fig. 2B), confirming their likely coexpression in human liver in vivo (21). *ABCG5* and *ABCG8* expression also correlated positively with the biliary cholesterol molar percentage ($r = 0.57$ and $r = 0.54$, $P < 0.05$) and the CSI ($r = 0.54$ and $r = 0.55$, $P < 0.05$).

Interestingly, the expression of *LXRα* was 102% higher in GS compared with GSF ($P < 0.01$) (Fig. 2A). It correlated with both *ABCG5* ($r = 0.58$, $P < 0.05$) (Fig. 2C) and *ABCG8* ($r = 0.59$, $P < 0.05$) mRNA levels and also with the biliary cholesterol molar percentage ($r = 0.45$, $P < 0.05$) and the CSI ($r = 0.43$, $P < 0.05$) (Fig. 2D). In contrast, the expression of *ABCB11* and *ABCB4*, measured as mRNA abundance, did not differ significantly between GS and GSF, as was also the case for 17 other genes involved in various aspects of the regulation of hepatic lipid metabolism (Fig. 2E).

Bile supersaturation in cholesterol may originate from a SR-BI-mediated pathway

To further elucidate the possible mechanisms behind the bile cholesterol supersaturation and the increased ex-

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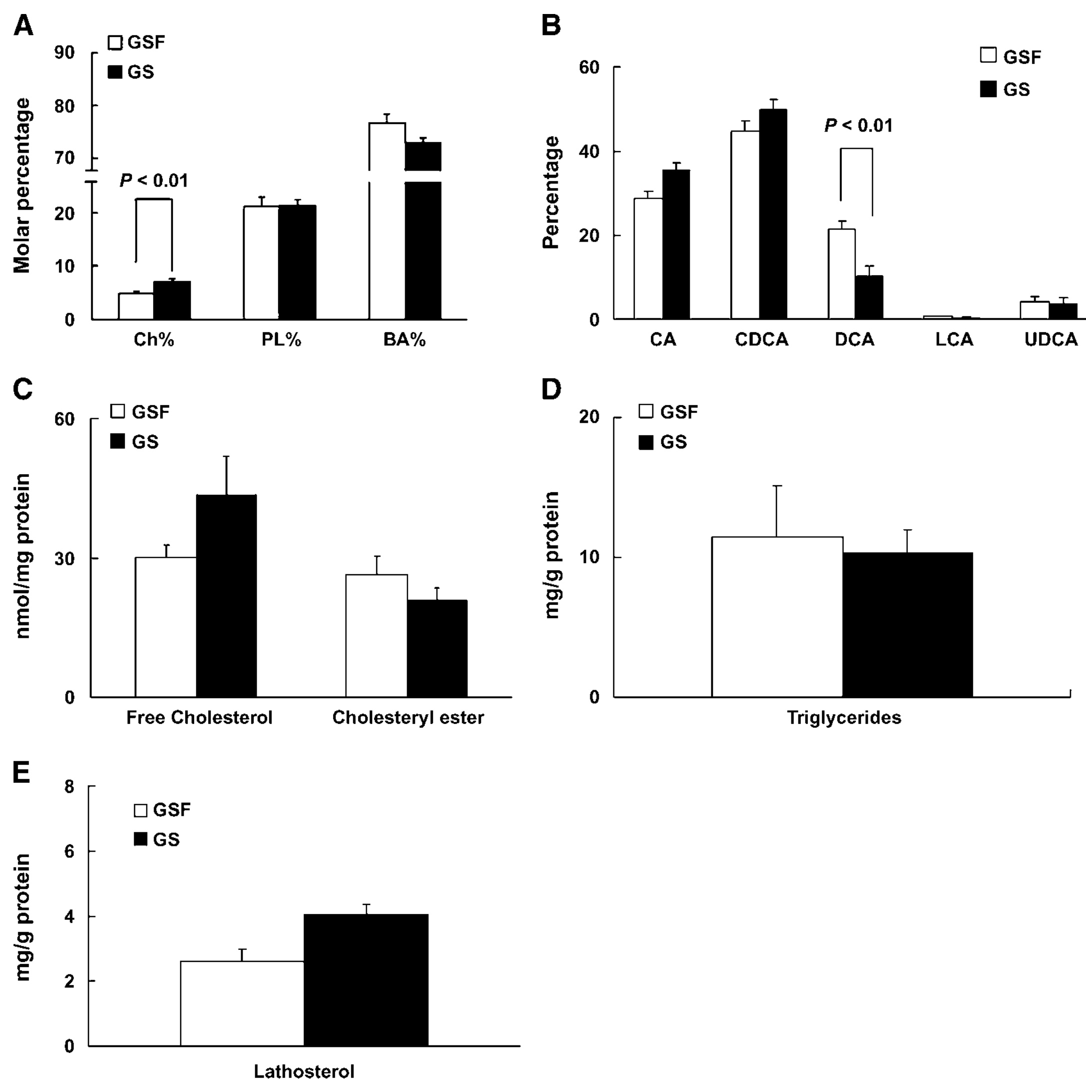

Fig. 1. Biliary and hepatic lipid composition in gallstone patients (GS) and gallstone-free patients (GSF). A: Biliary lipid composition of gallbladder bile (GS, n = 14; GSF, n = 9). Ch, cholesterol; PL, phospholipids; BA, bile acids. B: Bile acid composition of gallbladder bile (GS, n = 14; GSF, n = 9). CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid. C: Cholesterol content in liver lipid extract (GS, n = 20; GSF, n = 9). D: Triglyceride content in liver lipid extract (GS, n = 22; GSF, n = 13). E: Lathosterol content in liver lipid extract (GS, n = 22; GSF, n = 13). Data show means ± SEM.

pression of *ABCG5* and *ABCG8*, we examined genes considered to be key players in the regulation of the hepatic free cholesterol levels. The liver HDL receptor *SR-BI* displayed 38% higher levels of mRNA in GS compared with GSF ($P < 0.05$) (Fig. 2A). This was paralleled by a 74% increase of the SR-BI protein ($P < 0.05$) (**Fig. 3A, B**). The protein expression of SR-BI correlated significantly with the mRNA levels ($r = 0.46$, $P < 0.05$). In addition, the protein expression of SR-BI correlated with both the molar percentage of biliary cholesterol ($r = 0.56$, $P < 0.05$) (Fig. 3C) and the CSI ($r = 0.52$, $P < 0.05$) (Fig. 3D). No differences were seen in the mRNA of the genes for LDL receptor, LDL receptor-related protein, apoA-I, ABCA1, Niemann-Pick C1-like 1 protein, and HMGCR (Fig. 2E). Within the hepatocytes, the rate of cholesterol esterification has been proposed to determine the levels of free cholesterol available for biliary secretion and could, if so, be coupled to the pathogenesis of gallstone disease (5, 7). However, neither the activity nor the mRNA level of *ACAT2* differed between GS and GSF (Fig. 3E).

Expression of nuclear receptors and transcription factors regulating hepatic lipid metabolism

The hepatic mRNA levels of the nuclear receptor hepatocyte nuclear factor 4α (*HNF4A*) was 43% greater in the GS ($P < 0.05$) (Fig. 2A), and a positive correlation of *HNF4A* mRNA was found with *CYP7A1* mRNA ($r = 0.40$, $P < 0.05$) and cholesterol 27α-hydroxylase mRNA ($r = 0.34$, $P < 0.05$), but no correlation was seen with cholesterol 12α-hydroxylase mRNA. A strong positive correlation was also observed between *HNF4A* and *ABCG5* ($r = 0.73$, $P < 0.05$), between *HNF4A* and *ABCG8* ($r = 0.74$, $P < 0.05$), and between *HNF4A* and *SR-BI* ($r = 0.64$, $P < 0.05$). No differences in farnesoid X receptor

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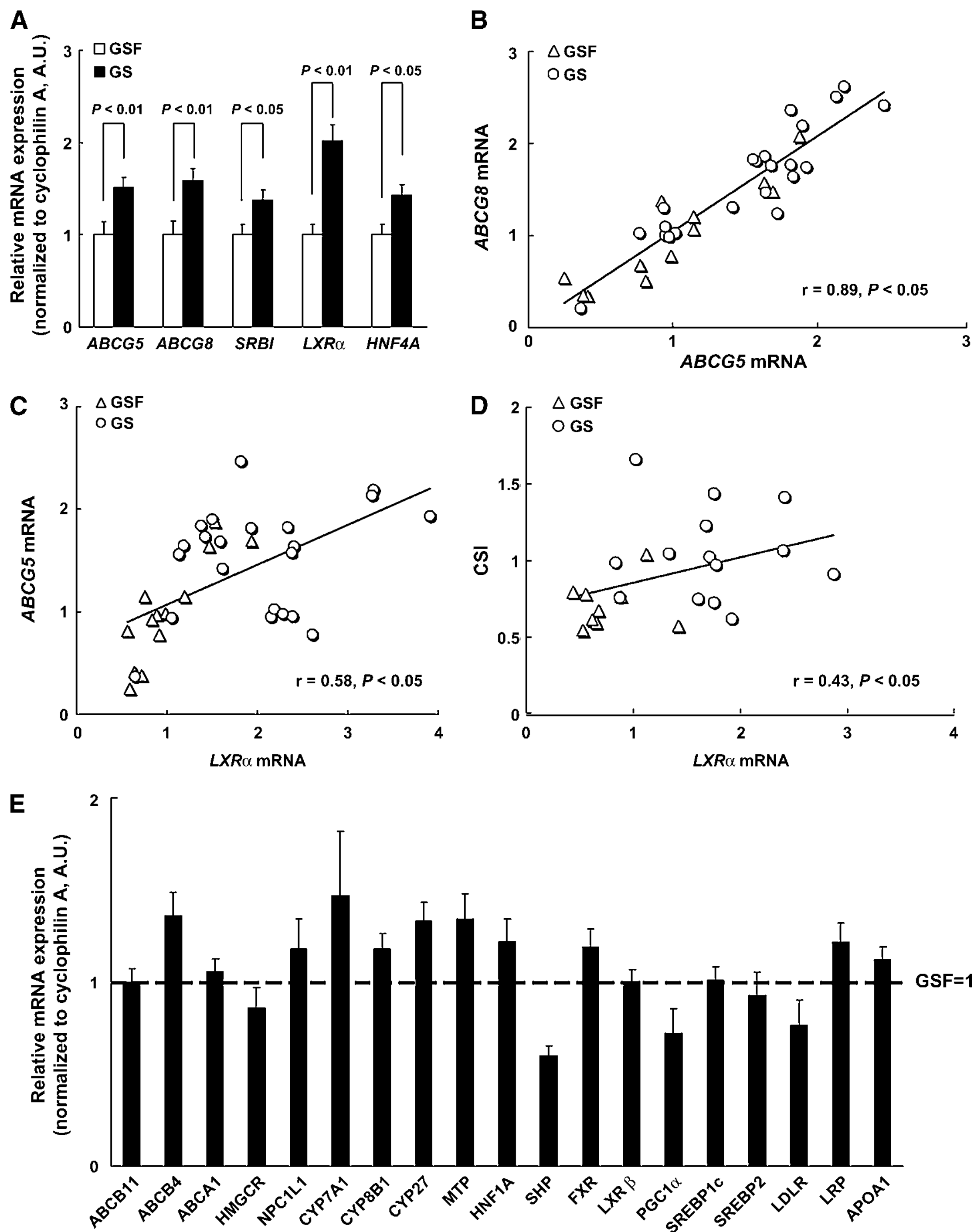

Fig. 2. Hepatic expression of genes involved in lipid metabolism in GS and GSF. A: Gene expression of *ABCG5*, *ABCG8*, scavenger receptor class B type I (*SR-BI*), liver X receptor α (*LXRα*), and hepatocyte nuclear factor 4α (*HNF4A*) (GS, n = 22; GSF, n = 13). Data show means ± SEM. B: Correlation between hepatic *ABCG5* and *ABCG8* mRNA levels (n = 35). C: Correlation between hepatic *LXRα* and *ABCG5* mRNA levels (n = 35). D: Correlation between hepatic *LXRα* mRNA levels and cholesterol saturation index (CSI) in gallbladder bile (n = 23). E: Relative gene expression between GS (n = 22) and GSF (n = 13). The dotted line at value 1 represents the mean gene expression level in GSF, which was arbitrarily set to 1 (A.U.); the black bars represent the gene expression levels in GS (means + SEM). For all genes, no difference was found between GS and GSF.

(*FXR*), small heterodimer partner, peroxisome proliferator-activated receptor γ coactivator 1α (*PGC1α*), *HNF1A*, *LXRβ*, sterol-regulatory element binding protein 1c (*SREBP1c*), and *SREBP2* mRNA levels were found between the two groups (Fig. 2E).

DISCUSSION

Most of the previous studies intended to better understand the molecular defects leading to gallstone disease have been carried out in animal models, and their

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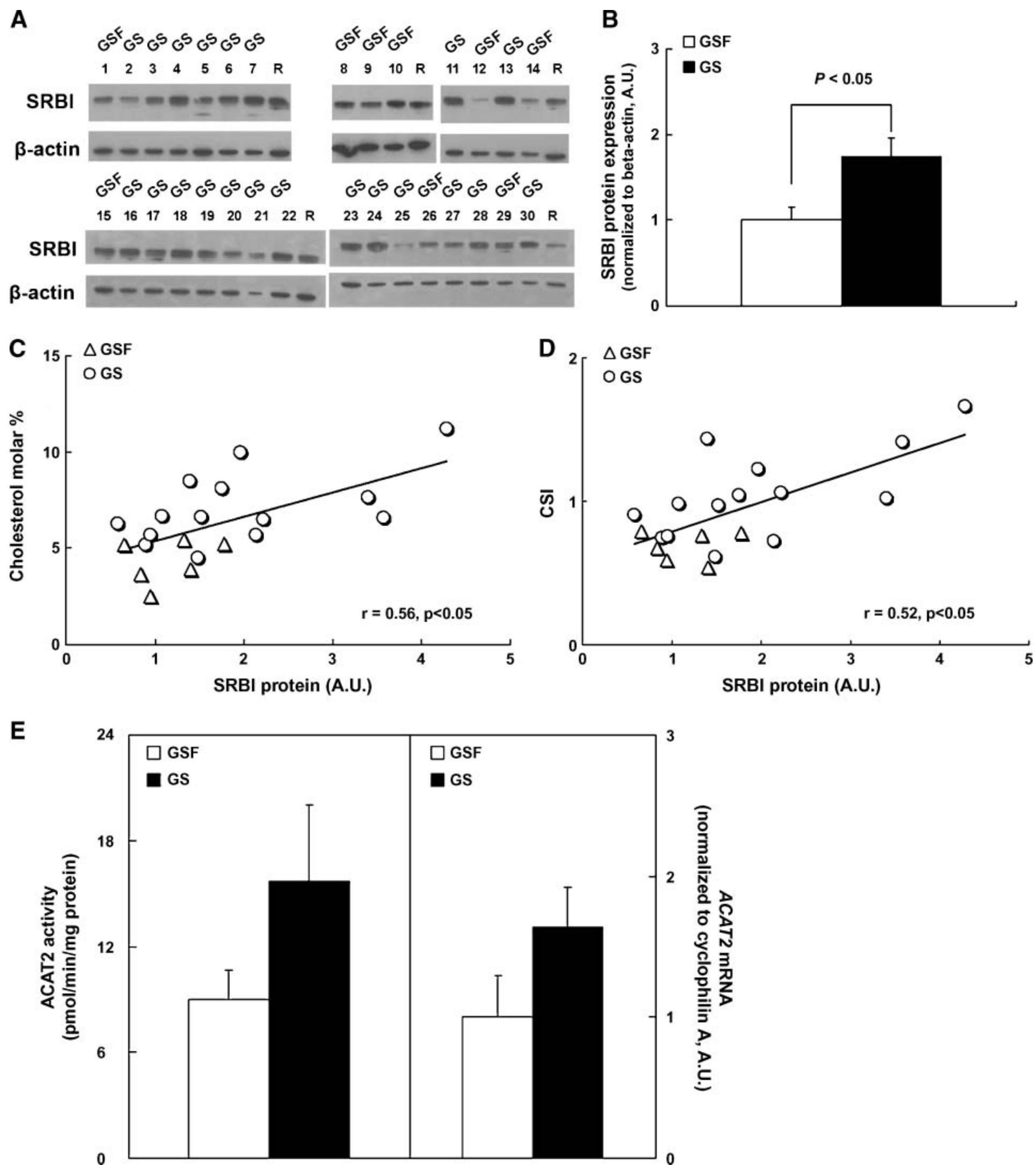

Fig. 3. Hepatic expression of SR-BI and hepatic expression and activity of ACAT2 in GS and GSF. A: Western blot analysis of SR-BI protein (molecular mass, 82 kDa) level normalized to β-actin (molecular mass, 42 kDa) as a loading control by 10% SDS-PAGE. R represents a human liver membrane sample that was used as a reference for each gel. B: SR-BI protein level was 74% higher in GS than in GSF. Data show means ± SEM of the value obtained from the quantitation of the blots shown in A. A.U., arbitrary units. C: Correlation between hepatic SR-BI protein and biliary cholesterol molar percentage in gallbladder bile (n = 20). D: Correlation between hepatic SR-BI protein and CSI in gallbladder bile (n = 20). E: Hepatic ACAT2 microsomal activity (GS, n = 19; GSF, n = 11) and hepatic ACAT2 mRNA (GS, n = 21; GSF, n = 13) expression in GS and GSF. Data show means + SEM.

relevance to the human condition needs confirmation. In this investigation carried out in nonobese, normolipidemic Chinese gallstone patients, a significantly greater molar percentage of biliary cholesterol occurred without simultaneous changes in the molar percentage of bile acids or phospholipids. This change results in an increased CSI, a known prerequisite for gallstone formation (3, 17). The increased mRNA expression of hepatic *ABCG5* and *ABCG8* in GS suggests one possible mechanism for the increase in CSI. Moreover, the observed correlation between the

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expression levels of these two transporters and the molar percentage of biliary cholesterol supports the likelihood of a link between the expression levels of *ABCG5* and *ABCG8* and biliary cholesterol secretion in humans. Unfortunately, we were not able to measure the protein levels of ABCG5 and ABCG8. In our GS, *ABCG5* and *ABCG8* also correlated with *LXRα* expression, and a pronounced increase (102%) in the mRNA of *LXRα* was seen in GS, consistent with the possibility that in humans, as in mice, both *ABCG5* and *ABCG8* are transcriptionally regulated by *LXRα*. Therefore, our data suggest that this nuclear receptor may have played a role in the pathogenesis of gallstone disease in our Chinese patients, although no correlations were found between two other LXR-regulated genes (*ABCA1* and *SREBP1c*) and *LXRα*.

In the human *ABCG5/ABCG8* transgenic mouse model, a correlation between the hepatic mRNA levels of these transmembrane transporters and biliary cholesterol was reported (22). Evidence for the correlation between *ABCG5/ABCG8* expression and biliary cholesterol secretion in humans has also been found in patients with sitosterolemia, a disease caused by mutations in either *ABCG5* or *ABCG8* (23), in whom biliary cholesterol secretion was markedly diminished (24). It has been proposed that pathways independent of ABCG5 and ABCG8 also exist and contribute to cholesterol secretion into bile (21). Our findings are in agreement with recent studies of inbred mice challenged with a lithogenic diet (25, 26). In this case, the expression of *Abcg5* and *Abcg8* was increased, the level of biliary cholesterol was higher, and gallstone formation occurred. In mice, *Abcg5* and *Abcg8* colocalized with the murine *Lith 9* gene locus. Furthermore, the murine *Abcg5* and *Abcg8* expression was induced by *LXRα* (10, 11), and this gene colocalized with murine *Lith 1* (27). Treatment of gallstone-resistant AKR mice with synthetic LXRα agonists induced gallstone formation, apparently as a result of an increased expression of *ABCG5* and *ABCG8*, which in turn enhanced biliary cholesterol secretion (28). However, it should be recognized that the bulk of data obtained on the transcriptional regulation by LXR has been generated in rodent models or cell systems, either by depletion of the LXR gene or by pharmacological treatment with LXR agonists. In this case, some discrepancies between the observations in human liver and the observations from other experimental models are not unexpected. Limitations in sample size and in the opportunity for experimental manipulation of human subjects may also play a role in the interpretation, although overall, the similarities identified between studies in mice and humans are encouraging.

In gallstone-susceptible C57L mice, synthetic FXR ligands are reported to prevent gallstone formation by inducing the expression of *ABCB11* and *ABCB4* (29). However, our gallstone patients did not show any differences in *FXR*, *ABCB11*, and *ABCB4* expression, in agreement with our findings on concentrations of biliary bile acids and phospholipids and consistent with the unchanged *CYP7A1* expression. These observations do not conflict with the proposed role for FXR in gallstone disease in that they do not exclude the possibility of FXR as a successful therapeutic target in humans aimed at reduction of the supersaturation of cholesterol in bile.

The increased expression of the HDL receptor SR-BI observed in our GS suggests that enhanced uptake of HDL cholesterol may have contributed to the increase of biliary cholesterol. This finding is consistent with our previous observations, in which we reported free cholesterol in HDL to be in rapid equilibrium with biliary cholesterol (30). Furthermore, in patients with a bile fistula, cholesterol from the HDL particles was more rapidly incorporated into biliary cholesterol than cholesterol from LDL particles (31). In complete agreement with the present report is the finding that *Srb1* expression regulates biliary cholesterol secretion in mice but not the secretion of phospholipids or bile acids (32, 33).

We did not observe any differences in plasma HDL cholesterol between GS and GSF, an observation consistent with another large study in Chinese gallstone patients (34). The increased SR-BI protein expression observed in GS was not paralleled by a decrease in HDL cholesterol. This would not be an expected finding if the major determinant of plasma HDL cholesterol levels is the hepatic expression of ABCA1, as was indicated by studies of ABCA1 liver-specific conditional knockout mice (35).

The excess in biliary cholesterol could also have originated from the de novo synthesis of cholesterol in liver, but neither *HMGCR* expression nor the hepatic levels of lathosterol showed any differences between GS and GSF, a finding consistent with observations by others (7, 36). Nevertheless, cholesterol de novo synthesis cannot be completely excluded, because in patients with a bile fistula, a condition that greatly induces bile acid synthesis, it was found that ∼30% of biliary cholesterol represents newly synthesized cholesterol (31, 37).

Another mechanism that might contribute to an increase in biliary cholesterol is a decrease in cholesterol esterification, leading to higher levels of free cholesterol available for secretion into the bile. However, our data do not support this hypothesis, as neither differences in hepatic ACAT2 activity between GS and GSF nor differences in free cholesterol concentrations in whole liver homogenates from GS and GSF were observed. Furthermore, previous observations suggested that cholesterol levels did not differ within the microsomal membrane in patients with and without gallstones (7).

Conversion of cholesterol into bile acids is one key mechanism for the disposal of excessive cholesterol from the body. Increased bile acid synthesis, measured as an increase in plasma 7α-hydroxy-4-cholesten-3-one, was observed in Chilean gallstone patients (38). It was suggested that this change could be secondary to increased intestinal losses of bile acids. The Chilean gallstone patients also had increased levels of triglycerides in plasma and liver, together with increased hepatic activity of the microsomal triglyceride transfer protein (MTTP). In contrast, we found no differences between our groups in *CYP7A1* and *MTTP* expression or in plasma and hepatic triglyceride levels. This discrepancy may be an indication that

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gallstone disease is not attributable to a single metabolic defect. The types of gallstone-free patients selected for the reference group (gallstone-free controls) likely affect the outcome of all studies of gallstone disease, because for ethical reasons, an ideal reference group of completely healthy subjects cannot be studied. In our study and in the study of Chilean patients, the reference groups were quite different. In our study, patients with gallbladder polyps were included among the gallstone-free controls. The increased levels of deoxycholic acid in the bile of these patients might be related to their disease, because an increase in deoxycholic acid has been described to be associated with an enhanced induction of gallbladder polyps in hamsters (39). However, in Asian subjects, a higher level of deoxycholic acid in healthy liver transplant donors has also been reported, suggesting that differences in biliary bile acid composition may exist between control groups of different ethnicity (40). In the Chilean study (38), the majority of the patients enrolled in the reference group underwent surgical procedures because of gastrointestinal cancer. All of the preoperative procedures, including the particular liquid or semiliquid diets and the "intestinal preparation" (i.e., eventual laxative and prophylactic antibiotic therapy), given to those patients before the surgical procedures could have influenced the outcome in unknown ways. Another limitation of the mechanistic studies performed in humans is the relatively small sample size. Thus, in our study as in other studies, a risk that some differences may be missed cannot be excluded.

Finally, in our GS, we found an increased expression of *HNF4A*, which is a nuclear receptor that regulates the tissue-specific expression of many genes in the liver and other organs originating from the endoderm (41). In addition to the correlation between the expression of *HNF4A* and *SR-BI*, as was reported previously (42), we found a correlation between the expression of *HNF4A* and of *ABCG5* and *ABCG8*. Recently, a regulatory element for *HNF4A* was identified in the human ABCG5/ABCG8 promoter region that could strongly regulate the expression of these two genes (43). A correlation between *HNF4A* and *PGC1α* and a decrease in the expression of the latter cofactor was described in a small number of Italian gallstone patients compared with patients with gastrointestinal cancer or with liver donors (44). We could not confirm these findings, but the ethnicity of our patients was different, as was that of the control group in our study. Another unexpected finding in our study was the significant correlation between the expression of *HNF4A* and the expression of *LXRα* ($r = 0.43$, $P < 0.05$). The possibility that altered HNF4A activity contributes to the pathogenesis of gallstone disease, as in our nonobese, normolipidemic Chinese subjects, is intriguing and should be addressed in future studies.

In conclusion, in our study of normolipidemic, nonobese Chinese gallstone patients, the supersaturation of the bile with cholesterol was associated with an increased expression of the canalicular transporters ABCG5 and ABCG8, which was possibly induced by the increased expression of hepatic LXRα. Our data also suggest that excess biliary cholesterol in gallstone patients may be derived, at least in part, from the plasma HDL cholesterol taken up via the hepatic HDL receptor SR-BI.

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