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Increased *NPC1L1* and *ACAT2* expression in the jejunal mucosa from Chinese gallstone patients

Zhao-Yan Jiang [a,b], Chong-Yi Jiang [a], Lei Wang [c], Jian-Cheng Wang [a], Sheng-Dao Zhang [a], Curt Einarsson [d], Mats Eriksson [e,f], Tian-Quan Han [a,*], Paolo Parini [b,f], Gösta Eggertsen [b,*]

[a] Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China
[b] Division of Clinic Chemistry, Department of Laboratory Medicine, Karolinska University Hospital Huddinge, Stockholm SE 14186, Sweden
[c] Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China
[d] Department of Gastroenterology, Karolinska University Hospital Huddinge, Stockholm SE 14186, Sweden
[e] Metabolism Unit, Department of Endocrinology, Metabolism and Diabetes, and Department of Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, SE 141 86 Stockholm, Sweden
[f] Molecular Nutrition, Department of Biosciences and Nutrition, Karolinska Institutet at NOVUM, SE 141 86 Stockholm, Sweden

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ABSTRACT

The incidence of cholesterol gallstones is a very common disease. The aim of this study is to probe for underlying intestinal molecular defects associated with development of gallstones. Twelve Chinese patients with cholesterol gallstone disease (GS) and 31 gallstone-free (GSF) patients were investigated. Quantitation of mRNA levels for individual genes in mucosal biopsies from jejunum was carried out with real-time PCR. The frequency of two SNPs in the ABCG8 gene (Y54C and T400K) was determined by allelic discrimination. The intestinal mRNA expression of NPC1L1 and ACAT2 were significantly higher in GS than GSF ($P < 0.05$). No differences were observed concerning the levels for plasma lipids, plant sterols and 7α-hydroxy-4-cholesten-3-one between GS and GSF. No correlations were observed between patients carrying the different genotypes for Y54C or T400K and their mRNA levels for ABCG5 or ABCG8. The increased NPC1L1 and ACAT2 mRNA levels in gallstone patients might indicate an upregulated absorption and esterification of cholesterol in the small intestine.

Hypersecretion of biliary cholesterol as well as supersaturation of the bile with cholesterol are considered to be the most important prerequisites for gallstone formation. In humans, biliary cholesterol is provided by the canalicular transporters ABCG5/G8, and comprises approximately about 2/3 of the daily intestinal cholesterol input (800–1000 mg); another 300 mg originates from the diet [1]. In a previous study, we observed that Chinese gallstone patients had an increased hepatic ABCG5/ABCG8 expression [2]. However, since defects in the cholesterol metabolism generally have multifactorial origins, other pathogenic mechanisms may participate, which might not be exclusively restricted to the liver [3,4].

Several evidences suggest that gallstone disease may involve alterations in the intestinal cholesterol absorption. Gallstones are prevalent in countries consuming a western diet [5]. In China, the incidence of gallstone disease increased during the last decades from less than 4% up to almost 10%, paralleling a national increase of dietary cholesterol intake [6]. In line with these observations, gallstone patients fed a cholesterol diet increased their biliary cholesterol [7,8], contrary to gallstone-free subjects [9,10]. In certain strains of mice, feeding a lithogenic diet containing cholesterol and cholic acid induces gallstone formation, but a gallstone-susceptible strain like C57BL seems to absorb more intestinal cholesterol than the gallstone-resistant AKR mice [11]. Finally, gallstone formation in mice could be prevented by inhibition of intestinal cholesterol absorption due to targeted disruption of the genes either for acyl-coenzyme A: cholesteryl acyltransferase (ACAT2) [12] or for ApoB-48 [13].

Presently, the pathways for the cholesterol absorption in the small intestine are incompletely understood. It has been suggested

Abbreviations: ACAT, acyl-coenzyme A: cholesteryl acyltransferase; ABC, ATP binding cassette; apoA1, apolipoprotein A1; CYP7A1, cholesterol 7α-hydroxylase; DGAT, diacyl glycerol acyl transferase; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LXR, liver X receptor; MTTP, microsomal triglycerides transport protein; NPC1L1, Niemann–Pick C1-like 1 protein; SRBI, scavenger receptor B type I

* Corresponding authors. Fax: +46 08 58581260 (G. Eggertsen); fax: +86 21 64373909 (T.-Q. Han).
E-mail addresses: digsurgrj@yahoo.com.cn (T.-Q. Han), Gosta.Eggertsen@ki.se (G. Eggertsen).

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that mixed micelles or unilamellar vesicles serve as cholesterol donors, interacting with membrane vesicles in the apical brush border. Recent studies (as reviewed in references [1,14]) conclude that absorption of luminal cholesterol occurs via a transporter protein called Niemann–Pick C1-Like1 protein (NPC1L1) [15]. Once inside the enterocyte, most of the free cholesterol is esterified by ACAT2 [16] and packaged into chylomicrons, together with triglycerides and apoB-48. Free cholesterol can also be secreted to HDL particles via ABCA1 [17], or transferred back to the intestinal lumen by the ATP binding cassette (ABC) G5/G8 heterodimer [18]. ABCG5/G8 also returns plant sterols to the intestinal lumen and in this way controls their plasma levels.

Very little is known about the relationships in humans between expression of intestinal genes, crucial for cholesterol absorption, and occurrence of gallstones. In this study, we have detected an increased expression of NPC1L1 and ACAT2 genes in the jejunum of gallstone patients (GS), suggesting that these patients may have an increased intestinal cholesterol absorption.

Materials and methods

Subjects. Twelve patients with cholesterol gallstone disease (GS, male:female = 6:6) and 31 gallstone-free (GSF, male:female = 22:9) patients were included in this study. The presence of gallstones was diagnosed by ultrasonography and confirmed during the operation. The patients were subjected to abdominal surgery to establish a jejunal-pancreatic anastomosis due of pancreatic tumors (GS = 11 and GSF = 24) or gastric-jejunal anastomosis due to gastric tumors (GS = 1 and GSF = 7). None of the patients were subjected to lipid-lowering therapy. No differences were found concerning age and BMI between GS and GSF patients (age: 54 ± 1.9 years vs 51 ± 4.2; BMI: 21.4 ± 0.5 vs 22.3 ± 0.7). Informed consent was obtained from all participants prior to enrollment in the study, including permission to collect an intestinal biopsy. The study protocol was approved by the Ethics Committees at the Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, and the Karolinska University Hospital at Huddinge, Stockholm, Sweden.

All patients utilized an identical diet program which started 7 days before surgery. All patients were also subjected to identical preoperative preparations: i.e., laxative, prophylactic antibiotic therapy, and overnight fasting. The surgical procedures began at 9 a.m. and one piece of jejunal mucosa (located at about 40 cm distal to the Treitz-ligament) was dissected from the annular muscular layer during the operation and immediately snapfrozen in liquid nitrogen and stored at −80°.

Analysis of plasma lipids, plant sterols, lathosterol, and 7α-hydroxy-4-cholesten-3-one (C4). Plasma lipoproteins were separated by size exclusion chromatography as previously described [19]. Plasma sitosterol, campesterol, and lathosterol were determined by isotope-mass spectrometry using deuterium-labeled sitosterol, campesterol [20], and lathosterol [21] as internal standards. Plasma 7α-hydroxy-4-cholesten-3-one (C4) levels were determined by the LC-MS/MS method with $^2H_6$-labelled C4 as internal standard as previously described [22].

Relative mRNA quantification. Intestinal total RNA was extracted with Trizol® (Invitrogen, Carlsbad, CA). cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Specific mRNA quantification was performed by real-time PCR using SYBR-Green (Power Master Mix Sybr Green, Applied Biosystem, Foster City, CA, USA) as previously described [2]. PCR primers (primer sequences available on request) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA), and were made to bridge an exon–exon boundary. Data were calculated by the ΔC_t method, expressed in arbitrary units, and normalized to the signals obtained when the same cDNA was assayed for Cyclophilin A, selected as endogenous reference gene. The fold change for each mRNA quantity in the GS material was expressed in relation to the obtained value for GSF, the mean value of which arbitrarily was set at 1.

Genotyping of ABCG8 polymorphisms. Single nucleotide polymorphism (SNP) analysis of the polymorphic sites Y54C and T400K in the ABCG8 gene were determined by allelic discrimination using intestinal cDNA as template. The following sequences were utilized for PCR primers and Taqman probes: Y54C: forward primer: ACA GTG GCC AGC CCA ACA; reverse primer: AGC CAG CTG CTC AAA CCA A. Probes: FAM-AGA GAC CTC AAC T***a***C CA-MGB, VIC-AGA GAC CTC AAC T***g***C CA-MGB. T400K: forward primer: GCC TCC CGA GTC CTA CGA A; reverse primer: CGG AAG TCG TTG GAA ATC TGA C. Probes: FAM-TGC AGC AGT TTA ***a***GA CGC TGA-MGB, VIC-TGC AGC AGT TTA ***c***GA CGC TGA-MGB. Analyses were carried out in duplex, using 4 μL of cDNA in each sample (equaling to 20 ng of intestinal RNA) (Applied Biosystems, Foster City, CA) in a total reaction volume of 10 μL. Final concentration for primers and probes were 0.2 mmol/L and 0.9 mmol/L, respectively. Required endpoint results were obtained by the Sequence Detection System Software (Applied Biosystems, Foster City, CA).

Stastistics. Data are reported as means ± SEM. Student's *t*-test was used to compare the differences of variables between gallstone patients and gallstone-free controls (Statistica 7.0 software, StatSoft Inc., Tulsa, USA). Statistical significance was set at $P < 0.05$.

Results

Plasma lipid levels in gallstone and gallstone-free patients

Determination of the fractional lipid content of the plasma lipoproteins did not demonstrate any differences between GS and GSF, neither for cholesterol or triglycerides (Fig. 1). Nor were any differences observed for the plasma levels of lathosterol or C4 between the two groups (Fig. 2A). Thus, cholesterol and bile acid synthesis did not differ between the GS and GSF, in line with previous observations made by us in another cohort of GS and GSF [2]. Neither were there any differences in plasma plant sterol concentrations—sitosterol and campesterol—between the two groups (Fig. 2B).

Intestinal gene mRNA levels

As shown in Fig. 3A, the mRNA levels for the cholesterol transporter NPC1L1 were +33% higher in the GS compared with the GSF ($P < 0.05$). However, there were no differences in either ABCG5 or ABCG8 mRNA expression between GS and GSF (Fig. 3B). The mRNA levels of these two genes correlated very well ($r = 0.95$, $P < 0.05$), as we previously have observed in liver tissue [2]. Interestingly, the mRNA for ACAT2 was also higher in the GS compared with the GSF (+41%, $P < 0.05$, Fig. 3A). No differences were found for the mRNA levels corresponding to HMG-CoA-R, DGAT1/DAGT2, MTTP, SRBI, ABCA1 or the nuclear receptors LXRα and LXRβ (Fig. 3B). Neither were there any differences of the intestinal gene expression between the patients with pancreatic tumors and gastric tumors (data not shown).

Genotypes of the ABCG8 gene and expression of ABCG5/G8

Four common nucleotide polymorphisms causing amino acid exchanges are reported for the ABCG8 gene [23]. Although two of the polymorphisms, D19H and A632V, frequently are occurring among Caucasian populations [23], they are rare in ethnic Chinese [24]. Thus, in the present study, genotyping was only carried out

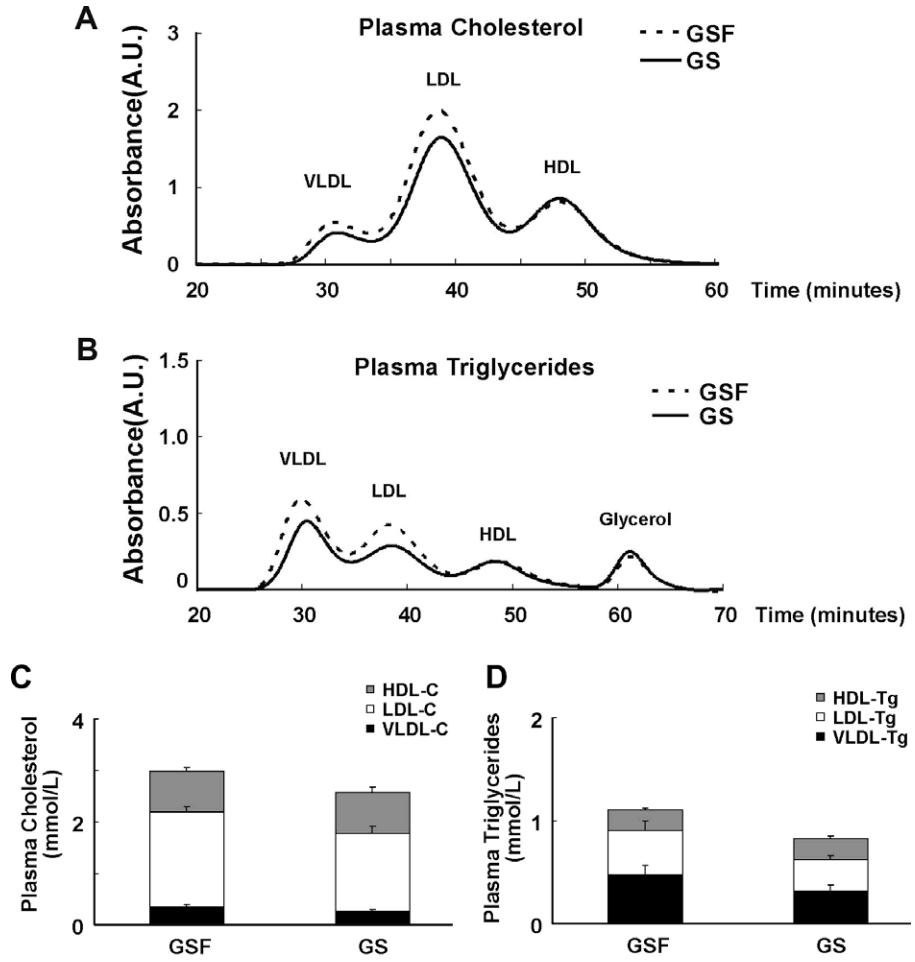

Fig. 1. Plasma lipoprotein profiles in the gallstone patients and gallstone-free controls. Data expressed as means ± SEM.

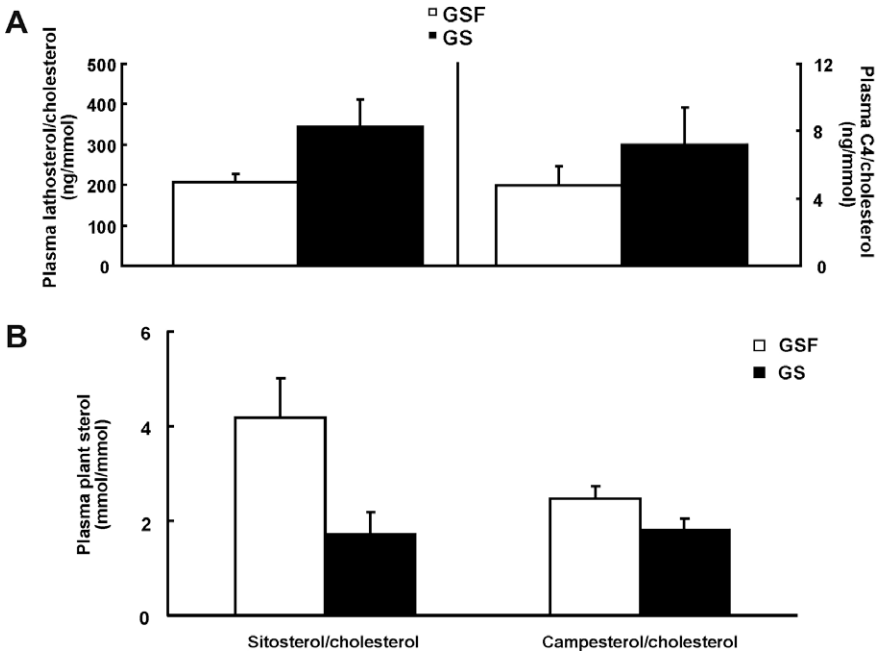

Fig. 2. Plasma lathosterol, 7α-hydroxy-4-cholesten-3-one (C4) and plant sterols levels in gallstone patients and gallstone-free controls. Data expressed as means ± SEM. (A) No significant differences of plasma lathosterol and C4 levels were observed. (B) No differences of plasma sitosterol and campesterol levels were found.

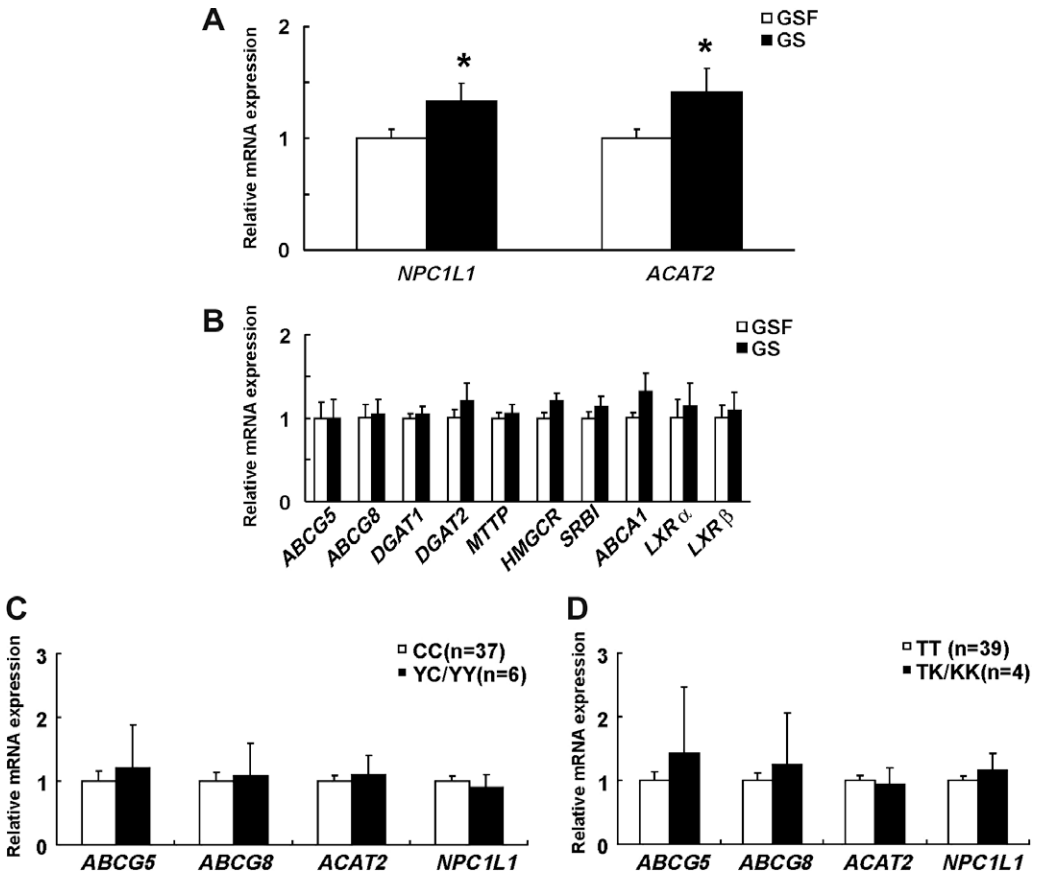

Fig. 3. Quantitative mRNA expression levels in genes from the jejunal mucosa. Data expressed as means ± SEM. (A) Comparison between GS patients and GSF controls. The mRNA levels of NPC1L1 and ACAT2 were significantly higher in GS than in GSF. $*P < 0.05$. (B) Comparison between GS patients and GSF controls. No differences were found for the mRNA levels corresponding to *ABCG5, ABCG8, DGAT1, DGAT2, MTTP, HMGCR, SRBI, ABCA1, LXRα*, and *LXRβ*. (C) Comparison between mRNA levels of the genes ABCG5/G8, NPC1L1 and ACAT2 in the individuals of the total material (GS + GSF) with reference to the Y54C polymorphic site in the ABCG8 gene. (D) Comparison between mRNA levels of the genes ABCG5/G8, NPC1L1, and ACCAT2 in the individuals of the total material (GS + GSF) with reference to the T400K polymorphic site in the ABCG8 gene.

for the Y54C and T400K polymorphisms in ABCG8 gene in the GS and GSF patients. Previously, T400K had been shown to associate with gallstone disease in Chinese patients [24], but this material was too small to allow a similar evaluation. Instead, we investigated whether the two polymorphic spots might influence the mRNA levels of ABCG8 or ABCG5, but no differences were found that related to the two genotypes (Fig. 3C and D). The campesterol and sitosterol levels were lower in subjects heterozygous or homozygous for the Y54C or the T400K polymorphism compared to individuals homozygous for the WT allele, but significance was only obtained for campesterol (Table 1, $P < 0.05$). No correlations were found for the cholesterol and triglyceride content in the fractioned plasma lipoproteins (VLDL, LDL and HDL; Table 1).

Discussion

This study is one of the first approaches to utilize mucosal biopsies from jejunum to evaluate differences in gene expression between patients with and without cholesterol gallstone disease. We find that in the GS patients, the mRNA levels for both the major intestinal cholesterol transporter NPC1L1, and the cholesterol esterifying enzyme ACAT2, were higher.

Table 1
Plasma lipid and plant sterol levels in the total material with reference to the polymorphic sites Y54C and T400K in the ABCG8 gene (means ± SEM).

	YC/YY ($n = 6$)	CC ($n = 37$)	TK/KK ($n = 4$)	TT ($n = 39$)
VLDL-C (mmol/L)	0.21 ± 0.04	0.35 ± 0.04	0.24 ± 0.06	0.34 ± 0.04
LDL-C (mmol/L)	1.71 ± 0.28	1.75 ± 0.10	1.70 ± 0.39	1.75 ± 0.09
HDL-C (mmol/L)	0.70 ± 0.07	0.81 ± 0.07	1.12 ± 0.40	0.76 ± 0.06
TC (mmol/L)	2.62 ± 0.36	2.91 ± 0.14	3.05 ± 0.65	2.85 ± 0.13
VLDL-Tg (mmol/L)	0.39 ± 0.09	0.44 ± 0.07	0.42 ± 0.13	0.43 ± 0.07
LDL-Tg (mmol/L)	0.31 ± 0.07	0.41 ± 0.08	0.34 ± 0.11	0.40 ± 0.07
HDL-Tg (mmol/L)	0.17 ± 0.03	0.20 ± 0.02	0.24 ± 0.06	0.19 ± 0.02
Tg (mmol/L)	0.87 ± 0.18	1.05 ± 0.16	0.99 ± 0.26	1.03 ± 0.15
Sitosterol/cholesterol	0.93 ± 0.26	3.97 ± 0.73	0.66 ± 0.33	3.74 ± 0.68
Campesterol/cholesterol	1.19 ± 0.30*	2.47 ± 0.21	0.71 ± 0.10**	2.45 ± 0.20

* $P < 0.05$.
** $P < 0.01$.

Alterations in the output of biliary lipids could have marked effects on the intestinal cholesterol absorption [25]. In the enterocyte, NPC1L1 is regarded as the major molecule for intestinal cholesterol absorption [15]. Mice devoid of the NPC1L1 protein display a dramatically decreased cholesterol absorption. In mice, gallstone formation could be prevented when ezetimibe, a potent inhibitor of intestinal cholesterol absorption, specifically targeting NPC1L1 [26], was added to a lithogenic diet, thereby displaying a clear dose–response effect and a clear link between intestinal cholesterol absorption and gallstone formation [27]. The functional impact of higher NPC1L1 mRNA production in our GS patients is difficult to estimate directly, but it could signify enhanced intestinal cholesterol absorption. Contrary to mice, humans express NPC1L1 also in the canalicular membranes of the hepatocytes, possibly for bringing biliary cholesterol back to the hepatocytes and thereby prevent supersaturation of the bile [28]. The mRNA levels for NPC1L1 in the liver of patients affected by gallstone disease were, however, not altered compared to GSF patients [2].

The higher expression levels of ACAT2 in the jejunum of GS would primarily indicate existence of a higher rate of intestinal cholesterol esterification. Recently, we have demonstrated that cholesterol upregulates the activity and transcription of the human ACAT2 *in vitro* [29] and, accordingly, in mice treated with ezetimibe, intestinal expression of ACAT2 decreases [27]. Furthermore, in mice, gallstone formation could be prevented following targeted disruption of the gene for ACAT2 [12]. Thus we cannot postulate whether the elevated mRNA levels of ACAT2 in the GS patients reflects an increased cholesterol uptake via elevated expression of NPC1L1, or if elevated levels of ACAT2 *per se* enhances intestinal cholesterol uptake (as seems to happen in mice). The increase in NPC1L1 and ACAT2 in gallstone patients would be in line with the fact that NPC1L1 facilitates the trafficking of apical membrane cholesterol to ACAT2 as shown in Caco2 cells [30]. Unfortunately, due to shortage of material, we were not able to measure ACAT2 enzyme activity in a sufficient number of individuals to perform a reliable statistical analysis. However, we observed that the intestinal activity of ACAT2 (423 ± 66 pmol/min/mg protein, $n = 18$) in the GSF patients was much higher than the hepatic activity found in another study of GSF patients (9.0 ± 5.6 pmol/min/mg protein, $n = 11$ [2]). Hence, in humans, the intestinal ACAT2 activity may have a very prominent mission, as its activity is 40-fold higher than found in liver tissue.

In the present study, no differences were observed between patients and controls for the intestinal ABCG5/ABCG8 mRNA expression. However, the exact significance of the intestinal ABCG5/G8 for the cholesterol homeostasis is unclear even if it is generally assumed that they mediate efflux of cholesterol to the lumen. In mice, overexpression of human ABCG5/ABCG8 protein did not affect the cholesterol absorption, but the plant sterol levels in plasma decreased [31], and thus the intestinal ABCG5/ABCG8 might be more of a gatekeeper for plant sterols rather than a discriminator for all sterols [32,33]. In a previous study, we found higher levels of hepatic ABCG5/G8 mRNA in gallstone patients [2], which could cause higher amounts of cholesterol to be delivered into the bile. The reason why our GS patients do not upregulate ABCG5/G8 (which are LXR-target genes), even if their cholesterol absorption should be increased, could be due to their higher ACAT2 activity, causing a reduction of the free cholesterol levels and in this way preventing an activation of LXR.

Polymorphisms within the ABCG5 and ABCG8 genes have been reported to associate with gallstone disease, as the frequency of D19H in the latter was higher in German and Chilean patients [23,34]. T400K was overrepresented in a Chinese GS patient material [24], and we also concluded that individuals with this SNP showed lower levels of plasma campesterol. The significance of these findings is difficult to interpret, but may indicate that GS patients have a higher activity of the intestinal ABCG5/G8. Nevertheless, in accordance with the concept that gallstone disease has a multifactorial origin, our results show that also alterations within the small intestine must be considered when investigating the pathogenesis.

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