Loci From a Genome-Wide Analysis of Bilirubin Levels Are Associated With Gallstone Risk and Composition

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BACKGROUND & AIMS: Genome-wide association studies have mapped loci that are associated with serum levels of bilirubin. Bilirubin is a major component of gallstones so we investigated whether these variants predict gallstone bilirubin content and overall risk for gallstones. **METHODS:** Loci that were identified in a meta-analysis to attain a genome-wide significance level of a *P* value less than 1.0×10^{-7} (*UGT1A1*, *SLCO1B1*, *LST-3TM12*, *SLCO1A2*) were analyzed in 1018 individuals with known gallstone composition. Gallstone risk was analyzed in 2606 German choleystecomized individuals and 1121 controls and was replicated in 210 cases and 496 controls from South America. **RESULTS:** By using the presence of bilirubin as a phenotype, variants rs6742078 (*UGT1A1*; $P = .003$), rs4149056 (*SLCO1B1*; $P = .003$), and rs4149000 (*SLCO1A2*; $P = .015$) were associated with gallstone composition. In regression analyses, only *UGT1A1* and *SLCO1B1* were independently retained in the model. *UGT1A1* (rs6742078; $P = .018$) was associated with overall gallstone risk. In a sex-stratified analysis, only male carriers of rs6742078 had an increased risk for gallstone disease ($P = 2.1\times10^{-7}$; odds ratio$_{recessive}$, 2.34; $P_{women} = .47$). The sex-specific association of rs6742078 was confirmed in samples from South America ($P_{men} = .046$; odds ratio$_{recessive}$, 2.19; $P_{women} = .96$). **CONCLUSIONS: The *UGT1A1* Gilbert syndrome variant rs6742078 is associated with gallstone disease in men; further studies are required regarding the sex-specific physiology of bilirubin and bile acid metabolism. Variants of *ABCG8* and *UGT1A1* are the 2 major risk factors for overall gallstone disease, they contribute a population attributable risk of 21.2% among men.**

Keywords: Pigment Gallstones; Cholelithiasis; Complex Disease.

Cholelithiasis represents a frequent and economically relevant health problem in both industrialized and developing countries.[1–3] In the Western world, the prevalence of cholelithiasis has increased from approximately 6% in the 1960s and 1970s to approximately 20% in the late 1990s.[1,4,5] Economically, gallstone disease has been identified as the second most costly digestive disorder,[6] and more than 170,000 cholecystectomies are performed annually in Germany alone.[7]

Demographic and environmental risk factors for gallstone disease include age, female sex, and body mass index.[2,8] A genetic component in the susceptibility to gallstones has been recognized as early as 1937.[9] The initial studies used pathologic section statistics,[9] whereas more recent studies have turned to ultrasound surveys to investigate familial clustering of cholelithiasis.[10] Despite the heterogeneous study designs, there is both compelling evidence for familial clustering and an increased concordance of the trait in monozygotic twins as compared with dizygotic twins.[11] Likewise, genome-wide linkage analyses of gallstone traits in inbred mouse strains have lead to a murine map of *Lith* loci.[12–14]

The most widely replicated human susceptibility gene for gallstone disease is *ABCG8*, which has been shown to confer an approximately 2-fold increase in gallstone risk in German, Sorbian (a Slavonic minority in Eastern Germany), Romanian, Swedish, Chilean, and Chinese patients.[15–19] This finding is also consistent with the identification of the murine orthologue of *ABCG8* as *Lith9*.[14] In addition, Rosmorduc et al[20] found point mutations in *ABCB4* in more than half of the patients with low phos-

Abbreviations used in this paper: OR, odds ratio; UDP, uridine 5′-diphosphate.

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pholipid-associated cholelithiasis, a syndrome characterized by cholesterol gallbladder stones before the age of 40 and recurrent biliary symptoms after cholecystectomy. A recent Norwegian study found that potentially deleterious *ABCB4* mutations were present in 2 of 140 patients with early onset gallstone disease.[21]

The recent progress in genotyping technologies has led to a number of studies using genome-wide association to investigate genetic predisposition for a number of quantitative laboratory traits measured in peripheral blood, including hematologic and metabolic parameters.[22,23] Along this paradigm, serum bilirubin levels have been studied using genome-wide linkage and association analysis.[24–26] This trait also has been investigated in a large meta-analysis from 3 genome-wide association studies (Framingham heart study, n = 3424; Rotterdam study, n = 3847; Age, Gene, Environment and Susceptibility-Reykjavik, n = 2193), constituting the largest report on genetics of serum bilirubin levels to date.[27] Because bilirubin is a common constituent of gallstones,[28,29] the loci identified by these investigators constitute natural candidate genes for the prediction of gallstone composition and possibly gallstone risk.

In this study, we therefore evaluated all loci attaining a genome-wide significance level of a *P* value less tan 1.0×10^{-7} in the large meta-analysis reported by Johnson et al[27] in a sample of 1018 gallstone patients with known stone composition.[30] By using the likely mode of inheritance extracted from the meta-analysis for each locus, we show that 3 of these loci (*UGT1A1*, *SLCO1B1*, and *SLCO1A2*) are associated with the presence of bilirubin in gallstones and show that overall gallstone risk is associated with *UGT1A1* genotype in patients with gallstones from Germany and South America.

Materials and Methods

Patients and Phenotypes

German cases and controls were recruited in Northern Germany through clinical centers at Kiel University, the POPGEN biobank and epidemiology project, and at Greifswald University, through the population-based Study of Health in Pomerania (SHIP). No significant population genetic differences exist between these 2 regions[31] so that patient samples were combined to construct genotyping panels on the basis of phenotypic criteria alone (Table 1) as in previous studies.[15,32,33] The recruitment of the Kiel and Greifswald patients has been reported previously in detail.[32,34] In brief, Kiel cases were recruited either postcholecystectomy or on the basis of the presence of gallstones in an ultrasound examination. Controls were free of gallstones as determined by history and abdominal ultrasound. Only patients and controls of German ethnicity were included. The self-reported ethnicity had to be German and the parental birthplaces had to be consistent with a German descent.[31] Written in-

Table 1. Overview of the Patient Samples Used in This Study

	Patients				Controls			
Patient sample	N	Age[a]	Age at diagnosis[a]	BMI[a]	N	Age[a]	BMI[a]	Female
Stone composition sample								
Total	1018	53 (±11)	45 (±11)	28 (±5)				75%
Presence of bilirubin (>5%)[b]	328	55 (±11)	46 (±12)	27 (±4)				66%
Bilirubin as main component (>30%)	52	55 (±13)	44 (±12)	27 (±5)				54%
Bilirubin as trace component (5%–10%)[c]	219	55 (±11)	46 (±11)	27 (±4)				70%
Gallstones without bilirubin[d]	690	53 (±11)	44 (±11)	29 (±6)				79%
Disease association sample								
German cohort[e]	2606	54 (±11)	45 (±11)	28 (±6)	1121	61 (±11)	27 (±4)	72%
Male sex	739	57 (±10)	49 (±9)	28 (±4)	708	64 (±11)	27 (±4)	0%
Female sex	1876	53 (±12)	42 (±12)	28 (±6)	793+	60 (±11)	26 (±5)	100%
South American cohort[f]								
Male sex	35	55 (±11)	N/A	28 (±4)	210	49 (±12)	28 (±4)	0%
Female sex	175	55 (±11)	N/A	31 (±6)	286	49 (±12)	29 (±5)	100%
Post hoc analysis in population-based cohort[g]								
Symptomatic male	98	63 (±11)	N/A	29 (±4)	297	67 (±11)	27 (±3)	0%
Nonsymptomatic male	140	63 (±12)	N/A	29 (±4)				
Symptomatic female	203	56 (±11)	N/A	30 (±6)	433	60 (±12)	27 (±5)	100%
Nonsymptomatic female	142	53 (±12)	N/A	28 (±5)				

N/A, not available.

[a]In years; mean (±SD) reported.

[b]All stones with bilirubin detected in Fourier transform infrared, 5% was the lower limit of detection.

[c]Other components of the stone: 94% cholesterol stones with bilirubin as trace component, 6% stones with calcium carbonate >50%.

[d]99.5% pure cholesterol stones.

[e]Controls were matched for sex.

[f]Pooled case-control samples from Chile and Argentina.

[g]Population-based cohort from Greifswald, Germany (SHIP).

formed consent was obtained from all patients, and the data handling procedures were monitored constantly by the local data protection authorities. In Greifswald, cases and controls were recruited through the SHIP project as previously described.[8,35] SHIP is a population-based study representative of the Northeast German adult population. A total of 4310 subjects aged 20–79 years underwent an abdominal ultrasound and a full physical examination, thereby determining either gallstone carrier status (N = 282) or a previous history of cholecystectomy (N = 301). Of the total study population, the 583 youngest cases and 730 controls were included in this study. In accordance with earlier studies from our group, German controls were sex-matched to the total case sample and selected to be of a median age 7 (±1) years older than the cases, to provide a more robust control population, bearing in mind that gallstone prevalence increases with age.

The Chilean samples represent a nested case-control study, embedded into an ongoing longitudinal ultrasonographic study on prevalence and risk factors of cholelithiasis in Chile, performed in La Florida.[2,36] For the present study, 144 unrelated patients with sonographically documented gallstones or a history of cholecystectomy and 281 unrelated control individuals who were consistently free of gallstone disease during the follow-up period were selected at random. The Argentinean samples represent a cross-sectional study performed in a county hospital of the city of Buenos Aires embedded into an ongoing longitudinal study of risk factors of nonalcoholic fatty liver disease. For the present study, 66 unrelated patients with sonographically documented gallstones or a history of cholecystectomy and 215 unrelated control individuals who were free of gallstone disease during the study period were included in the analysis. Both South American samples come from a mixed genetic heritage including European and native American ancestors. An overview of the study samples is given in Table 1.

Analysis of Gallstone Composition

Gallstone composition was determined using Fourier transform infrared spectrometry as described.[30] In brief, dry gallstone specimens were fragmented using a scalpel. Two samples were obtained from each gallstone. The sites of sampling of an individual stone were chosen to be as different in macroscopic appearance as possible. For example, the pigmented core and the yellow shell of a stone would have been sampled if present. If multiple stones were available for one patient, the largest stone was investigated. Substances were assigned as main components if they constituted more than 30% of the gallstone (weight/weight). Components were classified as trace components if the respective substance constituted less than 10% but more than 5% (weight/weight) of the gallstone. All bilirubinate salts were summarized as bilirubin (ie, the different bilirubin salts were not differentiated).

Genotyping

Genotyping for *UGT1A1* rs6742078 (hCV29367995); *SLCO1B1* rs4149056 (hCV30633906); *LST-3TM12* rs2417873 (hCV16237229); and *SLCO1A2* rs4149000 (hCV32325391) was performed using TaqMan allelic discrimination assays (Aplied Biosystems, Foster City, CA), as described before.[37] All process data were logged and administered through a database-driven Laboratory Information Management System (LIMS) system.[38] For all loci, there was no evidence of departure from Hardy–Weinberg equilibrium (all $P > .4$) in controls. The call rate for all markers was greater than 96%.

Statistical Analysis

All analyses were performed with SPSS (PASW 17; SPSS, Inc, Chicago, IL). Contingency tables were analyzed through χ^2 statistics or the Fisher exact test. Analyses of stone number and weight and genotypic predictors were performed using the Mann–Whitney *U* test and linear regression. Nominal *P* values are reported for all tests. The combined risk assessment and interaction of *ABCG8_D19H* (rs11887534) and *UGT1A1* (rs6742078) risk genotypes was assessed using a binary logistic regression model with carriership of rs11887534 (C) risk allele and homozygosity of rs6742078 (T/T) as predictor variables in the model, the combined odds ratios were obtained from the model parameters. Marker association with log-transformed bilirubin content in gallstones and overall presence of bilirubin in gallstones was assessed using a step-wise linear regression model and a logistic regression model, respectively. All models were adjusted for age and sex.

To quantify the proportion of severe phenotype manifestations due to a specific genotype, the percentage population attributable risk was calculated as follows:

$$PAR\% = \frac{f_{GT}(RR-1)}{f_{GT}(RR-1)+1} \cdot 100 \qquad (1)$$

Here, PAR% denotes percentage population attributable risk; f_{GT} denotes the frequency of the genotype in the at-risk population, and *RR* equals the genotypic relative risk as estimated by the corresponding odds ratio (OR).

Results

Selection of Bilirubin Loci From the Meta-analysis

All loci from the genome-wide meta-analysis[27] on serum bilirubin levels attaining a genome-wide significance level of a *P* value of less than 1.0×10^{-7} as an established threshold of genome-wide significance (ie, *UGT1A1* [uridine 5′-diphosphate (UDP)-glucuronosyl-

Table 2. Overview of the Results of the Association Analyses

	Locus SNP	UGT1A1 rs6742078	SLCO1B1 rs4149056	LST-3TM12 rs2417873	SLCO1A2 rs4149000
Meta-analysis of bilirubin levels according to Johnson et al[27]	*P* value	5.0×10^{-324}	6.7×10^{-13}	1.5×10^{-9}	2.8×10^{-8}
	β coefficient	(+) 0.234	(+) 0.053	(+) 0.05	(+) 0.06
	Mode of inheritance	Recessive	Dominant	Dominant	Dominant
Presence of bilirubin (>5%)	*P* value	.003	.003	.59	.015
	OR	1.73 (1.19–2.49)	1.57 (1.16–2.10)	1.07 (0.82–1.41)	1.43 (1.07–1.93)
Genotype counts[a]	>5%	141/118/59	208/97/8	183/110/23	218/93/8
	<5%/not detected	300/300/79	503/142/20	401/231/40	503/152/10
Presence of bilirubin as trace component (5%–10%)	*P* value	.007	.006	.58	.07
	OR	1.75 (1.15–2.64)	1.60 (1.14–2.23)	1.09 (0.79–1.48)	1.36 (0.97–1.90)
Genotype counts	Trace (5%–10%)	94/84/41	142/66/7	125/74/18	153/62/5
	<5%/not detected	300/300/79	503/142/20	401/231/40	503/152/10
Presence of bilirubin as main component (>30%)	*P* value	.044	.067	.87	.13
	OR	2.04 (1.01–4.12)	1.75 (0.95–3.19)	0.95 (0.53–1.71)	1.59 (0.86–2.94)
Genotype counts[a]	Main (>30%)	23/18/11	32/17/1	31/17/3	33/16/1
	<5%/not detected	300/300/79	503/142/20	401/231/40	503/152/10
Overall gallstone risk	*P* value	.018	.15	.42	.47
	OR	1.31 (1.04–1.64)	0.90 (0.76–1.04)	1.06 (0.92–1.22)	1.06 (0.91–1.24)
Genotype counts[a]	Cases	1137/1135/334	1751/689/70	1521/928/152	1848/698/47
	Controls	512/496/113	762/337/32	669/391/57	809/285/27

NOTE. The OR and *P* value were calculated under the mode of inheritance noted in the table.
SNP, single nucleotide polymorphism.
[a]Genotype order: rs6742078: GG/GT/TT, rs4149056:TT/TC/CC; rs2417873: CC/CT/TT; rs4149000: GG/GA/AA.

transferase], *SLCO1B1*, *LST-3TM12*, *SLCO1A2*) were selected. One tagging single nucleotide polymorphism with maximum effect size in the study reported by Johnson et al[27] was genotyped in the study cohorts. For the analysis, the likely mode of inheritance was extracted for each locus from the meta-analysis to minimize multiple testing. For example, 2 copies of the T allele of rs6742078 (*UGT1A1*) were associated with an increase in serum bilirubin of 61.3%, as compared with 27.0% for one copy of the T allele, suggesting a much stronger risk effect of rs6742078 T allele in the homozygous, recessive state. This information is summarized in the upper panel of Table 2. Two analyses per locus were performed as primary tests of hypotheses: (1) a test for association with the presence of bilirubin in the gallstones, and (2) a test for an overall association with gallstone risk in patients with symptomatic gallstone disease.

Association of the Presence of Bilirubin in Gallstones

For the analysis of a potential impact of the bilirubin serum loci on gallstone composition, patients with bilirubin present in their gallstones at least at the trace (5% wt/wt) level (N = 318 patients) were compared with patients with gallstones without detectable bilirubin (<5% bilirubin; N = 690 patients; Table 1) as a primary analysis. Variant rs6742078 in *UGT1A1* was associated with the presence of bilirubin in gallstones under a recessive model ($P = .003$; $OR_{recessive}$, 1.73; 95% confidence interval [CI], 1.19–2.49) for the T/T risk genotype. The single nucleotide polymorphism rs4149056, tagging *SLCO1B1* ($P = .003$; $OR_{dominant}$, 1.57; 95% CI, 1.16–2.10) and rs4149000 tagging *SLCO1A2* ($P = .015$; $OR_{dominant}$, 1.43; 95% CI, 1.07–1.93) were associated with the presence of bilirubin in the gallstone samples under a dominant model. The variant rs2417873 at the *LST-3TM12* (liver-specific organic anion transporter 3TM12) locus, showing a strong association ($P = 1.5 \times 10^{-9}$) with serum bilirubin levels in the meta-analysis,[27] was not associated with the presence of bilirubin in gallstones ($P = .59$; $OR_{dominant}$, 1.07). In a linear regression analysis including the log-transformed bilirubin content, sex, age, and genotypes of *UGT1A1*, *SLCO1B1*, *LST-3TM12*, and *SLCO1A2* under the genetic models noted in Table 2, independent association of *UGT1A1* ($P = .001$) and *SLCO1B1* ($P = .019$) with bilirubin content was detected. Similar results were obtained in a logistic regression analysis confirming *UGT1A1* ($P = .002$) and *SLCO1B1* ($P = .004$) as independent predictors of bilirubin presence. *SLCO1A2* was not retained in either model ($P > .50$). Indeed, substantial linkage disequilibrium ($r^2 = 0.66$) between markers in *SLCO1B1* and *SLCO1A2* was present. The genetic and physical structure of the locus containing these genes is depicted in Supplementary Figure 1.

In post hoc subgroup analyses, patients with different bilirubin contents of their stones were compared with the group of patients with stones without detectable bilirubin (N = 690, same control group as described earlier). The OR estimates in the subset of patients with high bilirubin contents (>30%; N = 52) were consistently higher than in the patients with low bilirubin contents (5%–10%; N = 219) for all variants with significant association in the primary analysis, thereby supporting a

Table 3. Post Hoc Risk Assessment Analysis for *UGT1A1* rs6742078

	Genotype counts		Frequency of risk genotype (T/T)			
	Case	Control	Case	Control	$OR_{recessive}$	*P*
Total sample						
G/G	1137	512				
G/T	1135	496				
T/T	334	113	12.8%	10.1%	1.31 (1.04–1.64)	.018
Males						
G/G	288	311				
G/T	323	339				
T/T	128	58	17.3%	8.2%	2.35 (1.69–3.26)	2.14×10^{-7}
Females						
G/G	849	368				
G/T	812	345				
T/T	206	80	11.0%	10.1%	1.10 (0.84–1.45)	.47

NOTE. All analyses were performed under a recessive mode of inheritance as before. Cases and controls were matched for sex in the total cohort. All cases had undergone surgery for symptomatic gallstone disease.

robust effect. The OR estimates increased for rs6742078 (*UGT1A1*) from 1.75 to 2.04, for rs4149056 (*SLCO1B1*) from 1.60 to 1.75, and for rs4149000 (*SLCO1A2*) from 1.36 to 1.59. In the patient group (N = 52) having bilirubin as the main gallstone component (>30% wt/wt), the frequency of the risk genotype T/T of rs6742078 (*UGT1A1*), for instance, was almost twice (21.2%) as high as in the subset of patients without bilirubin present in their gallstones (11.6%; *P* = .04; OR, 2.04; 95% CI, 1.01–4.12). Because of the low number of patients with stones with high bilirubin contents (N = 52), the power of these tests is much lower than in the primary hypothesis test and is reflected by the respective *P* values (Table 2). Gallstone number (*P* = .27) and weight (*P* = .37) were not associated with genotypes at the investigated loci.

Association With Gallstone Disease

As shown in Table 2, only *UGT1A1* (rs6742078, *P* = .018, recessive; OR, 1.3; 95% CI, 1.04–1.64) was also associated with overall gallstone risk in the total cohort comprising 2606 patients with surgery for symptomatic gallstone disease and 1121 matched sonographic gallstone-free controls. The frequency of the risk-genotype T/T was 12.8% in cases and 10.1% in controls. All other loci did not show nominal significance or suggestive evidence for association with the overall phenotype. All loci from Tables 1 and 2 from the meta-analysis of bilirubin loci[27] are listed together with their nominal significance in the 2007 genome-wide association scan for gallstone disease[15] in Supplementary Table 1. No additional significant associations are evident.

To obtain a better insight into the underlying risk structure, post hoc analyses were performed in patient subgroups. In a gender-specific post hoc analysis of *UGT1A1* locus, an association with gallstone disease risk was confined to men only ($P = 2.1 \times 10^{-7}$; $OR_{recessive}$, 2.34; 95% CI, 1.68–3.26) and was lacking in women (*P* = .47; $OR_{recessive}$, 1.10; 95% CI, 0.84–1.45) (Table 3). None of the other investigated loci showed significant differences in risk allele or genotype frequencies between men and women in the gallstone patients and control sample (data not shown). A total of 56 patients from the cohort underwent endoscopic retrograde cholangiopancreatography for bile duct stones. There was a trend for a higher frequency of the TT risk genotype in patients who underwent endoscopic retrograde cholangiopancreatography (18% vs 12% in patients without evidence of bile duct stones). However, this was not statistically significant in either the total cohort (*P* = .21) or in a sex-specific analysis (*P* = .94 for men, N = 13; and *P* = .11 in women, N = 43).

Replication in South American Samples

The earlier-described finding was replicated in a pooled case-control cohort from Argentina and Chile yielding a similar recessive genotypic OR of 2.19 (95% CI, 0.90–5.35; *P* = .046) and an allelic OR of 1.97 (95% CI, 1.18–3.28; *P* = .008) in the male-only analysis. The risk allele rs6742078 (T) and genotype T/T frequencies were not significantly different between female gallstone cases and controls (Table 4). The genotypic effect was consistent between both the Argentinean and Chilean part of the South American sample, as shown in Supplementary Table 2.

Association With Clinical Presentation

After the establishment of rs6742078 *UGT1A1* as a robust risk locus for gallstone bilirubin content and male gallstone disease, a further post hoc subgroup analysis was performed using the gallstone patients recruited in a population-based sample in Greifswald (N = 583). This sample, which included an approximately equal number of symptomatic (N = 301) and nonsymptomatic (N = 282) gallstone patients, revealed a disease association of

Table 4. Replication in South American Samples

	Genotype counts		Genotypes frequencies			
	Case	Control	Case	Control	OR	*P*
Male sex						
G/G	9	99	25.7%	47.7%		
G/T	18	86	51.4%	41.0%		
T/T	8	25	22.9%	11.9%	2.19 (0.90–5.35)[a]	.046[b]
Minor allele frequency						
T			48.6%	32.4%	1.97 (1.18–3.28)[c]	.008
Female sex						
G/G	96	138	54.9%	48.3%		
G/T	56	110	32.0%	38.5%		
T/T	23	38	13.1%	13.3%	0.98 (0.56–1.72)[a]	.96
Minor allele frequency						
T			29.1%	32.5%	0.85 (0.63–1.14)[c]	.28

NOTE. This was a pooled case-control study from Chile and Argentina.
[a]Genotypic OR for recessive model: T/T vs (G/G, G/T).
[b]Fisher exact test.
[c]Allelic odds ratio.

UGT1A1 rs6742078 for both patient groups. Here, the ORs for T/T genotype carriership were higher in symptomatic male gallstone cases (N = 98; OR, 3.25; 95% CI, 1.60–6.60; $P = 8.76 \times 10^{-4}$) than in nonsymptomatic male cases (N = 140; OR, 2.0; 95% CI, 0.98–4.05; $P = .023$), respectively (Table 3).

Combined Risk Assessment With ABCG8

The combined assessment of the established risk factor *ABCG8* (rs11887534) and *UGT1A1* (rs6742078) showed absence of genotypic interaction ($P = .763$) in the regression model, indicating multiplicative risk effects. The combined genotypic OR of the pooled heterozygotes and homozygotes for the *ABCG8* (rs11887534) risk allele and the homozygote *UGT1A1* (rs6742078) risk genotype in males was 5.22 (95% CI, 2.75–9.88; OR adjusted for age and body mass index, 5.91; 95% CI, 2.36–14.85). The population attributable risk of the combined risk factors *ACBG8* (rs11887534) and *UGT1A1* (rs6742078) was 21.2% and for the *ABCG8* risk factor was 11.2% in the absence of *UGT1A1* (rs6742078), and for the *UGT1A1* risk factor it was 9.9% in the absence of *ABCG8* (rs11887534) in males. In females, the population attributable risk of *ABCG8* was 11.7%.

Discussion

Gallstone Composition

In this report, we evaluated the 4 loci associated with serum bilirubin levels attaining genome-wide significance in a recent genome-wide meta-analysis[27] as predictors of gallstone composition and gallstone risk in a large German cohort of gallstone patients with known stone composition who underwent surgery (N > 1000),[30] the total sample of the Kiel gallstone patients who underwent surgery (N > 2600), and matched sonographic gallstone-free controls. Three (*UGT1A1*, *SLCO1B1*, and *SLCO1A2*) of 4 tested loci were associated significantly with the presence of bilirubin in gallstones in single-point analyses. In regression analyses, only *UGT1A1* and *SLCO1B1* were retained independently in the model owing to linkage disequilibrium ($r^2 = 0.66$) between markers in *SLCO1B1* and *SLCO1A2* (Supplementary Figure 1). Thus, *SLCO1B1* appears to be the phenotypically more relevant gene at this locus. The linkage disequilibrium between the 2 markers might indicate a functional relation or co-evolutionary selection within the solute carrier gene family. The frequency of the respective risk genotypes increased with higher bilirubin content in the stones, suggesting a robust association finding. However, only *UGT1A1* rs6742078 reached statistical significance in the high bilirubin content group—with the lack of formal significance most likely due to the low patient number (N = 52) in this group. For *LST-3TM12*, no association with stone composition was observed; allele and genotype frequencies were almost identical in the tested groups. It is thus unlikely that even in a larger patient sample, this locus might show significant association with the gallstone composition trait.

Association With Gallstone Disease

Although 3 of the reported serum bilirubin loci were associated with gallstone composition, only one, namely rs6742078 (*UGT1A1*), also was associated with overall gallstone risk. The tested variant rs6742078 previously was shown to be in high linkage disequilibrium ($r^2 = 0.88$) with the common functional TATA box TA repeat promoter variant *UGT1A1*28* that underlies Gilbert syndrome.[39,40] Gilbert syndrome is characterized by a mild, chronic unconjugated hyperbilirubinemia as a result of decreased hepatic bilirubin clearance in the absence of overt hemolysis.[41,42] An association of pigment gallstones with the promoter variation in the UDP-

glucuronosyltransferase 1A1 (*UGT1A1*) gene has been reported in patients with chronic hemolytic disorders[43,44] and cystic fibrosis.[45] Previous studies investigating the impact of *UGT1A1* variants on gallstone risk in unselected patients have yielded conflicting results. A Greek study in 198 gallstone carriers vs 152 controls detected association of the *UGT1A1*28* genotypes 6/7 and 7/7 (OR, 2.2; 95% CI, 1.37–3.61; $P = .001$; and OR, 2.1; 95% CI, 1.17–3.77, $P = .013$, respectively) with cholelithiasis risk.[46]

In contrast, a case-control study embedded in the recent meta-analysis by Johnson et al[27] that compared 515 gallstone carriers from the Framingham Study with 3783 self-reported gallstone-free controls and 161 gallstone carriers from the Rotterdam Study with 5813 controls did not detect association with single nucleotide polymorphisms in *UGT1A1*.

In this study, a strong and highly significant association with overall gallstone risk that was confined exclusively to males was seen with an OR of greater than 2.3 for homozygote carriers of the T/T risk genotype ($P = 2.14\times10^{-7}$). This sex-specific effect was confirmed in an independent patient sample from Argentina and Chile. The results of Borgna–Pignatti et al[47] who failed to detect an increased gallstone risk for *UGT1A1*28* in a female-only study in β-thalassemia are consistent with this sex-specific effect. Similarly, the study by Johnson et al[27] was limited in gallstone patient number, and patients were mostly of female sex, thus further reducing the effect size. The observed sex difference for gallstone risk is also consistent with the higher serum bilirubin levels in men as compared with women and the higher frequency of Gilbert syndrome in men.[48–50] In rat liver, lower bilirubin UDP-glucuronosyltransferase activity was observed in male as compared with female rats by Muraca and Fevery[51] already in 1984. In their study, gonadectomy decreased enzyme activity in female rats and increased it in male rats, suggesting that sex hormones may be an important regulator of bilirubin conjugation. Recently, sex-specific patterns of UDP-glucuronosyltransferase messenger RNA expression were established in mice.[52] UGT1A1 and a range of ABC membrane transporters are regulated by nuclear receptors with overlapping specificities in steroid-sensitive tissues, which may be the molecular basis of different enzyme and transporter induction patterns between males and females.[53–55]

Importantly, the increased gallstone risk conferred by the *UGT1A1* variant is not restricted to classic pigment stones. In fact, as noted in Table 2, the vast majority of gallstones present in homozygous risk allele carriers contain in excess of 90% cholesterol and, depending on the semantics, would be otherwise labeled as *cholesterol stones*. This effect would be consistent with the classic pathophysiological model of bilirubin serving as a nucleation core for the development of gallstones in general.[56–59] Along a more speculative note, the observed higher gallstone disease risk conferred by rs6742078 *UGT1A1* in male patients who underwent surgery for symptomatic gallstone disease (OR, 3.25) as compared with male patients with merely sonographic evidence of nonsymptomatic gallstones (OR, 2.0) in the German population-based SHIP cohort could reflect this pathomechanism with an increased abundance of nucleation cores and therefore a higher proportion of symptomatic stones.[60,61]

Association of SLCO1B1 and SLCO1A2 With Bilirubin Presence in Gallstones

SLCO1B1 and *SLCO1A2* (solute carrier organic anion transporter family, members 1B1 and 1A2) are members of the organic anion transporter gene family. One other member of this gene family (*SLC10A2*) has been reported previously as a risk factor for gallstone disease ($P = .007$; OR, 2.04),[62] but did not meet the genome-wide significance threshold for bilirubin levels in the meta-analysis by Johnson et al[27] and thus was not included in this experiment. Several studies have shown SLCO1B1 to be expressed exclusively on the basolateral membrane of hepatocytes.[63,64] SLCO1B1 is known to transport a broad variety of endogenous and exogenous substrates including taurocholate,[64] and mediates the cellular uptake of bilirubin and its glucuronide conjugates.[65] SLCO1A2 is expressed in the liver intestine, kidney, lung, and testes, with the highest expression being found in the brain.[66] Although some studies have shown expression of SLCO1A2 in the liver, immunohistochemical staining of proteins did not find any of these transporters in hepatocytes, but rather in cholangiocytes of the liver.[67] SLCO1A2 transports more amphipathic substrates, including bile salts[66] and bilirubin.[68] Several nonsynonymous polymorphisms have been identified in both genes, which alter the transport of several natural and xenobiotic substrates.[69–71] The exact mechanisms by which the variants in these genes contribute to increased bilirubin concentrations in serum and gallstones are not clear and will require further investigation. In accordance with our data on overall disease risk, variants in *SLCO1B1* were not associated with gallstone risk in a recent study from India[72] and in the nested phenotypic study of the meta-analysis.[27]

In conclusion, this study shows how genome-wide association data of a laboratory value, such as the bilirubin level, can be translated into a predictor of clinically relevant traits (ie, for gallstone composition and gallstone risk). Even if 2 of the variants do not translate into risk factors for overall gallstone disease risk, gallstone composition will likely be an important aspect of potential future nonsurgical interventions for gallstone treatment or prevention. In addition, the very clear-cut and replicated risk assignment of *UGT1A1* variants to males highlights the importance of considering the interaction between genes and sex in the etiology of common complex diseases[73] and has intriguing implications for the understanding of the

sex-specific physiology of bilirubin and bile acid metabolism. As a result of the overall field of gallstone genetics,[19,74,75] an increasingly clear picture on the genetic landscape of gallstone disease risk and composition is emerging. Notably, the 2 major genetic risk factors (*ABCG8* and *UGT1A1*) contribute a population attributable risk of 21.2% in males to overall gallstone risk, underlining the advanced stage of the study of gallstone risk in human beings.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.09.003.

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Conflicts of interest

The authors disclose no conflicts.

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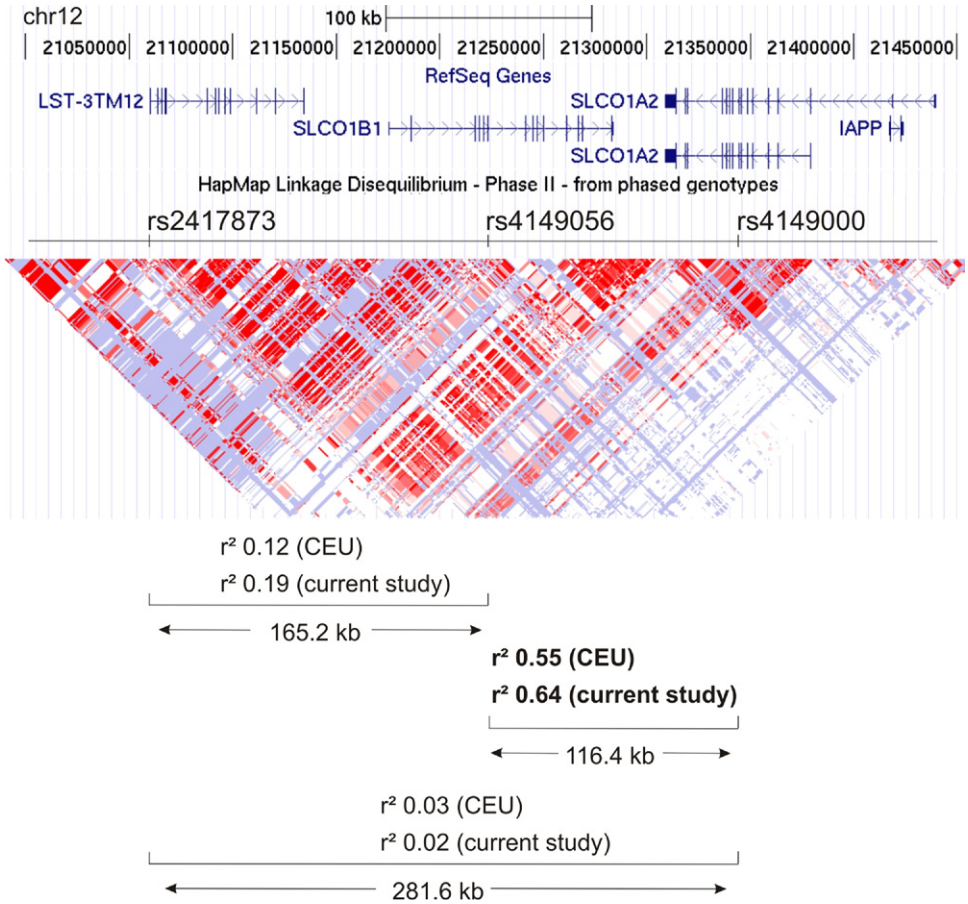

Supplementary Figure 1. Overview of the physical and genetic structure of the *LST-3TM12-SLCO1B-SLCO1A2* gene region on chromosome 12. The physical position of the investigated single nucleotide polymorphisms and a schematic illustration of the gene structure are shown in the *top panel.* The coordinates refer to the NCBI genome assembly build 36. The *middle panel* gives an overview of the linkage disequilibrium structure of the locus (D′) as generated by the University of California at Santa Cruz (UCSC) Genome Browser from the HapMap data (The International HapMap Consortium; www.hapmap.org). The *lower panel* shows the respective physical distances and r^2 values between the investigated SNP markers in the 3 genes of interest as calculated by Haploview (Barrett JC, Fry B, Maller J, et al. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263–265).

Supplementary Table 1. Side-by-Side Overview of the Loci From the Recent Meta-Analysis of Bilirubin Levels and the Results From an Earlier Genome-Wide Study for These Loci

Bilirubin meta-analysis				Gallstone Genome-wide Association Study			
Chromosome	Rs number	Gene	*P* value	SNP	Rs number	Distance, bp	*P* value
2	rs6742078	*UGT1A1*	5.0×10^{-324}	SNP_A-1923514	rs887829	−4.069	.1351
12	rs4149056	*SLCO1B1*	6.70×10^{-13}	SNP_A-4272900	rs11045819	−1.736	.2458
12	rs2417873	*LST-3TM12*	1.50×10^{-9}	SNP_A-2155956	rs2417876	−17.586	.1367
12	rs4149000	*SLCO1A2*	2.70×10^{-8}	SNP_A-2288781	rs16923647	3.398	.6681
11	rs16928809	*SLC22A18*	1.10×10^{-7}	SNP_A-2163041	rs451443	−106	.1443
2	rs12714207	*KRCC1*	5.30×10^{-7}	SNP_A-4259284	rs6735537	−4.803	.9297
6	rs12206204	*HIST1H2BC*	7.50×10^{-7}	SNP_A-1834929	rs933199	−4.089	.8552
4	rs1986655	*FAT4*	2.00×10^{-6}	SNP_A-1837818	rs1986655	0	.7927
7	rs4236644	*SEMA3C*	2.10×10^{-6}	SNP_A-1990316	rs2367090	10.590	.3356
13	rs4773330	*ARHGEF7*	7.70×10^{-6}	SNP_A-4241050	rs7334196	4.472	.4161
15	rs7173819	*SPATA8*	1.20×10^{-5}	SNP_A-2054208	rs758810	−2.432	.5535
9	rs12337836	*PRG-3*	1.30×10^{-5}	SNP_A-2064982	rs7855910	−531	.4611
16	rs12923103	*LOC283902*	1.40×10^{-5}	SNP_A-4245567	rs10500554	2.772	.9408
6	rs9380833	*KCNK5*	1.60×10^{-5}	SNP_A-1951752	rs2815090	−2.646	.5536
18	rs4410172	*SETBP1*	1.90×10^{-5}	SNP_A-1905954	rs2359894	−6.456	.4168
4*	rs2375971	*intergenic*	4.70×10^{-7}	SNP_A-2086119	rs2375953	−7.402	.8607
4*	rs2710818	*FAT4*	4.10×10^{-6}	SNP_A-4203744	rs2710818	0	.3315
1*	rs714839	*FMO4*	5.20×10^{-6}	SNP_A-1831610	rs12089574	1.231	.1818
1*	rs6655987	*RCSD1*	6.40×10^{-6}	SNP_A-1834927	rs7511792	285	.4373
10*	rs12414547	*C10orf18*	6.80×10^{-6}	SNP_A-1874639	rs9423726	−869	.535
11*	rs7120248	*MOGAT2*	9.70×10^{-6}	SNP_A-2136833	rs11236497	1.654	.9404
5*	rs10476123	*intergenic*	1.10×10^{-5}	SNP_A-2085336	rs11953588	−1.581	.6903

NOTE. The genome-wide gallstone data were generated using an earlier scanning instrument (Affymetrix 500K) on a limited number of patients (N = 280 patients who underwent surgery for gallstone disease). For each locus from the meta-analysis, the nearest marker and the nominal significance level from the gallstone 500K study is listed. Genomic coordinates refer to the NCBI build 36.6. The meta-analysis data stem from the reports by Johnson et al[27] and the results from an earlier genome-wide study from Buch et al.[15]
SNP, single nucleotide polymorphism.

Supplementary Table 2. Raw Data From Both Parts of the South American Replication Samples Indicating a Similar Genotypic Effect in Both Samples

	Genotype counts		Genotypes frequencies			
Male sex	Case	Control	Case	Control	$OR_{recessive}$	*P*
Chile						
G/G	7	76	28%	48.4%		
G/T	13	66	52%	42%		
T/T	5	15	20%	9.6%	2.36 (0.78–7.22)	.160
Argentina						
G/G	2	23	20%	43.4%		
G/T	5	20	50%	37.7%		
T/T	3	10	30%	18.9%	1.84 (0.40–8.40)	.417

NOTE. The individual post hoc analyses were not statistically significant, indicating insufficient power in the separated analyses. Fisher exact test P values are reported.