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Genome-wide association meta-analysis for total serum bilirubin levels

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Variation in serum bilirubin is associated with altered cardiovascular disease risk and drug metabolism. We aimed to identify genetic contributors to variability in serum bilirubin levels by combining results from three genome-wide association studies (Framingham heart study, $n = 3424$; Rotterdam study, $n = 3847$; Age, Gene, Environment and Susceptibility-Reykjavik, $n = 2193$). Meta-analysis showed strong replication for a genetic influence on serum bilirubin levels of the *UGT1A1* locus ($P < 5 \times 10^{-324}$) and a 12p12.2 locus. The peak signal in the 12p12.2 region was a non-synonymous SNP in *SLCO1B1* (rs4149056, $P = 6.7 \times 10^{-13}$), which gives rise to a valine to alanine amino acid change leading to reduced activity for a hepatic transporter with known affinity for bilirubin. There were also suggestive associations with several other loci. The top variants in *UGT1A1* and *SLCO1B1* explain ~18.0 and ~1.0% of the variation in total serum bilirubin levels, respectively. In a conditional analysis adjusted for individual genotypes for the top *UGT1A1* variant, the top *SLCO1B1* variant remained highly significant ($P = 7.3 \times 10^{-13}$), but no other variants achieved genome-wide significance. In one of the largest genetic studies of bilirubin to date ($n = 9464$), we confirm the substantial genetic influence of *UGT1A1* variants, consistent with past linkage and association studies, and additionally provide strong evidence of a role for allelic variation in *SLCO1B1*. Given the involvement of bilirubin in a number of physiological and disease processes, and the roles for *UGT1A1* and *SLCO1B1* in drug metabolism, these genetic findings have potential clinical importance. In analyses for association with gallbladder disease or gallstones, top bilirubin SNPs in *UGT1A1* and *SLCO1B1* were not associated.

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INTRODUCTION

Bilirubin is excreted as a major component of bile as the breakdown product of heme catabolism. Levels of serum bilirubin are significantly elevated in a number of diseases associated with jaundice, including gallstone disease. Bilirubin is an effective endogenous anti-oxidant (1,2), raising the prospect that increased levels in the nearly normal range may be partially protective against some diseases including atherosclerosis (3). An inverse relationship between bilirubin levels and atherosclerosis risk has been observed (4,5) and supported by meta-analysis (6), suggesting that it could be a protective factor. Family-based studies indicate that bilirubin levels are heritable and suggest that a single gene locus accounts for a significant proportion of variation (4,7–9). Furthermore, linkage studies identified the *UGT* locus on chromosome 2q37.1 as the major gene locus for bilirubin levels, but did not identify and replicate any additional loci (7,8). The UGT1A1 enzyme transcribed from 2q37.1 glucoronidates unconjugated bilirubin in hepatocytes prior to elimination, and rare mutations in this gene are responsible for Crigler–Najjar syndrome Types I and II. Further studies have shown that a common, functional TATA box TA repeat variant, *UGT1A1*28* (rs45557732), reduces UGT1A1 production and activity and is strongly associated with hyperbilirubinemia (10–12).

A number of clinical genetic disorders indicate additional gene influences on bilirubin physiology including Dubin–Johnson syndrome (*MRP2*/*ABCC2*), benign recurrent intrahepatic cholestasis and progressive familial intrahepatic cholestasis (*ABCB4*, *ABCB11*, *ATP8B1*). This suggests that common genetic variation in genes beyond *UGT1A1* that relate to bilirubin formation, transport, metabolism and excretion may additionally contribute to inter-individual variation in bilirubin levels. Hepatocellular pathways relating to bilirubin processing are also responsible for processing of a variety of endogenous substrates (steroids) and xenobiotics, including major drug treatments such as chemotherapeutics, statins and anti-retrovirals. Thus, there is considerable potential that gene associations with bilirubin levels may also have pharmacogenetic implications. This hypothesis is supported by pharmacogenetic associations of *UGT1A1* polymorphisms with myelosuppression and severe diarrhea in patients receiving irinotecan treatment, which eventually led to FDA-recommended changes in drug labeling (13). Furthermore, elevated bilirubin levels have been associated with gallstones (14), and prior studies have suggested a positive association of *UGT1A1* polymorphism with gallstones, particularly in persons with anemias (15).

To search for and confirm major genetic influences on bilirubin, we completed a meta-analysis of genome-wide association studies (GWAS) for total serum bilirubin levels in 9464 individuals from three prospective cohort studies: the Framingham Heart Study (FHS, $n = 3424$), the Rotterdam study (RS, $n = 3847$) and the Age, Gene, Environment and Susceptibility-Reykjavik study (AGES-Reykjavik, $n = 2193$). We further conducted analysis for association of top bilirubin SNPs with gallbladder disease or the presence of gallstones.

RESULTS

Total serum bilirubin levels among study participants

Characteristics of the study populations are presented in Supplementary Material, Tables S1 and S2. Bilirubin levels were measured in 3424 participants in FHS, 3847 participants in RS and 2193 participants in AGES-Reykjavik and differed between the studies, with higher levels in the RS. RS participants were significantly older than AGES-Reykjavik participants who were in turn older than FHS participants at the time of bilirubin measurement. All studies showed significantly higher bilirubin levels in males than females, as expected. Age- and sex-adjusted and multivariable-adjusted analyses (covariates described in Materials and Methods) were conducted. A subset of participants was used in multivariable analysis due to the availability of covariate information ($n = 3261$ FHS; $n = 3534$ RS, $n = 2193$ AGES-Reykjavik). Bilirubin levels and age and sex distributions did not differ significantly between the samples in the age- and sex-adjusted and multivariable analyses. The top GWA results were also similar for the age- and sex-adjusted models and the multivariable-adjusted modes. We present the results adjusted for age and sex.

GWA results for total serum bilirubin levels

We tested 2 555 103 imputed autosomal SNPs for association with serum bilirubin levels among FHS, RS and AGES-Reykjavik participants, representing three distinct GWA analyses. The independent GWAS, and the meta-analysis, did not show significant genomic inflation as seen in the QQ′ plot in Supplementary Material, Figure S1 (FHS $\lambda = 1.01$; RS $\lambda = 1.02$; AGES-Reykjavik $\lambda = 1.03$; meta-analysis $\lambda = 0.99$). Association results from the meta-analysis are displayed in Manhattan plots in Figure 1. We used a pre-determined genome-wide significance threshold of 5.0×10^{-8}. Results for the top loci by *P*-value in meta-analysis, with the most significant SNP per locus, are presented in Table 1. Two loci reached genome-wide significance in the meta-analysis: *UGT1A1* (chr 2q37.1) and *SLCO1B1* (chr 12p12.2) showed consistent results across the three populations in that the same allele was associated with bilirubin levels in the same direction. In the *UGT1A1* locus, rs6742078 has the most significant *P*-value ($P < 5.0 \times 10^{-324}$ in meta-analysis, $P = 9.2 \times 10^{-154}$ in FHS, $P = 2.6 \times 10^{-182}$ in RS, $P = 1.2 \times 10^{-92}$ in AGES-Reykjavik), whereas rs4149056 has the most significant *P*-value in the *SLCO1B1* locus ($P = 6.7 \times 10^{-13}$ in meta-analysis, $P = 4.0 \times 10^{-5}$ in FHS, $P = 1.3 \times 10^{-6}$ in RS, $P = 4.7 \times 10^{-4}$ in AGES-Reykjavik). There were four most significant *UGT1A1* SNPs in the proximal promoter region and intron 1, physically close to the *UGT1A1*28* TATA box polymorphism, all of which showed similar significant signals with nearly identical β coefficients (rs6742078, rs887829, rs4148324, rs4148325). These four SNPs are in perfect LD in the HapMap CEU population ($D' = 1.0$, $r^2 = 1.0$). These SNPs accounts for approximately 17.5, 18.1 and 16.7% of the variation in total serum bilirubin levels, in FHS, RS and AGES-Reykjavik, respectively. The most significant SNP (rs4149056) in *SLCO1B1* was located in exon 6 and results in an amino acid change (Val174Ala). This SNP

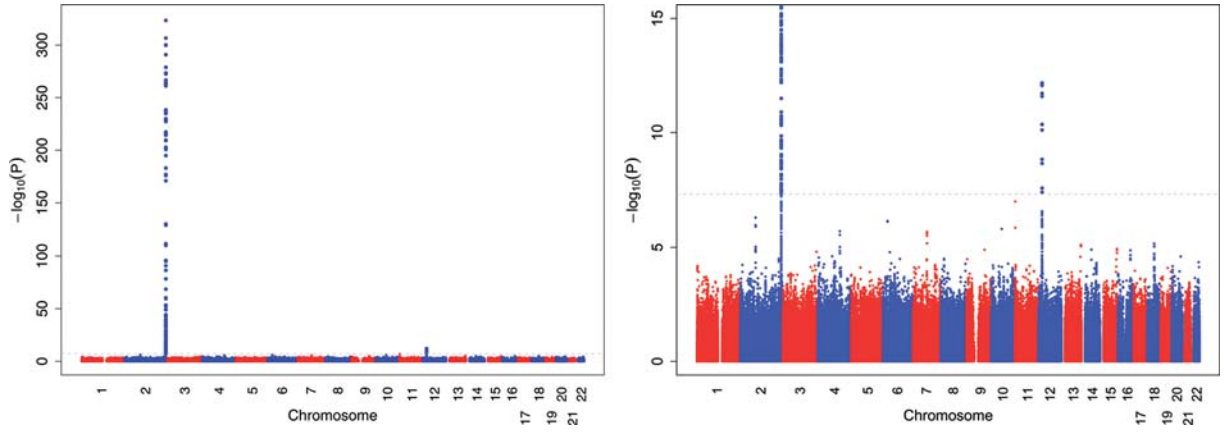

Figure 1. Genome-wide -$\log_{10}$ *P*-value plots for log total serum bilirubin levels (age–sex model) in meta-analysis including FHS, RS and AGES-Reykjavik. Full results are shown in the left panel and results with $P \geq 10^{-15}$ are shown in the right panel.

accounts for approximately 0.6, 0.5 and 0.5% of the variation in total serum bilirubin levels, in FHS, RS and AGES-Reykjavik, respectively. There were also SNPs in partial LD with rs4149056 and reaching genome-wide significance in other genes in the 12p12.2 region that contains *SLCO1B1*, including in *LST-3TM12* (rs2417873, $P = 1.5 \times 10^{-9}$, $r^2 = 0.06$ with rs4149056) and *SLCO1A2* (rs4149000, $P = 2.7 \times 10^{-8}$, $r^2 = 0.56$ with rs4149056) (Fig. 2).

In age–sex-adjusted regression models including these three 12p12.2 SNPs, rs4149056 (FHS $P = 0.11$; RS $P = 0.03$; AGES $P = 8.8 \times 10^{-5}$; meta-analysis $P = 6.3 \times 10^{-5}$) and rs2417873 (FHS $P = 2.3 \times 10^{-6}$; RS $P = 0.02$; AGES $P = 0.05$; meta-analysis $P = 1.2 \times 10^{-7}$), but not rs4149000 (FHS $P = 0.23$; RS $P = 0.35$; AGES $P = 0.03$; meta-analysis $P = 0.70$), were independently associated with bilirubin levels. We did not find strong evidence for gene–gene interaction between *UGT1A1* (rs6742078) and *SLCO1B1* (rs4149056) (FHS $P = 0.40$; RS $P = 0.39$; AGES $P = 0.48$) or for gene–gender interaction for *UGT1A1* (rs6742078: FHS $P = 0.03$; RS $P = 0.67$; AGES $P = 0.99$) or *SLCO1B1* (rs4149056: FHS $P = 0.40$; RS $P = 0.60$; AGES $P = 0.87$). Other loci had SNPs that approached but did not exceed the genome-wide significance threshold (*SLC22A18*, rs16928809, $P = 1.1 \times 10^{-7}$; the *KRCC1* region at 2p11.2, rs12714207, $P = 5.3 \times 10^{-7}$; a histone gene cluster at 6p22.1 near *HFE*, rs12206204, $P = 7.5 \times 10^{-7}$). We provide meta-analysis results for SNPs with $P < 5.0 \times 10^{-5}$ in Supplementary Material, Table S3. In formal tests for homogeneity of the effect size among studies for the top associated SNPs shown in Table 1, there was no significant evidence for heterogeneity among the studies (data not shown).

GWA results conditional on UGT1A1 (rs6742078) genotype

Because of the substantial proportion of variation in bilirubin levels explained by *UGT1A1*, we sought to determine whether additional loci remained significant after accounting for the *UGT1A1* effect. We added a covariate to the regression model representing *UGT1A1* genotype at one of the most highly associated SNPs (rs6742078) and performed conditional analysis for each cohort for all imputed SNPs and conducted subsequent meta-analysis (Table 2). The *SLCO1B1* gene variant (rs4149056, T > C) that changes amino acid 174 from valine to alanine showed a similar genome-wide significant signal to that observed in unconditional analysis. This SNP reached genome-wide significance after adjusting for *UGT1A1* genotype (FHS: $P = 4.5 \times 10^{-5}$, RS: $P = 7.8 \times 10^{-9}$, AGES-Reykjavik: $P = 1.2 \times 10^{-4}$, meta-analysis: $P = 7.3 \times 10^{-13}$). The most significant SNP in the *UGT1A1* region in conditional meta-analysis was ~2 kb upstream of *UGT1A5* and ~52 kb upstream from *UGT1A1* (rs4233633, FHS: $P = 2.0 \times 10^{-3}$, RS: $P = 2.9 \times 10^{-5}$, AGES-Reykjavik: $P = 0.43$, meta-analysis, $P = 5.3 \times 10^{-5}$). This indicates that our conditional analysis likely accounted for most, but not all, gene effects in this region. No additional loci reached genome-wide significance in the conditional analysis as compared with the initial analysis.

Association analysis with gallbladder disease etiology

We tested for association of top SNPs with gallbladder disease by routine exam questionnaire (FHS: 515 cases, 3783 controls) or with cholelithiasis-related admission (RS: 161 cases, 5813 controls). The top bilirubin SNPs in *UGT1A1* (rs6742078, $P = 0.20$) and *SLCO1B1* (rs4149056, $P = 0.42$) were not significantly associated with a history of gallbladder disease or gallbladder removal in FHS. Likewise, the *UGT1A1* (rs6742078, $P = 0.65$) and *SLCO1B1* (rs4149056, $P = 0.59$) SNPs were not significantly associated with evidence for cholelithiasis in RS.

Results for bilirubin association at *a priori* candidate loci

Based on previous genetic studies and bilirubin physiology, we derived an *a priori* list of 13 autosomal candidate genes, in addition to *UGT1A1* and *SLCO1B1*. Most of the genes on the list have not previously been studied in large samples for genetic associations with bilirubin levels and were included for reasons relating to bilirubin physiology, or because rare mutations in the genes cause bilirubinemias. The most significantly associated SNPs within 60 kb of each candidate are

Table 1. Associations of top SNPs ($P < 2.0 \times 10^{-5}$) with log total serum bilirubin levels

SNP information[a]	Modeled allele		FHS	RS	AGES-Reykjavik	Combined (RS, FHS, AGES-Reykjavik)
Loci with SNPs $< 5.0 \times 10^{-8}$						
rs6742078 (G>T)	T	*P*-value	9.2×10^{-154}	2.6×10^{-182}	1.2×10^{-92}	$< 5.0 \times 10^{-324}$
Chr 2: 234 337 378		β[b](se)	+0.239 (0.009)	+0.230 (0.008)	+0.238 (0.011)	
Gene: *UGT1A1*		MAF	32.0%	31.4%	32.2%	
rs4149056 (T>C), V174A	C	*P*-value	4.0×10^{-5}	1.3×10^{-6}	4.7×10^{-4}	6.7×10^{-13}
Chr 12: 21 222 816		β[b](se)	+0.05 (0.01)	+0.05 (0.01)	+0.05 (0.02)	
Gene: *SLCO1B1*		MAF	15.3%	15.2%	16.2%	
rs2417873 (G>A)	A	*P*-value	2.2×10^{-7}	0.0028	0.021	1.5×10^{-9}
Chr 12: 21 057 590		β[b](se)	+0.05 (0.01)	+0.03 (0.01)	+0.03 (0.01)	
Gene: *LST-3TM12*		MAF	28.0%	25.0%	20.9%	
rs4149000 (C>T)	T	*P*-value	7.9×10^{-5}	1.2×10^{-5}	0.396	2.8×10^{-8}
Chr 12: 21 339 264		β[b](se)	+0.06 (0.01)	+0.05 (0.01)	+0.01 (0.01)	
Gene: *SLCO1A2*		MAF	12.2%	13.7%	12.7%	
Loci with SNPs $> 5.0 \times 10^{-8}$						
rs16928809 (G>A)	A	*P*-value	7.5×10^{-4}	4.0×10^{-5}	0.133	1.1×10^{-7}
Chr 11: 2 893 528		β[b](se)	+0.06 (0.02)	+0.06 (0.01)	+0.03 (0.02)	
Gene: *SLC22A18*		MAF	10.9%	9.2%	8.8%	
rs12714207 (T>C)	T	*P*-value	0.126	2.7×10^{-3}	3.7×10^{-5}	5.3×10^{-7}
Chr 2: 88 096 908		β[b](se)	−0.03 (0.02)	−0.03 (0.01)	−0.05 (0.01)	
Gene: *KRCC1*		MAF	34.2%	32.3%	32.5%	
rs12206204 (C>T)	T	*P*-value	0.011	3.0×10^{-5}	0.136	7.5×10^{-7}
Chr 6: 26 224 961		β[b](se)	+0.14 (0.05)	+0.18 (0.04)	+0.09 (0.06)	
Gene: *histone cluster*		MAF	1.5%	1.8%	0.9%	
rs1986655 (A>G)	A	*P*-value	0.0013	0.048	2.0×10^{-4}	2.0×10^{-6}
Chr 4: 126 212 952		β[b](se)	−0.04 (0.02)	−0.02 (0.01)	−0.06 (0.02)	
Gene: *intergenic*		MAF	15.0%	16.6%	12.4%	
rs4236644 (G>A)	A	*P*-value	0.010	0.021	1.4×10^{-4}	2.1×10^{-6}
Chr 7: 80 437 293		β[b](se)	−0.04 (0.02)	−0.02 (0.01)	−0.05 (0.01)	
Gene: *SEMA3C*		MAF	24.9%	26.1%	30.3%	
rs4773330 (G>A)	A	*P*-value	0.021	0.0013	0.028	7.7×10^{-6}
Chr 13: 110 616 833		β[b](se)	−0.04 (0.02)	−0.04 (0.01)	−0.03 (0.02)	
Gene: *ARHGEF7*		MAF	10.7%	11.5%	15.4%	
rs7173819 (A>G)	A	*P*-value	0.0015	0.0083	0.073	1.2×10^{-5}
Chr 15: 95 253 682		β[b](se)	+0.05 (0.01)	+0.03 (0.01)	+0.03 (0.02)	
Gene: *intergenic*		MAF	12.6%	13.7%	9.5%	
rs12337836 (C>A)	A	*P*-value	0.540	2.8×10^{-4}	0.0049	1.3×10^{-5}
Chr 9: 103 116 417		β[b](se)	+0.02 (0.03)	+0.06 (0.02)	+0.07 (0.02)	
Gene: *PRG-3, BAAT*		MAF	6.7%	9.5%	5.5%	
rs12923103 (G>A)	A	*P*-value	0.168	7.5×10^{-5}	0.081	1.3×10^{-5}
Chr 16: 71 936 435		β[b](se)	+0.02 (0.01)	+0.03 (0.01)	+0.02 (0.01)	
Gene: *intergenic*		MAF	33.0%	30.7%	33.9%	
rs9380833 (C>T)	T	*P*-value	0.052	7.0×10^{-4}	0.054	1.6×10^{-5}
Chr 6: 39 247 442		β[b](se)	+0.07 (0.04)	+0.08 (0.02)	+0.08 (0.04)	
Gene: *KCNK5*		MAF	2.7%	3.0%	2.2%	
rs4410172 (G>C)	C	*P*-value	0.031	1.3×10^{-4}	0.317	1.9×10^{-5}
Chr 18: 40 315 726		β[b](se)	+0.02 (0.01)	+0.03 (0.01)	+0.01 (0.01)	
Gene: *BC051727*		MAF	25.4%	24.1%	23.1%	

[a]Major/minor alleles on forward strand of human genome reference sequence of NCBI build 36.2, the minor allele was modeled.
[b]The β coefficient represents the change in log-transformed bilirubin with one additional copy of the allele modeled, adjusting for age and sex. 'se' is the standard error of the β coefficient.

presented in Table 3. Except *UGT1A1* and *SLCO1B1*, no additional candidate gene regions had significant SNPs with $P < 0.05$ in all three studies; however, several SNPs had $P < 0.05$ in two studies with consistent direction of effect across all three studies (*GSTA4* region, *NR1H4* and *BLVRB*) (Table 3). After applying a resampling approach that tested 1000 permutations each of equal numbers of consecutive SNPs throughout the genome, the only candidate regions with significant SNPs beyond *UGT1A1* and *SLCO1B1* were the *GSTA4* region (rs10484408, $P = 0.039$) and the *SLC10A2* region (rs16962449, $P = 0.038$). Thus, the *GSTA4* and *SLC10A2* regions may be interesting to examine in further studies.

DISCUSSION

In a GWA meta-analysis including results from three studies on 9464 individuals and 2.5 million SNPs, we confirmed the substantial contribution of *UGT1A1* on human serum bilirubin levels and additionally found that a number of transporter genes, including *SLCO1B1*, *LST-3TM12* and *SLC22A18* may also influence variation in bilirubin levels. This is the largest

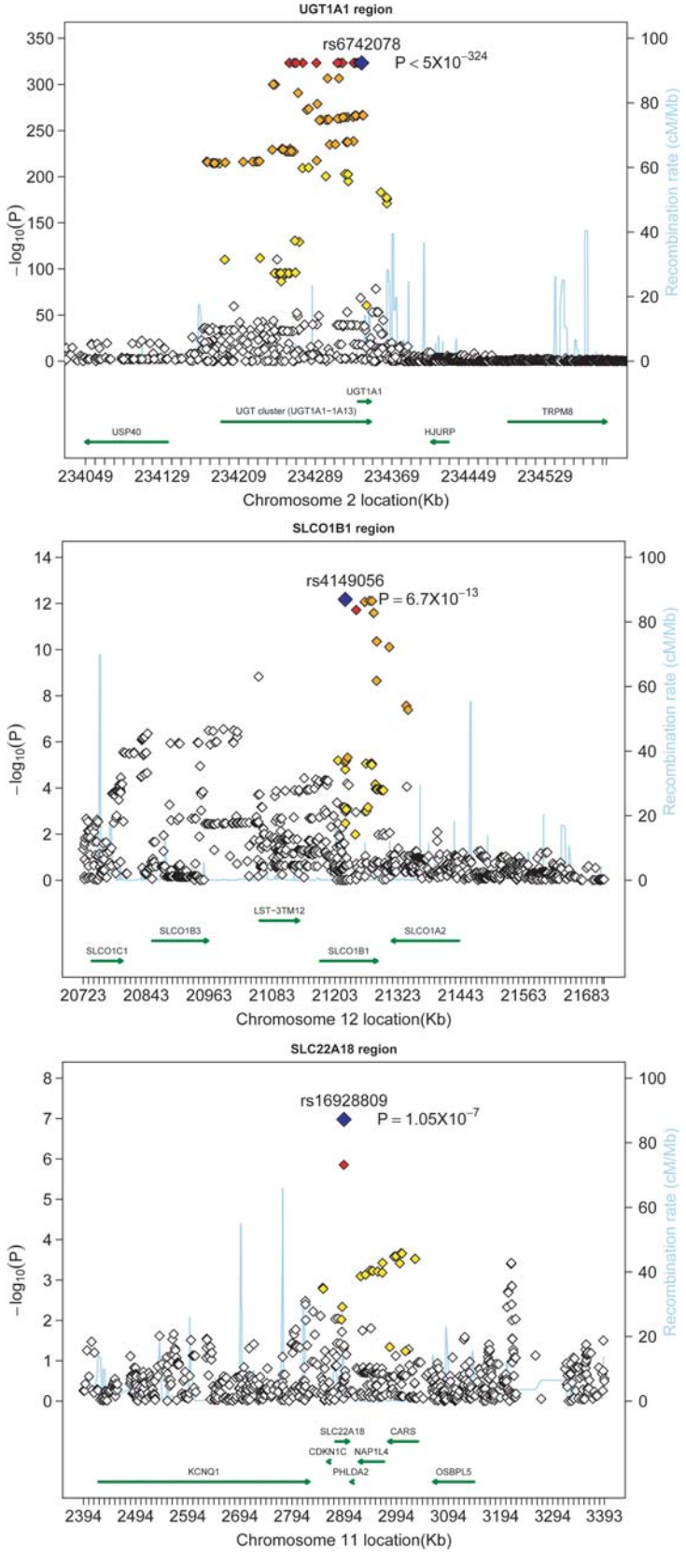

Figure 2. Regional view of selected loci (*UGT1A1, SLCO1B1, SLC22A18*) for associations with total serum bilirubin levels in the full meta-analysis, generated with SNAP (24). Top SNPs and *P*-values in each region are indicated (blue diamonds). Colour coding indicates the strength of LD of each SNP with the top SNP in each region: red ($r^2 \geq 0.8$), orange ($r^2 \geq 0.5$), yellow ($r^2 \geq 0.2$), white ($r^2 < 0.2$).

Table 2. Associations of top SNPs ($P < 2.0 \times 10^{-5}$) with log total serum bilirubin levels conditional on UGT1A1 genotype

SNP information[a]	Modeled allele		FHS	RS	AGES-Reykjavik	Combined (RS, FHS, AGES-Reykjavik)
Loci with SNPs $< 5.0 \times 10^{-8}$						
rs4149056 (T>C), V174A	C	*P*-value	4.5×10^{-5}	7.8×10^{-9}	1.2×10^{-4}	7.3×10^{-13}
Chr 12: 21 222 816		β^b (se)	+0.04 (0.01)	+0.06 (0.01)	+0.05 (0.01)	
Gene: *SLCO1B1*		MAF	15.3%	15.1%	16.2%	
Loci with SNPs $> 5.0 \times 10^{-8}$						
rs2375971 (C>T)	T	*P*-value	0.82	1.6×10^{-6}	0.047	4.7×10^{-7}
Chr 4: 36 204 248		β^b (se)	+0.02 (0.08)	+0.03 (0.007)	+0.02 (0.01)	
Gene: *intergenic*		MAF	43.8%	42.2%	43.0%	
rs4149000 (C>T)	T	*P*-value	3.8×10^{-4}	9.8×10^{-7}	0.346	1.4×10^{-6}
Chr 12: 21 339 264		β^b (se)	+0.04 (0.01)	+0.05 (0.01)	+0.01 (0.02)	
Gene: *SLCO1A2*		MAF	12.2%	13.7%	12.7%	
rs12206204 (C>T)	T	*P*-value	0.06	3.7×10^{-5}	0.047	3.8×10^{-6}
Chr 6: 26 224 961		β^b (se)	+0.08 (0.04)	+0.15 (0.04)	+0.11 (0.06)	
Gene: *histone cluster*		MAF	1.5%	1.8%	0.9%	
rs2710818 (T>C)	T	*P*-value	0.08	0.0029	1.5×10^{-4}	4.1×10^{-6}
Chr 4: 126 194 268		β^b (se)	−0.01 (0.01)	−0.02 (0.01)	−0.05 (0.01)	
Gene: *intergenic*		MAF	24.4%	25.1%	19.7%	
rs714839 (C>T)	T	*P*-value	0.23	3.6×10^{-4}	0.0018	5.2×10^{-6}
Chr 1: 169 565 564		β^b (se)	−0.01 (0.01)	+0.03 (0.01)	+0.03 (0.01)	
Gene: *FMO4*		MAF	40.4%	39.1%	40.4%	
rs6655987 (G>T)	T	*P*-value	0.81	7.4×10^{-4}	0.0015	6.4×10^{-6}
Chr 1: 165 918 611		β^b (se)	−0.00 (0.01)	+0.03 (0.01)	+0.05 (0.01)	
Gene: *RCSD1*		MAF	13.0%	15.4%	13.4%	
rs12414547 (T>C)	T	*P*-value	0.23	3.0×10^{-5}	0.037	6.8×10^{-6}
Chr 10: 5 778 854		β^b (se)	−0.03 (0.03)	+0.10 (0.02)	+0.08 (0.04)	
Gene: *C10orf18*		MAF	2.2%	2.5%	1.5%	
rs2417873 (G>A)	A	*P*-value	5.4×10^{-7}	4.7×10^{-4}	0.030	6.9×10^{-6}
Chr 12: 21 057 590		β^b (se)	+0.04 (0.01)	+0.03 (0.01)	+0.03 (0.01)	
Gene: *LST-3TM12*		MAF	28.0%	25.0%	20.9%	
rs7120248 (G>A)	A	*P*-value	0.84	4.5×10^{-4}	0.0043	9.7×10^{-6}
Chr 11: 75 091 934		β^b (se)	+0.00 (0.01)	−0.02 (0.01)	−0.03 (0.01)	
Gene: *MOGAT2*		MAF	37.6%	35.8%	32.6%	
rs10476123 (C>T)	T	*P*-value	0.89	7.3×10^{-5}	0.031	1.1×10^{-5}
Chr 5: 174 150 998		β^b (se)	+0.00 (0.01)	−0.03 (0.01)	−0.02 (0.01)	
Gene: *intergenic*		MAF	40.0%	36.4%	40.1%	
rs16928809 (G>A)	A	*P*-value	0.007	1.5×10^{-5}	0.25	1.3×10^{-5}
Chr 11: 2 893 528		β^b (se)	+0.04 (0.01)	+0.05 (0.01)	+0.02 (0.02)	
Gene: *SLC22A18*		MAF	10.9%	9.1%	8.8%	

[a]Effect allele on forward strand of human genome reference sequence of NCBI build 36.2.
[b]β coefficient represents the change in log-transformed bilirubin with one additional copy of the allele modeled, adjusting for age, sex, and rs6742078. 'se' is the standard error of the β coefficient.

genetic association study to date for bilirubin levels. In total, SNPs residing within four specific genes on two chromosomes were genome-wide significant ($P = 5.0 \times 10^{-8}$): *UGT1A1* (rs6742078, $P < 5.0 \times 10^{-324}$) in 2q37.1; *SLCO1B1* (rs4149056, $P = 6.7 \times 10^{-13}$), *LST-3TM12* (rs2417873, $P = 1.5 \times 10^{-9}$) and *SLCO1A2* (rs4149000, $P = 2.7 \times 10^{-8}$) in 12p12.2–12.1. Results were similar in age- and sex-adjusted and multivariable-adjusted models for the top loci. Top GWA results for the age–sex model are presented in Table 1. The top *UGT1A1* SNP, rs6742078, accounted for 16.7–18.1% of the variation in the circulating bilirubin in the three studies. In contrast, the top SNP in *SLCO1B1* (rs4149056) accounted for only 0.5–0.6% of the variation in the three studies. These results are consistent with previous linkage scans in which a single strong linkage signal was reported at 2q37.1 (the *UGT* region) (4,7,8) and past association studies which consistently showed association between bilirubin levels and the *UGT1A1*28* promoter TA repeat polymorphism. In a subset of individuals within the RS ($n = 490$) for which both *UGT1A1*28* and rs6742078 genotypes were available, we found these markers to be in high LD ($r^2 = 0.88$), suggesting that the GWA signal may be attributed to the functional *UGT1A1*28* polymorphism.

The direction of allelic effect is consistent with the hypothesis of an allele in LD with the T allele of rs6742078 that results in decreased production of UGT1A1 RNA and protein, and thus decreased modification and elimination of enzyme substrates including bilirubin. Accounting for the fact that log bilirubin levels were modeled, relative to zero copies of the T allele of rs6742078, one or two copies were associated with increases in bilirubin of 27.0 and 61.3%, respectively. Our findings provide strong confirmation of the major genetic effects of *UGT1A1* polymorphism and suggest that given the effect size these results support this locus as the main contributor to Gilbert's syndrome. Since a small portion of individuals with Gilbert's syndrome do not exhibit *UGT1A1* polymorphism, *SLCO1B1* or other genes may additionally contribute.

Table 3. Association results in the region of a priori candidate loci for bilirubin. Listed are the most significant SNP in meta-analysis either in or within 60 kb of each gene

SNP id	Modeled allele	Gene(s)	Chr	Number of SNPs tested	Framingham β (se)	*P*-value	Rotterdam β (se)	*P*-value	AGES-Reykjavik β (se)	*P*-value	Meta-analysis β (se)	*P*-value	Permutation *P*-value
rs6742078	T	*UGT1A1*	2q37.1	337	+0.239 (0.009)	9.2E−154	+0.230 (0.008)	2.6E−182	+0.238 (0.011)	1.2E−92	+0.234 (0.005)	5E−324	≤ 0.001
rs4149056	C	*SLCO1B1*	12p12.2	299	+0.054 (0.013)	4.0E−05	+0.052 (0.011)	1.3E−06	+0.053 (0.015)	0.00047	+0.053 (0.007)	6.7E−13	≤ 0.001
rs10484408	T	*GSTA* cluster	6p12.1	357	+0.031 (0.013)	0.017	+0.022 (0.011)	0.042	+0.026 (0.015)	0.085	+0.026 (0.007)	0.00046	0.039
rs16962449	C	*SLC10A2*	13q33.1	228	+0.014 (0.011)	0.20	+0.034 (0.008)	7.1E−05	+0.002 (0.012)	0.88	+0.021 (0.006)	0.00051	0.038
rs10492317	T	*NR1H4 (FXR)*	12q23.1	139	−0.061 (0.028)	0.034	−0.056 (0.027)	0.036	−0.087 (0.107)	0.42	−0.059 (0.019)	0.0022	0.07
rs8007929	A	*SLC10A1*	14q24.2	94	+0.001 (0.019)	0.96	+0.037 (0.013)	0.003	+0.036 (0.019)	0.06	+0.028 (0.009)	0.0026	0.07
rs2267332	C	*HMOX1*	22q12.3	127	+0.013 (0.017)	0.43	+0.037 (0.020)	0.065	+0.052 (0.021)	0.012	+0.031 (0.011)	0.0048	0.15
rs4803342	A	*BLVRB*	19q13.2	57	−0.008 (0.010)	0.41	−0.015 (0.008)	0.050	−0.023 (0.011)	0.046	−0.015 (0.005)	0.0069	0.11
rs10486752	A	*BLVRA*	7p13	104	−0.027 (0.015)	0.08	−0.019 (0.012)	0.13	−0.016 (0.018)	0.39	−0.021 (0.009)	0.016	0.30
rs319448	A	*ATP8B1*	18q21.31	162	+0.017 (0.011)	0.13	+0.015 (0.008)	0.06	+0.007 (0.012)	0.55	+0.014 (0.006)	0.016	0.41
rs11190297	T	*ABCC2 (MRP2)*	10q24.2	122	+0.030 (0.018)	0.09	+0.024 (0.014)	0.08	+0.011 (0.019)	0.54	+0.023 (0.010)	0.017	0.37
rs4148749	C	*ABCB4*	7q21.12	174	−0.055 (0.030)	0.07	−0.042 (0.027)	0.11	−0.008 (0.034)	0.82	−0.038 (0.017)	0.029	0.62
rs7605199	A	*ABCB11*	2q31.1	231	−0.011 (0.010)	0.27	−0.011 (0.008)	0.17	−0.011 (0.011)	0.32	−0.011 (0.006)	0.045	0.82
rs11712308	A	*NR1I2 (PXR)*	3q13.33	103	+0.013 (0.009)	0.17	−0.021 (0.008)	0.009	−0.016 (0.011)	0.17	−0.001 (0.005)	0.11	0.88
rs11645060	A	*HMOX2*	16p13.3	94	−0.003 (0.047)	0.96	−0.018 (0.016)	0.27	−0.035 (0.027)	0.19	−0.021 (0.014)	0.12	0.88

Our meta-analysis conditional on the *UGT1A1* SNP rs6742078 revealed that SNPs in *SLCO1B1* alone remained genome-wide significant (Table 2). Notably, the 12p12.2–p12.1 region harbors five organic anion transporters, including *SLCO1B1*, over ~750 kb. In orientation from 5′ to 3′, these genes are *SLCO1C1*, *SLCO1B3*, *LST-3TM12*, *SLCO1B1* and *SLCO1A2* (Fig. 2). Bilirubin and biliverdin are known substrates for at least two of the transporters, SLCO1B1 and SLCO1B3, both of which have been shown to be expressed in liver tissue (16). The *SLCO1B1* transporter is a logical candidate for effects on bilirubin since it has been shown to have transport affinity for bilirubin into hepatocytes, along with a number of other substrates including statins, methotrexate and antibiotics including rifampicin (16–18). Likewise, the *SLCO1B3* transporter shares greater than 80% amino acid identity with *SLCO1B1* and also exhibits substrate affinity for bilirubin and a wide range of therapeutics (16,18). Interestingly, the same non-synonymous polymorphism in *SLCO1B1* we identified (rs4149056) has previously been shown *in vitro* and *in vivo* to effect transporter function, including pharmacokinetics of various statins and variation in bilirubin levels (19–22). In our study, one or two copies of the C allele of rs4149056 were associated with a 4.9 and 9.5% increase in bilirubin levels, respectively, relative to no copies of the allele. Recently, a GWA identified and replicated an SNP associated with statin-induced myopathy, rs4363657, which is in high LD with rs4149056 ($r^2 = 0.97$) (23). Given the role of SLCO1B1 in drug processing and prior genetic evidence combined with our genome-wide results, this suggests that *SLCO1B1* polymorphism may play an important role in multiple clinically relevant phenotypes. We also evaluated the LD patterns in the HapMap CEU samples among the top genome-wide significant SNPs in the 12p12.2–p12.1 region using SNAP (24) (Fig. 2), finding modest LD between *SLCO1B1* and *SLCO1A2* SNPs (rs4149056<> rs4149000, $r^2 = 0.56$, $D' = 0.75$) and weaker LD between *SLCO1B1* and *LST-3TM12* (rs4149056<> rs2417873, $r^2 = 0.06$, $D' = 0.35$). Given the functional roles of the genes in this region as organic anion transporters and the similarity in protein sequence and gene expression patterns, including liver and kidney expression for *LST-3TM12* and *SLCO1A2* (25), this suggests that there are potentially multiple functional gene variants that influence bilirubin levels within this region, meriting further replication and functional studies of genes beyond *SLCO1B1*. In a regression model, SNPs in *SLCO1B1* (rs4149056) and *LST-3TM12* (rs2417873) were independently associated with bilirubin levels supporting this hypothesis.

The bilirubin associations with *UGT1A1* and *SLCO1B1* can be considered strongly and consistently replicated by previously reported criteria, including consistency of the direction of effect with prior associations, among the three studies, and evidence for associations with multiple SNPs in the region (26). Other gene regions showed associated SNPs with consistent directions of effect and significance in two or more of the cohorts that may warrant follow-up replication efforts. An organic cation transporter at 11p15.4, *SLC22A18*, shows high levels of expression in human kidney and liver tissues and displayed moderate association with serum bilirubin levels in our study (rs16928809, $P = 1.1 \times 10^{-7}$). Some additional functional evidence supports the hypothesis that this gene could play a role in bilirubin transport and metabolism. There is evidence in rodents and humans that *SLC22A18* is imprinted (27,28). In $APRT^{-/-}$ mice that develop kidney stone disease, a disease characterized by stones high in bilirubin content, it was noted that *SLC22A18* expression is markedly reduced in the cortical regions of kidney relative to wild-type mice, with imprinting a suggested cause (28). Given the relevance of liver tissue to bilirubin metabolism, we examined evidence for our top loci for eQTLs in available results from a large GWAS survey of 384 liver tissues (29). Notably, the same *SLC22A18* SNP associated with bilirubin levels here was associated as a *cis*-acting eSNP in that study (rs16928809, $P = 2.9 \times 10^{-7}$). Bilirubin is a major component of bile along with bile acid salts produced as a breakdown product of cholesterol. A liver enzyme, BAAT, is primarily responsible for bile acid salt conjugation prior to elimination. Mutations in this enzyme have been found to be partially responsible for familial hypercholanemia, resulting in excess accumulation of serum unconjugated bile acids (30). With the overlap between bile acid salt processing and bilirubin physiology, it is interesting to note that a variant near *BAAT* was also modestly associated with bilirubin levels in two of the cohorts here (rs12337836, meta-analysis, $P = 1.3 \times 10^{-5}$).

Further efforts are required to replicate associations in regions other than *UGT1A1* and *SLCO1B1*. The variants in *UGT1A1* and *SLCO1B1* identified in this study may serve as important markers in pharmacogenetics given their effects on the metabolism of a wide range of therapeutics, endogenous and exogenous substrates. These variants may also be of interest in bilirubin-related etiologies including cholestasis, neonatal jaundice and kernicterus, gall stones and choledocholithiasis. Although we did not note an association of our top SNPs with gallbladder disease or gallstones in a relatively small number of cases in two cohorts, this may be due to phenotypic heterogeneity (e.g. bilirubin stones represent a minority of gallstones) or insufficient statistical power. Further research is warranted in larger numbers of research subjects to explore clinical associations with *UGT1A1* and *SLCO1B1* gene variants.

MATERIALS AND METHODS

Study samples

An overview paper of the CHARGE consortium that includes FHS, RS and AGES-Reykjavik describes many of the details on the cohort samples and genotyping, imputation and analysis procedures (31). The FHS is a community-based, prospective, longitudinal study following three generations of participants, with a variety of measurements collected and genome-wide genotyping in a subset of participants. The Offspring cohort studied here represents the second generation of participants, including spouses, whose first exam cycle took place between 1971 and 1975 (32). Total serum bilirubin levels were measured ($n = 3{,}424$; 1633 men, 1791 women available for analysis) in the Offspring cohort of the FHS during exam 1 using an ultra-micromethod (33). All subjects provided informed consent for genetic studies.

The RS is a prospective population-based study on determinants of several chronic diseases in the elderly (34). The study comprised 7983 inhabitants of Ommoord, a district of Rotterdam in the Netherlands, who were 55 years or older. The first examination took place between 1990 and 1993. Total bilirubin levels were measured in non-fasting serum at baseline with a diazo-coupling method with 2,5-dichlorophenyl diazonium (35). The sample available for the bilirubin analysis included 3847 subjects (1483 men, 2364 women). All subjects provided informed consent for genetic studies.

The Reykjavik study cohort originally comprised a random sample of 30 795 men and women born in 1907–1935 and living in Reykjavik in 1967. A total of 19 381 attended, resulting in a 71% recruitment rate. The study sample was divided into six groups by birth year and birth date within a month. One group was designated for longitudinal follow-up and was examined in all stages. One group was designated a control group and was not included in examinations until 1991. Other groups were invited to participate in specific stages of the study. Between 2002 and 2006, the AGES-Reykjavik study re-examined 5764 survivors of the original cohort who had participated before in the Reykjavik study. The AGES-Reykjavik study GWAS was approved by the National Bioethics Committee (00-063-V8+1) and the Data Protection Authority. Total serum bilirubin was measured on the day of the first participant visit (between 1967 and 1977, with the median first visit taking place in 1971) for 2193 samples (1113 men, 1080 women) by a method adopted from Gambino and Schreiber (36), with standards prepared according to the recommendations of the College of American Pathologists Standards Committee.

Genotyping and imputation

DNA was extracted and genotyped for consenting FHS participants with the Affymetrix 500K array and an additional gene-focused 50K array as part of the SNP Health Association Resource (SHARe) project with final genotypes available from 8482 participants. Genotyping of Rotterdam subjects was conducted with the Illumina HumanHap550K array on 5974 participants. AGES-Reykjavik genotyping was conducted with the Illumina 370CNV BeadChip array on 3664 participants. Samples were excluded from the data set based on sample failure, genotype mismatches with a reference panel and sex mismatch, resulting in clean genotype data on 3219 AGES-Reykjavik individuals. Standard Illumina protocols were followed, requiring SNP clustering scores greater than 0.4. All cohorts used MACH 1.0 (37) to impute ~2.54 million SNPs based on the HapMap CEU phased haplotypes (build 22). SNPs used in imputation for FHS met the following criteria: MAF $\geq$ 1%, HWE $P > 1.0 \times 10^{-6}$, SNP call rate $\geq$97.0%, MISHAP test $P > 1.0 \times 10^{-9}$, Mendelian errors $\leq$100. SNPs included in imputation for RS met thresholds of MAF $\geq$ 1%, HWE $P \geq 1.0 \times 10^{-6}$ or SNP call rate $\geq$98.0%. SNPs included in imputation for AGES-Reykjavik excluded those with HWE $P < 1.0 \times 10^{-6}$, MISHAP test $P \leq 1.0 \times 10^{-9}$, position mismatches across databases (Illumina, dbSNP and/or HapMap) and call rate <97%. Quality control measures for individual cohorts indicated reliable imputation results [e.g. comparison of imputed results with actually genotyped results based on the previous FHS 100K studies (38)].

GWA replication meta-analysis

Imputed results were tested for association of estimated dosage of an allele for each SNP with log-transformed bilirubin levels using a linear mixed-effects model that accounts for familial correlation in FHS and a regression model in RS and AGEs-Reykjavik. All cohorts evaluated age–sex- and multivariable-adjusted models with similar covariates (age, sex, height, weight, HDL, total cholesterol, hematocrit, total protein, SGOT, smoking, alcohol, hypertension, diabetes status). The exception is that FHS included the first principal component from EIGENSTRAT 2.0 (39) as a covariate to account for population admixture since this component was significantly associated with log-transformed bilirubin levels. Meta-analysis was conducted using METAL (http://www.sph.umich.edu/csg/abecasis/Metal/) with sample size, and alternatively inverse variance, weighted methods and also adjusting for genomic control rates. Primary results presented in the manuscript are from the individual cohort age–sex-adjusted analyses and inverse variance-weighted meta-analyses.

Candidate gene loci analysis

Thirteen autosomal candidate gene regions aside from *UGT1A1* and *SLCO1B1* were selected before the analysis was conducted: *ATP8B1*, *HMOX1*, *HMOX2*, *BLVRA*, *BLVRB*, the *GSTA* cluster (chr 6p12.1), *SLC10A1*, *SLC10A2*, *ABCB4*, *ABCB11*, *MRP2*, *PXR* and *NR1H4*. SNPs within 60 kb of the boundaries of candidate loci were examined for association. The most significant meta-analysis SNP in or near each candidate gene was compared against the most significant SNPs in repeated resampling ($n = 1000$ permutations) of equal numbers of consecutive SNPs randomly positioned in the results across the genome in order to derive a permutation-based *P*-value.

Gallstone and gallbladder disease analysis

FHS and RS had different definitions of gall bladder disease and gallstones. FHS participants among Original Cohort and Offspring participants with GWA data were identified who answered positively to questions regarding a history of gallbladder disease and/or having had gallbladder surgery or having an abdominal scar indicating gallbladder removal ($n = 515$), and those who were not positive for any of these categories ($n = 3783$). In the RS, data on cholelithiasis were acquired using hospital discharge diagnosis records from 1991 to 2004. The diagnosis of cholelithiasis was mainly based on either gall bladder ultrasound or CT scan. Among RS subjects with available GWA data, there were 161 participants who were positive for cholelithiasis and 5813 who were not. In each study, an age- and sex-adjusted dichotomous GEE analysis was conducted for association of gallbladder disease history (FHS) or cholelithiasis (RS), respectively, with the top UGT1A1 (rs6742078) and SLCO1B1 (rs4149046) SNPs.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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