Extreme Bilirubin Levels as a Causal Risk Factor for Symptomatic Gallstone Disease

Stefan Stender, MD; Ruth Frikke-Schmidt, MD, DMSc; Børge G. Nordestgaard, MD, DMSc; Anne Tybjærg-Hansen, MD, DMSc

IMPORTANCE In individuals without blockage of their bile ducts, levels of plasma bilirubin likely reflect levels of biliary bilirubin; higher biliary bilirubin levels may increase the risk of gallstone disease.

OBJECTIVE To test the hypothesis that a lifelong increase in plasma bilirubin levels is a causal risk factor for symptomatic gallstone disease in the general population.

DESIGN, SETTING, AND PARTICIPANTS In a prospective study of the Danish general population (N = 61,212), we first tested whether elevated levels of plasma bilirubin predicted greater risk of symptomatic gallstone disease. Second, taking advantage of mendelian randomization, we tested whether a genetic variant in the bilirubin glucuronidating enzyme UGT1A1 (rs6742078) was associated with increased plasma bilirubin levels and, in turn, with an increased risk of symptomatic gallstone disease.

MAIN OUTCOMES AND MEASURES Plasma bilirubin level and symptomatic gallstone disease.

RESULTS During 34 years of follow-up, 3374 individuals developed symptomatic gallstone disease. In adjusted analyses, persons with plasma bilirubin levels in the 10th decile had a greater risk of symptomatic gallstone disease compared with those with plasma bilirubin levels in deciles 1 through 9, the hazard ratios (HRs) (95% CIs) were 1.57 (1.26-1.96) overall, 1.36 (1.02-1.82) in women, and 2.00 (1.41-2.83) in men. UGT1A1 genotype explained 20% of the total variation in plasma bilirubin levels and was associated with increases in the mean plasma bilirubin level overall of +16% (+0.09 mg/dL) in GT heterozygotes and +90% (+0.50 mg/dL) in TT homozygotes compared with GG homozygotes, with similar effects in women and men (P for trend <.001 for all). The corresponding HRs (95% CIs) for symptomatic gallstone disease were 1.09 (1.02-1.17) for GT heterozygotes and 1.22 (1.09-1.36) for TT homozygotes vs GG homozygotes and similar in women and men (P for trend = .04-.<.001).

CONCLUSIONS AND RELEVANCE These results are compatible with a causal association between extreme levels of plasma bilirubin and increased risk of symptomatic gallstone disease.
Biliary bilirubin has a role in the formation of gallstones, one of the most common and costly gastrointestinal tract diseases. Biliary bilirubin and calcium can combine to form calcium bilirubinate salts, which may grow and become symptomatic as pigment gallstones. In addition, a core of calcium bilirubinate is often found in cholesterol gallstones, the most common form of gallstones in the Western world. It has been hypothesized that calcium bilirubinate salts may act as a nucleating factor for the precipitation of biliary cholesterol, directly facilitating the formation of cholesterol gallstones. Factors that promote the formation of cholesterol gallstones include biliary cholesterol hypersecretion, faster precipitation of biliary cholesterol, gallbladder stasis, increased intestinal input of dietary or biliary cholesterol, and genetic background.

Increased production of bilirubin is associated with increased risk of gallstone disease. A range of hemolytic conditions, characterized by increased plasma levels of bilirubin secondary to the breakdown of free hemoglobin, have been associated with an increased risk of gallstone disease. Furthermore, a decreased rate of bilirubin conjugation in the liver, as seen in individuals with Gilbert syndrome, has been associated with increased bilirubin levels and increased risk of gallstone disease in several case-control studies. However, whether elevated levels of plasma bilirubin per se are causally associated with an increased risk of gallstone disease in the general population remains unknown. This question is important from both clinical and scientific viewpoints. Because plasma bilirubin is already routinely measured in the clinic, an elevated bilirubin level would alert a clinician of the elevated risk. From a scientific viewpoint, unraveling the pathways that lead to gallstone disease may provide new targets for treatment or prevention.

The random assortment of genes that occurs during gamete formation provides a relatively unbiased method of assessing whether risk factors that have a genetic component are in fact causally related to clinical outcomes. This phenomenon has been termed mendelian randomization and is often compared with that of the randomized clinical trial because the exposure is randomized (by the genetic process) and the outcome can be observed prospectively (Figure 1A). Thus, because genotypes are fixed at conception, genetic variants that specifically increase plasma levels of bilirubin (exposure) provide an ideal system to assess the consequences of lifelong high bilirubin levels, independent of other risk factors.

Using the mendelian randomization approach (Figure 1B), we tested the hypothesis that a lifelong increase in plasma bilirubin levels is a causal risk factor for symptomatic gallstone disease in the general population. First, we tested whether plasma bilirubin levels predicted risk of symptomatic gallstone disease in observational analyses (Figure 1B). Second, in genetic analyses we tested whether a variant in the bilirubin glucuronidating enzyme UGT1A1 (rs6742078) was associated with increased plasma bilirubin levels (Figure 1B). Third, we tested whether the UGT1A1 genotype was associated with an increased risk of symptomatic gallstone disease (Figure 1B). Fourth, we tested whether the observed genetic risk estimates were similar to those predicted based on the observational data, which is the theoretically predicted risk. If the first 3 factors are all documented firmly, it is likely that elevated plasma bilirubin is a causal risk factor for symptomatic gallstone disease (Figure 1B).

Methods

Studies were approved by institutional review boards and Danish ethical committees and conducted according to the Declaration of Helsinki. Written informed consent was obtained from participants. All participants were white and of Danish descent.

Participants

We included participants in 2 similar prospective studies of the Danish general population: the Copenhagen General Population Study (CGPS; n = 50,835) and the Copenhagen City Heart Study (CCHS; n = 10,377). Combining these 2 studies yielded a total of 61,212 participants. For study details, see the eMethods in the Supplement.

Symptomatic Gallstone Disease

We defined symptomatic gallstone disease as International Classification of Diseases (ICD) codes for cholelithiasis or choledocolithiasis (ICD-8: 574 and 575; ICD-10: K80 and K81) diagnosed at hospitals. Information about diagnoses of symptomatic gallstone disease was collected from the National Danish Patient Registry and the National Danish Causes of Death Registry. The National Danish Patient Registry has information on
all patient contacts with all clinical hospital departments and outpatient clinics in Denmark, including emergency wards (from 1994). The National Danish Causes of Death Registry contains data about the causes of all deaths in Denmark, as reported by hospitals and general practitioners.

**Covariates, Laboratory Analyses, and Genotyping**

For details on covariates, laboratory measurements, and genotyping, see the eMethods and the eTable in the Supplement.

**Statistical Analysis**

Data were analyzed using STATA/SE statistical software (release 12; StataCorp LP). The χ² tests evaluated Hardy-Weinberg equilibrium. The Mann-Whitney test or Pearson χ² test was used to compare characteristics in individuals by disease status. The Kruskal-Wallis analysis of variance was used to evaluate the association of the rs6742078 genotype with potential confounders. For statistical analyses, rs6742078 genotypes GG, GT, and TT were coded as 0, 16, and 90, respectively. This coding reflects the associations of the individual genotypes with mean levels of plasma bilirubin and was chosen to account for nonlinearity in the genotype-bilirubin association.

First, to examine the association between plasma bilirubin levels and risk of symptomatic gallstone disease in observational analyses (Figure 1B), Kaplan-Meier curves were used to estimate cumulative incidence, and Cox proportional hazards regression models with age as time scale and left truncation (delayed entry) were used to estimate hazard ratios (HRs) for gallstone disease in the CGPS. Analyses were conducted from the time of blood sampling (baseline) through 2011. To avoid reverse causation (ie, gallstones that influence baseline bilirubin levels), individuals with prevalent gallstones at blood sampling (n = 2034) were excluded, leaving 48 801 participants and 667 incident symptomatic gallstones. Because the distribution of bilirubin is positively skewed (eFigure 1 in the Supplement) and to gain information on the biological association between extremely high bilirubin levels and risk of gallstone disease, we defined bilirubin cut points a priori on the basis of deciles of the bilirubin distribution and estimated risk for adjusted age and sex or multifactorially for age, sex, body mass index (BMI), physical activity, hormone replacement therapy, and alcohol consumption. We also examined risk of gallstone disease as a function of combined deciles: 1 through 3, 4 through 6, 7 through 9, and 10, using deciles 1 through 3 as the reference group (eFigure 2 in the Supplement). Missing covariates were imputed based on age and sex (a maximum of 1.2% of any covariate was imputed). Competing risk of any death was accounted for by censoring at the date of death.

Second, to test whether the rs6742078 genotype was associated with elevated plasma bilirubin levels in the CGPS (Figure 1B), we used Cuzick's extension of a Wilcoxon rank sum test for trend.

Third, to test whether genetically elevated bilirubin levels were associated with an increased risk of gallstones (Figure 1B), we tested for an association between genotype and gallstones in the CGPS and the CCHS combined to obtain maximal power. Because genotype is constant throughout life and, hence, impervious to reverse causation, risk of symptomatic gallstone disease as a function of genotype was analyzed from 1977 to 2011 (ie, all 3374 symptomatic gallstones were included in this analysis). Cox proportional hazards regression models adjusted for age, sex, BMI, physical activity, hormone therapy, and alcohol consumption were used to estimate HRs. Finally, theoretically predicted risk of gallstone disease was estimated from delta bilirubin and the prospective association between plasma bilirubin and gallstone disease in the observational study (Figure 1B). Interaction of rs6742078 with all covariates listed above was evaluated by including 2-factor interaction terms between genotype and covariates, one at a time, in the Cox proportional hazards regression model.

**Results**

Characteristics of the 61 212 participants in the study by disease status are listed in the Table; 3374 developed symptomatic gallstone disease.

Genotyping UGT1A1 rs6742078 identified 28 610 GG homozygotes (47%), 26 459 GT heterozygotes (43%), and 6143 TT homozygotes (10%). Genotype frequencies did not deviate from those predicted by the Hardy-Weinberg equilibrium (P = .82).

In the CGPS, the distribution of plasma bilirubin was positively skewed (eFigure 1 in the Supplement). Geometric mean (95% CI) plasma bilirubin level was 0.63 mg/dL (0.63-0.63 mg/dL) (to convert bilirubin to micromoles per liter, multiply by 17.1). The corresponding values in women and men were 0.56 mg/dL (0.56-0.57 mg/dL) and 0.72 mg/dL (0.71-0.72 mg/dL), respectively.

**Plasma Bilirubin Level and Symptomatic Gallstone Disease**

The cumulative incidence of symptomatic gallstone disease during a mean of 4.7 years of follow-up (range, 0.0-7.5 years) was increased in individuals in the 10th decile (geometric mean bilirubin levels), individuals with prevalent gallstones at blood sampling (n = 2034) were excluded, leaving 48 801 participants and 667 incident symptomatic gallstones. Because the distribution of bilirubin is positively skewed (eFigure 1 in the Supplement) and to gain information on the biological association between extremely high bilirubin levels and risk of gallstone disease, we defined bilirubin cut points a priori on the basis of deciles of the bilirubin distribution and estimated risk for adjusted age and sex or multifactorially for age, sex, body mass index (BMI), physical activity, hormone replacement therapy, and alcohol consumption. We also examined risk of gallstone disease as a function of combined deciles: 1 through 3, 4 through 6, 7 through 9, and 10, using deciles 1 through 3 as the reference group (eFigure 2 in the Supplement). Missing covariates were imputed based on age and sex (a maximum of 1.2% of any covariate was imputed). Competing risk of any death was accounted for by censoring at the date of death.

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bilirubin, 1.34 mg/dL (95% CI 0.58-0.68 mg/dL) (Figure 2; log-rank test \( P < .001 \)). Mean (95% CI) age- and sex-adjusted HRs for symptomatic gallstone disease for individuals in the 10th decile vs individuals in deciles 1 through 9 of plasma bilirubin were 1.54 (1.23-1.92) for both sexes combined, 1.31 (0.98-1.75) in women, and 2.01 (1.42-2.84) in men (Figure 3). Corresponding estimates adjusted for age, sex, BMI, physical activity, hormone therapy, and alcohol consumption were 1.57 (1.26-1.96), 1.36 (1.02-1.82), and 2.00 (1.41-2.83), respectively (Figure 3). Bilirubin below the 10th decile (deciles 7-9 and 4-6 vs deciles 1-3) was not associated with the risk of symptomatic gallstone disease (Figure 2 in the Supplement).

To address potential reverse causation, we excluded all individuals with events \((n = 155)\) in the first year after baseline. The mean HRs (95% CIs) for individuals in the 10th decile of plasma bilirubin were 1.54 (1.23-1.92) for both sexes combined, 1.31 (0.98-1.75) in women, and 2.01 (1.42-2.84) in men, similar to the HRs above. Among the 155 individuals with symptomatic gallstones diagnosed in the first year after baseline, the geometric mean level of bilirubin (95% CI) was 0.63 mg/dL (0.58-0.68 mg/dL), comparable with the overall mean of 0.63 mg/dL.

**UGT1A1 Genotype and Plasma Bilirubin Level**

The UGT1A1 rs6742078 genotype explained 20% of the total variation in plasma bilirubin levels in the CGPS and was associated with increases in the geometric mean plasma bilirubin level of +16% (+0.09 mg/dL) in GT heterozygotes and +90% (+0.50 mg/dL) in TT homozygotes, respectively, compared with the GG homozygotes (Figure 4; \( P \) for trend < .001). Associations were similar in women and men (Figure 4; \( P < .001 \)).

**UGT1A1 Genotype and Symptomatic Gallstones**

Assuming that plasma bilirubin levels are causally associated with gallstone disease, lifelong genetically increased bilirubin levels due to the rs6742078 genotype should confer a similar increase in the risk of gallstones as that observed for bilirubin levels in the general population. For example, the 90% to 91% increase in plasma bilirubin levels for TT homozygotes would theoretically predict an increased risk of symptomatic gallstones with HRs (95% CIs) of 1.18 (1.05-1.34) in both sexes combined, 1.10 (0.94-1.28) in women, and 1.34 (1.10-1.63) in men (Figure 4). In accord with this, during a mean follow-up of 33.0 years (range, 0.0-34.4 years), the multifactorially adjusted HRs (95% CIs) for symptomatic gallstone disease were 1.09 (1.02-1.17) for GT heterozygotes and 1.22 (1.09-1.36) for TT homozygotes vs GG homozygotes. The corresponding HRs (95% CIs) were 1.10 (1.01-1.20) and 1.15 (1.01-1.32) in women and 1.07 (0.93-1.22) and 1.37 (1.12-1.67) in men (Figure 4). The corresponding HR (95% CI) after further adjustment for plasma bilirubin levels as a continuous trait was 1.18 (1.02-1.37) for TT homozygotes vs GG homozygotes in both sexes combined.

Risk factors for symptomatic gallstones did not differ by rs6742078 genotype, confirming that the effect of genotype on risk of symptomatic gallstones is unconfounded by these risk factors.

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**Table 1.** Cumulative Incidence of Symptomatic Gallstone Disease as a Function of Age and Plasma Bilirubin Level

<table>
<thead>
<tr>
<th>Bilirubin Deciles</th>
<th>Geometric Mean, mg/dL</th>
<th>Total, No.</th>
<th>No. of Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1-9</td>
<td>0.58</td>
<td>44105</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.34</td>
<td>4696</td>
</tr>
<tr>
<td>Women</td>
<td>1-9</td>
<td>0.52</td>
<td>23965</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.18</td>
<td>2569</td>
</tr>
<tr>
<td>Men</td>
<td>1-9</td>
<td>0.66</td>
<td>20140</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.56</td>
<td>1227</td>
</tr>
</tbody>
</table>

Prospective risk of symptomatic gallstone disease as a function of baseline plasma bilirubin level (10th decile vs deciles 1-9) in the general population in both sexes combined and in women and men separately. Hazard ratios (HRs) (95% CIs) were multifactorially adjusted for age, sex, body mass index, physical activity, hormone therapy, and alcohol consumption. To convert bilirubin to micromoles per liter, multiply by 171.
Discussion

To our knowledge, this is the first study to examine the association between elevated levels of plasma bilirubin and prospective risk of symptomatic gallstone disease in the general population and the first to use a mendelian randomization approach in this context. The main findings of this study are that both observationally and genetically elevated levels of plasma bilirubin are associated with an increased risk of symptomatic gallstone disease. These results are compatible with a causal association between elevated levels of plasma bilirubin per se and increased risk of symptomatic gallstone disease.

Possible links between plasma bilirubin level and gallstone disease have been described previously. First, bilirubin is a major constituent not only of pigment gallstones (primarily composed of calcium bilirubinate salts) but also of cholesterol gallstones, the most common form of gallstones in the Western world.1,2 Second, a range of hemolytic conditions (characterized by elevated plasma bilirubin level) is known to increase the risk of gallstone disease, presumably because of increased efflux of bilirubin into bile.2,3 Third, previous studies9,11,29-32 have reported an increased risk of gallstone disease in individuals with decreased hepatic conjugation of bilirubin due to genetic variation in the gene encoding the bilirubin-conjugating enzyme UGT1A1. In a large case-control study (n = 2606 gallstone cases), Buch et al9 found that UGT1A1 rs6742078 TT vs GG + GT was associated with gallstone disease in German men (odds ratio, 2.34; 95% CI, 1.68-3.26) but not in women (odds ratio, 1.10; 95% CI, 0.84-1.45). This sex-specific effect was replicated in a smaller cohort of South Americans (n = 210 gallstone cases).9 In contrast, in our general population study of 61,212 individuals, including 3374 with symptomatic gallstones, we observed an increased risk in both men (TT vs GG; HR, 1.37; 95% CI, 1.12-1.67) and women (HR, 1.15; 95% CI, 1.01-1.32), thus extending the effect of UGT1A1 rs6742078 to both sexes and to the general population. However, both observational and genetic estimates were attenuated among women compared with men in our study.

In the observational analyses, only extreme levels of plasma bilirubin in the 10th decile were associated with an increased risk of symptomatic gallstone disease, suggesting a threshold effect of elevated biliary bilirubin level on risk of gallstones. We suggest that extreme levels of plasma total bilirubin (unconjugated and conjugated), in individuals without cholestasis, mainly reflects increased levels of unconjugated bilirubin. Unconjugated bilirubin has been suggested to be more prone to precipitate in the bile compared with conjugated bilirubin4 and, therefore, may more readily cause gallstone disease.

It is well known that the TA repeat (UGT1A1*28) polymorphism underlying Gilbert syndrome is characterized by an approximately 70% reduced rate of bilirubin conjugation and, hence, lifelong moderate hyperbilirubinemia due to increased levels of unconjugated bilirubin.22,33 UGT1A1 rs6742078 and UGT1A1*28 are common, have a similar minor allele frequency of approximately 0.3, and are in complete linkage disequilibrium (r² = 0.88), implying that they are also extremely highly correlated.33 Thus, rs6742078 is an ideal single-nucleotide polymorphism for detecting the effect of UGT1A1*28 but is easier to genotype. In agreement, UGT1A1 rs6742078 explained 20% of the total variation in bilirubin levels in our study, and the genetic analyses revealed a stepwise increase in (unconjugated) bilirubin levels as a function of the UGT1A1 rs6742078 genotype. Therefore, the corresponding stepwise
increase in the observed genetic risk of gallstone disease may support that the threshold effect on risk in the observational study is due to an increase in plasma levels of mainly unconjugated bilirubin in the upper decile of the total bilirubin distribution.

The prevalence of 5.5% symptomatic gallstones based on ICD diagnoses fits well with the previously reported prevalences of 15% to 20% asymptomatic and symptomatic gallstones based on ultrasonography (only approximately 20% of all gallstones become symptomatic).\(^1\)

Our study has some potential limitations. Comparison of plasma bilirubin levels in the 10th decile vs deciles 1 through 9 might seem to be an a posteriori decision, introducing the risk of multiple testing problems. However, this approach was based on our previous studies of biomarkers with similarly positively skewed distributions (lipoprotein [a] and triglycerides). In these studies, we found that the extremes of these skewed biomarkers conferred a substantially greater increase in risk of cardiovascular disease and stroke than would be expected by assuming a linear relationship.\(^18,20\) The increased risk of symptomatic gallstone disease in carriers of rs6742078 might be mediated by pleiotropic effects of rs6742078, for instance, altered conjugation of hormones and/or drugs,\(^2,3,4\) rather than by an increase in biliary bilirubin levels. In apparent support of this, adjusting for bilirubin levels attenuated but did not remove the association between rs6742078 genotype and risk of symptomatic gallstones. However, a more likely explanation is that genotype is a far better marker of lifelong plasma bilirubin levels than a single measurement in adulthood. Importantly, gallstone disease cannot influence genotype, and therefore the observed association between genotype and risk of gallstone disease is causal.\(^13\)

Moreover, Buch et al\(^9\) observed an association between rs6742078 genotype and gallstone bilirubin content, strongly suggesting that the increased risk of symptomatic gallstones in carriers of rs6742078 is indeed caused by elevated biliary bilirubin levels. Our ICD-based definition of symptomatic gallstone disease might have included other hepatobiliary conditions apart from gallstones. However, because gallstone disease is extremely common compared with other conditions of the gallbladder and is a relatively hard clinical end point with well-defined diagnostic criteria (eg, colicky pain and ultrasonography) and ICD codes, misclassification was unlikely to have posed a major problem in our study. An inherent limitation to our definition of symptomatic gallstones (presence of gallstone and manifestation) is that we cannot study asymptomatic gallstones, which comprise approximately 80% of all gallstones.\(^1\) Finally, we did not have data on stone composition (cholesterol, mixed, or pigment), number, or size. It is possible that elevated biliary bilirubin level increases the number of nucleation cores, thus promoting the formation of numerous smaller gallstones (which may have a higher risk of complications).\(^7,8\) In support of this hypothesis, Buch et al\(^9\) found that rs6742078 was more strongly associated with symptomatic than with asymptomatic gallstones. In conclusion, both observationally and genetically elevated levels of plasma bilirubin are associated with an increased risk of symptomatic gallstone disease.

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