HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Elevated Body Mass Index as a Causal Risk Factor for Symptomatic Gallstone Disease: A Mendelian Randomization Study

Stefan Stender,[1] Børge G. Nordestgaard,[2,3,4] and Anne Tybjærg-Hansen[1,2,4]

Elevated body mass index (BMI) is associated with an increased risk of gallstone disease. Whether this reflects a causal association is unknown. Using a Mendelian randomization approach, we studied 77,679 individuals from the general population. Of these, 4,106 developed symptomatic gallstone disease during up to 34 years of follow-up. Subjects were genotyped for three common variants known to associate with BMI: *FTO*(rs9939609); *MC4R*(rs17782313); and *TMEM18*(rs6548238). The number of BMI-increasing alleles was calculated for each participant. In observational analyses, mean baseline BMI was 55% (11.6 kg/m^2) increased in individuals in the fifth quintile versus the first quintile, similar in women and men. The corresponding multifactorially adjusted hazard ratio (HR) for symptomatic gallstone disease was 2.84 (95% confidence interval [CI]: 2.32-3.46) overall, 3.36 (95% CI: 2.62-4.31) in women, and 1.51 (95% CI: 1.09-2.11) in men (*P* trend: 0.001 to <0.001; *P* interaction: BMI*sex on risk = 0.01). In genetic analyses, carrying 6 versus 0-1 BMI-increasing alleles was associated with a 5.2% (1.3 kg/m^2) increase in BMI overall and with increases of 4.3% in women and 6.1% in men (all *P* trend: <0.001). Corresponding HRs for symptomatic gallstone disease were 1.43 (95% CI: 0.99-2.05) overall, 1.54 (95% CI: 1.00-2.35) in women, and 1.19 (95% CI: 0.60-2.38) in men (*P* trend = 0.007, 0.02, and 0.26, respectively; *P* interaction allele score*sex on risk = 0.49). The estimated causal odds ratio (OR) for symptomatic gallstone disease, by instrumental variable analysis for a 1 kg/m^2 increase in genetically determined BMI, was 1.17 (95% CI: 0.99-1.37) overall and 1.20 (95% CI: 1.00-1.44) and 1.02 (95% CI: 0.90-1.16) in women and men, respectively. Corresponding observational HRs were 1.07 (95% CI: 1.06-1.08), 1.08 (95% CI: 1.07-1.10), and 1.04 (95% CI: 1.02-1.07), respectively. *Conclusion*: These results are compatible with a causal association between elevated BMI and increased risk of symptomatic gallstone disease, which is most pronounced in women. (HEPATOLOGY 2013;58:2133-2141)

Elevated body mass index (BMI) is associated with an increased risk of gallstone disease, one of the most common and costly of gastrointestinal diseases.[1-5] However, whether this association reflects a *causal* effect of obesity on gallstone disease is unclear. It may be that another factor simultaneously raises BMI and causes gallstone disease, and that elevated BMI is merely a marker of this other causal factor (in epidemiology, this common phenomenon is termed "confounding"). For instance, a high-fat diet

Abbreviations: BMI, body mass index; CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; CI, confidence interval; GWAS, genome-wide association study; HR, hazard ratio; ICD, International Classification of Disease; OR, odds ratio.

Contract grant sponsor: This work was supported by the Danish Medical Research Council (grant no.: 10-083788), the Research Fund at Rigshospitalet, Copenhagen University Hospital, Chief Physician Johan Boserup and Lise Boserup's Fund, Ingeborg and Leo Dannin's Grant, Henry Hansen and Wife's Grant, and a grant from the Odd Fellow Order. The funding organizations did not play a role in any of the following: design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

From the [1]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital and Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; [2]The Copenhagen General Population Study, Herlev Hospital, Copenhagen University Hospital and Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; [3]Department of Clinical Biochemistry, Herlev Hospital, Copenhagen University Hospital and Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; [4]The Copenhagen City Heart Study, Frederiksberg Hospital, Copenhagen University Hospital and Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

Received March 22, 2013; accepted May 29, 2013.

might cause obesity as well as changes in the bile composition that promote the formation of cholesterol gallstones.[6] Likewise, physical inactivity is known to be associated with both obesity and gallstone disease and thus constitutes another potential confounder.[7] Apart from confounding, reverse causation could also explain part of the association between BMI and gallstone disease in retrospective or cross-sectional studies (i.e., colicky pain associated with gallstone disease may lead to a more sedentary lifestyle and hence raised BMI). A sometimes underappreciated fact is that confounding and reverse causation can be difficult, if not impossible, to obviate by statistical methods in conventional observational epidemiology.[8]

Mendelian randomization is a new epidemiological approach that aims to avoid confounding and reverse causation by use of genetic variation in human populations.[8] Because of the random assortment of genotypes during conception, genetic variants with effect on a modifiable exposure of interest are randomly distributed in relation to potential confounders.[8] Put simply, genetic variants that associate with increased BMI can be used as unconfounded instruments to study the effect of raised BMI on outcomes. Thus, if raised BMI truly is a causal factor in the development of gallstone disease, genetic variants that increase BMI would be expected to also increase risk of gallstone disease. Furthermore, because genetic variants are determined at conception and remain constant throughout life, Mendelian randomization is not influenced by reverse causation (i.e., gallstone disease cannot change the genotype of an individual).

Using a Mendelian randomization design, we tested the hypothesis that there is a causal association between elevated BMI and increased risk of symptomatic gallstones (Fig. 1, arrows 1-4). First, we tested whether elevated BMI at baseline was associated prospectively with increased risk of symptomatic gallstones (Fig. 1, #1), second, whether BMI-increasing alleles of *FTO*(rs9939609), *MC4R(*rs17782313), and *TMEM18*(rs6548238), three common genetic variants with the largest known effects on BMI,[9] were associated with elevated BMI, as expected (Fig. 1, #2), third, whether BMI-increasing alleles were associated directly with an increased risk of symptomatic gallstones (Fig. 1, #3), and fourth, whether the causal effect of BMI-increasing alleles on risk of symptomatic gallstones, using instrumental variable analysis, was consistent with the observational association between BMI and risk of gallstone disease (Fig. 1, #4, compared with #1).

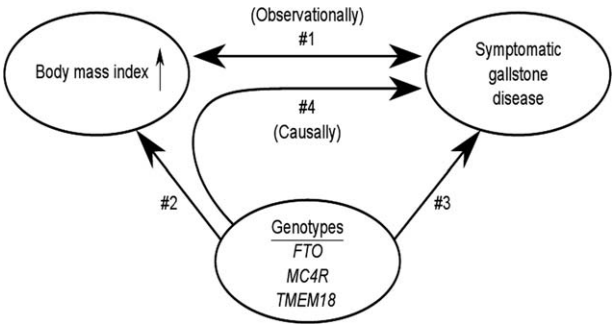

Fig. 1. Mendelian randomization design underlying the present study. If evidences #1-3 are all documented firmly, the interpretation would be that the data are compatible with a causal relationship between elevated BMI and risk of symptomatic gallstone disease. This is then tested directly using instrumental variable analysis (#4, causal association), which is compared with the observational estimate (#1).

Patients and Methods

Studies were approved by institutional review boards and Danish ethical committees and were 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 two similar prospective studies of the Danish general population: The Copenhagen General Population Study (CGPS; n = 67,314) and The Copenhagen City Heart Study (CCHS; n = 10,365).[10-12] Combining these two studies yielded a total of 77,679 participants, of whom 4,106 developed symptomatic gallstone disease.

The CGPS. The CGPS[10-12] is a prospective study of the Danish general population initiated in 2003 with ongoing enrollment. Individuals were selected based on the National Danish Civil Registration

Address reprint requests to: Anne Tybjærg-Hansen, M.D., D.M.Sc., Department of Clinical Biochemistry KB3011, Section for Molecular Genetics, Rigshospitalet, Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. E-mail: anne.tybjaerg.hansen@regionh.dk; fax: +45 3545 4160.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.26563
Potential conflict of interest: Nothing to report.
Additional Supporting Information may be found in the online version of this article.

System to reflect the adult Danish population at 20-100 years of age. Data were obtained from a self-administered questionnaire reviewed together with an investigator at the day of attendance, a physical examination, and from blood samples, including DNA extraction. We included 67,314 consecutive participants from this study in the present analysis. Of these, 3,435 developed symptomatic gallstone disease.

The CCHS. The CCHS[10-12] is a prospective study of the Danish general population initiated in 1976-1978 with follow-up examinations in 1981-1983, 1991-1994, and 2001-2003. Participants were recruited and examined exactly as in the CGPS. Blood samples for DNA extraction were drawn at the 1991-1994 and 2001-2003 examinations. We included 10,365 consecutive participants in the present analysis. Of these, 671 developed symptomatic gallstone disease.

Symptomatic Gallstone Disease. We defined symptomatic gallstone disease as International Classification of Disease (ICD) codes for cholelithiasis or cholecystitis (ICD8: 574 and 575; ICD10: K80 and K81) received at hospitals. Information on 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 on the causes of all deaths in Denmark, as reported by hospitals and general practitioners.

Follow-up time for gallstone disease for each participant in either study began at the establishment of the National Danish Patient Registry (January 1, 1977) or on the participant's birthday, whichever came last. Follow-up ended at the date of death (n = 6,490), occurrence of event (n = 4,106), emigration (n = 354), or on May 10, 2011 (last update of the registry), whichever came first. Follow-up was 100% complete, that is, no individual was lost to follow-up.

Genotyping. An ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and Taqman-based assays were used to genotype for *FTO*[rs9939609], *MC4R*[rs17782313], and *TMEM18*[rs6548238], as previously described.[10] These polymorphisms were selected as those with the largest known common effect sizes for association with BMI in European populations.[9] To act as an aggregate instrument for BMI, a simple allele score of 0-6 was constructed as the sum of BMI-increasing alleles across the three genotypes.[10] Individuals with 0 and 1 BMI-increasing alleles were combined into one group because there were very few individuals with 0 BMI-increasing alleles.[10] *ABCG8* D19H(rs11887534), the strongest genetic risk factor for gallstone disease, was genotyped by Taqman, as previously described.[11]

Covariates. BMI was measured as baseline weight in kilograms divided by measured height in meters squared (kg/m^2). Physical activity in leisure time, hormone replacement therapy and parity in women, alcohol, fast-food, and vegetable consumption were self-reported, dichotomized, and defined as physical activity (4 hours or more per week of light physical activity in leisure time versus less than 4 hours), hormone replacement therapy (yes/no), parity (any number of births versus nulliparity), alcohol consumption (>14/21 versus $\leq$14/21 units per week in women/men; 1 unit alcohol: $\sim$12 g), fast-food consumption (at least once per week versus less than once per week), and vegetable consumption (at least once per day versus less than once per day). Data on fast-food and vegetable consumption were only available in the CGPS (n = 67,314).

Statistical Analyses. Data were analyzed using STATA/SE 12 (StataCorp LP, College Station, TX). Chi-square tests were used to evaluate Hardy-Weinberg equilibrium. Mann-Whitney's U test or Pearson's chi-square test were used to compare characteristics in individuals by disease status. For statistical analyses, the allele score was coded as 1-6, BMI quintiles 1-5, and for individual genotypes, common homozygotes, heterozygotes, and rare homozygotes were coded as 0, 1, and 2, respectively.

A schematic of the Mendelian randomization model underlying the present study is shown in Fig. 1. We tested the four hypotheses described below.

First, to test whether elevated BMI associates observationally with an increased risk of symptomatic gallstone disease, Cox's regression models with age as the time scale and left truncation (delayed entry) were used to estimate hazard ratios (HRs) for symptomatic gallstone disease prospectively. Analyses were conducted from the time of blood sampling (baseline) through 2011. To avoid reverse causation (i.e., gallstones influencing baseline BMI), individuals with prevalent symptomatic gallstone disease at blood sampling (n = 2,941) were excluded from the prospective analysis, leaving 74,738 participants and 1,165 incident symptomatic gallstones. Risk of symptomatic gallstone disease was estimated as a function of BMI in quintiles adjusted for age and sex, or multifactorially for age, sex, physical activity, hormone replacement therapy, and alcohol consumption. Competing risk of any death was accounted for by censoring at

the date of death. Interaction of BMI with all covariates listed above was evaluated by including two-factor interaction terms between BMI and covariates, one at a time, in Cox's regression model.

Second, to test whether genotypes, individually or as an allele score, were associated with raised BMI, we used Cuzick's extension of a Wilcoxon rank-sum test for trend. For use of such genotypes as unconfounded instruments of increased BMI, it is essential to test that this assumption is indeed valid, whereas, at the same time, confounders likely associate both with BMI and/or gallstone disease. Therefore, logistic regression was used to assess whether observational BMI, symptomatic gallstone disease, or allele score were associated with potential confounders (e.g., age, sex, physical activity, consumption of alcohol, fast food, and/or vegetables, [for women] hormone replacement therapy and parity, and *ABCG8* D19H genotype).

Third, we tested for direct association between allele score and symptomatic gallstones. Because genotype is constant throughout life, and hence impervious to reverse causation, risk of symptomatic gallstone disease as a function of allele score was analyzed from 1977 through 2011 (i.e., all 4,106 symptomatic gallstones were included in this analysis). Cox's regression models multifactorially adjusted for age, sex, physical activity, hormone replacement therapy, and alcohol consumption were used to estimate HRs. Theoretically predicted risk[12] of symptomatic gallstone disease was estimated from delta BMI and the known prospective association of BMI with symptomatic gallstone disease.

Fourth, a potential causal relationship between genetically increased BMI and increased risk of symptomatic gallstone disease was assessed by instrumental variable analysis by two-stage least squares regression, using the *ivreg2* command in STATA.[13] In the first stage, we performed least squares regression of BMI on the allele score. In the second stage, we performed least squares regression of symptomatic gallstone disease on the predicted values from the first regression (the predicted values are the means of BMI within each allele score category).[8,13] Causal odds ratios (ORs) were estimated using the multiplicative generalized method of moments estimator implemented in the user-written STATA command, *ivpois*. Strength of the instrument (association of allele score with BMI) was evaluated by F-statistics from the first-stage regression, where $F > 10$ indicates sufficient strength to ensure the validity of the instrumental variable analysis, whereas R^2 (in percent) is used as a measure of percent contribution of allele score to the variation in BMI.[8] We used the method of Altman and Bland[14] to compare the causal genetic estimate obtained from the instrumental variable analysis with the corresponding risk in the observational study by Cox's regression.

Table 1. Baseline Characteristics of Participants by Disease Status

Characteristics	No Event	Symptomatic Gallstone Disease
No. of individuals (%)	73,573 (94.7)	4,106 (5.3)
Age, years (interquartile range)	57 (47-67)	62 (52-71)†
Women (%)	40,085 (54)	2,923 (71)†
Physical activity (%)	35,007 (48)	1,508 (37)†
Hormone replacement therapy (%)*	5,075 (13)	531 (18)†
Alcohol consumption (%)	13,353 (18)	536 (13)†

Values are median (interquartile range) or numbers (percentage). Physical activity was 4 hours or more per week of light physical activity in leisure time. Alcohol consumption was >14/21 units per week for women/men (1 unit of alcohol: ~12 g). *P* values by Mann-Whitney's U test or Pearson's chi-square test.

*In women only.

†$P < 0.001$ versus participants with no event.

Results

Baseline characteristics of study participants by disease status are shown in Table 1. Participants with symptomatic gallstone disease (n = 4,106) were older and more likely to be female, were less physically active, more often used hormone replacement therapy, and drank less alcohol than those without symptomatic gallstone disease (n = 73,573; all $P < 0.001$). *FTO* (rs9939609), *MC4R* (rs17782313), and *TMEM18* (rs6548238) genotypes were in Hardy-Weinberg's equilibrium ($P = 0.83$, 0.77, and 0.27, respectively).

BMI and Symptomatic Gallstones: Observational Estimates. Increasing BMI in quintiles was associated prospectively with stepwise increased risk of symptomatic gallstone disease (Fig. 2). During a mean follow-up of 5.3 years (range, 0.0-19.6), age- and sex-adjusted HRs for symptomatic gallstone disease for individuals in the fifth quintile for BMI (mean BMI = 32.5 kg/m^2) versus individuals in the first quintile (mean BMI = 20.9 kg/m^2) were 2.87 (95% confidence interval [CI]: 2.35-3.49; *P* for trend: <0.001; Fig. 2, left column). Corresponding estimates multifactorially adjusted (for age, sex, physical activity, hormonal replacement therapy, and alcohol consumption) were 2.84 (95% CI: 2.32-3.46; *P* for trend: <0.001; Fig. 2, right column).

Stratifying on sex revealed a stronger association of BMI with symptomatic gallstone disease in women, compared to men (Fig. 2). Multifactorially adjusted

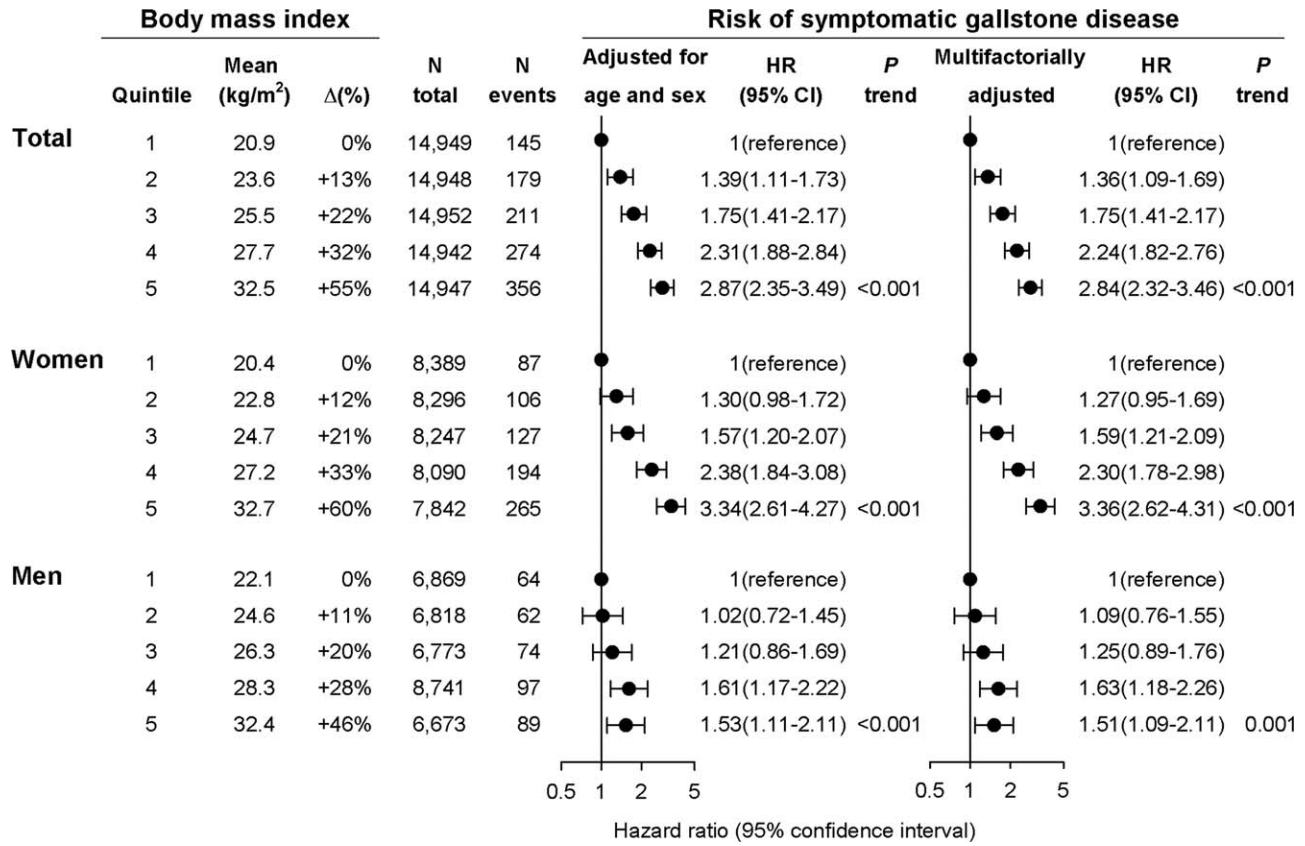

Fig. 2. Prospective risk of symptomatic gallstone disease as a function of baseline BMI in quintiles. Multifactorial adjustment was for age, sex, physical activity, hormone replacement therapy, and alcohol consumption. N, number. *P* values are for tests for trend of HRs.

HRs for individuals in the fifth versus the first BMI quintile were 3.36 (95% CI: 2.62-4.31) and 1.51 (95% CI: 1.09-2.11) in women and men, respectively (*P* for interaction between sex and BMI on risk of symptomatic gallstone disease = 0.01). BMI did not interact with age, physical activity, hormone replacement therapy, or alcohol consumption on risk of symptomatic gallstone disease (data not shown).

Allele Score and BMI. The association of the combined allele score with BMI is shown in Fig. 3 (left column). An increasing number of BMI-increasing alleles was associated with a stepwise increase in mean BMI of up to +5.2% (1.3 kg/m^2) for 6 versus 0-1 alleles, +4.3% (1.1 kg/m^2) in women and +6.1% (1.6 kg/m^2) in men (all *P* for trend: <0.001). The individual variants (*FTO* [rs9939609], *MC4R* [rs17782313], and *TMEM18* [rs6548238]) were associated with stepwise increases in BMI of up to +2.6% (all $P < 0.001$; Supporting Figure).

We tested whether potential confounding factors were associated with BMI, symptomatic gallstone disease, and allele score (Fig. 4). Age, sex, physical activity, consumption of alcohol, fast food, and vegetables, and (in women) hormone replacement therapy and parity were all strongly associated with BMI and risk of symptomatic gallstone disease and thus constitute potential confounders for the observational BMI-gallstone association (Fig. 4, left and middle columns). In contrast, allele score was not associated with any of these potential confounders (Fig. 4, right column). *ABCG8* D19H (a known genetic risk factor for gallstone disease) was associated with symptomatic gallstone disease in our cohort (Fig. 4, middle column, bottom, DH+HH versus DD; OR, 2.1 [95% CI: 1.9-2.2]), but not with BMI or with the BMI-increasing allele score ($P = 0.72$ and 0.77).[11]

Allele Score and Symptomatic Gallstone Disease. Assuming that increased BMI causes symptomatic gallstones, lifelong increased BMI levels resulting from genetic variation should confer a similar increase in risk of symptomatic gallstones as that observed for increased BMI in the general population. For example, the 5.2% increase in BMI for individuals with 6 versus 0-1 BMI-increasing alleles would theoretically predict an increased risk of symptomatic gallstones with an HR of 1.10 (95% CI: 1.08-1.11; Fig. 3, middle column). During a mean follow-up of 33.0 years (range, 0.0-34.4), the multifactorially adjusted HR for

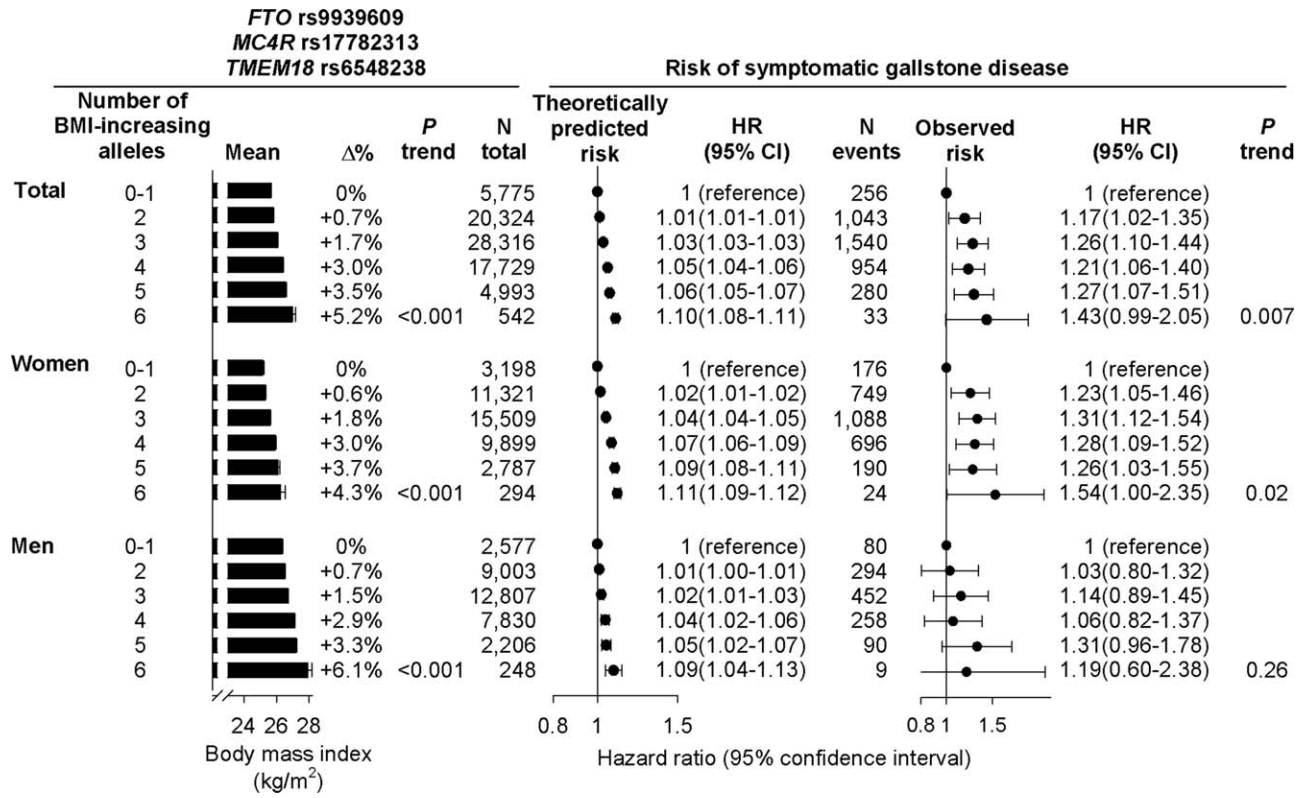

Fig. 3. BMI and theoretically predicted and observed risks of symptomatic gallstone disease as a function of a BMI-associated allele score. Allele score was constructed by summation of BMI-increasing alleles of the following genotypes: *FTO*(rs9939609), 0 (TT), 1 (TA), and 2 (AA); *MC4R*(rs17782313), 0 (TT), 1 (CT), and 2 (CC); and *TMEM18*(rs6548238), 0 (TT), 1 (CT), and 2 (CC). N, number. *P* values are for tests for trend of BMI or HRs.

symptomatic gallstone disease was 1.43 (95% CI: 0.99-2.05) in individuals with 6 versus 0-1 BMI-increasing alleles (*P* for trend = 0.007; Fig. 3, right column). The corresponding HRs were 1.54 (95% CI: 1.00-2.35) in women and 1.19 (95% CI: 0.60-2.38) in men (Fig. 3). There was no statistically significant interaction between allele score and sex on risk of symptomatic gallstone disease ($P = 0.49$). Associations of the individual genotypes are shown in the Supporting Figure.

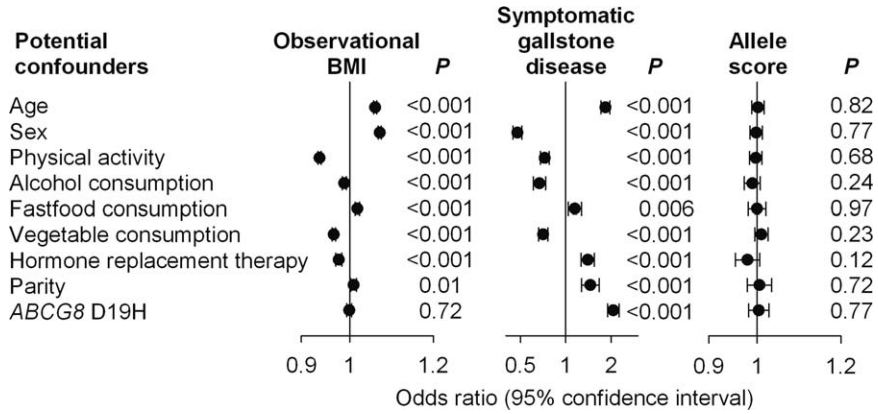

Fig. 4. Associations of potential confounders with BMI, symptomatic gallstone disease, and allele score. Potential confounders were dichotomized: age (≥55 versus <55 years); sex (male versus female) physical activity (≥4 versus <4 hours of light physical activity per week); alcohol consumption (>14/21 versus ≤14/21 units per week for women/men); fast-food consumption (≥1 versus <1 times per week); vegetable consumption (≥1 versus <1 times per day); hormone replacement therapy (yes/no); parity (any number of births versus nulliparity); and *ABCG8* D19H DH+HH versus DD. For each potential confounder, logistic regression analysis was used to calculate sex- and age-adjusted ORs and *P* values for, respectively, a 1-kg/m^2 increase in observational BMI, symptomatic gallstones versus no event, and a 1-unit increase in allele score.

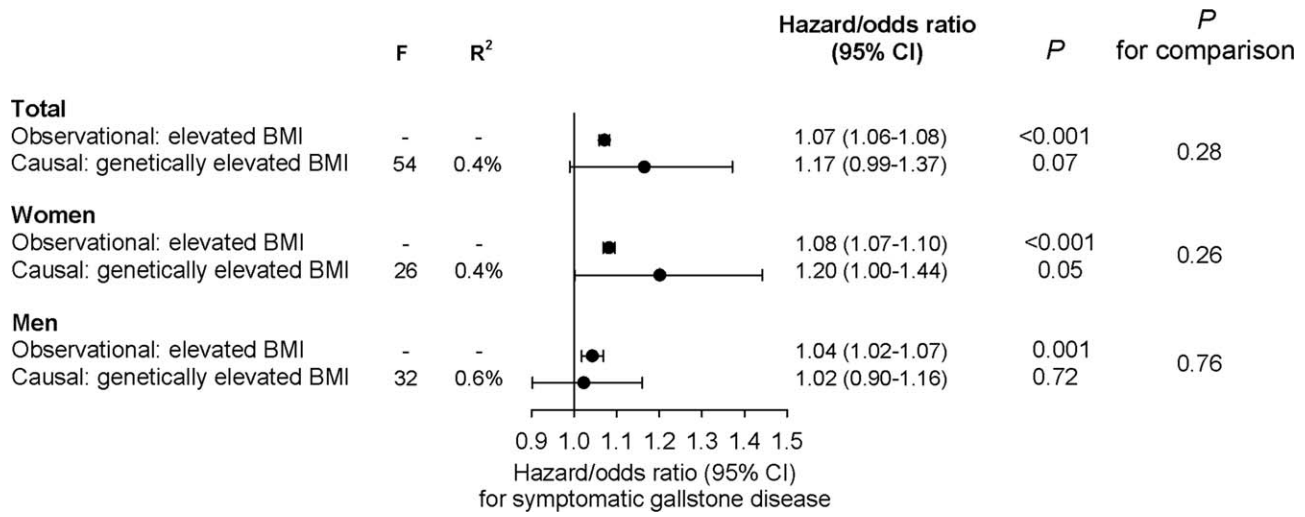

Fig. 5. Risk of symptomatic gallstone disease for a 1-kg/m^2 increase in, respectively, observational and causal, genetically determined BMI. The HR for a 1-kg/m^2 increase in observational BMI was calculated using Cox's regression, whereas the OR for genetically elevated BMI was derived from an instrumental variable analysis. *P* value: significance of HR or OR. *P* comparison: between the estimate from observational epidemiology and the causal, genetic estimate. F = strength of the genetic instrument (>10 indicates sufficient strength). R^2 = percent contribution of genetic instrument to the variation in BMI.

BMI and Symptomatic Gallstone Disease: Causal Estimates. We examined the potential causal effect of increased BMI on risk of symptomatic gallstone disease in instrumental variable analyses (Fig. 5). Causal ORs for a 1-kg/m^2 increase in genetically determined BMI were 1.17 (95% CI: 0.99-1.37) overall and 1.20 (95% CI: 1.00-1.44) and 1.02 (95% CI: 0.90-1.16) in women and men, respectively (Fig. 5). The corresponding observed multifactorially adjusted HRs for symptomatic gallstone disease for a 1-kg/m^2 increase in BMI were 1.07 (95% CI: 1.06-1.08) overall and 1.08 (95% CI: 1.07-1.10) and 1.04 (95% CI: 1.02-1.07) in women and men, respectively.

Discussion

The novel finding of this study of 77,679 individuals from the general population, including 4,106 with symptomatic gallstone disease, is that genetically elevated BMI is associated with increased risk of symptomatic gallstone disease, compatible with a causal association between elevated BMI and increased risk of symptomatic gallstones.

Obesity is known to be associated with gallstone disease in observational epidemiology.[1-5] However, a range of environmental, socioeconomic, and/or behavioural factors that are associated with obesity may also influence risk of gallstone disease. This makes it difficult to determine whether obesity *per se* is causally involved in gallstone disease. To overcome this inherent limitation of observational epidemiology, we used the Mendelian randomization approach,[8] a design that uses genetic variants that are associated with BMI, but not with potential confounding factors, as instruments to examine the effect of lifelong elevated BMI on risk of symptomatic gallstone disease.

In our Mendelian randomization study, the risk of symptomatic gallstone disease was increased 7% for every 1-kg/m^2 increase in measured BMI (the observational estimate). The corresponding risk increase for every 1 kg/m^2 in genetically determined BMI was 17% (the causal, genetic estimate). The concordance between the observational and genetic estimates supports that increased BMI *per se* is a causal risk factor for symptomatic gallstone disease, particularly because genetically elevated BMI even seemed to have a larger effect size on symptomatic gallstone disease than the effect size from observational analyses.

Numerous explanations for how obesity may influence gallstone formation have been proposed. Obesity may increase hepatic *de novo* cholesterol synthesis and hepatobiliary cholesterol efflux, a key event in the development of cholesterol gallstones.[15] Increased abdominal fat mass may cause gallbladder hypomotility and bile stasis, another risk factor for gallstone formation, a hypothesis that is supported by some,[16] but not all,[17] previous studies. Weight cycling (i.e., weight loss and regain) is a modest risk factor for gallstone disease.[18] Part of the BMI-gallstone association could be caused by weight cycling resulting from intentional weight loss, followed by unintentional weight regain, among overweight individuals. Also, factors that are secreted or metabolized by adipocytes may influence

gallstone formation. For example, estrogen is produced by adipocytes,[19] and estrogen therapy in women is known to increase the risk of gallstone disease.[20] Estrogen may promote gallstone formation by increasing the rate of hepatobiliary cholesterol efflux[21] and perhaps also by a direct pronucleating effect of estrogen molecules on biliary cholesterol.[22] It has been speculated that leptin, secreted by adipocytes and involved in appetite regulation and energy expenditure, could have lithogenic effects.[23] Furthermore, low levels of adiponectin, another hormone secreted by adipocytes and inversely associated with fat mass, have been associated with gallstone disease in animal and human studies.[24,25] Finally, obesity-associated hyperinsulinemia may have a causal effect on gallstone formation, perhaps mediated by hepatic insulin resistance and secretion of a more lithogenic bile.[26,27]

In agreement with our study, previous observational epidemiological studies have found that obesity is a stronger risk factor for gallstone disease in females than in males, in adults as well as in adolescents and children.[1,2,28] The biological mechanisms underlying this gender difference are unknown, but estrogen secreted by adipocytes may play a role, as discussed above.

Unraveling the genetic basis of gallstone disease has progressed rapidly during the last decade, but no BMI-associated lithogenic variants have, so far, been identified.[29] One implication of the data presented here is that any genetic variant that increases BMI should also theoretically increase the risk of symptomatic gallstone disease to the degree predicted by the effect of the genotype on BMI. The only genome-wide association study (GWAS) of gallstone disease did not report any BMI-associated lithogenic variants.[30] However, this GWAS[30] was not powered to detect modest associations (less than 5% power in the discovery cohort to detect ORs below ~1.2), as those reported on in the present study. One case-control study[31] did not observe associations between two BMI-associated variants in the leptin gene and gallstone disease, but the sample size (n = 54 cases, 43 controls) was too small to detect modest effects. Future studies will require very large sample sizes (thousands of gallstone cases) to detect the likely small effects of individual BMI-associated genetic variants on risk of gallstone disease, as also suggested by our study.

There are naturally potential limitations to our study. We defined "symptomatic gallstone disease" by ICD codes received in hospitals. The prevalence of 5.3% symptomatic gallstones in our study is comparable to results from previous studies that identified gallstones by similar registry-based methods.[32] Because gallstone disease is a hard clinical endpoint with well-defined diagnostic critera, the risk of misclassification is likely minor, and individuals receiving ICD codes for gallstones in hospitals likely had symptomatic gallstones. In support of this, approximately 68% of individuals with symptomatic gallstone disease in our cohort underwent cholecystectomy.[11] However, we cannot rule out that a small fraction of symptomatic gallstones defined this way were, in fact, asymptomatic gallstones diagnosed incidentally. Another potential limitation to our definition of symptomatic gallstone disease is that treating physicians might be more suspicious of gallbladder disease in obese than in lean individuals. Such an ascertainment bias might have led to a slight overestimation of the BMI-gallstone association in the present study. However, the estimates of the BMI-gallstone association reported here are in agreement with those from previous studies that used ultrasound to diagnose gallstones in asymptomatic individuals (i.e., studies unlikely to suffer from ascertainment bias).[1,2] Also, we did not have data on stone composition (i.e., cholesterol/mixed/pigment). Thus, the pathophysiological mechanisms by which obesity influences gallstone formation could not be assessed here. Finally, we only studied white individuals of Danish descent. Because ethnic differences in gallstone prevalence are well known, the results reported here may not necessarily translate to other ethnicities.

There are also potential limitations to the use of the Mendelian randomization approach.[8] For example, the genetic variants used may have influenced risk of symptomatic gallstone disease by other pathways than BMI (i.e., pleiotropy). However, this concern is lessened by the use of multiple genetic variants, each associated with increased BMI and each influencing BMI independently and by different pathways.[8,10] Also, the effect of lifelong genetically elevated BMI may have been buffered by compensatory biological mechanisms (i.e., canalisation). Canalization might theoretically obscure effects of BMI-associated genetic variants on symptomatic gallstone disease and would thus tend to drive associations toward the null, but is unlikely to account for positive associations, as those reported in the present study.

In conclusion, elevated BMI as measured at baseline, as well as genetically (lifelong and unconfounded) elevated BMI, is associated with increased risk of symptomatic gallstone disease. Taken together, this indicates that elevated BMI *per se* is likely a causal risk factor for symptomatic gallstone disease, which is most pronounced in women. These data reemphasize obesity as a major cause of human morbidity and provide

additional impetus for lifestyle interventions aimed at weight loss among overweight and obese individuals in the general population.

Acknowledgment: The authors are indebted to the staff and participants of the Copenhagen General Population Study and the Copenhagen City Heart Study for their important contributions to our study.

References

1. Attili AF, Capocaccia R, Carulli N, Festi D, Roda E, Barbara L, et al. Factors associated with gallstone disease in the MICOL experience. Multicenter Italian Study on Epidemiology of Cholelithiasis. HEPATOLOGY 1997;26:809-818.
2. Jørgensen T. Gall stones in a Danish population. Relation to weight, physical activity, smoking, coffee consumption, and diabetes mellitus. Gut 1989;30:528-534.
3. Katsika D, Tuvblad C, Einarsson C, Lichtenstein P, Marschall HU. Body mass index, alcohol, tobacco and symptomatic gallstone disease: a Swedish twin study. J Intern Med 2007;262:581-587.
4. Grundy SM. Cholesterol gallstones: a fellow traveler with metabolic syndrome? Am J Clin Nutr 2004;80:1-2.
5. Portincasa P, Moschetta A, Palasciano G. Cholesterol gallstone disease. Lancet 2006;368:230-239.
6. Hayes KC, Livingston A, Trautwein EA. Dietary impact on biliary lipids and gallstones. Annu Rev Nutr 1992;12:299-326.
7. Leitzmann MF, Rimm EB, Willett WC, Spiegelman D, Grodstein F, Stampfer MJ, et al. Recreational physical activity and the risk of cholecystectomy in women. N Engl J Med 1999;341:777-784.
8. Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey SG. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. Stat Med 2008;27:1133-1163.
9. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet 2010;42:937-948.
10. Nordestgaard BG, Palmer TM, Benn M, Zacho J, Tybjaerg-Hansen A, Davey SG, Timpson NJ. The effect of elevated body mass index on ischemic heart disease risk: causal estimates from a Mendelian randomisation approach. PLoS Med 2012;9:e1001212.
11. Stender S, Frikke-Schmidt R, Nordestgaard BG, Tybjaerg-Hansen A. Sterol transporter adenosine triphosphate-binding cassette transporter G8, gallstones, and biliary cancer in 62,000 individuals from the general population. HEPATOLOGY 2011;53:640-648.
12. Zacho J, Tybjaerg-Hansen A, Jensen JS, Grande P, Sillesen H, Nordestgaard BG. Genetically elevated C-reactive protein and ischemic vascular disease. N Engl J Med 2008;359:1897-1908.
13. Baum C, Schaffer M, Stillman S. Instrumental variables and GMM: estimation and testing. Stata J 2003;3:1-31.
14. Altman DG, Bland JM. Interaction revisited: the difference between two estimates. BMJ 2003;326:219.
15. Ståhlberg D, Rudling M, Angelin B, Bjorkhem I, Forsell P, Nilsell K, Einarsson K. Hepatic cholesterol metabolism in human obesity. HEPATOLOGY 1997;25:1447-1450.
16. Mathus-Vliegen EM, Van Ierland-Van Leeuwen ML, Terpstra A. Determinants of gallbladder kinetics in obesity. Dig Dis Sci 2004;49:9-16.
17. Vezina WC, Paradis RL, Grace DM, Zimmer RA, Lamont DD, Rycroft KM, et al. Increased volume and decreased emptying of the gallbladder in large (morbidly obese, tall normal, and muscular normal) people. Gastroenterology 1990;98:1000-1007.
18. Tsai CJ, Leitzmann MF, Willett WC, Giovannucci EL. Weight cycling and risk of gallstone disease in men. Arch Intern Med 2006;166:2369-2374.
19. Key TJ, Appleby PN, Reeves GK, Roddam A, Dorgan JF, Longcope C, et al. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. J Natl Cancer Inst 2003;95:1218-1226.
20. Cirillo DJ, Wallace RB, Rodabough RJ, Greenland P, LaCroix AZ, Limacher MC, Larson JC. Effect of estrogen therapy on gallbladder disease. JAMA 2005;293:330-339.
21. Wang HH, Liu M, Clegg DJ, Portincasa P, Wang DQ. New insights into the molecular mechanisms underlying effects of estrogen on cholesterol gallstone formation. Biochim Biophys Acta 2009;1791:1037-1047.
22. Brown AC, Wrenn SP, Suresh N, Meyers WC, Abedin MZ. Gender differences in cholesterol nucleation in native bile: estrogen is a potential contributory factor. J Membr Biol 2009;232:35-45.
23. Méndez-Sánchez N, Ponciano-Rodrigoez G, Chavez-Tapia N, Uribe M. Effects of leptin on biliary lipids: potential consequences for gallstone formation and therapy in obesity. Curr Drug Targets Immune Endocr Metabol Disord 2005;5:203-208.
24. Ogiyama H, Kamada Y, Kiso S, Araki H, Yamada T, Nishihara T, et al. Lack of adiponectin promotes formation of cholesterol gallstones in mice. Biochem Biophys Res Commun 2010;399:352-358.
25. Wang SN, Yeh YT, Yu ML, Wang CL, Lee KT. Serum adiponectin levels in cholesterol and pigment cholelithiasis. Br J Surg 2006;93:981-986.
26. Misciagna G, Guerra V, Di Leo A, Correale M, Trevisan M. Insulin and gall stones: a population case control study in southern Italy. Gut 2000;47:144-147.
27. Biddinger SB, Haas JT, Yu BB, Bezy O, Jing E, Zhang W, et al. Hepatic insulin resistance directly promotes formation of cholesterol gallstones. Nat Med 2008;14:778-782.
28. Koebnick C, Smith N, Black MH, Porter AH, Richie BA, Hudson S, et al. Pediatric obesity and gallstone disease. J Pediatr Gastroenterol Nutr 2012;55:328-333.
29. Krawczyk M, Wang DQ, Portincasa P, Lammert F. Dissecting the genetic heterogeneity of gallbladder stone formation. Semin Liver Dis 2011;31:157-172.
30. Buch S, Schafmayer C, Völzke H, Becker C, Franke A, von Eller-Eberstein H, et al. A genome-wide association scan identifies the hepatic cholesterol transporter ABCG8 as a susceptibility factor for human gallstone disease. Nat Genet 2007;39:995-999.
31. Méndez-Sánchez N, Bermejo-Martinez L, Chávez-Tapia NC, Zamora-Valdes D, Sánchez-Lara K, Uribe-Ramos MH, et al. Obesity-related leptin receptor polymorphisms and gallstones disease. Ann Hepatol 2006;5:97-102.
32. Katsika D, Grjibovski A, Einarsson C, Lammert F, Lichtenstein P, Marschall HU. Genetic and environmental influences on symptomatic gallstone disease: a Swedish study of 43,141 twin pairs. HEPATOLOGY 2005;41:1138-1143.