



The Role of Gallstones in Gallbladder Cancer in India: A Mendelian Randomization Study **A C**

Sharayu Mhatre^{1,2}, Rebecca C. Richmond^{3,4}, Nilanjan Chatterjee^{5,6,7}, Preetha Rajaraman⁸, Zhaoming Wang^{9,10}, Haoyu Zhang¹¹, Rajendra Badwe^{2,12}, Mahesh Goel^{2,12}, Shraddha Patkar^{2,12}, Shailesh V. Shrikhande^{2,13}, Prachi S. Patil^{2,14}, George Davey Smith^{3,4}, Caroline L. Relton^{3,4}, and Rajesh P. Dikshit^{1,2}

ABSTRACT

Background: Past history of gallstones is associated with increased risk of gallbladder cancer in observational studies. We conducted complementary observational and Mendelian randomization (MR) analyses to determine whether history of gallstones is causally related to development of gallbladder cancer in an Indian population.

Methods: To investigate associations between history of gallstones and gallbladder cancer, we used questionnaire and imaging data from a gallbladder cancer case-control study conducted at Tata Memorial Hospital, Mumbai, Maharashtra, India (cases = 1,170; controls = 2,525). We then used 26 genetic variants identified in a genome-wide association study of 27,174 gallstone cases and 736,838 controls of European ancestry in an MR approach to assess causality. The association of these genetic variants with both gallstones and gallbladder cancer was examined in the gallbladder cancer case-control study. Various complementary MR approaches

were used to evaluate the robustness of our results in the presence of pleiotropy and heterogeneity, and to consider the suitability of the selected SNPs as genetic instruments for gallstones in an Indian population.

Results: We found a strong observational association between gallstones and gallbladder cancer using self-reported history of gallstones [OR = 4.5; 95% confidence interval (CI) = 3.5–5.8] and with objective measures of gallstone presence using imaging techniques (OR = 2.0; 95% CI = 1.5–2.7). We found consistent causal estimates across all MR techniques, with ORs for gallbladder cancer in the range of 1.3–1.6.

Conclusions: Our findings indicate a causal relationship between history of gallstones and increased risk of gallbladder cancer, albeit of a smaller magnitude than those found in observational analysis.

Impact: Our findings emphasize the importance of gallstone treatment for preventing gallbladder cancer in high-risk individuals.

Introduction

Although gallbladder cancer is rare in most parts of the world, it is more common in certain populations, including some parts of India, Chile, and Mexico (1). While the aetiology of gallbladder cancer remains poorly understood, increased risk of gallbladder cancer has been associated with *Salmonella Typhi* infection (2), possession of certain genetic variants (3), obesity (4), and high consumption of mustard oil (5). In addition, a personal history of gallstones has long been found to be strongly related to gallbladder cancer risk (6).

While a causal effect of gallstones on gallbladder cancer is probable, variation in estimates of risk have been observed in previous studies, most likely due to differences in study design and the methods used to collect information on gallstones (7). In particular, case-control studies with questionnaire-based retrospective ascertainment of history of gallstones could result in differential misclassification and a biased estimate of risk because cases might report history of gallstones more frequently than controls as they undergo various investigative procedures, including ones that may uncover silent gallstones. The case-control design also raises the issue of temporality, that is, it cannot be determined whether the gallstone occurred prior to, or after, the development of gallbladder cancer.

It is of clinical importance to confirm causality for the association of gallstones with gallbladder cancer, which could direct intervention strategies in countries with high risk of gallbladder cancer because treatment for gallstones is considerably lower in cost, complexity, and risk compared with treatment of gallbladder cancer. To better elucidate the role of gallstones history in the aetiology of gallbladder cancer, and to determine the magnitude of any causal effect, we examined potential biases in the estimation of gallbladder cancer risk using both

¹Centre for Cancer Epidemiology, Tata Memorial Centre, Kharghar, Navi Mumbai, Maharashtra, India. ²Homi Bhabha National Institute (HBNI), Mumbai, Maharashtra, India. ³MRC Integrative Epidemiology Unit, University of Bristol, Bristol, United Kingdom. ⁴Department of Population Health Sciences, Bristol Medical School, University of Bristol, Bristol, United Kingdom. ⁵Division of Cancer Epidemiology and Genetics, NCI, Bethesda, Maryland. ⁶Department of Biostatistics, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland. ⁷Department of Oncology, School of Medicine, Johns Hopkins University, Baltimore, Maryland. ⁸Office of Global Affairs, Department of Health and Human Services, Washington, DC. ⁹Department of Epidemiology and Cancer Control, St. Jude Children's Research Hospital, Memphis, Tennessee. ¹⁰Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, Tennessee. ¹¹School of Materials Science and Engineering, Shenyang University of Technology, Shenyang, China. ¹²Department of Surgical Oncology, Tata Memorial Centre, Mumbai, Maharashtra, India. ¹³Division of Cancer Surgery, GI & HPB Surgical Services, Tata Memorial Hospital, Mumbai, Maharashtra, India. ¹⁴Department of Medical Gastroenterology, Tata Memorial Hospital, Mumbai, Maharashtra, India.

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S. Mhatre and R.C. Richmond contributed equally as co-first authors of this article.

C.L. Relton and R.P. Dikshit contributed equally as co-last authors of this article.

Corresponding Author: Rajesh P. Dikshit, Centre for Cancer Epidemiology, Tata Memorial Centre, Kharghar, Navi Mumbai, India. Phone: 91-2230435151; E-mail: dixr24@hotmail.com

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self-report and objective measures of gallstone history (from imaging techniques) in a large Indian case-control study of gallbladder cancer. In addition, we used genetic variants strongly associated with risk of gallstones (8) to estimate the causal effect of gallstones on risk of gallbladder cancer within a Mendelian randomization (MR) framework. MR is a technique that uses genetic variants as unconfounded proxies for an exposure of interest, which are less susceptible to problems of measurement error and reverse causation, as a method to ascertaining better evidence for causality (9).

Materials and Methods

Study design

A case-control study was conducted at the Tata Memorial Hospital (TMH), Mumbai, Maharashtra, India, from 2010 to 2015 to evaluate the relationship between lifestyle and genetic risk factors and gallbladder cancer. Details of the methodology are published elsewhere (10). In brief, a total of 1,170 gallbladder cancer cases and 2,525 visitor controls were enrolled into the study. All cases of gallbladder cancer [International Classification of Diseases for Oncology Version 3 (ICD-O-3) site code C23] were microscopically confirmed. Controls were recruited from friends, neighbours, colleagues, in-laws, spouses, and relatives (other than first-degree relatives) visiting TMH (Mumbai, Maharashtra, India). Controls were frequency matched to cases on age (± 10 years), gender, and region. Matching by geographical region (north, north-east, west, central, and south) was conducted using reported place of current residence at the time of enrolment. We obtained written informed consent from all study participants before enrolment and obtained ethical approval from all relevant local and hospital-based institutional review boards.

A structured questionnaire was used to obtain self-reported history of gallstones along with other lifestyle and environmental risk factors. To procure more objective measures of gallstone history, we examined the case records of all gallbladder cancer cases for imaging techniques such as ultrasonography (USG), MRI, CT scan and accordingly classified them as being with or without gallstones. As the visitor controls that were used for the observational study did not undergo similar kinds of imaging investigations, we used breast cancer cases as a second control group as a comparator for this aspect of the study (11). Breast cancer cases were recruited as a control group as virtually all breast cancer cases undergo imaging evaluation (particularly USG) and thus it is possible to look for evidence of the presence of gallstones in their case records. This allowed an additional comparison of female gallbladder cancer cases with female breast cancer cases as a control for the presence of gallstones confirmed by imaging. Breast cancer cases were enrolled during the same study period using the same questionnaire and case-control study design as used for the gallbladder cancer study.

Genetic data

Participants in the gallbladder cancer study were genotyped on the HumanOmniExpress-24 version 1.1 IlluminaBeadChip array (Illumina) at the Centre for Cancer Epidemiology, Tata Memorial Centre (Mumbai, Maharashtra, India; ref. 3). SNPs with a call rate of less than 95% (cut-off level empirically determined), failure to meet Hardy-Weinberg equilibrium at $P < 10^{-6}$ or a minor allele frequency (MAF) of < 0.005 were excluded in quality control procedures. The study also excluded samples with a call rate of less than 90% (cut-off level empirically determined), no intensity, gender discordance ($> 5\%$ heterozygosity on the basis of the X chromosome SNPs for males or $< 15\%$ heterozygosity on the basis of the X chromosome SNPs for

females), first-degree relatives who were also genotyped in the study on the basis of identity by descent ($\pi\text{-hat} > 0.48$), and unexpected duplicates. After genotyping, we performed genome-wide imputation to statistically infer untyped variants using IMPUTE2 software version 2.2.2 (12) and version 3 of the 1000 Genomes Project data as the reference set (13). We included 10.4 million SNPs, which were imputed with an imputation information (info) score of more than 0.3 and a MAF of more than 0.5%, for analysis. A genome-wide association study (GWAS) of gallbladder cancer was performed among cases and controls, as described previously (3). The GWAS estimates were adjusted for age, sex, and five significant eigenvectors of the derived principal components.

A total of 32 SNPs associated with gallstones at a genome-wide level of significance ($P < 5 \times 10^{-8}$) have been identified in a previous GWAS (8). Two SNPs (rs756082276, rs756935975) were neither genotyped nor imputed in the gallbladder cancer study (3). Two SNPs (rs34851490, rs45575636) which were in linkage disequilibrium with other SNPs used in the analysis ($r^2 > 0.001$) were excluded. Similarly, two palindromic SNPs (rs2469991, rs1935) were excluded. The remaining 26 SNPs were used in both one-sample and two-sample MR. All 32 SNPs, their location and nearby gene, with reasons for exclusion are tabulated in Supplementary Table S1.

Statistical analysis

Observational analysis

The association between gallstones and gallbladder cancer was initially assessed using self-reported gallstone history. Logistic regression models were adjusted for the following potential confounders: age, current residential region (north, north-east, west, central, and south), education (< 5 years, ≥ 5 years), gender, tobacco chewing and/or smoking (yes/no), and waist-to-hip ratio (continuous variable), per capita per month mustard oil consumption (continuous), and per capita per week fresh fish consumption (continuous). Mustard oil and fresh fish consumption have been included as potential confounders because both have been associated with gall bladder cancer in the same dataset under investigation (5). To avoid bias due to reporting of gallstones as a consequence of development of cancer, we conducted sensitivity analysis by removing participants with a history of gallstones within 1 year of diagnosis or interview for cases and controls, respectively.

To obtain a more objective measure of gallstones, we used information on the presence of gallstones among gallbladder cancer cases confirmed by imaging techniques. The information about the presence of gallstones among breast cancer cases was similarly ascertained and breast cancer cases were used as a control. Logistic regression was then used to estimate risk of image-confirmed gallstones on gallbladder cancer among females only.

The associations obtained were multiplied by 0.693 to observe the change in odds of gallbladder cancer per 2-fold increase (doubling) in the odds of gallstones, to be compared with the results of MR. All observational analyses were performed using the statistical package STATA version 15.0.

MR analysis

Two-sample MR

We used a two-sample MR approach to estimate the risk of gallstones on the development of gallbladder cancer, using the Two-SampleMR package in R version 3.5.1 (14). For each of the SNPs robustly associated with gallstones, information on the SNP-exposure (gallstones) and SNP-outcome (gallbladder cancer) effects were obtained from genome-wide association analyses conducted in

separate studies (3, 8). Specifically, we performed a look-up of the 26 SNPs associated with gallstones from the GWAS of gallbladder cancer (3) and extracted the following summary data for each SNP: the effect estimate (log OR) for gallbladder cancer per copy of the effect allele and its SE, the reference allele, and the effect allele along with its frequency. We then combined information on the SNP-gallstone associations from the previous GWAS with information on the SNP-gallbladder cancer associations and performed MR, using the inverse variance weighted (IVW) approach. For this, we first calculated the causal effect of gallstones on gallbladder cancer by calculating the SNP-specific Wald ratios $[\beta(\text{outcome} - \text{SNP})/\beta(\text{exposure} - \text{SNP})]$; where β reflects the log odds). SEs of the Wald ratios were approximated by the delta method. Wald ratios for each SNP were combined in a fixed effects meta-analysis after weighting each ratio estimate by the inverse variance of their associations with the outcome. We also conducted random effects meta-analysis to allow for heterogeneity in the individual SNP effect estimates. The causal estimate obtained was multiplied by \log_2 ($=0.693$) to yield the change in log odds of gallbladder cancer per doubling in the odds of gallstone (15).

To account for horizontal pleiotropy, whereby the genetic variants might affect gallbladder cancer through pathways other than via gallstones, we used different MR methods, namely, the weighted median (16), MR Egger (17), and modal estimates (18). The weighted median estimator takes the median value of the Wald ratio over the SNPs, and is robust to outliers as the estimator is consistent for the causal effect if at least half the information in the analysis comes from valid instruments (16). The MR-Egger approach is similar to the IVW approach except that the intercept is not fixed to zero, and so allows for directional pleiotropy. An intercept term that differs from zero is indicative of overall directional pleiotropy (17). The MR-Egger approach will return a valid causal estimate in the presence of directional pleiotropy. The MR modal estimates assume that the most common causal effect is consistent with the true causal effect (18). As each of these methods make different assumptions regarding horizontal pleiotropy, a consistent effect across multiple MR methods strengthens causal evidence (19).

For the graphical representation of the effect of each SNP on gallbladder cancer, we constructed a forest plot of Wald ratios, with summary effect estimates derived using the MR-Egger and IVW approaches, as well as a scatter plot of the SNP associations with both gallstones and gallbladder cancer. For assessing asymmetry of SNP-specific causal estimates and thus directional pleiotropy, we provide a funnel plot for visual assessment.

To further detect and correct causal estimates for potential violation of the MR assumptions, we assessed heterogeneity in the causal effects estimated by each SNP by generating Cochran Q statistics (20). We performed radial MR (21) and the MR pleiotropy residual sum and outlier (MR-PRESSO) test (22) to detect and correct for influential outliers. Both approaches can be used to identify outliers which may represent strong and potentially highly pleiotropic instruments. Using radial MR, SNPs with the largest contribution to Cochran Q for heterogeneity were identified removed and the data reanalyzed. We also plotted a Galbraith radial plot for straightforward detection of outliers and influential data points. Three parts of the MR-PRESSO test were also conducted: (i) the MR-PRESSO global test which identifies horizontal pleiotropy, (ii) the outlier corrected causal estimates which correct for detected horizontal pleiotropy, and (iii) the MR-PRESSO distortion test which determines whether causal estimates differ after adjustment for outliers. We also performed the contamination mixture method for MR which can (i) identify subgroups of genetic variants with similar causal estimates and therefore similar mechanisms of

action, and (ii) obtain robust causal estimates in the presence of invalid instruments (23).

One-sample MR

One assumption of two-sample MR is that the exposure and outcome datasets are homogeneous with respect to the underlying populations (24). As the GWAS for gallstones was conducted in a European population and the GWAS for gallbladder cancer in an Indian population, we carried out further analyses to assess the suitability of the selected SNPs as genetic instruments for gallstones in the Indian population.

We investigated associations of the previously identified gallstone SNPs in relation to self-reported history of gallstones in our study. We also performed a one-sample MR (25) using data on SNPs, gallstones, and gallbladder cancer status for all participants in our case-control study to address the issue of population heterogeneity. For this, we generated a genetic risk score (GRS) for gallstones, calculated as the sum of the number of gallstone-increasing alleles for each of the individuals in our study (26).

To estimate the risk of gallbladder cancer using a one-sample MR approach, we used the two-stage least squares method (27, 28). In the first stage, the self-reported history of gallstones (exposure) was regressed on the GRS. The predicted values of the gallstones were taken from the first-stage regression model. In the second stage, the gallbladder cancer (outcome) was regressed over the predicted values of the exposure by logistic regression. We calculated the F-statistic of the GRS on gallstone to assess instrument strength. To test the MR assumption that genetic variants should not be associated with confounders of exposure-outcome relation, we investigated associations between GRS and potential confounders (age, gender, residential region, waist-to-hip ratio, mustard oil consumption, and fresh fish consumption).

Results

Conventional epidemiologic analyses

The overall prevalence of self-reported gallstone history was 40.0% among cases and 2.1% among controls. Gallstone prevalence was higher in the north and north-eastern geographical regions compared with other geographical regions in India (Table 1). The OR per doubling of exposure to gallstones based on self-reported gallstone history and gallbladder cancer was observed to be 10.0 (95% CI = 8.0–12.8; Table 2). After removing participants with history of gallstones 1 year prior to diagnosis/interview, the OR was attenuated but still large (OR = 4.5; 95% CI = 3.5–5.8). The objective measure of gallstone history with 580 female gallbladder cancer cases and 787 female breast cancer cases treated as “controls” revealed a similar increase in the risk of developing gallbladder cancer, albeit of a much smaller magnitude (OR = 2.0; 95% CI = 1.5–2.7).

Genetic associations

The genetic variants used for MR were obtained from a GWAS of gallstones conducted in Europeans. A comparison between European and Indian populations with respect to allele frequencies, risk of developing gallstones and gallbladder cancer for the genetic variants was made and results are shown in Supplementary Table S1. The allele frequencies between the two populations were similar, although with striking differences for some SNPs (e.g., for rs601338, rs1260326, rs174567, rs2469991, rs2290846, where the difference in MAF was >15%). The risk for developing gallstones and gallbladder cancer were in broadly the same direction for the SNPs in the Indian population

Table 1. Prevalence of gallstones among gallbladder cancer case-control study participants.

Variable	Presence of gallstone	
	GBC (n = 1,170) N (%)	Controls (n = 2,525) N (%)
Self-reported gallstone using stringent definition ^a	145 (12.4)	54 (2.1)
Self-reported gallstone history ^b	468 (40.0)	60 (2.4)
Gender	Male	120 (25.6)
	Female	348 (74.4)
Geographical regions	North	207 (44.2)
	Northeast	181 (38.7)
	South	1 (0.2)
	West	47 (10.0)
	Central	32 (6.8)

Abbreviation: GBC, gallbladder cancer.

^aSelf-reported gallstone using stringent definition. Gallstone history was ascertained using definition of self-reported gallstone; however, those gallstones diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of interview for controls were excluded from the analysis.

^bSelf-reported gallstone; reported by study participant as either present/not present.

(consistently increased risk for 80% of SNPs in relation to gallstones and 70% SNPs in relation to gallbladder cancer). When assessed individually, one SNP (rs11887534) showed an increased risk for gallstones and two SNPs (rs11887534, rs686030) showed an increased risk for gallbladder cancer in the Indian population which surpassed Bonferroni correction ($P < 0.002$).

Two-sample MR

When 26 SNPs were used to estimate risk between gallstones and gallbladder cancer, the results of MR were similar when we compared causal effects using the IVW and pleiotropy-robust methods, and ranged from OR = 1.34–1.62 (Table 3; Supplementary Figs. S1–S3). The analysis of the intercept in the MR-Egger test did not provide strong evidence for directional pleiotropy (Egger intercept = -0.014 , $P = 0.77$; Table 3; Supplementary Figs. S1–S3). However, the Cochrane Q test indicated heterogeneity in the individual SNP effects ($Q_{IVW} = 72.95$, $P = 1.4 \times 10^{-6}$ and $Q_{MR\text{-}Egger} = 72.69$, $P = 8.0 \times 10^{-7}$). The radial MR method indicated five outlier SNPs (Supplementary Table S2; Supplementary Fig. S4) contributing to heterogeneity in the causal estimates. We repeated the analysis using the various MR methods after removing these outlier SNPs and IVW and MR-Egger effect esti-

mates were largely unchanged (Table 3). The MR-PRESSO analysis also provided no strong evidence of distortion in causal estimates after adjustment for outliers ($P = 0.303$; Table 3). In addition, the contamination mixture method did not identify any clusters of variants representing distinct causal mechanisms.

One-sample MR

Because the genetic variants were determined from a population of non-Indian ancestry (European ancestry), we performed one-sample MR approach using a GRS derived in the Indian case-control to confirm that the estimates in the two-sample MR analyses were not affected by subtle differences in the genetic architecture of the different populations.

Using a GRS, the risk of developing gallstones was found to increase with an increasing number of risk alleles (OR = 1.03; $P = 0.034$, per allele increase), suggesting that these SNPs contributed to risk of developing gallstones in Indians as well as Europeans. The GRS was a strong instrument for the presence of gallstones (F-statistic = 11.62, $r^2 = 0.024$). None of the potential confounders for gallbladder cancer considered in this study were associated with the GRS (Supplementary Table S3). In two-stage least squares analysis, using the GRS as an instrument for gallstones, we observed an OR of 1.26 (95% CI = 0.88–

Table 2. OR and 95% CI for gallbladder cancer in relation to gallstone using self-reported and image-verified gallstone.

Gallstone history	Self-reported gallstone history ^a			Self-reported gallstone using stringent definition ^b			Gallstone history as determined by imaging techniques ^c		
	N (cases controls)	OR (95% CI) ^d	P ^d	N (cases controls)	OR (95% CI) ^d	P ^d	N (cases controls)	OR (95% CI) ^e	P ^e
Never	693 2,455	Reference		693 2,455	Reference		580 787	Reference	
Ever	468 60	10.0 (8.0–12.8)	≤0.001	145 54	4.5 (3.5–5.8)	≤0.001	208 58	2.0 (1.5–2.7)	≤0.001

Note: Missing values were excluded from analysis.

Abbreviations: CI, confidence interval; OR, odds ratio.

^aSelf-reported gallstone; as per reported by study participant either present/not present.

^bSelf-reported gallstone using stringent definition. Gallstone history was ascertained using definition of self-reported gallstone; however, those gallstones diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of interview for controls were excluded from the analysis.

^cGallstone history as determined by imaging techniques (USG, MRI, and CT scans) and using breast cancer cases as a control group.

^dAdjusted for age (continuous), education (less than 5 years schooling, ≥5 years of education), current residential region (north, northeast, central, west, and south), gender, waist-to-hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no), per capita per month mustard oil consumption (continuous), per capita per week fresh fish consumption (continuous).

^eSame as d, except not adjusted for gender and additionally adjusted for the number of full-term pregnancies.

Table 3. Estimates from various MR methods for the association between gallstone and gallbladder cancer.

Method	OR	95% LCI	95% UCI	P
MR estimates (SNPs = 26)				
MR Egger	1.62	1.00	2.63	0.062
Weighted median	1.49	1.15	1.94	0.003
Inverse variance weighted	1.53	1.16	2.02	0.003
Simple mode	1.59	0.96	2.65	0.084
Weighted mode	1.34	1.09	1.65	0.011
Test for directional horizontal pleiotropy test				
Egger intercept (SE)	-0.014 (0.046)			0.774
MR estimates after removing outlier SNPs (<i>n</i> = 21)				
MR Egger	1.54	1.12	2.13	0.016
Weighted median	1.60	1.22	2.10	≤0.001
Inverse variance weighted	1.50	1.23	1.83	≤0.001
Simple mode	1.86	1.07	3.24	0.040
Weighted mode	1.62	1.24	2.11	0.002
Test for directional horizontal pleiotropy test after removing outlier SNPs (<i>n</i> = 21)				
Radial Egger intercept (SE)	-0.007 (0.028)			0.814
MR-PRESSO raw estimates (SD)	1.53 (0.21)			0.006
MR-PRESSO outlier corrected estimate (SD)	1.39 (0.14)			0.003
Distortion coefficient				0.303
Contamination mixture method	1.57	1.00	1.99	0.050

Abbreviations: LCI, lower confidence interval; OR, odds ratio; UCI, upper confidence interval.

Analytic methods:

Observation study:

Self-reported gallstone history*

8 10 12.8

Self-reported gallstone using stringent definition**

3.5 4.5 5.8

Gallstone history as determined by imaging techniques

1.5 2 2.7

Two-sample MR:

MR Egger

1.0 1.6 2.6

Weighted median

1.1 1.5 1.9

Inverse variance weighted

1.2 1.5 2.0

Simple mode

1.0 1.6 2.6

Weighted mode

1.1 1.3 1.7

One-sample MR:

One-sample MR: Unweighted

0.9 1.3 1.8

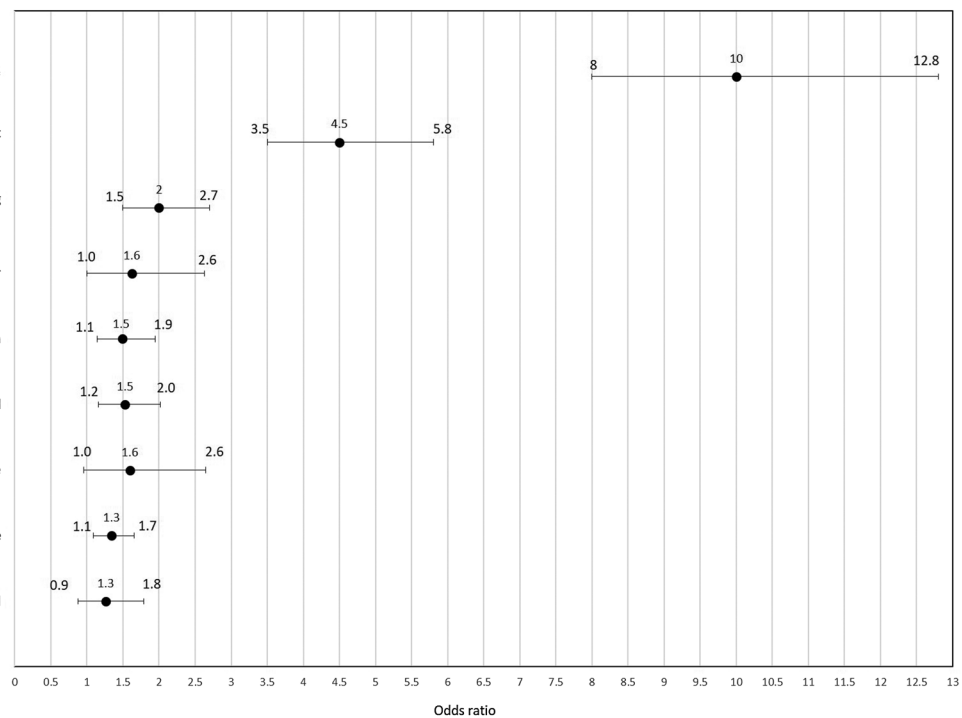


Figure 1.

Summary of results from analytic methods assessing gallstones and gallbladder cancer used in this study. ORs and CIs shown represent the risk of gallbladder cancer per doubling in liability to gallstone formation. *Self-reported gallstones, reported by study participant as either present or not present. **Self-reported gallstones using stringent definition of self-report, where those diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of interview for the controls were excluded from the analysis.

1.79) per doubling of exposure to gallstones. Findings from the one-sample MR were largely consistent with those from the two-sample MR, although CIs crossed the null (Fig. 1).

Discussion

We consistently observed an increased risk of gallbladder cancer for individuals with a history of gallstones using various methods to assess personal history of gallstones. Estimates varied in line with the robustness of the exposure data (i.e., measurement of the presence of gallstones) and risk of bias in case-control analysis, as has observed previously (7, 29, 30). The association remained although attenuated when we conducted analysis using more stringent and more objective assessments of the presence of gallstones. Furthermore, evidence of a causal effect was also provided by MR, which is less susceptible to issues of confounding, reverse causation, and measurement error, although estimates were of a smaller magnitude to those found in the observational analysis. The effect estimates obtained from both imaging techniques (OR = 2.0; 95% CI = 1.5–2.7) and MR analysis (ORs ranging from 1.3–1.6) were similar, and largely consistent with findings from a prospective cohort study of screen-detected gallstone disease and gastrointestinal cancer (HR = 1.41; 95% CI = 1.01–1.97; ref. 30) as well as a cohort study of self-reported gallstones and gallbladder cancer (HR = 3.10; 95% CI = 1.55–6.19; ref. 31).

The use of the MR approach is particularly useful in this setting because genetic variants robustly associated with liability to form gallstones can be used to appraise their causal role in gallbladder cancer development. In the absence of any reliable, unbiased objective measures of the presence or absence of actual gallstones, inference can be made about their likely role in gallbladder cancer. Genetic proxies for gallstones have been identified in several large GWAS (8) and these genetic proxies were tested for association in 1,042 gallbladder cancer cases and 1,709 controls. We applied several MR methods that rely on different underlying assumptions to evaluate the causal relationship between liability to form gallstones and risk of gallbladder cancer. We observed consistent estimates of increased risk of gallbladder cancer using these different MR approaches. An increased risk of gallbladder cancer was similarly observed when we conducted one-sample MR using a GRS.

The MR findings supporting a causal role for gallstones in gallbladder cancer has strong biological plausibility. Of note, many of the genetic variants identified to proxy the presence of gallstones are associated with cholesterol metabolism (8). Cholesterol metabolism is known to play a fundamental role in gallstone formation. In addition, some variants (rs56398830 and rs55971546) are located proximal to the bile acid transporter, *SLC10A2* gene. The main function of these transporters is to reabsorb bile salts from the terminal ileum into ileocytes, after which the bile salts are transported back to the liver through the enterohepatic circulation. As some of the identified variants relates to gallstone risk through pathways which are modifiable (cholesterol levels and bile salts) and relate to genetic liability to gallstones, any strategy to intervene on these pathways has the potential to reduce gallbladder cancer risk. For example, previous studies have demonstrated a reduction in gallstone disease and biliary tract cancer among long-term users of statins (32–34). However, it should be acknowledged that targeting a single pathway such as cholesterol or bile salts is unlikely to be entirely sufficient for minimizing risk and the contamination mixture method did not identify any clusters of variants representing distinct causal pathways.

Several limitations in the study require acknowledgement. While the use of breast cancer cases as “controls” provided us with an opportunity to compare cases and controls who have gone through similar imaging technique procedures, this approach has some limitations. The breast cancer cases might have come from a different source population to the gallbladder cancer cases. Furthermore, it is possible that some risk factors are common between gallbladder cancer and breast cancer which might attenuate effect estimates, although we adjusted for additional confounders such as adiposity in the analysis. In addition, even with more objective measure of gallstones, we cannot completely rule out misclassification in measurement. The imaging techniques may not completely capture gallstone history and may detect silent gallstones which are not present years later (35). This is an important consideration because a long duration of gallstones may be necessary to induce chronic trauma to the mucosa, which initiates the sequence of pathologic changes resulting in cancer development (36).

While MR offers several advantages to observational analysis, it also relies of various assumptions and any violation of these may bias causal estimates. The core MR assumptions are that: (i) the genetic instrument is strongly associated with the exposure; (ii) the genetic instrument is independent of confounding factors; and (iii) the genetic instrument is only related to the outcome via the exposure of interest (i.e., no horizontal pleiotropy; ref. 9). The genetic variants used in MR may be imperfect at capturing gallstone formation. In particular, because the SNPs were identified in a large GWAS of gallstones among individuals of European ancestry, they may not provide an adequate instrument for gallstone formation in Indians. We carried out analyses to assess the suitability of the selected SNPs as genetic instruments for gallstones in the Indian population. Although the risk for developing gallstones was in the same direction for 80% of SNPs, when assessed individually just one SNP (rs11887534) showed a strong association with gallstones in Indians. While we demonstrated adequate instrument strength of the genetic variants when combined into an GRS in this Indian population ($F\text{-stat} > 10$), the identification of genetic variants robustly related to gallstone formation among Indians would be of particular use for confirming the effects observed. With respect to the second assumption, we demonstrated that this genetic instrument was not strongly associated with any potential confounders, unlike self-reported gallstone history. The use of complementary MR approaches allowed us to assess the third assumption and infer that any pleiotropic effects do not seriously distort effect estimates. Using radial plots, we were able to identify and then remove five outlier SNPs, potentially involving pleiotropic pathways to gallbladder cancer which did not involve gallstone formation. These variants have been most consistently associated with blood metabolite levels and measures of cholesterol, as identified in PhenoScanner (37). Two of these SNPs were found in the *HNF4* region which is associated with diabetes, while two variants were found in the *FUT2* and *FUT6* regions, which are responsible for glycosylation in the gastrointestinal tract (38). We repeated the MR analysis after removing these outliers and the results were broadly consistent, in term of the direction and strength of association observed.

Implications of findings

The consistency of the association between history of gallstones and gallbladder cancer using a range of analytic methods leads us to believe that this association reflects a causal effect of gallstones on gallbladder cancer. The findings provide important evidence that is otherwise not feasible to ascertain given the challenges inherent in conducting a randomized controlled trial of gallstone

treatment in populations at risk of gallbladder cancer. Our findings have major implications in developing preventive strategies for gallbladder cancer, including the early detection and/or prophylactic treatment of gallstones in high-risk individuals. As USG may be difficult to conduct in the field for identification of gallstones especially in resource-poor settings, a panel of SNPs associated with gallstone formation could potentially be used as triage for identifying liability to form gallstones. This could be used to plan interventional strategies to reduce the risk of gallstone formation and consequently reducing the risk of gallbladder cancer.

Authors' Disclosures

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Authors' Contributions

S. Mhatre: Conceptualization, formal analysis, writing-original draft, writing-review and editing. **R.C. Richmond:** Conceptualization, formal analysis, writing-original draft, writing-review and editing. **N. Chatterjee:** Writing-review and editing. **P. Rajaraman:** Writing-review and editing. **Z. Wang:** Writing-review and editing. **H. Zhang:** Writing-review and editing. **R. Badwe:** Data curation, writing-review and editing. **M. Goel:** Data curation, writing-review and editing. **S. Patkar:** Data curation, writing-review and editing. **S.V. Shrikhande:** Data curation, writing-original draft. **P.S. Patil:** Data curation, writing-review and editing. **G. Davey Smith:** Conceptualization, supervision, writing-original draft, writing-review and editing. **C.L. Relton:** Conceptualization, supervision, writing-original draft, writing-review and editing.

R.P. Dikshit: Conceptualization, data curation, supervision, writing-original draft, writing-review and editing.

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Sharayu Mhatre, Rebecca C. Richmond, Nilanjan Chatterjee, et al.

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