

ORIGINAL ARTICLE

Insights into modifiable risk factors of cholelithiasis: A Mendelian randomization study

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Abstract

Background and Aims: The risk factors of cholelithiasis have not been clearly identified, especially for total cholesterol. Here, we try to identify these causal risk factors.

Approach and Results: We obtained genetic variants associated with the exposures at the genome-wide significance ($p < 5 \times 10^{-8}$) level from corresponding genome-wide association studies. Summary-level statistical data for cholelithiasis were obtained from FinnGen and UK Biobank (UKB) consortia. Both univariable and multivariable Mendelian randomization (MR) analyses were conducted to identify causal risk factors of cholelithiasis. Results from FinnGen and UKB were combined using the fixed-effect model. In FinnGen, the odds of cholelithiasis increased per 1-SD increase of body mass index (BMI) (OR = 1.631, $p = 2.16 \times 10^{-7}$), together with body fat percentage (OR = 2.108, $p = 4.56 \times 10^{-3}$) and fasting insulin (OR = 2.340, $p = 9.09 \times 10^{-3}$). The odds of cholelithiasis would also increase with lowering of total cholesterol (OR = 0.789, $p = 8.34 \times 10^{-5}$) and low-density lipoprotein-cholesterol (LDL-C) (OR = 0.792, $p = 2.45 \times 10^{-4}$). However, LDL-C was not significant in multivariable MR. In UKB, the results of BMI, body fat percentage, total cholesterol, and LDL-C were replicated. In meta-analysis, the liability to type 2 diabetes mellitus and smoking could also increase the risk of cholelithiasis. Moreover, there were no associations with other predominant risk factors.

Conclusions: Our MR study corroborated the risk factors of cholelithiasis from previous MR studies. Furthermore, lower total cholesterol level could be an independent risk factor.

Abbreviations: BMI, body mass index; FDR, false discovery rate; GWAS, genome-wide association study; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; IV, instrumental variable; IVW, inverse variance-weighted; LDL-C, low-density lipoprotein cholesterol; MICOL, Multicenter Italian Study on Epidemiology of Cholelithiasis; MR, Mendelian randomization; MVMR, multivariable MR; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus; UKB, UK Biobank.

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INTRODUCTION

Cholelithiasis (gallstone disease) harasses about 10%–20% of the adults globally and is among the hepatobiliary diseases associated with the highest socioeconomic costs.^[1] In addition, cholelithiasis is also an important risk factor of gallbladder cancer^[2] and it is increasingly recognized as a public health concern that needs much more attention. Generally, cholelithiasis can be categorized into two types, including cholesterol and pigment gallstones. Therein, cholesterol gallstones, consisting of the majority, are caused by the disturbance of biliary cholesterol homeostasis, and pigment gallstones result from abnormal bilirubin metabolism.^[1] Lammert et al. have summarized several exogenous risk factors in their review, including factors associated with metabolic syndrome, dietary factors, factors causing gallbladder hypomotility, factors increasing enterohepatic bilirubin cycling, and drugs.^[1] Metabolic factors may be the predominant ones, as environmental factors should affect cholelithiasis through modulation of metabolism.

Several metabolic risk factors have been established to be associated with the risk of cholelithiasis, including obesity,^[3] higher fasting insulin,^[4,5] type 2 diabetes mellitus (T2DM),^[6] and NAFLD.^[7] However, the controversy has been unsettled with regard to the true association between total cholesterol, low-density lipoprotein–cholesterol (LDL-C) and cholelithiasis, as Atamanalp et al. suggested a positive correlation^[8] while the Multicenter Italian Study on Epidemiology of Cholelithiasis (MICOL) study indicated a negative correlation.^[9] Additionally, a Mendelian randomization (MR) study suggested a null association between plasma LDL-C and symptomatic gallstone disease.^[10] Moreover, there is still disagreement about the effect of smoking and drinking on cholelithiasis, as a recent study demonstrated that smoking is a risk factor of cholelithiasis and alcohol intake is a protective one,^[11] while an MR study suggested alcohol intake was not associated with this disease.^[6] Although the risk of cholelithiasis was associated with higher leptin level^[12] or lower adiponectin level,^[13] whether they are causal is still unknown.

Thus, it is necessary to disentangle the causal relationship between total cholesterol, LDL-C, smoking, drinking, leptin, adiponectin and cholelithiasis, especially for total cholesterol and LDL-C. As an emerging method used for causal inference in epidemiology, MR has achieved great success in finding risk factor for diseases. It uses genetic variants, which are randomly allocated at conception, as the instrumental variables to estimate the causal effect of exposure on outcome, and can reduce the bias caused by confounders or reverse causation.^[14]

Here, we included 20 predominant risk factors, including both definite and controversial, to explore the causal relationship between them and cholelithiasis

using MR. The ultimate aims of this MR are to clarify the causal relationship between serum cholesterol and cholelithiasis, and to corroborate previous findings.

METHODS

Summary statistics of 20 predominant risk factors from a genome-wide association study

The 20 predominant risk factors can be categorized into six groups, including anthropometric traits, lipidemic traits, glycemic traits, adipokines, smoking and drinking, and metabolic diseases.

We extracted instrumental variables (IVs) of anthropometric traits from the GIANT (Genetic Investigation of ANthropometric Traits) consortium. For body mass index (BMI) GWAS, they included 234,069 European individuals and the covariates were sex, age, age squared, and principal components.^[15] For waist circumference, hip circumference, and waist-to-hip ratio genome-wide association studies (GWASs), the participants were 210,088 Europeans and the researchers adjusted for age, age square, and study-specific covariates if necessary.^[16] In our MR analysis, we included GWASs adjusting for BMI and not adjusting for BMI. The GWAS summary statistics of body fat percentage were from a meta-analysis with 65,831 European participants and adjusted for sex, age, age squared, and study-specific covariates (e.g., genotype-based principle components, study center).^[17]

The GWAS summary statistics of lipidemic traits were from the Global Lipids Genetics Consortium (GLGC), including four lipid phenotypes total cholesterol, high-density lipoprotein–cholesterol (HDL-C), LDL-C, and triglycerides.^[18] The GLGC consortium is made up of 188,577, with 18,678 from non-European ancestry, and the covariates were sex, age, age squared, BMI, and genotyping chips.

The GWAS summary statistics of glycemic traits were from MAGIC (Meta-Analyses of Glucose and Insulin-related traits Consortium).^[19] This study included 281,416 samples and adjusted for study-specific covariates with 70% from European ancestry, and we only used the European summary statistics. We included fasting glucose, fasting insulin, glycated hemoglobin (HbA1c), and 2-hour glucose post-challenge in an oral glucose tolerance test.

The GWAS summary statistics of adipokines were from two different GWASs. The adiponectin GWAS included 39,883 individuals of European ancestry.^[20] This study was adjusted for age, sex, BMI, principal components, and study site if necessary. However, for leptin, GWAS included 33,987 European participants and was adjusted for age, age squared, and any necessary study-specific covariates.^[21]

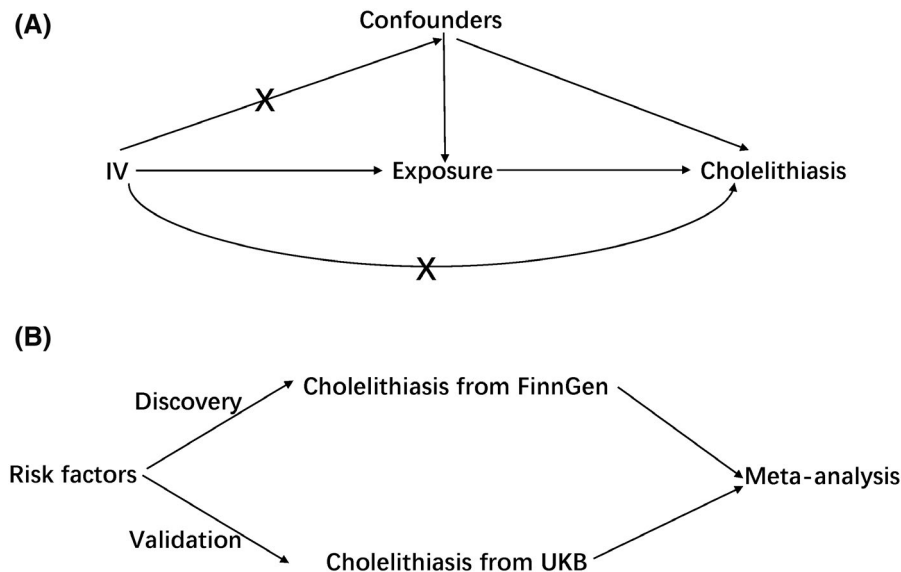


FIGURE 1 (A) Basic assumptions of Mendelian randomization. (B) Main design of this study. IV, instrumental variable

The GWAS summary statistics of smoking and drinking were obtained from the GWAS and Sequencing Consortium of Alcohol and Nicotine use, with 249,752 European participants for smoking and 335,394 European participants for drinking.^[22] The smoking is defined as the average number of cigarettes smoked per day, and drinking is the average number of drinks a participant reported drinking each week, aggregated across all types of alcohol. They included age, sex, age \times sex interaction, and the first 10 genetic principle components as the covariates and applied genomic control to the GWAS.

The GWAS summary statistics of T2DM include 26,488 cases and 83,964 controls, with 21,491 cases and 55,647 controls of European ancestry, and this study adjusted for study-specific components.^[23] For NAFLD, its GWAS included 7,176 European participants, adjusting for age, age squared, sex, alcohol consumption, and first 10 principal components.^[24] We extracted IV for coronary heart disease from the CARDIoGRAM (Coronary ARtery Disease Genome wide Replication and Meta-analysis) plus the Coronary Artery Disease (C4D) Genetics consortium, with 63,746 cases and 130,681 controls adjusting for sex and age.^[25]

GWAS summary statistics of cholelithiasis from FinnGen and UKB consortia

We used the cholelithiasis GWAS summary statistics from FinnGen (<https://r4.finngen.fi/>). This GWAS consisted of 7,737 cases and 87,135 controls, and about 16,000,000 single nucleotide polymorphisms (SNPs) were analyzed using SAIGE (<https://github.com/weizhouUMICH/SAIGE>), adjusting for sex, age, first 10 principal components, genotyping batch, and genetic

relatedness. In UKB, the GWAS was performed in 6,986 cases and 330,213 controls by Neale Lab (<http://www.nealelab.is/uk-biobank>) using Hail (<https://hail.is/>), with adjustment of first 20 principal components, sex, age, age squared, interaction between sex and age, and interaction between sex and age squared.

This MR study was performed using GWAS summary statistics, and ethical approval was obtained by each GWAS. Therein, Neale Lab received approval from the Ethics Advisory Committee of the UKB to perform the GWAS. The release of summary statistics pertaining to UKB has been approved by the UKB, and these data are publicly downloadable from the Neale Lab website. The FinnGen Biobank GWAS was performed by the FinnGen team and was approved by the FinnGen Steering Committee. The summary statistics are publicly downloadable in the website. All of these data are de-identified, freely downloadable, and can be used without restriction.

MR design

The MR should be performed under three basic assumptions: (1) The genetic variants are closely associated with exposure; (2) the genetic variants are not associated with any potential confounders; and (3) the genetic variants are not associated with outcome except via the way of exposure (Figure 1). Moreover, additional assumptions should be satisfied, including linearity and no statistical interactions.^[14] We included SNP reaching GWAS (GWAS $p < 5 \times 10^{-8}$) whose minor allele frequency > 0.01 . Then, these SNPs were clumped based on the linkage disequilibrium ($r^2 < 0.01$) in the given genome region. We then evaluated the remaining SNPs' power using the F statistics ($F = \beta^2 /$

se²) for each SNP and calculated a general F statistic for all SNPs. SNPs with less statistical power would be removed (F statistics < 10).

When assessing the causal relationship between risk factors and cholelithiasis, the FinnGen GWAS was initially used as the discovery set and UKB GWAS was the validation set, considering FinnGen has a relatively higher proportion of cases (Figure 1). Both univariable and multivariable MR analyses were performed to disentangle the potential risk factors of cholelithiasis. In univariable MR analysis, we simply tested the causation between each risk factor and cholelithiasis. However, in multivariable MR analysis, we included the significant risk factors from the univariable analysis and tried to identify the independent risk factors, especially for blood lipids.

Statistical analysis and data visualization

We used the Wald ratio to estimate the effect of exposure on outcome for each IV and then adopted the inverse variance-weighted (IVW) method to combine each IV's effect size. In addition, MR-Egger and weighted-median methods were used as supplements to IVW. The Cochran's Q value was used to assess the heterogeneity. The MR-Egger intercept^[26] and MR-PRESSO^[27] methods were used to detect horizontal pleiotropy. If the outliers were detected, they would be

removed and we would reassess the MR causal estimation. The MR-PRESSO-corrected results are reported in the main results as well, as they adopted the IVW method. If heterogeneity still existed, the median-based estimation was adopted as the main effect size. A false discovery rate (FDR) was used to adjust for multiple testing. In multivariable MR (MVMR) analysis, the IVW model was also the main method and the MR-Egger method was the complementary method. A fixed-effect model was used to combine the MR results derived from FinnGen and UKB.

The univariable MR analysis was performed using the R packages "TwoSampleMR" and "MendelianRandomization." The MR-PRESSO was conducted using the R package "MRPRESSO." The MVMR was performed using the R packages "MendelianRandomization" and "MVMR."

The mRnd was used to calculate the statistical power for Mendelian randomization (<https://cnsngomics.shinyapps.io/mRnd/>). All statistical analyses and data visualization were performed in R software 3.4.0 (<https://www.r-project.org/>).

RESULTS

The number of SNPs ranged from 5 to 826, and the explained variances varied from 0.82% to 21.17% (Table 1). The F statistics for each SNP and the general

TABLE 1 Summary of modifiable risk factors

Exposure	NSNP	Unit	Sample	R ² (%)	F	PMID
2h Glucose	15	SD	281,416	2.03	364.42	34059833
Adiponectin	15	SD	39,883	21.17	669.15	22479202
BMI	97	SD	234,069	2.86	70.29	25673413
Body fat percentage	10	SD	65,831	1.24	75.13	26833246
Coronary heart disease	10	1 unit in logOR	194,427	1.04	185.74	23202125
Drinking	39	SD	335,394	2.7	232.65	30643251
Fasting glucose	16	SD	281,416	2.08	351.61	34059833
Fasting insulin	43	SD	281,416	1.34	86.85	34059833
HbA1c	99	SD	281,416	4.35	127.94	34059833
HDL-C	126	SD	188,577	10.62	176.31	24097068
Hip circumference	55	SD	210,088	1.42	54.03	25673412
LDL-C	101	SD	188,577	9.86	202.12	24097068
Leptin	11	SD	33,987	1.26	36.13	26833098
NAFLD	5	1 unit in logOR	7,176	5.82	73.85	21423719
Smoking	28	SD	249,752	3.52	314.17	30643251
Total cholesterol	119	SD	188,577	10.02	174.89	24097068
Triglycerides	72	SD	188,577	9.63	275.17	24097068
T2DM	35	1 unit in logOR	110,452	2.64	83.17	24509480
Waist circumference	45	SD	210,088	1.34	62.02	25673412
Waist-to-hip ratio	34	SD	210,088	0.82	49.62	25673412

Abbreviations: F, F statistics; logOR, logarithm of OR; PMID, ID of publication in PubMed; R², phenotype variance explained by genetics; T2DM, type 2 diabetes mellitus.

F statistics were all greater than the empirical threshold 10, suggesting that all SNPs had sufficient validity.

Discovery result of cholelithiasis in FinnGen consortium

In the discovery stage, genetically predicted higher BMI, waist circumference, hip circumference, body fat percentage, and fasting insulin could increase the risk of cholelithiasis, whereas lower total cholesterol and LDL-C might elevate the risk of it after FDR control (FDR < 0.05) (Figure 2). Additionally, smoking and waist-to-hip ratio were suggestively associated with risk of cholelithiasis (FDR > 0.05 and IVW $p < 0.05$).

The odds of cholelithiasis would increase per 1-SD increase of BMI (OR = 1.631, $p = 2.16 \times 10^{-7}$), waist circumference (OR = 1.929, $p = 6.70 \times 10^{-6}$), hip circumference (OR = 1.653, $p = 1.19 \times 10^{-4}$), body fat percentage (OR = 2.108, $p = 4.56 \times 10^{-3}$), and fasting insulin (OR = 2.340, $p = 9.09 \times 10^{-3}$). The waist circumference and hip circumference were not significant after adjustment of BMI. Moreover, a 1-SD increase of total cholesterol could help reduce the risk of cholelithiasis (OR = 0.789, $p = 8.34 \times 10^{-5}$), together with LDL-C (OR = 0.792, $p = 2.45 \times 10^{-4}$). The multivariable MR analysis suggested that lower total cholesterol might be the independent risk factor of cholelithiasis (adjusted OR = 0.488, $p = 0.046$), whereas LDL-C was not significant in the multivariable MR model (adjusted OR = 1.264, $p = 0.468$).

There were heterogeneity and outliers in hip circumference, waist circumference, body fat percentage, fasting insulin, total cholesterol, and LDL-C. All of the results of these risk factors were MR-PRESSO-corrected results if outliers were detected. There was horizontal pleiotropy for hip circumference, but the MR-Egger results suggested that the causal relationship still holds (MR-Egger $p = 7.94 \times 10^{-5}$). The original results of heterogeneity and pleiotropy tests can be found in Table 2, together with weighted-median and MR-Egger results.

However, T2DM was not associated with increase in odds of cholelithiasis (OR = 1.061 for T2DM vs. non-T2DM, $p = 0.196$), together with NAFLD (OR = 0.811 for NAFLD vs. non-NAFLD, $p = 0.168$). There was no pleiotropy in them, but outliers were found in NAFLD. Moreover, no significant causal relationship was found in this stage.

The statistical power for FinnGen outcome was 100%.

Validation results of cholelithiasis in the UKB consortium

In the UKB data set, we successfully replicated the MR results of BMI, waist circumference, hip circumference, body fat percentage, total cholesterol, and LDL-C.

Therein, higher BMI, waist circumference, hip circumference, and body fat percentage could increase the risk of cholelithiasis, whereas lower total cholesterol and LDL-C could elevate the risk of it (Figure 2). No horizontal pleiotropy was found for these risk factors, but there was heterogeneity for total cholesterol and LDL-C. After removing outliers, the odds of cholelithiasis would decrease per 1-SD increase of total cholesterol (OR = 0.996, $p = 2.35 \times 10^{-5}$) and LDL-C (OR = 0.997, $p = 1.53 \times 10^{-4}$).

In addition, the UKB results suggested that genetic liability to T2DM, smoking, and higher waist-to-hip ratio could increase the risk of cholelithiasis ($p < 0.05$). Therein, T2DM was associated with increase in odds of cholelithiasis (OR = 1.002 for T2DM vs. non-T2DM, $p = 5.25 \times 10^{-3}$).

It should be noted that the effect sizes of UKB were smaller than those of FinnGen, and we deemed that it might result from low statistical power in UKB, as it had fewer cases. The statistical power for UKB outcome ranged from 5% to 44%, suggesting that the power was not sufficient.

Combined result of cholelithiasis from meta-analysis

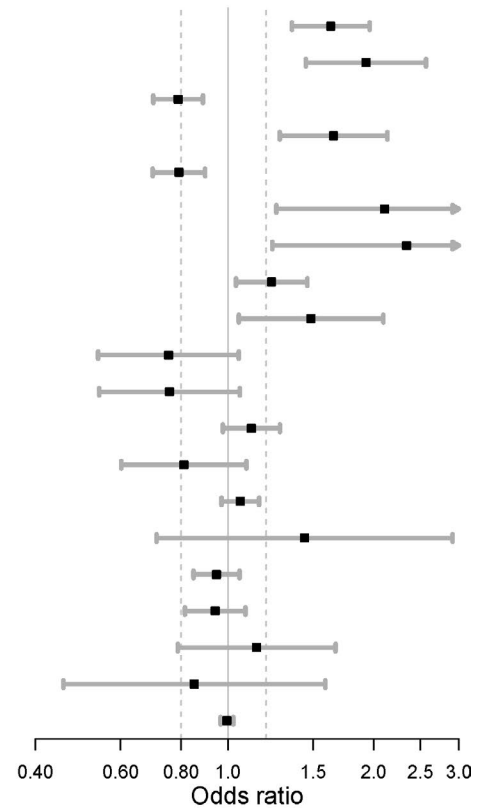
The meta-analysis of MR results from FinnGen and UKB further confirmed that previous risk factors that could increase the risk of cholelithiasis, including higher BMI (OR = 1.010, $p = 2.97 \times 10^{-11}$), waist circumference (OR = 1.012, $p = 1.01 \times 10^{-7}$), hip circumference (OR = 1.008, $p = 5.43 \times 10^{-6}$), and body fat percentage (OR = 1.009, $p = 0.016$) (Figure 3). It also confirmed that the lower total cholesterol (OR = 0.996, $p = 6.94 \times 10^{-5}$) and LDL-C (OR = 0.997, $p = 7.62 \times 10^{-5}$) could increase the risk of cholelithiasis.

In addition, the combined results suggested that cholelithiasis could be affected by the other three risk factors, which were not discovered in FinnGen, including T2DM (OR = 1.002 for diabetic vs. not diabetic, $p = 8.33 \times 10^{-3}$), smoking (OR = 1.003, $p = 0.017$), and waist-to-hip ratio (OR = 1.007, $p = 0.020$). It should be noted that both smoking and waist-to-hip ratio were also suggestively significant in the FinnGen results, although failing to pass FDR correction (smoking OR = 1.231, $p = 0.017$; waist-to-hip ratio OR = 1.49, $p = 0.024$). Thus, the results of smoking and waist-to-hip ratio should be deemed consistent. The discrepancy of T2DM between FinnGen and UKB might be attributed to the different IVs, as these two GWASs consisted of five different genotyped SNPs.

Overall, our MR study found that genetically predicted higher BMI, waist circumference, hip circumference, waist-to-hip ratio, and body fat percentage were significant modifiable risk factors of cholelithiasis. Additionally, the liability to smoking and T2DM could

(A)

Exposure	NSNP	OR	95%LCI	95%UCI
BMI	79	1.631	1.356	1.963
Waist circumference	37	1.929	1.449	2.567
Total cholesterol	91	0.789	0.701	0.888
Hip circumference	43	1.653	1.280	2.136
LDL-C	80	0.792	0.699	0.897
Body fat percentage	9	2.108	1.259	3.528
Fasting insulin	35	2.340	1.235	4.434
Smoking	20	1.231	1.039	1.459
Waist-to-hip ratio	28	1.485	1.053	2.095
2h Glucose	10	0.754	0.539	1.053
Fasting glucose	11	0.757	0.542	1.058
Coronary heart disease	8	1.118	0.976	1.281
NAFLD	4	0.811	0.602	1.092
Type 2 diabetes	22	1.061	0.970	1.160
Leptin	7	1.440	0.712	2.913
HDL-C	103	0.947	0.849	1.057
Triglycerides	56	0.941	0.815	1.087
HbA1c	72	1.146	0.788	1.669
Drinking	29	0.852	0.457	1.590
Adiponectin	7	0.995	0.966	1.026



(B)

Exposure	NSNP	OR	95%LCI	95%UCI
BMI	95	1.010	1.007	1.013
Waist circumference	44	1.012	1.007	1.016
Total cholesterol	116	0.996	0.994	0.998
Hip circumference	54	1.008	1.004	1.011
LDL-C	98	0.997	0.995	0.998
Type 2 diabetes	25	1.002	1.001	1.004
Smoking	28	1.003	1.001	1.006
Waist-to-hip ratio	33	1.007	1.001	1.013
body fat percentage	10	1.009	1.002	1.017
Coronary heart disease	10	0.998	0.995	1.000
Fasting insulin	42	1.008	0.999	1.018
2h Glucose	14	0.998	0.994	1.001
Fasting glucose	15	0.998	0.994	1.002
NAFLD	5	0.998	0.994	1.002
HDL-C	125	0.999	0.998	1.001
Triglycerides	71	0.999	0.997	1.001
HbA1c	95	0.998	0.992	1.004
Drinking	38	1.002	0.994	1.010
Adiponectin	9	1.000	0.999	1.000
Leptin	9	1.000	0.994	1.005

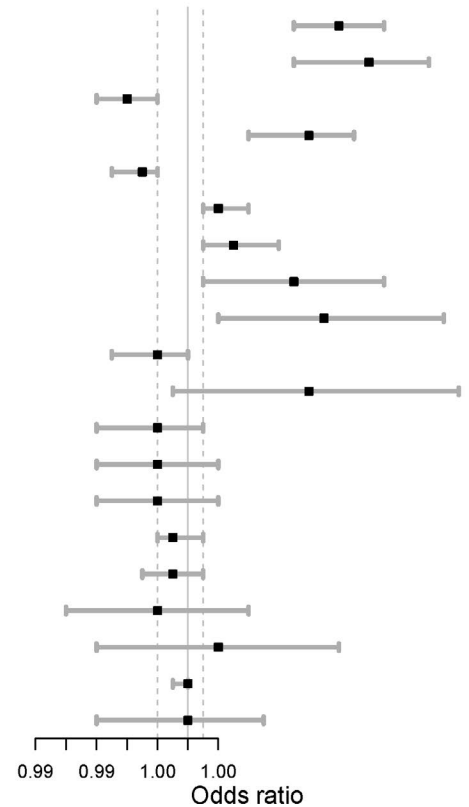


FIGURE 2 Forest plot of Mendelian randomization results. (A) Results derived from FinnGen consortium. (B) Results from the UK Biobank. 95%LCI, lower limit of 95% CI; 95%UCI, upper limit of 95% CI; 2h Glucose, 2-hour glucose after oral glucose tolerance test; BMI, body mass index; HbA1c, glycated hemoglobin; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; NSNP, number of single nucleotide polymorphisms

also increase the risk of it. More importantly, we identified that lower total cholesterol and LDL-C might be risk factors of cholelithiasis, and lower total cholesterol could be independent of LDL-C using the MVMR method.

DISCUSSION

Our MR study substantiates the conclusion that obesity, T2DM, and smoking are risk factors of cholelithiasis, and rules out the causal effect of alcohol intake on cholelithiasis, as reported by Yuan et al.^[6] Furthermore, this study found that lower total cholesterol and LDL-C levels can increase the risk of cholelithiasis, and lower total cholesterol might be independent of LDL-C.

BMI, an indicator for general obesity, has been reported to be causally associated with increased risk of cholelithiasis by two MR studies,^[3,6] and this finding was further corroborated by our study. Another two indicators for general obesity, body fat percentage and waist-to-hip ratio, could increase the risk of cholelithiasis in our study as well. Up until now, only one study found body fat percentage was only associated with increased risk of cholelithiasis in women,^[28] and another study suggested waist-to-hip ratio might be only associated with it in women as well.^[29] However, higher waist circumference, hip circumference, and waist-to-hip ratio were not significant after adjustment of BMI. Thus, we deemed that general obesity might be a more important risk factor of cholelithiasis than central obesity in both sexes, by way of causing cholesterol supersaturation in the bile, gallbladder hypomotility, and excessive bile mucin concentration.^[30] The effect of central obesity on cholelithiasis might be sex-specific, as pregnancy was a risk of cholelithiasis,^[31] and we deemed that the null or negative effect in the male might cancel out the positive effect in the female. Further research is needed to clarify this.

The relationship between blood lipids and cholelithiasis has been unsettled for years, especially for total cholesterol and LDL-C, as mentioned in the Introduction. Our MR study found that lower total cholesterol and LDL-C were associated with increased risk of cholelithiasis, whereas the association of LDL-C was not significant after adjustment of total cholesterol and HDL-C, suggesting that lower total cholesterol might be the independent risk factor of cholelithiasis. The result was consistent with the MICOL study, which unveiled an inverse relationship between total cholesterol and gallstone disease,^[9] and we revealed such association was causal. The null association between LDL-C

and cholelithiasis was consistent with the previous MR study.^[10]

Total cholesterol level in the body pool of adult is constant, and the hepatic cholesterol biosynthesis can be suppressed if the amount of cholesterol in the diet is increased.^[32] Considering that elevated hepatic cholesterol secretion can usually lead to cholesterol supersaturation and promote gallstone formation,^[31] we postulated that elevation of serum total cholesterol can inhibit the hepatic cholesterol biosynthesis, and further decrease the risk of cholelithiasis. Higher serum total cholesterol might enhance bile acid synthesis, and elevated bile acid, such as ursodeoxycholic acid, could inhibit gallstone formation via assembly of simple micelles that can solubilize cholesterol, whereas lower total cholesterol could inhibit the output of bile acid, promoting gallstone formation.^[33] On the other hand, weight loss, which can be caused by lower cholesterol diet, could cause hepatic cholesterol hypersecretion, as the total cholesterol level is low in the body pool.^[34] In addition, the risk of developing cholelithiasis should be higher with the increasing speed of weight loss, especially for very-low-calorie diet and bariatric surgery. Thus, the association between lower total cholesterol and increased risk of cholelithiasis can be explained by the compensative secretion of hepatic cholesterol and decreased secretion of bile acid. However, the effect of weight loss alone might be subtle, as weight loss is slower in low-calorie diet.^[35]

Although definitive conclusions are hard to be drawn due to different powers of FinnGen and UKB consortia, the possibility of false-positive and reverse causation should be low in our study because of the application of strict IV selection procedure and MR-Steiger test, and the consistent findings in FinnGen, UKB, and meta-analysis. Meanwhile, the MICOL study also supported these findings.^[9] Although a previous MICOL study revealed that higher serum triglycerides were associated with increased risk of cholelithiasis, a recent cohort study found no significant association between triglycerides and cholelithiasis.^[36] In addition, patients with hypertriglyceridemia are often overweight and insulin resistant, and they are at risk for gallstone formation.^[37,38] Combined with our findings, it is likely that high triglycerides level should not directly lead to gallstone formation, and previous observed association might be confounded by insulin resistance and obesity. Regardless, further investigations should be carried out to verify these findings and hypotheses.

Insulin resistance can precipitate lithogenesis in both healthy and obese individuals,^[30,39] and higher fasting insulin has been reported to be associated

TABLE 2 Mendelian randomization results of weighted median and MR-Egger methods

	Weighted median				MR-Egger				$P_{\text{heterogeneity}}$	$P_{\text{pleiotropy}}$	
	NSNP	OR	95%LCI	96%UCI	OR	95%LCI	95%UCI	P			
											P
FinnGen											
2h Glucose	10	0.776	0.580	1.039	0.088	1.669	0.625	4.455	0.337	<0.001	0.139
Adiponectin	137	0.986	0.942	1.032	0.539	0.912	0.833	0.999	0.050	0.001	0.058
BMI	79	1.691	1.281	2.233	<0.001	2.245	1.454	3.465	<0.001	0.085	0.116
Body fat percentage	9	2.198	1.241	3.894	0.007	1.875	0.170	20.731	0.624	0.045	0.925
Coronary heart disease	8	1.095	0.930	1.289	0.278	0.973	0.697	1.359	0.879	0.824	0.407
Drinking	29	0.735	0.324	1.667	0.461	3.598	0.270	47.906	0.341	<0.001	0.320
Fasting glucose	11	0.721	0.514	1.012	0.059	1.590	0.461	5.489	0.463	<0.001	0.243
Fasting insulin	35	2.067	1.014	4.214	0.046	5.395	0.807	36.053	0.091	0.001	0.497
HbA1c	72	1.529	0.858	2.724	0.150	0.961	0.342	2.701	0.939	<0.001	0.559
HDL-C	103	0.927	0.793	1.085	0.346	0.933	0.711	1.225	0.619	<0.001	0.763
Hip circumference	43	1.836	1.334	2.526	<0.001	4.300	2.240	8.256	<0.001	0.012	0.009
LDL-C	80	0.827	0.712	0.962	0.014	0.770	0.541	1.095	0.150	<0.001	0.869
Leptin	7	1.153	0.591	2.250	0.677	1.091	0.047	25.298	0.959	0.002	0.834
NAFLD	4	0.884	0.739	1.057	0.177	1.344	0.837	2.160	0.346	<0.001	0.203
Smoking	20	1.160	0.955	1.409	0.134	1.070	0.813	1.409	0.635	0.141	0.225
T2DM	22	1.017	0.899	1.150	0.787	0.884	0.618	1.264	0.506	0.050	0.322
Total cholesterol	91	0.850	0.727	0.994	0.042	0.812	0.532	1.241	0.339	<0.001	0.728
Triglycerides	56	0.935	0.768	1.139	0.505	0.829	0.610	1.126	0.236	<0.001	0.376
Waist circumference	37	1.811	1.266	2.590	0.001	3.105	1.086	8.875	0.042	0.007	0.381
Waist-to-hip ratio	28	1.705	1.093	2.659	0.019	3.122	0.953	10.230	0.071	0.094	0.314
UKB											
2h Glucose	14	0.998	0.993	1.003	0.395	1.002	0.985	1.020	0.808	<0.001	0.471
Adiponectin	166	0.999	0.999	1.000	0.241	0.999	0.997	1.000	0.130	<0.001	0.122
BMI	95	1.010	1.006	1.015	<0.001	1.010	1.003	1.018	0.007	0.705	0.954
Body fat percentage	10	1.009	0.999	1.019	0.063	1.027	0.991	1.065	0.187	0.201	0.364
Coronary heart disease	10	0.998	0.995	1.001	0.180	0.998	0.992	1.005	0.656	0.383	0.807
Drinking	38	0.999	0.989	1.010	0.896	0.999	0.978	1.021	0.945	<0.001	0.431
Fasting glucose	15	0.998	0.992	1.004	0.519	1.003	0.983	1.023	0.808	<0.001	0.655

TABLE 2 (Continued)

	NSNP	Weighted median				MR-Egger				p _{pleiotropy}	
		OR	95%LCI	96%UCI	p	OR	95%LCI	95%UCI	p		p _{heterogeneity}
Fasting insulin	42	1.012	1.000	1.025	0.059	1.042	1.011	1.075	0.012	0.001	0.061
HbA1c	95	0.996	0.987	1.005	0.361	0.994	0.982	1.007	0.380	0.002	0.411
HDL-C	125	0.998	0.996	1.001	0.264	0.998	0.993	1.002	0.316	<0.001	0.214
Hip circumference	54	1.007	1.002	1.013	0.009	1.017	1.007	1.028	0.002	0.467	0.061
LDL-C	98	0.997	0.995	1.000	0.020	0.994	0.987	1.000	0.051	<0.001	0.742
Leptin	9	1.002	0.993	1.012	0.620	0.992	0.944	1.042	0.757	0.016	0.604
NAFLD	5	0.997	0.995	1.000	0.090	1.001	0.991	1.011	0.903	<0.001	0.466
Smoking	28	1.003	0.999	1.006	0.129	1.002	0.997	1.006	0.491	0.523	0.320
T2DM	25	1.003	1.001	1.005	0.015	1.001	0.995	1.008	0.728	0.398	0.764
Total cholesterol	116	0.996	0.993	0.998	0.001	0.992	0.986	0.999	0.020	<0.001	0.387
Triglycerides	71	1.000	0.996	1.003	0.820	0.996	0.991	1.000	0.076	<0.001	0.190
Waist circumference	44	1.014	1.008	1.020	<0.001	1.024	1.009	1.040	0.003	0.770	0.094
Waist-to-hip ratio	33	1.007	0.999	1.015	0.072	1.005	0.981	1.030	0.675	0.113	0.868

Abbreviations: p_{heterogeneity}, p value of Cochrane's Q value in heterogeneity test; p_{pleiotropy}, p value of MR-Egger intercept.

with cholelithiasis, especially in women.^[5] Although we did not observe such causation in the UKB and meta-analysis, higher fasting insulin could elevate the risk of cholelithiasis in FinnGen. However, because the statistical power of the UKB was relatively low, the causal effect of fasting insulin on cholelithiasis might not be detected in the UKB. Previous association between blood glucose and cholelithiasis was null^[40] or positive.^[41] Our MR suggested no causal association between blood glucose and cholelithiasis. Here, we postulated that a previously observed association might be confounded by insulin resistance and obesity, as they usually harass glucose metabolism, thus elevating one's blood glucose level.

Previous studies suggested that higher leptin level could contribute to the formation of gallstones, and such effect might be mediated by alteration of lipid profiles.^[12] However, another study indicated that such association was insignificant in the obese patients.^[42] Our MR results found leptin level was not causally associated with cholelithiasis, and we deemed that the previously observed association might be confounded by insulin resistance, obesity, and serum total cholesterol. Decreased adiponectin level was observed in patients with cholelithiasis,^[13] but this conclusion was challenged, as the researchers found the knockout of adiponectin could not promote the formation of cholesterol stone.^[43] This MR study appeared to support the latter, and observation studies should be confounded by weight loss, as it can elevate plasma adiponectin level.^[44] Considering that higher adiponectin level might increase the risk of cholesterol gallstone formation while decreasing the risk of pigment gallstone formation,^[45] cholelithiasis should be sophisticatedly classified to investigate the effect of adiponectin on different types of gallstone formation.

There exist disparities in the relationship between smoking and cholelithiasis, as already mentioned. Yuan et al suggested that smoking can increase the risk of cholelithiasis,^[6] and this was corroborated in our study. However, we cannot rule out the causal relationship between them, as a weak nonlinear relationship was reported.^[46] Furthermore, nonlinear MR analysis should be carried out to explain it.

The causal relationship between T2DM and cholelithiasis has been well established by both observational studies and MR studies, and it was confirmed in our study. That obesity and T2DM can contribute to gallstone formation might share metabolic mechanism like insulin resistance.^[30] Moreover, diabetes can lead to increased biliary saturation index and gallbladder hypomotility through visceral neuropathy, thus promoting gallstone formation.^[47] As for NAFLD, a recent meta-analysis revealed that NAFLD was associated with increased risk of cholelithiasis.^[48] Our MR suggested no causal relationship between NAFLD and cholelithiasis; however, the causal effect of NAFLD on cholelithiasis

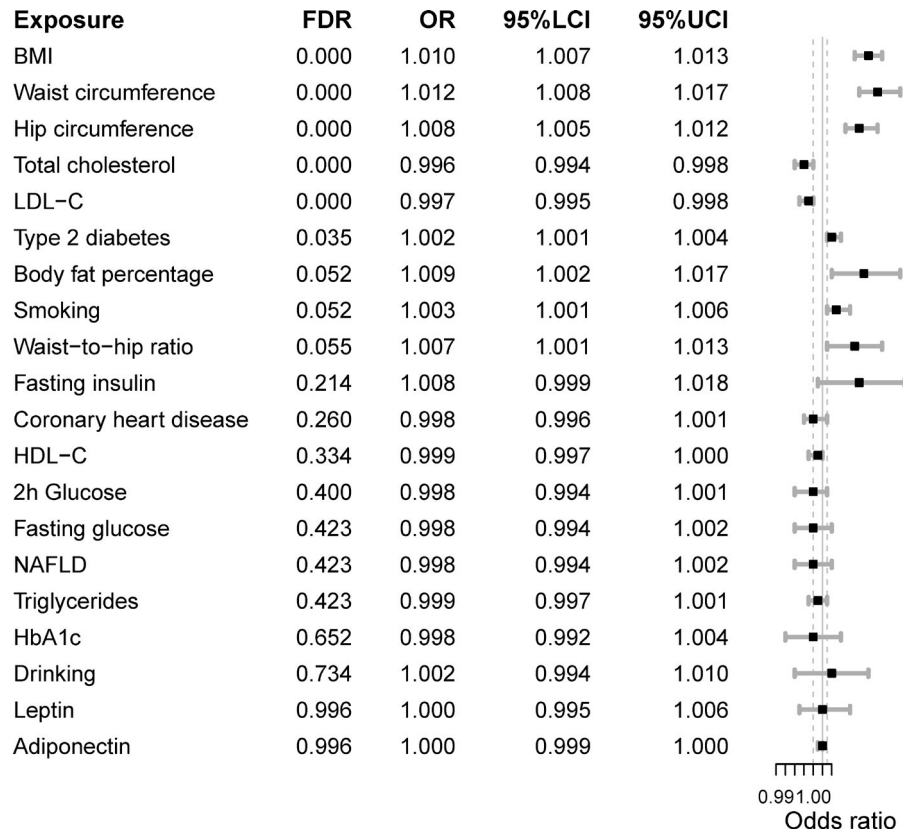


FIGURE 3 Forest plot of results from meta-analysis

might be canceled out because of co-existence of the protective effect of higher total cholesterol and hazardous effect of diabetes. Moreover, the sample size of NAFLD GWAS was relatively low, and it may lead to less statistical power. Thus, further investigation should be carried out with a larger sample size to elucidate their causal relationship. The association between gallstone and coronary heart disease is still unsettled, as the association was either positive^[49] or null.^[50] Our MR study tended to support the null association between them. However, like NAFLD, we cannot completely rule out their causal relationship, as dyslipidemia plays an important role in cholelithiasis and coronary heart disease.

Our study has several major strengths. First, this is a MR design and suitable for causal inference. Second, we included some factors that were not investigated in the MR setting, such as serum cholesterol, fasting insulin, leptin, and adiponectin. Third, this study consisted of three parts, including discovery, validation and meta-analysis stages, adding much more confidence to our research. Finally, the participants of all GWAS studies were primarily from European ancestry and all studies have genomic control, suggesting that population stratification and genomic inflation are unlikely to bias our results.

However, there are several limitations in this MR study that should be noted. The biggest concern is

pleiotropy in the MR setting. Pleiotropy can be classified into vertical pleiotropy and horizontal pleiotropy, in which the former means the SNP influences one trait (exposure), which in turn influences another (outcome), and the latter means the SNP influences two traits independently. The vertical pleiotropy can be tested by MR analysis, whereas the horizontal pleiotropy should be avoided in MR. It is hard to prove that the vertical pleiotropy mediated by the exposure cannot be biased due to SNPs influencing the two traits through independent pathways. Thus, we applied two main methods to detect the horizontal pleiotropy, including the MR-Egger intercept^[26] and MR-PRESSO,^[27] hoping to minimize the bias caused by it.

In addition, the proportion of cases in UKB is relatively low and could bring compromised statistical power, failing to detect true causal relationship. For example, we observed that higher fasting insulin can lead to the increased risk of cholelithiasis, while such causation did not hold in the UKB consortium. Considering the evident impact of insulin resistance on gallstone formation, we need another data set to verify the effect of fasting insulin on cholelithiasis in future research. Also, considering that we cannot obtain the individual-level data, the selection bias and exclusion-restriction bias might distort our results, as binary traits were included as exposures, such as T2DM, NAFLD, and coronary heart diseases. Last but not least, we

should take care when expanding our conclusions to other populations, as the participants of the included GWAS studies are primarily Europeans.

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CONFLICT OF INTEREST

Nothing to report.

AUTHOR CONTRIBUTIONS

Guoyue Lv and Lanlan Chen proposed the idea and elaborated the research. Lanlan Chen performed the main data analysis and wrote the draft of the manuscript. Both Hongqun Yang and Haitao Li contributed to the data analysis and manuscript revision. Chang He and Liu Yang reviewed and revised the manuscript. Guoyue Lv supervised the whole research and is responsible for the integrity of data analysis. All authors have given consent to the publication of this study.

DATA AVAILABILITY STATEMENT

GIANT: (http://portals.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files). MAGIC: (<https://magicinvestigators.org/downloads/>). GLGC: (<http://lipidgenetics.org/#data-downloads-title>). CARDIoGRAMplusC4D: (<http://www.cardiogramplusc4d.org/data-downloads/>). UKB: (<http://www.nealelab.is/uk-biobank>). FinnGen: (<https://r4.finnngen.fi/>)

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