MR. FELIX BOEKSTEGERS (Orcid ID : 0000-0002-0587-7624)

PROF. ELISABETE WEIDERPASS (Orcid ID : 0000-0003-2237-0128)

DR. JUSTO LORENZO BERMEJO (Orcid ID : 0000-0002-6568-5333)

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Gallstones, Body Mass Index, C-reactive Protein and Gallbladder Cancer – Mendelian Randomization Analysis of Chilean and European Genotype Data

Carol Barahona Ponce, MS[1,2], Dominique Scherer, PhD[1], Regina Brinster[1], Felix Boekstegers, MS[1], Katherine Marcelain, PhD[2], Valentina Gárate, MS[1,2], Bettina Müller, MD[3], Gonzalo de Toro, MD[4], Javier Retamales, MD[3], Olga Barajas, MD [2,5], Monica Ahumada, MD [2,5], Erik Morales, MD[6], Armando Rojas, PhD[7], Verónica Sanhueza, MD[8], Denisse Loader, MD[8], María Teresa Rivera, MD[9], Lorena Gutiérrez, MD[10], Giuliano Bernal, PhD[11], Alejandro Ortega, MD[12], Domingo Montalvo, MD[13], Sergio Portiño, MD[5], Maria Enriqueta Bertrán, MD[14], Fernando Gabler, MD[15], Loreto Spencer, MD [16], Jordi Olloquequi, PhD[17], Christine Fischer, PhD[18], Mazda Jenab, PhD [19], Krasimira Aleksandrova, PhD[20], Verena Katzke, PhD[21], Elisabete Weiderpass, MD, PhD[22], Catalina Bonet, PhD[23] Tahereh Moradi, PhD[24], Krista Fischer, PhD[25], Willem Bossers, PhD[26], Hermann Brenner, PhD[27], Kristian Hveem, PhD[28], Niina Eklund, PhD[29], Uwe Völker, PhD[30], Melanie Waldenberger, PhD[31], Macarena Fuentes Guajardo, MS[32], Rolando Gonzalez-Jose, PhD[33], Gabriel Bedoya, MS[34], Maria C Bortolini, PhD[35], Samuel Canizales, PhD[36], Carla Gallo, PhD[37], Andres Ruiz Linares, PhD[38], Francisco Rothhammer, PhD[32] and Justo Lorenzo Bermejo, PhD[1*]

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[1]Statistical Genetics Group, Institute of Medical Biometry and Informatics, University of Heidelberg, Germany
[2]Department of Basic and Clinical Oncology, Medical Faculty, University of Chile, Chile
[3]Servicio de Oncología Médica, Instituto Nacional del Cáncer, Santiago, Chile
[4]Servicio de Anatomía Patológica, Hospital de Puerto Montt, Puerto Montt, Chile
[5]Oncology, Hospital Clínico Universidad de Chile, Santiago, Chile
[6]Servicio de Anatomía Patológica, Hospital Regional, Talca, Chile
[7]Biomedical Research Labs, Medicine Faculty, Catholic University of Maule, Talca, Chile
[8]Servicio de Anatomía Patológica, Hospital Padre Hurtado, Santiago, Chile
[9]Servicio de Anatomía Patológica, Hospital del Salvador, Santiago, Chile
[10]Servicio de Anatomía Patológica, Hospital San Juan de Dios, Santiago, Chile
[11]Laboratory of Molecular and Cellular Biology of Cancer (CancerLab), Department of Biomedical Sciences, Faculty of Medicine, Universidad Católica del Norte, Coquimbo, Chile
[12]Servicio de Anatomía Patológica, Hospital Regional, Arica, Chile
[13]Department of Surgery, Hospital Juan Noe Crevani, Arica, Chile
[14]Secretaría Regional Ministerial de Salud, Ministerio de Salud, Valdivia, Chile
[15]Servicio de Anatomía Patológica, Hospital San Borja Arriarán, Santiago, Chile
[16]Servicio de Anatomía Patológica, Hospital Regional Guillermo Grant Benavente, Concepción, Chile
[17]Laboratory of Cellular and Molecular Pathology, Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile
[18]Institute of Human Genetics, University of Heidelberg, Germany
[19]International Agency for Research on Cancer (IARC), Lyon, France
[20]Department of Epidemiology, German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany
[21]Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany
[22]International Agency for Research on Cancer, World Health Organization, Lyon, France
[23]Unit of Nutrition and Cancer, Cancer Epidemiology Research Programme, Catalan Institute of Oncology (ICO), Barcelona, Spain
[24]Division of Epidemiology, Department of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

[25]Estonian Genome Centre, Institute of Genomics, University of Tartu, Estonia
[26] The Lifelines Cohort Study, Groningen, Netherlands
[27]Division of Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Heidelberg, Germany; Division of Preventive Oncology, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Heidelberg, Germany; German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany
[28]The Nord-Trøndelag Health (HUNT) Research Centre, Norwegian University of Science and Technology (NTNU), Trondheim, Norway; K.G. Jebsen Centre for Genetic Epidemiology, Department of Public Health and Nursing, NTNU, Norwegian University of Science and Technology, Norway
[29]Genomics and biobank, National Institute for Health and Welfare (THL), Helsinki, Finland
[30]Interfakultäres Institut für Genetik und Funktionelle Genomforschung, Universitätsmedizin Greifswald, Germany
[31]Research Unit of Molecular Epidemiology and Institute of Epidemiology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
[32]Instituto de Alta Investigación, Tarapacá University, Chile
[33]Instituto Patagónico de Ciencias Sociales y Humanas, Centro Nacional Patagónico, CONICET, Puerto Madryn, Argentina
[34]Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Medellín, Colombia
[35]Instituto de Biociências, Universidad Federal do Rio Grande do Sul, Puerto Alegre, Brazil
[36]Facultad de Química, Universidad Nacional Autónoma de México, Ciudad de México, México
[37]Unidad de Neurobiología Molecular y Genética, Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru
[38]Ministry of Education Key Laboratory of Contemporary Anthropology and Collaborative Innovation Center of Genetics and Development, School of Life Sciences and Human Phenome Institute, Fudan University, Shanghai 200433, China; Aix-Marseille Univ, CNRS, EFS, ADES, Marseille 13007, France

***Corresponding author:** Justo Lorenzo Bermejo, Statistical Genetics Group, Institute of Medical Biometry and Informatics, University of Heidelberg, Im Neuenheimer Feld 130.3, 69120 Heidelberg, Germany. E-mail address: lorenzo@imbi.uni-heidelberg.de. Phone: +496221564180.

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CONFLICT OF INTEREST

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Abstract

Background & aims: Gallbladder cancer (GBC) is a neglected disease with substantial geographical variability: Chile shows the highest incidence worldwide, while GBC is relatively rare in Europe. Here we investigate the causal effects of risk factors considered in current GBC prevention programmes as well as C-reactive protein (CRP) level as a marker of chronic inflammation.

Approach & results: We applied two-sample Mendelian randomization (MR) using publicly available data and our own data from a retrospective Chilean and a prospective European study. Causality was assessed by inverse variance weighted (IVW), MR-Egger regression and weighted median estimates complemented with sensitivity analyses on potential heterogeneity and pleiotropy, two-step MR and mediation analysis. We found evidence for a causal effect of gallstone disease on GBC risk in Chileans ($p = 9 \times 10^{-5}$) and Europeans ($p = 9 \times 10^{-5}$). A genetically elevated body mass index (BMI) increased GBC risk in Chileans ($p = 0.03$), while higher CRP concentrations increased GBC risk in Europeans ($p = 4.1 \times 10^{-6}$). European results suggest causal effects of BMI on gallstone disease ($p = 0.008$); public Chilean data were not, however, available to enable assessment of the mediation effects among causal GBC risk factors.

Conclusions: Two risk factors considered in the current Chilean programme for GBC prevention are causally linked to GBC risk: gallstones and BMI. For Europeans, BMI showed a causal effect on gallstone risk, which was itself causally linked to GBC risk.

KEYWORDS: Gallbladder cancer, Mendelian randomization, Gallstones, C-reactive protein, Native American ancestry

Background

Each year, gallbladder cancer (GBC; ICD-10 diagnosis code C23) kills more than 70,000 people worldwide (globocan.iarc.fr). Most GBC diagnoses occur in low- and middle-income countries, and research into this aggressive disease has been largely neglected.(1) A strong association between gallstone disease and GBC has been found in observational studies, with a relative GBC risk of 2.4 for gallstones 2.0-2.9 cm in diameter and 9.2-10.1 for gallstones larger than 3 cm.(2) Potential confounding by female gender and other GBC risk factors, however, makes it difficult to infer causality. Around 20% of the GBC burden can be attributed to excess body weight, with a substantial risk increase of 25% to 31% for every five body mass index (BMI) units.(1, 3-5) To date, however, very little is known about the causal mechanisms that underlie the association between body fatness and GBC. Obesity is linked to chronic inflammation, as reflected in elevated levels of C-reactive protein (CRP), and strong associations between the CRP concentration in serum and the risk of GBC have been reported for Chileans (odds ratio [OR] for the fourth versus the first concentration quartile = 18.6) and Chinese (OR = 7.6).(6-9) The strong observed associations could, however, be related to reverse causation (elevated CRP concentration caused by GBC tumours rather than CRP → GBC). Women are at higher risk of developing GBC, in particular those with early age at menarche, early age at first childbirth and high numbers of pregnancies and childbirths.(10-12) Additional risk factors include advanced age, a family history of GBC or gallstones, chronic inflammatory conditions affecting the gallbladder, diabetes, a low educational level and chronic infections with *Helicobacter* and *Salmonella* spp.(2) Lifestyle factors such as cigarette smoking and alcohol consumption, as well as environmental pollution (waste gas emission and pollutant plants), also seem to increase GBC risk (13-15).

Current GBC care, from prevention and early detection to diagnosis and therapy, does not take full account of ethnic, cultural, environmental and health-care system disparities. The identification of possible differences in GBC aetiology between regions of high and low incidence could potentially translate into more efficient prevention policies. The genome of modern Chileans is a genetic admixture of Europeans, Native Americans from two major indigenous peoples (Mapuche and Aymara) and Africans.(16) It is well established that individuals with a high proportion of Mapuche ancestry are at high risk of developing GBC: We found that each added 1% of Mapuche ancestry

represents a 3.7% increase in the GBC mortality risk.(16) The Chilean government currently supports prophylactic cholecystectomy for men and women aged 35–49 years, and multiparous women with BMI over 27 kg/m^2, 8 years' education or less and at least one Mapuche surname are considered to be at a particularly high risk of developing GBC.(17) Each year 50,000 gallbladders are removed in Chile, at an average cost of $1000 per cholecystectomy, in the framework of this prevention policy.(18)

GBC is rare in most countries, and publicly available genotype data from genetic association studies are sparse. We conducted a retrospective Chilean (277 patients, 2107 controls) and a prospective European (103 cases, 168 controls) study on GBC, and applied Mendelian randomization (MR) to investigate the causal relationship between risk factors considered in the current Chilean GBC prevention programme, CRP concentration as a marker of chronic inflammation and GBC risk. The available sample size was small compared with traditional MR studies, but the strong associations found in observational studies – gallstones increase the GBC risk by up to tenfold, and the ORs associated with increased serum CRP levels vary from 7.6 to 18.6 – and the urgent necessity to optimize GBC prevention motivated this study. We utilized genetic variants robustly associated with gallstone disease, BMI, CRP concentration, age at menarche and age at first childbirth as instrumental variables, and tested the causal effect of these risk factors on GBC risk. Our ultimate goal is to unravel the complex aetiology of GBC and discriminate between non-causal and causal risk factors, striving to improve the efficiency of current GBC prevention programmes in regions of high and low GBC incidence.

Methods

All Chilean and European cases were patients with a diagnosis of gallbladder cancer (International Classification of Diseases for Oncology Version 3, site code C23). The majority (79%) of Chilean GBC patients were diagnosed incidentally after a prophylactic cholecystectomy to treat gallstone disease. Population controls included individuals affected by gallstone disease. European controls did not include individuals affected by any type of cancer, but information on cancer history was not available for Chilean controls. The proportions of Chilean controls affected by gallstone disease and

cancer should, however, be representative of the corresponding proportions in the general population that gave rise to the cases. The gender and age distributions of the investigated cases and controls are shown in **Supplementary Table 1**. Additional details on the recruitment strategy in Chile are available in our recent publication.(16) **Supplementary Table 2** describes the arrays used for genotyping of Chilean and European study participants.

We used two-sample MR with genetic variants – specifically, single-nucleotide polymorphisms – as instrumental variables to investigate the causal effects on the risk of GBC exerted by (1) risk factors considered in the current Chilean GBC prevention programme (gallstone disease, BMI and age at menarche) and (2) CRP level as a marker of chronic inflammation.(9, 17, 18) Summary statistics on the association between the genetic variants and GBC risk adjusted for age, gender and the first five genetic principal components were obtained using our own demographic and genotype data from the retrospective Chilean (277 cases, 2107 controls) and the prospective European (103 cases, 168 controls) study (**Supplementary Table 1**). Summary statistics on the association between the genetic variants and the risk factors were retrieved from the studies listed in **Table 1**, while detailed information on the original genetic association analyses including investigated phenotypes, association models and covariates, and analysis tools is provided in the **Supplementary Methods.** In addition to overall analyses, calculations for Chileans were stratified by the median proportion of Mapuche ancestry in the Chilean study (34%).

The under-representation of non-European populations is an important problem in current human genetic research.(19) To assess the potential impact of this limitation on our results, we compared the variance explained by the genetic instruments in different populations. We also calculated the F statistic as a measure of the strength of the instrumental variables, with a low value (i.e. <10) indicative of possible weak instrument bias. Please see the **Supplementary Methods** for details on these calculations. Our primary objective was to identify causal associations (to test causality), which requires weaker modelling assumptions than estimation of the magnitude of the causal effects.(20) We calculated Cochran's Q statistic using first-order inverse variance weights to detect heterogeneity, which indicates a possible violation of the instrumental variable or modelling assumptions, of which

pleiotropy is a likely major cause.(21) We visually inspected scatter and funnel plots and removed genetic variants with outlying MR estimates in the case of excessive heterogeneity.(22) More precisely, when the initial Q *p*-value was smaller than 0.10, genetic variants with departing MR estimates were removed one after another until heterogeneity disappeared (Q *p*-value > 0.10). As a secondary objective we estimated the causal effect sizes and assessed their robustness by comparing the inverse variance weighted (IVW), MR-Egger regression and weighted median estimates for BMI and age at menarche. MR analyses were conducted using the R-version of MR-Base, which provides convenient tools for the harmonization of the summary statistics, including standardization of the effect alleles and removal of the problematic palindromic genetic variants, and implements a random-effect IVW model by default.(23)

We also conducted comprehensive sensitivity analyses, which are described in the **Supplementary methods.** For the risk factors that showed a causal effect on GBC ($p < 0.05$ and no evidence of violation of instrumental variable assumptions), two-step MR was applied to assess mediation.(24) In the first step of the procedure, genetic instruments for the exposure were used to estimate the causal effect of the exposure on the potential mediator. In the second step, genetic instruments for the mediator were used to assess the causal effect of the mediator on GBC risk. Evidence of association in both steps (for example, BMI → gallstone disease and gallstone disease → GBC) implies some degree of mediation between the exposure and the outcome by the intermediate trait. We also applied bootstrapping for testing the hypothesis that gallstone disease is a mediator which explains the underlying mechanism of the relationship between BMI and GBC risk. We considered the three regression models "BMI → GBC", "BMI → gallstone disease", and "BMI + gallstone disease → GBC" with age, gender and the first five genetic principal components as adjustment covariates, and used the CAUSALMED procedure in SAS version 14.3 to perform causal mediation analyses.

Results

We used the six genetic variants robustly associated with the risk of gallstone disease in the study by Joshi et al. as instrumental variables, under the assumption that they act on GBC risk only through the conferred susceptibilities to gallstone development, and used the heterogeneity between the six MR

estimates as a proxy for pleiotropy.(21, 25) Summary statistics on the association between the six instruments and the risk of gallstone disease were obtained from the studies described in **Table 1** and are provided in **Supplementary Table 6**.(25, 26)

Figure 1 panel A/B shows the variance in liability to gallstone disease explained by the used genetic instruments in Europeans, Hispanic Americans and Chileans. The variance explained by the missense variant rs11887534 at the *ABCG8* locus was 0.02 for Europeans, 0.002 for Hispanic Americans and 0.04 for Chileans. The variance attributable to rs4245791 – non-coding transcript exon variant in *ABCG8* – was similar for Europeans and Hispanic Americans, but twice as high for Chileans. The total variance explained by the six genetic instruments for gallstone disease was 0.04 for Europeans, 0.01 for Hispanic Americans and 0.07 for Chileans. We found no heterogeneity among instruments (IVW Q $p = 0.56$ for Chileans and 0.47 for Europeans; **Figure 2** and **Table 2**). Evidence for a causal effect of gallstones on GBC was detected in Chileans (OR = 1.97, $p = 9 \times 10^{-5}$) and Europeans (OR = 5.02, $p = 9 \times 10^{-5}$). The estimated causal effect sizes were higher for Europeans than Chileans, but the difference between the ORs did not reach statistical significance (overlapping 95% confidence intervals). Stratified results for Chileans according to the median proportion of Mapuche ancestry (34%) were consistent with the causal effect of gallstone disease on GBC risk increasing with a decreasing proportion of Mapuche ancestry (OR = 1.60 for Mapuche ancestry > 34% vs OR = 2.88 for Mapuche ancestry ≤ 34%).

For the two-sample MR of the impact of BMI on GBC risk we utilized the 289 genetic variants identified by Hoffmann et al. and their reported summary statistics on the association with BMI (**Table 1**).(27) **Figure 1 panel C** depicts the variance in BMI explained by the genetic instruments in Europeans and Latinos. The variance explained by the intergenic variant rs13021737 was 0.003 for Latinos but only 0.0008 for Europeans, whereas rs6567160 explained 0.001 of the BMI variance in both Latinos and Europeans. The total variance explained by the considered instruments for BMI was 0.04 for Europeans and 0.07 for Latinos. The lower sample size and incomplete parameter information for Latinos, however, motivated us to use exclusively European summary statistics in this study. We excluded instruments with a p for the association with BMI higher than 5×10^{-8} or a low

imputation accuracy, resulting in selection of 202 variants. The harmonization of publicly available summary statistics and our own data using MR-Base resulted in 192 instrumental variables for Chileans and 199 instruments for Europeans. We detected slight heterogeneity among the instruments for Chileans (IVW Q $p = 0.08$); this decreased to $p = 0.14$ after the visual inspection of scatter and funnel plots and removal of one outlying variant (rs3783890; **Supplementary Table 5** and **Supplementary Figure 1 panel A**). No heterogeneity (IVW Q $p = 0.18$) and no directional bias (MR-Egger intercept $p = 0.65$) were detected for Europeans. Evidence for a causal effect of BMI on GBC was detected in Chileans ($p = 0.03$), with consistent causal effect estimates using IVW (OR per inverse normally transformed BMI unit = 2.47) and weighted median (OR = 2.70). Stratified results for Chileans were compatible with a stronger causal effect of BMI on GBC risk with increasing proportion of Mapuche ancestry (OR = 3.83 for Mapuche proportion > 34% vs. OR = 1.13 for Mapuche proportion ≤ 34%).

We found no evidence for a causal effect of BMI on GBC risk in Europeans, but BMI showed a causal effect on gallstone risk according to IVW, MR-Egger regression and weighted median estimates, and gallstone risk was in turn causally associated with GBC risk in Europeans as described above **(Figure 3**, **Tables 2 and 3**, **Supplementary Table 3, Supplementary Figure 1 panel B)**. Results from causal mediation analysis using our own European data did not indicate any effect of BMI on GBC risk through gallstones: the estimated OR for the natural indirect effect was 1.001 ($p = 0.60$). This was an expected result given the absence of a direct causal effect of BMI on GBC risk for Europeans, the limited size of the European cohort and the low quality of available gallstone data (self-reported gallstone history, which was missing for 47% of the study participants). These limitations motivated us to analyse UK Biobank data, which include complete BMI and gallstone information for 271,539 study participants of white British ancestry, 22 GBC cases and 271,517 histories of self-reported gallstone. Consistently with our results from two-step MR, causal mediation analysis using UK Biobank data identified an indirect effect of BMI on GBC risk through gallstones (OR natural indirect effect 1.03 [$p = 0.004$]). The OR natural direct effect was 0.98 ($p = 0.75$), and the adjusted OR of GBC associated with a gallstone history was 12.3 ($p = 0.0001$).

Figure 1 panel D shows the variance in log-transformed CRP levels in serum explained by the genetic instruments in European Americans and Hispanics relying on Kocarnik et al. 2014.(28) For example, the variance explained by the 3' UTR variant rs1205 was 0.01 in European Americans and 0.02 in Hispanics. The splice region variant rs1800947 explained 0.01 for European Americans and 0.005 for Hispanics. In our main analyses, we used four tagging genetic variants in the *CRP* gene (rs1205, rs1800947, rs1130864, rs2808630) as instrumental variables to infer causality between CRP as a marker of chronic inflammation and GBC risk. Summary statistics on the association between the four instruments and CRP concentrations were obtained from the study by Nimptsch et al. (**Table 1**).(29) Evidence for a causal effect of CRP on GBC risk was detected in Europeans (OR = 4.44 per mg/L, $p = 4 \times 10^{-6}$) but not in Chileans (**Table 2**). As an alternative to the four variants in the *CRP* gene, for Chileans we also used the summary statistics recently reported by Kocarnik et al. 2018 for Hispanic Americans. We found no heterogeneity among the instruments (IVW Q $p = 0.29$) and no evidence for a causal effect of CRP on GBC risk ($p = 0.11$, OR = 0.55, 95% CI 0.27 to 1.14).(30)
To test causality between age at menarche and GBC risk in women, we used the 389 genetic variants identified by Day et al. and their reported summary statistics for the association with age at menarche (all $p < 5 \times 10^{-8}$; **Table 1**).(31) Exclusion of the variants with low imputation accuracy and problematic palindromic polymorphisms using MR-Base resulted in selection of 339 instruments for Chileans and 342 instruments for Europeans. Heterogeneity as a proxy for pleiotropy was evident for Chileans (IVW Q $p = 0.02$); this decreased to $p = 0.11$ after removal of three outlying genetic variants. No heterogeneity and no directional bias were detected for Europeans (**Table 2** and **Supplementary Table 3**). No evidence for a causal effect of age at menarche on GBC was detected either in Chileans or in Europeans.

We used the 10 genetic variants and the summary statistics reported by Barban et al. to test the causal effect of age at first childbirth on GBC risk.(32) The limitation of the study to parous women only translated into F statistics of 14.6 for Chileans and 5.29 for Europeans; the detectable ORs were 4.2 for Chileans and 10.1 for Europeans (**Table 1**). Due to the substantially lower statistical power for age at first childbirth compared to the other investigated exposures, age at first childbirth was not considered further.

Results from the conducted sensitivity analyses were consistent with robust rejections of the causal null hypotheses and with robust estimates of the causal effect sizes (**Supplementary Tables 4 and 5**). The estimated causal effect of gallstone disease on GBC for Chileans (OR = 1.97) varied from OR = 1.95 (adjustment for the first 20 genetic principal components) to OR = 2.16 (LD clumping). The estimated causal effect of BMI on GBC risk for Chileans varied from OR = 1.70 (radial MR) to 2.79 (LD clumping). The Wald ratios for the genetic variant rs9939609 in the *FTO* gene as instrumental variable of BMI were OR = 3.73 (association statistic for Latinos in Hoffmann et al., n = 8322) and OR = 1.18 (statistic for Chileans in Petermann et al., n = 409). In agreement with our primary results in **Table 2**, the corresponding Wald ratio for Europeans was OR = 0.40, 95% confidence interval 0.14 to 1.12 (data not shown). The estimated causal effect of gallstone disease on GBC for Europeans (OR = 5.02) varied from OR = 2.47 (integration of summary statistics from UK Biobank) to OR = 5.66 (exclusion of instruments associated with multiple risk factors). The OR for the causal effect of CRP concentration on GBC risk for Europeans varied from OR = 2.41 (summary statistics for CRP reported by Dehghan et al.) to 5.91 (LD clumping).

Discussion

Gallbladder cancer is a very aggressive disease with considerable potential for prevention. The tumour develops over a period of 10–20 years, and preventive gallbladder removal (prophylactic cholecystectomy) can be offered to individuals at high GBC risk. Maintenance of an ideal body weight by means of a healthy diet and regular physical activity may prevent gallstone formation in the general population, and treatment with ursodeoxycholic acid can be recommended for patients at high risk of gallstones, for example obese patients during rapid weight loss after bariatric surgery and patients on long-term therapy with somatostatin (33). In spite of the poor prognosis and the substantial prevention potential, research on the disease has been largely neglected and the mechanisms underlying GBC aetiology are not yet well understood. The present study takes advantage of MR to assess the causal relationship between established risk factors and GBC risk.

Gallstones are found in up to 90% of neoplastic gallbladders, and their presence is a major risk factor for developing GBC.(34) The co-occurrence of gallstone disease and GBC differs strongly by ethnicity. East Indian women and Native American Mapuche and Pima often develop both gallstones and GBC. In contrast, North Indian women are at high risk of developing GBC but are rarely affected by gallstones. Around 15% of Caucasian women carry gallstones, but they are at low risk of GBC.(35) Our data provide evidence for a causal association between gallstone disease and GBC risk in Europeans and Chileans. Furthermore, MR results suggest that gallstones mediate the effect of body fatness, marked by BMI, on GBC risk in Europeans. We found a causal effect of BMI on GBC risk in Chileans, and the effect seemed more pronounced in those with a large proportion (> 34%) of Mapuche ancestry. Two recent meta-analyses reported an increased risk of GBC for overweight (OR = 1.10 to 1.14) and obese individuals (OR = 1.56 to 1.58), with a 4% risk increase per BMI unit.(36, 37) Accordingly, the World Cancer Research Fund concluded that there is strong evidence for a causal role of body fatness on GBC development, which we were able to confirm in the present study.

The association between elevated BMI and gallstone formation has long been established from observational studies. Compared with normal-weight people (BMI < 25 kg/m^2), individuals with a BMI between 25 and 30 kg/m^2 are at 20% increased risk of developing gallstones, while the risk excess increases to 73% for obese individuals (BMI larger than 30 kg/m^2).(38, 39) In agreement with our study, a causal association between BMI and the risk of gallstone disease was recently identified in a large population-based European MR study; the estimated size of the causal effect on gallstone disease was OR = 1.17 per BMI unit.(40) The formation and growth of cholesterol-based gallstones is a multifactorial process resulting from the complex interplay between systemic factors (age, gender, genetic predisposition, chronic inflammation) and gallbladder-related factors accompanying cholesterol supersaturation of the bile (hypomotility of the gallbladder, hypersecretion of mucin in the gallbladder with local inflammation, rapid precipitation of solid cholesterol crystals) (41, 42). Biliary cholesterol supersaturation could also be partially related to poor dietary habits, hyperinsulinaemia and insulin resistance, which are in turn associated with body composition. Hyperinsulinaemia promotes two conditions predisposing to cholesterol-supersaturated lithogenic bile: hepatic uptake of cholesterol resulting in an increased secretion of biliary cholesterol, and decreased secretion of bile

acids (33). While it is not yet possible to separate the direct and gallstone-mediated effects of body fatness on GBC risk, the present study adds to the current understanding of GBC development, suggesting that the relative contributions of obesity and gallstones to GBC risk depend on ethnicity (43, 44). Taking full advantage of these differences may translate into more efficient GBC prevention.

Despite the evidently strong association between obesity and GBC risk, very little is known about the causal mechanisms that underlie this association. Obesity is causally linked to chronic inflammation, as reflected by increased levels of circulating inflammatory proteins such as CRP.(6-8) Elevated circulatory levels of inflammatory markers are also associated with an increased risk of GBC, as was recently shown in a study of Chinese and Chilean individuals.(9) The study investigated immune-related markers in GBC and gallstone disease patients from China and validated associated markers in serum samples from Chilean patients. Six inflammation markers, including CRP, were associated with an increased risk of GBC in the two study populations; the estimated OR for GBC associated with increased CRP levels was 18.6 for Chileans and 7.6 for Chinese. Similar associations were observed in a European study, which did not, however, investigate GBC as a distinct cancer entity; rather, GBC was combined with other biliary tract cancers.(45) The study included 137 cases of biliary tract cancer (among them, 51 of GBC) and found a 22% increased risk of biliary tract cancer for elevated CRP levels. It is well established that GBC often develops along the sequence gallstones and inflammation $\rightarrow$ dysplasia $\rightarrow$ GBC. Our data provide evidence that genetically increased CRP levels are associated with GBC risk in Europeans, consolidating the causal role of chronic inflammation in GBC development. The unavailability of summary statistics on CRP for women only was, however, a limitation of our study.

Two-step MR and mediation analyses consistently pointed to an indirect effect of BMI on GBC risk through gallstones for Europeans. According to GLOBOCAN (globocan.iarc.fr), the incidence of GBC is progressively decreasing in most European countries, for example Germany, Italy and Spain, where an increase in overweight and obesity has been noted. To examine the discrepancy between our results and the decreasing GBC rates in combination with increasing population BMI, we retrieved and plotted existing data for Germany on GBC incidence (krebsdaten.de), BMI (46) and

cholecystectomy rates (gbe-bund.de). The results are shown in **Figure 4**. The incidence of GBC has been decreasing and the percentage of the population with a BMI of 25 kg/m^2 or more has been increasing among both men and women in Germany. At the same time, and possibly reflecting the identified causal effect of BMI on gallstone disease, the number of cholecystectomies in Germany has been increasing, potentially resulting in the avoidance of some GBC cases. The likely contribution of common external (i.e. environmental) factors that modulate epigenetic mechanisms and the individual inherited predisposition to overweight and obesity, inflammation, gallstones and GBC, together with the time lag from weight gain to gallstone formation, local and chronic inflammation and GBC development, adds additional complexity to the interdependence among the investigated risk factors and GBC as the final outcome. Besides gallbladder cancer, gallstones and chronic inflammation have been also linked to other types of cancers, including right-sided colon, colorectal, pancreatic, hepatic, prostate and gastric cancer (47-51). The development of gallstones and the concomitant elevated CRP levels may thus indicate both local (in the gallbladder) and systemic (via circulating pro-inflammatory proteins) inflammatory effects of gallstones. However, in contrast to our finding for GBC, a causal effect of CRP on the risk of developing these other types of cancer has not been reported (52, 53), and neither has the causal effect of gallstones on these cancers been investigated by means of MR.

The relatively low numbers of investigated GBC patients and cases represented a limitation of the study, especially in view of the large sample sizes usually required for MR. For illustration, assuming that 4% of the variation in BMI is explained by the genetic variants used as instruments, the present study had 80% statistical power to reject the causal null hypothesis for a true OR of GBC per standard deviation of the BMI higher than 1.95 for Chileans and 3.70 for Europeans (type I error rate of 5%).(27) The difference in statistical power between the Chilean and the European study was probably larger, because women are at a higher risk of GBC than men, and Latino women show a higher proportion of BMI variation explained by known genetic variants than non-Hispanic white women (4.1% vs. 3.2%) and also greater BMI variability (standard deviation 6.3 vs. 6.0 kg/m^2).(27) On the other hand, the Chilean results were limited by the sparse public data on the association between the used instruments and GBC risk factors for the Chilean population and by the population stratification due to genetic admixture of Chileans. With the exception of CRP and age at first

childbirth for Europeans, F statistics were high (> 10) for the investigated risk factors. We conducted extensive sensitivity analyses to examine the potential influence of the genetic admixture on the estimated causal effects, but further methodological research is needed to deal adequately with stratification in MR studies of admixed populations.(54) In both high- and low-incidence regions, collaborative research is crucial to maximize sample sizes and fully exploit the potential of MR to investigate GBC risk factors with weaker associations found in observational studies, such as diabetes, educational level, smoking and alcohol consumption.

In addition to low statistical power, another major limitation of MR studies is pleiotropy. Regardless of the number and strength of the instrumental variables used, first-order inverse variance weights preserve the type I error rate under the causal null.(21) We calculated Cochran's Q statistic using first-order weights to detect heterogeneity, which often reflects pleiotropy. We applied a rather conservative heterogeneity cut-off (Q *p*-value = 0.10) and utilized a random-effect IVW model, but results based on a fixed-effect model were identical for gallstone disease and CRP, and practically identical for BMI (random-effect OR = 2.47, 95% CI 1.10 to 5.54, fixed-effect OR = 2.47, 95% CI 1.14 to 5.32). We visually inspected scatter and funnel plots, performed MR-Egger regression for BMI and age at menarche as exposures to quantify the amount of bias due to horizontal pleiotropy, used radial MR and conducted sensitivity analyses by excluding genetic variants associated with multiple GBC risk factors.

In conclusion, to our knowledge this is the first MR study on GBC, a neglected disease with considerable potential for individualized prevention. The investigated sample size was limited compared with traditional MR analyses, but it is important to consider that strong associations have been reported for established GBC risk factors in observational studies, and that GBC is a rare disease in most countries. To put numbers into context, the present MR results for Europeans rely on 103 GBC cases in comparison with the 22 cases of white British ancestry among the 500,000 participants in the UK Biobank. Other novelties were the investigation of genetically admixed Chileans, the examination of the transferability of the genetic instruments among populations with European and Latin American ancestry, and the examination of ethnic differences in GBC causation. We found that two risk factors currently considered in the Chilean programme for GBC prevention are causally

linked to GBC risk: gallstones and BMI. For Europeans, the effect of BMI on GBC risk seems to be exerted through gallstones. Further collaborative research is needed to identify and quantify ethnic differences in GBC causation and finally improve the performance of GBC prevention programmes.

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Table Legends

Table 1: Studies used to retrieve summary statistics for the two-sample Mendelian randomization analyses

Table 2: Mendelian randomization results for GBC (outcome) using genetic variants as instrumental variables for established risk factors (exposures)

Table 3: Results from two-step Mendelian randomization to assess the potential mediation effects of gallstone disease, BMI and CRP on GBC in Europeans

Figure Legends

Figure 1: Variance in liability to gallstone disease explained by the considered instruments in Europeans, Hispanic Americans and Chileans (panels A and B), explained variance in BMI in Europeans and Latinos (panel C) and explained variance in CRP in European Americans and Hispanics (panel D).

Figure 2: Scatter and funnel plots for the association between gallstone disease and GBC in Chileans (panel A) and Europeans (panel B).

Figure 3: Causal effects of established risk factors on GBC for Chileans (Panel A). Causal effects of established risk factors on GBC and mediation effects of gallstone disease, BMI and CRP for

Europeans (Panel B). Thin lines depict the investigated causal associations, while orange arrows show the identified causal effects.

Figure 4: Incidence of GBC, proportion of persons with BMI ≥ 25 kg/m^2 and number of cholecystectomies in Germany from 1999 to 2016 in women (red lines) and men (blue lines).

Table 1: Studies used to retrieve summary statistics for the two-sample Mendelian randomization analyses

Trait	Outcome/ Exposure	Study population	Study size	Gender	Number of variants	Explained variance	F statistic / Detectable OR*		Authors	Year	Ref
							Chileans	Europeans			
Gallbladder	Outcome	Admixed Chilean	277 cases and 2107 controls	Both	-				-	-	-
cancer	Outcome	European	103 cases and 168 controls	Both	-				-	-	-
Gallstone	Exposure	European	15,209 cases and 117,949 controls	Both	6	4%		12.3 / 3.70	Joshi et al.	2016	(31)
disease	Exposure	Admixed Chilean	529 cases and 566 controls	Both	6	7%	180 / 1.71		Bustos et al.	2019	(32)
	Outcome	European	361,194 individuals	Both	-				UK Biobank, Neale	2018	(†)
Body mass index	Exposure	Europeans	334, 487 individuals	Both	289	4%	100 / 1.95	12.3 / 3.70	Hoffmann et al.	2018	(26)
	Outcome	Europeans	359,983 individuals	Both	-				UK Biobank, Neale	2017	(§)
C-reactive protein	Exposure	Europeans	727 individuals	Both	4	3%	74.7 / 2.11	9.38 / 4.20	Nimptsch et al.	2015	(34)
	Exposure	Europeans	12,400 individuals	Both	18	5%		15.3 / 3.37	Kocarnik et al.	2014	(33)
	Exposure	Hispanic Americans	15,895 individuals	Both	9	4%	100 / 1.96		Kocarnik et al.	2018	(35)
	Outcome	Europeans	3,301 individuals	Both	-				Sun et al.	2018	(57)
Age at menarche	Exposure	Europeans	329,345 individuals	Women	389	7%	99.9 / 1.85	15.6 / 3.50	Day et al.	2017	(36)
Age at first birth	Exposure	Europeans	250,941 individuals	Parous women	10	4%	14.6 / 4.20	5.29 / 10.11	Barban et al.	2016	(37)

*Detectable true odds ratio with 80% statistical power and type I error rate of 5% considering the actual study size, proportion of cases and proportion of explained variance

† http://www.nealelab.is/uk-biobank/

§ http://www.nealelab.is/blog/2017/7/19/rapid-gwas-of-thousands-of-phenotypes-for-337000-samples-in-the-uk-biobank

Table 2: Mendelian randomization results for GBC (outcome) using genetic variants as instrumental variables for established risk factors (exposures).

Exposure	Population	Inverse variance weighted				
		Q p-value	β p-value	OR[a]	95% CI	
Gallstone disease	Chileans	0.56	$9\ 10^{-5}$	**1.97**	1.40	2.77
	Chileans (Mapuche > 34%)	0.45	0.03	**1.60**	1.04	2.46
	Chileans (Mapuche ≤ 34%)	0.23	0.002	**2.88**	1.49	5.58
	Europeans	0.47	$9\ 10^{-5}$	**5.02**	2.23	11.3
Body mass index	Chileans	0.14	0.03	**2.47**	1.10	5.54
(per unit)	Chileans (Mapuche > 34%)	0.53	0.007	**3.83**	1.46	10.1
	Chileans (Mapuche ≤ 34%)	0.20	0.86	1.13	0.30	4.27
	Europeans	0.18	0.89	0.91	0.22	3.78
C-reactive protein	Chileans	0.42	0.99	1.00	0.71	1.41
(per mg/L)	Chileans (Mapuche > 34%)	0.98	0.50	1.16	0.75	1.79
	Chileans (Mapuche ≤ 34%)	0.19	0.72	0.87	0.41	1.85
	Europeans	0.64	$4\ 10^{-6}$	**4.44**	2.35	8.37
Age at menarche	Chileans	0.11	0.79	0.94	0.61	1.46
(per year)	Chileans (Mapuche > 34%)	0.47	0.85	0.95	0.56	1.60
	Chileans (Mapuche ≤ 34%)	0.15	0.53	0.79	0.38	1.64
	Europeans	0.69	0.33	0.70	0.34	1.43

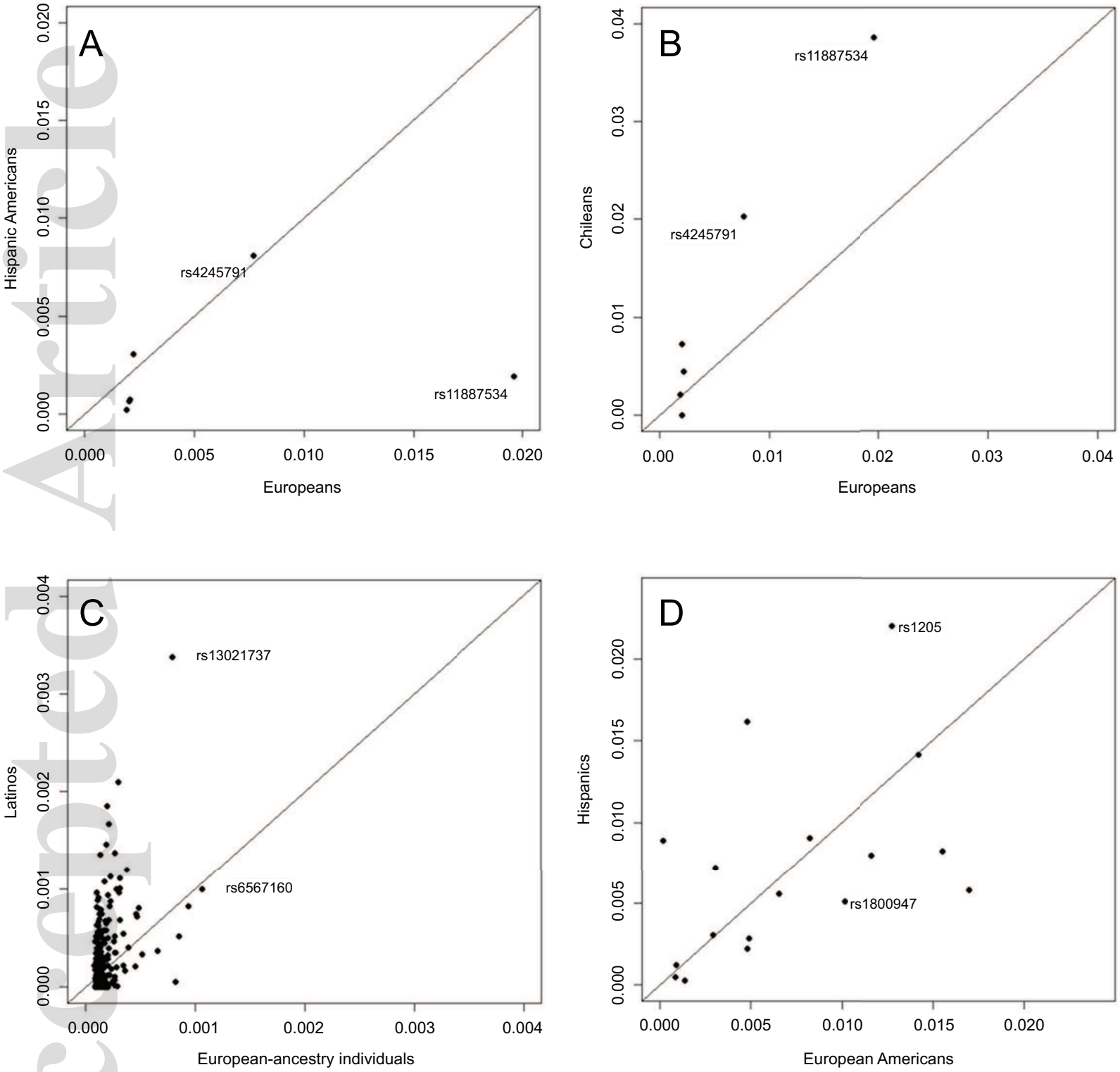
[a]Bold type for inverse variance weighted OR denotes Q p-value > 0.10 and β p-value < 0.05.

Table 3: Results from two-step Mendelian randomization to assess the potential mediation effects of gallstone disease, BMI and CRP on GBC in Europeans.

Exposure	Potential Mediator	Inverse variance weighted				
		Q p-value	β p-value	OR/β[a]	95% CI	
Gallstone disease[b]	C-reactive protein	0.97	0.54	0.96	0.86	1.08
	Body mass index	0.14	0.27	0.99	0.98	1.01
Body mass index	Gallstone disease	0.11	0.008	**1.01**	1.00	1.01
(per unit)	C-reactive protein	0.37	0.07	0.17	-0.01	0.36
C-reactive protein[b]	Gallstone disease	0.37	0.82	1.00	0.99	1.00
(per mg/L)	Body mass index	0.99	0.09	-0.01	-0.01	0.00

[a]Bold type for inverse variance weighted-β denotes Q p-value > 0.10 and β p-value < 0.05.

[b]Data for women only was not available.



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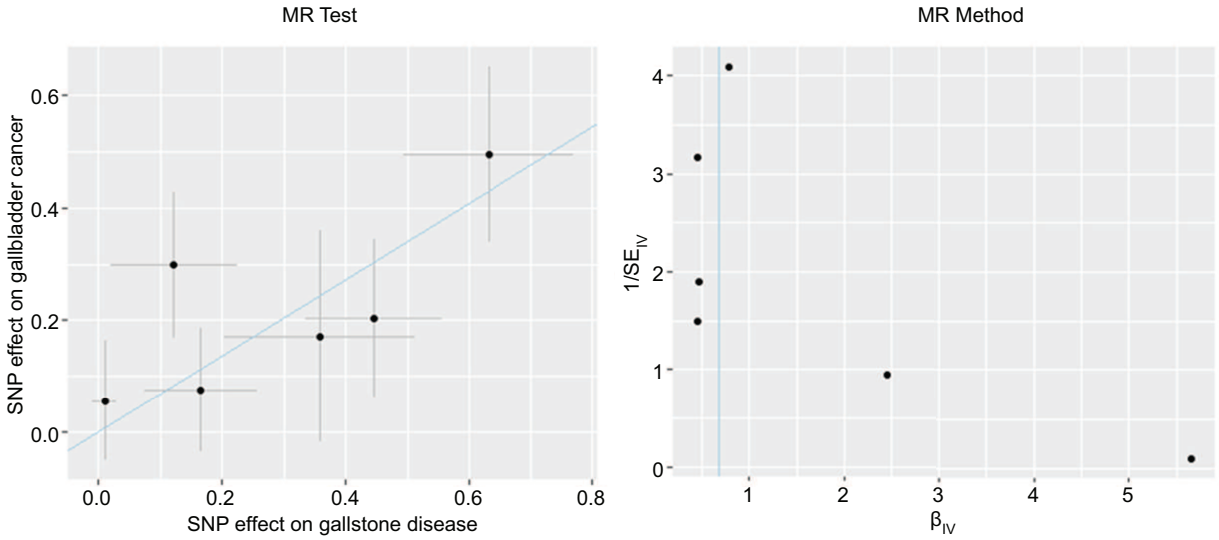
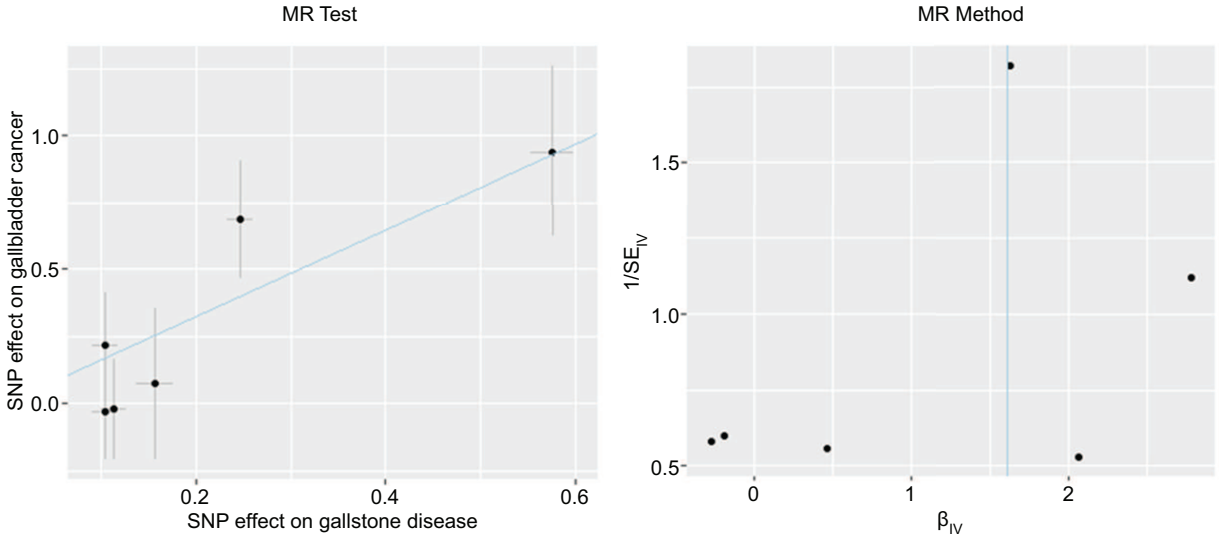
Exposure: Gallstone disease
Outcome: Gallbladder cancer

Panel A: Chileans



Panel B: Europeans



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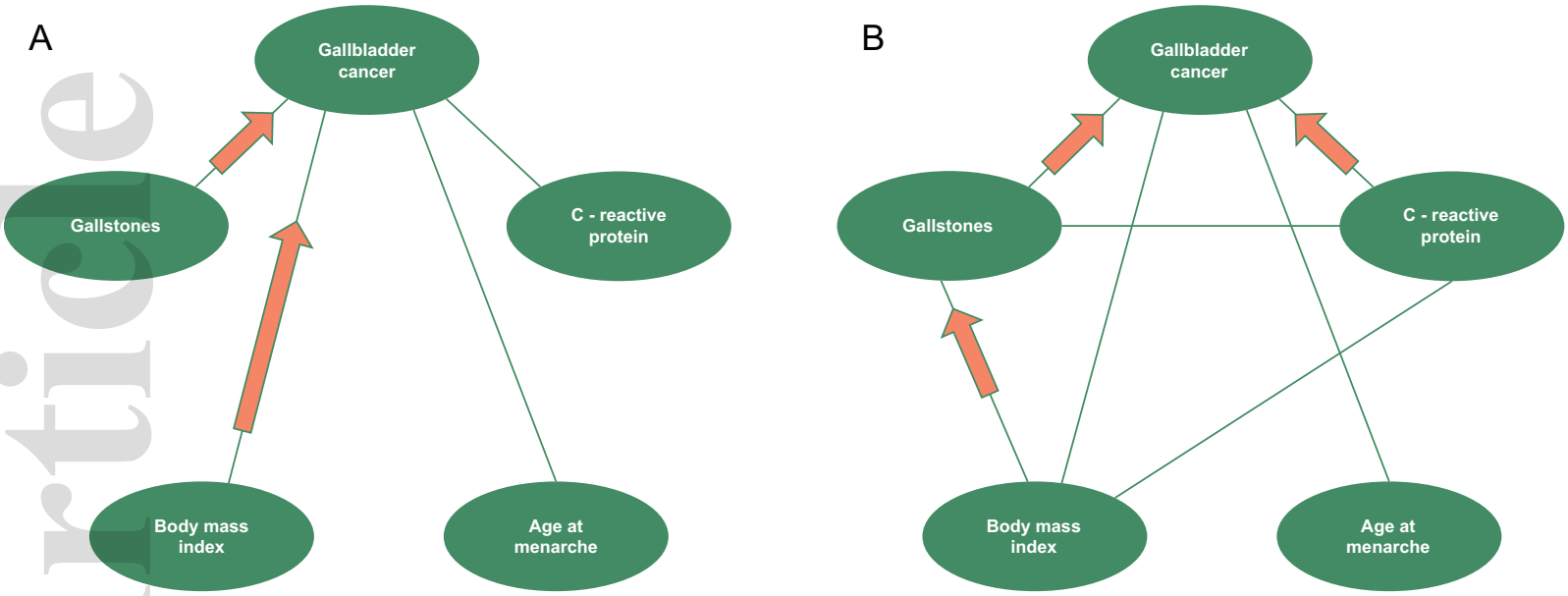

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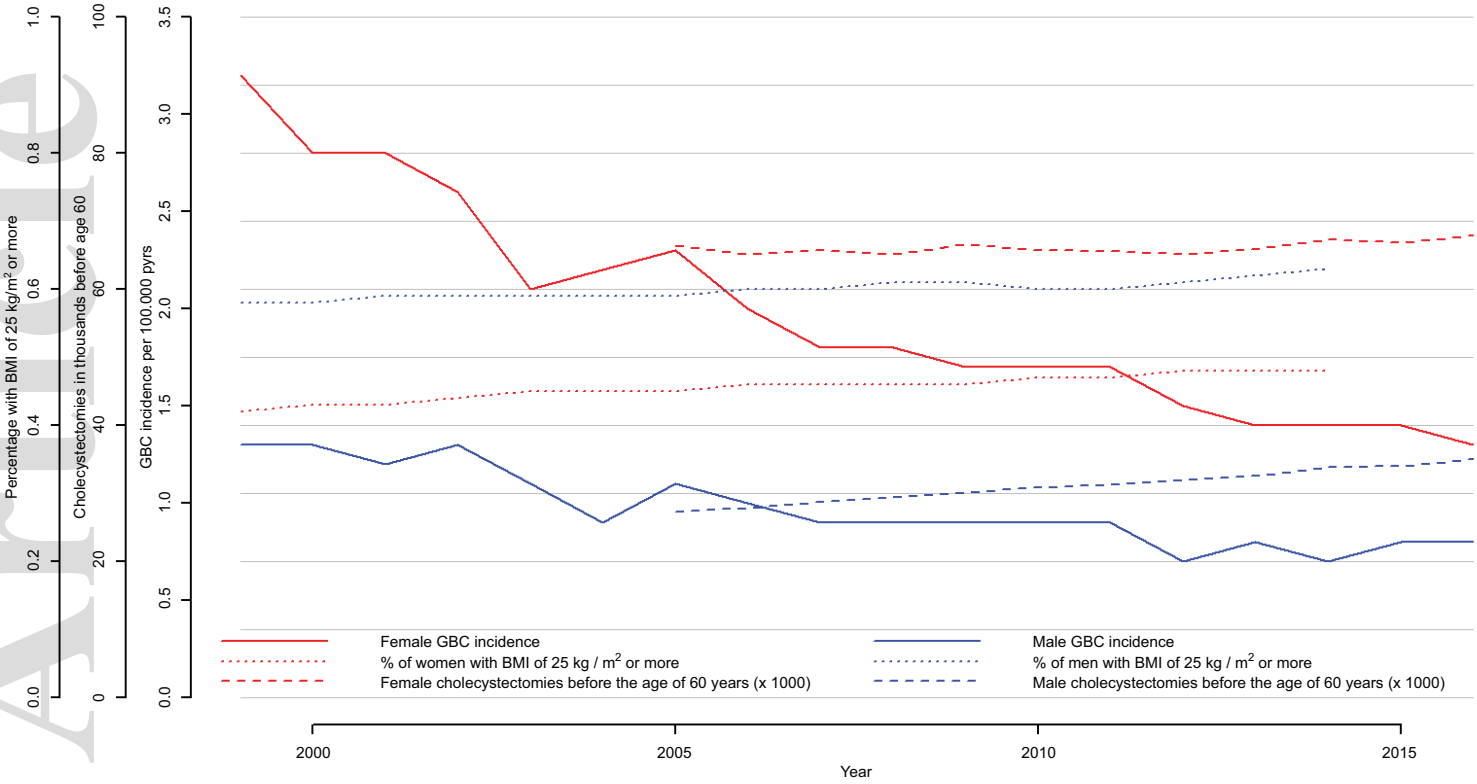

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