MENDELIAN RANDOMIZATION: APPROACH AND APPLICATIONS

EDITED BY: Lei Zhang and Yaozhong Liu
PUBLISHED IN: Frontiers in Genetics

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-674-6
DOI 10.3389/978-2-88971-674-6

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MENDELIAN RANDOMIZATION: APPROACH AND APPLICATIONS

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Citation: Zhang, L., Liu, Y., eds. (2021). Mendelian Randomization: Approach and Applications. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-674-6

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frontiers in Genetics

EDITORIAL
published: 28 September 2021
doi: 10.3389/fgene.2021.752146

Editorial: Mendelian Randomization: Approach and Applications

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Keywords: Mendelian randomization, causal inference, horizontal pleiotropy, genome-wide association study, confounding factor

Editorial on the Research Topic

Mendelian Randomization: Approach and Applications

Mendelian randomization (MR) is a valuable approach to assess potential causal relationships between exposures and outcomes in an observational study, especially when traditional randomized controlled trials or observational studies are not feasible (Davey Smith and Ebrahim, 2003). Genetic variants, such as single-nucleotide polymorphism (SNP), are selected as instrumental variables due to random allocation at birth, which can effectively address unmeasured confounding bias and reverse causation. The productive findings of published genome-wide association studies (GWAS) for screening suitable instrumental variables of various exposure traits contributes to MR's increasing popularity as a major approach to infer potential causal associations (Sekula et al., 2016). However, the endeavor of MR is still limited in methodological development and empirical application.

Although MR analysis provides a time- and cost-efficient solution to infer causal relationships without additional recruitment or experimental design, certain threats, especially pleiotropy, weak instrument, and linkage disequilibrium, could violate core assumptions, leading to biased causal links (Burgess et al., 2015; Lawlor, 2016). Hence, it is urgent to develop and apply novel MR methodologies and assess existing methods to address those issues. Moreover, the application of MR has also extended to new data types and scenarios, such as mRNA, protein, metabolic biomarkers, molecular phenotypes, and microbiome. All these applications will further explore the etiological mechanisms behind human diseases.

Here, we organized a Research Topic on "*Mendelian Randomization: Approach and Applications*" which gathered a collection of 14 high-quality studies made up of 13 original articles and one review that deal with approaches and applications of MR. These studies emphasize on novel methodological development, comparison of existing statistical methods, causal inference between traditional traits/diseases or novel data types, and drug target application.

Five contributions in this issue clarified whether there is a causal relationship between traditional traits/diseases and identified their potential risk factors. Zhu et al. utilized a MR study to fill the gap on causal links between alcohol use and mental health in East Asian populations. Their study reported that alcohol consumption was causally associated with a lower risk of depression. The study by Cui, Hou, et al. employed the bidirectional causal association between inflammatory bowel disease and Ankylosing Spondylitis with a two-sample MR based on GWAS summary statistics. It indicated that inflammatory bowel disease was the causal factor of an increased risk of Ankylosing Spondylitis. Similarly, Gao X. et al. investigated the causal link between sleep-related phenotypes and type 2 diabetes

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Edited and reviewed by:
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *02 August 2021*
Accepted: *12 August 2021*
Published: *28 September 2021*

Citation:
Wu G-L, Liu Y-Z and Zhang L (2021) Editorial: Mendelian Randomization: Approach and Applications. Front. Genet. 12:752146. doi: 10.3389/fgene.2021.752146

mellitus and showed an adverse effect of insomnia on type 2 diabetes mellitus. On the other hand, evidence from the MR approach did not support a causal relation between certain associated conditions, which possibly represented other confounders in previous observational studies, as shown in two articles by Yang et al. and Cui, Feng, et al., respectively.

Six contributions in this issue explored possibilities in using new data types in MR analysis and application of MR in new scenarios. Zhang et al. utilized summary statistics of the gut microbiome to assess causal relationships in inflammatory bowel disease, which is a novel attempt to identify specific pathogenic bacteria taxa for complex diseases by MR. In a similar vein, Ha et al. examined the causal effects of 69 environmental factors on asthma in the MR analysis and found that body mass index causally affects the development of asthma. For protein level, Zheng et al. demonstrated that fibroblast growth factor 23 level was significantly and causally associated with large-artery atherosclerotic stroke, which offers potential therapeutic targets for the disease. Yet Wang B. et al. did not find evidence to support the causal relationship of C-reactive protein and fibrinogen with an increased risk of intracerebral hemorrhage. Moreover, Gao Y. et al. applied the genetic risk score and MR framework to assess the causality of leukocyte telomere length on reviewed around 100 MR studies of different types of exposures with risk of stroke and provided perspectives to reviewed around 100 MR studies of different types of exposures with risk of stroke and provided perspectives to future novel approaches, including drug development and repurposing.

Although the application of MR has become more popular, existing MR statistical methods still cannot completely solve the issues that violate the core assumptions, such as pleiotropy and linkage disequilibrium. Thereby, novel methodological development is warranted. In particular, three contributions have showed novel statistical methods and compared studies to optimize MR methods and address existing problems. The study by Schooling et al. has demonstrated theoretically and empirically that multivariable MR may effectively mitigate selection bias due to survival before recruitment and performed an example simulation after amelioration. Wang Y. et al. proposed a novel mixed-effects regression model-based method, Pleiotropic and linkage disequilibrium adaptive Mendelian randomization (PLDMR), which corrected linkage disequilibrium and pleiotropic effect in the causal statistical inference. The simulation results showed the validity and advantage of PLDMR compared with others. Finally, the contribution by Lin et al. provided an improved MR approach, Mendelian Randomization with Refined Instrumental Variable from Genetic Score (MR-RIVER), to integrate summary data of multiple instrumental variables into a single genetic score. Through statistical simulations, it indicated that this novel approach possessed more statistical power as well as smaller biases and mean squared errors than the competing methods.

In summary, articles in this issue have comprehensively illustrated that MR framework is an effective and powerful solution to causal inference in various application scenarios. We hope that our special issue will stimulate further research and promote the development of more efficient and accurate MR approaches in the near future.

AUTHOR CONTRIBUTIONS

Y-ZL and LZ conducted this topic issue. G-LW, LZ, and Y-ZL wrote the manuscript. All authors contributed to the article and approved the submission.

FUNDING

LZ was partially supported by a project funded by the Priority Academic Program Development (PAPD) of Jiangsu higher education institutions.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
published: 19 October 2020
doi: 10.3389/fgene.2020.585351

Alcohol Use and Depression: A Mendelian Randomization Study From China

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Background: Alcohol use has been linked to a number of physical conditions, but the relationship between alcohol drinking and depression, one of the most common mental disorders that is a significant contributor to the global burden of disease, is still under debate. We aim to help fill the literature gap on the causal effect of alcohol use on depression by using genetic instruments of *ALDH2* rs671 and *ADH1B* rs1229984 in the Mendelian randomization (MR) framework.

Materials and Methods: We collected a sample of 476 middle-aged and older adults from mainland China. The 10-item Center for Epidemiologic Studies Depression Scale (CESD-10) was used to measure the status of depression. The frequency and intensity of alcohol consumption were measured by (1) a binary indicator of drinking or not, (2) the total number of drinking occasions during the past 30 days, and (3) the weekly ethanol consumption in grams.

Results: MR estimates indicated that alcohol use was causally associated with a lower risk of depression. Parameter estimates of drinking or not ($b = -0.127$, $p = 0.048$), number of drinking occasions ($b = -0.012$, $p = 0.040$), and weekly ethanol consumption ($b = -0.001$, $p = 0.039$) were all negative and statistically significant. The results were robust after adjustments for potential confounders (e.g., income, smoking, and parental drinking behaviors), and the exclusion of heavy or former drinkers.

Conclusions: This is one of the first study to investigate the causal relationship between alcohol use and mental health using an MR design in East Asian populations. Further studies are needed to clarify the mechanisms of this causal link.

Keywords: alcohol use, depression, Mendelian randomization, genetic instruments, *ALDH2* rs671, *ADH1B* rs1229984, China, alcohol consumption

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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *20 July 2020*
Accepted: *28 September 2020*
Published: *19 October 2020*

Citation:
Zhu C, Chen Q, Si W, Li Y, Chen G and Zhao Q (2020) Alcohol Use and Depression: A Mendelian Randomization Study From China. Front. Genet. 11:585351. doi: 10.3389/fgene.2020.585351

INTRODUCTION

Alcohol use has been linked to a large number of physical conditions, and recent work has challenged the conventional view that low-to-moderate alcohol consumption has a beneficial health effect on coronary artery disease and diabetes (GBD 2016 Alcohol Collaborators, 2018).

However, there is much less research about the causal relationship between alcohol consumption and mental health. One common but serious mental disorder, in particular, is depression, which is a significant contributor to the global burden of disease and affects one in 15 people in any given year (American Psychiatric Association, 2013). Earlier observational studies have found that alcohol use was associated with several psychological benefits (Baum-Baicker, 1985; Peele and Brodsky, 2000; Marchand et al., 2003). Whereas other studies have reported an overall null effect of moderate drinking on depression in different populations (Paschall et al., 2005; Almeida et al., 2014; Polimanti et al., 2019) and even a positive association between alcohol use disorders and major depression (Boden and Fergusson, 2011). These inconsistent findings have raised debate about the causal link between alcohol use and depression. Since alcohol use is *not* randomly assigned, one major threat of the previous observational study designs is the endogeneity issue originated from unobserved confounders and/or reverse causality. This study aims to help fill the literature gap on the causal relationship between alcohol use and depression by using genetic variants of *ALDH2* rs671 and *ADH1B* rs1229984 to instrument for alcohol use in the Mendelian randomization (MR) framework.

MR is a causal research design that uses genetic variants as instrumental variables (IV; DiPrete et al., 2018). The genetic basis of the MR approach relies on the random allocation of genes at meiosis in humans, resembling the random assignment into treatment groups in randomized controlled trials (RCT) that may be infeasible or unethical in this setting (Yeung et al., 2012; Holmes et al., 2014). We collected a sample of 476 middle-aged and older adults from mainland China, with demographics, socioeconomic status (SES), drinking behaviors, and mental health conditions linked to individual genotyping data. China is an interesting and important country in which to study this research question for several reasons: first, China has the highest alcohol-related deaths in the world (GBD 2016 Alcohol Collaborators, 2018), but studies on the causal links between alcohol use and mental health outcomes among Chinese populations are still very limited (Yeung et al., 2012). Second, depression has become a significant public health concern in China. It was estimated that the disability-adjusted life years (DALYs) of depression in China had increased by 36.5% from 1990 to 2017 (Ren et al., 2020). But little is known about the link between depression and alcohol drinking among Chinese populations. Third, both the spending and consumption of alcohol in China are still increasing rapidly. The per capita alcohol consumption in China went up from 1.7 L in 1980 to 5.7 L in 2016 (OECD, 2019) and is projected to jump to more than 10 L by 2030, exceeding the United States per capita consumption.[1] Fourth, the proposed genetic instruments, *ALDH2* rs671 and *ADH1B* rs1229984, have a strong association with alcohol consumption and dependence, but are only prevalent in East Asian populations, which makes the MR research design more applicable in this region.

To strengthen the MR framework, we tested the instrument validity of *ALDH2* rs671 and *ADH1B* rs1229984 and dealt with threats from the pleiotropy effect, dynastic effect, and population stratification by controlling for a number of potential confounders that have not been included in most previous MR studies (e.g., parental drinking behaviors and individual genetic ancestral compositions). Besides, to avoid biased results due to inadequate separation of alcohol use levels, such as the sick-quitter bias (Paschall et al., 2005), we separated *former drinkers* from *never drinkers* and *heavy drinkers* from *moderate drinkers*.

MATERIALS AND METHODS

Sample Collection

The survey was designed and implemented by the China Center for Genoeconomic Studies (CCGS) at China Agricultural University in the summer of 2019. The Institutional Review Board of China Agricultural University approved the protocol. Prior to data collection, all participants signed an informed consent form after receiving a careful explanation about the purpose of this study. All participants were informed that their responses were completely voluntary and confidential and were invited to contact the research team later if they had any further questions regarding any aspect of the study. Fifty villages from seven provinces in mainland China (Heilongjiang, Henan, Zhejiang, Yunnan, Xinjiang, Shandong, and Anhui) were selected; in each village, 10 households were then randomly selected. The survey collected information on participants' regular demographic/socioeconomic status as well as detailed information about their alcohol consumption. We also collected participants' parental drinking behaviors (i.e., father drinking or not and mother drinking or not). Additionally, 1 ml saliva samples were collected from all participants during the face-to-face interview. Excluding individuals who did not pass the quality control yielded, a total of 476 observations were collected. As reported in **Table 1**, the average respondent in our sample was 49.4 years old, completed 8.2 years of education, and earned CNY 70,232 (1 US Dollar ≈ CNY 7) annually.

Genotyping

DNA was extracted from saliva samples using the Illumina WeGene V2 Array. Imputation and quality control were performed using PLINK (1.90 Beta), SHAPEIT (v2.17), and IMPUTE2 (v2.3.1).

Measures of Alcohol Use

We surveyed respondents about three complementary measures of the frequency and intensity of alcohol consumption. First, we asked for a binary measure of drinking-or-not status, where 0 and 1 represent *current non-drinkers* (54.5%) and *current drinkers* (45.5%), respectively. Second, we asked participants the total number of occasions that they consumed any alcohol during the past 30 days (mean = 4.7, SD = 6.4). Third, by

[1]Source: https://www.theguardian.com/society/2019/may/08/world-alcohol-consumption-on-the-rise-as-chinas-thirst-grows (Accessed: June 24, 2020).

TABLE 1 | Demographic, socioeconomic and genetic characteristics of participants according to their groups of alcohol use (*N* = 476).

		Groups by alcohol use			
	Pooled	**Never drinkers**	**Former drinkers**	**Moderate drinkers**	**Heavy drinkers**
	***N* = 476**	***N* = 184**	***N* = 76**	***N* = 165**	***N* = 51**
	(100%)	**(38.7%)**	**(16.0%)**	**(34.7%)**	**(10.7%)**
	% or mean (SD)	**% or mean (SD)**	**% or mean (SD)**	**% or mean (SD)**	**% or mean (SD)**
Age	49.4 (11.6)	50.0 (11.4)	51.5 (13.6)	47.7 (10.6)	50.0 (12.3)
Gender					
Male	74.0%	48.7%	86.6%	89.0%	100.0%
Female	26.0%	51.3%	13.4%	11.0%	0.0%
Drinking times during the past 30 days	4.7 (6.4)	0.0 (0.0)	0.0 (0.0)	8.8 (5.8)	14.9 (2.3)
Weekly ethanol consumption (g)	62.2 (113.7)	0.0 (0.0)	0.0 (0.0)	71.6 (55.0)	347.9 (96.0)
Depression or not					
Yes	9.0%	16.7%	10.4%	2.1%	2.2%
No	91.0%	83.3%	89.6%	97.9%	97.8%
Years of schooling	8.2 (3.5)	7.8 (3.9)	7.9 (3.4)	8.8 (3.1)	8.8 (2.9)
Annual earnings (in 10,000 CNY)	7.0 (10.8)	6.1 (8.8)	6.7 (8.2)	8.2 (13.9)	6.7 (9.1)
Smoking or not					
Yes	38.3%	18.5%	40.3%	52.1%	62.2%
No	61.7%	81.5%	59.7%	47.9%	37.8%
No. of parents that drink					
0	19.8%	26.5%	22.4%	12.3%	15.6%
1	65.0%	59.3%	59.7%	72.6%	68.9%
2	15.2%	14.2%	17.9%	15.1%	15.5%
***ALDH2* rs671 (no. of effect alleles)**					
AA (2):	4.5%	11.1%	1.5%	0.0%	0.0%
AG (1):	31.0%	41.4%	46.3%	17.8%	13.3%
GG (0):	64.5%	47.5%	52.2%	82.2%	86.7%
***ADH1B* rs1229984 (no. of effect alleles)**					
AA (2):	42.4%	47.5%	40.3%	39.0%	37.8%
AG (1):	44.8%	39.5%	41.8%	52.1%	44.4%
GG (0):	12.9%	13.0%	17.9%	8.9%	17.8%
Ancestral composition					
Northern Han	49.9%	48.9%	43.7%	53.1%	52.7%
Southern Han	19.0%	21.2%	21.3%	16.9%	14.5%
Mongolian	10.0%	8.3%	11.9%	10.4%	12.1%
Japanese	2.5%	2.5%	2.2%	2.8%	2.2%
Province					
Heilongjiang	94	34	10	35	15
Henan	22	8	5	7	2
Zhejiang	41	18	6	12	5
Yunnan	182	78	33	54	17
Xinjiang	52	13	11	26	2
Shandong	16	7	1	6	2
Anhui	69	26	10	25	8

combining the information on drinking frequency and the average amount that a participant drank on one occasion, we calculated the weekly ethanol (i.e., pure alcohol) consumption in grams as a continuous measure of alcohol use (mean = 62.2, SD = 113.7). While self-reported data often raise concerns of misreporting, it has been demonstrated that self-reported recent alcohol consumption suffers less from misreporting when multiple closed-ended questions are used and can be reliable measures of alcohol consumption (Lintonen et al., 2004). To avoid biases elevated from the inadequate separation of alcohol use levels (Paschall et al., 2005), we also classified participants into four distinct alcohol use groups of *never drinkers* (38.5%), *former drinkers* (16.0%), *heavy drinkers* (10.7%; defined as having more than 210 g of ethanol per week; Yeung et al., 2012), and *moderate drinkers* (34.8%; defined as *current drinkers* who that have less than or equal to 210 g of ethanol per week).

Genetic Instruments

There are two genetic variants commonly used in MR studies of alcohol use: the alcohol dehydrogenase 1B gene (*ADH1B* rs1229984) and the aldehyde dehydrogenase 2 gene (*ALDH2* rs671), both of which encode enzymes involved in the metabolic pathway for ethanol and can change the metabolic balance of acetaldehyde in human body (Peng and Yin, 2009). In the

human body, ethanol is first converted to acetaldehyde by alcohol dehydrogenase (ADH) and then to acetate by aldehyde dehydrogenase (ALDH).

The enzyme activity of ADH and ALDH are largely determined by the number of effect alleles (i.e., A-allele) in both *ADH1B* rs1229984 and *ALDH2* rs671. In East Asian populations, *ALDH2* rs671 alleles exist with three genotypes, GG (# of A allele = 0), AG (# of A allele = 1), and AA (# of A allele = 2), where the presence of A allele can significantly decrease the detoxification of acetaldehyde generated during alcohol metabolism in humans as noted above (Peng and Yin, 2009; Edenberg and McClintick, 2018). From **Table 1**, 35.5% of respondents in our sample are A-allele carriers (i.e., genotypes of AA and AG). Specifically, the percentages of genotype AA and AG are 4.5 and 31.0%, respectively. In European populations, *ADH1B* rs1229984 has been used as the principal genetic instrument in MR studies of alcohol use (Holmes et al., 2014). But because the proportion of A-allele carriers is very low (around 3% in Europeans), these MR studies require much larger sample sizes.[2] In comparison, a majority of participants are A-allele carriers of the *ADH1B* rs1229984 in our sample (AA: 42.4% and AG: 44.8%).

Measures of Depression

The 10-item Center for Epidemiologic Studies Depression Scale (CESD-10) was used as a reliable and valid survey instrument to screen for symptoms of depression (Radloff, 1977; Boey, 1999). Following Cheng and Chan (2005), we adopted the cut-off score of 12 as the optimal threshold for screening for depression. **Figure 1** shows a scatter plot of CESD-10 scores over age by different combinations of *ALDH2* rs671 (horizontal) and *ADH1B* rs1229984 (vertical) genotypes. Each dot represents a single subject. Different shapes and colors demote for distinct genders (female and male) and alcohol use groups (never drinkers, former drinkers, and moderate drinkers/heavy drinkers). As expected, most of the participants with two effect alleles (AA) in *ALDH2* rs671 are never drinkers (right column).

Statistical Analysis

Multivariable linear regression was used to examine the relationship between different measures of alcohol use and depression. In the MR analyses, we first verified the validity of genetic instruments and then evaluated the causal relationship between alcohol use and depression using two-stage least squares (2SLS). Given that demographic characteristics, SES, and smoking might be highly correlated with both alcohol use and depression (Room, 2004), we adjusted age, gender, income, years of schooling, smoking, and province fixed effects in all regressions. **Figure 2** illustrates the relationships between the genetic instruments (*ALDH2* rs671 and *ADH1B* rs1229984), the exposure (alcohol consumption), the health outcome (depression), and the (observed or unobserved) confounders in our MR framework. We dealt with potential threats from the pleiotropy effect, dynastic effect, and population stratification by further adjusting for parental drinking behaviors (i.e., number of parents that drink) and the individual genetic ancestral composition[3] in MR estimations. We also performed the mediation analysis to evaluate the associations of alcohol drinking on depression explained by the years of schooling, income, and smoking. Results were reported as beta coefficients and 95% confidence intervals. All values of p were two-sided.

RESULTS

Table 2 panel *a* reports critical estimates of alcohol use on depression from separate multivariable linear regressions on all participants (column 1), the subsample excluding heavy drinkers (column 2), and the subsample excluding former drinkers (column 3). From estimates of the full sample (panel *a*, column 1), both drinking or not ($b = -0.068$, $p = 0.022$, 95% CI = −0.126 to −0.010) and the number of drinking times during the past 30 days ($b = -0.005$, $p = 0.017$, 95% CI = −0.010 to −0.001) were found to be significantly associated with a lower risk of depression, suggesting a protective effect of alcohol drinking in the prevention of depression. Exclusion of heavy alcohol drinkers (panel *a*, column 2) or former drinkers (panel *a*, column 3) did not change the estimates substantially. However, alcohol use can still be confounded by various factors even after adjustments, such as socioeconomic classes, diet patterns, physical activity, BMI, etc. Thus, the estimated associations presented in this section were not causal and needed to be interpreted with caution.

The validity of using *ALDH2* rs671 and *ADH1B* rs1229984 as genetic instruments relied on the critical assumption of relevance and the exclusion restriction (Davies et al., 2018). In our research design, the instrumental relevance was satisfied with *a priori* given the robust associations previously documented (Yeung et al., 2012; Peng et al., 2019). We confirmed these correlations hold in our sample without and with the adjustments for additional controls.[4] R-squared suggested that *ALDH2* rs671 and *ADH1B* rs1229984 together could explain 9.6–13.7% of the total phenotypic variation in different measures of alcohol consumption, indicating strong genetic instruments. We also tested for weak instruments by using Cragg-Donald F statistics in the estimation (Burgess et al., 2017; Davies et al., 2018). Another crucial concern was the potential pleiotropic effect, which occurs when a genetic IV can directly influence the outcome variable (Davies et al., 2018). There are several reasons to think pleiotropy is unlikely in our setting. First, Yeung et al. (2012) and Peng et al. (2019) considered this assumption and provided epidemiological evidence for the credibility of *ALDH2* rs671 as IV for alcohol

[2]Source: https://www.ncbi.nlm.nih.gov/snp/rs1229984#frequency_tab

[3]Individual genetic ancestral composition gave the percentage of DNA that comes from different populations by comparing an individual genome to hundreds and thousands people with known ancestry and was calculated by using the ADMIXTURE program. The top four ancestries included in the MR estimation are (from high to low) Northern Han, Southern Han, Mongolian, and Japanese.

[4]Detailed first-stage estimation results are available from the corresponding author on request.

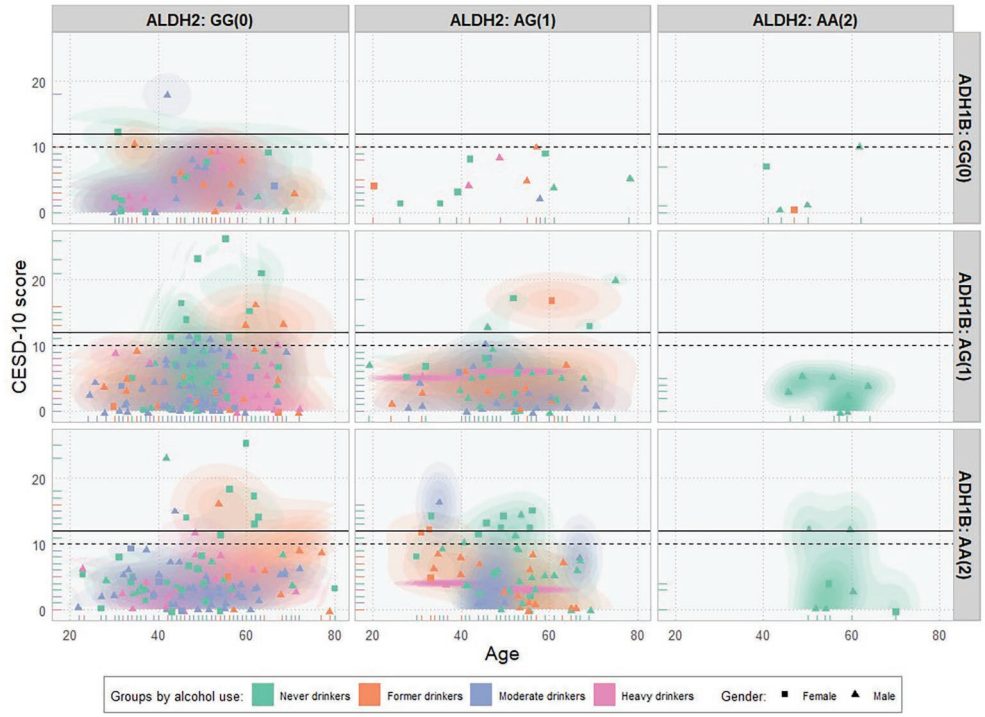

FIGURE 1 | Scatter plot of CESD-10 scores over age by gender, genotypes, and groups of alcohol use (N = 476). CESD-10 scores over age were plotted by different combinations of *ALDH2* rs671 (horizontal) and *ADH1B* rs1229984 (vertical) genotypes (with the number of effect alleles in parenthesis). Each dot represents a single subject. The solid and dashed black horizontal lines denote for cut-off scores of 12 (optimal) and 10 for the depression, respectively. Different genders (female/male) and groups of alcohol use (never drinkers/former drinkers/moderate drinkers/heavy drinkers) were represented by distinct shapes and colors, respectively.

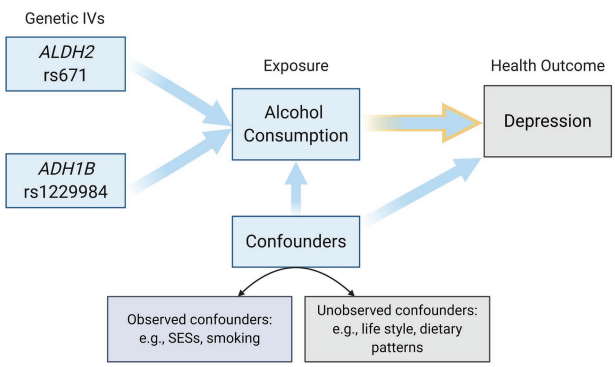

FIGURE 2 | Directed acyclic graph (DAG) of the Mendelian randomization (MR) framework. This DAG illustrated the relationships between the genetic instruments (*ALDH2* rs671 and *ADH1B* rs1229984), the exposure (Alcohol Consumption), the health outcome (Depression), and the (observed or unobserved) confounders.

use. Second, we consulted with PhenoScanner (v2) and found no evidence of direct links of *ALDH2* rs671 and *ADH1B* rs1229984 with depression-related phenotypes. Third, we formally conducted the overidentification test based on Sargan statistics in the estimation, which revealed no violation of the exclusion restriction, lending further support to the validity of these genetic instruments (Burgess et al., 2017).

Table 2 panel *b* reports MR results by incorporating both *ALDH2* rs671 and *ADH1B* rs1229984 as instrumental variables. The First-stage Cragg-Donald F statistics from all models exceeded the conventional cut-off of 10 for weak instruments, indicating that the two genetic IVs are jointly strong instruments in our MR design. The Sargan statistics and values of p of overidentification tests suggested no evidence that the genetic IVs were correlated with unobserved confounders. In all MR models, we additionally adjusted for parental drinking behaviors and individual ancestral compositions to further validate the MR settings (Willage, 2018). We found that under the MR design, alcohol use was *causally* associated with a lower risk of depression in the full sample (panel *b*, column 1). Parameter estimates of drinking or not (b = −0.127, p = 0.048, 95% CI = −0.253 to −0.001), the number of drinking times during the past 30 days (b = −0.012, p = 0.040, 95% CI = −0.023 to −0.001), and the weekly ethanol consumption (b = −0.001, p = 0.039, 95% CI = −0.002 to −0.000) were all negative and statistically significant at the 5% level.[5] The results were robust

[5]Results remained robust when we used the Two-stage instrumental variables (2SIV) approach that replaced the second-stage least-squares regression in 2SLS by logistic regression (Palmer et al., 2008). Detailed estimation procedures and results are available from the corresponding author on request.

TABLE 2 | Effects of alcohol use on depression – OLS and 2SLS estimation results.

	(1) All participants	(2) Excluding heavy drinkers	(3) Excluding former drinkers
	***N* = 476**	***N* = 425**	***N* = 400**
(a.) Multivariable linear regressions			
(1) Key explanatory variable: drinking or not			
b (95% CI)	−0.068 (−0.126, −0.010)	−0.082 (−0.148, −0.016)	−0.065 (−0.129, −0.001)
p	0.022	0.015	0.046
(2) Key explanatory variable: drinking times during the past 30 days			
b (95% CI)	−0.005 (−0.010, −0.001)	−0.006 (−0.011, −0.001)	−0.006 (−0.012, −0.001)
p	0.017	0.012	0.019
(3) Key explanatory variable: weekly ethanol consumption (g)			
b (95% CI)	−0.000 (−0.000, 0.000)	−0.000 (−0.000, 0.000)	−0.001 (−0.001, 0.000)
p	0.191	0.19	0.092
(b.) Mendelian randomization			
(1) Key explanatory variable: drinking or not			
b (95% CI)	−0.127 (−0.253, −0.001)	−0.136 (−0.267, −0.004)	−0.149 (−0.295, −0.003)
p	0.048	0.043	0.045
Cragg-Donald F statistics of weak instrument tests (*p*)	44.491 (0.000)	55.917 (0.000)	34.221 (0.000)
Sargan statistics of overidentification tests (*p*)	1.837 (0.175)	1.036 (0.309)	2.061 (0.151)
(2) Key explanatory variable: drinking times during the past 30 days			
b (95% CI)	−0.012 (−0.023, −0.001)	−0.012 (−0.024, −0.001)	−0.015 (−0.030, −0.000)
p	0.040	0.036	0.047
Cragg-Donald F statistics of weak instrument tests (*p*)	28.664 (0.000)	36.403 (0.000)	19.967 (0.000)
Sargan statistics of overidentification tests (*p*)	1.605 (0.205)	1.595 (0.207)	2.190 (0.139)
(3) Key explanatory variable: weekly ethanol consumption (g)			
b (95% CI)	−0.001 (−0.002, −0.000)	−0.001 (−0.002, −0.000)	−0.000 (−0.001, −0.000)
p	0.039	0.027	0.044
Cragg-Donald F statistics of weak instrument tests (*p*)	18.113 (0.000)	20.818 (0.000)	20.386 (0.000)
Sargan statistics of overidentification tests (*p*)	1.240 (0.265)	2.559 (0.110)	2.092 (0.148)

Depression was defined as CESD-10 score ≥ 12 (Cheng and Chan, 2005). Abbreviations: 95% CI represents 95% confidence interval. All models were adjusted for age, gender, education, income, smoking, and province fixed effects. MR results were additionally adjusted for the number of drinking parents and individual genetic ancestral compositions of Northern Han, Southern Han, Mongolian, and Japanese. The First-stage Cragg-Donald F statistics (with values of p) and Sargan statistics (with values of p) are test statistics of the weak instrument and overidentification tests, respectively, which indicate that genetic instruments of ALDH2 rs671 and ADH1B rs1229984 used in MR satisfied with the relevance assumption and exclusion restriction.

after the exclusion of either heavy drinkers (panel *b*, column 2) or former drinkers (panel *b*, column 3). Further mediation analysis showed that the association of drinking or not with depression was mediated by approximately 11.8% through years of schooling, but not *via* income or smoking.

DISCUSSION

In this analysis, using a MR research design in a sample of 476 participants from mainland China, we found that the observed protective effect of alcohol use against depression was likely to be causal. The results were robust after adjustments for SES, smoking, parental drinking behaviors, genetic ancestral compositions, province fixed effects, and the exclusion of heavy or former drinkers.

This is one of the first studies to investigate the causal relationship between alcohol use and mental health using an MR design. The findings are in line with previous research that reported regular alcohol consumption was associated with better mental health conditions and lower levels of depression (Baum-Baicker, 1985; Peele and Brodsky, 2000; Marchand et al., 2003; Polimanti et al., 2019). Our study contributed further evidence that among a sample of middle-aged and older adults (with an average age of 49.4) from mainland China, alcohol use was causally associated with the prevention of depression. Paschall et al. (2005) reported no statistically significant associations between moderate alcohol use and depression, but the sample they used was young United States adults with an average age of 21.8 years old. Boden and Fergusson (2011) reported a link between alcohol use disorders and major depression based on a meta-analysis. However, they defined alcohol use disorders as a variety of alcohol misuse measures, which beyond the scope of regular alcohol use as in the current study. Almeida et al. (2014) found no significant causal effect of alcohol consumption on depression by using *ADH1B* rs1229984 as the single instrumental variable, but the analysis was based on a different population that contained 3,874 elderly (age 65–83) male participants from

the metropolitan region of Perth in Australia, and the results may suffer from a weak instrument problem and lack of power since *ADH1B* rs1229984 was reported to explain only 0.24% of the variance in alcohol consumption (Rees et al., 2019).

The mechanism underlying the detected beneficial association of alcohol use and depression is still under debate. The main explanations include the psychological benefits of stress reduction and mood enhancement resulting from low to moderate drinking (Baum-Baicker, 1985; Peele and Brodsky, 2000). Hence, the role of alcohol drinking in depression and overall mental health may be a balance of the beneficial effects (likely from low to moderate drinking) and harmful effects (likely from excessive drinking; Boden and Fergusson, 2011). Among certain groups of people, such as middle-aged and older Chinese adults in our sample with limited options of entertaining and stress-relieving activities, the beneficial effects of drinking may offset the harmful effects on depression symptoms.

Before closing, we noted several caveats to our results. First, the sample used in this study was not representative of the entire Chinese population. Second, the current study was based on a sample of 476 participants, which may lack statistical power due to the small sample size, and research with a larger sample size would be preferred to confirm our findings in the future.[6,7] Third, as noted before, the underlying mechanism of the detected beneficial impact of drinking on depression is still unclear. Further studies are needed in order to clarify the mechanisms of this causal link.

[6]Nevertheless, it should be noted that the bias resulting from small sample sizes tends to underestimate (rather than overestimate) the effect size (Dumas-Mallet et al., 2017).

[7]We replicated the results in a separate sample (N = 2,216) collected from China, but without available genotyping data. By using alcohol flushing reaction (i.e., a proxy to *ALDH2* rs671) as IV, we found that alcohol consumption was also related to the lower risk of depression, consistent with our main MR results. Detailed estimation procedures and results are available from the corresponding author on request.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://doi.org/10.6084/m9.figshare.13010567.v2.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of China Agricultural University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CZ and QZ designed this study. CZ, YL, and GC performed research and analyzed the data. CZ, QC, and WS drafted the manuscript. All the authors read and approved the final version of the manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (No. 71973136, 71773122, and 71603261) and China Agricultural University.

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Conflict of Interest: GC and YL are employees of WeGene. WeGene provided data support of collecting and analyzing genotyping data, but did not have any additional role in financially supporting the current study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 23 October 2020
doi: 10.3389/fgene.2020.583106

Assessing the Relationship Between Leukocyte Telomere Length and Cancer Risk/Mortality in UK Biobank and TCGA Datasets With the Genetic Risk Score and Mendelian Randomization Approaches

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OPEN ACCESS

Edited by:
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Soochow University, China

Reviewed by:
Jian Gu,
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Yuehua Cui,
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: 14 July 2020
Accepted: 24 September 2020
Published: 23 October 2020

Citation:
Gao Y, Wei Y, Zhou X, Huang S, Zhao H and Zeng P (2020) Assessing the Relationship Between Leukocyte Telomere Length and Cancer Risk/Mortality in UK Biobank and TCGA Datasets With the Genetic Risk Score and Mendelian Randomization Approaches. Front. Genet. 11:583106. doi: 10.3389/fgene.2020.583106

Background: Telomere length is an important indicator of tumor progression and survival for cancer patients. Previous work investigated the associations between genetically predicted telomere length and cancers; however, the types of cancers investigated in those studies were relatively limited or the telomere length-associated genetic variants employed often came from genome-wide association studies (GWASs) with small sample sizes.

Methods: We constructed the genetic risk score (GRS) for leukocyte telomere length based on 17 associated genetic variants available from the largest telomere length GWAS up to 78,592 individuals. Then, a comprehensive analysis was undertaken to evaluate the association between the constructed GRS and the risk or mortality of a wide range of cancers [i.e., 37 cancers in the UK Biobank and 33 cancers in The Cancer Genome Atlas (TCGA)]. We further applied the two-sample Mendelian randomization (MR) to estimate the causal effect of leukocyte telomere length on UK Biobank cancers via summary statistics.

Results: In the UK Biobank dataset, we found that the GRS of leukocyte telomere length was associated with a decreased risk of nine types of cancer (i.e., significant association with multiple myeloma, chronic lymphocytic leukemia, kidney/renal cell cancer, bladder cancer, malignant melanoma, basal cell carcinoma, and prostate cancer and suggestive association with sarcoma/fibrosarcoma and Hodgkin's lymphoma/Hodgkin's disease). In addition, we found that the GRS was suggestively associated with an increased risk of leukemia. In the TCGA dataset, we observed suggestive evidence that the GRS was associated with a high death hazard of rectum adenocarcinoma (READ), sarcoma (SARC), and skin cutaneous melanoma (SKCM), while the GRS was associated with a low death hazard of kidney renal papillary cell

carcinoma (KIRP). The results of MR further supported the association for leukocyte telomere length on the risk of malignant melanoma, Hodgkin's lymphoma/Hodgkin's disease, chronic lymphocytic leukemia and multiple myeloma.

Conclusion: Our study reveals that telomere played diverse roles in different types of cancers. However, further validations in large-scale prospective studies and deeper investigations of the biologic mechanisms are warranted.

Keywords: leukocyte telomere length, cancer, genetic risk score, UK Biobank, TCGA, Mendelian randomization

INTRODUCTION

Telomere is a special structure with a 6-bp TTAGGG repeat sequence and plays an important role in genomic stability by protecting DNA against damage and fusion 0 (de Lange, 2005). Due to the inability of DNA polymerase to fully extend the 3′ end of DNA strand, the telomere becomes progressively shorter during each round of cell division. The length of telomere is thus a biomarker of cellular and overall biological aging. Once a critically short telomere length is reached, the cell would be triggered to enter senescence, which would ultimately lead to cell growth arrest or apoptosis (Shay and Wright, 2019). In stem and progenitor cells, the length of telomere is maintained by enzyme telomerase (Hackett and Greider, 2002; Shawi and Autexier, 2008). It is shown that enzyme telomerase is activated in almost all human tumors; such an activation can result in the continuous division of cancer cells and is the key component of the tumorigenic phenotype of human cancer cells (Stewart and Weinberg, 2006; O'Sullivan and Karlseder, 2010).

Prior studies have demonstrated that telomere length is associated with a lot of age-related diseases and disorders (e.g., cancers and neurodegenerative disorders) (Zhu et al., 2011) and that a shorter telomere length in tumor tissues is an important indicator of tumor progression and survival for cancer patients (Ma et al., 2011; Xu et al., 2016). However, not all studies reported consistent findings (**Supplementary Table S1**), partly reflecting the complicated function of telomere on human cancers. The diversity in cancer types, ethnicities, study designs, measurement methods, and selected tissues for telomere length in previous work further complicates the observed association. Given the severe disease burden of cancers worldwide (Siegel et al., 2019), understanding the association between telomere length and cancers can provide valuable insights into the development of cancers and has the potential to improve the prevention and treatment strategies for cancers.

On the other hand, in the past few years, a number of single nucleotide polymorphisms (SNPs) have been identified to be associated with leukocyte telomere length through genome-wide association studies (GWASs) (Levy et al., 2010; Gu et al., 2011; Mangino et al., 2012; Codd et al., 2013; Pooley et al., 2013; Dorajoo et al., 2019). Relying on associated genetic variants, many studies have been undertaken to investigate the association between genetically predicted leukocyte telomere length and cancers. However, the types of cancers investigated in previous studies (Zhang et al., 2015; Li et al., 2020) were relatively limited. In addition, the telomere length-associated SNPs employed in previous studies (Zhang et al., 2015; Rode et al., 2016; Haycock et al., 2017) often came from GWASs with small sample sizes (Levy et al., 2010; Codd et al., 2013).

Recently, a large-scale GWAS of leukocyte telomere length was conducted with the largest sample size to date (up to ∼80,000) (Li et al., 2020), which allows us to choose more appropriate SNPs to study the multilocus genetic profile of leukocyte telomere length *via* the genetic risk score (GRS) approach (Ripatti et al., 2010; Dudbridge et al., 2013; Eusden et al., 2015; Guo et al., 2016; Goldman, 2017; Tosto et al., 2017; Bogdan et al., 2018; De La Vega and Bustamante, 2018; Zeng et al., 2019b). Briefly, GRS is an efficient and powerful genetic method to explore the association between an exposure and complex diseases by integrating multiple genetic variants with weak effects, and it dramatically enhances the predictability of complex diseases through genetic polymorphisms (Belsky et al., 2013; Khera et al., 2018; Duncan et al., 2019; Khera et al., 2019). Moreover, several cancer-relevant cohorts, such as The UK Biobank (Bycroft et al., 2018) and The Cancer Genome Atlas (TCGA) (Hoadley et al., 2018), have collected a variety of cancer-related omics and clinical information, which makes it feasible to systematically investigate a large number of types of cancers.

Based on these valuable data resources, in the present work, we evaluated the association between leukocyte telomere length and 37 cancers from the UK Biobank cohort as well as 33 cancers from the TCGA dataset using the genetic risk score method. We further applied the two-sample Mendelian randomization (MR) (Burgess et al., 2017; Hartwig et al., 2017) to assess the association between leukocyte telomere length and multiple cancers, for which the summary statistics can be available from the UK Biobank cohort. Our study revealed that telomere played cancer-specific roles and that a shorter leukocyte telomere length can either increase or decrease the risk/mortality of cancers. However, further validations in large-scale prospective studies and deeper investigations of the biological mechanism of leukocyte telomere length on various types of cancers are warranted.

MATERIALS AND METHODS

Selection of Instrumental Variables for Leukocyte Telomere Length

We obtained the summary statistics (e.g., effect size and effect allele) of leukocyte telomere length from the ENGAGE consortium as well as the EPIC-CVD and EPIC-InterAct cohorts

(**Supplementary Table S2**; Li et al., 2020), which was the largest GWAS of telomere length (N = 78,592) undertaken in the European population to date. In this study, leukocyte telomere length was measured as a continuous variable and the linear additive regression was implemented to investigate the association for each genetic variant (Li et al., 2020). Particularly, in the association analysis, the age of participants was considered as a covariate to remove the influence of biological age. We selected 17 independent index SNPs that were strongly associated with leukocyte telomere length (p < 5.00E-8; see **Table 1**) to construct GRS. Note that, given the fact that the length of telomere would shorten progressively with age, to facilitate the explanation of our results, we made a sign transformation for the effect sizes of these used SNPs so that the relationship under investigation corresponded to a *shorter* leukocyte telomere length.

Construction of Genetic Risk Score

The genetic risk score for leukocyte telomere length is calculated in a weighted way (Ripatti et al., 2010; Guo et al., 2016; Zeng et al., 2019b).

$$\mathrm{GRS} = \sum_{j=1}^{17} G_j \hat{\beta}_j \quad (1)$$

where $\hat{\beta}_j$ is the estimated marginal SNP effect on the shorter leukocyte telomere length for the jth selected index SNP (e.g., **Table 1**) (Li et al., 2020). G_j is the individual-level genotype of the same SNP in the UK Biobank (Bycroft et al., 2018) or TCGA dataset (Hoadley et al., 2018) and is coded to be 0, 1, and 2, representing the number of effect allele. Following prior work (Zeng et al., 2019b), we do not directly rescale the GRS as its p-value would not be altered regardless of whether the GRS is scaled or not. We instead standardize the GRS so that its mean is zero and the variance is equal to 1.

Two-Stage Regression Model in the UK Biobank and TCGA Using GRS

To link GRS with the risk of cancers from the UK Biobank (**Table 2**; Bycroft et al., 2018), we apply an additive logistic regression while adjusting for a set of available covariates (i.e., age, gender, smoke, drink, and BMI).

$$\mathrm{logit}(\mu_i) = \mathrm{GRS}_i \times \theta + X_i^T \alpha \quad (2)$$

where μ_i is the expectation of y_i, with y_i = 1 or 0 representing the status of individual i with or without cancer; θ is the effect size of GRS; and $\boldsymbol{X}_i$ is the vector of standardized covariates with effect sizes α. Of note, we assume that all of the entries in the first column of $\boldsymbol{X}$ are 1, representing the intercept term.

We next evaluate the effect of GRS on the mortality of cancers from TCGA (**Table 3**; Hoadley et al., 2018) with the Cox proportional hazards model (Cox, 1972) while controlling for available clinical covariates (i.e., age at diagnosis, gender, and stage).

$$h(t_i|\mathrm{GRS}_i,\ X_i) = h_0(t_i) e^{\mathrm{GRS}_i \times \theta + X_i^T \alpha} \quad (3)$$

where t_i is the observed survival time and $h_0(t)$ is an arbitrary baseline hazard function. Cancer-specific covariates are considered for some cancers in TCGA [e.g., the status of estrogen and progesterone receptors for breast invasive carcinoma (BRCA)]. In the logistic or Cox model, we are mainly

TABLE 1 | Independent index single nucleotide polymorphisms (SNPs) associated with leukocyte telomere length in the European population.

SNP	Chr	Position	Gene	A1/A2	EAF	Beta	SE	*p*	PVE	*F*
rs3219104	1	226,562,621	*PARP1*	C/A	0.83	−0.042	0.006	9.60E-11	6.23E-04	49.0
rs55749605	3	101,232,093	*SENP7*	A/C	0.58	0.037	0.007	2.45E-08	3.55E-04	27.9
rs10936600	3	169,514,585	*TERC*	T/A	0.24	0.086	0.006	7.18E-51	2.61E-03	205.4
rs13137667	4	71,774,347	*MOB1B*	C/T	0.96	−0.077	0.014	2.43E-08	3.85E-04	30.2
rs4691895	4	164,048,199	*NAF1*	C/G	0.78	−0.058	0.006	1.58E-21	1.19E-03	93.4
rs7705526	5	1,285,974	*TERT*	A/C	0.33	−0.082	0.006	5.34E-45	2.37E-03	186.8
rs34991172	6	25,480,328	*CARMIL1*	G/T	0.07	0.061	0.011	6.19E-09	3.91E-04	30.8
rs2736176	6	31,587,561	*PRRC2A*	C/G	0.31	−0.035	0.006	3.53E-10	4.33E-04	34.0
rs59294613	7	124,554,267	*POT1*	A/C	0.29	0.041	0.006	1.17E-13	5.94E-04	46.7
rs9419958	10	105,675,946	*OBFC1*	C/T	0.86	0.064	0.007	5.05E-19	1.06E-03	83.6
rs228595	11	108,105,593	*ATM*	A/G	0.42	0.029	0.005	1.43E-08	4.28E-04	33.6
rs2302588	14	73,404,752	*DCAF4*	C/G	0.10	−0.048	0.008	1.68E-08	4.58E-04	36.0
rs3785074	16	69,406,986	*TERF2*	G/A	0.26	−0.035	0.006	4.64E-10	4.33E-04	34.0
rs62053580	16	74,680,074	*RFWD3*	G/A	0.17	0.039	0.007	4.08E-08	3.95E-04	31.0
rs7194734	16	82,199,980	*MPHOSPH6*	T/C	0.78	0.037	0.006	6.94E-10	4.84E-04	38.0
rs8105767	19	22,215,441	*ZNF208*	G/A	0.30	−0.039	0.005	5.42E-13	7.74E-04	60.8
rs75691080	20	62,269,750	*STMN3*	T/C	0.09	0.067	0.009	5.99E-14	7.05E-04	55.4

Chr, chromosome; A1, effect allele; A2, alternative allele; EAF, frequency of the effect allele; PVE, proportion of variance explained by the SNP [i.e., $\mathrm{PVE}_j = (\hat{\beta}_j^X)^2/((\hat{\beta}_j^X)^2 + \mathrm{var}(\hat{\beta}_j^X) \times N_j)$*, where* $\hat{\beta}_j^X$ *and* $\mathrm{var}(\hat{\beta}_j^X)$ *are the estimated effect size and variance, respectively, for instrument j (Shim et al., 2015)]; F, F statistic [i.e.,* $F_j = \mathrm{PVE}_j(N_j - 1 - k)/(k - k \times \mathrm{PVE}_j)$*, where Nj is the sample size for instrument j (i.e., Nj = 78,592) and k is the number of instruments (Burgess et al., 2011; Burgess and Thompson, 2012). Both PVE and F statistic are calculated to validate the issue of weak instruments].*

TABLE 2 | Association between the genetic risk score (GRS) of leukocyte telomere length and the risk of 37 UK Biobank cancers.

Types of cancer	OR (95%CI)	*p*	FDR	Case	M/F	Age (years)
Leukemia	1.20 (1.02–1.41)	**0.025**	0.058	147	79/68	67.99 ± 8.17
Rectal cancer	1.10 (0.96–1.25)	0.165	0.193	231	134/97	70.64 ± 6.17
Tongue cancer	1.06 (0.88–1.29)	0.526	0.407	102	65/37	68.89 ± 7.35
Squamous cell carcinoma	1.04 (0.93–1.15)	0.514	0.401	332	168/164	70.89 ± 6.21
Testicular cancer	1.02 (0.95–1.11)	0.549	0.417	595	595/0	64.78 ± 8.02
Primary bone cancer	1.02 (0.81–1.29)	0.845	0.524	72	44/28	67.56 ± 8.02
Non-melanoma skin cancer	1.02 (0.93–1.12)	0.648	0.458	472	280/192	70.01 ± 6.97
Large bowel cancer/Colorectal cancer	1.02 (0.93–1.12)	0.739	0.490	440	260/180	71.54 ± 5.80
Rodent ulcer	1.01 (0.92–1.11)	0.893	0.538	437	203/234	70.50 ± 5.77
Esophageal cancer	1.01 (0.85–1.19)	0.946	0.552	137	110/27	71.98 ± 6.30
Cervical cancer	1.01 (0.95–1.06)	0.857	0.527	1273	0/1,273	66.16 ± 7.65
Non-Hodgkin's lymphoma	0.99 (0.92–1.08)	0.945	0.552	593	355/238	69.40 ± 7.34
Pre-cancer cells cervix	0.99 (0.94–1.06)	0.922	0.546	1117	1/1,116	63.99 ± 7.98
Breast cancer	0.98 (0.96–1.01)	0.164	0.193	7330	37/7,293	70.08 ± 6.48
Colon cancer/sigmoid cancer	0.97 (0.92–1.04)	0.399	0.342	1055	631/424	72.30 ± 5.68
Uterine/endometrial cancer	0.95 (0.89–1.02)	0.176	0.200	752	0/752	71.27 ± 5.89
Ovarian cancer	0.95 (0.87–1.03)	0.222	0.224	512	0/512	69.11 ± 7.33
Brain cancer/primary malignant brain tumor	0.95 (0.80–1.13)	0.539	0.412	128	62/66	64.77 ± 8.81
Prostate cancer	0.94 (0.91–0.98)	**0.005**	**0.020**	2410	2,410/0	73.96 ± 4.08
Skin cancer	0.94 (0.88–1.00)	0.065	0.106	943	478/465	71.21 ± 6.38
Basal cell carcinoma	0.94 (0.90–0.97)	**0.001**	**0.010**	2916	1,206/1,710	70.02 ± 6.84
Stomach cancer	0.94 (0.77–1.14)	0.516	0.402	96	56/40	71.33 ± 6.22
Malignant melanoma	0.91 (0.88–0.95)	**4.57E-06**	**9.56E-05**	2526	1,031/1,495	68.95 ± 7.41
Larynx/throat cancer	0.91 (0.80–1.04)	0.161	0.191	228	190/38	71.12 ± 6.43
Bladder cancer	0.91 (0.84–0.98)	**0.010**	**0.030**	725	548/177	72.30 ± 5.87
Eye and/or adnexal cancer	0.90 (0.74–1.11)	0.325	0.297	95	44/51	68.82 ± 7.48
Thyroid cancer	0.90 (0.80–1.01)	0.067	0.108	293	52/241	67.27 ± 7.63
Small intestine/small bowel cancer	0.90 (0.76–1.06)	0.206	0.216	133	77/56	72.28 ± 5.55
Hodgkin's lymphoma/Hodgkin's disease	0.89 (0.79–0.99)	**0.033**	0.069	321	184/137	64.96 ± 8.13
Chronic myeloid leukemia	0.88 (0.71–1.10)	0.273	0.262	81	44/37	68.35 ± 7.98
Lung cancer	0.88 (0.74–1.06)	0.172	0.197	123	82/41	72.60 ± 5.70
Kidney/renal cell cancer	0.86 (0.78–0.95)	**0.003**	**0.017**	401	261/140	70.22 ± 6.36
Cancer of lip/mouth/pharynx/oral/cavity	0.86 (0.68–1.09)	0.213	0.220	69	43/26	70.42 ± 5.98
Sarcoma/fibrosarcoma	0.84 (0.72–0.98)	**0.028**	0.063	164	76/88	66.73 ± 7.58
Chronic lymphocytic leukemia	0.82 (0.71–0.94)	**0.005**	**0.020**	206	131/75	71.28 ± 6.28
Lymphoma	0.80 (0.64–1.01)	0.057	0.098	78	51/27	68.69 ± 8.27
Multiple myeloma	0.77 (0.63–0.93)	**0.006**	**0.021**	108	62/46	70.37 ± 7.08

The cancers were sorted by the estimated odds ratios (ORs). CI, confidence internal; p, the original p-value; FDR, false discovery rate; M, male; F, female. In bold are significant (i.e., FDR < 0.05) or suggestive associations (i.e., p < 0.05).

interested in estimating θ and testing for the null hypothesis H_0: θ = 0. We further examine the interaction effect between GRS and each of the clinical covariates (e.g., GRS × gender) if GRS is detected to be associated with some cancer.

Two-Sample MR Analysis

Besides the GRS method, we also perform the two-sample MR analysis to estimate the causal effect of leukocyte telomere length on cancers in the UK Biobank using summary statistics (Sudlow et al., 2015). In observational studies, MR is a flexible approach for causal inference to avert confounding and reverse causality (Zeng et al., 2019a; Yu et al., 2020). In brief, we estimate the causal effect of leukocyte telomere length (again, denoted as θ) relying on all the available instrumental variables (**Table 1**) through the commonly employed inverse-variance weighted (IVW) method (Burgess et al., 2017; Hartwig et al., 2017).

$$\begin{aligned} \hat{\theta} &= \frac{1}{\sum_{j=1}^{17} \mathrm{var}(\hat{\beta}_j^{\mathrm{Y}})^{-1}(\hat{\beta}_j^{\mathrm{X}})^2} \sum_{j=1}^{17} \mathrm{var}(\hat{\beta}_j^{\mathrm{Y}})^{-1} \hat{\beta}_j^{\mathrm{Y}} \hat{\beta}_j^{\mathrm{X}} \\ \mathrm{var}(\hat{\theta}) &= \frac{1}{\sum_{j=1}^{17} \mathrm{var}(\hat{\beta}_j^{\mathrm{Y}})^{-1}(\hat{\beta}_j^{\mathrm{X}})^2} \end{aligned} \quad (4)$$

where $\hat{\beta}_j^{\mathrm{X}}$ and $\mathrm{var}(\hat{\beta}_j^{\mathrm{X}})$ are the effect size and the variance, respectively, of the instrumental variable *j* for the exposure X (i.e., leukocyte telomere length; Li et al., 2020), and $\hat{\beta}_j^{\mathrm{Y}}$ and $\mathrm{var}(\hat{\beta}_j^{\mathrm{Y}})$ are the effect size and the variance, respectively, for the same instrumental variable *j* on the outcome Y (i.e., cancer in the UK Biobank; Sudlow et al., 2015).

TABLE 3 | Association between the genetic risk score (GRS) of leukocyte telomere length and the mortality of 33 TCGA cancers.

Cancer	HR (95%CI)	*p*	FDR	*N*	Median survival time			M/F	Age at diagnosis (years)	Stage or grade (1/2/3/4/5)
					All	Event	Censor			
DLBC	2.24 (0.88–5.67)	0.090	0.317	42	31.85	19.83	32.4	19/23	55.33 ± 14.39	8/17/5/12
PCPG	2.16 (0.95–4.92)	0.068	0.283	178	25.28	15.08	25.6	78/100	47.30 ± 15.12	NA
READ	1.72 (1.09–2.73)	**0.020**	0.138	157	21.2	24.33	21.02	85/72	64.34 ± 11.67	30/51/51/25
UVM	1.47 (0.94–2.30)	0.092	0.320	79	25.77	19.68	27.37	44/35	61.68 ± 13.94	0/39/36/4
PRAD	1.44 (0.72–2.87)	0.306	0.610	501	30.8	29.17	30.87	501/0	60.93 ± 6.81	NA
SARC	1.29 (1.06–1.58)	**0.011**	0.138	260	31.77	21.6	36.4	119/141	60.80 ± 14.61	NA
ESCA	1.28 (0.99–1.66)	0.063	0.274	162	13.57	13.38	13.57	137/25	62.40 ± 11.74	18/79/56/9
TGCT	1.23 (0.10–15.59)	0.870	0.816	81	37.53	116.48	37.53	81/0	32.85 ± 10.18	55/12/14/0
SKCM	1.19 (1.03–1.37)	**0.018**	0.138	411	33.2	31.93	34.5	256/155	58.82 ± 15.51	77/140/171/23
KICH	1.17 (0.46–2.99)	0.743	0.792	65	74.93	28.5	90.43	38/27	51.15 ± 13.99	20/25/14/6
CESC	1.14 (0.90–1.45)	0.274	0.583	295	21.27	20.23	23.12	0/295	47.88 ± 13.47	160/69/46/20
THCA	1.12 (0.66–1.89)	0.676	0.776	503	31.67	34.03	31.47	136/367	47.28 ± 15.78	284/52/113/54
BRCA	1.11 (0.93–1.32)	0.258	0.569	924	26.38	44.13	24.23	0/924	58.84 ± 13.14	156/523/219/14/12
LUSC	1.06 (0.93–1.21)	0.378	0.659	487	21.77	18.13	24.58	359/128	67.31 ± 8.58	239/157/84/7
MESO	1.05 (0.82–1.35)	0.705	0.783	86	17.1	15.23	38.93	70/16	63.08 ± 9.72	10/16/44/16
LUAD	1.04 (0.90–1.21)	0.584	0.749	503	21.87	20.47	22.33	232/271	65.16 ± 10.07	277/121/80/25
UCEC	1.04 (0.84–1.28)	0.742	0.791	546	30.47	23.63	32.2	0/546	63.99 ± 11.13	338/52/127/29
KIRC	1.04 (0.90–1.20)	0.639	0.766	532	39.2	27.35	48.1	342/190	60.57 ± 12.07	267/57/125/83
HNSC	1.03 (0.90–1.19)	0.655	0.770	450	21.37	14.08	27.4	324/126	60.90 ± 12.13	27/73/82/268
LIHC	1.00 (0.83–1.21)	0.963	0.831	350	19.43	13.67	21.47	239/111	59.03 ± 13.30	174/86/85/5
GBM	1.00 (0.91–1.10)	0.949	0.829	595	12.27	12.7	8.67	364/231	57.87 ± 14.41	NA
ACC	0.99 (0.68–1.44)	0.967	0.832	88	37.93	18.38	48.45	29/59	47.07 ± 16.43	9/43/18/18
OV	0.98 (0.88–1.08)	0.629	0.763	569	33.57	35.77	28.57	0/569	59.71 ± 11.46	16/30/437/86
PAAD	0.97 (0.80–1.19)	0.792	0.802	182	15.55	13.13	16.92	100/82	64.92 ± 11.06	21/152/4/5
STAD	0.96 (0.83–1.11)	0.576	0.746	407	14.53	11.6	18.87	260/147	65.37 ± 10.70	55/128/181/43
BLCA	0.96 (0.82–1.12)	0.576	0.746	411	17.87	13.68	21.27	303/108	68.10 ± 10.58	3/131/141/136
COAD	0.93 (0.76–1.13)	0.448	0.696	458	22.32	13.47	24.33	239/219	67.03 ± 13.06	79/183/131/65
LGG	0.89 (0.73–1.09)	0.270	0.580	512	22.47	27.13	20.97	284/228	42.99 ± 13.34	0/247/265/0
LAML	0.89 (0.73–1.09)	0.252	0.563	186	12.17	9.1	23.3	102/84	55.53 ± 16.06	NA
UCS	0.85 (0.59–1.23)	0.395	0.669	56	20.25	16.72	27.6	0/56	69.38 ± 8.89	21/5/20/10
THYM	0.85 (0.41–1.76)	0.654	0.770	121	41.77	28.43	42.33	62/59	58.37 ± 12.94	37/61/15/8
KIRP	0.66 (0.47–0.93)	**0.019**	0.138	257	24.67	20.8	25.37	190/67	61.50 ± 12.03	171/20/51/15
CHOL	0.64 (0.38–1.08)	0.097	0.331	36	21.5	16.67	31.42	16/20	63.03 ± 12.67	19/9/1/7

The cancers were sorted by the estimated hazard ratios (HRs). CI, confidence internal; p, the original p-value; FDR, false discovery rate; M, male; F, female. Cancer types: DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; PCPG, pheochromocytoma and paraganglioma; READ, rectum adenocarcinoma; UVM, uveal melanoma; PRAD, prostate adenocarcinoma; SARC, sarcoma; ESCA, esophageal carcinoma; TGCT, testicular germ cell tumor; SKCM, skin cutaneous melanoma; KICH, kidney chromophobe; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; THCA, thyroid carcinoma; BRCA, breast invasive carcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; LUAD, lung adenocarcinoma; UCEC, uterine corpus endometrial carcinoma; KIRC, kidney renal clear cell carcinoma; HNSC, head and neck squamous cell carcinoma; LIHC, liver hepatocellular carcinoma; GBM, glioblastoma multiforme; ACC, adrenocortical carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; STAD, stomach adenocarcinoma; BLCA, bladder urothelial carcinoma; COAD, colon adenocarcinoma; LGG, brain lower grade glioma; LAML, acute myeloid leukemia; UCS, uterine carcinosarcoma; THYM, thymoma; KIRP, kidney renal papillary cell carcinoma; CHOL, cholangiocarcinoma. In bold are suggestive associations (i.e., $p < 0.05$).

To guarantee the validity of our MR analysis, before the formal analysis, we examine the pleiotropic effects of instruments by removing index SNPs that may be potentially related to individual cancers if the Bonferroni-adjusted *p*-values are less than 0.05. We also conduct a series of sensitivity analyses: (i) weighted median-based (Bowden et al., 2016b) and maximum likelihood methods (Burgess et al., 2013), which are robust when some instrumental variables might be invalid; (ii) MR-Egger regression (Bowden et al., 2016a; Burgess and Thompson, 2017), which guards against horizontal pleiotropic effects; and (iii) leave-one-out (LOO) analysis (Noyce et al., 2017) and Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) test (Verbanck et al., 2018) to examine potential instrumental outliers.

UK Biobank and TCGA Cancer Datasets

The UK Biobank dataset consists of approximately 500,000 individuals (Bycroft et al., 2018). We selected age, gender, smoke, drink, and BMI as covariates and originally chose 79 self-reported cancers up to 337,198 independent individuals (28,820 cases and 308,378 controls) of European ancestry, but only included

cancers with at least 60 cases (to some extent, this cutoff value was used arbitrarily) and treated cancer-free individuals to be controls. Finally, a total of 37 cancers were left up to 335,036 individuals (27,641 cases for various cancers and 307,395 shared cancer-free controls after removing individuals with missing values). The genotypes were provided by the UK Biobank after the research application was approved. However, we can only obtain 15 SNPs because two were missing (i.e., rs3219104 on *PARP1* and rs55749605 on *SENP7*) in the UK Biobank. In addition, because summary-level statistics are necessary for the two-sample MR analysis, herein we can only consider 28 cancers from the UK Biobank (n = 420,473) (Sudlow et al., 2015; **Supplementary Table S6**). The summary statistics of these cancers were obtained from https://pan.ukbb.broadinstitute.org/.

Then, we obtained the survival and clinical information of 33 cancers from TCGA (Hoadley et al., 2018). We selected the overall survival time and status as the outcome and primarily included age at diagnosis, gender, and pathologic tumor stage as covariates because many other important clinical covariates were missing for most of the patients. When the pathologic tumor stage cannot be available, we instead employed the clinical stage (i.e., for CESC, DLBC, OV, THYM, UCEC, and UCS) or histological grade (i.e., for LGG). It needs to be stated that all three stage variables were missing in five cancers (i.e., GBM, LAML, PCPG, PRAD, and SARC). For each cancer, we only kept samples from the primary cancer tissue and excluded those with missing values in clinical covariates. More details about these TCGA cancers are demonstrated in **Table 3** and **Supplementary Table S3**. For each cancer, we filtered out SNPs that had a missingness rate >0.95 across individuals, genotype calling rate <0.95, minor allele frequency (MAF) > 0.01, or Hardy–Weinberg equilibrium (HWE) p-value < 10^{-4}. We next performed an imputation procedure by first phasing the genotypes with SHAPEIT (Delaneau et al., 2013), then imputed the SNPs based on the Haplotype Reference Consortium panel (McCarthy et al., 2016) on the Michigan Imputation Server using minimac3 (Das et al., 2016). The filtering procedure for the imputed genotypes included an HWE p-value < 10^{-4}, a genotype call rate <95%, a MAF < 0.01, and an imputation score <0.30. After the imputation of genotypes, all of the 17 SNPs were yielded in TCGA.

Power Evaluation

Finally, we performed power calculation to detect a non-zero causal effect for GRS with regards to cancers based on the UK Biobank and TCGA datasets. Firstly, we simulated genotypes for 17 independent SNPs with varying MAFs (**Table 1**) and then calculated the GRS. Two independent covariates (i.e., one was binary and the other was continuous) were also included, with each having an effect size of 0.5. We generated a case–control variable y with the probability of $\exp(\eta)/(1+\exp(\eta))$ and $\eta = \text{GRS} \times \theta + 0.5X_1 + 0.5X_2$. We created 2,000,000 individuals to be the population and then randomly sampled 50 (or 100 and 150) cases and 300,000 controls (as well as their GRS and covariates) to be a subset for the final simulation analysis.

Secondly, to simulate survival datasets, we first generated genotypes and calculated the GRS in the same way as described above. Again, two independent covariates were included, with each having an effect size of 0.5. Then, we employed the inverse probability method (Bender et al., 2005) to create survival time which followed a Weibull distribution, with the shape parameter being 1 and the scale parameter being 0.01. The location parameter of this Weibull distribution was determined by the GRS and the two covariates [i.e., $\mu = \exp(\eta)$, with $\eta = \text{GRS} \times \theta + 0.5X_1 + 0.5X_2$]. The censored rate was fixed to be 50% in a random manner (the high censored rate corresponded to a similar situation observed in the TCGA cancer dataset). The sample size varied from 100, 300, to 500.

In both simulations, the effect size of GRS θ was set to 0.05, 0.10, or 0.20, approximately corresponding to odds ratios (ORs) [or hazard ratio (HR)] of 1.05, 1.10, and 1.20. The simulation was repeated 1,000 times, and the power calculated by the proportion of the p-value of GRS was less than 1.67E-3, approximately equal to the significance level after the Bonferroni correction of 30 types of cancers.

Throughout our study, we utilized the R software (version 3.6.1) to implement all the analyses. The association was declared to be statistically significant if the false discovery rate (FDR) is <0.05 (Benjamini and Hochberg, 1995), while the association was deemed to be suggestive if the unadjusted p-value is <0.05.

RESULTS

Association Between GRS and UK Biobank Cancers

The 17 selected index SNPs collectively explain about 1.37% phenotypic variance of leukocyte telomere length, and all the F statistics are above 10 (ranging from 27.9 to 205.4, with an average of 63.3) (**Table 1**), largely ruling out the possibility of weak instrument bias (Cragg and Donald, 1993; Burgess et al., 2017; Zeng and Zhou, 2019a). Based on the constructed GRS, we first investigate the association between leukocyte telomere length and the risk of UK Biobank cancers (**Table 2**). We detect that the GRS of leukocyte telomere length is significantly associated with a decreased risk of seven types of cancers (**Table 2**), including multiple myeloma [OR = 0.77, 95% confidence interval (CI) = 0.63–0.93, FDR = 0.021], chronic lymphocytic leukemia (OR = 0.82, 95%CI = 0.71–0.94, FDR = 0.020), kidney/renal cell cancer (OR = 0.86, 95%CI = 0.78–0.95, FDR = 0.017), bladder cancer (OR = 0.91, 95%CI = 0.84–0.98, FDR = 0.030), malignant melanoma (OR = 0.91, 95%CI = 0.88–0.95, FDR = 9.56E-05), basal cell carcinoma (OR = 0.94, 95%CI = 0.90–0.97, FDR = 0.010), and prostate cancer (OR = 0.94, 95%CI = 0.91–0.98, FDR = 0.020). Suggestive associations are observed for two types of cancers including sarcoma/fibrosarcoma (OR = 0.84, 95%CI = 0.72–0.98, FDR = 0.063) and Hodgkin's lymphoma/Hodgkin's disease (OR = 0.89, 95%CI = 0.79–0.99, FDR = 0.069). In addition, we discover that the GRS of leukocyte telomere length is also marginally related to an increased risk of leukemia (OR = 1.20, 95%CI = 1.02–1.41, FDR = 0.058).

We further examine the interaction effect of GRS and one of the covariates (e.g., age, gender, smoke, drink, or BMI)

for each of the 10 cancers. We observe that the interaction term is statistically significant between smoke and GRS for sarcoma/fibrosarcoma (OR = 0.83, 95%CI = 0.71–0.97) as well as between drink and GRS for leukemia (OR = 0.82, 95%CI = 0.69–0.97) (**Supplementary Table S4**).

Association Between GRS and TCGA Cancers

We now examine the effect size of GRS on 33 TCGA cancers through the Cox proportional hazards model. We observe suggestive evidence that the GRS of leukocyte telomere length is related to a higher death hazard of READ (HR = 1.72, 95%CI = 1.09–2.73, $p = 0.020$), SARC (HR = 1.29, 95%CI = 1.06–1.58, $p = 0.011$), and SKCM (HR = 1.19, 95%CI = 1.03–1.37, $p = 0.018$) and is associated with a lower death hazard of KIRP (HR = 0.66, 95%CI = 0.47–0.93, $p = 0.019$), suggesting that a genetically decreased leukocyte telomere length can lead to a worse overall survival of READ, SARC, and SKCM while can result in a better overall survival of KIRP. However, all these associations become non-significant after accounting for multiple comparisons (FDR > 0.05). Neither suggestive nor significant associations are identified between GRS and the remaining cancers (**Table 3**). We further examine the interaction effect of GRS and each of the covariates (e.g., age at diagnosis, gender, or stage) for each of the four cancers. We do not identify any statistically significant interactions (**Supplementary Table S5**).

Association Between Leukocyte Telomere Length and UK Biobank Cancers Using the Two-Sample MR

With the selected 17 instrumental variables, we further perform MR analysis to investigate the causal effect of leukocyte telomere length on each of the 28 cancers from the UK Biobank. As no evidence of effect heterogeneity is presented across instruments (all the *p*-values for the Cochran's *Q* test are greater than 0.05), thus, only the results estimated *via* the fixed-effects IVW method are displayed below. Among the 28 cancers, we identify that leukocyte telomere length is associated with a decreased risk of nine cancers (**Supplementary Table S6**), including basal cell carcinoma, malignant melanoma, skin cancer, bladder cancer, kidney/renal cell cancer, Hodgkin's lymphoma/Hodgkin's disease, thyroid cancer, chronic lymphocytic leukemia, and multiple myeloma. We also observe that leukocyte telomere length is associated with an increased risk of leukemia (**Supplementary Table S6**).

We now validate the observed causal associations shown above through various sensitivity analyses (**Supplementary Table S6**). Here, we focus on the associations that are significant in all sensitivity analyses (i.e., $P_{\text{Weighted median}}$ and $P_{\text{Likelihood}} < 0.05$) and have no horizontal pleiotropic effects (i.e., $P_{\text{Egger-intercept}} > 0.05$). Then, four types of cancers are left, including malignant melanoma (OR = 0.58, 95%CI = 0.44–0.79, FDR = 0.004), Hodgkin's lymphoma/Hodgkin's disease (OR = 0.30, 95%CI = 0.13–0.69, FDR = 0.008), chronic lymphocytic leukemia (OR = 0.20, 95%CI = 0.08–0.54, FDR = 0.004), and multiple myeloma (OR = 0.18, 95%CI = 0.05–0.66, FDR = 0.018). Of note is that both the weighted median method and the maximum likelihood method generate consistent causal effect estimates compared with the IVW method (**Supplementary Table S6**). In addition, we create scatter plots for the SNP effect sizes of leukocyte telomere length and these four cancers (**Figure 1**); we find that no instruments may be potential outliers. The finding is also supported by MR-PRESSO, which displays the absence of instrument outliers at the significance level of 0.05.

To further examine whether a single instrumental variable may strongly influence the causal effects of leukocyte telomere length on these four cancers, we performed the LOO analysis. Again, the LOO analysis results demonstrate that none of the 17 instruments can substantially impact the estimated casual effect. Therefore, we can conclude that it is likely that a shorter leukocyte telomere length can decrease the risk of malignant melanoma, Hodgkin's lymphoma/Hodgkin's disease, chronic lymphocytic leukemia, and multiple myeloma. This finding here is also consistent with the results derived by the GRS regression above.

Power Calculation for the Association Between GRS and Cancers in the UK Biobank/TCGA Datasets

In terms of our simulations, we have sufficient power to detect the association in the UK Biobank as the total sample size is large, although only a few of the cancer cases are included. Specifically, we observe that the estimated power approaches 100% even when the number of cases is only 50 and the OR is only 1.05. In contrast, due to the relatively weak effect size and small sample size in the simulated TCGA cancer dataset, under our simulation settings, we have only low to moderate power to detect the association between GRS and the survival risk of cancer (**Figure 2**). For example, when the sample size is 300, the statistical power is only 3.0 or 10.7% when the HR was set to be 1.05 or 1.10. As can be expected, the power improves with the increase in the sample sizes and effect sizes.

DISCUSSION

Summary of the Results of the Present Study

The main objective of our study was to investigate whether there existed associations between genetically predicted leukocyte telomere length and various types of cancers. To achieve this, we first constructed the GRS of leukocyte telomere length based on associated SNPs from a large-scale GWAS and evaluated the effect of GRS on the risk and mortality of cancers. We found statistical evidence supporting the existence of associations between GRS and cancers in the UK Biobank and TCGA. Briefly, based on the GRS, a shorter leukocyte telomere length was identified to be associated with the decreased risk of some cancers (i.e., multiple myeloma, chronic lymphocytic leukemia, kidney/renal cell cancer, bladder cancer, malignant melanoma, basal cell carcinoma, prostate cancer, sarcoma/fibrosarcoma, and Hodgkin's lymphoma/Hodgkin's disease) as well as related to the decreased mortality of KIRP. In addition, inverse associations

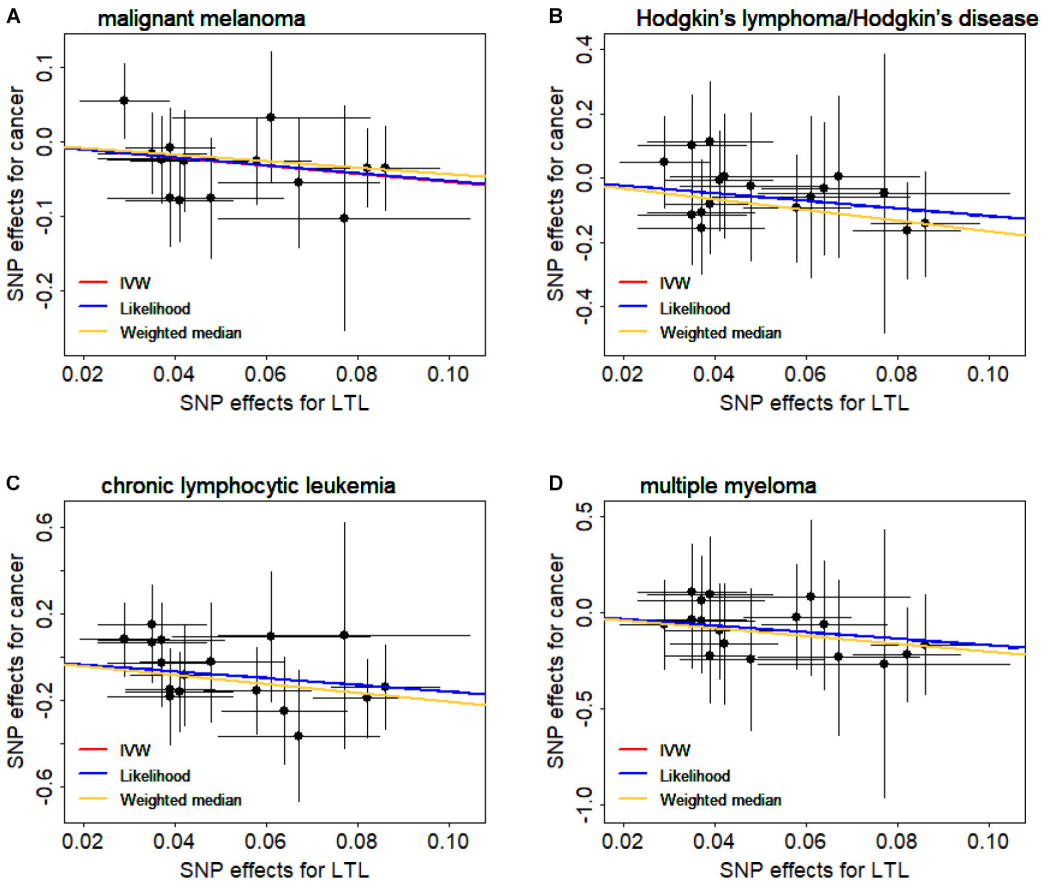

FIGURE 1 | Relationship between the single nucleotide polymorphism (SNP) effect sizes of leukocyte telomere length (LTL) (*x*-axis) and the corresponding effect sizes of cancer (*y*-axis). **(A)** Malignant melanoma. **(B)** Hodgkin's lymphoma/Hodgkin's disease. **(C)** Chronic lymphocytic leukemia. **(D)** Multiple myeloma. In the plot, horizontal/vertical lines represent the 95% confidence interval.

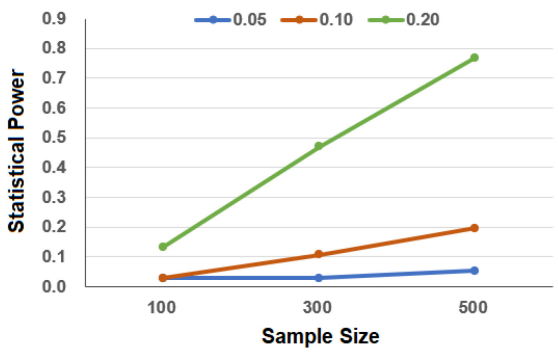

FIGURE 2 | Estimated power in the simulation to evaluate the association between genetic risk score (GRS) and cancers in The Cancer Genome Atlas (TCGA). In the simulation, the effect sizes of GRS were set to 0.05, 0.10, and 0.20 and the sample sizes of cancer were set to 100, 300, and 500.

were observed for shorter leukocyte telomere length on the risk of leukemia as well as on the mortality of READ, SARC, and SKCM. The results of the MR analysis also supported the existence of an association between leukocyte telomere length and various cancers, including malignant melanoma, Hodgkin's lymphoma/Hodgkin's disease, chronic lymphocytic leukemia, and multiple myeloma. The diverse associations between leukocyte telomere length and cancers may in part reflect the different carcinogenic mechanisms acted by telomere in specific cancer types, further suggesting that telomere length is a valuable indicator of cancer risk and prognosis.

Discoveries Combined With the Previous Study

We found that the observed associations between leukocyte telomere length and cancers in the present study (i.e., multiple myeloma, chronic lymphocytic leukemia, kidney/renal cell cancer, bladder cancer, malignant melanoma, basal cell carcinoma, and prostate cancer) are greatly consistent with prior findings obtained in terms of MR (**Supplementary Table S1**; Zhang et al., 2015; Ojha et al., 2016; Haycock et al., 2017; Machiela et al., 2017; Li et al., 2020; Went et al., 2020). Particularly, several previous studies demonstrated that a shorter telomere length was associated with a decreased lung cancer risk or mortality and that the association was present in adenocarcinoma while absent in squamous cell carcinoma (**Supplementary Table S1**;

Zhang et al., 2015; Haycock et al., 2017; Kachuri et al., 2018; Yuan et al., 2018), which may be attributed to the discrepancy in the biological characteristics of various subtypes of lung cancer. In the present study, inconsistent correlations were also identified within different subtypes of cancer. For example, we discovered that leukocyte telomere length had an opposite effect on the risk of leukemia and chronic lymphocytic leukemia. However, we observed that leukocyte telomere length displayed similar effects on the risk of malignant melanoma and basal cell carcinoma. These findings suggest that leukocyte telomere may influence the risk or mortality of cancer in a histologic way and also emphasize the unique roles of leukocyte telomere in the development of cancers.

Although the molecular mechanism remains unclear, some prior studies implied that both short and long telomere length played an important role in the etiology of cancers (Cui et al., 2012; Cheng et al., 2017; Nelson and Codd, 2020). Cells with longer telomere lengths have greater proliferative potential and more probability of accruing mutations (Hanahan and Weinberg, 2011); therefore, telomere shortening is generally considered to be a protective mechanism against tumorigenesis (Rode et al., 2016; Zhang et al., 2017; Kuo et al., 2019). However, it has been proposed that telomere shortening can generally give rise to end-to-end chromosome fusions and attenuates DNA damage response, thus increasing genomic instability and finally initiating carcinogenesis (Wu et al., 2003). These findings indicate that telomere plays a dual role in cancer development, and such role seems to depend on the types of cancers and the balance of the proliferation and senescence of cells in cancers.

Strengths and Limitations of Our Study

One advantage of our study is that more than 50 diverse types of cancers were investigated; it is thus feasible to undertake a systematic evaluation in the present analysis. In addition, methodologically, the GRS analysis can be viewed to be a two-stage regression model within the framework of instrumental variable-based causal inference (Baum et al., 2003; Hernán and Robins, 2006; Zeng et al., 2019a). Specifically, leukocyte telomere length is the exposure of interest and the associated SNPs are the carefully selected instrumental variables which are supposed to satisfy the necessary assumptions of instruments (Lawlor et al., 2008; Sheehan et al., 2008; Zeng et al., 2019a; Zeng and Zhou, 2019a,b). In the first stage, the effect size of each instrumental variable is estimated with an external large-scale GWAS dataset; in the second stage, the influence of leukocyte telomere length on various cancers is assessed based on the genetically determined leukocyte telomere length which is predicted with the chosen instrumental variables. Therefore, in terms of the principle of instrumental variable inference, the estimated effect of GRS can be interpreted as causal. In this sense, besides the MR method, we are actually investigating the causal association between leukocyte telomere length and cancers by constructing a GRS.

Finally, some shortcomings of this study should also be mentioned. Firstly, the majority of the individuals of the UK Biobank and TCGA were of European ancestry, so our results may not be applicable to other populations. Secondly, in our study, telomere length measured in blood leukocytes was employed and not in all cell types *in vivo*; however, leukocyte telomere length was demonstrated to be highly correlated with that in cells from other tissues (Friedrich et al., 2000; Wilson et al., 2008; Butt et al., 2010). Thirdly, as described before, the effect sizes of leukocyte telomere length on the mortality of TCGA cancers were only suggestive and the sample size of these cancers was not sufficiently large to maintain high power to detect weak associations. Therefore, further investigations with a larger sample size are required to validate our results.

CONCLUSION

Our study reveals that telomere played diverse roles in different types of cancers; however, further validations in large-scale prospective studies and deeper investigations of the biologic mechanisms are warranted.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

PZ conceived the idea for the study. PZ, YW, XZ, SH, and HZ obtained the data. PZ and YG cleared up the datasets, performed the data analyses, and drafted the manuscript. PZ, YG, and YW interpreted the results of the data analyses. All authors approved the manuscript and provided relevant suggestions.

FUNDING

The research of PZ was supported in part by the Youth Foundation of Humanity and Social Science funded by Ministry of Education of China (18YJC910002), the Natural Science Foundation of Jiangsu Province of China (BK20181472), the China Postdoctoral Science Foundation (2018M630607 and 2019T120465), the QingLan Research Project of Jiangsu Province for Outstanding Young Teachers, the Six-Talent Peaks Project in Jiangsu Province of China (WSN-087), the Training Project for Youth Teams of Science and Technology Innovation at Xuzhou Medical University (TD202008), the Postdoctoral Science Foundation of Xuzhou Medical University, the National Natural Science Foundation of China (81402765), and the Statistical Science Research Project from National Bureau of Statistics of China (2014LY112). The research of SH was supported in part by the Social Development Project of Xuzhou City (KC19017). The research of YW was supported in part by the National Natural Science Foundation of China (81402764 and 81973142).

ACKNOWLEDGMENTS

We thank the ENGAGE Telomere Consortium as well as the EPIC-CVD and EPIC-InterAct cohorts for making the leukocyte telomere length summary data publicly available and are grateful to all the investigators and participants who contributed to this study. This study has been conducted using the UK Biobank resource under application number 30686. The UK Biobank was established by the Wellcome Trust Medical Charity, Medical Research Council, Department of Health, Scottish Government, and the Northwest Regional Development Agency. It has also had funding from the Welsh Assembly Government, British Heart Foundation, and Diabetes United Kingdom. The UK Biobank GWAS data can be accessed from the UK Biobank repository (https://biota.osc.ox.ac.uk/). The genetic and phenotypic UK Biobank data are available through application to the UK Biobank (https://www.ukbiobank.ac.uk). The UK Biobank summary statistics can be accessed from https://pan.ukbb.broadinstitute.org/. The TCGA data are publicly available from https://portal.gdc.cancer.gov/legacy-archive/. We also thank the editor, the associate editor, and two reviewers for their constructive comments, which substantially improved our manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.583106/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 19 November 2020
doi: 10.3389/fgene.2020.587876

Bidirectional Causal Associations Between Inflammatory Bowel Disease and Ankylosing Spondylitis: A Two-Sample Mendelian Randomization Analysis

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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *27 July 2020*
Accepted: *19 October 2020*
Published: *19 November 2020*

Citation:
Cui Z, Hou G, Meng X, Feng H, He B and Tian Y (2020) Bidirectional Causal Associations Between Inflammatory Bowel Disease and Ankylosing Spondylitis: A Two-Sample Mendelian Randomization Analysis. Front. Genet. 11:587876. doi: 10.3389/fgene.2020.587876

Background: Associations between inflammatory bowel disease (IBD) [including ulcerative colitis (UC) and Crohn's disease (CD)] and ankylosing spondylitis (AS) were discovered in observational studies, but no evidence supported the causal relationship between the two diseases.

Methods: We employed two-sample Mendelian randomization (MR) to estimate the unconfounded bidirectional causal associations between IBD (including UC and CD) and AS. We selected single-nucleotide polymorphisms (SNPs) from genome-wide association studies (GWAS) after strictly assessing the quality of the studies in the IEU GWAS database. Sensitivity analyses were also conducted to verify whether heterogeneity and pleiotropy can bias the MR results.

Results: We found positive causal effects of genetically increased UC, CD, and IBD risk on AS (e.g., UC and AS, IVW OR: 1.0256, 95% CI: 1.0130∼1.0385, p = 6.43E-05). However, we did not find significant causal associations of AS with UC, CD, or IBD (e.g., AS and UC, IVW OR: 1.1858, 95% CI: 0.8639∼1.6278, p = 0.2916). The sensitivity analysis also confirmed that horizontal pleiotropy was unlikely to bias the causality (e.g., UC and AS, MR-Egger: intercept p = 0.1326). The leave-one-out analysis also demonstrated that the observed links were not driven by SNP. No evidence of heterogeneity was found between the genetic variants (e.g., UC and AS, MR-Egger: Q statistic = 43.1297, I^2<0.0001, p = 0.7434).

Conclusion: Our results provide new evidence indicating there are positive causal effects of IBD on AS in the European population. We suggest that the features of inflammatory bowel disease in particular should be assessed in the diagnosis of ankylosing spondylitis. We also provide some advice for preventing and treating the two diseases.

Keywords: ankylosing spondylitis, inflammatory bowel disease, ulcerative colitis, Crohn's disease, Mendelian randomization

INTRODUCTION

Ankylosing spondylitis (AS) is a type of immune-mediated inflammatory rheumatic and spinal disease in the axial spondyloarthritis (SpA) spectrum, and it is also termed radiographic axial SpA (Raychaudhuri and Deodhar, 2014; Sieper and Poddubnyy, 2017). Ulcerative colitis (UC) and Crohn's disease (CD) are two main forms of inflammatory bowel disease (IBD) (Abraham and Cho, 2009; Ordás et al., 2012; Torres et al., 2012). It has been suggested that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host.

It has long been recognized that there is a close relationship between IBD and AS. Patients with IBD frequently suffer from extraintestinal symptoms, the most common symptoms of which are musculoskeletal manifestations (Larsen et al., 2010). It has been estimated that the prevalence of AS in IBD patients is approximately 3%, as reported by a meta-analysis (Karreman et al., 2016). Because both diseases likely occur concomitantly, some researchers suggest that AS and IBD might share a similar pathogenesis, but there is no evidence showing that the two conditions have a causal relationship. Exploring the causal relationship between the diseases is of great significance and may increase the current knowledge of the pathogeneses of AS and IBD and improve treatments.

Observational studies conducted to estimate causal inference have numerous inherent limitations, such as remaining limited to known and properly measured confounders (Greenland and Morgenstern, 2001). Therefore, we used Mendelian randomization (MR), which uses instrumental variables (IVs) in the analysis of genetic variants, to determine whether an observational association between exposures and outcomes exists and is consistent with a causal effect. MR rests on three assumptions: (a) the genetic variant is associated with the exposures; (b) the genetic variant is not associated with confounders; and (c) the genetic variant influences the outcomes only through the exposures. The genetic variants used in MR are available due to genome-wide association studies (GWAS) being conducted and high-throughput genomic technologies being developed. In this study, we used single-nucleotide polymorphisms (SNPs) strongly associated with IBD (including UC and CD) and AS as IVs. We performed two-sample MR using the effects of IVs on the exposures (UC, CD, and IBD) and outcomes (AS) from two independent samples. We analyzed the summary-level data and used statistical methods to obtain quantitative estimates of the effects of UC, CD, and IBD on AS. Moreover, we also used reverse MR to investigate the bidirectional causal relationship between IBD and AS.

MATERIALS AND METHODS

Data Source

In our study, a crucial step in performing MR was to choose appropriate genetic variants from the publicly available GWAS database to serve as valid IVs for IBD. We selected SNPs as IVs for all exposures (UC, CD, and IBD) and outcomes (AS) from the IEU GWAS database, a database of genetic associations from GWAS summary datasets[1] (Hemani et al., 2018). SNPs associated with IBD were derived from a transancestry association study on IBD, which was performed by the International Inflammatory Bowel Disease Genetics Consortium (IIBDGC) (Liu et al., 2015). In the association study, IBD was diagnosed on the basis of the accepted radiological, endoscopic, and histopathological evaluations. The summary-level data on the impact of the IBD-associated SNPs on AS were derived from GWAS, which were performed by the International Genetics of Ankylosing Spondylitis Consortium (IGAS) (International Genetics of Ankylosing Spondylitis Consortium[IGAS] et al., 2013). In the GWAS, AS was diagnosed on the basis of the modified New York Classification Criteria (van der Linden et al., 1984). Population stratification is a potential source of bias for MR analyses. Because there are differences in allele frequencies, one SNP can be associated with ancestry, which itself can be associated with disease risk (Emdin et al., 2017; Larsson et al., 2019). To prevent population stratification bias, we selected SNPs and their corresponding summary statistics (p-value, beta effect, and standard error) from studies that included only individuals of European descent for both the exposures and outcomes.

SNP Selection

From the GWAS summary results, we conducted a series of quality control steps to select eligible SNPs. We selected SNPs with a genome-wide association ($p < 5E{-}08$), with independent inheritance ($r^2 < 0.01$), and without linkage disequilibrium (LD) in summary statistics. When the target SNPs were not available in the outcomes of a study, we used proxy SNPs that had high LD ($r^2 > 0.8$) with the SNPs of interest. We selected the reference sample derived from European ancestral individuals from the 1,000 Genomes Project to estimate the allele frequency and LD level[2] (1000 Genomes Project Consortium et al., 2010). The palindromic SNPs with intermediate allele frequencies (palindromic SNPs referred to the SNPs with A/T or G/C alleles and "intermediate allele frequencies" referred to $0.01 <$ allele frequency < 0.30) were excluded from the above selected instrument SNPs. SNPs with a minor allele frequency (MAF) of < 0.01 were also excluded. We also calculated the F statistics for the SNPs to measure the strength of the instruments. IVs with an F statistic less than 10 were excluded and were often labeled as "weak instruments" (Burgess et al., 2015). These rigorously selected SNPs were used as the final instrumental SNPs for the subsequent MR analysis. The proportion of phenotypic variation explained by IV SNPs was also estimated.

Effect Size Estimate

We applied the principles of two-sample MR to assess the role of exposures (UC, CD, and IBD) in the susceptibility of the outcomes (AS). We chose the SNPs according to the selection criteria listed above as our instrumental variables and estimated the effects of the selected SNPs on the exposures and outcomes. We verified the stability of the results by comparing the effect

[1] https://gwas.mrcieu.ac.uk/

[2] http://www.internationalgenome.org/

directions across different two-sample MR filtering methods (Emdin et al., 2017; Larsson et al., 2019). The causal associations between exposures (UC, CD, and IBD) and outcomes (AS) were estimated with inverse variance weighted (IVW), MR-Egger, and the weighted median (WM). The IVW method uses a meta-analysis approach to combine the Wald ratios of the causal effects of each SNP and can provide the most precise estimates. The WM estimate provides a reliable effect estimate of the causal effect when at least 50% of the weight in the analysis comes from effective IVs. MR-Egger regression is used to create a weighted linear regression of the outcome coefficients with the exposure coefficients. The WM method offers some important advantages over MR-Egger because it has improved precision and is more robust to violations in the causal effects. MR-Egger estimates may be inaccurate and can be strongly influenced by outlying genetic variants (Bowden et al., 2016).

We also performed a recently developed method called the Robust Adjusted Profile Score (MR.RAPS) to estimate the causal effects, which can lead to a considerably higher statistical power than the conventional MR analysis can, which only uses a small set of strong instruments (Zhao et al., 2019). MR.RAPS considers the measurement error in SNP-exposure effects and is unbiased when there are many weak instruments, and is robust to systematic and idiosyncratic pleiotropy (Zhao et al., 2019). The MR.RAPS method can alleviate but cannot solve the problem of horizontal pleiotropy (Zhao et al., 2019).

Sensitivity Analyses

To exclude possible violations of the MR assumptions, we conducted multiple sensitivity analyses to verify whether heterogeneity and pleiotropy within the genetic instruments tested can bias the MR results. Pleiotropy refers to the phenomenon in which a single locus affects multiple phenotypes. Horizontal pleiotropy arises when a genetic variant is associated with more than one phenotype on separate pathways, which can invalidate the results from MR analyses. We performed MR-Egger regression to assess and adjust for horizontal pleiotropy, as it is a method that can identify confounders that may distort the MR results. We evaluated the MR-Egger regression intercept and conducted the MR-PRESSO (Pleiotropy RESidual Sum and Outlier) global test (Verbanck et al., 2018) to estimate the presence of pleiotropy. MR-PRESSO is an extension of previous approaches that utilize the general model of multi-instrument MR on summary statistics and is best used to identify inconsistencies between genetic associations of different genetic variants and remove outlying genetic variants (Verbanck et al., 2018). In addition, to test for the presence of pleiotropy, we evaluated the pleiotropic effects of UC, CD, and IBD on osteoarthritis (OA), as these effects might distort the effects of UC, CD, and IBD on AS. Summary statistics for OA were extracted from studies performed by the GWAS of European descent performed by Arthritis Research UK Osteoarthritis Genetics (arcOGEN) Consortium (arcOGEN Consortium et al., 2012). We assessed the potential associations between the SNPs that were extracted for the MR analysis and OA. Variants with detectable associations with OA were removed from the MR analysis, and the remaining non-pleiotropic variants were taken as instruments for the MR analysis. Associations of the SNPs with OA were considered statistically significant at a Bonferroni-corrected $p < 0.05/N$, with N representing the number of SNPs in each exposure trait.

We used the IVW, WM, and maximum likelihood methods to evaluate the heterogeneity among SNPs. The level of heterogeneity was quantified by Cochran Q statistics and I^2 statistics. The Cochran Q statistic was calculated as the weighted sum of the squared differences between individual SNP effects and the pooled effect across all SNPs.

An I^2 statistic calculation adapted for meta-analyses was used to quantify the strength of the violation for MR-Egger. The values are between 0 and 1 and indicate the expected relative bias of the MR-Egger causal estimate in the two-sample MR context (Bowden et al., 2016). Moreover, the causal directions between the exposures and outcomes were tested by the MR-Steiger method (Hemani et al., 2017). To guarantee that the MR estimates are not influenced by the inclusion of proxy SNPs, we implemented a "leave-one-out" sensitivity analysis by removing a different SNP in each iteration when performing the MR. All statistical tests were two-sided, and the results of the MR analyses and sensitivity analyses regarding the causal effects of UC, CD, and IBD on AS were considered statistically significant at $p < 0.05$.

Bidirectional Mendelian Randomization

We also sought to explore whether AS influenced UC, CD, and IBD. Therefore, we reversed the functions of the exposures and outcomes to perform a bidirectional MR analysis and determine the effects of a genetically increased risk of AS on UC, CD, and IBD. To that end, we selected SNPs that were significant genome-wide ($p < 5E{-}08$) and independently inherited ($r^2 < 0.01$) without LD for AS from IGAS (International Genetics of Ankylosing Spondylitis Consortium[IGAS] et al., 2013). We then used the corresponding effect estimates from IIBDGC as the outcomes (Liu et al., 2015). We then applied the same MR methods as above. The statistical tests of the bidirectional MR analysis were two-sided, and the results of the MR analyses and sensitivity analyses regarding the causal effects of AS on UC, CD, and IBD were considered statistically significant at a Bonferroni-corrected $p < 0.0167$ (e.g., 0.05/3 outcomes).

All statistical tests were performed using the "TwoSampleMR" package for R language, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). The "TwoSampleMR" codes in our study were available here: https://mrcieu.github.io/TwoSampleMR.

RESULTS

We incorporated 52, 59, and 82 significant ($p < 5E{-}08$) and independent SNPs ($r^2 < 0.01$) as IV SNPs for UC, CD, and IBD, respectively. However, three SNPs (rs3135501, rs11641016, and rs2564117) for IBD that were palindromic with intermediate allele frequencies were excluded. Finally, a total of 52, 59, and 79 IVs of UC, CD, and IBD were carefully selected (**Table 1**). Overall, the selected instruments explained approximately 21.49,

TABLE 1 | MR estimates from each method of assessing the causal effects of ulcerative colitis, Crohn's disease, and IBD on ankylosing spondylitis risk.

Exposure traits	MR methods	Ankylosing spondylitis				
		Number of SNPs	OR (95% CI)	SE	MR *p*-value	MR-Steiger test
Ulcerative colitis	MR-Egger	52	0.9927 (0.9502∼1.0370)	0.0223	0.7426	Direction: TRUE *p*-value <0.0001
	Inverse variance weighted	52	1.0256 (1.0130∼1.0385)	0.0063	*6.43E-05*	
	Weighted median	52	1.0241 (1.0054∼1.0432)	0.0094	*0.0115*	
	MR.RAPS	52	1.0280 (1.0146∼1.0414)	0.0067	*3.59E-05*	
	MR-PRESSO test	52	1.0256 (1.0137∼1.0377)	0.0060	*9.63E-05*	
Crohn's disease	MR-Egger	59	1.0015 (0.9702∼1.0337)	0.0162	0.9282	Direction: TRUE *p*-value <0.0001
	Inverse variance weighted	59	1.0194 (1.0088∼1.0302)	0.0054	*0.0003*	
	Weighted median	59	1.0235 (1.0069∼1.0404)	0.0083	*0.0054*	
	MR.RAPS	59	1.0214 (1.0103∼1.0327)	0.0056	*0.0002*	
	MR-PRESSO test	59	1.0194 (1.0096∼1.0293)	0.0049	*0.0002*	
IBD	MR-Egger	79	0.9920 (0.9512∼1.0345)	0.0214	0.7078	Direction: TRUE *p*-value <0.0001
	Inverse variance weighted	79	1.0259 (1.0133∼1.0387)	0.0063	*5.25E-05*	
	Weighted median	79	1.0352 (1.0153∼1.0556)	0.0099	*0.0005*	
	MR.RAPS	79	1.0292 (1.0160∼1.0427)	0.0066	*1.33E-05*	
	MR-PRESSO test	79	1.0238 (1.0123∼1.0355)	0.0058	*0.0001*	

MR, Mendelian randomization; RAPS, Robust Adjusted Profile Score; PRESSO, Pleiotropy RESidual Sum and Outlier; SNP, single nucleotide polymorphism; IBD, inflammatory bowel disease; OR, odds ratio; CI, confidence interval; SE, standard error (the standard error is an estimate of the standard deviation (SD) of the coefficient). The italic values mean the statistical significance.

28.94, and 21.69% of the phenotypic variation in UC, CD, and IBD, respectively, on the observed scale. For these instrumental variables, all the *F*-values were larger than 10 (ranging from 29.7576 to 110.7637 for UC; ranging from 30.7373 to 349.9869 for CD and ranging from 30.9495 to 232.7940 for IBD) with average *F*-values of 45.7381, 66.9439, and 52.0463 for UC, CD, and IBD, respectively; these results indicate that the variables satisfy the strong relevance assumption of MR and that the instrument bias is weak and cannot substantially influence the estimations of causal effects (**Supplementary Tables S1–S3**).

The causal associations between UC and AS determined using the full set of 52 SNPs were not consistent among the three MR methods. The IVW and WM MR results showed that the per unit increase in the log-odds of having UC was significantly associated with an increased risk of having AS at $p < 0.05$ (IVW OR = 1.0256, 95% CI 1.0130–1.0385, p = 6.43E-05; and WM OR = 1.0241, 95% CI 1.0054–1.0432, p = 0.0115), while the MR-Egger regression method did not suggest a significant association between CD and AS (OR = 0.9927, 95% CI 0.9502–1.0370, p = 0.7426) (**Table 1** and **Figure 1**). Given that the IVW estimates were consistent with the WM estimates and that the IVW estimates may be unbiased estimates of causal effects and are considerably more powerful than the MR-Egger regression estimates (Bowden et al., 2016), we believe that UC had a positive causal effect on AS risk. The causal effects of CD and IBD on AS were the same as those of UC at $p < 0.05$ (For CD, IVW OR = 1.0194, 95% CI 1.0088–1.0302, p = 0.0003; WM OR = 1.0235, 95% CI 1.0069–1.0404, p = 0.0054 and MR-Egger OR = 1.0015, 95% CI 0.9702–1.0337, p = 0.9282. For IBD, IVW OR = 1.0259, 95% CI 1.0133–1.0387, p = 5.25E-05; WM OR = 1.0352, 95% CI 1.0153–1.0556, p = 0.0005 and MR-Egger OR = 0.9920, 95% CI 0.9512–1.0345, p = 0.7078) (**Table 1** and **Figure 1**). Moreover, the MR.RAPS results were found to be consistent with the MR IVW and WM results, showing that UC, CD, and IBD were significantly associated with an increased risk of having AS at $p < 0.05$ (For UC, MR.RAPS OR = 1.0280, 95% CI 1.0146–1.0414, p = 3.59E-05. For CD, the MR.RAPS results were as follows: OR = 1.0214, 95% CI 1.0103–1.0327, p = 0.0002. For IBD, the MR.RAPS results were as follows: OR = 1.0292, 95% CI 1.0160–1.0427, p = 1.33E-05) (**Table 1** and **Figure 1**). Therefore, we found positive causal associations of UC, CD, and IBD with an increased risk of AS with the MR IVW, WM, MR-Egger, and MR.RAPS methods.

We conducted MR-Egger regression to assess pleiotropy, and the results revealed that horizontal pleiotropy was unlikely to bias the causality of UC (p = 0.1326), CD (p = 0.2484), and IBD (p = 0.1044) with AS (**Table 2**). The "leave-one-out" analysis also revealed that no single SNP was driving the MR estimates (see **Supplementary Figures S1–S3**). The associations between these genetic variants and confounding factors OA were also analyzed. None of the genetic variants of the UC, CD, or IBD traits were significantly associated with OA at the Bonferroni-corrected significance threshold of $p < 0.0010$ (e.g., 0.05/52), $p < 0.0008$ (e.g., 0.05/59), or $p < 0.0006$ (e.g., 0.05/79) (**Supplementary Tables S4–S6**). Cochran *Q*-value and the I^2-value indicated there was no heterogeneity between the IV estimates determined with the IVW, MR-Egger, and maximum likelihood methods (For UC, MR-Egger Q = 43.1297, $I^2 < 0.0001$, p = 0.7434; IVW Q = 45.4670, $I^2 < 0.0001$, p = 0.6923; maximum likelihood Q = 45.2706, $I^2 < 0.0001$, p = 0.6996. For CD, the MR-Egger results were as follows: Q = 47.4296, $I^2 < 0.0001$, p = 0.8129; IVW Q = 48.7894, $I^2 < 0.0001$, p = 0.8002; maximum likelihood Q = 48.6807, I^2<0.0001, p = 0.8034. For IBD, the MR-Egger results were as follows: Q = 63.1835, $I^2 < 0.0001$, p = 0.8715; IVW *Q* = 65.8841, $I^2 < 0.0001$, p = 0.8343; maximum likelihood *Q* = 65.7086, $I^2 < 0.0001$, p = 0.8382.) (**Table 2**). The MR-Steiger

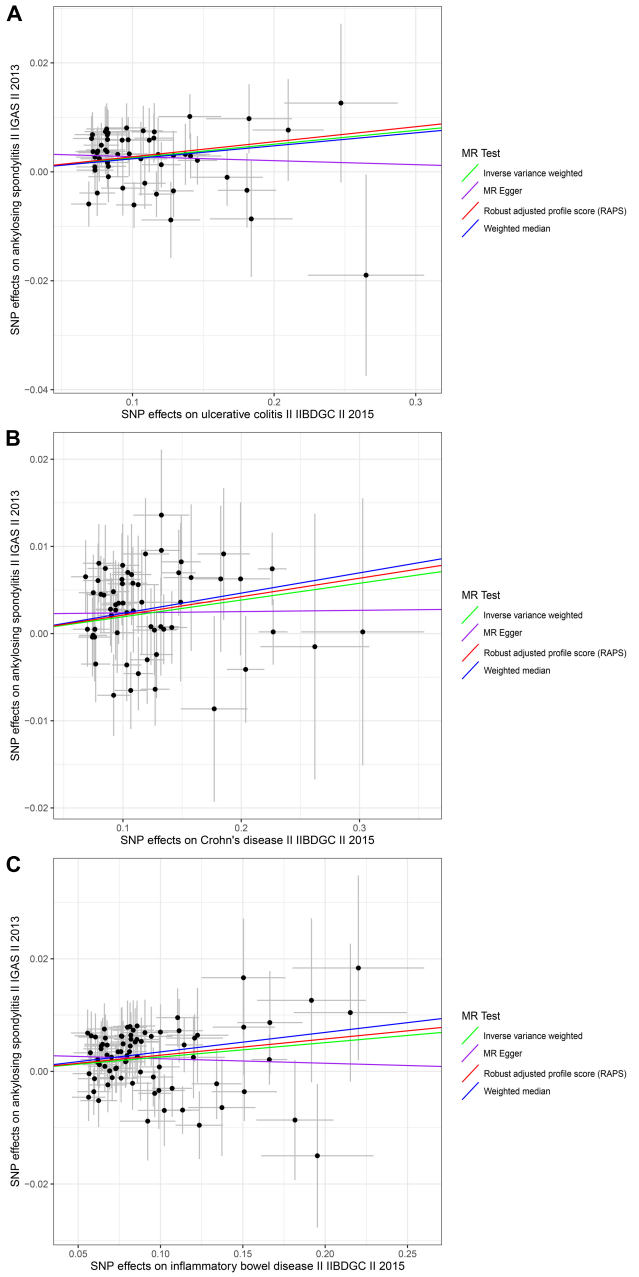

FIGURE 1 | Scatter plots of the genetic associations with inflammatory bowel disease against ankylosing spondylitis risk using different MR methods. **(A)** Ulcerative colitis against ankylosing spondylitis risk; **(B)** Crohn's disease against ankylosing spondylitis risk; and **(C)** inflammatory bowel disease against ankylosing spondylitis risk. The slopes of each line represent the causal association for each method. The green line represents the inverse variance weighted estimate, the purple line represents the MR-Egger estimate, the red line represents the MR.RAPS estimate, and the blue line represents the weighted median estimate.

results supported a causal association between the IBD traits and AS (**Table 1**). Moreover, we did not detect any outlier SNPs or a horizontal pleiotropic effect of UC, CD, or IBD on the risk of AS when we used the MR-PRESSO global test (p-values for UC, CD, and IBD were 0.7020, 0.8060, and 0.7300, respectively). The MR results determined with the outlier-corrected MR-PRESSO method were similar to the MR IVW results reported above (For UC, OR = 1.0256, 95% CI 1.0137–1.0377, p = 9.63E-05. For CD, OR = 1.0194, 95% CI 1.0096–1.0293, p = 0.0002. For IBD, OR = 1.0238, 95% CI 1.0123–1.0355, p = 0.0001) (**Table 1**). Therefore, the MR-PRESSO results suggested there are causal effects of UC, CD, and IBD on AS.

To explore the causal effects of AS on UC, CD, and IBD, we incorporated 8, 8, and 6 significant and independent IV SNPs for AS, respectively, which were retrieved from IGAS (**Supplementary Tables S7–S9**). Overall, the selected instruments explain approximately 0.37, 0.16, and 1.80% of the phenotypic variation of AS on the observed scale. For the instrumental variables of AS, all the F-values were greater than 10 (ranging from 30.0213 to 252.1853 with UC; ranging from 30.0213 to 748.6627 with CD and ranging from 30.0213 to 1314.9257 with IBD) with average F-values of 105.6131, 156.4408, and 375.1283 for UC, CD, and IBD, respectively (**Supplementary Tables S7–S9**). There was no evidence suggesting causal associations of an increased risk of AS with changes in the risk of UC, CD, or IBD, based on the IVW, WM, and MR-Egger regression methods and the Bonferroni-corrected significance threshold of $p < 0.0167$ (e.g., 0.05/3) (**Supplementary Table S10** and **Figure 2**). The MR.RAPS and MR-PRESSO test results were consistent with the IVW, WM, and MR-Egger regression results (**Supplementary Table S10** and **Figure 2**). We conducted MR-Egger regression to assess pleiotropy, and the results revealed that the presence of horizontal pleiotropy was unlikely to bias the causality of AS with UC (p = 0.2931), CD (p = 0.2895), and IBD (p = 0.5554) (**Supplementary Table S11**). The leave-one-out method demonstrated that the observed links were not driven by SNP (see **Supplementary Figures S4–S6**). Cochran Q-value and the I^2-value also indicated there was no heterogeneity across the IV estimates determined with the IVW, MR-Egger, and maximum likelihood methods (**Supplementary Table S11**). In summary, we did not find significant causal associations of AS with UC, CD, or IBD.

DISCUSSION

To the best of our knowledge, our study is the first to illustrate the bidirectional causal relationship between IBD and AS using MR analysis and large-scale GWAS data. Our findings provided evidence that IBD (including UC and CD) had positive causal effects on AS risk but did not suggest that there are causal effects of AS on IBD risk in individuals of European descent. We found that suffering from IBD was the causal factor of an increased risk of AS, which suggests that IBD and AS might share a similar pathogenesis.

Although the exact mechanisms linking IBD and AS are not fully understood, the joint-gut axis hypothesis was proposed to explain the pathogenic link (Brakenhoff et al., 2010). Various environmental (gut bacteria-dysbiosis) factors and host factors (migration of activated gut-T cells and macrophages) lead to inflammation in genetically susceptible individuals, which may

TABLE 2 | Heterogeneity and pleiotropy analysis of ulcerative colitis, Crohn's disease and IBD with ankylosing spondylitis risk using different analytic methods.

Exposure traits	MR methods	Ankylosing spondylitis			
		Cochran *Q* statistic	I^2	Heterogeneity *p*-value	MR-Egger Intercept *p*-value
Ulcerative colitis	MR-Egger	43.1297	< 0.0001	0.7434	0.1326
	Inverse variance weighted	45.4670	< 0.0001	0.6923	
	Maximum likelihood	45.2706	< 0.0001	0.6996	
Crohn's disease	MR-Egger	47.4296	< 0.0001	0.8129	0.2484
	Inverse variance weighted	48.7894	< 0.0001	0.8002	
	Maximum likelihood	48.6807	< 0.0001	0.8034	
IBD	MR-Egger	63.1835	< 0.0001	0.8715	0.1044
	Inverse variance weighted	65.8841	< 0.0001	0.8343	
	Maximum likelihood	65.7086	< 0.0001	0.8382	

MR, Mendelian randomization; IBD, inflammatory bowel disease.

act as triggers of inflammatory responses against gut and joint components (Brakenhoff et al., 2010; Fragoulis et al., 2019). In one study Tito et al. (2016), investigated the association between intestinal microbiota and spondyloarthritis and demonstrated a significant difference in the intestinal microbial composition between patients with spondyloarthritis who had microscopic gut inflammation and those without microscopic gut inflammation. This study indicated that gut bacteria-dysbiosis might play an important role in the pathogenesis of both diseases. Genetic factors also seem to have a significant impact on linking the two diseases. Laukens et al. (2005) reported that CARD15 gene polymorphisms are associated with an increased risk for chronic gut inflammation in patients with SpA. Peeters et al. (2004) included 102 patients with CD in a study and found that CARD15 variants are genetic predictors of CD-related sacroilitis.

Many studies have shown that the risk of IBD is high in patients with AS (Stolwijk et al., 2014, 2015), but those results did not indicate there are causal effects of AS on the risk of IBD. The findings of some studies were consistent with our findings. With the data from a large population-based public health database in Spain, Muñoz-Ortego et al. (2014) found no significant associations between AS and IBD. In a preliminary cohort study conducted using data from the 2005–2012 database of the Taiwan National Health Insurance Programme in Taiwan, the overall incidence of IBD was lower in the AS group than in the non-AS group, but the difference did not reach statistical significance (Lai et al., 2019). Because there are confounding factors in observational studies, it is unclear whether they are etiologically relevant to each other. The results of our study can provide new information on the similarity of the pathogeneses of the two diseases.

The causal effects of IBD on AS are of great significance for the classification of SpA and the diagnosis and treatment of AS. Traditionally, SpA can be classified as axial SpA or as peripheral SpA. Axial SpA is subclassified as radiographic SpA and non-radiographic SpA based on the presence or absence of definite sacroilitis according to the modified New York Classification Criteria (van der Linden et al., 1984; Rudwaleit et al., 2009). According to the ASAS classification criteria for axial SpA, patients with > 3 months of back pain and age of onset of < 45 years confirmed sacroilitis on imaging examinations, and more than one SpA features (including IBD) or those with HLA-B27 combined with more than two SpA features (including IBD) can be diagnosed with axial SpA. Since we found that IBD is the cause of AS, we recommend that the significance of IBD is emphasized in the axial SpA classification criteria. We also recommend that the features of IBD are included in the modified New York Classification Criteria (van der Linden et al., 1984) for the diagnosis of AS. Our study is also an important addition to IBD and AS research, and the results have important implications for public health. We will predict the occurrence of AS in IBD patients and will provide strategies for preventing and treating AS in IBD patients. For example, surveillance examinations for IBD patients should include not only a regular colonoscopy but also a regular spine X-ray. We also suggest that IBD patients take measures to prevent back injuries that may result in spinal fractures, especially those who have low back pain, because patients with AS are at high risk of fractures (Muñoz-Ortego et al., 2014).

The present study has several limitations. First, the summary-level statistics approach does not allow us to perform analyses stratified by covariates that were adjusted by the original GWAS. Second, we only assumed a linear effect relationship between IBD and AS in the MR model. The summary statistics also did not permit us to explore the non-linearity of the association between IBD and AS. Although linearity is a first-order approximation of any -linear relationship, a simple linearity assumption may not always be reasonable in practice (Burgess et al., 2014). Third, we did not stratify the causal effects between IBD and AS by gender or age, although previous studies revealed that causal effects between IBD and AS can be age and gender dependent (Van Praet et al., 2013). It is difficult to obtain individual-level data in original GWAS. The study population included in the exposure and outcome analyses were of European ancestry,

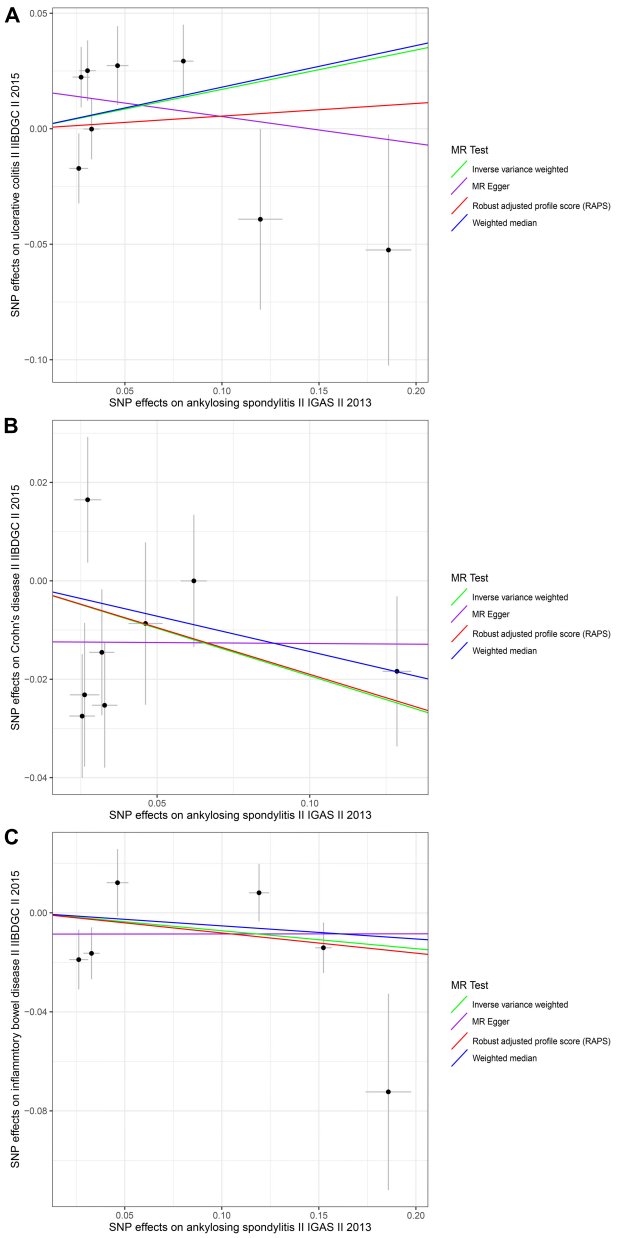

FIGURE 2 | Scatter plots of the genetic associations with ankylosing spondylitis against inflammatory bowel disease risk using different MR methods. **(A)** Ankylosing spondylitis against ulcerative colitis risk; **(B)** ankylosing spondylitis against Crohn's disease risk; and **(C)** ankylosing spondylitis against inflammatory bowel disease risk. The slopes of each line represent the causal association for each method. The green line represents the inverse variance weighted estimate, the purple line represents the MR-Egger estimate, the red line represents the MR.RAPS estimate, and the blue line represents the weighted median estimate.

which may have mitigated population stratification. However, the conclusions made based on the European study population are not representative of individuals of other ancestries, such as Asians and Americans. Fourth, the small variance for the exposures, especially for AS with the SNP instruments, might affect the power of the causal effects. The variance might be affected by the small amounts of SNP instruments. However, based on the very large sample size and strongly relevant instruments, we still have been powered to rule in or rule out the causal relationship.

A further limitation is our use of binary risk factors (IBD). IBD is a dichotomization of a continuous risk factor which can lead to violation of the exclusion restriction assumption and limit the inferences drawn from an MR study. In particular, the effect estimate of IBD (yes/no) on AS represents the average effect among individuals for whom the presence or absence of the included genetic effects determines their IBD status. We further assume that the effect of IBD on AS is constant for all individuals, which may not be the case. However, it is important to note that the MR test for an association between IBD and AS is still valid if the instrumental variable assumptions are satisfied (Burgess and Labrecque, 2018). Additionally, we did not propose a physiological mechanism to explain the causal associations between IBD and AS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YT, ZC, and GH conceptualized and designed the study. XM provided the "TwoSampleMR" package codes in R language and analyzed the data in the study. ZC drafted the manuscript. HF and BH gave constructive suggestions when writing the manuscript. All authors have read the manuscript.

FUNDING

This work was supported by grants from the Beijing Municipal Science and Technology Commission (Z181100001718195).

ACKNOWLEDGMENTS

We thank the DSC (Wuhan) Scientific Co. Ltd. for consulting services on data management, statistical analysis, and scientific writing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.587876/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 15 December 2020
doi: 10.3389/fgene.2020.607865

Investigating Causal Relations Between Sleep-Related Traits and Risk of Type 2 Diabetes Mellitus: A Mendelian Randomization Study

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Objective: Extensive literature put forward the link between sleep and type 2 diabetes mellitus (T2DM), however, little is known about the underlying causality of the associations. Here we aim to assess the causal relationships between five major sleep-related traits and T2DM.

Design, Setting, and Participants: Two-sample Mendelian randomization (MR) was utilized to investigate the potential causal relations. Independent genetic variants associated with five sleep-related phenotypes—insomnia, sleep duration, short sleep duration, long sleep duration, and morningness—were chosen as instrumental variables to estimate the causal associations with T2DM. Summary statistics were acquired from the genome-wide association studies of UK Biobank and 23andMe (for sleep-related measures), the DIAbetes Genetics Replication And Meta-analysis and the FinnGen (for T2DM).

Main Methods: Individual Cochran's Q statistic was applied to remove the pleiotropic instruments, global Q statistics and MR-Egger regression were adopted to test for the global heterogeneity and horizontal pleiotropy of the screened instruments, respectively. Two T2DM cohorts were selected to analyze their associations with sleep traits. A modified inverse variance weighted (IVW) estimate was performed to combine the ratio estimators from each instrument and acquire the causal estimate, alternative methods including IVW with first-order weights, simple and weighted median estimations, and MR-Egger regression were conducted as sensitivity analyses, to ensure the robustness and solidity of the findings.

Results: Two-sample MR supported findings for an adverse effect of genetically predicted insomnia on T2DM risk (odds ratio [OR] = 1.14, 95% confidence interval [CI]: 1.09–1.19, $p = 1.29E{-}08$) at the Bonferroni-adjusted level of significance ($p < 0.005$). We further investigated the causal role of T2DM on insomnia but obtained a non-significant estimation. There was also little evidence for the causal effect of other sleep-related measures on T2DM. Results were largely consistent when leveraging two different T2DM cohorts, and were robust among various sensitivity analyses.

OPEN ACCESS

Edited by:
Lei Zhang,
Soochow University, China

Reviewed by:
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Hong-Yu Zhang,
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *18 September 2020*
Accepted: *24 November 2020*
Published: *15 December 2020*

Citation:
Gao X, Sun H, Zhang Y, Liu L, Wang J and Wang T (2020) Investigating Causal Relations Between Sleep-Related Traits and Risk of Type 2 Diabetes Mellitus: A Mendelian Randomization Study. Front. Genet. 11:607865. doi: 10.3389/fgene.2020.607865

Conclusion: Findings provide significant evidence for an adverse effect of insomnia on T2DM risk. The study extends fundamental knowledge to further understanding of the pathophysiological mechanisms of T2DM, and points out the non-negligible role of insomnia on epidemiologic intervention and clinical therapeutics of T2DM.

Keywords: insomnia, sleep duration, morningness, type 2 diabetes mellitus, causal relations, Mendelian randomization

INTRODUCTION

Type 2 diabetes mellitus (T2DM), is a chronic condition that describes a group of metabolic disorders characterized by insulin resistance (Arnold et al., 2018). The global prevalence of T2DM was estimated to be around 450 million. If current trends continue, the cases will rise to 700 million by 2045 (Saeedi et al., 2019). In the past three decades, the prevalence of T2DM has risen doubled worldwide, leading to a heavy health burden of disability and mortality (Roglic et al., 2005).

Quantity and quality of sleep are considerable lifestyle factors that influence the development of T2DM. Several studies have reported the associations of sleep-related traits with T2DM (Kawakami et al., 2004; Meisinger et al., 2005). One retrospective cohort study indicated that insomnia imparts an increased risk of T2DM (LeBlanc et al., 2018). Moreover, a systematic review of prospective studies showed a U-shaped association between sleep duration and the risk of T2DM (Brady et al., 2018). Consistent with this, a cross-sectional study demonstrated that both short and long sleep duration are associated with an increased risk of T2DM (Chaput et al., 2007). Besides, sleep chronotype may also play an important role in the risk of T2DM. One study found that morningness was associated with lower HbA1c, which induced a high risk of T2DM (Iwasaki et al., 2013).

Despite previous observational evidence of the relationship between sleep and T2DM, there are also inconsistent results (Björkelund et al., 2005; Lai et al., 2013). Also, several studies showed that T2DM may also lead to sleep disorders, it is not certain whether sleep causally influences the risk for T2DM, or T2DM reversely affects sleep (Hein et al., 2018; Dong et al., 2020). Moreover, observational studies are open to confounding, which can hardly be ruled out. Therefore the causal relationship between sleep and T2DM remains unclear.

Mendelian randomization (MR) can offer essential evidence for the causal inference (Emdin et al., 2017). Commonly, MR utilizes genetic variants associated with the exposure but does not directly affect the disease outcome as instrumental variables. The alleles of the variants are distributed randomly at conception, thus the predisposition for the exposure that is proxy by the genetic variants is distributed randomly, in this way the MR framework can approximate the RCT design. Besides, since the formation of the genetic variants is stable from conception on, they always precede the development of disease outcomes and other possibly confounding factors. Based on the distinct nature of genetic variants, MR can overcome the downsides of traditional observational studies such as unmeasured confounding and reverse causation, and provide a more valid estimation for the causal relationship between the exposure and the outcome (Agoritsas et al., 2017). Benefiting from the Genome-wide association studies (GWAS) which unraveled the association of genetic variants with phenotypes, the summarized data-based MR methods promote the causal inference of various traits with diseases considerably, and are prevailingly suggested and extensively applied in recent studies (Gao et al., 2019; Porcu et al., 2019; Sun et al., 2020).

There have been some studies exploring the causality between sleep and T2DM based on MR design. For example, Wang et al. (2019) examined the relationship of sleep duration with the risk of diabetes but found that they were not causally related. Bos et al. (2019) investigated the effect of total, short and long sleep duration on glycemic traits but the results provided little evidence for the causal role. Despite this, a comprehensive causal relationship of different sleep measures with T2DM has not been identified. As sleep is potentially modifiable, identifying the causal association of sleep and T2DM has substantial implications for preventing T2DM and improving population health. Herein, in the current study, we utilized the MR framework to investigate the causal relations between a wide range of sleep-related phenotypes (including insomnia, sleep duration and morningness) and T2DM.

MATERIALS AND METHODS

Study Sample and Data Sources

A total of five cohorts (three for sleep-related traits and two for T2DM) were included in our study. Summary-level data had been made publicly available, and ethical approval had been obtained in the original studies (**Table 1**).

Summary-level data for insomnia were derived from the largest available meta-analysis of GWAS, including unrelated European descent individuals from UK Biobank (UKB, $N = 386{,}533$, 46.0% female) and 23andMe ($N = 944{,}477$, 53.1% female) (Jansen et al., 2019). Insomnia cases were measured with questionnaire data and defined as participants who usually have trouble in falling asleep at night or wake up in the middle of the night in the UKB cohort, and were diagnosed with participants affirming no less than one phenotypic concept concerning inferior sleep status in the 23andMe cohort. The prevalence of insomnia was 29.9% in the combined sample of UKB and 23andMe and was higher in females (34.6%) than males (24.5%).

Genetic association estimates with sleep duration were obtained from the UKB participants of European ancestry ($N = 446{,}118$, 54.1% female) (Dashti et al., 2019). The GWAS examined the following three sleep duration phenotypes: self-reported habitual sleep duration (continuous variable), which

TABLE 1 | GWAS cohorts used in this study.

Phenotype	First author (year)	Sample size	Consortium	PubMed ID
Insomnia	Jansen et al. (2019)	397,959 cases, 933,051 controls	UKB/23andMe	30804565
Sleep duration	Dashti et al. (2019)	446,118	UKB	30846698
Short sleep duration	Dashti et al. (2019)	106,192 cases, 305742 controls	UKB	30846698
Long sleep duration	Dashti et al. (2019)	34,184 cases, 305742 controls	UKB	30846698
Morningness	Jones et al. (2019)	372,765 cases, 278,530 controls	UKB/23andMe	30696823
T2DM	Scott et al. (2017)	26,676 cases, 132,532 controls	DIAGRAM	28566273
T2DM	2020	17,616 cases, 114,000 controls	FinnGen	NA

T2DM, Type 2 diabetes mellitus; UKB, UK Biobank; DIAGRAM, DIAbetes Genetics Replication And Meta-analysis.

was assessed by the question: "About how many hours sleep do you get in every 24 h? (please include naps)." The answer was responded in hour increments and could only contain integer values; short and long sleep duration (binary variable), categorized as <7 h and >8 h relative to 7–8 h sleep duration, respectively. The mean sleep duration was 7.2 h (1.1 standard deviation) per day, and the prevalence for short and long sleep duration ware 25.8 and 10.1%, respectively.

Full summary statistics for morningness were acquired from the largest meta-analysis of GWAS among adults of European ancestry, including 449,734 participants from UKB and 248,098 participants from 23andMe (Jones et al., 2019). The participants in the UKB cohort were promoted to answer the question "Do you consider yourself to be?" with six possible answers, persons answering "Definitely a 'morning' person" or "More a 'morning' than 'evening' person" were assigned to cases of morningness, and persons answering "More an 'evening' than a 'morning' person" or "Definitely an 'evening' person" were assigned to controls. The participants in the 23andMe cohort responded to the question "Are you naturally a night person or a morning person?" with two possible answers. 57.2% of the individuals were coded as a morning person in the pooled cohort, and the percentages were 62.6% and 48.6% for UKB and 23andMe, respectively.

GWASs for T2DM were selected to extract genetic association information for the outcome. When using genetic consortia that have significant overlapping sets in the exposure and outcome GWAS, the two-sample summary data-based MR may develop biased estimates (Burgess et al., 2016). Thus, we excluded the GWASs that involving UKB or 23andMe as main or sub cohort. Furthermore, to reduce the possible confounding derived from population stratification, we restricted the T2DM cohort to European-descent adults. According to the above criteria, we drew on summary statistics from the largest GWAS of T2DM, which was conducted by the DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) consortium and contained 18 study cohorts and a total of 159,208 participants (Scott et al., 2017). The T2DM diagnosis criteria, control selection principles, and study characteristics for each cohort had been described in more detail in the original article. To examine the solidity of the findings, we also selected another newly released T2DM GWAS, which was derived from FinnGen cohort and contained a total of 131,616 individuals, as the validation sample.

Selection of Instruments

The first core assumption for MR is that the instruments are robustly and strongly associated with the exposure of interest. When the relationship of the instruments with exposure is weak, the causal estimate will be biased toward the null, which is referred to as weak instrument bias (Davies et al., 2015). To address the potential weak instrument bias, we included the independent lead single nucleotide polymorphisms (SNPs) that are genome-wide significantly ($P < 5 \times 10^{-8}$, $r^2 < 0.1$) associated with the sleep traits as preliminary instruments. Then we extracted the summary statistics for the associations of the selected instruments with T2DM from the T2DM GWAS database and matched the two groups of sample data based on the SNP ID. To make sure that the effect of an instrument on the exposure and the effect of that instrument on the outcome each correspond to the same allele, we performed harmonization of the direction of effects (Hemani et al., 2018). To further ensure the independence of the instruments, SNPs that were in linkage disequilibrium (LD) were excluded from the instrument variable set using the clumping algorithm (r^2 threshold $= 0.01$ and window size $= 1$ Mb) (Chang et al., 2015).

Investigation of Pleiotropy

Other main identifying assumptions for an MR analysis are that the instrument is not associated with the confounding factors, and it influences the outcome only through the exposure. These two assumptions can be together summarized as independent of pleiotropy (Emdin et al., 2017). Pleiotropy occurs when the genetic variant influences the target outcome via any pathway other than the exposure. The nature of pleiotropy could invalidate an instrument, thus bias the estimate in the MR analysis (Zhu et al., 2018). Pleiotropy is commonplace in practice, however, a complete understanding of the effect of genetic variants on the phenotypes is lacking. Therefore, it is necessary to take full advantage of statistical findings to assess and identify the potential pleiotropic instruments (Swerdlow et al., 2016). We first investigated the association of the instruments with major confounders such as body mass index, alcohol use and physical activity, and excluded the SNPs that were associated with these known confounders at the genome-wide significance level (Chen et al., 2011; Van Reen et al., 2011; Bayon et al., 2014; Semplonius and Willoughby, 2018). Known potentially pleiotropic effects of the chosen SNPs were obtained with PhenoScanner, a database

that provides massive human genotype-phenotype associations (Staley et al., 2016). We then performed the individual Cochran's Q outlier test to detect the unknown pleiotropic effects of the instruments (Del Greco et al., 2015). The ratio estimate (i.e., the estimate for the SNP–outcome association divided by the estimate for the SNP-exposure association) of each valid instrument will only vary by chance, and a significant heterogeneity would hint at the violation of assumptions in the instrument, most likely as a result of pleiotropy. These outlier SNPs with significant contributions to the Q statistic for heterogeneity were removed from the instrument variable set.

After the above steps to eliminate the potential pleiotropy, global Q test was implemented to examine if there is still heterogeneity among the screened instruments, and MR-Egger regression was also conducted to evaluate the directional pleiotropy of the instruments (Bowden et al., 2015). Directional pleiotropy here refers to the pleiotropic effects of genetic variants are not balanced about the null. In this situation, the estimations from MR analyses inevitably suffer from bias (Burgess and Thompson, 2017). A significant deviation of the MR-Egger intercept from 0 indicated directional pleiotropy in the instruments.

Main MR Analysis

After a series of examinations for the validity of the instruments, we evaluated the causal estimations with the inverse-variance weighted (IVW) method, which essentially models the weighted regression of SNP-outcome effects on SNP-exposure effects where the intercept is constrained to zero (Burgess et al., 2013). The modified weights in the IVW framework take account of the uncertainty of SNP–exposure associations and move beyond the "NO Measurement Error" (NOME) assumption, therefore leveraging more power compared with the IVW using the first-order weights (Bowden et al., 2019). Given this, we adopted the modified IVW approach to obtain the estimates for the causal effect of the sleep traits on T2DM.

Sensitivity Analyses and Other Elements

To assess the extent to which findings were robust to potential pleiotropy, we also performed sensitivity analysis with four other established MR methods: IVW with first-order weights (Burgess et al., 2013), simple weighted median (SME), weighted median estimates (WME) (Bowden et al., 2016), and MR-Egger (Bowden et al., 2015). IVW with first-order weights could produce consistent estimates when there are no pleiotropic instruments, whereas the median based and MR-Egger estimates allow the inclusion of the pleiotropic instruments and are relatively robust to pleiotropy, although at the cost of reduced statistical power. Consistent estimates across multiple methods strengthen the robustness of the causal findings. Furthermore, we conducted the analyses by combining the five major sleep traits with both the main DIAGRAM T2DM cohort and the alternative FinnGen T2DM cohort respectively, to assess the consistency of the results. Lastly, we exploited the three largest GWAS cohorts from the MAGIC consortium and investigated the causal relationships of five sleep-related traits with Hemoglobin A1c(Hb1Ac), fasting glucose (FG), and fasting insulin (FI).

To account for multiple testing, we employed a Bonferroni-corrected threshold of $P < 0.005$ (0.05/10 to correct for five sleep traits in relation to two T2DM outcomes). A *P*-value between 0.005 and 0.05 was considered as suggestive evidence of causality and needs to be further confirmed. The statistical analyses were performed in two-tailed, with the use of TwoSampleMR package (perform data extraction, harmonization, and clumping), RadialMR package (perform modified IVW and Q test), and MendelianRandomization package (query the genotype-phenotype associations, perform sensitivity analysis and MR-Egger intercept test) in R project 3.5.0.

RESULTS

Insomnia and T2DM

We extracted summary association statistics for the 248 genome-wide significant SNPs previously demonstrated to be associated with insomnia. We then matched and harmonized the effects for the SNPs on insomnia and on T2DM to each be for the same reference allele. Thirty-seven SNPs were excluded because of high LD with the other SNPs, 22 SNPs were excluded due to their significant relationships with the known confounders, and 22 outliers were identified by the individual Q test and were removed from the instrument variable set. Global Q test ($Q = 138.04$, $P = 0.94$) and MR-Egger test (intercept $P = 0.32$) did not support any evidence for heterogeneity or directional pleiotropy for the rest of the instruments. Detailed information on the instruments for insomnia was shown in **Supplementary Table 1** and **Supplementary Figure 1**. Modified IVW supported the findings of a significant adverse effect of insomnia on the risk of T2DM [odds ratio (OR) = 1.14, 95% confidence interval (CI): 1.09–1.19, $P = 1.29E{-}08$], and the estimates were broadly consistent between the main analysis and sensitivity analysis (**Table 2** and **Figure 1**).

Since a significant effect of insomnia on T2DM was observed, we further conducted a complimentary analysis to assess the causal effect of T2DM on the risk of insomnia. Each genome-wide significant T2DM-related instrument and its association estimates with T2DM and insomnia are presented in **Supplementary Table 2** and **Supplementary Figure 2**. However, across all MR methods, we found no evidence of the

TABLE 2 | Main MR analysis for the causality of sleep traits with the risk of T2DM.

Phenotypes		MR results		
Exposure	**Outcome**	**N SNPs**	**OR (95% CI)**	***P*-value**
Insomnia	T2DM	167	1.14 (1.09, 1.19)	1.29E–08
Sleep duration		56	1.00 (1.00, 1.00)	0.43
Short sleep duration		17	1.15 (0.96, 1.38)	0.09
Long sleep duration		5	1.10 (0.79, 1.51)	0.43
Morningness		284	1.03 (0.98, 1.08)	0.29

MR, Mendelian randomization; T2DM, Type 2 diabetes mellitus; N SNPs, number of SNPs retained and used in the MR analysis after filtered by individual Q outlier test; OR, odds ratio; CI, confidence interval.

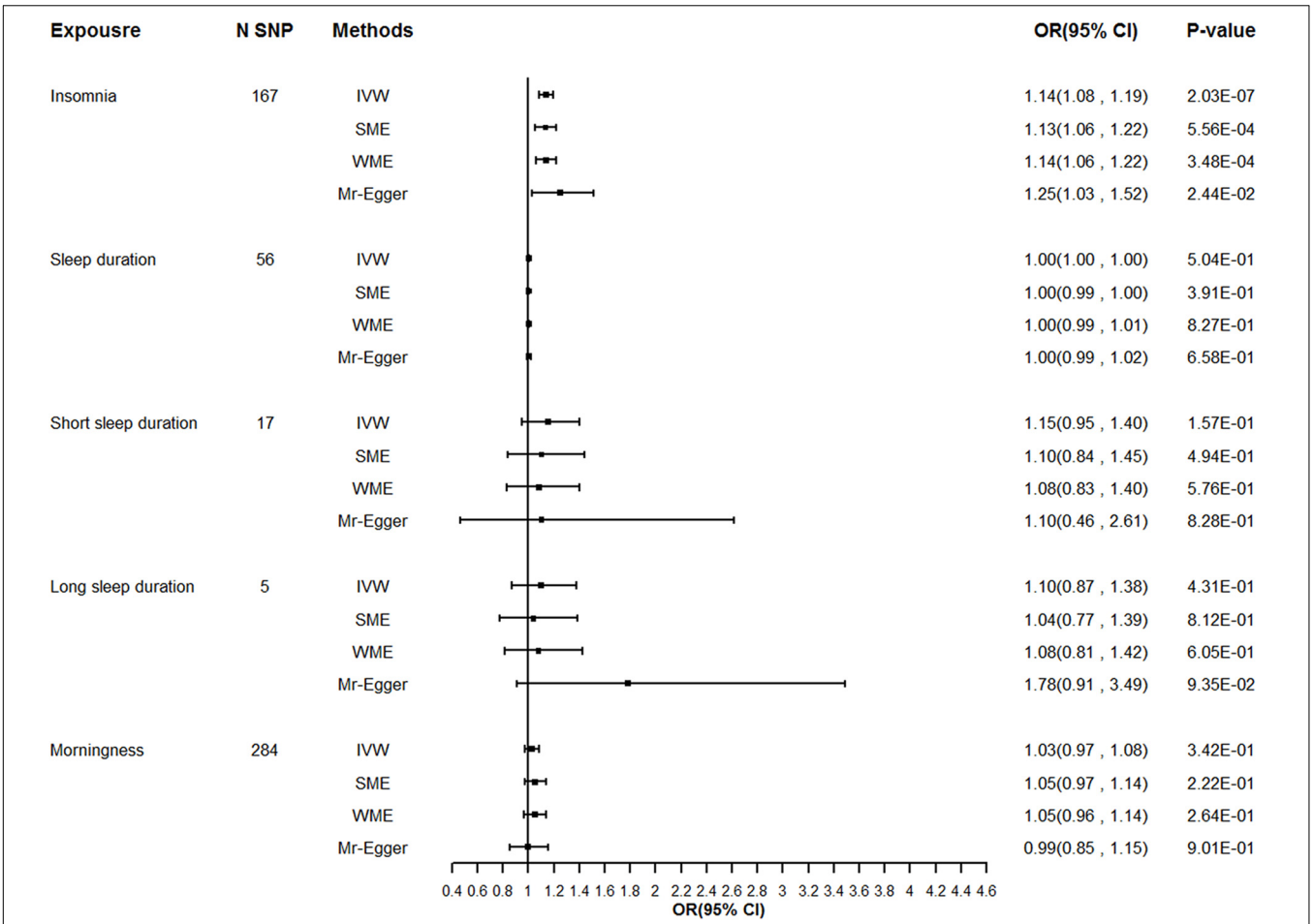

FIGURE 1 | Sensitivity analysis for the causality of sleep traits with the risk of T2DM. T2DM, Type 2 diabetes mellitus; N SNPs, number of SNPs retained and used in the MR analysis after filtered by individual Q outlier test; OR, odds ratio; CI, confidence interval.

causal relationship of T2DM with insomnia (**Supplementary Table 3**).

Sleep Duration and T2DM

After harmonization of the SNP effects in the two summary datasets (UKB for sleep duration and DIAGRAM for T2DM), there were 77, 27, and 6 SNPs used to instrument sleep duration, short sleep duration, and long sleep duration. After clumping for the selected SNPs, we removed rs7115226 and rs142180737 from the sleep duration and short sleep duration instrument set, respectively, due to their LD with other SNPs. We then queried the left SNPs for their associations with the phenotypes and filtered 10, 4, and 1 SNPs which were significantly correlated with the given confounders. Subsequently, the individual Q test detected 10 outliers and 5 outliers from the sleep duration and short sleep duration instrument set, respectively. The removal of these outliers resulting in a final number of 56, 17, and 5 SNPs that acted as instruments for sheep duration, short sleep duration, and long sleep duration, respectively. Evidence for heterogeneity provided by global Q test did not indicate any violation of the MR assumptions ($Q = 39.70$, $P = 0.94$ for sleep duration; $Q = 11.35$, $P = 0.79$ for short sleep duration; $Q = 4.37$, $P = 0.36$ for long sleep duration), and the directional pleiotropy estimated by MR-Egger test was consistent with the null for all of the models (intercept $P = 0.51$ for sleep duration; intercept $P = 0.92$ for short sleep duration; intercept $P = 0.14$ for long sleep duration). Resulting lists of instrument SNPs are given in **Supplementary Tables 4–6** and **Supplementary Figures 3–5**.

Little evidence for a causal effect of sleep duration on T2DM was observed either with the modified IVW (OR 1.00, 95% CI: 1.00–1.00, $P = 0.43$), first-order IVW or the pleiotropy robust methods (simple median, weighted median, MR-Egger) applied. Similarly, we found no causal relationship of either short sleep duration (OR 1.15, 95% CI: 0.96–1.38, $P = 0.09$) or long sleep duration with T2DM in the primary analysis (OR 1.10, 95% CI: 0.79–1.51, $P = 0.43$), and the sensitivity analysis yielded a similar pattern of results (**Table 2** and **Figure 1**).

Morningness and T2DM

We implemented MR analysis using 343 SNPs that are strongly associated with morningness as instruments. Among the instruments, 6 SNPs were ruled out after the clumping process,

19 SNPs were significantly associated with the confounders thus been excluded from the instrument set, and 34 potential outlier SNPs were picked out with the individual Q test. When restricting the MR model to the remaining 284 instruments, the global Q statistic indicated no notable heterogeneity ($Q = 228.37$; $P = 0.99$), and the MR-Egger test also suggested no horizontal pleiotropy (intercept $P = 0.62$). Details of the selected instruments are provided in **Supplementary Table 7** and **Supplementary Figure 6**. No significant causal relationship of genetically determined morningness with T2DM was suggested with the modified IVW (OR 1.03, 95% CI: 0.98–1.08, $P = 0.29$), and the effect estimates with other sensitivity analyses methods were largely unchanged compared to the main analysis (**Table 2** and **Figure 1**).

Other Analyses

We acquired quite similar results when replicating the causal estimations using the FinnGen T2DM cohort. After excluding the correlated and pleiotropic SNPs, the valid instruments for insomnia exhibited a significant effect on the risk of T2DM. Furthermore, we found suggestive evidence for the effect of sleep duration and morningness on T2DM risk with the modified IVW model, whereas the nominally significant effects disappeared when utilizing alternative MR models in the sensitivity analyses. One possible reason for the inconsistency is that the modified IVW method accounts for the sampling errors in the estimated effect sizes of the instruments on the exposure, therefore it is more powerful than other MR methods. Meanwhile, the pleiotropy-corrected approaches such as median-based estimations and MR-Egger regression introduce noise to the causal association, which means that the statistical power will be reduced (Bowden et al., 2019; Dudbridge, 2020). Since the causation of sleep duration and morningness on T2DM failed to reach the Bonferroni-adjusted significance, the suggestive evidence for the relationships should be investigated further (**Supplementary Figure 7**). Also, we acquired a similar pattern of results for the causality of sleep with Hb1Ac, FG, FI (**Supplementary Figure 8**).

DISCUSSION

The biological mechanisms of the relationship between sleep and diabetes are complicated, sleep habits and sleep disturbance, such as sleep duration, insomnia, and different kinds of circadian rhythms may act on T2DM through different biological mediations. Defining the relationship between these sleep traits and glycemic health is of great importance in understanding the detailed mechanisms and discovering potential treatment strategies for T2DM disease (Anothaisintawee et al., 2016; Ding et al., 2019). Therefore, we investigated the causality of different sleep traits with T2DM in this study and found significant evidence for an adverse effect of insomnia on T2DM risk. However, no evidence of causal association was found in other sleep-related phenotypes with T2DM. The study enhances the understanding of T2DM and opens new potential avenues for T2DM intervention and therapy, thus making a positive endeavor on public health and medical care.

Previous epidemiological studies provide inconsistent results in terms of the relationship between sleep and T2DM (Yaggi et al., 2006; Chaput et al., 2007; Shan et al., 2015). A cohort study observed that both short or long sleep duration increase the risk of developing T2DM independently (Chaput et al., 2007). While another study found that there is no significant association between sleep duration and a higher risk of T2DM after adjusting for possible factors (Hayashino et al., 2007). The mixed results can be attributed partly to the existence of unmeasured confounders and reverse causation, which has distorted effect estimates of the observational studies. However, when the studies are designed reasonably and the core assumptions are tested rigorously, valid causal evidence can always be achieved through the MR approaches. Considering this, we leverage a series of MR methods to investigate the causal relation of sleep with T2DM and acquire credible results.

Recent studies have offered some supporting evidence for the causal link between insomnia and T2DM. A longitudinal observation study demonstrated that insomnia patients were more likely to develop T2DM than the comparison cohort at about 16% higher. Furtherly, with an increased duration of insomnia symptoms, the risk of T2DM also tended to increase (Lin et al., 2018). Besides, an experimental study induced sleep deprivation in healthy individuals and found that insomnia led to hyperglycemia and insulin resistance, which was reversed subsequently when their sleep returned to normal (Benedict et al., 2011; Rao et al., 2015). Moreover, a large retrospective cohort study including more than 80,000 pre-diabetic people indicated that after adjusting for traditional risk factors, people who suffer from insomnia were 28% more likely to develop T2DM than those without insomnia symptoms (LeBlanc et al., 2018). The external evidence strengthens our confidence in the generalizability and validity of the present findings. All of these findings, including our study, have provided ample and credible evidence for a causal effect of insomnia on T2DM.

Furtherly, several potential mechanisms may contribute to the causal relationship between insomnia and T2DM. A previous study showed that insomnia may promote activation of the sympathetic nerve thereby increasing insulin resistance, which plays an important role in the risk of developing T2DM (Irwin et al., 2003). Furthermore, insomnia is also associated with the activation of chronic systemic inflammation, leading to the presence of insulin resistance which eventually develops into T2DM (Wang et al., 2013; Irwin et al., 2016). Despite these findings, further work to uncover the in-depth causal mechanisms is required.

It has long been uncertain whether the association between insomnia and T2DM is owed potentially to a negative causal relationship of insomnia on T2DM, and/or a relationship between T2DM and more serious insomnia symptoms. Some studies have reported that about half of the participants with T2DM also suffered from insomnia, indicating that insomnia itself is a further complication of T2DM (Luyster and Dunbar-Jacob, 2011; Koopman et al., 2020). To better understand the direction of the relationship, we also evaluated

the causal effect of T2DM on insomnia but found non-significant results. The potential reason for the observed T2DM-insomnia relationship maybe that observational studies cannot control for all confounding factors like physical activities, which related both sleep and T2DM and thus induce a major bias for the estimation (Semplonius and Willoughby, 2018).

This study has important strengths. First, this study explored the causality between a broad range of sleep-related traits and T2DM, contributing to filling the gaps over the existing observational studies and extending the relevant research notably. Second, we exploited the MR design and analysis to control for reverse causality and unmeasured confounding, which might lead to biased results in the traditional observational studies. Third, we took a series of steps to make sure the MR core assumptions are satisfied and the estimates are valid. Specifically, the instruments of sleep-related phonotypes came from large scale GWASs, which provided strongly and robustly associated SNPs and averted the potential weak instrument bias. Furthermore, the pleiotropic SNPs were identified and the validity of the reserved instruments was examined through different tests to correct for the bias deriving from pleiotropy. Fourth, the replication study for the relationship of sleep with another T2DM cohort ensured the solidity of the results. Last, the analyses included a large number of sample sizes and SNPs leveraging from GWASs, thereby offering sufficient statistical power for the causal estimation. These measures together help increase confidence in the results.

Nevertheless, our study has several limitations. First, we could not investigate the non-linear effects of sleep traits on T2DM due to the summary statistics we used, resulting in hardly assessable U-shaped associations. Second, the sleep-related data were obtained from self-reported questionnaires surveys, some of which may be less exact than directly objective measurements, such as sleep duration. Although previous studies have proved the validity of subjective sleep, people often overestimated their sleep duration time by up to 1 h, which may lead to imprecise results (Lockley et al., 1999). Further work can attempt to use the device-measured sleep duration to evaluate its association with T2DM. Moreover, recent studies indicated that there are gender or age differences in people with T2DM, but we could not investigate these differences due to the limitation of a lack of data (Lai et al., 2013).

Overall, we concluded from this study that there is strong evidence for a causal effect of insomnia on T2DM risk. The potentially modifiable sleep traits should be added to the prevention strategies of T2DM to improve public health. Moreover, this study highlights the need for further research regarding the mechanisms underlying these causal associations and leads to optimized medical care and management of T2DM.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. The data can be found at GWAS Catalog (https://www.ebi.ac.uk/gwas/), DIAGRAM (http://diagram-consortium.org/index.html), FinnGen (https://finngen.gitbook.io/documentation/), and MAGIC (https://www.magicinvestigators.org/).

AUTHOR CONTRIBUTIONS

TW, XG, and HS conceived and designed the whole study. YZ acquired and interpreted the data. XG, JW, and LL conducted the statistical analysis. XG and HS drafted the initial manuscript. TW supervised the whole study and attested to the integrity of the data and the accuracy of the data analysis. All authors reviewed and revised the manuscript, and approved the final version.

FUNDING

This study received funding from the National Natural Science Foundation of China (Grant No. 81872715).

ACKNOWLEDGMENTS

We sincerely acknowledge the original GWASs and the related consortiums (UKB, 23andMe, DIAGRAM, FinnGen, and MAGIC) for the collection and management of the large-scale data resources. We also want to acknowledge the participants and investigators of the studies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.607865/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 23 December 2020
doi: 10.3389/fgene.2020.608517

Association of Fibroblast Growth Factor 23 With Ischemic Stroke and Its Subtypes: A Mendelian Randomization Study

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Fibroblast growth factor 23 (FGF23), which is involved in the regulation of vitamin D, is an emerging independent risk factor for cardiovascular diseases. Previous studies have demonstrated a positive association between FGF23 and stroke. In this study, we aimed to assess the association of FGF23 with ischemic stroke and its subtypes by applying a Mendelian randomization (MR) framework. Five genetic variants obtained from a genome-wide association study involving 16,624 European subjects were used as valid instruments of circulating FGF23 levels. MR was applied to infer the causality of FGF23 levels and the risk of ischemic stroke using data from the MEGASTROKE consortium. Subsequently, several MR analyses, including inverse-variance weighted meta-analysis, MR-Egger, weighted median estimate (WME), MR Pleiotropy Residual Sum and Outlier were performed. The heterogeneity test analysis, including Cochran's Q, I^2 test and leave-one-out analysis were also applied. Furthermore, potential horizontal/vertical pleiotropy was assessed. Lastly, the power of MR analysis was tested. Three validated variants were found to be associated with circulating FGF23 levels and were used for further investigation. We found that high expression level of FGF23 was not associated with any ischemic stroke. However, a causal association between genetically predicted FGF23 levels and the risk of large-artery atherosclerotic stroke (LAS) was significant, with an odds ratio of 1.74 (95% confidence interval = 1.08–2.81) per standard deviation increase in circulating FGF23 levels. Our findings provide support for the causal association between FGF23 and LAS, and therefore, offer potential therapeutic targets for LAS. The specific roles of FGF23 in LAS and associated molecules require further investigation.

Keywords: ischemic stroke, large-artery atherosclerotic stroke, Mendelian randomization, fibroblast growth factor 23, MEGASTROKE consortium, vitamin D regulation

OPEN ACCESS

Edited by:
Lei Zhang,
Soochow University, China

Reviewed by:
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *21 September 2020*
Accepted: *01 December 2020*
Published: *23 December 2020*

Citation:
Zheng K, Lin L, Cui P, Liu T, Chen L, Yang C and Jiang W (2020) Association of Fibroblast Growth Factor 23 With Ischemic Stroke and Its Subtypes: A Mendelian Randomization Study. Front. Genet. 11:608517. doi: 10.3389/fgene.2020.608517

Abbreviations: AIS, any ischemic stroke; CES, cardioembolic stroke; CI, confidence interval; FGF23, fibroblast growth factor 23; GWAS, whole-genome association studies; IS, ischemic stroke; IVW, inverse-variance weighted; LAS, large-artery atherosclerotic stroke; MR, Mendelian randomization; MR-PRESSO, MR pleiotropy residual sum and outlier; OR, odds ratio; SNPs, single-nucleotide polymorphisms; SVS, small-vessel stroke; WME, weighted median estimate.

INTRODUCTION

Stroke is one of the major causes of death and long-term disability worldwide (GBD 2016 Stroke Collaborators, 2019). Approximately 70% of strokes are ischemic stroke (IS), which is usually caused by the occlusion of the middle cerebral artery (The GBD 2016 Lifetime Risk of Stroke Collaborators et al., 2018). The increasing global burden and limited therapy options for stroke have led to urgent demands for more effective preventive and therapeutic measures (Avan et al., 2019).

Fibroblast growth factor 23 (FGF23), a bone-derived hormone, plays an important role in the regulation of calcium, phosphate, and active vitamin D levels (Vervloet, 2019). Recently, increasing evidence has indicated a strong relationship between FGF23 and cardiovascular diseases (Panwar et al., 2018). Several studies have demonstrated that an increased circulating FGF23 level was correlated with a higher risk (Wright et al., 2014) and a poorer outcome (Seiler et al., 2010) for stroke. Other studies have indicated that plasma FGF23 was associated with carotid atherosclerosis in patients who suffered from stroke as well as in the normal population (Shah et al., 2015; Yan et al., 2017; Chang et al., 2020). Meanwhile, higher FGF23 level also correlated with increased instability of carotid plaques (Biscetti et al., 2015). However, a case–cohort study indicated that there was a graded association of FGF23 with the risk of cardioembolic stroke, but there was no significant association between FGF23 and other IS subtypes or with hemorrhagic strokes in community-dwelling adults (Panwar et al., 2015). In addition, a Multi-Ethnic Study of Atherosclerosis (MESA) showed that FGF-23 was not associated with carotid intima-media thickness or stroke (Kestenbaum et al., 2014). Until now, it is unclear whether FGF23 levels are causally associated with risk of IS. Therefore, in this study, we aimed to investigate the possible causal relationships of FGF23 with IS and its subtypes and the potential research value of FGF23.

Recently, with the development of whole-genome association studies (GWAS), an increasing number of single-nucleotide polymorphisms (SNPs) related to human diseases have been identified (Pei et al., 2019; Liu et al., 2020a,b). Meanwhile, Mendelian randomization (MR) has been widely used for causal inference (Davies et al., 2018; Larsson et al., 2019). Since genetic variants such as SNPs are randomly allocated during conception and the genotypes are determined in the zygote stage, the MR framework can detect causality by minimizing the impacts of confounders and reverse causality (Davies et al., 2018). In this study, an MR design was used to investigate the association of circulating FGF23 levels with IS and its subtypes.

MATERIALS AND METHODS

Study Design

MR was performed based on three primary assumptions as described previously (Yang et al., 2019; He et al., 2020). The first assumption was that the SNPs identified to be the instrumental variables (IVs) should be significantly related to the exposure (FGF23) (**Figure 1**). The second assumption was that genetic variants should be unrelated to the confounding factors of an outcome (IS) (Liu et al., 2018). The third assumption was that the genetic variants must only affect the risk of the disease (IS) through the exposure (FGF23) but not via other routes. Meanwhile, both the second and third assumptions were identified to be independent of pleiotropic effects. As the large-scale datasets from the published genome-wide meta-analysis were publicly available, no additional ethical approval was required.

Selection of SNPs and Validation

The circulating FGF23-associated variants were collected from a meta-analysis comprising 16,624 individuals of European-descent after excluding those whose estimated glomerular filtration rate was less than 30 mL/min/1.73 m^2 (Robinson-Cohen et al., 2018). The selected genetic instruments from the GWAS of FGF23 were composed of top five significant ($P < 5 \times 10^{-8}$) SNPs near *CYP24A1*, *ABO*, *RGS14*, *LINC01506*, and *LINC01229* genes, and were located in five genomic regions, accounting for approximately 3% of FGF23 variation. Detailed information is provided in **Supplementary Table 1**. The strength of the IVs was evaluated using the mean F-statistic, defined as the ratio of the mean square of effect size to the mean square of standard error for each genetic instrument (Bowden et al., 2016b). The rule of thumb threshold of F value is greater than 10 to avoid potential bias from weak instruments (Burgess and Thompson, 2011). The F statistics for each of the five instruments was greater than 10 (**Supplementary Table 1**). Subsequently, we verified the independence among these SNPs by linkage disequilibrium ($R^2 < 0.1$) through the 1000 Genomes Phase 3 (European) reference panel.

Data Sources

The summary-level data for IS and its subtypes were obtained from the MEGASTROKE consortium. Any ischemic stroke (AIS) group (n = 34,217), regardless of the subtype of European ancestry, was selected and compared with 406,111 control subjects. The three main subtypes of IS were acquired mainly on the basis of the Trial of ORG 10172 in Acute Stroke Treatment criteria, including LAS (n = 4,373), cardioembolic stroke (CES; n = 7,193), and small-vessel stroke (SVS; n = 5,386) (Malik et al., 2018). As all the five genetic instruments associated with FGF23 levels were available in the MEGASTROKE consortium, no proxy variant was needed. The MEGASTROKE-matched data are shown in **Supplementary Table 2**.

Statistical Analysis

The principal analyses assessing the causal associations of FGF23 with IS and its subtypes were performed using the inverse-variance-weighted (IVW) method (Davies et al., 2018). For each of the five SNPs, we computed an Wald's ratio estimates by dividing the beta-coefficients (log odds ratio) for the SNP–stroke association by the beta coefficient for the SNP–FGF23 association. Moreover, to improve the reliability of causal effect estimates, we also carried out the MR Pleiotropy Residual Sum and Outlier (MR-PRESSO) test (Verbanck et al., 2018).

To further evaluate the impact of potential pleiotropy on causal estimates, we performed sensitivity analyses using several

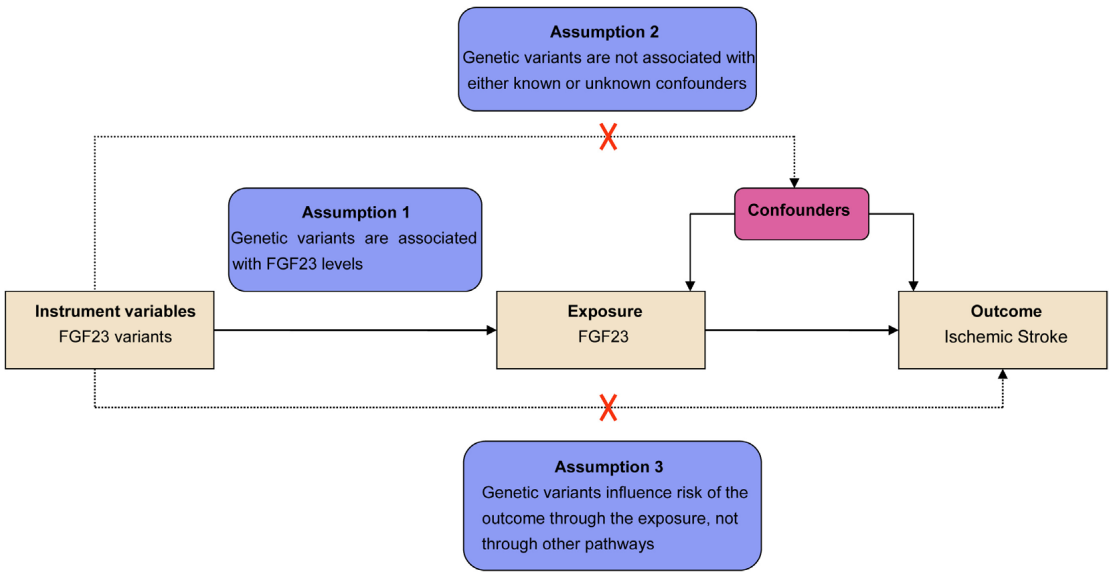

FIGURE 1 | Assumptions for the Mendelian randomization (MR) and study design. The MR was based on three principal assumptions, including: (1) the genetic variants selected to be instrumental variables should be correlated with the exposure [fibroblast growth factor 23 (FGF23) levels]; (2) the genetic variants should be unrelated to confounding factors; (3) genetic variants must influence the risks of the outcome [ischemic stroke (IS)] only through the exposure (FGF23 levels).

other methods. First, we used the MR-Egger regression to assess the presence of directional pleiotropy (Bowden et al., 2015). A statistically significant intercept term from the MR-Egger regression suggests the possibility that genetic variants may not affect the outcome via the exposure of interest. We also conducted the weighted median estimate (WME) (Bowden et al., 2016a), which provides an effective estimate of causality when at least 50% of genetic IVs is valid. Furthermore, to evaluate the potential heterogeneity due to pleiotropy or other causes, we conducted the Cochran's *Q*-test (together with the I^2 statistic), as reported in a previous study (Liu et al., 2013). In addition, we selected the leave-one-out sensitivity method to sequentially remove each SNP from the MR analysis and assess the impact of single-gene variants on the causal estimates (He et al., 2020). Moreover, vertical pleiotropy was assessed using the Steiger test to verify the causal direction between FGF23 and stroke (Hemani et al., 2017).

Lastly, we excluded those SNPs associated with potential confounders (Bonferroni correction, $P < 0.05/5$ SNPs) by using PhenoScanner V2 in March 2020 (Staley et al., 2016; Kamat et al., 2019), and repeated the MR analysis using the IVW, MR-Egger regression, and weighted-median estimate. To correct for potential pleiotropic bias, we performed multivariable MR following Sanderson's method (Sanderson et al., 2020). We also calculated the power of MR estimates using the mRnd platform[1] and the effect size based on a 5% type 1 error rate and enough power (>80%). Statistical analyses were performed using Mendelian Randomization (version 0.4.1) (Yavorska and Burgess, 2017) and TwoSampleMR (version 0.5.1) (Hemani, 2019) on R 3.6.2 (The R Foundation for Statistical Computing, Vienna, Austria). All statistical tests were two-sided and the statistical significance was set at the level of $P < 0.05$.

[1]https://shiny.cnsgenomics.com/mRnd/

RESULTS

Primary MR Analysis of the Association of FGF23 With IS and Its Subtypes

As listed in **Supplementary Table 1**, five SNPs were used as the IVs for FGF23 levels. We identified significant association of high FGF23 levels with increased LAS risk (OR = 1.94, 95% CI 1.35–2.27; p = 3.04E−04) but not with the other IS subtypes or AIS using the IVW method (**Supplementary Table 4**). However, a potential heterogeneity was identified using the Cochran's Q test and I^2 for causal estimates of five SNPs in the conventional IVW model for AIS (14.34, p = 0.0063, I^2 = 72.10%), LAS (14.12, p = 0.0069, I^2 = 71.70%), and CES (16.79, p = 0.0021, I^2 = 76.20%) (**Supplementary Table 4**), suggesting the possibility that the obtained effect estimates of these associations from the IVW method may be biased by outlier SNPs.

Sensitivity Analysis of the Association of FGF23 With IS and Its Subtypes

To assess the robustness of the causal effect of FGF23 on IS and its subtypes, we performed several sensitivity analyses as follows. First, WME suggested significant association between FGF23 levels and LAS risk with an odds ratio of 1.75 (95% CI 1.06–2.90; p = 0.029), but not with the other IS subtypes or AIS (**Supplementary Table 4**). Second, the intercept term from MR-Egger analysis revealed no evidence of directional pleiotropy in the analysis of LAS (p = 0.81), SVS (p = 0.97), CES (p = 0.22), or AIS (p = 0.26).

However, MR-PRESSO test identified horizontal pleiotropic outliers in AIS (p = 0.0066), LAS (p = 0.0134), and CES (p = 0.0034). The leave-one-out permutation analysis further indicated that the direction and precision of the genetics estimates between increased FGF23 levels and risk of IS and its subtypes changed largely with the deletion of *rs2769071* (**Supplementary Table 7**).

We next searched the PhenoScanner V2 database (Kamat et al., 2019) for possible pleiotropic associations of individual SNPs with risk factors for IS. Among the FGF23-associated SNPs, associations were observed for the *rs2769071* variant with low-density lipoprotein (P = 3.06E−10), total cholesterol (P = 7.48E−13), diastolic blood pressure (P = 2.80E−10), and type 2 diabetes (P = 2.30E−05). The *rs11741640* variant was significantly related to self-reported hypertension (P = 2.22E−04) and alcohol intake frequency (P = 3.74E−03). Detailed information is provided in **Supplementary Table 3**.

In total, we excluded two SNPs (*rs2769071* near the *ABO* gene and *rs11741640* near the *R*GS14 gene) that were potentially associated with at least one secondary phenotype and repeated the MR analyses. Based on the remaining three effective SNPs, FGF23 levels were significantly associated with LAS but not with the other IS subtypes or AIS (**Figure 2**). In the standard MR analysis-IVW method, the odds ratios per standard deviation of the genetically predicted increase in FGF23 levels was 1.74 (95% CI 1.08–2.81; p = 0.023) for LAS. Importantly, the results obtained for LAS were similar in the WME analysis (OR = 1.76, 95% CI = 1.04–2.99; p = 0.036), while the Egger estimate was less precise despite having the same direction and a similar size (OR = 1.80, 95% CI = 0.26–12.46; P = 0.549). The single variant causal ratio and results of all three variants for the association of FGF23 and LAS are shown in **Supplementary Table 6**. No heterogeneity among these three instruments was found using Cochran's Q analysis (Q = 0.02, P = 0.992, I^2 = 0.00%) in LAS (**Supplementary Table 5**). The leave-one-out sensitivity analysis also showed the same direction and estimates between the increased FGF23 levels and the risk of LAS, although the deletion of IV *rs17216707* near *CYP24A1* gene was not statistically significant (**Supplementary Table 8**). No directional pleiotropy in LAS was found according to the Egger intercept test (–0.003, 95% CI = –0.150 to 0.145; P = 0.970). Considered the potential effects of obesity and smoking-the two most important confounders for both heart disease and circulating metabolites, we then applied the multivariable MR analysis. The BMI or smoke adjusted data by three validated instruments also verifies our results (**Supplementary Table 9**).

Besides, the direction of causality inferred by the Steiger test showed that the SNPs–FGF23 association (r^2 = 1.04E−02) was more significantly correlated ($p_{Steiger} = 3.20 \times 10^{-14}$) than the SNPs-LAS association (r^2 = 1.26E−05), suggesting that higher FGF23 levels leads to the increased risk of LAS, consistent with expectation. We had enough power (>80%) to detect 1.59 OR of LAS risk per SD increased log FGF23 levels (cases n = 4,373; non-cases n = 406,111); and the power of causal estimate for FGF23 to LAS here was 94%.

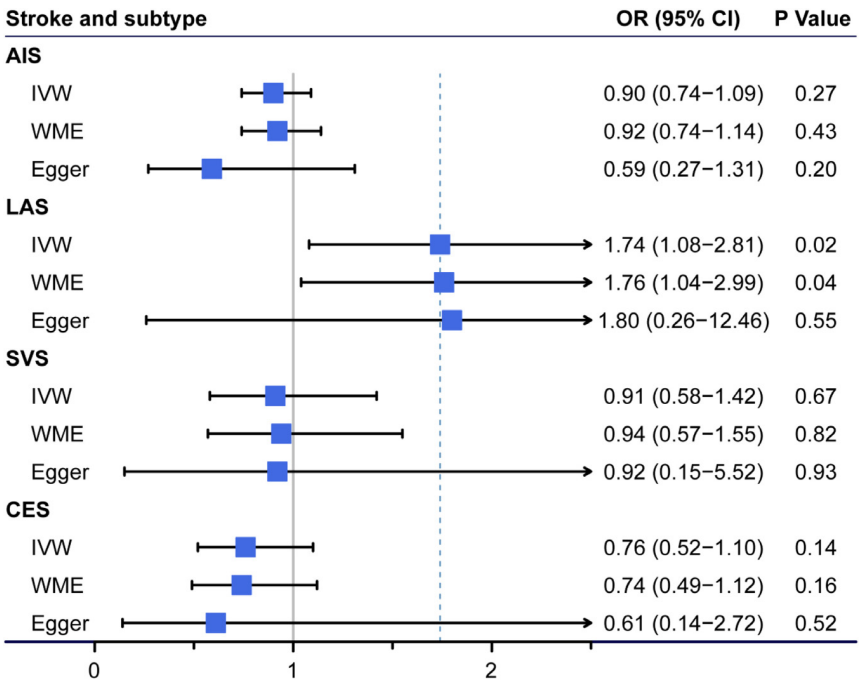

FIGURE 2 | Association of genetically predicted circulating fibroblast growth factor 23 (FGF23) levels with ischemic stroke (IS) and other stroke subtypes. The odds ratio (OR) represented at the center of each box was the risk of genetically predicted one standard deviation increase in FGF23 levels. AIS, any ischemic stroke; LAS, large-artery atherosclerotic stroke; SVS, small-vessel stroke; CES, cardioembolic stroke; CI, confidence interval; IVW, Mendelian randomization (MR) inverse-variance weighted method; WME, weighted median estimate; Egger, the MR-Egger method.

DISCUSSION

Until now, it has remained unclear whether the circulating FGF23 levels is genetically associated with risk of IS. In this study, we found a potential association of genetically predicted high levels of FGF23 and the increased risk of LAS. The risk of LAS increased by 74% with a 23 pg/mL per SD increase in circulating FGF23 levels. This effect size was similar to previously reported sizes of low-density lipoprotein cholesterol (OR = 1.28, 95% CI = 1.07–1.53) (Hindy et al., 2018), fasting blood glucose (OR = 1.42, 95% CI = 1.08–1.85) (Larsson et al., 2017), systolic blood pressure (OR = 1.56, 95% CI = 1.37–1.78) (Parish et al., 2019), and waist-to-hip ratio (OR = 1.75, 95% CI = 1.44–2.13) (Marini et al., 2020).

Our results are consistent with those of previous epidemiological studies (Seiler et al., 2010; Shah et al., 2015; Yan et al., 2017; Chang et al., 2020). In patients with acute IS, the plasma FGF23 concentration was positively correlated with the presence and burden of intracranial carotid atherosclerosis (Chang et al., 2020). FGF23 seems to be mainly involved in vessel calcification, vascular stiffness, and inflammation (Mirza et al., 2009; Kim et al., 2011; Libby et al., 2019; Vervloet, 2019). In mice, excessive plasma FGF23 directly stimulates the production of inflammatory factors such as interleukin-6 (Singh et al., 2016). Meanwhile, inflammatory factors in turn promote the production of FGF23 and exacerbate LAS progression (Feger et al., 2017; Durlacher-Betzer et al., 2018; Egli-Spichtig et al., 2019; McKnight et al., 2020). Our analysis implied that reducing FGF23 levels may be a potential therapeutic strategy for IS, especially for LAS. However, the potential mechanisms that correlate FGF23 with LAS still require further investigations.

Considering the role of FGF23 in regulation of vitamin D levels, some previous studies argued that the pathophysiological effects of FGF23 were partially through decreasing the level of active vitamin D. FGF23 inhibits the functions of vitamin D by promoting its degradation via 24-hydroxylase encoded by the *CYP24A1* gene and inhibiting its production by 1α-hydroxylase encoded by the *CYP27B1* gene (Vervloet, 2019). The physiological roles of vitamin D, including anti-inflammation and inhibition of artery calcification, are contrary to the effects of FGF23 (Han et al., 2016; Wang et al., 2018). In addition, vitamin D receptor activation enables the recovery of αKlotho, an anti-aging protein, while this recovery is inhibited in an inflammatory environment (Lim et al., 2012). FGF23 induces vessel damage and inflammation through an αKlotho-independent pathway when αKlotho is insufficient (Komaba and Fukagawa, 2012; Navarro-González et al., 2014; Krick et al., 2018). The aforementioned studies collectively suggest that proper calcitriol supplements might reduce the risk of LAS in people or those with intracranial atherosclerosis. The effects of calcitriol supplements involved in the process of vasomotion and immune modulation have been reported by several studies (Chitalia et al., 2014; Ojeda López et al., 2018). In this study, the validated genetic variant (*rs17216707*) near the *CYP24A1* gene showed a strong association with LAS (**Supplementary Table 6**), which supports the critical role of active vitamin D in the regulation of FGF23 level and the risk of LAS.

To our knowledge, this is the first MR study to clarify the genetic causalities between FGF23 levels and IS with MR methods. Considering the ethical care of patients and the high cost of randomized controlled trials, the MR framework is effective in the discovery of potential targets of intervention and can indicate potential therapeutic strategies. In addition, our findings in this study were especially prospective, as analyzed data were extracted from the database with the largest number of participants currently known.

However, this study also has several limitations. The different methods for FGF23 measurement could have potentially caused bias in the results. The FGF23 levels were detected in two forms: intact and C-terminal FGF23 (Robinson-Cohen et al., 2018). In patients with chronic kidney diseases, the production of FGF23 (intact FGF23) was separated from its cleaved form (C-terminal FGF23) (Edmonston and Wolf, 2020). Meanwhile, the FGF23-associated GWAS data were obtained from individuals whose estimated glomerular filtration rate was above 30 mL/min/1.73 m^2. In addition, log-transformed FGF23 levels, applied in each cohort and the following meta-analysis, could reflect the relative change in circulating FGF23 levels.

In our study, only three SNPs accounting for 1.13% of the total variation in FGF23 levels were identified as genetic instruments, causing a possible limitation in the results. Thus, additional influential loci are necessary as IVs in the future if new GWAS data are available. As this limited number of IVs restricted the application of PRESSO, the sensitive analysis of potential horizontal pleiotropy could not be performed completely. However, similar results were obtained from WME and IVW estimate, while no signs of heterogeneity (Cochran's Q test) and directional pleiotropy (MR-Egger intercept analysis) were discovered. Therefore, the above results indicated that confounders are unlikely to explain the observed associations.

Population stratification also potentially restricted the accuracy of this study. The MR inference depended on three instrumental assumptions that rely on the same genetic backgrounds in the exposure and outcome data. In this study, we used European-descent genotypes to assess the association between FGF23 levels and IS. This result may be altered in different populations due to different genetic backgrounds, such as linkage disequilibrium. Moreover, the MR framework was not able to infer the association during specific periods of the life cycle or conditions. Thus, further animal experiments and possible intervention trials are needed.

In summary, our results provide support for a suggestive causal association between higher circulating FGF23 levels and an increased risk of LAS. Our findings may offer new therapeutic targets for LAS. Further studies are necessary to investigate whether genetic variants at or near the *CYP24A1* gene influence the risk of LAS through downstream effects or pathways related to vitamin D.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

LL, CY, KZ, and PC contributed to the conception and design of the study. LL collected data and performed the MR framework. KZ and LL drafted the manuscript. KZ, LL, PC, TL, LC, CY, and WJ participated in the analysis of the results and provided critical review. All authors approved the submitted article.

FUNDING

This work was supported by the National Natural Science Foundation of China (81801197 to WJ) and the Natural Science Foundation of Tianjin (19JCQNJC10500 to WJ).

ACKNOWLEDGMENTS

We sincerely thank the MEGASTROKE consortium and Cassianne Robinson-Cohen for the FGF23-associated summary statistics. We also thank the Global Lipids Genetic Consortium, the Meta-Analyses of Glucose and Insulin-related traits Consortium, the Diabetes Genetics Replication and Meta-analysis Consortium, the Genetic Investigation of Anthropometric Traits Consortium, the International Consortium of Blood Pressure, the CARDIoGRAMplusC4D Consortium, and the UK Biobank for other GWAS summary statistics.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.608517/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 13 January 2021
doi: 10.3389/fgene.2020.597876

Type 2 Diabetes and Glycemic Traits Are Not Causal Factors of Osteoarthritis: A Two-Sample Mendelian Randomization Analysis

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Background: It remains unclear whether an increased risk of type 2 diabetes (T2D) affects the risk of osteoarthritis (OA).

Methods: Here, we used two-sample Mendelian randomization (MR) to obtain non-confounded estimates of the effect of T2D and glycemic traits on hip and knee OA. We identified single-nucleotide polymorphisms (SNPs) strongly associated with T2D, fasting glucose (FG), and 2-h postprandial glucose (2hGlu) from genome-wide association studies (GWAS). We used the MR inverse variance weighted (IVW), the MR–Egger method, the weighted median (WM), and the Robust Adjusted Profile Score (MR.RAPS) to reveal the associations of T2D, FG, and 2hGlu with hip and knee OA risks. Sensitivity analyses were also conducted to verify whether heterogeneity and pleiotropy can bias the MR results.

Results: We did not find statistically significant causal effects of genetically increased T2D risk, FG, and 2hGlu on hip and knee OA (e.g., T2D and hip OA, MR–Egger OR = 1.1708, 95% CI 0.9469–1.4476, $p = 0.1547$). It was confirmed that horizontal pleiotropy was unlikely to bias the causality (e.g., T2D and hip OA, MR–Egger, intercept = −0.0105, $p = 0.1367$). No evidence of heterogeneity was found between the genetic variants (e.g., T2D and hip OA, MR–Egger $Q = 30.1362$, $I^2 < 0.0001$, $p = 0.6104$).

Conclusion: Our MR study did not support causal effects of a genetically increased T2D risk, FG, and 2hGlu on hip and knee OA risk.

Keywords: Osteoarthritis, type 2 diabetes, fasting glucose, 2-h postprandial glucose, Mendelian randomization

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *22 August 2020*
Accepted: *07 December 2020*
Published: *13 January 2021*

Citation:
Cui Z, Feng H, He B, Xing Y, Liu Z and Tian Y (2021) Type 2 Diabetes and Glycemic Traits Are Not Causal Factors of Osteoarthritis: A Two-Sample Mendelian Randomization Analysis. Front. Genet. 11:597876. doi: 10.3389/fgene.2020.597876

INTRODUCTION

Osteoarthritis (OA) and type 2 diabetes (T2D) are two pandemic chronic diseases and have significant impact on quality of life, social expenditure, and life expectancy (Martel-Pelletier et al., 2016; Hunter and Bierma-Zeinstra, 2019). OA is the most common chronic joint disease, and its main characteristic is the loss of chronic irreversible articular cartilage (Martel-Pelletier et al., 2016; Hunter and Bierma-Zeinstra, 2019). T2D is one category of diabetes, which is a chronic metabolic

syndrome characterized by increased blood glucose levels as a consequence of insulin resistance (Chatterjee et al., 2017). The epidemiological association between T2D and OA was confirmed by different analyses showing that the prevalence of OA is significantly higher among patients with T2D than among those without the condition, and vice versa (Cheng et al., 2012; Alenazi et al., 2019). Cheng et al. (2012) found that the unadjusted prevalence of arthritis was 48.1% among United States adults with diabetes. Alenazi et al. (2019) also reported that the overall prevalence of T2D among patients with OA was about 16.6%. OA and T2D share common risk factors, such as obesity, physical activity, and genetic factors (Martel-Pelletier et al., 2016; Reyes et al., 2016; Chatterjee et al., 2017), which could explain why there was increasing prevalence of OA in T2D. However, the strength of such an association may have been heterogeneous by considering the heterogeneity of age, sex, ethnic group, duration of T2D, and body weight (Cannata et al., 2020). Since the exact mechanism involving T2D and OA was still debatable, it was controversial whether there was a causal relationship between T2D and OA. Recognizing the causal associations between the two diseases would have clinical implications for diseases management and be of great value for the design of specific therapeutic interventions targeting T2D and OA main pathogenic hallmarks.

Observational studies to estimate the causal inference have numerous inherent limitations, such as remaining limited to known and properly measured confounders (Greenland and Morgenstern, 2001). Therefore, we used Mendelian randomization (MR), an application of the method of instrumental variables (IVs), to the analysis of genetic data to assess the causal associations of T2D and related glycemic traits [fasting glucose (FG) and 2-h postprandial glucose (2hGlu)] with hip and knee OA. The genetic variants in MR are available with the progress of genome-wide association studies (GWAS) and high-throughput genomic technologies. MR method aims to give estimates of the causal effect free from biases due to confounding. Confounding factors are mitigated due to random assortment of genetic variants during meiosis yielding a random distribution of genetic variants in a population. MR method can also prevent reverse causality because genetic variants are fixed at conception and cannot be affected by disease processes. Moreover, the causal associations tested based on the MR method likely reflect the longstanding effect of exposures on outcomes for the genetic instruments that generally represent lifelong exposures (Emdin et al., 2017). In this study, we used single-nucleotide polymorphisms (SNPs) strongly associated with T2D and glycemic traits as IVs. We performed a two-sample MR and used statistical methods to obtain quantitative estimates to investigate the effect of T2D, FG, and 2hGlu upon hip and knee OA.

MATERIALS AND METHODS

SNP Selection

In our study, we selected SNPs as IVs for all exposures (T2D, FG, and 2hGlu) and outcomes (hip and knee OA) from the IEU GWAS database, a database of genetic associations from GWAS summary datasets[1] (Hemani et al., 2018). When target SNPs were not available in the outcome study, we used proxy SNPs that were in high linkage disequilibrium (LD) ($r^2 > 0.8$) with the SNPs of interest. We selected the reference sample formed by the European ancestral individuals from the 1000 genomes project to estimate the allele frequency and LD level[2] (1000 Genomes Project Consortium et al., 2010). The palindromic SNPs with intermediate allele frequencies (palindromic SNPs referred to the SNPs with A/T or G/C alleles and "intermediate allele frequencies" referred to 0.01 < allele frequency < 0.30) were excluded from the above selected instrument SNPs. SNPs with a minor allele frequency (MAF) of < 0.01 were also excluded. We also calculated the F statistics for the SNPs to measure the strength of the instruments. IVs with an F statistic less than 10 were excluded and were often labeled as "weak instruments" (Burgess et al., 2015). These rigorously selected SNPs were used as the final instrumental SNPs for the subsequent MR analysis.

Data Source

Single-nucleotide polymorphisms associated with T2D were derived from a meta-analysis of GWAS in a very large sample of T2D (62,892 cases and 596,424 controls) of European ancestry (Xue et al., 2018). SNPs associated with FG and 2hGlu were derived from genome-wide association meta-analyses of up to 133,010 and 42,854 individuals, respectively, with males and females, of European ancestry without diabetes, performed by the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) (Scott et al., 2012). The summary-level data for the impact of the exposures-associated SNPs on hip and knee OA were extracted from a genome-wide meta-analysis for OA of European descent with the UK Biobank and Arthritis Research UK Osteoarthritis Genetics (arcOGEN) resources (Tachmazidou et al., 2019). The self-reported OA status established during interview with a nurse and the Hospital Episode Statistics International Classification of Diseases, 10th edition (ICD 10) primary and secondary codes were used in the UK Biobank cases (Tachmazidou et al., 2019). The arcOGEN case samples were collected on the basis of clinical evidence of disease to a level requiring joint replacement or radiographic evidence of disease (Kellgren–Lawrence grade ≥ 2) (Tachmazidou et al., 2019). The detailed characteristics of GWAS associated with exposures (T2D, FG, and 2hGlu) and outcomes (hip and knee OA) are shown in **Supplementary Table 1** in the **Supplementary Material**.

Effect Size Estimate

We applied the two-sample MR to assess the role of exposures (T2D, FG, and 2hGlu) in the susceptibility of outcomes (hip and knee OA) (Hemani et al., 2018). We assessed the independent association of SNPs with T2D, FG, and 2hGlu and selected SNPs that were strongly associated ($p < 5E\text{-}08$) and independent inheritance ($r^2 < 0.01$) without any LD with the exposures. Then, we obtained the effect estimates for the selected SNPs

[1] https://gwas.mrcieu.ac.uk/

[2] http://www.internationalgenome.org/

on hip and knee OA from genome-wide meta-analysis for OA in 2019. The causal associations between exposures (T2D, FG, and 2hGlu) and outcomes (hip and knee OA) were estimated with inverse variance weighted (IVW), MR–Egger, and weighted median (WM) (Burgess et al., 2013; Bowden et al., 2015). The IVW method uses a meta-analysis approach to combine the Wald ratios of the causal effects of each SNP and can provide the most precise estimates. However, it can be influenced by invalid IVs and pleiotropic effects. The WM estimate provides a reliable effect estimate of the causal effect when at least 50% of the weight in the analysis comes from effective IVs. MR–Egger regression is based on the assumption that the pleiotropic associations are independent, performs a weighted linear regression of the outcome coefficients on the exposure coefficients. MR–Egger estimates may be inaccurate and can be strongly influenced by outlying genetic variants (Bowden et al., 2016). We also performed a recently developed method called the Robust Adjusted Profile Score (MR.RAPS) to estimate the causal effects, which can lead to a considerably higher statistical power than the conventional MR analysis can, which only uses a small set of strong instruments (Zhao et al., 2019). MR.RAPS considers the measurement error in SNP-exposure effects, is unbiased when there are many weak instruments, and is robust to systematic and idiosyncratic pleiotropy (Zhao et al., 2019).

Sensitivity Analyses

We used the IVW, WM, and maximum likelihood methods to evaluate the heterogeneity between SNPs. The heterogeneity was quantified by Cochran Q statistics and I^2 statistics (Bowden et al., 2016). Moreover, we used the MR–Steiger method to compute the amount of variance instrumenting SNPs explained in the exposure and outcome variable and test if the variance in the outcome is less than the exposure. In case of a true causal direction, SNPs should be more predictive of the exposure than of the outcome (Hemani et al., 2017). Pleiotropy refers to the phenomenon in which a single locus affects multiple phenotypes. Horizontal pleiotropy arises when a genetic variant associates with more than one phenotype on separate pathways, which can invalidate the results from MR analyses (Stearns, 2010; Larsson et al., 2019). In order to explore and adjust for horizontal pleiotropy, we evaluated the pleiotropic effects of T2D and glycemic traits on weight-associated factors, including body mass index (BMI), weight, and obesity, as these confounding effects might distort the effects of T2D and glycemic traits on OA. Summary statistics for BMI were extracted from studies performed by the Genetic Investigation of ANthropometric Traits (GIANT) consortium (Locke et al., 2015), weight (male and female) from GIANT Consortium (Randall et al., 2013), and obesity from GIANT Consortium (Berndt et al., 2013). The detailed characteristics of studies associated with confounding factors are shown in **Supplementary Table 1**. We assessed the potential associations between SNPs that were extracted for the MR analysis and those confounding factors. Associations of the SNPs with the four confounding factors were considered statistically significant at a Bonferroni-corrected $p < 0.05/(4 \times N)$, with N representing the number of SNPs in each exposure trait. In addition to evaluating the associations with the risk factors, we performed MR–Egger regression to explore and adjust for horizontal pleiotropy, which was a method that can provide evidence for confounders that would distort the MR results. The intercept represents the average pleiotropic effect across the genetic variants.

Power Assessment

We also used an online tool mRnd[3] to calculate the power to detect causal effect. The equations using an approximate linear model on the observed binary (0–1) scale were adapted for binary outcomes, which needs several parameters to estimate. These parameters include the proportion of phenotypic variation explained by IV SNPs, the effect size of the exposure to the outcome at the epidemiological level, sample size, and standard deviation of exposure and outcome (Brion et al., 2013).

The results of the MR analyses were considered statistically significant at a Bonferroni-corrected $p < 0.025$ (e.g., 0.05/2 outcomes). All statistical tests were two-sided and performed using the "TwoSampleMR" package for R language, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Causality Between T2D and OA

For T2D, we used 37 genome-wide significant ($p < 5E-08$) SNPs associated with increased T2D risk identified in the largest meta-analysis of T2D GWAS (Xue et al., 2018). For each of the susceptibility variants for T2D, we sought summary-level data for OA from the GWAS performed by arcOGEN Consortium. After removing two T2D variants (rs6494307, rs7619041) that were palindromic with intermediate allele frequencies, 35 SNPs remained to perform the MR analysis for hip and knee OA. None of the proxy SNPs were used in the analysis. For these IVs, all the F values were larger than 10, ranging from 30.6746 to 256.3266, with an average F value of 53.4983 (**Supplementary Table 2**).

In our analysis using the full set of 35 SNPs, we did not find causal associations of per unit increase in the log-odds of having T2D with risk changes of having OA, based on the IVW, WM, MR–Egger regression, and MR.RAPS methods at the Bonferroni-corrected significance threshold $p < 0.025$ (e.g., 0.05/2). (For hip OA, MR–Egger OR = 1.1708, 95% CI 0.9469–1.4476, $p = 0.1547$; IVW OR = 1.0022, 95% CI 0.9329–1.0767, $p = 0.9517$; WM OR = 1.0454, 95% CI 0.9369–1.1664, $p = 0.4274$; MR.RAPS OR = 0.9957, 95% CI 0.9239–1.0731, $p = 0.9094$. For knee OA, MR–Egger OR = 0.9046, 95% CI 0.7880–1.1085, $p = 0.4426$; IVW OR = 0.9809, 95% CI 0.9265–1.0385, $p = 0.5084$; WM OR = 1.0053, 95% CI 0.9213–1.0971, $p = 0.9050$; MR.RAPS OR = 0.9833, 95% CI 0.9247–1.0457, $p = 0.5920$.) (**Table 1** and **Figure 1**). We assessed the horizontal pleiotropy by checking the association of T2D-associated SNPs with confounders, and no significant association signal was detected among the 35 SNPs we selected at the Bonferroni-corrected significance threshold $p < 3.57E-04$ (e.g., 0.05/140) (**Supplementary Table 3**). We also assessed the horizontal pleiotropy with the MR–Egger regression

[3]http://cnsgenomics.com/shiny/mRnd/

TABLE 1 | MR estimates from each method of assessing the causal effects of T2D, FG and 2hGlu on OA risk.

Exposure traits	MR methods	Hip OA					Knee OA				
		Number of SNPs	OR (95% CI)	SE	MR *p*-value	MR-Steiger test	Number of SNPs	OR (95% CI)	SE	MR *p*–value	MR-Steiger test
T2D	MR-Egger	35	1.1708 (0.9469∼1.4476)	0.1083	0.1547	Direction: TRUE *p*-value < 0.0001	35	0.9046 (0.7880∼1.1085)	0.0871	0.4426	Direction: TRUE *p*-value < 0.0001
	Inverse variance weighted	35	1.0022 (0.9329∼1.0767)	0.0366	0.9517		35	0.9809 (0.9265∼1.0385)	0.0291	0.5084	
	Weighted median	35	1.0454 (0.9369∼1.1664)	0.0559	0.4274		35	1.0053 (0.9213∼1.0971)	0.0446	0.9050	
	Robust adjusted profile score	35	0.9957 (0.9239∼1.0731)	0.0382	0.9094		35	0.9833 (0.9247∼1.0457)	0.0314	0.5920	
FG	MR-Egger	10	0.4634 (0.1848∼1.1617)	0.4690	0.1396	Direction: TRUE *p*-value < 0.0001	10	0.5890 (0.3697∼1.0943)	0.1665	0.1559	Direction: TRUE *p*-value < 0.0001
	Inverse variance weighted	10	0.9820 (0.6545∼1.4734)	0.2070	0.9301		10	0.8158 (0.6009∼1.1077)	0.1560	0.1921	
	Weighted median	10	0.6670 (0.4176∼1.0651)	0.2388	0.0899		10	0.6491 (0.4060∼1.1897)	0.1721	0.3417	
	Robust adjusted profile score	10	0.8996 (0.5811∼1.3926)	0.2229	0.6350		10	1.0374 (0.4699∼2.2900)	0.4040	0.9299	
2hGlu	MR-Egger	3	1.3062 (0.2540∼2.8190)	0.6140	0.8309	Direction: TRUE *p*-value < 0.0001	3	1.3652 (0.7171∼2.5993)	0.3285	0.5171	Direction: TRUE *p*-value < 0.0001
	Inverse variance weighted	3	1.1976 (0.9437∼1.5199)	0.1216	0.1380		3	1.0594 (0.9067∼1.2378)	0.0794	0.4673	
	Weighted median	3	1.3301 (1.0348∼1.7098)	0.1281	*0.0260*		3	1.0786 (0.8998∼1.2930)	0.0925	0.4133	
	Robust adjusted profile score	3	1.2125 (0.9817∼1.4976)	0.1077	0.0737		3	1.0598 (0.9020∼1.2451)	0.0822	0.4804	

MR, Mendelian randomization; SNP, single nucleotide polymorphism; OA, osteoarthritis; T2D, type 2 diabetes; FG, fasting glucose; 2hGlu, 2-h postprandial glucose; OR, odds ratio; CI, confidence interval; SE, standard error (the standard error is an estimate of the standard deviation (SD) of the coefficient).

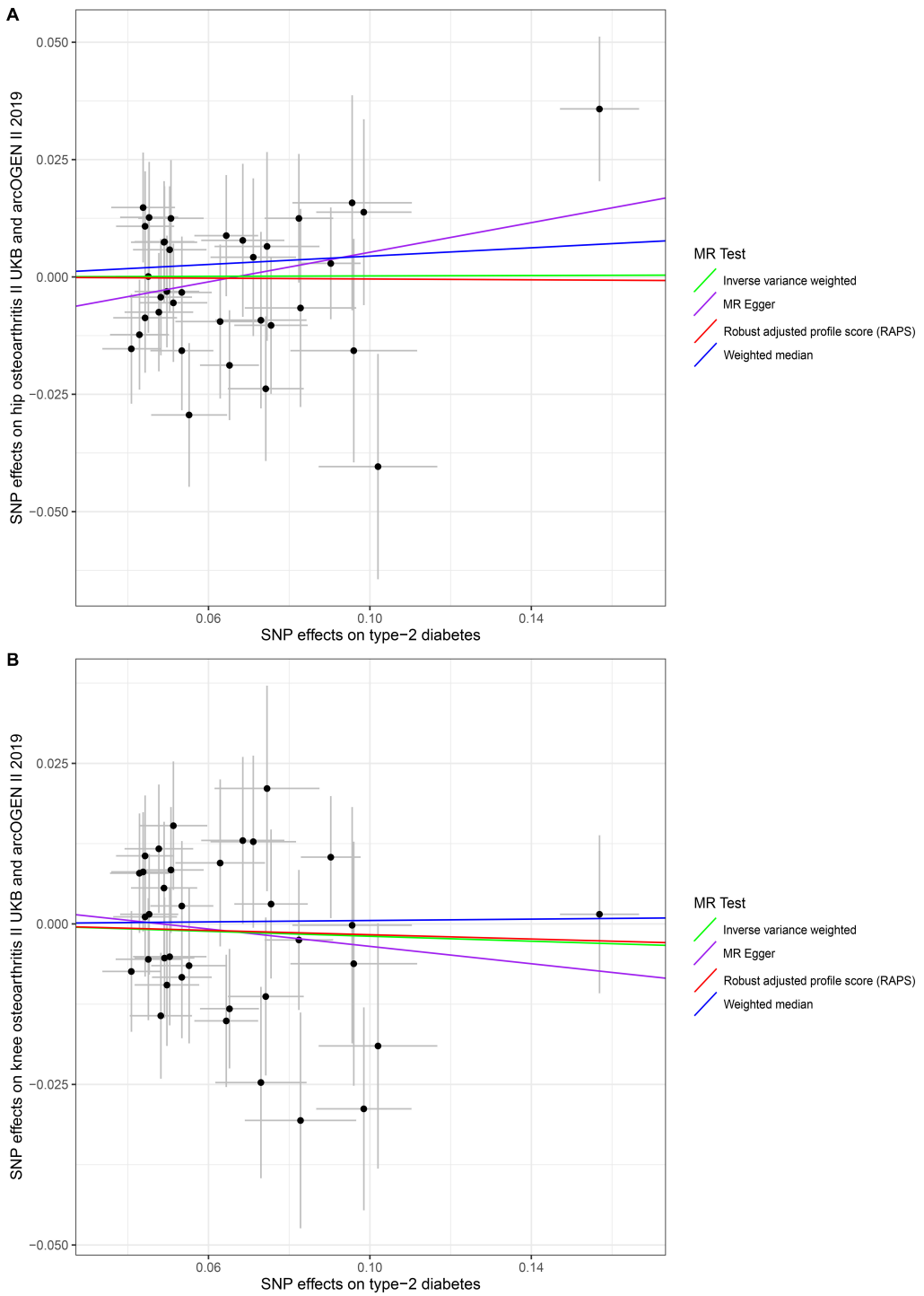

FIGURE 1 | Scatter plots of genetic associations with type 2 diabetes risk against osteoarthritis using different MR methods. **(A)** Type 2 diabetes and hip osteoarthritis results; **(B)** type 2 diabetes and knee osteoarthritis results. The slopes of each line represent the causal association for each method. The green line represents the inverse variance weighted estimate, the purple line represents the MR–Egger estimate, the red line represents the MR.RAPS estimate, and the blue line represents the weighted median estimate.

and found that no horizontal pleiotropy would bias the causality with hip OA (intercept = −0.0105, p = 0.1367) and knee OA (intercept = 0.0033, p = 0.5589) (**Table 2**). The heterogeneity test demonstrated that there is no evidence of heterogeneity in the MR analysis. For hip OA, MR–Egger Q = 30.1362, I^2 < 0.0001, p = 0.6104; IVW Q = 32.4634, I^2 < 0.0001, p = 0.5430; maximum

likelihood Q = 32.4633, I^2 < 0.0001, p = 0.5430. For knee OA, MR–Egger Q = 33.5130, I^2 = 0.0153, p = 0.4424; IVW Q = 33.8671, I^2 < 0.0001, p = 0.4741; maximum likelihood Q = 33.8592, I^2 < 0.0001, p = 0.4745. (**Table 2**). The MR–Steiger results supported that the SNPs in the analysis were more predictive of the exposure than of the outcome (**Table 1**). Power calculations showed that our sample provided sufficient statistical power (>80%) for causal analysis of T2D on hip and knee OA (**Table 3**).

Causality Between FG in Non-diabetic Individuals and OA

Based on independent and LD analyses, we selected 10 genome-wide significant (p < 5E-08) SNPs associated with FG in non-diabetic individuals to analyze the causality with hip and knee OA, and no palindromic SNPs were found. None of the proxy SNPs were used in the analysis. The F values of the 10 SNPs ranged from 32.6531 to 442.4495, with an average value of 124.7024 (**Supplementary Table 4**).

No evidence supported that the genetically increased FG was causally associated with the hip and knee OA risk changes in non-diabetic individuals based on the IVW, WM, MR–Egger regression, and MR.RAPS methods (p < 0.025). For hip OA, MR–Egger OR = 0.4634, 95% CI 0.1848–1.1617, p = 0.1396; IVW OR = 0.9820, 95% CI 0.6545–1.4734, p = 0.9301; WM OR = 0.6670, 95% CI 0.4176–1.0651, p = 0.0899; MR.RAPS OR = 0.8996, 95% CI 0.5811–1.3926, p = 0.6350. For knee OA, MR–Egger OR = 0.5890, 95% CI 0.3697–1.0943, p = 0.1559; IVW OR = 0.8158, 95% CI 0.6009–1.1077, p = 0.1921; WM OR = 0.6491, 95% CI 0.4060–1.1897, p = 0.3417; MR.RAPS OR = 1.0374, 95% CI 0.4699–2.2900, p = 0.9299. (**Table 1** and **Figure 2**). None of the 10 SNPs were significantly associated with known confounders at the Bonferroni-corrected significance threshold (p < 0.0013) (e.g., 0.05/40) (**Supplementary Table 5**). We also conducted the MR–Egger regression to assess the horizontal pleiotropy, and the results revealed that the horizontal pleiotropy was unlikely to bias the causality with hip OA (intercept = 0.0243, p = 0.1189) and knee OA (intercept = −0.0078, p = 0.5348) (**Table 2**).

We also found no significant heterogeneity between FG and OA. For hip OA, MR–Egger Q = 11.6746, I^2 = 0.2291, p = 0.1663; IVW Q = 16.1241, I^2 = 0.4418, p = 0.0643; maximum likelihood Q = 16.1240, I^2 = 0.4418, p = 0.0643. For knee OA, MR–Egger Q = 13.8053, I^2 = 0.4205, p = 0.0870; IVW Q = 14.5310, I^2 = 0.3806, p = 0.1047; maximum likelihood Q = 14.5175, I^2 = 0.3801, p = 0.1051. (**Table 2**). The MR–Steiger directionality test showed that the SNPs in the analysis were more predictive of the exposure than of the outcome (**Table 1**). We also have sufficient statistical power (>80%) to detect the true causal effect between FG and hip and knee OA (**Table 3**).

Causality Between 2hGlu in Non-diabetic Individuals and OA

We chose three independent SNPs associated with 2hGlu in European ancestry from summary statistics datasets of GWAS meta-analyses, and no palindromic SNPs were found. The F

TABLE 2 | Heterogeneity and pleiotropy analysis of T2D, FG and 2hGlu with hip and knee OA risk using different analytic methods.

Exposure traits	MR methods	Hip OA					Knee OA				
		Cochran Q statistic	I^2	Heterogeneity p-value	MR-Egger		Cochran Q statistic	I^2	Heterogeneity p-value	MR-Egger	
					Intercept	Intercept p-value				Intercept	Intercept p-value
T2D	MR-Egger	30.1362	<0.0001	0.6104	−0.0105	0.1367	33.5130	0.0153	0.4424	0.0033	0.5589
	Inverse variance weighted	32.4634	<0.0001	0.5430			33.8671	<0.0001	0.4741		
	Maximum likelihood	32.4633	<0.0001	0.5430			33.8592	<0.0001	0.4745		
FG	MR-Egger	11.6746	0.2291	0.1663	0.0243	0.1189	13.8053	0.4205	0.0870	−0.0078	0.5348
	Inverse variance weighted	16.1241	0.4418	0.0643			14.5310	0.3806	0.1047		
	Maximum likelihood	16.1240	0.4418	0.0643			14.5175	0.3801	0.1051		
2hGlu	MR-Egger	2.2054	0.5466	0.1375	0.0303	0.6639	0.0204	<0.0001	0.8863	−0.0222	0.5722
	Inverse variance weighted	2.9551	0.3232	0.2282			0.6533	<0.0001	0.7213		
	Maximum likelihood	2.8701	0.3032	0.2381			0.6503	<0.0001	0.7224		

MR, Mendelian randomization; OA, osteoarthritis; T2D, type 2 diabetes; FG, fasting glucose; 2hGlu, 2-hour postprandial glucose.

TABLE 3 | Power calculation for the MR analysis in current study.

Exposure		Outcome			OR	Proportion of variance explained by the SNPs on exposure	Power
Trait	Sample size	Trait	Sample size	Proportion of cases			
T2D	659,316	Hip OA	382,833	0.0336	1.1708	0.0508	99%
T2D	659,316	Knee OA	391,904	0.0559	0.9046	0.0508	87%
FG	133,010	Hip OA	382,833	0.0336	0.4634	0.0029	91%
FG	133,010	Knee OA	391,904	0.0559	0.5890	0.0029	91%
2hGlu	42,854	Hip OA	382,833	0.0336	1.3062	0.0074	83%
2hGlu	42,854	Knee OA	391,904	0.0559	1.3652	0.0074	99%

T2D, type 2 diabetes; FG, fasting glucose; 2hGlu, 2-h postprandial glucose; OA, osteoarthritis; OR, odds ratio; SNP, single nucleotide polymorphism.

values of the three SNPs were 32.8017, 33.5180, and 39.0625, with an average value of 35.1274 (**Supplementary Table 6**).

Our results did not suggest causal associations of genetically increased 2hGlu with hip and knee OA risk changes in non-diabetic individuals ($p < 0.025$). (For hip OA, MR–Egger OR = 1.3062, 95% CI 0.2540–2.8190, $p = 0.8309$; IVW OR = 1.1976, 95% CI 0.9437–1.5199, $p = 0.1380$; WM OR = 1.3301, 95% CI 1.0348–1.7098, $p = 0.0260$; MR.RAPS OR = 1.2125, 95% CI 0.9817–1.4976, $p = 0.0737$. For knee OA, MR–Egger OR = 1.3652, 95% CI 0.7171–2.5993, $p = 0.5171$; IVW OR = 1.0594, 95% CI 0.9067–1.2378, $p = 0.4673$; WM OR = 1.0786, 95% CI 0.8998–1.2930, $p = 0.4133$; MR.RAPS OR = 1.0598, 95% CI 0.9020–1.2451, $p = 0.4804$.) (**Table 1** and **Figure 3**). We conducted the MR–Egger regression to assess the pleiotropy, and the results revealed that the horizontal pleiotropy was unlikely to bias the causality with hip OA (intercept = 0.0303, $p = 0.6639$) and knee OA (intercept = −0.0222, $p = 0.5722$) (**Table 1**).

The associations between these genetic variants and confounding factors were analyzed. None of the three genetic variants were significantly associated with the confounding factors mentioned above at the Bonferroni-corrected significance threshold ($p < 0.0042$) (e.g., 0.05/12) (**Supplementary Table 7**). Cochran's Q value and I^2 value indicated no evidence of heterogeneity between IV estimates with the IVW, MR–Egger, and maximum likelihood methods. (For hip OA, MR–Egger $Q = 2.2054$, $I^2 = 0.5466$, $p = 0.1375$; IVW $Q = 2.9551$, $I^2 = 0.3232$, $p = 0.2282$; maximum likelihood $Q = 2.8701$, $I^2 = 0.3032$, $p = 0.2381$. For knee OA, MR–Egger $Q = 0.0204$, $I^2 < 0.0001$, $p = 0.8863$; IVW $Q = 0.6533$, $I^2 < 0.0001$, $p = 0.7213$; maximum likelihood $Q = 0.6503$, $I^2 < 0.0001$, $p = 0.7224$.) (**Table 2**). The MR–Steiger results supported that the SNPs in the analysis were more predictive of the exposure than of the outcome (**Table 1**). Power calculations showed that our sample provided sufficient statistical power (>80%) for causal analysis of 2hGlu on hip and knee OA (**Table 3**).

DISCUSSION

To our knowledge, this is the first MR study on the effect of T2D and other glycemic traits on OA. We obtained sufficient sample sizes and thus had sufficient power (>80%) to detect causal effects. We did not distinguish statistical causality between exposures and outcomes based on our MR results. The F values of IVs indicated that the variables satisfy the strong relevance assumption of MR, and that the instrument bias was weak and could not substantially influence the estimations of causal effects. We used the MR–Egger method to detect and adjust for pleiotropy of the genetic variants. We also performed heterogeneity and did not find significant heterogeneity between SNPs, which indicated the reliability of the MR results. Some studies reported similar results that there was no evidence to support the causal associations between T2D and OA. Frey et al. (2016) conducted one case–control study and provided evidence that T2D is not an independent risk factor for hand OA regardless of T2D severity, duration, or pharmacological treatment. Funck-Brentano et al. (2019) analyzed the individual-level data in UK Biobank study and performed MR analysis. They found no significant causality for T2D with all OA, knee OA, hip OA, and hand OA. Zengini et al. (2018) also found no causal association of T2D with self-reported OA or hospital-diagnosed OA with the MR analysis.

Some studies provided a few suggestive pathophysiological mechanisms for the development of OA in T2D patients. One of them was hyperglycemia-induced accelerated synthesis of Advanced Glycation End products (AGEs), which leads to an increase in oxidative stress. These AGEs have been regarded as one of the factors responsible for healing impairment and loss of elasticity of the cartilage (DeGroot et al., 2001). Another mechanism was that chronic high glucose environment had noxious effects on chondrocytes metabolism (Laiguillon et al., 2015). High glucose environment would induce diabetic cartilages to produce more interleukin-6 and prostaglandin E2. High glucose exposure also increased the metalloproteinases production especially in human OA chondrocytes and decreased the production of collagen II. Vaamonde-Garcia et al. (2017) demonstrated that high glucose environment favored the suppression of heme oxygenase-1, which led to an increase in the oxidative stress and cartilage damage. Chen et al. (2015) found that high glucose diminished the synthesis of type II collagen and peroxisome proliferator-activated receptor gamma (PPARγ) by chondrocytes, which could result in the development of cartilage defects.

Many studies reported the suggestive evidence to support the associations between T2D and OA. Eymard et al. (2015) demonstrated that T2D was a predictor of joint space reduction in men with established knee OA. Davies-Tuck et al. (2012) reported

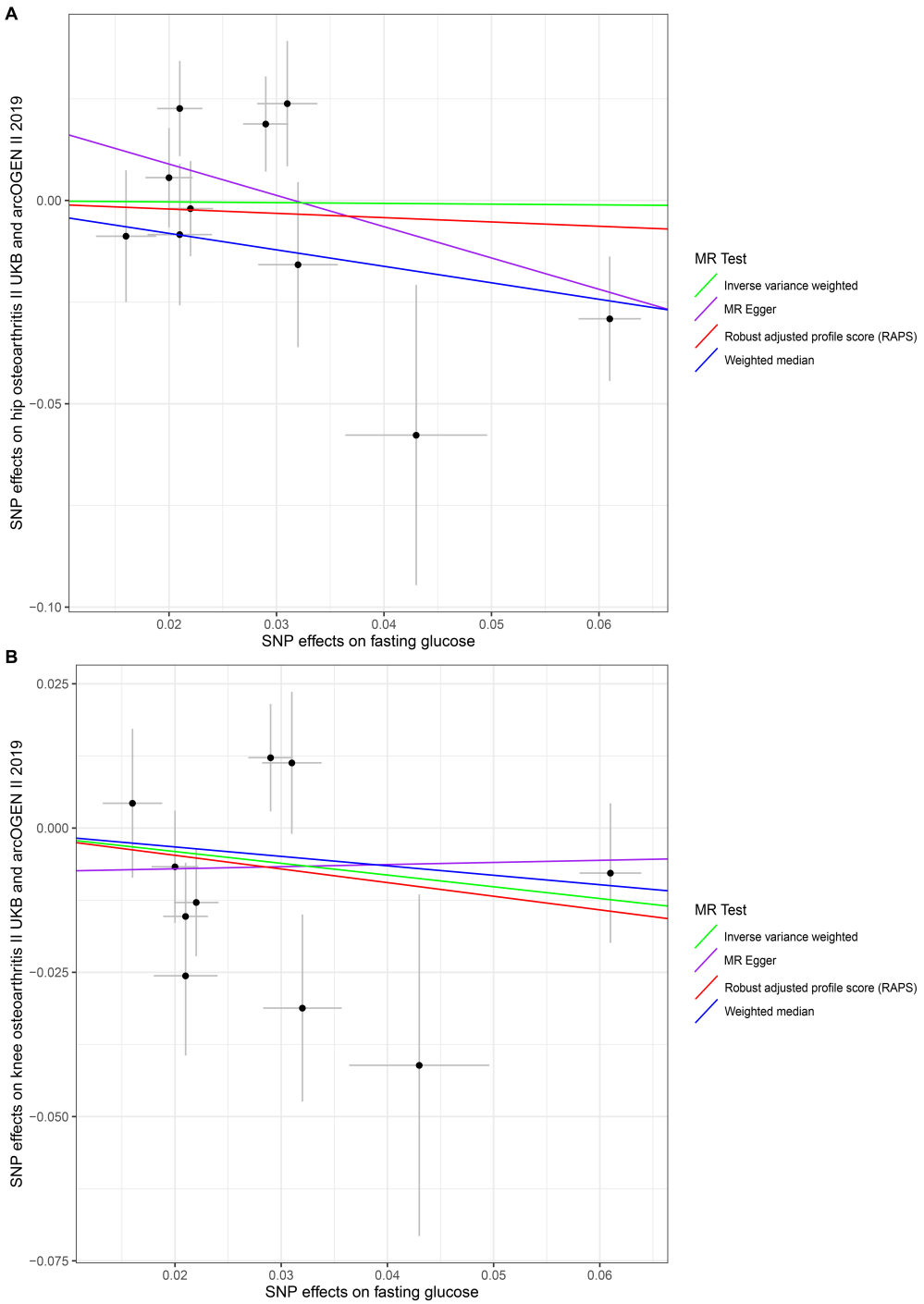

FIGURE 2 | Scatter plots of genetic associations with fasting glucose against osteoarthritis using different MR methods. **(A)** Fasting glucose and hip osteoarthritis results; **(B)** fasting glucose and knee osteoarthritis results. The slopes of each line represent the causal association for each method. The green line represents the inverse variance weighted estimate, the purple line represents the MR–Egger estimate, the red line represents the MR.RAPS estimate, and the blue line represents the weighted median estimate.

the evidence that increased FG concentration in non-diabetic individuals was associated with adverse structural changes at the knee in women based on one prospective cohort study. Williams et al. (2016) executed one meta-analysis including 10 observational studies with 16,742 patients in total. They revealed that T2D was associated with radiographic and symptomatic OA even after controlling the BMI and weight. Schett et al. (2013) reported that T2D could predict the progress of OA,

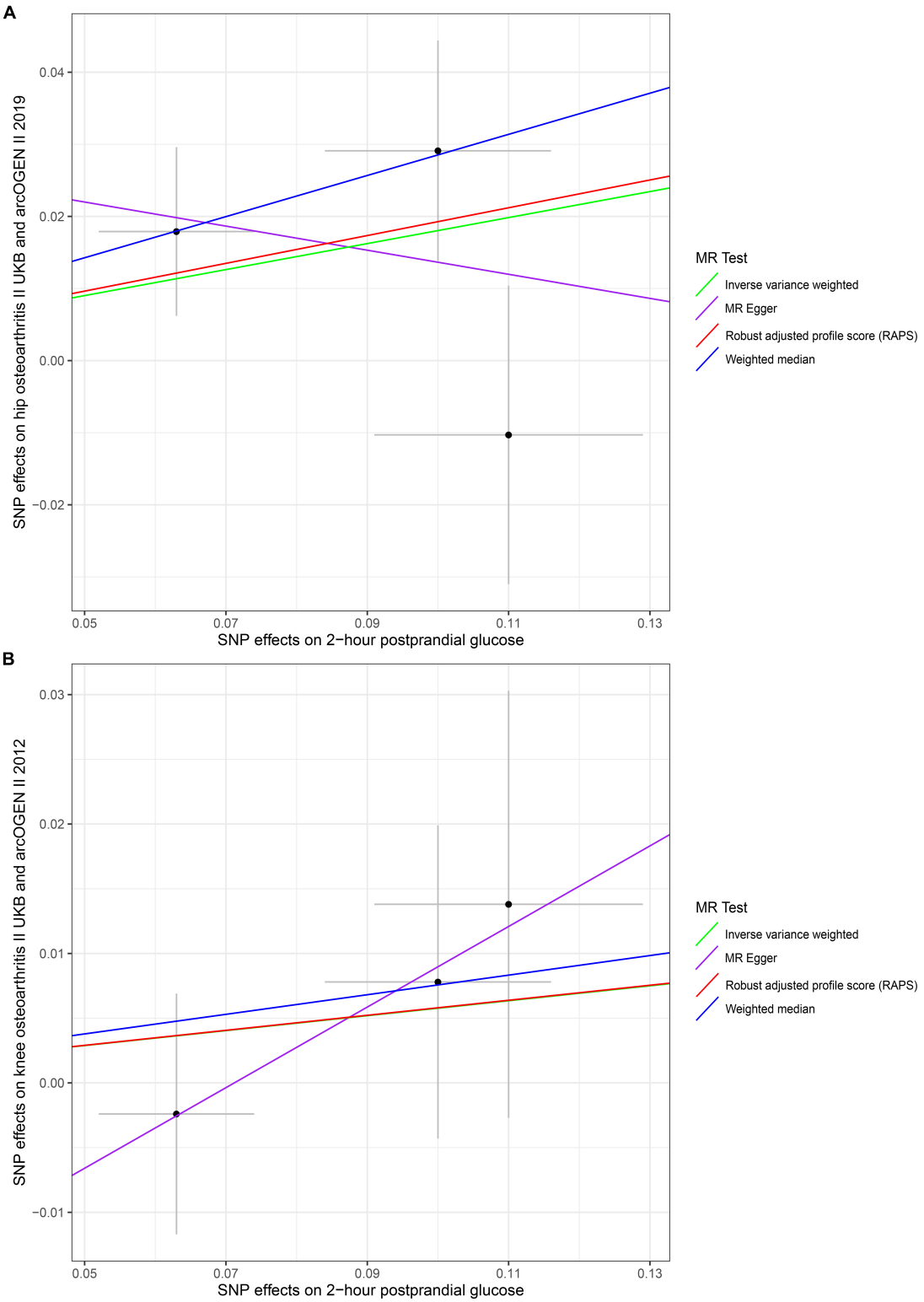

FIGURE 3 | Scatter plots of genetic associations with 2-h postprandial glucose against osteoarthritis using different MR methods. **(A)** 2-h postprandial glucose and hip osteoarthritis results; **(B)** 2-h postprandial glucose and knee osteoarthritis results. The slopes of each line represent the causal association for each method. The green line represents the inverse variance weighted estimate, the purple line represents the MR–Egger estimate, the red line represents the MR.RAPS estimate, and the blue line represents the weighted median estimate.

independent of age and BMI, based on one cohort study followed up over 20 years. Although many studies suggested the associations, those evidences were too weak to indicate the causal associations between T2D and OA. Confounders might interfere with the associations between T2D and OA. Since T2D and OA share common risk factors, some studies even suggested that OA was the component of metabolic syndrome (Schett et al., 2013); metabolic factors, such as obesity, inflammatory factors, physical activity, and diabetic medication might have impacts on OA. For example, obesity is a pandemic condition defined as the abnormal or the excessive accumulation of fat, which is characteristic of T2D (Kolb and Martin, 2017). Obesity would promote the progress of OA through mechanical load and inflammatory reaction. Mechanical load means that the increase load of weight-bearing joint caused by obesity could accelerate the development of OA. Inflammatory reaction indicated that the increased systemic and local inflammation caused by obesity would damage the integrity of the extracellular matrix of the cartilage (Martel-Pelletier et al., 2016; Reyes et al., 2016). Furthermore, some confounders that cannot be entirely ruled out, such as socioeconomic status, occupation, and nutrition, also had impacts on the association between T2D and OA (Frey et al., 2016). Some studies (Schett et al., 2013) used joint arthroplasty due to OA as the study endpoint, which precludes the ability to assess temporality, because joint arthroplasty was the terminal event of OA. This would be the reason that affected the association between T2D and OA. Besides, reverse causation bias between T2D and OA (Kendzerska et al., 2018) would also limit the ability to provide causal estimates of the effect of exposures on outcomes in the observational studies.

The present study has several limitations. The criteria for OA were limited in the GWAS included in the study. The radiographic OA or the mild symptomatic OA was not included. Additionally, there were only two types of OA, hip and knee OA, involved in the study. The hand OA was not analyzed in the MR study, which might distinguish the pathogenesis mechanism from hip and knee OA due to the absence of weight-bearing factors. The samples included in the exposures and outcomes were of European ancestry, which could mitigate the population stratification. However, the conclusions based on the European sample were not representative of other ancestries, such as Asians and Americans. Moreover, we only evaluated the associations between SNPs and weight-associated confounders due to the limited publicly available GWAS databases. The associations between these instruments and other potential confounders, such as physical activity, were not evaluated in our study.

CONCLUSION

In summary, our two-sample MR analysis did not suggest the significant causal effects of genetic increases in T2D risk, FG, and 2hGlu with hip and knee OA. The complicated effects of T2D risk, FG, and 2hGlu with OA might be influenced by other confounding factors, which still need further investigation in the future. In addition, future studies should additionally seek to investigate the effect of T2D and glycemic traits on hand OA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YT, ZC, and BH conceptualized and designed the study. ZC and HF provided the "TwoSampleMR" package codes in R language and analyzed the data in the study. ZC drafted the manuscript. YX and ZL gave constructive suggestions when writing the manuscript. All authors have read the manuscript.

FUNDING

This work was supported by grants from the Beijing Municipal Science and Technology Commission (Z181100001718195).

ACKNOWLEDGMENTS

The authors wish to thank Xikun Han from the University of Queensland in Australia for providing the summary-level data about exposures and outcomes in the study. This manuscript has been released as a pre-print at research square (Cui et al., 2020).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.597876/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 15 January 2021
doi: 10.3389/fgene.2020.610852

Use of Multivariable Mendelian Randomization to Address Biases Due to Competing Risk Before Recruitment

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OPEN ACCESS

Edited by:
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Reviewed by:
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Icahn School of Medicine at Mount Sinai, United States
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *27 September 2020*
Accepted: *01 December 2020*
Published: *15 January 2021*

Citation:
Schooling CM, Lopez PM, Yang Z, Zhao JV, Au Yeung SL and Huang JV (2021) Use of Multivariable Mendelian Randomization to Address Biases Due to Competing Risk Before Recruitment. Front. Genet. 11:610852. doi: 10.3389/fgene.2020.610852

Background: Mendelian randomization (MR) provides unconfounded estimates. MR is open to selection bias when the underlying sample is selected on surviving to recruitment on the genetically instrumented exposure and competing risk of the outcome. Few methods to address this bias exist.

Methods: We show that this selection bias can sometimes be addressed by adjusting for common causes of survival and outcome. We use multivariable MR to obtain a corrected MR estimate for statins on stroke. Statins affect survival, and stroke typically occurs later in life than ischemic heart disease (IHD), making estimates for stroke open to bias from competing risk.

Results: In univariable MR in the UK Biobank, genetically instrumented statins did not protect against stroke [odds ratio (OR) 1.33, 95% confidence interval (CI) 0.80–2.20] but did in multivariable MR (OR 0.81, 95% CI 0.68–0.98) adjusted for major causes of survival and stroke [blood pressure, body mass index (BMI), and smoking initiation] with a multivariable Q-statistic indicating absence of selection bias. However, the MR estimate for statins on stroke using MEGASTROKE remained positive and the Q statistic indicated pleiotropy.

Conclusion: MR studies of harmful exposures on late-onset diseases with shared etiology need to be conceptualized within a mechanistic understanding so as to identify any potential bias due to survival to recruitment on both genetically instrumented exposure and competing risk of the outcome, which may then be investigated using multivariable MR or estimated analytically and results interpreted accordingly.

Keywords: selection bias, competing risk, Mendelian randomization, shared etiology, instrumental variable analysis

INTRODUCTION

Mendelian randomization (MR), i.e., instrumental variable analysis with genetic instruments, is an increasingly popular and influential analytic technique (Davies et al., 2018; Taubes, 2018), which can be used to investigate causal effects even when no study including both exposure and outcome of interest exists. Invaluably, MR studies have provided estimates more consistent with results from randomized controlled trials (RCTs) than conventional observational studies, even foreshadowing the results of major trials (Holmes et al., 2017). MR studies are often presented as observational studies analogous to RCTs (Davey Smith and Ebrahim, 2005; Burgess et al., 2012) because they take advantage of the random assortment of genetic material at conception, while observational studies are open to biases from confounding and selection bias (Bareinboim and Pearl, 2016). Instrumental variable analysis is described in health research as addressing confounding (Greenland, 2000; Maciejewski and Brookhart, 2019), i.e., bias from common causes of exposure and outcome (Bareinboim and Pearl, 2016). MR is currently described as "less likely to be affected by confounding or reverse causation than conventional observational studies" (Davies et al., 2018).

Mendelian randomization was originally thought to be less open to selection bias than conventional observation studies (Smith and Ebrahim, 2004). Selection bias is now increasingly widely recognized as a limitation of MR (Nitsch et al., 2006; Boef et al., 2015; Canan et al., 2017; Munafo et al., 2017; Swanson et al., 2017; Gkatzionis and Burgess, 2018; Munafo and Smith, 2018; Vansteelandt et al., 2018; Hughes et al., 2019; Swanson, 2019), which may violate the instrumental variable assumptions. Sources of potential selection bias in MR have been specifically identified as selecting an unrepresentative sample (Munafo et al., 2017; Munafo and Smith, 2018; Hughes et al., 2019), attrition from an initially representative sample, such as a birth cohort (Munafo et al., 2017), and selecting a sample strongly on surviving the exposure (Gkatzionis and Burgess, 2018) or genotype of interest (Vansteelandt et al., 2018; Smit et al., 2019). What has not explicitly been considered is selecting the underlying sample(s) on surviving the genotype of interest in the presence of competing risk of the outcome. MR studies are particularly vulnerable to sample selection on survival because of the time lag between genetic randomization (at conception) and typical recruitment into genetic studies of major diseases in middle to old age. MR studies also often concern major causes of death thought to share considerable etiology. For example, lipids, blood pressure, diabetes, lifestyle (such as smoking, diet, physical activity, and sleep), and socioeconomic position cause both ischemic heart disease (IHD) and ischemic stroke, with death from IHD typically occurring at younger ages than death from stroke (Kesteloot and Decramer, 2008; Menotti et al., 2019). As a result, a study of the association of lipid modifiers with stroke among the living will automatically select on surviving high lipids and on surviving competing risk of prior death from IHD due to shared etiology between IHD and stroke. Some people dying from genetically high lipids and others dying from IHD before recruitment into a stroke study will leave a shortage of people available to recruit with genetically high lipids and susceptibility to stroke, thereby obscuring any effect of lipids or lipid modifiers on stroke. Correspondingly, MR studies suggest less effect of lipids and lipid modifiers on stroke than IHD (Hopewell et al., 2018; Valdes-Marquez et al., 2019), although RCTs suggest similar effects (Mills et al., 2011; Chou et al., 2016; Schmidt et al., 2017). Similarly, MR studies do not consistently show detrimental effects of body mass index (BMI) on stroke (Marini et al., 2020). In this study, we explain how potential violations of the instrumental variable assumptions due to inadvertently recruiting survivors of the genetically predicted exposure and competing risk of the outcome may bias MR estimates. We explain how this bias might be corrected using multivariable MR and provide a simple means of estimating how large the bias is likely to be.

MATERIALS AND METHODS

Potential Biasing Pathways Due to Recruiting on Selective Survival

Figure 1A shows the directed acyclic graph for MR illustrating the instrumental variable assumptions typically referred to as relevance, independence, and exclusion restriction. Relevance is explicitly indicated by the arrow from instrument to exposure. Independence is implicitly indicated by the lack of an arrow from confounders of exposure on outcome (or of instrument on outcome) to instrument. Exclusion restriction is implicitly indicated by the lack of arrows linking instrument to outcome, sometimes illustrated as no arrow from instrument to outcome indicating no pleiotropy (Bowden et al., 2015, 2016; Hartwig et al., 2017; Verbanck et al., 2018) (**Figure 1B**). **Figure 1C** shows selection on survival of both instrument and common causes of the outcome (U_2) (Hughes et al., 2019; Swanson, 2019), which also violates the exclusion restriction assumption, particularly when stated as "*every unblocked path connecting instrument and outcome must contain an arrow pointing into the exposure*" (Pearl, 2009). **Figure 1D** explicitly shows survival on instrument, and another disease (Y_2) sharing etiology (U_2) with the outcome (Y). **Figure 1E** shows the exclusion restriction assumption with both no pleiotropy and no selection bias from competing risk (U_2) made explicit. Notably, **Figures 1C–E** are very similar in structure to a well-known example of selection bias, which occurs when conditioning on an intermediate (or covariable adjustment) reverses the direction of effect: the "birth weight" paradox (Hernandez-Diaz et al., 2006). In the birth weight paradox adjusting the association of maternal smoking with infant death for birth weight makes maternal smoking look protective; further adjusting for all common causes of birth weight and infant death, thought to be birth defects, should remove this bias (Hernandez-Diaz et al., 2006) by blocking the path from maternal smoking to infant death via birth weight and birth defects. Similarly, bias due to inadvertently selecting the underlying sample in an MR study on surviving the genetically instrumented exposure and surviving competing risk of the outcome should be ameliorated by adjusting for major causes of survival and the outcome (**Figure 2**). The recent development of multivariable MR (Sanderson et al., 2019) provides the means

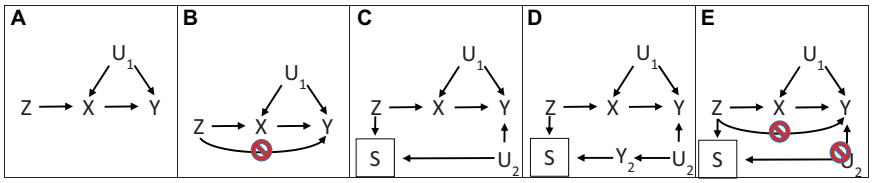

FIGURE 1 | Directed acyclic graphs with instrument (Z), outcome (Y), exposure (X), confounders (U_1), and survival (S), where a box indicates selection, for **(A)** a valid Mendelian randomization study and **(B)** a Mendelian randomization study with an invalid instrument through violation of the exclusion-restriction assumption via pleiotropy, **(C)** a Mendelian randomization study with an invalid instrument through violation of the exclusion-restriction assumption via survival on instrument and shared etiology with the outcome (U_2), **(D)** a Mendelian randomization study with an invalid instrument through violation of the exclusion restriction assumption via survival (S), competing risk of another disease (Y_2) and shared causes (U_2) with (Y_2) and the outcome (Y), and **(E)** a Mendelian randomization illustrating both conditions which have to be met to satisfy the exclusion restriction assumption.

to do so. Specifically, as indicated in **Figures 1C,D**, where univariable MR may be biased, using multivariable MR adjusting for the main determinants of survival and outcome may reduce bias by at least partially blocking any backdoor paths from instrument to outcome.

In addition, to provide triangulation, the level of selection bias due to surviving to recruitment on genetically instrumented exposure in the presence of competing risk of the outcome can also be thought of as depending on the proportion of the exposed who are not available for recruitment because of prior death due to the genetically predicted exposure and the proportion of those who could have experienced the outcome who are not available for recruitment because of prior death from a competing risk. Assuming these proportions are independent and their corresponding probabilities do not sum to more than 1, then for an observed odds ratio (OR) greater than 1, the true OR for genetically predicted exposure on disease can be estimated as the observed OR multiplied by the ratio of the probability of surviving the exposure and the competing risk to the probability of surviving the exposure or the competing risk, as shown in **Appendix Table 1**.

Examples of Selection Bias and Amelioration

We investigated effects of lipid modifiers and BMI on ischemic stroke as possible exemplars, because previous MR studies of these exposures on stroke have not always given the expected results (Hopewell et al., 2018; Marini et al., 2020). Statins and PCSK9 inhibitors are very well-established interventions for cardiovascular disease, which reduce low-density lipoprotein (LDL)-cholesterol, IHD (Mills et al., 2011; Chou et al., 2016; Schmidt et al., 2017), stroke (Mills et al., 2011; Chou et al., 2016; Schmidt et al., 2017), and atrial fibrillation (AF) (Peng et al., 2018). BMI is also known to be harmful. IHD, stroke, and AF also share major causes independent of lipid modifiers, such as blood pressure (Emdin et al., 2015; Ettehad et al., 2016), smoking, lifestyle, and socioeconomic position. Death from IHD typically occurs at earlier ages than death from stroke in Western populations (Kesteloot and Decramer, 2008; Menotti et al., 2019). AF may also be a consequence of IHD. **Figure 2A** suggests bias would be expected for harmful exposures on stroke or AF in any sample of survivors, such as middle-aged or older adults. Adjusting for major factors causing survival to recruitment into the underlying studies of stroke or AF, as shown for lipid modifiers on stroke (**Figure 2B**) or BMI on stroke (**Figure 2C**), should reduce the bias. As such, univariable MR, even with well-defined genetic instruments free from genetic pleiotropy, might generate biased estimates due to selection bias violating the exclusion-restriction assumption, but appropriate use of multivariable MR might ameliorate the problem.

We used well-established independent genetic variants to mimic effects of statins (rs12916) and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors (rs11206510, rs2149041, and rs7552841) (Ference et al., 2019), and for BMI (96 variants) (Locke et al., 2015). Using two-sample univariable MR, we applied these variants to major GWAS, in people largely of European descent, of IHD (CARDIoGRAMplusC4D 1000 Genomes) (Nikpay et al., 2015), stroke (MEGASTROKE) (Malik et al., 2018), and AF (Nielsen et al., 2018). We also used the UK Biobank summary statistics for IHD and stroke (Zhou et al., 2018), but not for AF because the AF GWAS includes the UK Biobank data (Nielsen et al., 2018). We obtained univariable MR estimates by meta-analyzing the Wald estimates (genetic variant on outcome divided by genetic variant on exposure) using inverse variance weighting, with multiplicative random effects, after aligning variant estimates on the same-effect allele in each study.

We used multivariable two-sample MR to obtain MR estimates for the lipid modifiers on stroke and AF adjusted for major causes of survival (smoking initiation, blood pressure, and BMI) (Forouzanfar et al., 2015; Sakaue et al., 2020) and stroke, and to obtain an MR estimate for BMI on stroke adjusted for smoking initiation. We used published independent genetic instruments for smoking initiation (327 variants) (Larsson et al., 2020), systolic blood pressure (SBP) and diastolic blood pressure (DBP) [all replicated variants (SBP 215, DBP 219)] (Evangelou et al., 2018), and BMI (96 variants) (Locke et al., 2015). Genetic associations, for all the instruments selected, with LDL-cholesterol, ever smoking, SBP, DBP, and BMI, were obtained from the UK Biobank summary statistics[1] adjusted for age, sex, age^2, sex*age, and sex*age^2 and the first 20 principal components. We used the MR-Base clump_data R package

[1]http://www.nealelab.is/uk-biobank

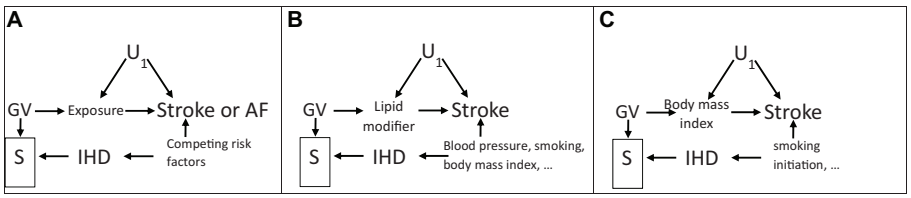

FIGURE 2 | Directed acyclic graphs showing how selection bias could occur because of selection on survival (S), indicated by a box, on the instrument (GV) and on competing risk of ischemic heart disease (IHD) which shares causes with the outcome of interest, i.e., stroke, with U_1 as confounders of exposure and outcome, when assessing **(A)** effects of an exposure on stroke or AF, **(B)** effects of lipid modifiers on stroke, and **(C)** effects of body mass index on stroke.

with $r^2 < 0.05$ to obtain independent genetic variants across exposures and the MendelianRandomization package to obtain IVW multivariable estimates. Here, we used summary statistics, meaning we assumed linear and homogenous effects for all exposures. We reported the multivariable conditional F-statistic as a measure of instrument strength and the multivariable Q-statistic as a measure of instrument pleiotropy (Sanderson et al., 2019), obtained using the MVMR package (Sanderson et al., 2019). Calculation of the conditional F-statistic and the multivariable Q-statistic requires the covariance between the effects of genetic variants on each exposure or use of non-overlapping samples for the exposure GWAS (Sanderson et al., 2019). Use of summary statistics for the exposures makes it difficult to obtain their covariance, so we largely selected genetic instruments for exposures from non-overlapping samples; however, some overlap exists, for example, the GWAS used to obtain genetic instruments for smoking initiation and blood pressure both included the UK Biobank (as 33 and ∼40% of the sample, respectively) (Forouzanfar et al., 2015; Locke et al., 2015; Evangelou et al., 2018; Larsson et al., 2020; Sakaue et al., 2020). As such, the conditional F-statistic gives a lower bound for strength of the instruments and the modified Q-statistic gives an upper bound on bias from pleiotropy (Sanderson et al., 2019). Notably, in this context, a significant multivariable Q statistic may indicate genetic pleiotropy or violation of the exclusion restriction assumption by selection bias, because both might inflate the multivariable Cochran Q. If the same instruments give very different multivariable Cochran's Q for the same outcomes in different studies or for related outcomes in the same study, it would suggest that estimates with higher Cochran's Q are more likely open to selection bias than genetic pleiotropy. We also reported the multivariable MR-Egger intercept which may indicate genetic pleiotropy (Rees et al., 2017).

This study only used publicly available genetic summary statistics, collected with consent, and so does not require ethical approval.

RESULTS

As expected, the cases recruited into the underlying GWAS (Nikpay et al., 2015; Malik et al., 2018; Nielsen et al., 2018) seemed to be youngest for IHD and oldest for AF with stroke somewhere in between (**Supplementary Table 1**). In univariable MR, genetically mimicking statins or PCSK9 inhibitors reduced IHD, while genetically instrumented BMI increased IHD (**Table 1**). Estimates were similar using CARDIoGRAMplusC4D 1000 Genomes and the UK Biobank. IHD is not expected to be majorly open to competing risk, so it was not considered further. In univariable MR, genetically mimicking statins or PCSK9 inhibitors was not associated with a lower risk of stroke or AF; some estimates for statins were in the direction opposite to expected (**Table 1**). In univariable MR, genetically instrumented BMI did not consistently increase stroke but did increase AF (**Table 1**). Univariable MR estimates for the major causes of survival considered are shown in **Supplementary Table 2**.

In multivariable MR, the conditional F-statistics for each exposure were similar in each analysis, suggesting similar instrument strength (**Table 1**). The Q-statistics were not significant for lipid modifiers on UK Biobank stroke (**Table 1**). The multivariable MR estimates in the UK Biobank, in contrast to the corresponding univariable MR estimates, showed that genetically instrumented lipid modifiers protected against stroke and that genetically instrumented BMI caused stroke (**Table 1**). The multivariable MR-Egger intercepts were significant, with largely similar MR-Egger estimates for statins [OR 0.70, 05% confidence interval (CI) 0.56–0.88] and PCSK9 inhibitors (OR 0.66, 95% CI 0.53–0.83) but not BMI (OR 1.00, 95% CI 0.83–1.20). The Q-statistics were highly significant for lipid modifiers and BMI on MEGASTROKE stroke and AF (**Table 1**), indicating that these estimates were likely still biased by pleiotropy probably from selection bias given the same instruments gave estimates apparently unbiased by genetic pleiotropy for stroke in the UK Biobank. Correspondingly, the multivariable MR estimates were similar to the univariable estimates, and for lipid modifiers differed from those expected from RCTs (**Table 1**). The multivariable MR-Egger intercepts were not significant for MEGASTROKE estimates or for BMI on AF but were significant for statins and PCSK9 inhibitors on AF. The corresponding multivariable MR-Egger estimates gave directionally similar estimates to the inverse variance weighted estimates for genetically mimicked statins (OR 1.06, 95% CI 0.92–1.23) and PCSK9 inhibitors (OR 1.01, 95% CI 0.87–1.17).

To provide triangulation, we estimated whether the level of selection bias for statins on stroke, from surviving genetically instrument statins and IHD, was consistent with the univariable estimate, using the formula given in **Appendix Table 1**. The OR for the protective allele of the statin single-nucleotide polymorphism (rs12916) on IHD used to obtain the Wald

TABLE 1 | Effect of genetically mimicking statins and PCSK9 inhibitors use (Ference et al., 2019) (in effect size of LDL-cholesterol) and BMI (Locke et al., 2015) on IHD using the CARDIoGRAMplusC4D 1000 Genomes based GWAS (Nikpay et al., 2015) and the UK Biobank on all ischemic stroke using MEGASTROKE (Malik et al., 2018) and the UK Biobank and on AF using a study by Nielsen et al. (2018) from univariable Mendelian randomization and from multivariable Mendelian randomization, with genetically mimicked statins and PCSK9 inhibitors adjusted for systolic blood pressure (Evangelou et al., 2018), diastolic blood pressure (Evangelou et al., 2018), smoking initiation (Larsson et al., 2020) and BMI (Locke et al., 2015), and BMI adjusted for smoking initiation.

			Univariable		Multivariable				
Disease	**Source of genetic associations with disease**	**Exposure**	**OR**	**95% CI**	**OR**	**95% CI**	**MR-Egger Intercept**	**Conditional F**	**Q *p*-value**
Ischemic heart disease	CARDIoGRAMplusC4D 1000 Genomes	Statin	0.56	0.41–0.75					
		PCSK9 inhibitor	0.32	0.22–0.46					
		BMI	1.57	1.36–1.81					
	UK Biobank (SAIGE)	Statin	0.69	0.52–0.93					
		PCSK9 inhibitor	0.47	0.34–0.65					
		BMI	1.38	1.18–1.61					
All ischemic stroke	MEGASTROKE	Statin	1.17	0.84–1.65	1.05	0.91–1.21	0.56	5.8	1.4e–10
		PCSK9 inhibitor	0.94	0.65–1.37	1.02	0.88–1.18	0.47	5.7	1.3e–10
		BMI	1.18	1.04–1.34	1.16	1.05–1.28	0.12	18.0	0.0001
	UK Biobank (SAIGE)	Statin	1.33	0.80–2.20	0.79	0.65–0.97	0.04	5.8	0.09
		PCSK9 inhibitor	0.96	0.55–1.69	0.76	0.62–0.92	0.02	5.7	0.11
		BMI	1.13	0.93–1.36	1.27	1.10–1.47	<0.001	18.0	0.02
Atrial fibrillation	Nielsen et al. (including UK Biobank)	Statin	1.22	0.97–1.54	1.16	1.03–1.32	0.02	5.9	1.3e–80
		PCSK9 inhibitor	0.79	0.62–1.01	1.12	0.99–1.27	0.01	5.7	1.3e–81
		BMI	1.46	1.34–1.59	1.44	1.35–1.56	0.70	17.9	4.6e–18

estimate was 0.96. Assuming statins have the same effect on IHD and stroke, it would only take 10% with that harmful allele and 25% of potential stroke cases to have died from IHD or other competing risks before recruitment into a stroke study for the observed OR to be exactly 1.0, which would give a null MR estimate. If instead 40% of potential stroke cases had died from competing risk before recruitment, then the OR would reverse to 1.04 and give an MR estimate similar to the univariable estimate from MEGASTROKE.

DISCUSSION

Here, we have shown theoretically, empirically, and analytically that univariable MR studies can be open to quite severe selection bias likely arising from selective survival on genetically instrumented exposure when other causes of survival and outcome exist, i.e., competing risk before recruitment. We have also explained the relevance of this situation to the assumptions of MR, as a violation of the exclusion restriction assumption, how to mitigate this bias using multivariable MR, how to assess the success of this mitigation (using the multivariable Q statistic), and how to make an assessment of the possible level of bias using an approximation based on contextual knowledge (**Appendix Table 1**). Notably, genetic studies are particularly vulnerable to bias because most genetic estimates are of small magnitude; the closer the true estimate is to the null, the easier it is for a reversal to occur (**Appendix Figure 1**).

Our study differs from many other studies suggesting that MR is open to selection bias by specifically identifying when such bias can occur in the context of a typical MR study using existing GWAS, and by showing how any such bias may be addressed along with a means of checking whether the bias has been successfully addressed. For participants selected on surviving the genetically instrumented exposure and competing risk of the outcome, our study is similar to other studies about bias in MR in showing that bias can occur from using GWAS summary statistics with "covariable adjustment" (Hartwig et al., 2020). We add by explaining that selecting from the living is common in MR studies and may engender covariable adjustment on survival. Rather than suggesting that such situations should be avoided (Hartwig et al., 2020), precluding MR studies of a harmful exposure on a late-onset disease subject to competing risk, we show how such situations can be addressed. Specifically, external knowledge can be used to identify potential common causes of survival and outcome, followed by multivariable MR to adjust for them and thereby possibly obtain a less biased estimate, bearing in mind the Q statistic. We also show that when, in this situation, it is not possible to adjust comprehensively for factors causing survival and the outcome, the level of potential bias can be estimated (**Appendix Table 1**). Alternatively, restricting MR studies to younger people will usually reduce bias because death prior to recruitment is less common in younger people. However, these studies may need to consider competing risk after recruitment. Our study also implies that care should be taken in interpreting phenome-wide association studies

identifying the effect of a specific genetically instrumented exposure across the phenome, because the effects of harmful exposures observed will vary depending of the level of competing risk of the outcome.

Despite the strengths of our study in explicating and providing means of addressing a relatively common bias in univariable MR, limitations exist. First, use of multivariable MR to address bias arising from sample selection on survival requires knowledge of the underlying causal structure and suitable genetic instruments for all sources of bias. In all observational studies, knowledge of the underlying causal structure is needed to identify potential sources of confounding and selection bias. For example, here our results could also be due to removing the harmful effects of statins and PCSK9 inhibitors via body composition by adjusting for BMI, although these effects are still under investigation (Nelson et al., 2019). Alternative methods to recover from selection bias due to surviving the genetically instrumented exposure and competing risk of the outcome that do not require knowledge of the underlying causal structure or additional data would be easier to use. Second, our study did not conduct simulations of the level of bias. Simulations including research questions with the same underlying directed acyclic graph s as investigated here have been done (Hartwig et al., 2020), and simulation of a similar situation is available (Glymour and Vittinghoff, 2014). The key issue in making use of these simulations is appreciating when these biasing situations might arise and how serious the issues can be in practice, which is the gap addressed by this study. As such, we address appreciating which real-life situations will result in the simulated bias, and what to do to ameliorate it. Third, we provide a means of addressing any such selection bias using multivariable MR (adjusting for common causes of survival and outcome) as well as a means of assessing the likely validity of the revised estimate (non-significant multivariable Q-statistic). However, application and interpretation may not always be straightforward. As with any bias correction by adjustment, it may not be feasible to recover the correct estimate, due to lack of contextual knowledge, a highly interrelated causal structure, such as the genetic instruments causing common causes of survival and outcome, or a lack of relevant information. Fourth, we also provide an approximation to estimate the likely effects of such bias (**Appendix Table 1**). However, given that the role of selection bias due to death before recruitment from the genetically predicted exposure or from a competing risk of the outcome has rarely been explicitly considered previously, the information needed to identify the sources of bias and estimate the likely level of bias is not easily available. More research concerning the effects of genetic exposures on longevity and the sequence of death from different diseases in different populations would be helpful, as well as easily accessible information about the age and sex structure of participants in genetic studies by case status. Fifth, we do not provide an exhaustive list of examples of when this bias has occurred, because few MR studies have been validated against RCTs. For example, Alzheimer's disease usually occurs in old age and appears to share causes with determinants of longevity (Deelen et al., 2019), so MR studies of harmful exposures on Alzheimer's disease could be open to selection bias but the true causes of Alzheimer's disease are unknown making any determination of whether the MR studies are biased or not difficult. Finally, the issue of obtaining valid estimates in the presence of selective survival on exposure and competing risk of the outcome is similar to the issue of obtaining valid genetic estimates in other studies of survivors, i.e., patients. The current solution for obtaining valid estimates in genetic studies of patients relies on the assumption that the factors causing disease and disease progression differ (Dudbridge et al., 2019). Use of multivariable MR to adjust observational studies in patients suitably might bear consideration.

Specifically, as regards the example here, for the MR estimate for statins on stroke, we were able to recover a plausible estimate in the UK Biobank but not in MEGASTROKE. The UK Biobank participants are younger (∼57 years) than the MEGASTROKE participants (**Supplementary Table 1**), so the confounders of survival to recruitment and stroke used to adjust for survival could also be more biased by survival in MEGASTROKE making adjustment less effective in MEGASTROKE than in the UK Biobank, possibly as indicated by **Supplementary Table 2**. In addition, the Q-statistic represents both genetic pleiotropy and pleiotropy due to selection bias, so it is possible that the Q-statistic in MEGASTROKE is larger due to MEGASTROKE having more cases than UK Biobank rather than more severe selection bias, although the same instruments were used in both studies. The conditional F-statistics were quite low for lipid modifiers; however, they did not differ by outcome, so they are unlikely to fully explain the difficulty in fully recovering plausible estimates. The multivariable Q-statistics could also be somewhat larger because some samples used to obtain instruments for the exposures overlapped (Sanderson et al., 2019). However, given the very large Q-statistics for the multivariable estimates for stroke using MEGASTROKE and for AF (**Table 1**), this overlap is unlikely to affect the interpretation. Finally, the multivariable MR-Egger intercepts were not always significant even when the estimates did not look plausible, perhaps because MR-Egger detects exposure specific directional pleiotropy. In contrast, the multivariable Q-statistic assesses heterogeneity across several exposures which if different due to differing selection bias by exposure could contribute to a larger multivariable Cochran's Q as well as biased estimates.

CONCLUSION

Here, we have shown theoretically, empirically, and analytically that univariable MR studies can be open to quite severe selection bias arising from selecting on survival of genetically instrumented exposure when other causes of survival and outcome exist, i.e., competing risk before recruitment. Bias from such selection bias is likely to be least for MR studies of harmless exposures recruited shortly after genetic randomization with no competing risk, i.e., studies using birth cohorts with minimal attrition. Conversely, such bias is likely to be most evident for MR studies recruited at older ages examining the effect of a harmful exposure on an outcome subject to competing risk from shared etiology with other common conditions that occur earlier in life. Use of multivariable MR to adjust for major causes of survival

and outcome may ameliorate this bias, while simple sensitivity analysis based on information about the exposure and the natural history of disease may help quantify the magnitude of the bias. Infallible, methods of obtaining valid MR estimates, when the exclusion restriction is invalidated by selection bias stemming from competing risk, that do not require external knowledge, would be helpful.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author/s. This study only uses publicly available R packages to conduct the analysis. The code used to arrange the data for analysis is available on request.

ETHICS STATEMENT

This study only used publicly available genetic summary statistics, collected with consent, and so does not require ethical approval.

AUTHOR CONTRIBUTIONS

CS originated the study concept. PL, SAY, and JH explicated the concepts. JZ and ZY contributed substantially to the analysis, and implementation of the concepts. PL and CS wrote the first draft. SAY and JH contributed to the interpretation and presentation. All authors contributed to drafting and revising the article for intellectual content and approved the final version. All authors are accountable for all aspects of the work.

ACKNOWLEDGMENTS

This manuscript has been released as a pre-print at biorxiv (Schooling et al., 2020).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.610852/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX

A possible solution for recovering the causal effect in the presence of selection bias due to selecting on surviving the exposure and competing risk of the exposure in a case-control study.

The fundamental issue of the selection bias in a case–control study is unknown information for the "missing" (or unselected) participants. **Appendix Table 1** shows the possible mechanism generating a biased causal effect due to selection on surviving the exposure (E) and surviving competing risk (CR) of the outcome (D) in a case–control study.

Based on the observed data a′, b′, c′, and d′, the observed causal effect of E on D using an OR (OR^{obs}) is,

$$OR^{obs} = \frac{a'/b'}{c'/d'} = \frac{a'd'}{b'c'}$$

To obtain the true causal effect, we have to recover the data for the whole population, i.e., the birth cohorts who formed the population. Let P_E denote the proportion of participants unselected due to E, and let P_{CR} denote the proportion of participants unselected due to CR. Suppose P_E and P_{CR} are additive, and $0 < P_E + P_{CR} < 1$. We can construct the pattern of the unselected participants, as shown in **Appendix Table 1**. As such, the causal effect of E on D for the whole population can be estimated as follows,

$$OR^{true} = \frac{ad}{bc} = \frac{\frac{a'/(1-P_E)}{b'/(1-P_E-P_{CR})}}{\frac{c'}{d'/(1-P_{CR})}} = OR^{obs} \times \frac{(1-P_E)(1-P_{CR})}{(1-P_E-P_{CR})}$$

This relationship will be invalid if we replace the OR with a risk ratio.

TABLE A1 | Possible mechanism for biased causal effects in a case-control study due to selection bias from surviving the exposure and competing risk of the outcome.

		S = 1		**S = 0**	
		D = 1	**D = 0**	**D = 1**	**D = 0**
E = 1	CR = 1	a'	b'	P_E	$P_E + P_{CR}$
	CR = 0				
E = 0	CR = 1	c'	d'	0	P_{CR}
	CR = 0				
Observation		OR^{obs}			
Target		OR^{true}			

S indexes selection status of participants, i.e., S = 1 indicates those selected and S = 0 indicates those unselected. D indexes outcome status, i.e., D = 1 indicates disease and D = 0 indicates no disease. E indexes exposure status, i.e., E = 1 indicates the exposed, and E = 0 indicates unexposed. CR indicates competing risk (CR) of the outcome D; i.e., CR = 1 then D = 0, and if D = 1 and CR = 0. a′, b′, c′, and d′ are observed data about the selected participants.

Notably, the level of bias depends on the magnitude of the OR. A small OR, of the order of 1.05, as is typical in a genetic study, is much more vulnerable to a reversal of effect from selection bias due to selecting on surviving the exposure and surviving competing risk of the outcome than a larger OR, of the order of 1.50, as is typical in traditional observational studies. To clarify Appendix **Figure 1** shows the observed OR plotted against the true OR for different combinations of selection on survival (P_E) and selection on competing risk of surviving the outcome (P_{CR}).

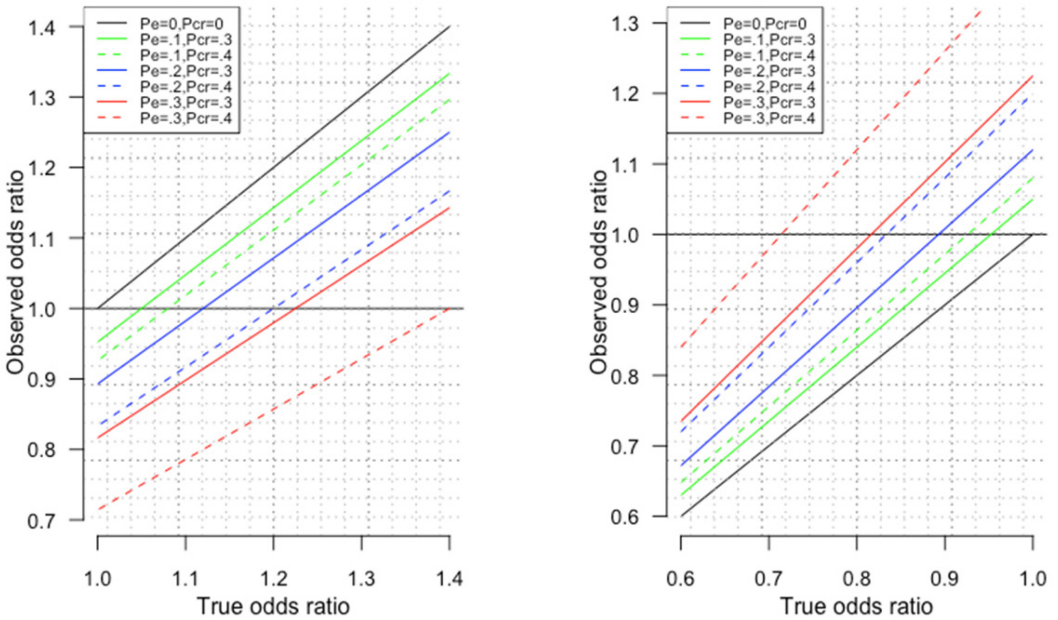

FIGURE A1 | Observed odds ratio against the True odds ratio in the presence of different proportions of death before recruitment due to the exposure (P_E) and different proportions of death before recruitment due to competing risk of the outcome (P_{CR}) for true odds ratios large than 1 (left hand side) and smaller than 1 (right hand side, obtained by taking the inverse of the odds ratio).

frontiers in Genetics

ORIGINAL RESEARCH
published: 04 February 2021
doi: 10.3389/fgene.2021.608714

The Role of C-Reactive Protein and Fibrinogen in the Development of Intracerebral Hemorrhage: A Mendelian Randomization Study in European Population

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: 02 October 2020
Accepted: 06 January 2021
Published: 04 February 2021

Citation:
Wang B, Zhang X, Liu D, Zhang J, Cao M, Tian X, Maranga IE, Meng X, Tian Q, Tian F, Cao W, Wang W, Song M and Wang Y (2021) The Role of C-Reactive Protein and Fibrinogen in the Development of Intracerebral Hemorrhage: A Mendelian Randomization Study in European Population. Front. Genet. 12:608714. doi: 10.3389/fgene.2021.608714

Background: The causal association of C-reactive protein (CRP) and fibrinogen on intracerebral hemorrhage (ICH) remains uncertain. We investigated the causal associations of CRP and fibrinogen with ICH using two-sample Mendelian randomization.

Method: We used single-nucleotide polymorphisms associated with CRP and fibrinogen as instrumental variables. The summary data on ICH were obtained from the International Stroke Genetics Consortium (1,545 cases and 1,481 controls). Two-sample Mendelian randomization estimates were performed to assess with inverse-variance weighted and sensitive analyses methods including the weighted median, the penalized weighted median, pleiotropy residual sum and outlier (MR-PRESSO) approaches. MR-Egger regression was used to explore the pleiotropy.

Results: The MR analyses indicated that genetically predicted CRP concentration was not associated with ICH, with an odds ratio (OR) of 1.263 (95% CI = 0.935–1.704, $p = 0.127$). Besides, genetically predicted fibrinogen concentration was not associated with an increased risk of ICH, with an OR of 0.879 (95% CI = 0.060–18.281; $p = 0.933$). No evidence of pleiotropic bias was detected by MR-Egger. The findings were overall robust in sensitivity analyses.

Conclusions: Our findings did not support that CRP and fibrinogen are causally associated with the risk of ICH.

Keywords: C-reactive protein, fibrinogen, single-nucleotide polymorphisms, Mendelian randomization, intracerebral hemorrhage

INTRODUCTION

Globally, stroke is a leading cause of death with a high societal burden in most regions (GBD 2015 Mortality and Causes of Death Collaborators, 2016). Among adults, the risk of stroke from the age of 25 years is approximately 25% (Feigin et al., 2018). Hemorrhagic stroke (HS) as a subtype of stroke carries high morbidity and mortality rates (Stokum et al., 2015),

and intracerebral hemorrhage (ICH) is by far the most common type of HS (Qureshi et al., 2009). Inflammation plays an important part in pathogenesis of stroke by influencing the development of atherosclerosis and plaque instability (Barone and Feuerstein, 1999; Scirica and Morrow, 2006).

C-reactive protein (CRP) and fibrinogen, considered as well-proven clinical markers of systemic inflammation, are acute-phase protein synthesized by hepatocytes against inflammation (Dalmon et al., 1993; Pepys and Hirschfield, 2003) and can increase the risk of cardiovascular disease (Scirica and Morrow, 2006; Zhang et al., 2014) and stroke (Coull et al., 1991; Cao et al., 2007; Jiménez et al., 2015). Significantly increased levels of fibrinogen are commonly found in patients with stroke, suggesting that fibrinogen is elevated before thrombotic incidents occur and is a risk factor for stroke (Coull et al., 1991). However, Roudbary et al. (2011) revealed that CRP concentration was not improved in patients with HS. No associations of CRP and fibrinogen with ICH were identified in a nested case-control study (Karim et al., 2020). The observational epidemiologic studies on the associations of CRP and fibrinogen with ICH showed inconsistent results (Coull et al., 1991; Roudbary et al., 2011). Furthermore, potential unmeasured confounders and reverse causation bias in observational studies limit the ability to ascertain causal inferences.

Mendelian randomization (MR) is a genetic epidemiological method to explore the association between the exposure and outcome, using genetic variants as instrumental variables (IVs) for the exposure (Smith and Ebrahim, 2003). Because of the independent segregation and randomized assignment of alleles at meiosis, MR approach can control potential confounders and reverse causation, making stronger causal inference (Lawlor et al., 2008). Therefore, we conducted two-sample MR analysis to assess the causal relationships of CRP and fibrinogen in the development of ICH in European population.

MATERIALS AND METHODS

Study Design and Data Sources

A two-sample MR approach was used to investigate the causal effects of CRP and fibrinogen on the risk of ICH. The study design is under the assumption that the genetic variants are associated with CRP and fibrinogen, but not with confounders. Besides, the genetic variants affect risk of ICH only through exposure and not through any alternative pathways.

Information on genetic variants associated with level of CRP was collected from a meta-analysis of genome-wide association study (GWAS), which is currently the largest study attempted to identify genetic variants in relation to CRP concentration involving 204,402 individuals from 88 previous population-based cohort studies (Ligthart et al., 2018). In genetic variants associated with fibrinogen, we used previously published genetic variants of a GWAS meta-analysis involving more than 100,000 subjects (Sabater-Lleal et al., 2013).

Summary statistics data on associations of genetic variants with ICH were obtained from the published GWAS meta-analysis by the International Stroke Genetics Consortium (ISGC) of 3,026 participants (1,545 cases and 1,481 controls; Woo et al., 2014). All data in our MR analyses were restricted to individuals of European ancestry only.

Genetic Variants

We used single-nucleotide polymorphisms (SNPs) published previously, which reached genome-wide significance ($p < 5 \times 10^{-8}$) for CRP and fibrinogen concentrations as MR IVs. The selected SNPs were independent, namely, not in linkage disequilibrium ($r^2 < 0.2$). Nineteen SNPs (11 for CRP and 8 for fibrinogen) were not presented in ISGC datasets. For the unavailable SNPs in outcome datasets, we replaced them with proxy SNPs. The proxy SNPs in linkage disequilibrium ($r^2 > 0.8$) were identified for two SNPs. Accordingly, 42 SNPs for CRP and 16 SNPs for fibrinogen were included in the analysis of ICH. The summary genetic association data are reported in **Supplementary Table S1**.

Mendelian Randomization Analysis

We performed two-sample MR analyses to estimate the associations of CRP and fibrinogen with ICH using summarized data. Causal effects on ICH of CRP and fibrinogen concentrations were estimated using the conventional inverse-variance weighted (IVW) method (Burgess et al., 2013). We also conducted sensitivity analyses using the weighted median (WM), the penalized weighted median (PWM), and pleiotropy residual sum and outlier (MR-PRESSO) methods (Bowden et al., 2015, 2016; Verbanck et al., 2018). For MR-Egger regression analysis, we assessed directional pleiotropy based on its intercepts (Burgess and Thompson, 2017). A leave-one-out analysis (omitted one SNP in turn) was performed to test the influence of outlying values (Burgess and Thompson, 2017). Heterogeneity of individual genetic variants was evaluated by Cochran's *Q* test. All results are presented as an odds ratio (OR) with 95% confidence interval (CI) of the outcomes per predicted increase in CRP and fibrinogen concentrations. The associations of each SNP with CRP and fibrinogen concentrations are further plotted compared to their effects for the outcomes. All analyses were performed by the TwoSampleMR and MR-PRESSO packages with R version 4.0.2.

RESULTS

Causal Association of CRP With ICH

The results of associations between genetically determined CRP and fibrinogen and the risk of ICH were presented in **Table 1**. Genetic predisposition to CRP levels were not observed to be statistically significantly associated with ICH by performing IVW method (OR = 1.263, 95% CI = 0.935–1.704, $p = 0.127$). The lack of causal association remained in all sensitivity analyses (all $p > 0.05$; **Table 1**).

The MR-Egger method showed no evidence of directional pleiotropy for the association of CRP with ICH [odds (intercept), −0.010; $p = 0.480$; **Table 2**]. For IVs, MR-PRESSO did not detect any potential outliers. Likewise, no heterogeneity was observed among individual SNPs of CRP for ICH ($Q = 36.775$,

p = 0.616, **Table 2**). We calculated the individual and pooled MR estimates between each CRP-related SNP and the risk for ICH shown as forest plots and scatter plots in **Figure 1**. The result of leave-one-out sensitivity analysis showed that the association between CRP and ICH was not substantially driven by any individual SNP (**Supplementary Figure S1**).

Causal Association of Fibrinogen With ICH

Regarding fibrinogen, we found no causal effect of genetically instrumented fibrinogen on ICH (OR = 0.879, 95% CI = 0.042–18.281, p = 0.933). No significant association was observed for ICH in sensitivity analyses that were performed by WM, PWM, MR-PRESSO, and MR-Egger methods (**Table 1**).

The MR-Egger method showed no evidence of directional pleiotropy for the association of fibrinogen with ICH (**Table 2**). For IVs, MR-PRESSO did not detect any potential outliers. We calculated the individual and pooled MR estimates between each fibrinogen-related SNP and risk for ICH shown as forest plots and scatter plots in **Figure 2**. Furthermore, analysis on leaving out each SNP revealed that the inverse association between fibrinogen concentrations and ICH was not substantially driven by any individual SNP (**Supplementary Figure S2**). However, the Cochran Q statistic was 28.028 with an associated $p < 0.05$, suggesting some heterogeneity in the effect estimates of fibrinogen and ICH (**Table 2**), but there was no clear evidence of directional pleiotropy (p for intercept > 0.05, **Table 2**).

TABLE 1 | Mendelian randomization (MR) estimates of exposure with intracerebral hemorrhage from the inverse-variance weighted (IVW) and sensitivity analysis.

Phenotype	IVs (SNPs)	OR (95% CI)	p
CRP			
IVW	42	1.263 (0.935–1.704)	0.127
Weighted median	42	1.458 (0.977–2.175)	0.065
Penalized weighted median	42	1.466 (0.957–2.247)	0.079
MR_Egger	42	1.432 (0.906–2.266)	0.133
MR-PRESSO	42	1.236 (0.950–1.522)	0.154
Fibrinogen			
IVW	16	0.879 (0.042–18.281)	0.933
Weighted median	16	0.438 (0.016–11.771)	0.623
Penalized weighted median	16	0.438 (0.014–13.561)	0.637
MR_Egger	16	1.663 (0.004–746.651)	0.872
MR-PRESSO	16	1.221 (−1.893 to 4.335)	0.901

CRP, C-reactive protein; IVW, inverse-variance weighted; MR, Mendelian randomization; WM, weighted median; PWM, penalized weighted median; OR, odds ratio; MR-PRESSO, pleiotropy residual sum and outlier; CI, confidence interval.

DISCUSSION

In the present study, we assessed whether high circulating levels of CRP and fibrinogen are causally associated with ICH using two-sample MR analysis in European population. In the present study using publicly available summary statistics data, we did not find CRP and fibrinogen levels might increase ICH risk. The findings were overall robust in sensitivity analyses.

Apart from being markers of systemic inflammation, CRP and fibrinogen are acute-phase protein induced by proinflammatory cytokine contributing to host defense against infection (Dalmon et al., 1993; Pepys and Hirschfield, 2003). Previous studies investigated associations between CRP and fibrinogen and ICH but reported inconsistent results. A large-scale cohort study found that CRP and fibrinogen were not associated with a significantly greater risk of HS (Jiménez et al., 2016), while a retrospective cohort study suggested that increased CRP was a significant risk factor for in-hospital mortality among patients with cardiovascular disease including ICH (Yoshinaga et al., 2017).

In our analysis, we did not observe the relationships of CRP and fibrinogen with ICH. These findings suggested that the role of CRP and fibrinogen may be less important in causing the risk of ICH. A previous MR study indicated that CRP concentration itself was unlikely to be even a modest causal factor in coronary heart disease (Wensley et al., 2011). Our findings corroborate earlier studies that showed CRP had no clear effect on ICH risk (Liu et al., 2014). Similar results were also found in a meta-analysis consisting of six population-based prospective studies (Georgakis et al., 2019). Another meta-analysis has also suggested that elevated baseline CRP levels exhibited no clear effect on HS (Zhou et al., 2016). However, evidence from a few prospective studies showed that CRP level in HS patients was significantly elevated (Das et al., 2014; Xue et al., 2017).

Fibrinogen participates in platelet aggregation, thrombogenic activity, atherogenesis, and inflammation, and the role of fibrinogen is probably various in the different subtypes of stroke. Our findings were supported by previous studies, which also reported

TABLE 2 | Heterogeneity tests and MR-Egger intercept of CRP and fibrinogen causally linked to ICH.

Outcome	Exposure	Intercept	p^a	Cochran's Q	Q_df	p^b
ICH	CRP	−0.010	0.480	36.775	40	0.616
ICH	Fibrinogen	−0.008	0.815	28.028	15	0.045

[a]*Value of p for MR-Egger intercept.*

[b]*Value of p for heterogeneity tests by performing inverse-variance weighted method.*

CRP, C-reactive protein; ICH, intracerebral hemorrhage.

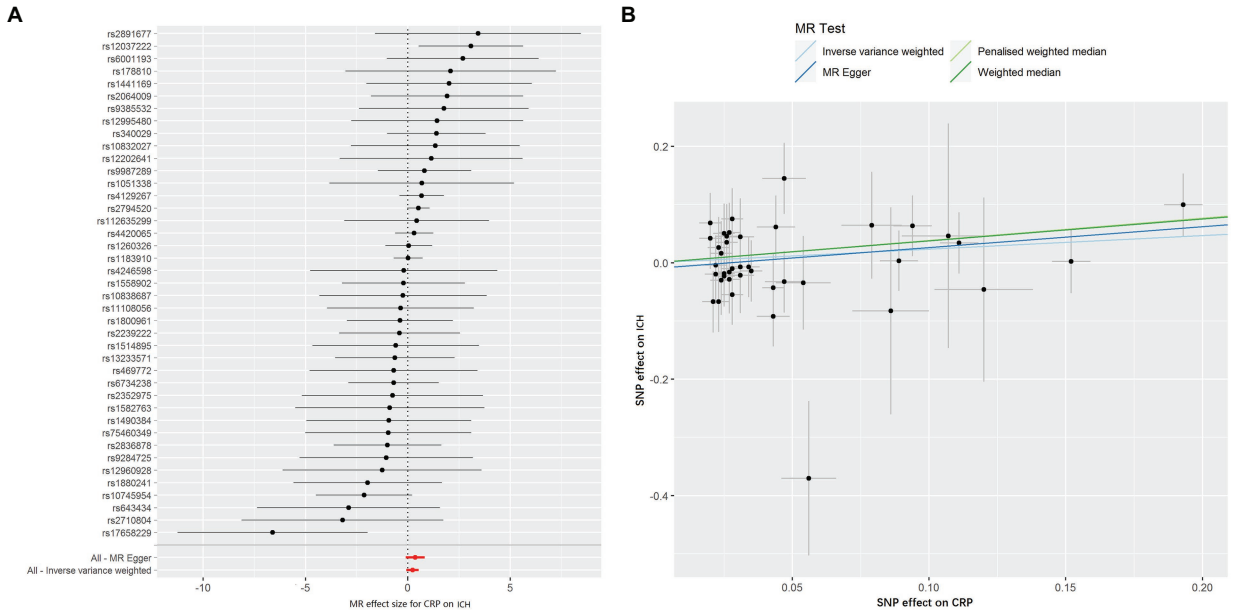

FIGURE 1 | Forest plots and scatter plots of CRP-associated SNPs potential effects on intracerebral hemorrhage (ICH). Forest plot **(A)** shows the odds ratio (OR) with a horizontal line representing 95% CI for the CRP-associated SNP allele for ICH risk. Scatter plot **(B)** shows the per-allele association with ICH risk plotted against the per-allele association with 1 SD of CRP (vertical and horizontal black lines presenting the 95% CI of OR for each SNP), with the slope of each line corresponding to estimated Mendelian randomization (MR) effect per method.

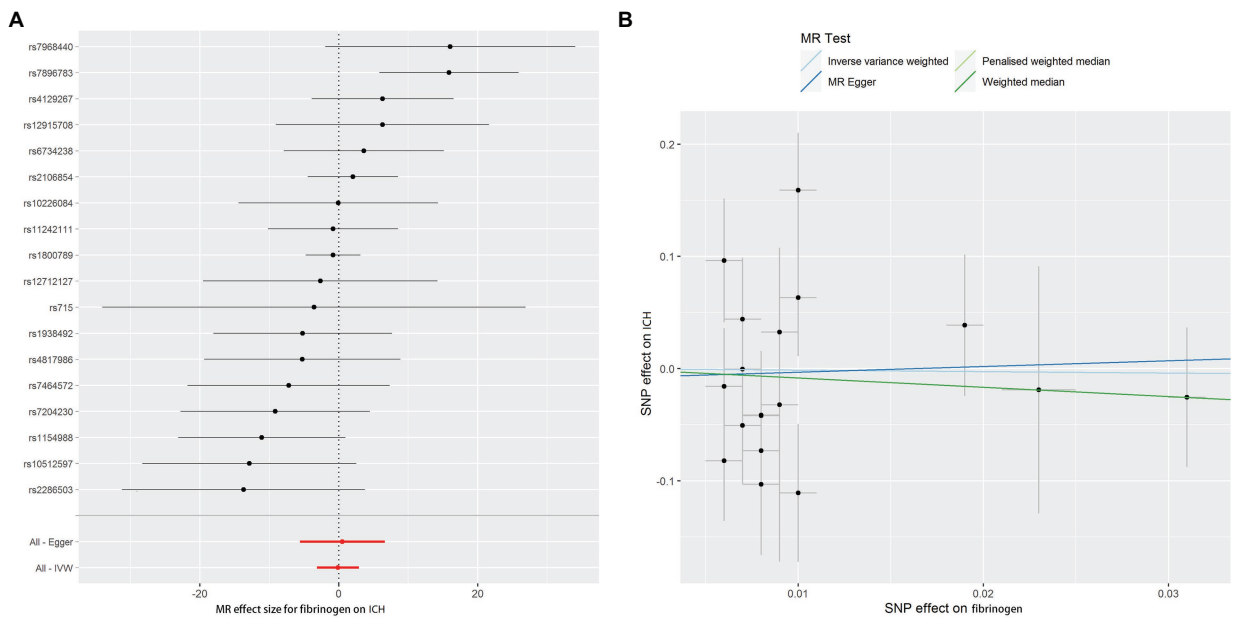

FIGURE 2 | Forest plots and scatter plots of fibrinogen-associated SNPs potential effects on ICH. Forest plot **(A)** shows the odds ratio (OR) with a horizontal line representing 95% CI for the fibrinogen-associated SNP allele for ICH risk. Scatter plot **(B)** shows the per-allele association with ICH risk plotted against the per-allele association with 1 SD of fibrinogen (vertical and horizontal black lines presenting the 95% CI of OR for each SNP), with the slope of each line corresponding to estimated Mendelian randomization (MR) effect per method.

that elevated levels of fibrinogen did not exhibit suggestive evidence of association with HS (Alvarez-Perez et al., 2011). In line with our results, no significant association between fibrinogen and ICH was observed in observational studies (Woodward et al., 2005; Welsh et al., 2008; Folsom et al., 2016). However, greater plasma fibrinogen concentration was associated

with increased risk of ICH in these prospective studies (Sato et al., 2006; Sturgeon et al., 2008). These findings should be interpreted cautiously as higher CRP and fibrinogen levels may reflect subclinical infection, chronic infectious diseases, preexisting disease, and socioeconomic or lifestyle characteristics. Besides, these opposite results may be due to different study populations and ethnic groups (Iso et al., 2012; Shi et al., 2016).

The pathogenesis of the associations of CRP and fibrinogen with the risk of ICH is unclear. CRP plays a direct role in the pathogenesis of atherosclerosis and is upregulated significantly in atheromatous plaques, where it may promote low-density lipoprotein cholesterol uptake by macrophages (Torzewski et al., 2000). Moreover, these inconsistent previous results may be due to reverse causal bias or confounders from atherosclerosis (Libby et al., 2011) or inflammation (Hartwig et al., 2017). One possible explanation is that the previous finding was a false-positive outcome because the effect of confounding was not controlled for, whereas in our studies, the genetic variants associated with exposure explained a larger proportion of variance, showing the true relationship of CRP and fibrinogen with ICH. Another possible explanation is that a mass of variants resulted in greater pleiotropy potential, which may have diluted the association in our analysis.

The major strengths of this study are using data from large-scale GWAS studies and ISGC collaboration. We used a two-sample MR approach assessing CRP and fibrinogen levels in relation to the risk of ICH in European-descent individuals, which reduces bias of population stratification. Moreover, in terms of the MR analysis, we performed conventional IVW, WM, PWM, MR-PRESSO, and MR-Egger methods to avoid reverse causation and to reduce other confounding factors. Lastly, there is no strong evidence of pleiotropic effects for the genetic instruments, suggesting there was less likelihood of CRP and fibrinogen-related SNPs are associated with other phenotypes.

The present study also has some limitations. Interpreting the magnitude of estimates for the effect of CRP and fibrinogen on ICH risk requires caution. First, stratified analyses or analyses adjusted for other covariates were not possible on the account of using the available summary statistics datasets. In addition, the genetic IVs accounted for approximately 7.0% of the total variation in CRP and 3.7% of plasma fibrinogen variation (Sabater-Lleal et al., 2013; Ligthart et al., 2018), which might be low for the use as IVs, and any bias from weak instruments was in the direction of the null (Pierce and Burgess, 2013). Nevertheless, MR analysis likely reflects lifelong exposure to elevated CRP and fibrinogen levels. However, it is possible that only exposure in a specific window of time (e.g., early life) affects ICH risk. Lastly, we used a relatively small sample size to explore the causal relationship between CRP, fibrinogen, and ICH with the power of less than 0.90. Thus, the nonsignificant but still suggestive associations between CRP and fibrinogen levels and ICH risk should be further validated in future studies with larger independent populations and larger datasets offering greater statistical power.

In conclusion, these MR analyses did not find evidence to support the causal relationship between CRP and fibrinogen with ICH. The results add to the burgeoning evidence that refutes the harmful role of CRP and fibrinogen in ICH. Further research is required to clarify this finding, using larger samples for undertaking "adjusted" MR analyses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

BW and XZ drafted the manuscript. YW, MS, DL, JZ, MC, XT, IM, XM, QT, FT, WC, and WW critically revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The study was supported by grants from the National Natural Science Foundation of China (81673247, 81872682, and 81773527) and the China-Australian Collaborative Grant (NSFC 81561128020-NHMRC APP1112767).

ACKNOWLEDGMENTS

Data on CRP and fibrinogen associated single-nucleotide polymorphisms have been derived from published articles (Sabater-Lleal et al., 2013; Ligthart et al., 2018). Data on intracerebral hemorrhage were accessed through the ISGC Cerebrovascular Disease Knowledge Portal (Woo et al., 2014).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.608714/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 18 February 2021
doi: 10.3389/fgene.2021.631061

Assessment of Causal Direction Between Gut Microbiota and Inflammatory Bowel Disease: A Mendelian Randomization Analysis

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Background: Recent studies have shown that the gut microbiota is closely related to the pathogenesis of Inflammatory Bowel Disease (IBD), but the causal nature is largely unknown. The purpose of this study was to assess the causal relationship between intestinal bacteria and IBD and to identify specific pathogenic bacterial taxa via the Mendelian randomization (MR) analysis.

Materials and Methods: MR analysis was performed on genome-wide association study (GWAS) summary statistics of gut microbiota and IBD. Specifically, the TwinsUK microbiota GWAS (N = 1,126 twin pairs) was used as exposure. The UK inflammatory bowel disease (UKIBD) and the Understanding Social Program (USP) study GWAS (N = 48,328) was used as discovery outcome, and the British IBD study (N = 35,289) was used as replication outcome. SNPs associated with bacteria abundance at the suggestive significance level ($\alpha = 1.0 \times 10^{-5}$) were used as instrumental variables. Bacteria were grouped into families and genera.

Results: In the discovery sample, a total of 30 features were available for analysis, including 15 families and 15 genera. Three features were nominally significant, including one family (*Verrucomicrobiaceae,* 2 IVs, beta = −0.04, p = 0.05) and two genera (*Akkermansia*, 2 IVs, beta = 0.04, p = 0.05; *Dorea*, 2 IVs, beta = −0.07, p = 0.04). All of them were successfully replicated in the replication sample (*Verrucomicrobiaceae* and *Akkermansia* $P_{\text{replication}}$ = 0.02, *Dorea* $P_{\text{replication}}$ = 0.01) with consistent effect direction.

Conclusion: We identified specific pathogenic bacteria features that were causally associated with the risk of IBD, thus offering new insights into the prevention and diagnosis of IBD.

Keywords: mendelian randomization, gut microbiota, inflammatory bowel disease, ulcerative colitis, causal relationship

OPEN ACCESS

Edited by:
Lei Zhang,
Soochow University, China

Reviewed by:
Feng Zhang,
Xi'an Jiaotong University, China
Sumei Xiao,
Sun Yat-sen University, China

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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *19 November 2020*
Accepted: *04 January 2021*
Published: *18 February 2021*

Citation:
Zhang Z-J, Qu H-L, Zhao N, Wang J, Wang X-Y, Hai R and Li B (2021) Assessment of Causal Direction Between Gut Microbiota and Inflammatory Bowel Disease: A Mendelian Randomization Analysis. Front. Genet. 12:631061. doi: 10.3389/fgene.2021.631061

Abbreviations: FDR, false discovery rate; GWAS, genome-wide association study; IBD, inflammatory bowel disease; IV, instrumental variable; IVW, inverse-variance weighted; LD, linkage disequilibrium; MGWAS, microbiome genome-wide association study; MR, Mendelian randomization; OTU, operational taxonomic unit; UKB, UK Biobank; USP, understanding social program.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic non-specific inflammatory disease that invades colonic mucosa without gender advantage (Matsuoka et al., 2018). The peak age of IBD is between 20 and 40 years old (Loftus, 2004; Bernstein et al., 2006; Cosnes et al., 2011). The main symptoms of IBD are abdominal pain, diarrhea, mucous bloody stool, as well as extra-intestinal symptoms. IBD is mostly common in developed countries including North America, Europe, Australia, and New Zealand, with incidence rate as high as 20–100 per million people. It is estimated that as many as 1 million Americans suffer from IBD (Cohen et al., 2010; Magro et al., 2012). In recent decades, the incidence of IBD has been rising all over the world, especially in East Asian (Loftus et al., 2007; Bengtson et al., 2009; Cosnes et al., 2011; Molodecky et al., 2012).

The pathogenesis of IBD has not been fully elucidated. It has a strong genetic determinant. For instance, first-degree relatives of patients with IBD are 4 to 20 times more likely to develop IBD (Kevans et al., 2016). Recent genome-wide association studies (GWASs) have identified more than 200 responsible genomic loci associated with IBD (Turpin et al., 2018). Despite these fruitful findings, its pathogenic mechanism has not been fully understood yet. On the other hand, gut microbiota may be related to the pathogenesis of IBD (Nishida et al., 2018). Imbalance of gut microbiota coupled with impaired intestinal bacterial clearance could enhance the invasiveness of pathogens, disrupt intestinal immune response, accelerate intestinal inflammation, and eventually lead to IBD. In a recent controlled trial, patients in the fecal microbiota transplantation group showed significant clinical improvement, indicating that high-dose fecal microbiota transplantation is an effective method for the treatment of active IBD (Paramsothy et al., 2017). Another study indicates that the low abundance of *Phascolarctobacterium* is positively correlated with the occurrence of IBD (Bajer et al., 2017).

Although previous extensive studies have established observational associations between gut microbiota and IBD developing risk, the causal nature is largely unclear. Mendelian randomization (MR) analysis is a statistical approach that aims to infer causal relationship from observational association results (Lee and Lim, 2019). With the rapidly increasing genetic data at both microbiota and complex disease sides, MR has been widely applied in recent years. MR approach has three essential assumptions: (1) Instrumental variable (IV) is strongly associated with exposure; (2) IV is not associated with any confounders of exposure; and (3) The association of IV with outcome is only through exposure. It has been used to infer the causal relationship from gut microbiota to type 2 diabetes, neurodegenerative diseases, and bone density (Burgess et al., 2013; Bowden et al., 2015; Goodrich et al., 2016; Quigley, 2017; Verbanck et al., 2018).

In the present study, in order to explore the causal relationship from gut microbiota to IBD, and to identify specific pathogenic bacteria taxa, we conducted a two-sample MR study based on GWAS summary data. In brief, summary data from the microbiota GWAS (MGWAS) of the TwinsUK study were used as exposure, and GWAS summary statistics from two IBD GWAS were used as discovery and replication outcomes.

MATERIALS AND METHODS

GWAS Summary Statistics

The MR analysis was performed on GWAS summary statistics of both microbiota and IBD. All data were retrieved from previously published studies that were released to the public.

The microbiota GWAS summary statistics from the TwinsUK study (Goodrich et al., 2016) served as exposure. In brief, The TwinsUK study collected 3,261 fecal samples from 1,126 twin pairs from the TwinsUK Registry in British. Microbiota 16S rRNA was sequenced using Illumina Miseq 2×250 bp sequencing platform, followed by host genome genotyping using Illumina HumanHap610 Quad Chip. For genotype imputation, the 1,000 Genomes project (Phase 3) reference panel was used. Sixty-one bacteria taxa were found to be associated with 307 host SNPs with p-values ranging from 7.33×10^{-5} to 4.94×10^{-9} (**Supplementary Table 1**).

The discovery outcome sample UK IBD and Understanding Social Program (UKIBD and USP) is a GWAS study based on a general prospective population cohort of European ancestry with 12,924 cases and 35,391 controls. Host genome was genotyped by the HumanCyto SNP-12 BeadChip and the Immunochip arrays, and was imputed into the UK IBD Genetics Consortium and UK10K Consortium reference panel (Burgess et al., 2013). A total of 38 genomic loci were identified at the genome-wide significance level ($p < 5.0 \times 10^{-8}$), increasing the number of known IBD risk sites to 200.

The replication British IBD sample was the GWAS of 16,452 IBD British cases and 18,837 controls. Participants were genotyped on the Human Core Exome v12.1, the Affymetrix 500K, or the Affymetrix 6.0 genotyping array.

Instrumental Variable Selection

The same criteria were used for IV selection in both discovery and replication samples. IVs were grouped at family or genus level. Specifically, a bacterial feature was defined as a family or genus. SNPs associated with bacterial taxa in one feature were grouped together for that feature. As a QC step, palindrome SNPs, which are defined as SNPs with ambiguous strand information (e.g., A/T or G/C polymorphisms), were removed. SNP-feature association threshold was set to be 1.0×10^{-5}. In order to eliminate the effect of linkage disequilibrium (LD), SNPs within each feature were clumped with PLINK (v1.9). The LD threshold was set to be $r^2 < 0.1$, and the clustering window was set to be 500 kb. LD was estimated on the 1,000 Genome Project sequencing data (Phase 3).

In order to minimize the effect of horizontal pleiotropy. MR-PRESSO global test and outlier test were applied (Verbanck et al., 2018). The MR-PRESSO outlier test calculates the p-value for the significance of pleiotropy for each SNP, while the MR-PRESSO Global test calculates the p-value for the overall level of pleiotropy. Each individual SNP was deleted in turn and the MR-PRESSO Outlier test was applied to the set of remaining

SNPs (Verbanck et al., 2018). All significant SNPs were removed. A MR-PRESSO Global test was finally performed to monitor the overall pleiotropic effect. Non-significant SNPs were used for subsequent MR analysis.

MR Analysis

Upon the selection of qualified SNPs, MR analysis was then performed for a causal relationship from microbiota feature to IBD risk. Specifically, each microbiota feature was tested for its association. For features with multiple IVs, the inverse-variance weighted (IVW) test (Burgess et al., 2013) was applied. For features with only one IV, the Wald ratio test was applied. The results of IVW were also cross-validated by three alternative tests, including the MR-Egger regression (Bowden et al., 2015), the weighted median estimator (Bowden et al., 2016) and the MR-PRESSO (Verbanck et al., 2018).

Nominally significant results identified in the discovery sample were subjected to be replicated in the replication sample, with the same analysis procedures.

The horizontal heterogeneity effect was examined by the IVW test and the MR-Egger regression. Meanwhile, a leave-one-out sensitivity analysis was performed to monitor if significant associations were dominated by a single SNP.

All the above analyses (including sensitivity analysis and MR analysis) were implemented within the R packages TwoSampleMR[1] (Hemani et al., 2018) and MRPRESSO[2] (Sanna et al., 2019).

RESULTS

The flow chart of the present study is displayed in **Figure 1**. In the discovery sample, there are 237 host SNPs that are associated with gut microbiota features at the significance threshold $p < 1.0 \times 10^{-5}$. After clumping, 168 and 80 SNPs are left for 15 families and 15 genera, respectively (**Supplementary Table 2**). Two families with the largest number of SNPs are *Lachnospiraceae* (51 SNPs) and *Ruminococcaceae* (51 SNPs), followed by *Bacteroidaceae* (36 SNPs). There are five families, *Barnesiellaceae*, *Enterobacteriaceae*, *Rikenellaceae*, *Streptococcaceae*, and *Veillonellaceae*, each having only one SNP. At the genus level, the genus with the largest number of SNPs is *Bacteroides* (36 SNPs), followed by *Faecalibacterium* (9 SNPs) and *Coprococcus* (6 SNPs). There are four genera each having only one SNP, *Anaerostipes*, *Dorea*, *Streptococcus*, and *Veillonella*. Of note, genus is a child taxon of family, therefore SNPs contained in both features may overlap. For example, the genus *Faecalibacterium* is

[1]https://github.com/MRCIEU/TwoSampleMR
[2]https://github.com/rondolab/MR-PRESSO

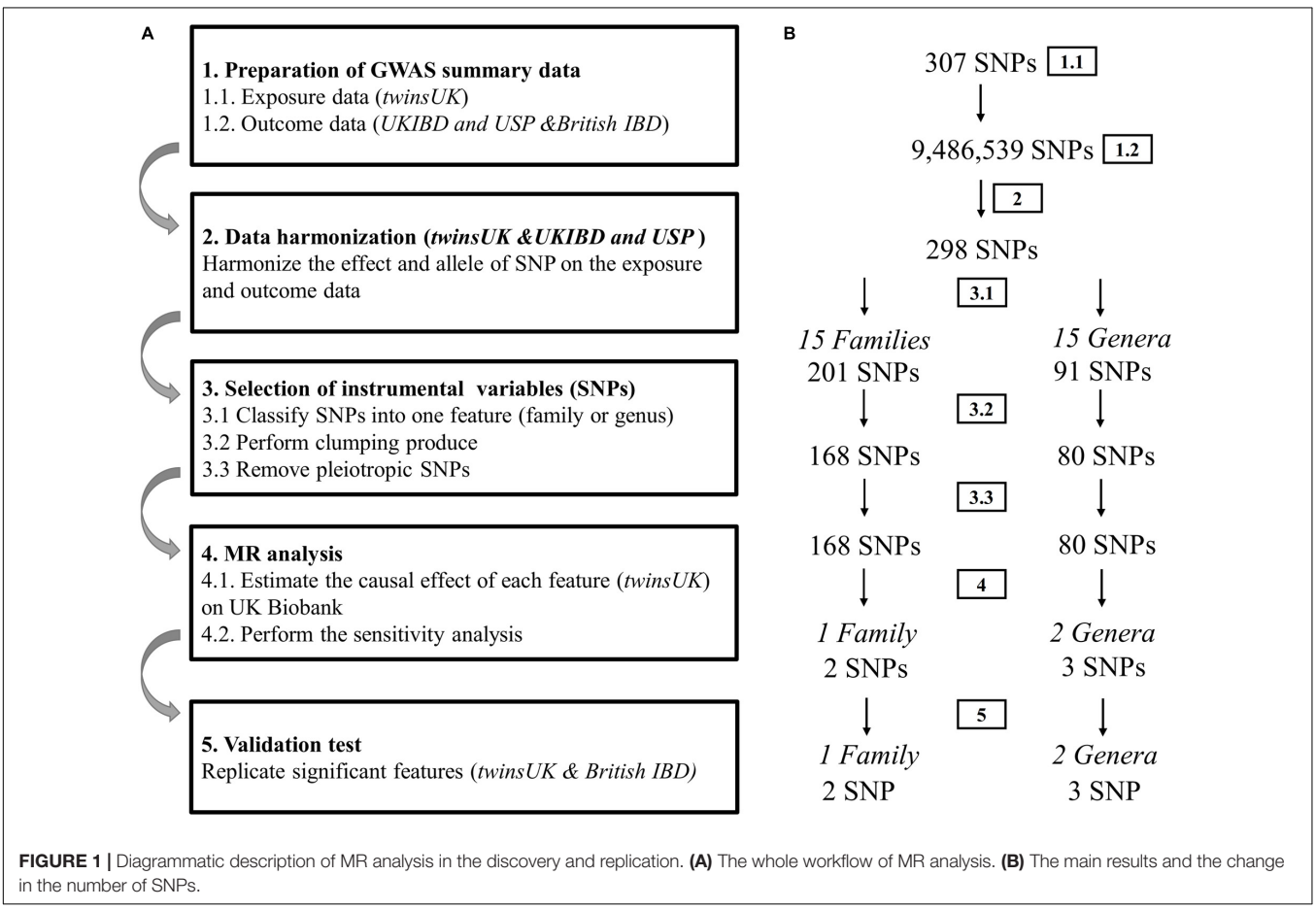

FIGURE 1 | Diagrammatic description of MR analysis in the discovery and replication. **(A)** The whole workflow of MR analysis. **(B)** The main results and the change in the number of SNPs.

a child taxon of the family *Ruminococcaceae*. The SNPs in them are partly identical.

For features containing multiple IVs, no outliers were detected using the MR-PRESSO outlier test and no evidence of horizontal pleiotropy (both MR-PRESSO Global test $p > 0.05/15 = p > 3.3 \times 10^{-3}$ and MR-Egger regression $p > 0.05$) was observed.

MR Analysis

In the discovery sample, after removing potentially pleiotropic SNPs, one family and two genera are significant at the nominal level ($p <= 0.05$): family *Verrucomicrobiaceae* (2 IVs, beta = 0.04, $p = 0.05$), genus *Akkermansia* (2 IVs, beta = 0.04, $p = 0.05$) and genus *Dorea* (1 IV, beta = −0.07, $p = 0.04$).

In sum, three features (one family ++ two genera) are causally associated with IBD in the discovery sample. These three features were replicated in the British IBD replication sample. The same IVs are available in the replication sample. Using the same IVW test, the replication *p*-value is significant ($p = 0.02$) and the effect direction is consistent for family *Verrucomicrobiaceae* and genus *Akkermansia* (**Table 1**). For the other genus *Dorea*, only one SNP rs10743315 is qualified as the IV. Using the Wald ratio test, the MR *p*-value is 0.01, again with consistent effect direction. Moreover, there is no evidence of heterogeneity at the three identified features in both discovery and replication sample. Detailed information of the 3 IVs is listed in **Table 2**.

DISCUSSION

In this study, we used MR analysis to evaluate the causal relationship between gut microbiota and IBD. Using the summary statistics of one microbiota GWAS and 2 IBD GWASs, we identified and replicated three bacterial taxa, one family *Verrucomicrobiaceae* and two genera *Akkermansia* and *Dorea*, that may have causal relationship with IBD. Our study confirmed that gut microbiota can aggravate IBD, suggesting that gut microbiota plays a regulatory role in IBD.

The gut microbiota is an intricate and dynamic collective of ecological microbial communities that are colonized in the human gut, even called a "forgotten organ" (O'Hara and Shanahan, 2006; Backhed et al., 2015). Gut microbiota is not only an important part of immune and metabolic health, but also regulate central nervous system and relevant disorders, including movement disorders, neurodegenerative diseases, behavioral disorders, neuroimmune-mediated diseases, and Cerebrovascular accident (Strandwitz, 2018). More than 90% of the gut microbiota that maintain intestinal health and balance in adults consist of four phylums of *Firmicutes*, *Bacteroides*, *Actinobacteria*, and *Proteobacteria* (Matsuoka and Kanai, 2015). The large intestine comprises the densest and metabolism-active microorganism in healthy individuals, which is predominated by anaerobic microbiota, two phyla *Firmicutes* and *Bacteroidetes*, apart from *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Eckburg et al., 2005).

The *Dorea* identified in this study belongs to the *Lachnospiraceae* family, which mainly exists in the gut microbiota of mammals and humans. One previous study has established a link between *Lachnospiraceae* and IBD (Lee et al., 2020). Another recent studies has also confirmed that the level of *Lachnospiraceae* and butyric acid gets decreased in IBD patients (Sasaki et al., 2019). The genus *Akkermansia* is present abundantly in the human gastrointestinal tract where it is believed to be a key symbiont member of the microbiota (Collado et al., 2007; Derrien et al., 2008; van Passel et al., 2011; Clarke et al., 2014; Guo et al., 2016). Extensive studies demonstrate that the lower level of *Akkermansia* is found in patients with IBD and other metabolic disorders, suggesting that *Akkermansia* may have potential anti-inflammatory properties (Zhang et al., 2016).

Previous studies have shown that the imbalance of gut microbiota is one of the pathogenic factors of IBD, but the specific regulatory mechanism is yet poorly understood. One possible mechanism, among others, is that the anti-inflammatory activity of IBD model is related to the regulation of inflammatory cytokines such as iNOS, MPO, IL-4, IL-10, EGF, MUC2, IL-6 and so on (Ma et al., 2018). However, this needs to be confirmed by further functional studies, which is beyond the scope of this study.

Mendelian randomization analysis is an effective method to explore causality from exposure to outcome while controlling confounding factors. The MR analysis in this study has the following advantages. First, it is a new attempt to speculate the causal relationship from gut microbiota to IBD, which provides a theoretical basis for the follow-up study of the regulation

TABLE 1 | MR analysis of gut microbiota on IBD in both discovery and replication samples.

Stage	MR Tests	Family			Genus					
		Verrucomicrobiaceae			*Akkermansia*			*Dorea*		
		No. SNP	b_{xy}	*p*-value	No. SNP	b_{xy}	*p*-value	No. SNP	b_{xy}	*p*-value
Discovery										
	IVW	2	0.04	**0.05**	2	0.04	**0.05**	–	–	–
	Wald ratio test	–	–	–	–	–	–	1	−0.07	**0.04**
Replication										
	IVW	2	0.02	**0.02**	2	0.02	**0.02**	–	–	–
	Wald ratio test	–	–	–	–	–	–	1	−0.08	**0.01**

No. SNP is the number of SNPs being used as IVs. b_{xy} is the estimated effect coefficient. Significant p-values were marked in bold. IVW, inverse-variance weighted.

TABLE 2 | Instrumental variables used in both discovery and replication studies.

Stage	Snp	Chr	Position	Locus	A1	A0	Closest Gene	Exposure			Outcome (Discovery)			Outcome (Replication)		
								Beta	SE	*p*-value	Beta	SE	*p*-value	Beta	SE	*p*-value
Family *Verrucomicrobiaceae*/	rs10081087	6	141858751	6q24.1	A	G	RN7SKP106	0.57	0.12	2.16×10^{-7}	0.04	0.02	0.045	0.04	0.02	0.05
Genus Akkermansia	rs692899	18	43316270	18q12.3	C	T	SLC14A1	−0.55	0.11	1.83×10^{-8}	−0.01	0.02	0.42	−0.02	0.02	0.16
Genus *Dorea*	rs12607607	18	39082090	18q12.3	T	C	KC6	−0.63	0.11	3.69×10^{-9}	0.04	0.02	0.04	0.05	0.02	0.01

Chr is Chromosome of SNP. Physical position is based on the human genome GRCH37 assembly. A1 is the effect allele and A0 is the other allele. Beta is the estimate coefficient of the effect allele. SE is the standard error of the estimate coefficient. Closest gene is the closest gene to which the SNP is mapped. Family Verrucomicrobiaceae and genus Akkermansia have the same SNPs.

mechanism of single strain on IBD. Second, it is based on publicly available large-scale GWAS summary statistics, so it provides an effective choice for mining reliable genetic information without additional experimental cost.

Obviously, our study has certain limitations. First, due to limited sample size, the genetic loci identified in gut microbiota GWAS are still limited, which limits the statistical power of MR analysis. Second, MR analysis based on one single IV is less robust, which may bias the interpretation of our findings.

In conclusion, we evaluated the causal relationship from gut microbiota to IBD and identified specific bacterial taxa that may affect the pathogenesis of IBD by a two-sample MR analysis using publicly available GWAS summary statistics. Our results provide a basis for revealing the causal relationship from gut microbiota to IBD, and thus offer new insights into its development and treatment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: ftp://ftp.sanger.ac.uk/pub/project/humgen/summary_statistics/human/2016-11-07/. The accession code EGAS00001000924. Key data were supplied in the **Supplementary Tables 1, 2**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the Local Legislation and Institutional Requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Z-JZ: conceptualization, formal analysis, data curation, and writing – original draft. H-LQ: validation and writing – original draft. NZ: formal analysis and writing – original draft. JW: resources and writing – original draft. X-YW: data curation and writing – original draft. BL: data curation, supervision, methodology, writing, and revising. RH: conceptualization, methodology, software, data curation, writing, review, editing, and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was partially supported by the funding from National Natural Science Foundation of China (81460223 to RH) and a project funded by the Natural Science Foundation of Inner Mongolia Autonomous Region (2016MS0879 to JW). BL was partially supported by grants from Jiangsu Provincial Medical Youth Talent (No. QNRC2016249), Suzhou Science and

Technology Bureau (Nos. SYSD2017041 and SYS201788), Gusu Health Top-Notch Youth Talent of Suzhou Health Commission (No. GSWS2019086), and Wujiang District Health Commission (Nos. WWK201609 and WWK201806).

ACKNOWLEDGMENTS

We appreciate all the volunteers who participated in this study. We are grateful to the TwinsUK study for releasing the gut microbiota GWAS summary statistics, the UKIBD and USP study and the British IBD study for releasing the IBD GWAS summary statistics.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.631061/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

METHODS
published: 17 March 2021
doi: 10.3389/fgene.2021.618829

Mendelian Randomization With Refined Instrumental Variables From Genetic Score Improves Accuracy and Reduces Bias

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Edited by:
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *18 October 2020*
Accepted: *01 February 2021*
Published: *17 March 2021*

Citation:
Lin L, Zhang R, Huang H, Zhu Y, Li Y, Dong X, Shen S, Wei L, Chen X, Christiani DC, Wei Y and Chen F (2021) Mendelian Randomization With Refined Instrumental Variables From Genetic Score Improves Accuracy and Reduces Bias. Front. Genet. 12:618829. doi: 10.3389/fgene.2021.618829

Mendelian randomization (MR) can estimate the causal effect for a risk factor on a complex disease using genetic variants as instrument variables (IVs). A variety of generalized MR methods have been proposed to integrate results arising from multiple IVs in order to increase power. One of the methods constructs the genetic score (GS) by a linear combination of the multiple IVs using the multiple regression model, which was applied in medical researches broadly. However, GS-based MR requires individual-level data, which greatly limit its application in clinical research. We propose an alternative method called Mendelian Randomization with Refined Instrumental Variable from Genetic Score (MR-RIVER) to construct a genetic IV by integrating multiple genetic variants based on summarized results, rather than individual data. Compared with inverse-variance weighted (IVW) and generalized summary-data-based Mendelian randomization (GSMR), MR-RIVER maintained the type I error, while possessing more statistical power than the competing methods. MR-RIVER also presented smaller biases and mean squared errors, compared to the IVW and GSMR. We further applied the proposed method to estimate the effects of blood metabolites on educational attainment, by integrating results from several publicly available resources. MR-RIVER provided robust results under different LD prune criteria and identified three metabolites associated with years of schooling and additional 15 metabolites with indirect mediation effects through butyrylcarnitine. MR-RIVER, which extends score-based MR to summarized results in lieu of individual data and incorporates multiple correlated IVs, provided a more accurate and powerful means for the discovery of novel risk factors.

Keywords: Mendelian randomization, multiple correlated instrumental variables, genetic score, metabolomics, educational attainment

INTRODUCTION

Observational studies have long been utilized to detect associations between the exposures of interest and the risk of complex diseases. However, the estimated effects are typically biased and causality cannot be directly inferred because of unobserved confounders or reverse causality (Ebrahim and Davey Smith, 2008). Double-blind randomized controlled trials with perfect adherence, which use randomization allocation to avoid potential confounding, are often considered as the gold standard to infer causality (Bothwell et al., 2016). However, logistical difficulties limit the use in real-world studies.

Instrumental variable (IV) analysis provides unbiased causal estimates in the presence of observed and unobserved confounders under certain assumptions (Burgess et al., 2017). A valid IV should (1) be associated with the exposure of interest; (2) not be associated with any confounders of the exposure–outcome association; and (3) affect the outcome only through its impact on the exposure of interest (**Figure 1A**; Martens et al., 2006). Because human germline genetic variants usually form at fertilization and remain unchanged after birth (Ference et al., 2019), they are less likely to be correlated with the environmental or clinical factors but can be correlated with susceptibility to these factors that are associated with outcomes and thus are ideal candidates for IVs.

Mendelian randomization (MR), which uses genetic variants as IVs, has emerged recently as a powerful tool to estimate the causal effects of risk factors in observational settings (Smith and Ebrahim, 2003; Yavorska and Burgess, 2017; Burgess and Labrecque, 2018; Bowden et al., 2019) and has been increasingly used in genome-wide association studies (GWAS) (Welter et al., 2014; Burgess et al., 2015; Pickrell et al., 2016). However, as a single variant typically explains only a small proportion of variability, a large sample size is often required to power the traditional MR (Pierce et al., 2011). A variety of generalized MR methods have been proposed to integrate results arising from multiple IVs in order to increase power (Burgess and Thompson, 2013; Burgess et al., 2013). These methods include generalized summary-data-based Mendelian randomization (GSMR) (Zhu et al., 2018) and inverse-variance weighted method (IVW) (Burgess et al., 2013, 2016). GSMR integrates estimates from single IVs by using a generalized least-square approach (Zhu et al., 2018), whereas IVW combines estimates by using weights based on the variance–covariance matrix (Burgess et al., 2016). However, these existing methods are based on the summarized results of single-variant analysis and commonly prune IVs based on linkage disequilibrium to obtain relatively independent IVs, resulting in loss of information. Even with adjustment of the correlation structure, the results may still be inefficient. Notably, Burgess et al. (2017) introduced a multivariate regression method, which regresses the exposure factor on multiple IVs at the first stage to construct genetic scores (GSs). GS can be viewed as a linear combination of multiple IVs weighted by the strength of the association between an IV and the exposure, adjusted for all the other IVs. In the ensuing MR analysis, GS will be passed along as a single IV. The method was recently implemented in a study of *ACLY* and cardiovascular disease which incorporated multiple germline genetic variants (IVs) to construct GS as single IV and further inferred the causal relationship between *ACLY* inhibitors and the reduced risk of cardiovascular disease (Ference et al., 2019).

Thus, we propose an alternative method called Mendelian Randomization with Refined Instrumental Variable from Genetic Score (MR-RIVER) (**Figure 1B**) to construct a genetic score summarizing multiple genetic variants based on summarized results rather than individual-level data. Our method, which accounts for correlations among multiple genetic variants by borrowing linkage disequilibrium (LD) information from public databases (such as 1000 Genomes Project), provides a useful framework to integrate estimates obtained by using various genetic IVs and improves the performance of the summarized genetic score for the correlated genetic variants. Simulation studies suggested improved performance of our proposed method, compared to GSMR and IVW. We further applied the proposed method to estimate the effects of blood metabolites on educational attainment, by integrating results from several publicly available resources (Shin et al., 2014; Okbay et al., 2016).

METHOD

MR-RIVER Algorithm

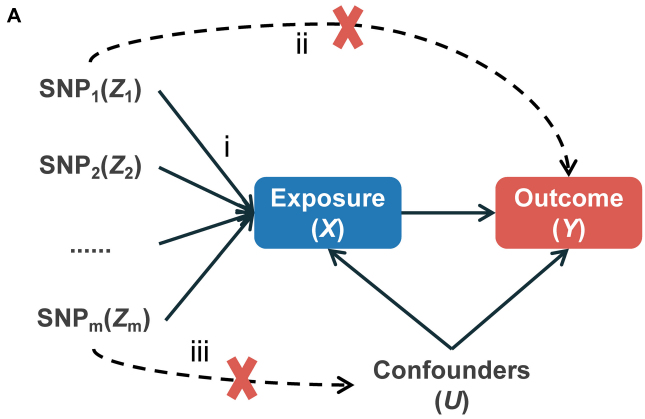
We propose a method to infer the causal relationship between risk factor X (e.g., blood metabolites) and outcome Y (e.g., years of schooling) given a set of IVs, denoted by $\mathbf{Z} = (Z_1, Z_2, \ldots, Z_p)$ (e.g., a set of genetic variants). The major components of our framework are depicted in **Figure 1A**. More specifically, we use b_{XZ_i}, along with standard error $se(b_{XZ_i})$, to quantify the association of each Z_i with the risk factor X from the traditional single-locus association analysis model, and likewise for b_{YZ_i} and $se\left(b_{YZ_i}\right)$ for each Z_i with the outcome Y.

The unified weighted GS incorporating multiple IVs could be estimated by the linear combination of multiple IVs:

$$GS = \sum_{i=1}^{p} \tilde{b}_{XZ_i} Z_i \tag{1}$$

Where $\tilde{b}_{XZ_i}$ denotes the direct effect of Z_i on X after controlling for the other IVs that derived from multivariable regression. However, in practice, the published-available summarized data were derived from single-variant analysis; it is unlikely to get genetic association estimates from a multivariable regression model in a large independent dataset due to issues of practicality and confidentiality of data sharing on such a large scale. Here, we propose an estimator by borrowing the idea of coefficient decomposition to estimate $\tilde{b}_{XZ_i}$ by using summarized results rather than individual-level data.

Specifically, under the assumption that (X, $\mathbf{Z}$) follow a multivariate normal distribution, regressing X on each Z_i will yield an estimate of b_{XZ_i}. Without loss of generality, we assume that there is a linear relationship between X and $\mathbf{Z}$. As $E(X|Z_i) = b_0 + b_{XZ_i} Z_i$, b_{XZ_i} represents the total effect of Z_i on X.



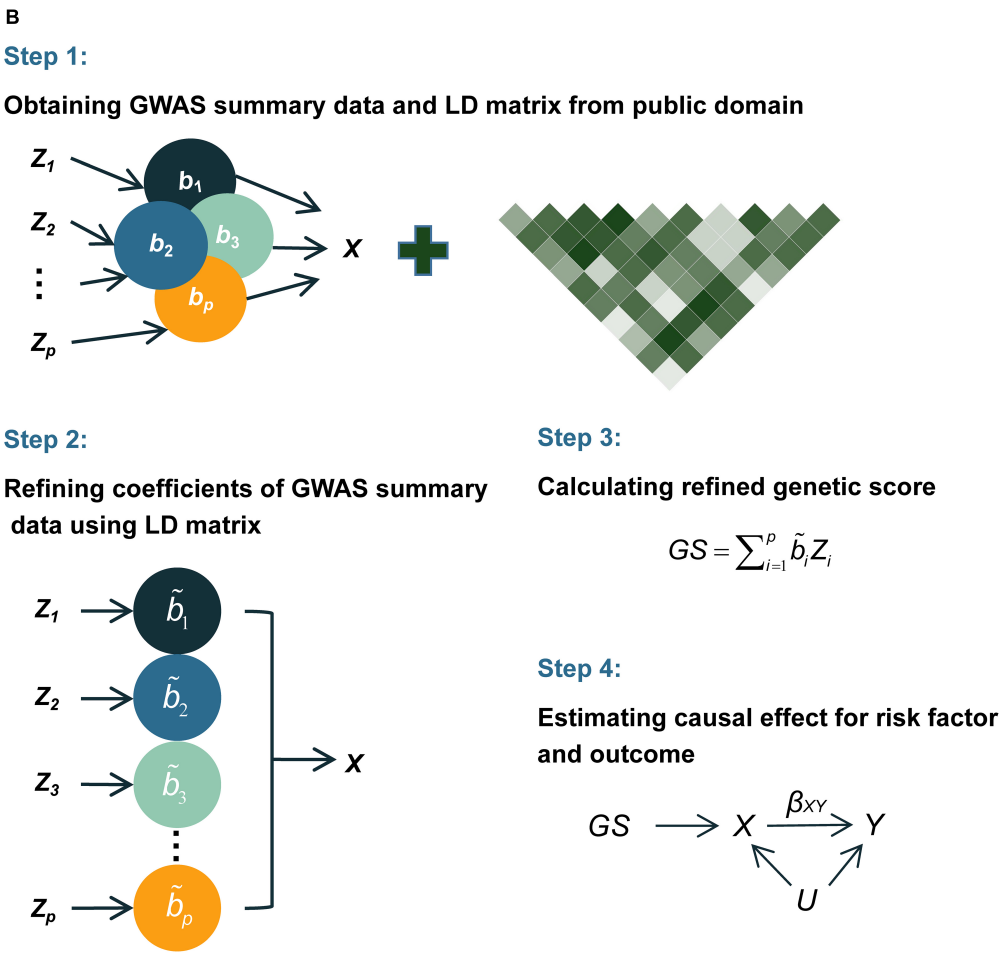


FIGURE 1 | Diagram of Mendelian randomization and flowchart of the proposed MR-RIVER method. **(A)** Mendelian randomization inferring the causal association of the exposure and outcome: (i) IVs are associated with the exposure X; (ii) IVs and outcome *Y* are independent, conditional on exposure *X* and unmeasured confounders *U*; (iii) IVs and confounders *U* are independent. **(B)** Flowchart of the proposed MR-RIVER method for multiple genetic variants in causal inference.

After adjusting the effect of all the other IVs, the relationship between X and Z_i can be expressed as $E\left(X|Z_1, \cdots, Z_p\right) = b_0 + \tilde{b}_{XZ_1}Z_1 + \cdots + \tilde{b}_{XZ_p}Z_p$, where $\tilde{b}_{XZ_i}$ is the direct effect of Z_i on X under the control of other IVs. Therefore, b_{XZ_i} can be decomposed into the direct effect and indirect effect via other correlated IVs:

$$b_{XZ_i} = \tilde{b}_{XZ_i} + \sum_{j\neq i}^{p} \tilde{b}_{XZ_j}\theta_{Z_jZ_i} \quad (2)$$

Here, $\theta_{Z_jZ_i}$ is the regression coefficient of Z_j on Z_i, and $\tilde{b}_{XZ_i}$ is the direct effect of Z_i on X, after controlling for the other IVs. Equation 2 can be rewritten as:

$$\tilde{\mathbf{b}}_{XZ} = \boldsymbol{\theta}^{-1}\mathbf{b}_{XZ} \tag{3}$$

where $\mathbf{b}_{XZ}$ is the p-length vector containing b_{XZ_i}, $\tilde{\mathbf{b}}_{XZ}$ is the vector of refined coefficients $\tilde{b}_{XZ_i}$, and θ is a $p \times p$ matrix with $\theta_{Z_j\,Z_i}$ being the ij-th entry. It follows that $\theta_{Z_iZ_j} = \rho_{Z_jZ_i}\sqrt{var\left(Z_j\right)/var\left(Z_i\right)}$ where $\rho_{Z_j\,Z_i}$ is the correlation between Z_j and Z_i, $var(Z_i)$ is the variance of Z_i. $var(Z_i)$ and $\rho_{Z_jZ_i}$ can be obtained from the public GWAS resources (e.g., 1000 Genomes Project).

We note that Eq. 3 is crucial as it enables us to compute GS defined in Eq. 1 with only summary data, in lieu of individual-level data. With GS as a single IV, we can estimate the association between the risk factor X and outcome Y with:

$$\begin{aligned}\hat{\beta}_{XY} &= \frac{\beta_{YGS}}{\beta_{XGS}} \\ &= \frac{cov(Y, GS)}{cov(X, GS)} = \frac{cov(Y, \sum_i^p \tilde{b}_{XZ_i}Z_i)}{cov(X, \sum_i^p \tilde{b}_{XZ_i}Z_i)} \\ &= \frac{\sum_i^p \tilde{b}_{XZ_i}\, cov(Y, Z_i)}{\sum_i^p \tilde{b}_{XZ_i}\, cov(X, Z_i)} = \frac{\sum_i^p \tilde{b}_{XZ_i}b_{YZ_i}\, var(Z_i)}{\sum_i^p \tilde{b}_{XZ_i}b_{XZ_i}\, var(Z_i)}\end{aligned} \tag{4}$$

As mentioned by Burgess et al. (2016), $var\left(Z_i\right)$ is approximately proportional to $1/var\left(b_{YZ_i}\right)$; thus, Eq. 4 can be simplified as:

$$\hat{\beta}_{XY} = \frac{\sum_i^p \tilde{b}_{XZ_i}b_{YZ_i}\Big/ var\left(b_{YZ_i}\right)}{\sum_i^p \tilde{b}_{XZ_i}b_{XZ_i}\Big/ var\left(b_{YZ_i}\right)} \tag{5}$$

The asymptotic standard error for $\hat{\beta}_{XY}$ can be estimated by the delta method (Thomas et al., 2007):

$$se\left(\hat{\beta}_{XY}\right) = \sqrt{\frac{\sum_i^p \sum_j^p \rho_{Z_iZj}\tilde{b}_{XZ_i}\tilde{b}_{XZ_j}\Big/\left(se\left(b_{YZ_i}\right)se\left(b_{YZ_j}\right)\right)}{\left(\sum_i^p \tilde{b}_{XZ_iXZ_j}\Big/ var\left(b_{YZ_i}\right)\right)^2}} \tag{6}$$

The association between X and Y can be further tested by using the Wald test statistic $u = \hat{\beta}_{XY}\Big/ se\left(\hat{\beta}_{XY}\right)$, which asymptotically follows a standard normal distribution under the null hypothesis.

We stress that, though Eqs.5, 6 resemble the estimator proposed in Burgess et al. (2017), our estimator differs from that in Burgess et al.'s (2017) required individual data, while our estimator, with the introduction of the refined estimates in Eq. 3, can be computed even with the summary data. Therefore, our estimator is applicable in more broad settings, where only summary data are available. Simulations have confirmed the utility of our method.

Design of Statistical Simulations

Two sets of simulation studies were designed to investigate MR-RIVER.

Evaluation of the Estimates of the Refined Coefficients of IVs on *X*

We generated six IVs, $Z_1, Z_2, \ldots, Z_6$, from a multivariate normal distribution $MVN\ (\mathbf{0},\ \Sigma)$, where Σ is a correlation matrix with an equal correlation structure. We varied the correlation coefficient and set it to be 0, 0.1, 0.3, 0.5, 0.7, and 0.9, corresponding to various scenarios: from the independent case to the highly correlated case. We generated X using the following models:

$$\begin{aligned}&X_i = \textstyle\sum_{j=1}^{6} Z_{ij}\tilde{b}_j + \varepsilon_{Xi} \\ &\tilde{b}_j \sim N\left(\mu,\ 1\right), \quad \mu = -1,\ -0.5,\ 0.5,\ 1,\ 1.5,\ 2 \\ &\varepsilon_{X_i} \sim N\left(0, 1\right)\end{aligned} \tag{7}$$

The sample size was fixed at 1,000. In addition, we simulated 5,000 additional individuals to provide an external correlation structure for IVs. For each simulation configuration, 2,000 datasets were produced.

We first regressed X on each Z_j separately to obtain the summarized effect of Z_j on X, and based on these results, we applied Eq. 2 to obtain the estimates of the refined coefficients. The estimated refined coefficients, along with the corresponding standard errors, were compared to those from traditional GWAS summarized results under different correlation structures and effect sizes of Z.

Investigation of the Statistical Properties of MR-RIVER

Let X_i and Y_i denote the exposure and outcome variables of the ith subject, and Z_{ij} the jth IV (j = 1, …, J). The data were generated from the following model:

$$\begin{aligned}&Z_i \sim MVN\left(0,\ \Sigma\right), \quad b_j \sim U\left(0,\ 0.5\right) \\ &X_i = \textstyle\sum_{j=1}^{J} Z_{ij}b_j + \varepsilon_{Xi} \\ &Y_i = X_ib_{XY} + \varepsilon_{Yi} \\ &where \quad \varepsilon_{Xi} \sim N\left(0, var\left(\textstyle\sum_{j=1}^{J} Z_{ij}b_j\right)\left(R_{ZX}^{-2} - 1\right)\right) \\ &and \quad \varepsilon_{Yi} \sim N\left(0, var\left(X_ib_{XY}\right)\left(R_{XY}^{-2} - 1\right)\right)\end{aligned} \tag{8}$$

where Σ is the correlation matrix of IVs with an equal correlation structure. We varied the correlation parameter from 0 to 0.9 by 0.1. Each IV explains 0.005 of the variance of X, and we considered J = 5, 10, 15, 20. Moreover, R_{ZX}^2 is the proportion of variance of X explained by all IVs, which was set to be 0.025, 0.05, 0.075, and 0.1, while R_{XY}^2 is the proportion of variance of Y explained by X, which was set to be 0.05, 0.1, 0.15, and 0.2. Sample sizes for the IV-exposure association study (N_1) and the IV-outcome association study (N_2) were set to be 1,000 and 1,500, respectively. In addition, 5,000 (N_3) individuals were generated to provide an external correlation structure for genetic variants.

For each parameter configuration, a total of 2,000 datasets were produced. Under all the scenarios examined, MR-RIVER was found to outperform GSMR and IVW by maintaining the Type I error, possessing more statistical power, as well as having smaller biases and mean squared errors.

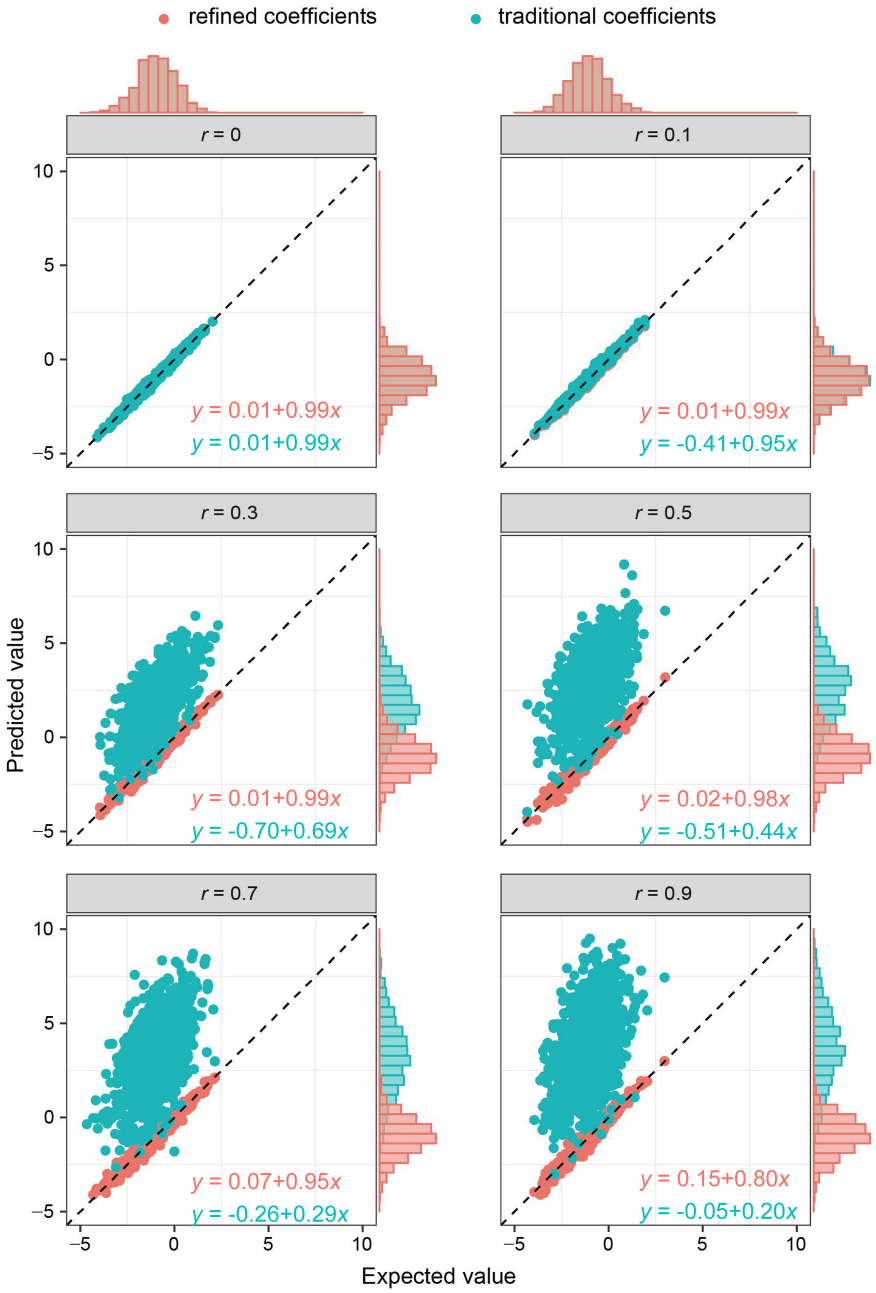


FIGURE 2 | Comparison of refined and traditional coefficients under different correlation structures. Expected values are the regression coefficients obtained from the multivariable regression model with all the variables used to generate dependent variable Y plotted against predicted values obtained from the refined method (refined coefficients) and traditional single-locus analyses (traditional coefficients). Refined and traditional coefficients were compared with the bias from expected coefficients under different correlation structures through a regression model. Red equation represents the relationship between expected coefficients and refined coefficients, and green equation represents traditional coefficients.

RESULTS

Statistical Properties of Refined Coefficients

We investigated the accuracy of refined coefficients. With the obtained correlation structure of IVs from the internal analysis set, the estimated refined coefficients (along with the standard errors) based on the summarized results were in consistent with the corresponding estimates from multivariable regression (**Supplementary Figures 1A,B**), suggesting that the estimates of the refined coefficients were unbiased.

As the key of the approach lies in borrowing the correlation information from public resources, we further evaluated

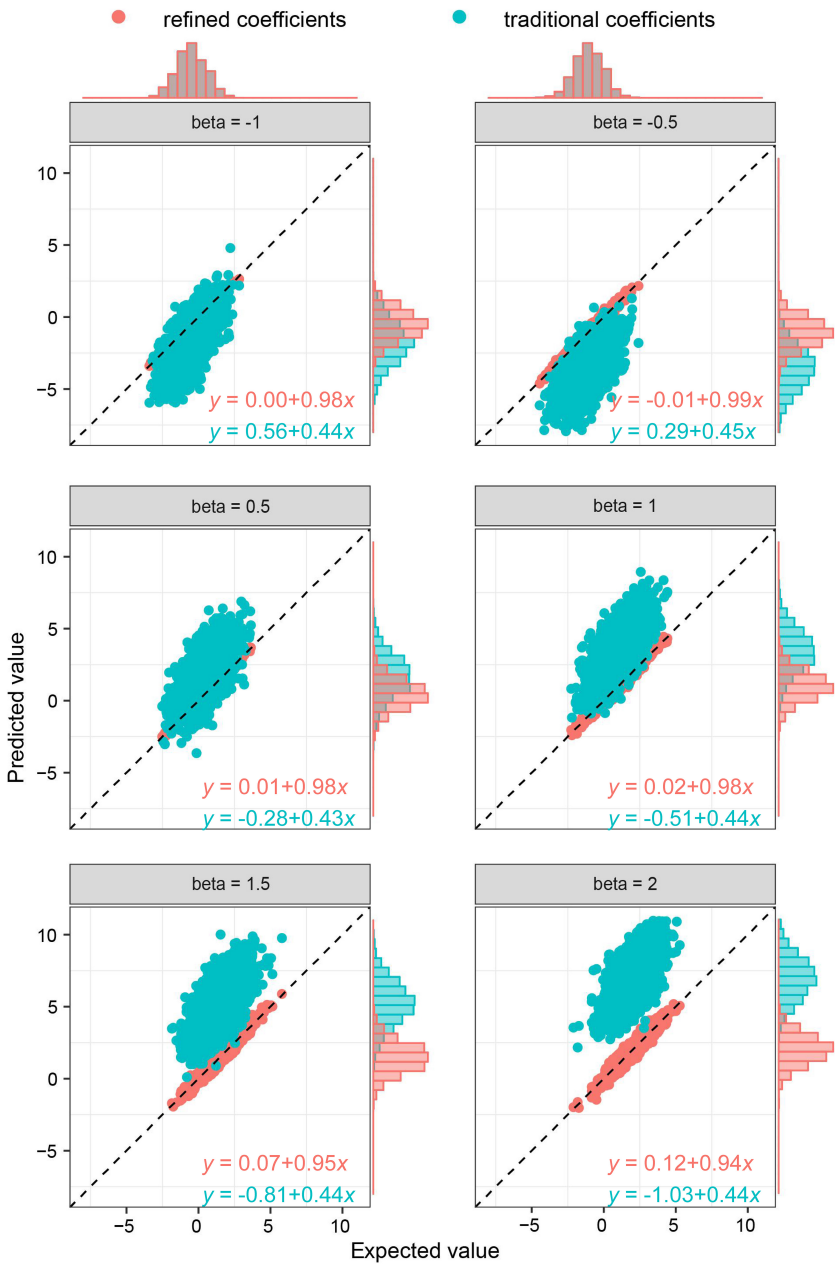


FIGURE 3 | Comparison of refined and traditional coefficients under different effect sizes. Expected values are the regression coefficients obtained from the multivariable regression model with all the variables used to generate dependent variable *Y* plotted against predicted values obtained from the refined method (refined coefficients) and traditional single-locus analyses (traditional coefficients). Refined and traditional coefficients were compared with the bias from expected coefficients under different effect sizes through a regression model. Red equation represents the relationship between expected coefficients and refined coefficients, and green equation represents traditional coefficients.

the method by obtaining the correlation structure from the simulated external samples. According to different levels of correlation among IVs, refined coefficients outperformed traditional coefficients obtained from single-locus analysis, especially when the correlations among IVs were relatively high (**Figure 2**). Similarly, under the specific correlation structure (with a correlation coefficient of 0.5), refined coefficients remained approximately unbiased, while traditional coefficients showed increased biases with increased effect sizes (**Figure 3**).

Statistical Properties of MR-RIVER

With various strengths of correlations among IVs, MR-RIVER maintained the type I error at the 0.05 level, compared to the IVW (with type I error around 0.04) and GSMR (with the most conservative control of the type I error) (**Figure 4A**). The results

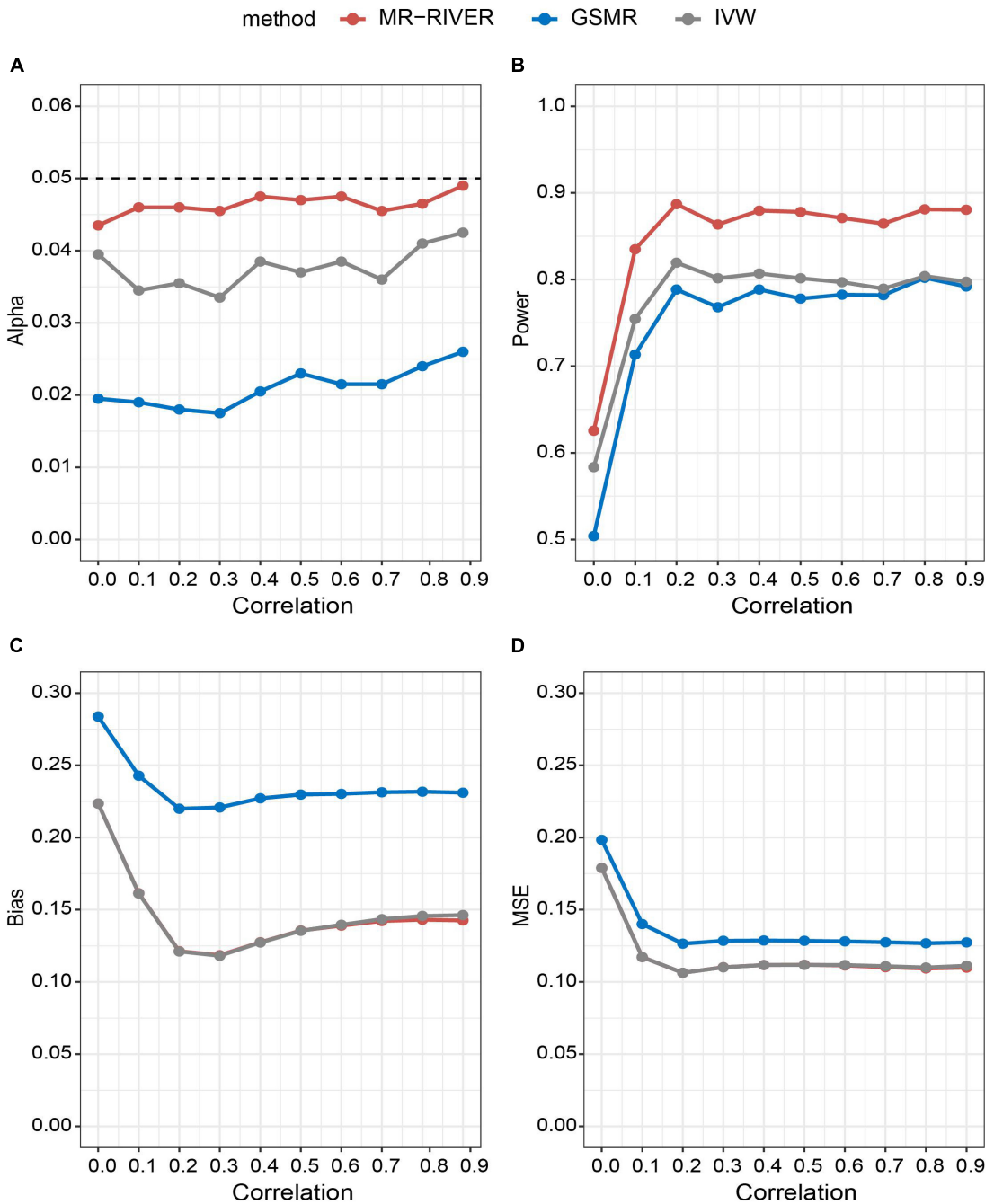


FIGURE 4 | Statistical properties of MR methods under different correlations. Correlation between IVs plotted against: **(A)** type I error under the null hypothesis; **(B)** performance of power under the alternative hypothesis with b_{xy} = 1; **(C)** bias under the alternative hypothesis; and **(D)** mean square error.

held when we varied the sample size (**Supplementary Figure 2A**) or the number of IVs (**Supplementary Figure 3A**). Further, increasing correlation strengths among IVs (**Figure 4B**), or increasing sample size (**Supplementary Figure 2B**), or increasing the numbers of IVs (**Supplementary Figure 3B**) led to increased power for all MR methods. Overall, the power of MR-RIVER was higher than that of GSMR and IVW under different parameter settings.

Estimates of b_{xy} from the three MR methods were approximately unbiased, while the biases of the MR-RIVER and IVW estimates were lower than that of the GSMR estimate (**Figure 4C**). The bias increased with the increased effect size (**Supplementary Figure 4**) and so was true for the MSE (**Figure 4D**). MR-RIVER and IVW had lower biases and MSEs, compared to GSMR.

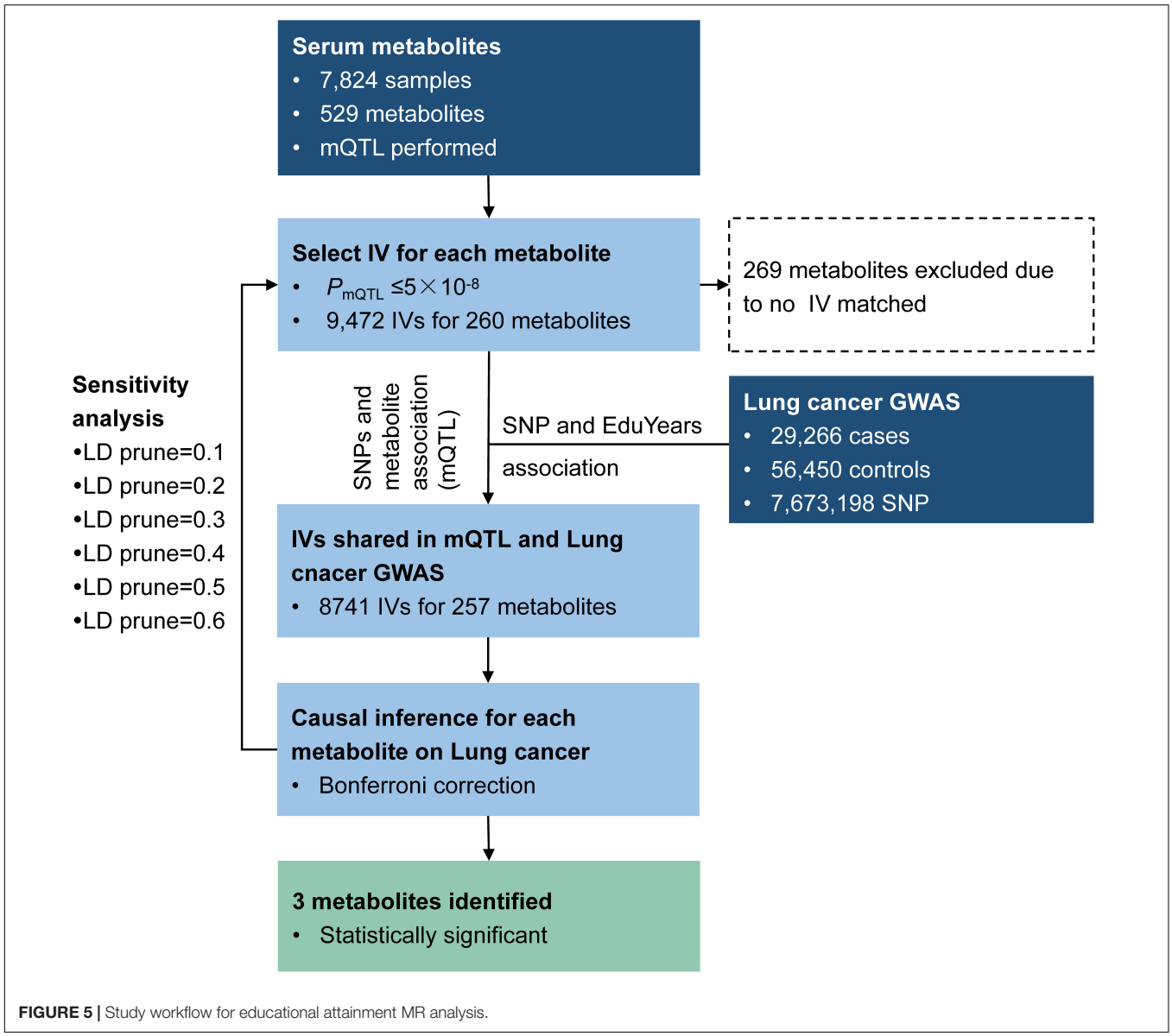


FIGURE 5 | Study workflow for educational attainment MR analysis.

REAL DATA APPLICATION

Motivation

Educational attainment is moderately heritable and has been recognized as a proxy phenotype for intelligence, cognition, and neuropsychiatric disorders (Berry et al., 2006; Esch et al., 2014). Discovery of the causal factors linking to the educational attainment could shed light on the biological pathways underlying human behavioral and health-related outcomes (Rietveld et al., 2013). Blood metabolites, which closely represent the physiological status of an organism, have garnered significant interest in biomedical research (Simpson et al., 2016). However, few studies have focused on a causal relationship between metabolites and educational attainment in the presence of multiple IV variables. Taking advantage of the proposed MR-RIVER, this application aims to systematically evaluate the causal relationship between blood metabolites and educational attainment using multiple GWAS summary results.

Materials

Genome-wide association studies summary results for educational attainment were obtained based on various studies from the Social Science Genetic Association Consortium[1] (Berry et al., 2006; Rietveld et al., 2013). Educational attainment was measured as the year of schooling completed (EduYears) among 293,723 individuals (with a mean of 14.3 years) (**Supplementary Table 1**). Approximately, 9.3 million SNPs were included in the association analysis, and minor allele frequencies were obtained from the 1000 Genomes Project. Details of the SNPs included in our analysis are displayed in **Supplementary Table 2**.

[1]https://www.thessgac.org/data

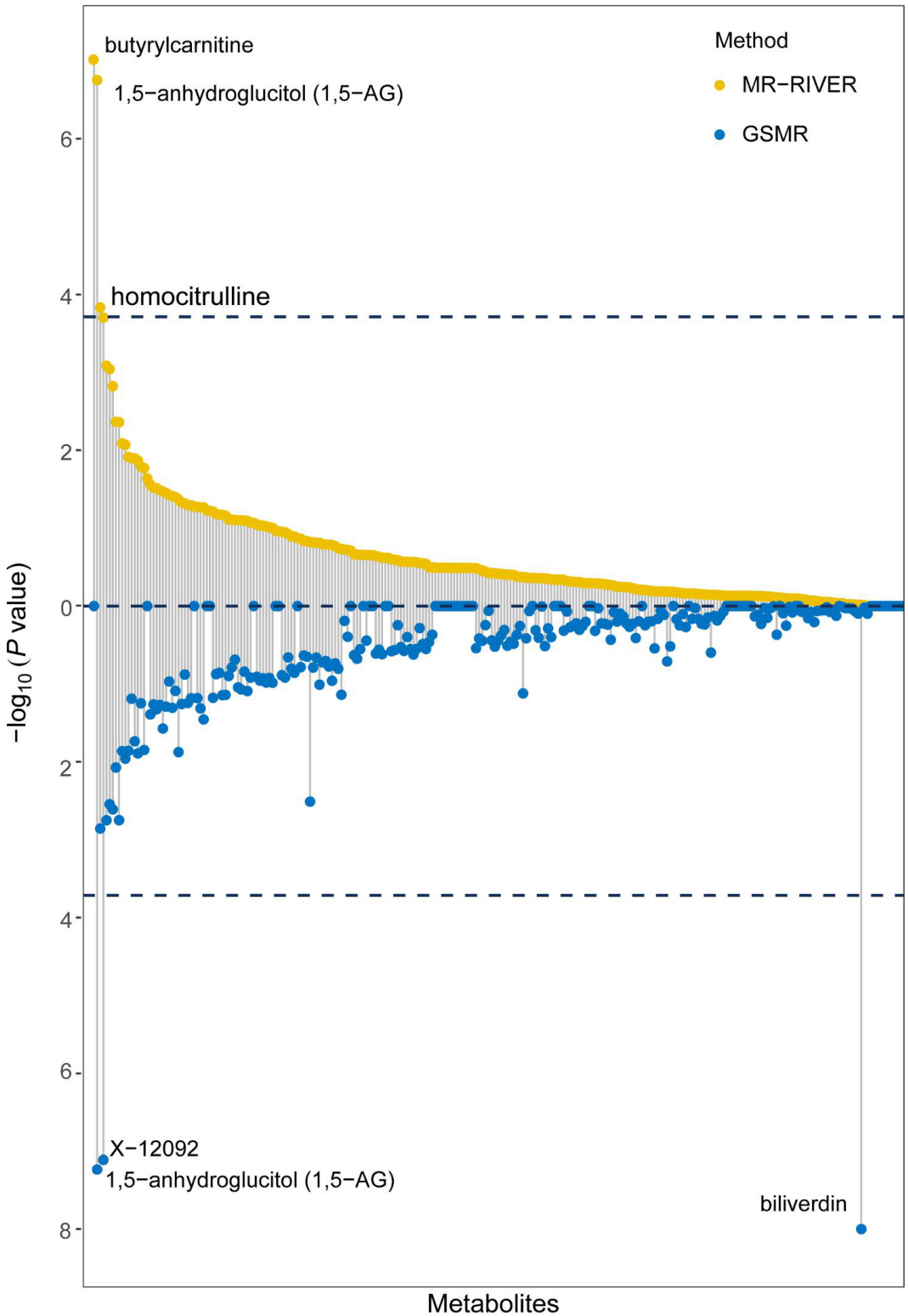


FIGURE 6 | MR-RIVER and GSMR analysis for causal association between metabolites and educational attainment. Relationship between individual metabolites with $-\log_{10}$ (*P*-value) of the association. Upper yellow values represent MR-RIVER results, and lower blue values represent GSMR results. Associated metabolites are annotated.

Summary results of quantitative trait locus (QTL) analysis of SNPs on corresponding metabolites were obtained from 7,824 European adult individuals (**Supplementary Table 3**) (Shin et al., 2014). Specifically, the metabolite QTL (mQTL) data contained all of the summarized association statistics for 529 metabolites with *P*-values less than 1×10^{-5}[2].A total of 196 metabolites out of 529 (37%) were unknown because their chemical identity was not yet determined at the time of analysis. Detailed information of metabolites can be found in **Supplementary Table 4**.

MR Analysis Results

We applied the method to explore the causal effect of blood metabolites on educational attainment as depicted in **Figure 5**. Based on assumption (1) of IV, SNPs were required to have an mQTL relationship with the corresponding metabolites with *P-values* less than 5×10^{-8}. As a result, 9,472 SNPs were selected as IVs, matched with 260 metabolites. Among these, 9,329 SNPs were available in the educational attainment GWAS.

Causal inference for each metabolite on quantitative education years was evaluated through MR-RIVER and GSMR. To obtain sufficient IVs to increase the power of MR, IVs were pruned by LD at 0.5; The HEIDI-outlier test was used to detect pleiotropic SNPs and remove them from the MR analysis; see **Figure 6**. Bonferroni correction was used to control for false positives. MR-RIVER identified three metabolites associated with education years: butyrylcarnitine ($b_{xy} = -0.043$, $P = 1.08 \times 10^{-7}$), 1,5-anhydroglucitol (1,5-AG) ($b_{xy} = -0.192$, $P = 1.77 \times 10^{-7}$), and homocitrulline ($b_{xy} = -0.269$, $P = 1.47 \times 10^{-4}$). GSMR identified biliverdin ($b_{xy} = -0.028$, $P = 2.92 \times 10^{-15}$), 1,5-AG ($b_{xy} = -0.183$, $P = 5.83 \times 10^{-8}$), and an unknown metabolite, X-12092 (retention time, 1.130; mass-to-charge ratio, 144.2; spectra, 84.2:0.8) ($b_{xy} = 0.028$, $P = 3.85 \times 10^{-7}$) (**Table 1**). In addition, sensitivity analyses with different LD prune criteria (0.1–0.7, in 0.1 increments) showed robust results for MR-RIVER, but not for GSMR (**Supplementary Tables 5, 6**).

We performed additional analyses to explore whether the remaining metabolites affected education years through the above-identified candidate metabolites. SNPs associated with the remaining metabolites were treated as IVs to infer potential causal associations between the identified metabolites and remaining metabolites (**Figure 7A**). The results indicated 28 additional metabolites were associated with the three candidate metabolites. Among these, 24 metabolites (including six unknown metabolites) were associated with butyrylcarnitine, three unknown metabolites were associated with 1,5-AG, and one unknown metabolite was associated with homocitrulline (**Supplementary Table 7**).

Further, mediation analysis was used to evaluate potential metabolic regulatory pathways for education years by Sobel test (Baron and Kenny, 1986). The 15 metabolites indirectly mediated the effect on education years through butyrylcarnitine (**Figure 7B** and **Supplementary Table 7**). Most metabolites were located in the carnitine metabolism pathway (8/15, 53.0%). Blood metabolic biomarkers overall formed a potential causal network (**Figure 7C**).

[2]http://metabolomics.helmholtz-muenchen.de/gwas

TABLE 1 | Relative bias of imputed datasets with three imputation methods.

Method	Metabolite	b_{xy}	se of b_{xy}	*P-value*
MR-RIVER	Butyrylcarnitine	−0.0430	0.0081	1.08×10^{-07}
	1,5-Anhydroglucitol (1,5-AG)	−0.1916	0.0367	1.77×10^{-07}
	Homocitrulline	−0.2687	0.0708	1.47×10^{-04}
GSMR	Biliverdin	−0.0284	0.0036	2.92×10^{-15}
	1,5-Anhydroglucitol (1,5-AG)	−0.1838	0.0339	5.83×10^{-08}
	X-12092	0.0283	0.0056	3.85×10^{-07}

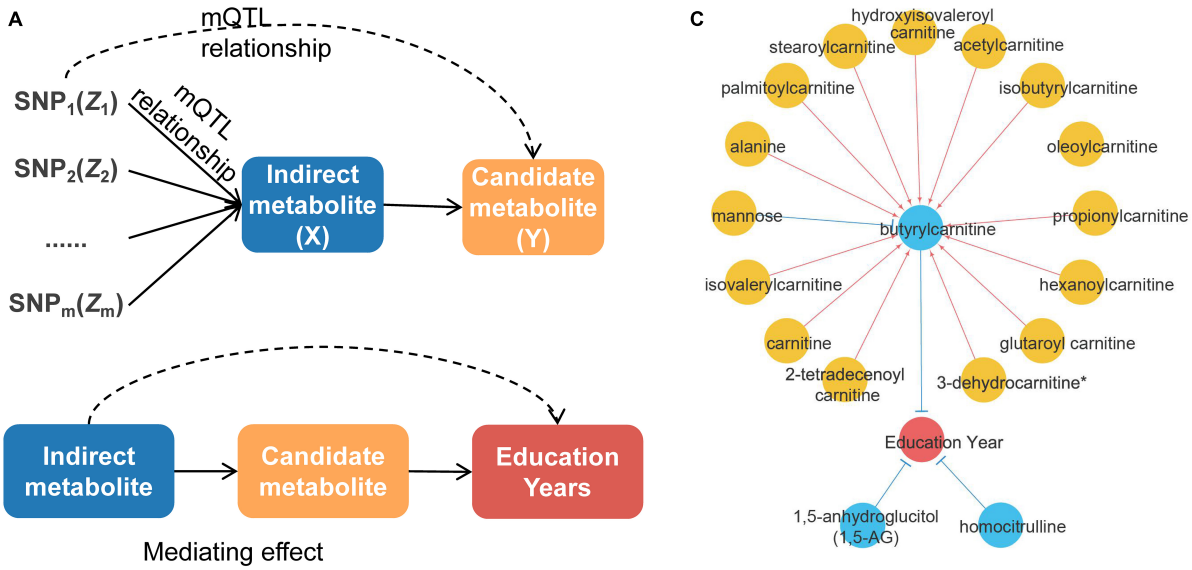
b_{xy}: causal effect of metabolite and educational attainment.
se of b_{xy}: standard error of causal effect.
P value: P-value of causal effect.
X-12092: unknown metabolite (retention time, 1.130; mass-to-charge ratio, 144.2; spectra, 84.2:0.8).

DISCUSSION

We proposed an improved MR approach, MR-RIVER, to combine summarized results of multiple IVs into a single GS and to estimate the unbiased causal effect of a risk factor on an outcome. The publicly accessible summary-level data were obtained from single-locus analyses without consideration of the correlation between IVs. MR-RIVER provides a novel way to refine the effect size of genetic variants account for the correlation based on summary data and makes it efficient to perform summarized data genetic score MR when the correlation between IVs are unignorable. MR-RIVER closely maintains the type I error around the nominal level while it has higher power, lower bias, and smaller variation compared to GSMR and IVW.

Genome-wide association studies uses original GWAS summarized results for IV exposure and IV outcome obtained from single-locus analyses and then derives the causal effect by the generalized least-square approach weighted by the variance–covariance matrix to adjust for correlations among IVs (Zhu et al., 2018). MR-RIVER instead first modifies the summarized results, accounting for correlations among IVs, and then integrates the results. Thus, there are several differences between MR-RIVER and GSMR. First, MR-RIVER adjusts summarized results for each genetic IV by borrowing external LD information to obtain more accurately estimate IV-exposure effect—therefore, MR-RIVER has an advantage in accuracy. Second, MR-RIVER aggregates multiple IVs by weighted linear combination weighted by refined coefficients, which reduces the dimension for IVs and simplifies the following calculation.

Interestingly, MR-RIVER and IVW showed similar performance in bias and MSE. If the weights used to aggregate multiple IVs are equal to the original GWAS summary results ($\tilde{b}_{XZ_i} = b_{XZ_i}$ in Eq. 5), then MR-RIVER is the same as IVW. On the one hand, estimates of MR-RIVER are approximately identical to IVW because point estimates are robust toward the weights (**Supplementary Figure 5A**). On the other hand, different weights result in different standard errors



B

metabolites	b_CI	IE_CI	IE_pval
3−dehydrocarnitine*	1.06(0.98,1.15)	−0.05(−0.06,−0.03)	2.02e−07
hexanoylcarnitine	1(0.88,1.11)	−0.04(−0.06,−0.03)	3.98e−07
isovalerylcarnitine	0.51(0.47,0.55)	−0.02(−0.03,−0.01)	2.07e−07
propionylcarnitine	1.13(1.03,1.23)	−0.05(−0.07,−0.03)	2.38e−07
carnitine	1.33(1.22,1.44)	−0.06(−0.08,−0.04)	2.22e−07
alanine	1.26(0.83,1.69)	−0.05(−0.08,−0.03)	9.24e−05
acetylcarnitine	1.25(1.14,1.36)	−0.05(−0.07,−0.03)	2.39e−07
isobutyrylcarnitine	0.44(0.35,0.53)	−0.02(−0.03,−0.01)	3.30e−06
oleoylcarnitine	0.94(0.83,1.06)	−0.04(−0.06,−0.02)	4.66e−07
mannose	−0.32(−0.48,−0.16)	0.01(0.01,0.02)	1.64e−03
palmitoylcarnitine	1.13(0.59,1.67)	−0.05(−0.08,−0.02)	1.14e−03
hydroxyisovaleroyl carnitine	1.02(0.66,1.38)	−0.04(−0.07,−0.02)	1.24e−04
stearoylcarnitine	1.07(0.57,1.56)	−0.05(−0.07,−0.02)	9.50e−04
glutaroyl carnitine	1.56(1.38,1.75)	−0.07(−0.09,−0.04)	4.36e−07
2−tetradecenoyl carnitine	0.93(0.5,1.37)	−0.04(−0.06,−0.02)	9.36e−04

FIGURE 7 | Diagram of MR analysis between metabolites and mediation analysis. **(A)** MR inferring the causal association of remaining metabolites (*X*) on previously identified metabolites (*Y*). Mediation analysis of the rest of metabolites on risk of education years through the identified metabolites. **(B)** Metabolites that indirectly mediate the effect on education years through butyrylcarnitine in mediation analysis. b_CI represents effect of metabolites on butyrylcarnitine and 95% confidence interval (95% CI). IE_CI represents indirect effect of metabolites on education years and 95% CI, and IE_pval represents *P*-values. **(C)** Causal network of blood metabolites on education years. Blue circles indicate metabolites that are directly identified, while yellow circles have indirect effect through blue metabolites. Red lines represent positive effects, and blue lines indicate negative effects.

(**Supplementary Figure 5B**), which in turn lead to different statistics (**Supplementary Figure 5C**). This may explain why the bias and MSE of MR-RIVER and IVW are similar, but the performance of power and type I error is different. To summarize, MR-RIVER improves upon IVW and is powerful to infer a causal relationship between an exposure and outcome.

There has been much discussion on the potentials and limitations of MR, as the IV assumptions cannot be fully tested (VanderWeele et al., 2014; Paternoster et al., 2017). Horizontal pleiotropy is a common phenomenon in the human genome that some genetic variants affect the outcome through other traits or pathways rather than exclusively through the risk factor (Solovieff et al., 2013). It is a violation of the instrumental variable assumptions and may induce a major source of potential bias in causal inference. There are several methods are proposed to detect pleiotropy (Slob and Burgess, 2020). The MR-Egger method is able to assess the pleiotropic effects as well as to provide a consistent estimate of the causal effect (Bowden et al., 2017), while the estimates were generally imprecise with low power (Slob and Burgess, 2020). The HEIDI-outlier test was proposed to detect heterogeneity at multiple correlated instruments (Zhu et al., 2018). It will be powerful and valuable when only some proportion of the SNPs have a horizontal pleiotropy effect. In our proposed method, we ensembled the HEIDI-outlier test to detect potential pleiotropy and then remove them from the MR-RIVER analysis.

Notably, after GWAS significant threshold screening, LD prune, and HEIDI-outlier filtering, MR-RIVER analysis suggested three causal metabolites that are associated with education years. The first metabolite is butyrylcarnitine, classified as an acylcarnitine. Previous studies have shown that abnormally increased levels of acylcarnitines, including butyrylcarnitine, are associated with fatty acid oxidation disorders (Jones et al., 2010). Elevated butyrylcarnitine concentration in plasma is associated with short-chain acyl-CoA dehydrogenase deficiency (van Maldegem et al., 2006), which may cause failure to thrive, developmental and cognitive delay, seizures, and neuromuscular (Corydon et al., 2001). Moreover, fatty acid oxidation disorders may lead to mitochondrial dysfunction and further affect the energy supply of the brain (Kölker et al., 2004; Wajner and Amaral, 2015). Therefore, high levels of acylcarnitines may be involved in potential metabolic regulatory pathways affecting cognitive status or brain energy supplement and, in turn, increased education years (mannose→butyrylcarnitine→education years). Mannose easily crosses the blood–brain barrier and is converted to fructose-6-phosphate that enters the glycolytic pathway (Sharma et al., 2014). Cerebral tissue can utilize mannose directly and rapidly from the blood to restore or maintain normal metabolic functions in the absence of glucose (Sloviter and Kamimoto, 1970). Taken altogether, mannose levels appear to be a potential beneficial factor for education years.

The second metabolite, 1,5-AG, is a monosaccharide structurally similar to glucose and is a validated marker of short-term glycemic control (Buse et al., 2003). Low levels of 1,5-AG, indicative of glycemic peak, are associated with dementia and cognitive decline (Rawlings et al., 2017). Finally, elevated homocitrulline, the third metabolite, is structurally similar to but one methylene group longer than citrulline, and impairs bioenergetics in the brain cortex, by reducing velocity of the citric acid cycle and creatine kinase activity. Consequently, it decreases energy production and transfer (Viegas et al., 2009). Therefore, administration of 1,5-AG and homocitrulline may improve educational attainment.

In conclusion, the proposed MR-RIVER method appears to outperform the existing commonly used MR methods. With publicly accessible summary-level data, MR-RIVER provides a more accurate and powerful mean for novel discoveries and identifies several blood metabolites as biomarkers and interventional targets for educational attainment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: GWAS summary results for education attainment are available at https://www.thessgac.org/data; summary results of quantitative trait locus (QTL) analysis of SNPs on metabolites are available at http://metabolomics.helmholtz-muenchen.de/gwas.

AUTHOR CONTRIBUTIONS

LL, RZ, YW, and FC contributed the study design. LL, YW, and RZ performed the statistical analysis and interpretation and drafted the manuscript. YW, LL, RZ, YL, XD, SS, HH, YZ, LW, XC, and DC revised the manuscript. RZ, YW, and FC provided the financial support and study supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the National Key Research and Development Program of China (2016YFE0204900 to FC), National Natural Science Foundation of China (81530088 to FC and 81973142 to YW), Natural Science Foundation of Jiangsu Province (BK20191354 to RZ), Natural Science Foundation of Jiangsu Higher Education Institutions of China (18KJB310011 to RZ), and Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). The sponsors had no role in design of the study, collection and analysis of data, or preparation of the manuscript.

ACKNOWLEDGMENTS

We would like to thank SSGAC for providing the educational attainment summary data and the Metabolomics GWAS Server for providing mQTL results.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.618829/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 20 May 2021
doi: 10.3389/fgene.2021.639905

Association Between Environmental Factors and Asthma Using Mendelian Randomization: Increased Effect of Body Mass Index on Adult-Onset Moderate-to-Severe Asthma Subtypes

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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *10 December 2020*
Accepted: *07 April 2021*
Published: *20 May 2021*

Citation:
Ha T-W, Jung H-U, Kim DJ, Baek EJ, Lee WJ, Lim JE, Kim HK, Kang J-O and Oh B (2021) Association Between Environmental Factors and Asthma Using Mendelian Randomization: Increased Effect of Body Mass Index on Adult-Onset Moderate-to-Severe Asthma Subtypes. Front. Genet. 12:639905. doi: 10.3389/fgene.2021.639905

Although asthma is one of the most common chronic diseases throughout all age groups, its etiology remains unknown, primarily due to its heterogeneous characteristics. We examined the causal effects of various environmental factors on asthma using Mendelian randomization and determined whether the susceptibility to asthma due to the causal effect of a risk factor differs between asthma subtypes, based on age of onset, severity of asthma, and sex. We performed Mendelian randomization analyses (inverse variance weighted, weighted median, and generalized summary-data-based Mendelian randomization) using UK Biobank data to estimate the causal effects of 69 environmental factors on asthma. Additional sensitivity analyses (MR-Egger regression, Cochran's Q test, clumping, and reverse Mendelian randomization) were performed to ensure minimal or no pleiotropy. For confirmation, two-sample setting analyses were replicated using BMI SNPs that had been reported by a meta-genome-wide association study in Japanese and European (GIANT) populations and a genome-wide association study in control individuals from the UK Biobank. We found that BMI causally affects the development of asthma and that the adult-onset moderate-to-severe asthma subtype is the most susceptible to causal inference by BMI. Further, it is likely that the female subtype is more susceptible to BMI than males among adult asthma cases. Our findings provide evidence that obesity is a considerable risk factor in asthma patients, particularly in adult-onset moderate-to-severe asthma cases, and that weight loss is beneficial for reducing the burden of asthma.

Keywords: asthma, environmental factors, body mass index, mendelian randomization, moderate-to-severe asthma

Abbreviations: AM, adult-onset mild; AM-S, adult-onset moderate-to-severe; BMI, body mass index; BTS, British Thoracic Society; CI, confidence interval; CM, child-onset mild; CM-S, child-onset moderate-to-severe; FC, female control; FAM, female adult-onset mild; FAM-S, female adult-onset moderate-to-severe; GIANT Consortium, The genetic investigation of anthropometric traits consortium; GSMR, generalized summary-data-based mendelian randomization; GWAS, genome-wide association study; HEIDI, heterogeneity in dependent instrument; IVW, inverse-variance weighted; MR, mendelian randomization; MC, male control; MAM, male adult-onset mild; MAM-S, male adult-onset moderate-to-severe; OR, odds ratio; SD, standard deviation; SNP, single-nucleotide polymorphism.

INTRODUCTION

Asthma is one of the most common chronic diseases, affecting children and adults; yet, much remains to be determined with regard to its etiology (Subbarao et al., 2009). Approximately 300 million persons have been afflicted by asthma worldwide (Dharmage et al., 2019), and it is estimated that 250,000 people die prematurely each year due to asthma (Bousquet et al., 2010). If current trends continue, roughly 100 million more persons will develop asthma by 2025 (Dharmage et al., 2019). However, asthma has considerable heterogeneity, including its pathophysiological mechanisms, environmental exposure, comorbidities, underlying disease severity, age of onset, medical accessibility, psychological factors, and medical responsiveness (Bousquet et al., 2010). For this reason, the identification of causal risk factors in a specific asthma case has been a challenging task.

Asthma is characterized by variable narrowing of the airway due to the interaction of airway inflammation and remodeling. Several studies have classified asthma by various clinical criteria, such as age of onset (early onset, late onset) (Ferreira et al., 2019), disease severity (mild, moderate-to-severe) (Shrine et al., 2019), and allergy (atopic, non-atopic) (Zhu et al., 2020). In addition, a gender-specific asthma cluster analysis has reported heterogeneous characteristics of asthma between sexes (Hsiao et al., 2019). T-helper type 2 (Th2) inflammation is regarded as the central molecular mechanism of asthma, and based on Th2 profiles, asthma is classified as Th2-high and Th2-low (Woodruff et al., 2009; Sterk and Lutter, 2014).

The severe form of asthma is aggravated by marked thickening of the airway walls and widespread inflammation (Busse et al., 2000). These changes can reduce lung function and cause the airway to be narrowed rapidly due to smooth muscle contraction (Busse et al., 2000). Thus, individuals with severe asthma present with symptoms that are distinct from those in mild asthma patients, including debilitating lung function, frequent exacerbation of asthma, and increased hospital admissions despite significant use of medicines (corticosteroids) (Shrine et al., 2019). Classification of asthma by phenotype provides a foundation on which to understand disease causality and develop management approaches that improve the control of asthma while avoiding adverse effects and decreasing the risk of serious outcomes (Moore et al., 2010).

Many environmental factors, such as BMI (Taylor et al., 2008), smoking (Hedman et al., 2011), physical inactivity (Cordova et al., 2017; Vlashki et al., 2018), and dietary habits (Protudjer et al., 2012), have been epidemiologically and clinically related to the occurrence or severity of asthma. But, observational studies encounter limitations in determining a causal link, due to their various potential confounding factors (Sun et al., 2020). Randomized controlled trials (RCTs), if appropriately conducted, are the standard for determining causal inferences in health sciences. However, RCTs are expensive and time-consuming, involving thousands of patients, some of whom might experience unwanted side effects of drugs (Roberts, 2018).

With some similarities to RCTs, Mendelian randomization (MR) is an alternative approach for establishing evidence of causal relationships for which RCTs are practically unavailable (Smith and Ebrahim, 2003). MR is a method from genetic epidemiology that uses randomly inherited genetic instruments [single-nucleotide polymorphisms (SNPs)] that are robustly associated with a risk factor as proxies for environmental exposure to assess causal inferences for the effects of exposure on an outcome (Smith and Ebrahim, 2003; Rosoff et al., 2019). The increasing availability of summary-level data from genome-wide association studies (GWASs) in the public domain allows MR to make inferences on causality by integrating summary-level GWAS data from various studies (Bowden et al., 2016).

The aim of our study was to estimate causal inferences of environmental factors on asthma using MR and to examine the differences in the susceptibility to asthma with regard to the causal effect of an environmental factor between asthma subtypes, classified by the age of onset, disease severity, and sex.

MATERIALS AND METHODS

Study Population and Design

The UK Biobank is a population-based cohort that recruited over 487,409 individuals aged 40–69 years from 2006–2010 (Collins, 2012). For quality control of the samples, we used the following filter parameters of the Neale lab[1]: PCA calculation filter for selection of unrelated samples; sex chromosome filter for removal of aneuploidy; filter of principal components (PCs) for European sample selection to determine British ancestry; and filters for selection of self-reported 'white British,' 'Irish,' and 'White.' The UK Biobank has been granted ethical approval to collect data on participants by the North West Multicentre Research Ethics Committee, the National Information Governance Board for Health & Social Care, and the Community Health Index Advisory Group.

Asthma cases (n = 35,926) were determined as those that had been diagnosed with asthma by a doctor and had checked for age of onset. Participants who had been diagnosed with chronic obstructive pulmonary disease (COPD) were excluded. Controls (n = 227,924) were defined those who had not been diagnosed with asthma, rhinitis, eczema, allergy, or emphysema/chronic bronchitis. In addition, those who had diagnostic records of hay fever, allergic rhinitis, emphysema, chronic bronchitis, or COPD and those who had J40-47 records in the ICD 10 codes (the 10th revision of the International Statistical Classification of Diseases and Related Health Problems) were excluded (**Supplementary Material** and **Supplementary Figure 1**).

For the study of specific asthma subtypes, cases were divided into 4 groups by the age of onset and disease severity: child-onset mild (CM, n = 9,758), child-onset moderate-to-severe (CM-S, n = 1,875), adult-onset mild (AM, n = 19,415), and adult-onset moderate-to-severe (AM-S, n = 4,878). Individuals with an age of onset before 19 years were defined as child-onset asthma cases (Ferreira et al., 2019), and those with an age of onset after 20 years were considered adult-onset asthma. Moderate-to-severe asthma cases were selected from individuals for whom, in addition to the conditions above, medication information was available and

[1]https://github.com/Nealelab/UK_Biobank_GWAS

the British Thoracic Society (BTS) stage 3-5 criteria were met, as described (Shrine et al., 2019). Asthma cases that did not satisfy these conditions were classified as mild asthma.

To determine whether gender differences had causal effects, adult-onset asthma cases and controls were separated by gender: female adult-onset mild (FAM, n = 12,208), female adult-onset moderate-to-severe (FAM-S, n = 3075), male adult-onset mild (MAM, n = 7207), male adult-onset moderate-to-severe (MAM-S, n = 1803), female control (FC, n = 119,515), and male control (MC, n = 108,409).

Genetic Instrumental Variants for Environmental Factors and Asthma

We studied 93 environmental factors in 18 categories that were associated with asthma (**Supplementary Table 1**, references attached). Association test was performed between asthma and each of the 93 factors by logistic linear regression in R, with adjustments for age, sex, and each environmental factor. As a result, 69 environmental factors were associated with asthma [P < 5.38E-04 (0.05/93)] (**Supplementary Table 2**). For each of the 69 environmental traits, we extracted SNPs that were significantly associated with each factor (e.g., SNP-environmental factor) (P < 5E-08) using genome-wide summary statistics, provided by the Neale lab UK Biobank GWAS summary data (**Supplementary Table 3**)[2]. The SNPs-environmental factor were subject to clumping (r^2 > 0.05, 1-Mbp boundary distance) using FUMA (Watanabe et al., 2017) to ensure the independence of environmental factor-associated loci. As an additional quality control of SNPs for Mendelian randomization, we removed strand-ambiguous SNPs (e.g., A/T and C/G, MAF > 0.42) (Tang et al., 2020) and SNPs in the MHC region (chromosome 6:25-34M) due to their strong pleotropic effects (**Supplementary Figure 2** and **Supplementary Table 4**; Zhu et al., 2020).

For asthma, we performed a genome-wide association analysis using UK Biobank data. Genotyping imputation was performed using the UK10K Project and 1000 Genome Project Phase 3 reference panels (UK10K Consortium, Walter et al., 2015; Genomes Project et al., 2015). General quality control procedures for exclusion (P for Hardy-Weinberg equilibrium test <1E-06, missing genotype call rate >0.05, minor allele frequency <0.01) were applied to 7,402,791 SNPs. In total, 5,664,578 SNPs were retained for further analysis. A GWAS for asthma was performed using PLINK 1.9, with adjustments for age, sex, genetic array, and 10PCs. A list of independent asthma-associated loci (e.g., asthma SNPs) were determined by clumping (P < 5E-08, r^2 > 0.05, 1-Mbp boundary distance), and SNPs that were strand-ambiguous and in the MHC region were excluded. The resulting quantile-quantile (QQ) plot and Manhattan plot are shown in **Supplementary Figure 3**. Thus, 142 SNPs were selected for genetic instruments of asthma (**Supplementary Table 5**).

For the two-sample MR setting of BMI → asthma, we extracted summary association statistics for the 158 genome-wide significant SNPs (P < 5E-08) that were associated with BMI in a trans-ethnic meta-GWAS of 173,430 Japanese subjects (the BioBank Japan project, the Japan Public Health Center-based Prospective Study, and the Tohoku Medical Megabank Project) and 339,224 Europeans (the GIANT consortium) (total N_{max} = 480,438) (Akiyama et al., 2017). Of 158 BMI SNPs, we removed SNPs with palindromes (e.g., A/T and C/G) (Tang et al., 2020) and SNPs in the MHC region (chromosome 6:25-34M) in the MR analyses (Zhu et al., 2020), retaining 149 SNPs.

To avoid the biases of one-sample settings, such as reverse causality and overfitting, we performed a GWAS for BMI only in controls (n = 227,924) from the UK Biobank data. The resulting genome-wide significant BMI SNPs (P < 5E-08) were subject to clumping using FUMA (Watanabe et al., 2017). The 170 independent BMI SNPs were further subject to the removal of SNPs with strand ambiguity, SNPs in the MHC region, and SNPs that were associated with asthma, leaving 159 SNPs (**Supplementary Table 10**). The resulting quantile-quantile (QQ) and Manhattan plots are shown in **Supplementary Figure 4**.

Mendelian Randomization

To assess the causal relationship between environmental factors and asthma, we applied 3 methods: inverse variance weighted (IVW) random effects model (Burgess et al., 2013), weighted median regression (Bowden et al., 2016; Censin et al., 2017), and generalized summary-data-based Mendelian randomization (GSMR) (Zhu et al., 2018). The causal effect estimate by IVW is liable to be biased if any SNP exhibits horizontal pleiotropy. As a complementary method to reduce heterogeneity, we performed GSMR, in which genetic variants were pruned at a high threshold of r^2 < 0.05 (Pasman et al., 2019) and filtered for pleiotropic effects on exposure and outcome [Heterogeneity In Dependent Instrument (HEIDI) filtering] (Zhu et al., 2018). The weighted median method provides an unbiased estimate of the causal effect even when up to 50% of the information comes from invalid genetic variants (Bowden et al., 2016; Censin et al., 2017).

To ensure minimal or no pleiotropy in our results, we performed additional sensitivity analyses. First, we estimated the intercept by MR-Egger regression, with an intercept that differs significantly from 0 (P < 0.05) as an indication of residual heterogeneity due to directional pleiotropy (Bowden et al., 2015). Next, we evaluated the residual heterogeneity using Cochran's Q statistic, with significant heterogeneity (P < 0.05) due to horizontal pleiotropy. Then, we removed SNPs with any evidence of pleiotropy by clumping both environmental factor and asthma SNPs (r^2 > 0.05, 1-Mbp boundary distance) and excluded SNPs that were potentially associated with asthma (P < 0.05/number of environmental factor SNPs) for forward MR and SNPs that were linked to environmental factors (P < 0.05/number of asthma SNPs) for reverse MR and then repeated the MR analyses. Finally, only unidirectional causal effects were determined by performing the reverse MR of asthma → environmental factor (P < 5E-08).

The estimates from the IVW, weighted median, and GSMR were defined as causal effects only when meeting significance after Bonferroni correction for multiple tests as the threshold for the true causal estimate (P < 0.05/number of environmental factors x number of asthma subtypes).

[2]http://www.nealelab.is/uk-biobank

Statistical Analysis

For the genome-wide association analyses, we used a logistic regression model and assumed an additive genetic model of trait status with genotype dose, fitted using the R package and adjusted for covariates, including age, sex, environmental factor, and PC10s. t-tests were used to compare characteristics between control and asthma cases using R.

We used the TwoSampleMR package for performing Mendelian randomization methods, such as IVW, weighted median, and MR-Egger; the gsmr package for GSMR, the qqman package for drawing Manhattan plot and quantile-quantile (QQ) plots; and the ggplot2 package for drawing odds ratio plots, available in the R stats package, version 3.6.3[3].

RESULTS

Characteristics of Study Population for Asthma Case and Control Subjects

The basic characteristics of the 263,850 UK Biobank participants (35,926 asthma cases and 227,924 controls) in this study are described in **Table 1**. BMI, obesity, eosinophil parameters, and female frequency were significantly higher in asthma cases than controls, and there was no difference in smoking status between groups.

Effects of 69 Environmental Factors on Asthma

Forward MR analyses (environmental factor → asthma) were conducted using IVW, weighted median, and GSMR for 69 factors (**Figure 1** and **Supplementary Table 4**). Factors that were related to white blood cells and anthropometry (leukocyte, eosinophil parameters, BMI, and waist circumference) satisfied the threshold by Bonferroni correction for multiple tests ($P < 7.25E{-}04$, 0.05/69) in all 3 methods. The intercept *P*-value from the MR-Egger regression suggests that there was relatively balanced pleiotropy ($P = 0.08$ for leukocyte count, $P = 0.27$ for eosinophil count, $P = 0.47$ for eosinophil percentage, $P = 0.88$ for BMI, and $P = 0.19$ for waist circumference). However, the Cochran's Q values indicated significant residual heterogeneity in all 4 analyses (Q = 394.9, *P*-value = 1.65E–30 for leukocyte count; Q = 1095.7, *P*-value = 3.22E–149 for eosinophil count; Q = 1181.2, *P*-value = 6.41E–169 for eosinophil percentage; Q = 383.4, *P*-value = 8.25E–14 for BMI; and Q = 309.6, *P*-value = 2.46E–10 for waist circumference), which must be improved to determine the true causal effects.

For the sensitivity analyses, we clumped all 873 environmental factor SNPs (123 for leukocyte count, 138 for eosinophil count, 131 for eosinophil percentage, 200 for BMI, 170 for waist circumference, and 111 for asthma; $r^2 < 0.05$, 1-Mbp boundary distance; **Supplementary Table 15**). In addition, we removed environmental factor SNPs that were associated with asthma [$P < 5.05E{-}04$ (0.05/99) for leukocyte count SNPs, $P < 5.32E{-}04$ (0.05/94) for eosinophil count SNPs, $P < 6.17E{-}04$ (0.05/81) for eosinophil percentage SNPs, $P < 2.99E{-}04$ (0.05/167) for BMI SNPs, and $P < 5.21E{-}04$ (0.05/96) for waist circumference SNPs] and asthma SNPs that were related to environmental factors [$P < 5.38E{-}04$ (0.05/93) for asthma SNPs]. RE–analysis of MR using SNPs that were retained after pruning showed that the overall heterogeneity improved, as indicated by the lower Cochran's Q values (**Figure 2A** and **Supplementary Table 6**; Q = 221.7, *P*-value = 1.03E–12 for leukocyte count; Q = 173.1, *P*-value = 3.61E–09 for eosinophil count; Q = 98.0, *P*-value = 8.01E–04 for eosinophil percentage; Q = 254.7, *P*-value = 4.48E–06 for BMI; and Q = 167.0, *P*-value = 5.33E–06 for waist circumference). These additional analyses suggest that eosinophil count, eosinophil percentage, and BMI have causal inferences on asthma, whereas the effects of leukocyte count and waist circumference are not significant, because they failed to reach the threshold for significance [$P < 1.00E{-}02$ (0.05/5)].

To determine unidirectional causal effects (environmental factor → asthma), we conducted reverse MR analyses (asthma → environmental factor) using asthma SNPs (**Figure 2B** and **Supplementary Table 6**). We found that asthma had causal effects on leukocyte count, eosinophil count, and eosinophil percentage, whereas asthma did not causally affect BMI or waist circumference. The reverse MR results indicate that only BMI causally increases the risk of asthma.

Effects of BMI on the Susceptibility of Asthma Subtypes

Asthma is a highly heterogeneous disease that can be classified by various clinical criteria (Haldar et al., 2008; Zhu et al., 2020). Recent studies indicate that specific asthma subtypes are related to metabolic traits, such as obesity (Jeong et al., 2017). In our study, asthma cases were divided into 4 subsets, based on 2 criteria, combining age of onset and severity: child-onset mild (CM), child-onset moderate-to-severe (CM-S), adult-onset mild

[3]www.r-project.org

TABLE 1 | Characteristics of the asthma cases and controls from the UK Biobank.

	Control (*n* = 227,924)	Asthma (*n* = 35,926)
Age (years) *	57.00 ± 7.91	55.86 ± 8.19
Onset age (years)	–	31.07 ± 18.71
Male (%) *	108,409 (47.56%)	15,562 (43.32%)
BMI (kg/m^2) *	27.26 ± 4.59	28.10 ± 5.28
Obesity (%) *	52,342 (22.96%)	10,468 (29.14%)
Hay fever (%)	–	16,304 (45.38%)
Eosinophil percentage (%) *	2.39 ± 1.67	3.25 ± 2.39
Eosinophil count (10^9 cells/L) *	0.16 ± 0.12	0.23 ± 0.18
Medicine use (%)	–	16,933 (47.13%)
Smoking status	193,627	30,389
Never smoker	90,354 (46.66%)	14,086 (46.35%)
Previous smoker	80,157 (41.40%)	12,962 (42.65%)
Current smoker	23,116 (11.94%)	3,341 (10.99%)

Obesity, BMI ≥ 30 kg/m^2; eosinophil percentage, proportion of eosinophils in the leukocytes; and medicine use is used to distinguish between mild and moderate-to-severe asthma.
**P < 7.14E-03 (0.05/7).*

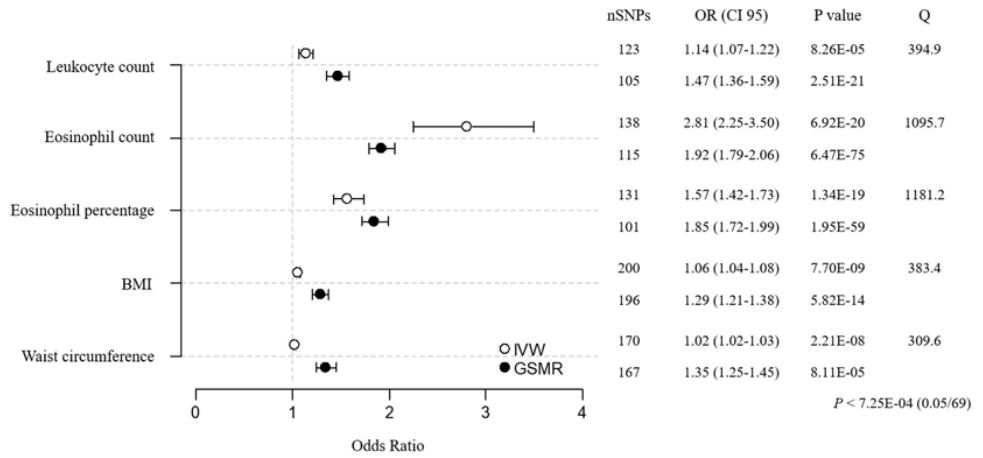

FIGURE 1 | MR estimates for associations between 5 environment factors and asthma. The odds ratio is plotted as dots with the 95% confidence interval. White dots are the IVW method, and black dots are the GSMR method. The *P* value indicates significance when $P < 7.25E-04$ (0.05/69) by Bonferroni correction. The Q value by Cochran's Q statistic indicates residual heterogeneity in the MR estimates.

(AM), and adult-onset moderate-to-severe (AM-S) (**Table 2**). The child- and adult-onset moderate-to-severe subgroups had significantly higher BMI, obesity, and eosinophil parameters than their respective mild subgroups (**Table 2**).

Mendelian randomization (BMI → asthma) was performed using 4 asthma subtypes. As a result, the causal impact of BMI on asthma was stronger in moderate-to-severe versus mild cases before sensitivity analysis, with the adult-onset moderate-to-severe subtype the most susceptible to BMI ([CM versus CM-S] IVW OR/CI95 = 1.02/0.99–1.05 versus 1.10/1.04–1.17, weighted median = 1.02/0.98–1.06 vs. 1.09/1.00–1.20, GSMR = 1.09/0.97–1.23 vs. 1.62/1.24–2.12; [AM vs. AM-S] IVW = 1.06/1.03–1.08 vs. 1.12/1.07–1.16, weighted median = 1.02/0.99–1.05 vs. 1.11/1.05–1.17, GSMR = 1.271.17–1.39 vs. 1.67/1.41–1.97; **Supplementary Tables 7, 15**). Additional sensitivity analyses improved the heterogeneity, as evidenced by the Cochran's Q values, establishing BMI as having its strongest effect on asthma in the adult-onset moderate-to-severe subgroup ([CM vs. CM-S] IVW OR/CI95 = 1.02/0.99–1.05 vs. 1.12/1.05–1.20, weighted median = 1.01/0.97–1.06 vs. 1.10/1.00–1.22, GSMR = 1.09/0.96–1.25 vs. 1.74/1.30–2.32; [AM vs. AM-S] IVW = 1.06/1.03–1.08 vs. 1.10/1.06–1.15, weighted median = 1.02/0.99–1.06 vs. 1.09/1.03–1.16, GSMR = 1.25/1.14–1.38 vs. 1.51/1.26–1.82; **Figure 3** and **Supplementary Table 7**). The child-onset moderate-to-severe and adult-onset mild subgroups were marginally susceptible to asthma, based on the IVW and GSMR analyses, but not weighted median analysis, suggesting the weak causality of BMI. These findings suggest that the causality of BMI in asthma increases as the severity of asthma rises in child- and adult-onset asthma cases and that the causal effect of BMI is the strongest in the adult-onset moderate-to-severe subgroup.

Gender-Specific Effect of BMI in Adult Onset Asthma Cases

Observational studies have suggested that BMI causes the development of adult-onset asthma and that the effect of BMI on asthma is greater in female versus male adults (Chen et al., 2002; Guerra et al., 2002; Beuther and Sutherland, 2007), although the gender-specific effect of BMI on asthma is unknown in children (Castro-Rodriguez et al., 2001; Gold et al., 2003). To study the sex-specific causality of BMI, we divided adult-onset asthma cases into 4 subgroups and controls into 2 subgroups by gender: male adult-onset mild (MAM, $n = 7207$), male adult-onset moderate-to-severe (MAM-S, $n = 1803$), female adult-onset mild (FAM, $n = 12{,}208$), female adult-onset moderate-to-severe (FAM-S, $n = 3075$), male control (MC, $n = 108{,}409$), and female control (FC, $n = 119{,}515$). FAM-S and MAM-S patients had higher BMI, obesity, and eosinophil parameters compared with FAM and MAM cases, respectively (**Table 3**).

Prior to the sensitivity analysis, the results in the adult subgroups showed that female adult-onset cases were more susceptible to the causal effect of BMI than male cases ([MAM versus FAM] IVW OR = 1.05 versus 1.06, weighted median OR = 1.05 versus 1.04, GSMR OR = 1.26 versus 1.35; [MAM-S versus FAM-S] IVW OR = 1.08 versus 1.14, weighted median OR = 1.11 versus 1.09, GSMR OR = 1.43 versus 1.83; **Supplementary Tables 8, 15**). All 3 MR results in the female moderate-to-severe subgroup support the robust inference of BMI on asthma, with the heterogeneity by Cochran's test implying little pleiotropy. In the sensitivity analyses using clumping of SNPs, the susceptibility to asthma in the female moderate-to-severe cases was marginally significant, because the weighted median regression failed to satisfy the threshold by Bonferroni correction ($P < 1.25E-02$, 0.05/4) (**Figure 4** and **Supplementary Table 8**).

Effect of BMI on Asthma and Its Subtypes in Two-Sample Mendelian Randomization

To confirm the causal inference of BMI on asthma subtypes without the bias that often occurs in a one-sample setting, we

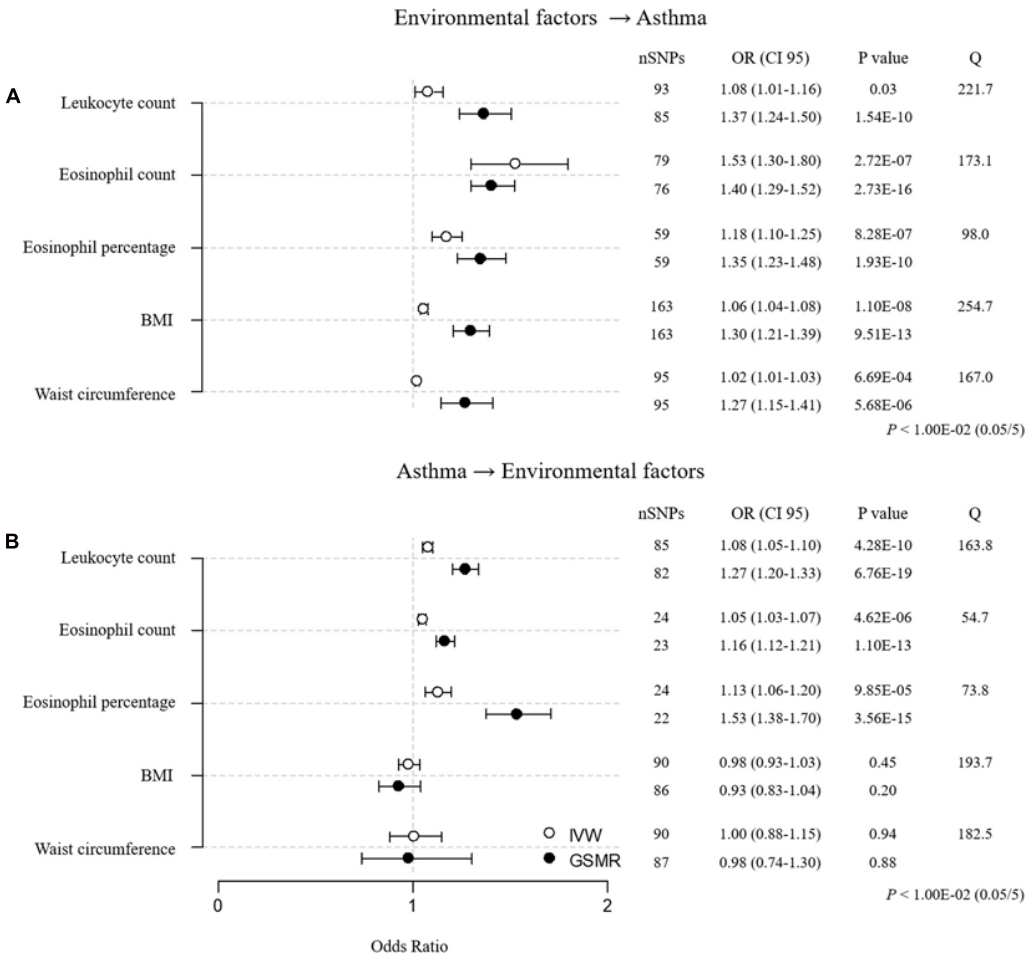

FIGURE 2 | MR estimates for associations between 5 environmental factors and asthma after sensitivity analysis. **(A)** Forward MR: environmental factors → asthma. **(B)** Reverse MR: asthma → environmental factors. The odds ratio is plotted as dots with the 95% confidence interval. White dots are the IVW method, and black dots are the GSMR method. The *P* value indicates significance when $P < 1.00E-02$ (0.05/5) by Bonferroni correction. The Q value by Cochran's Q statistic indicates residual heterogeneity in the MR estimates.

performed two-sample MR using BMI SNPs from another data resource. A total of 149 BMI SNPs were selected from the 158 genome-wide significant SNPs ($P < 5E-08$) that were associated with BMI in a trans-ethnic meta-GWAS of Japanese individuals (the BioBank Japan consortium) and Europeans (the GIANT consortium) (total N_{max} = 480,438) (Akiyama et al., 2017). Then, we excluded 2 SNPs that were potentially associated with asthma [$P < 3.36E-4$ (0.05/149)] to mitigate heterogeneity as a sensitivity analysis.

The MR (BMI → asthma) results of the two-sample setting using 147 BMI SNPs were consistent with the one-sample MR result, implicating BMI as a causal risk factor for asthma [IVW OR = 1.18 (1.07–1.30), $P = 5.97E-04$; weighted median OR = 1.14 (1.01–1.29), $P = 3.68E-02$; GSMR OR = 1.17 (1.08–1.28), $P = 2.15E-04$; **Figure 5A** and **Supplementary Table 9**]. Further, in the two-sample MR of the 4 asthma subtypes, the most susceptible was adult-onset moderate-to-severe asthma [IVW OR = 1.35 (1.11–1.64), $P = 2.81E-03$; weighted median OR = 1.52 (1.12–2.06), $P = 6.76E-03$; GSMR OR = 1.40 (1.13–1.73), $P = 2.24E-03$; **Figure 5B** and **Supplementary Table 9**], replicating the one-sample MR findings.

To avoid reverse causality or overfitting bias in the one-sample setting, we performed an additional MR analysis using 170 BMI SNPs that were acquired from a genome-wide association analysis with BMI alone in controls (no asthma cases) from the UK Biobank. SNPs were further subjected to clumping by FUMA and exclusion of SNPs that were strand-ambiguous and in the MHC region and SNPs that were potentially associated with asthma ($P < 3.05E-4$ (0.05/164)) as a sensitivity analysis. Ultimately, 159 BMI SNPs were used for the MR (BMI → asthma). The results confirmed BMI as a causal factor for asthma [IVW OR = 1.16 (1.07–1.26), $P = 2.89E-04$; weighted median OR = 1.18 (1.07–1.32), $P = 1.71E-03$; GSMR OR = 1.18 (1.11–1.26), $P = 4.37E-08$; **Figure 5C** and **Supplementary Table 11**]. Additional MR analyses of the 4 asthma subtypes confirmed that the adult-onset moderate-to-severe cases were the most susceptible to the development of asthma due to the causal effect of BMI [IVW OR = 1.37 (1.16–1.62), $P = 2.60E-04$; weighted

TABLE 2 | Characteristics of the asthma subtypes and controls from the UK Biobank.

	Control (*n* = 227,924)	Child-onset mild (*n* = 9,758)	Child-onset moderate-to-severe (*n* = 1,875)	Adult-onset mild (*n* = 19,415)	Adult-onset moderate-to-severe (*n* = 4,878)
Age (years)	57.00 ± 7.91	53.86 ± 8.31[a]	55.74 ± 8.45[ab]	56.10 ± 8.01[ad]	58.96 ± 7.42[ace]
Onset age (years)	–	8.62 ± 4.88	8.16 ± 5.37[b]	41.41 ± 12.13[d]	43.62 ± 11.92[ce]
Male (%)	108,409 (47.56%)	5,608 (57.47%)[a]	944 (50.35%)[ab]	7,207 (37.12%)[ad]	1,803 (36.96%)[ace]
BMI (kg/m^2)	27.26 ± 4.59	27.30 ± 4.78	28.26 ± 5.33[ab]	28.22 ± 5.32[ad]	29.11 ± 5.83[ace]
Obesity (%)	52,342 (22.96%)	2,224 (22.73%)[a]	533 (28.43%)[ab]	5,907 (30.42%)[ad]	1,804 (36.98%)[ace]
Hay fever (%)	–	5,350 (54.68%)	1,099 (58.61%)[b]	7,896 (40.67%)[d]	1,959 (40.16%)[ce]
Eosinophil percentage (%)	2.39 ± 1.67	3.32 ± 2.47[a]	3.74 ± 2.84[ab]	3.13 ± 2.23[ad]	3.39 ± 2.60[ace]
Eosinophil count (10^9 cells/L)	0.16 ± 0.12	0.23 ± 0.17[a]	0.27 ± 0.23[ab]	0.22 ± 0.17[ad]	0.25 ± 0.20[ace]
Medicine use (%)	–	3,020 (30.86%)	1,875 (100%)	7,160 (36.88%)	4,878 (100%)
Smoking history	193,627	8,081[a]	1,628[a]	16,389[d]	4,291[ace]
Never smoker	90,354 (46.66%)	4,078 (50.46%)	846 (51.97%)	7,503 (45.78%)	1,659 (38.66%)
previous smoker	80,157 (41.40%)	3,076 (38.06%)	615 (37.78%)	7,198 (43.92%)	2,073 (48.31%)
Current smoker	23,116 (11.94%)	927 (11.47%)	167 (10.26%)	1,688 (10.30%)	559 (13.03%)

Onset age: child-onset <20, adult-onset ≥20; Obesity, BMI ≥30 kg/m^2; Eosinophil percentage, proportion of eosinophils in the leukocytes; and Medicine use is used to distinguish between mild and moderate-to-severe asthma. [a]P < 7.81E–04 (0.05/64) compared with the asthma control group, [b]P < 7.81E–04 (0.05/64) vs. the child-onset mild group, [c]P < 7.81E–04 (0.05/64) vs. the child-onset moderate-to-severe group, [d]P < 7.81E-04 (0.05/64) vs. the child-onset mild group, [e]P < 7.81E–04 (0.05/64) vs. the adult-onset mild group.

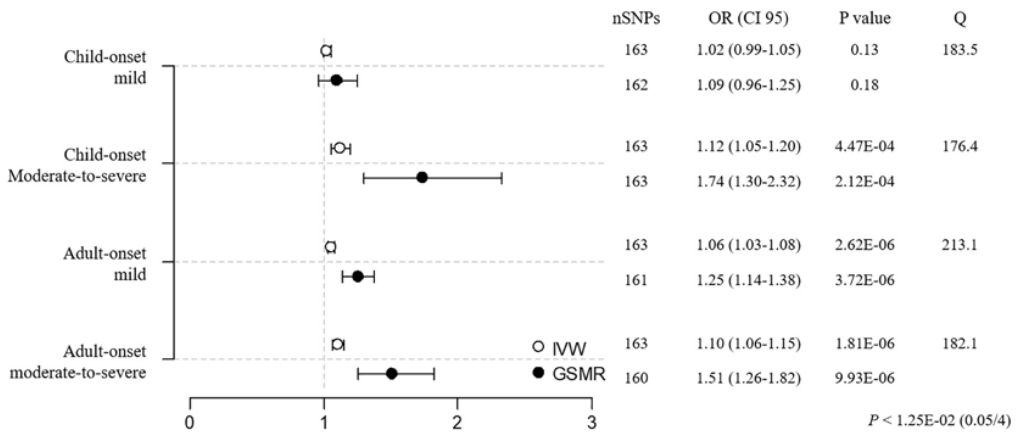

FIGURE 3 | MR estimates of associations between BMI and asthma subtypes. The odds ratio is plotted as dots with the 95% confidence interval. White dots are the IVW method and black dots are the GSMR method. The *P* value indicates significance when *P* < 1.25E-02 (0.05/4) by a Bonferroni correction. The Q value by Cochran's Q statistic indicates residual heterogeneity in the MR estimates.

median OR = 1.60 (1.25–2.06), *P* = 2.41E–04; GSMR OR = 1.36 (1.17–1.58), *P* = 8.84E–05; **Figure 5D** and **Supplementary Table 11**].

To determine the effect of childhood BMI on asthma in our setting, we extracted 25 childhood BMI SNPs with associated statistical values from a previous study (Vogelezang et al., 2020). These BMI SNPs were subject to the removal of SNPs with strand ambiguity (e.g., A/T and C/G), SNPs in the MHC region (chromosome 6:25-34M), SNPs with no proxy (r^2 > 0.8) in UKB, and SNPs that were associated with asthma (*P* < 2.17E–03, 0.05/23) leaving 21 SNPs. Two-sample MR was performed using these 21 childhood BMI SNPs, and the results are described in **Supplementary Table 12**. The estimates of childhood BMI in total asthma showed significant effects by IVW (OR/CI95 = 1.11/1.03-1.19, *P*-value = 3.43E–03) and GSMR (OR/CI95 = 1.12/1.04–1.20, *P*-value = 3.43E-03) but not by the weighted median method (OR/CI95 = 1.09/0.99–1.21, *P*-value = 0.07). However, no significant causal effect was found between childhood BMI and the 4 asthma subtypes by IVW, GSMR, and weighted median, consistent with the previous report (Au Yeung et al., 2021). Based on the previous study (Au Yeung et al., 2021) and our result, childhood BMI has a weak causal impact on asthma but not on any specific subtype.

DISCUSSION

In this study, we used MR to examine the causal relationship between 69 environmental factors and asthma and noted the

TABLE 3 | Characteristics of the adult-onset asthma cases and controls from the UK Biobank.

Environment	Male control (*n* = 108,409)	Male adult-onset mild (*n* = 7,207)	Male adult-onset moderate-to-severe (*n* = 1,803)	Female control (*n* = 119,515)	Female adult-onset mild (n = 12,208)	Female adult-onset moderate-to-severe (n = 3,075)
Age (years)	57.21 ± 8.00	56.29 ± 8.19[a]	59.71 ± 7.36[ab]	56.81 ± 7.83	55.99 ± 7.89[a]	58.51 ± 7.42[abc]
Onset age	–	41.74 ± 12.22	44.65 ± 12.03[b]	–	41.21 ± 12.06[d]	43.01 ± 11.81[bc]
BMI (kg/m^2)	27.80 ± 4.12	28.33 ± 4.50[a]	28.92 ± 4.87[ab]	26.77 ± 4.93	28.16 ± 5.75[a]	29.21 ± 6.33[ab]
Obesity (%)	26,944 (24.85%)	2,156 (29.92%)[a]	625 (34.66%)[ab]	25,398 (21.25%)	3,751 (30.73%)[ad]	1,179 (38.34%)[abc]
Hay fever (%)	–	2,618 (36.33%)	571 (31.67%)[b]	–	5,278 (43.23%)[d]	1,388 (45.14%)[bc]
Eosinophil percentage (%)	2.57 ± 1.75	3.40 ± 2.31[a]	3.64 ± 2.69[a]	2.22 ± 1.57	2.97 ± 2.17[ad]	3.25 ± 2.54[abc]
Eosinophil count (10^9 cells/L)	0.17 ± 0.13	0.24 ± 0.17[a]	0.27 ± 0.22[ab]	0.15 ± 0.12	0.21 ± 0.16[ad]	0.24 ± 0.19[abc]
Medicine use (%)	–	2,640 (36.63%)	1,803 (100%)	–	4,520 (37.02%)	3,075 (100%)
Smoking history	92,770	6,103	1,600[ab]	100,857	1,0286[ad]	2,691[abc]
Never smoker	37,483 (40.40%)	2,373 (38.88%)	448 (28.00%)	52,871 (52.42%)	5,130 (49.87%)	1,211 (45.00%)
previous smoker	42,282 (45.58%)	3,058 (50.11%)	916 (57.25%)	37,875 (37.55%)	4,140 (40.25%)	1,157 (43.00%)
Current smoker	13,005 (14.02%)	672 (11.01%)	236 (14.75%)	10,111 (10.03%)	1,016 (9.88%)	323 (12.00%)

Age onset: adult-onset ≥20; Obesity, BMI ≥ 30 kg/m^2; Eosinophil percentage, proportion of eosinophils in the leukocytes; and Medicine use are used to distinguish between mild and moderate-to-severe asthma. [a]P < 8.93E–04 (0.05/56) vs. its respective gender control, [b]P < 8.93E–04 (0.05/56) vs. its gender-specific mild group, [c]P < 8.93E–04 (0.05/56) vs. the male moderate-to-severe group, [d]P < 8.93E-04 (0.05/56) vs the male mild group.

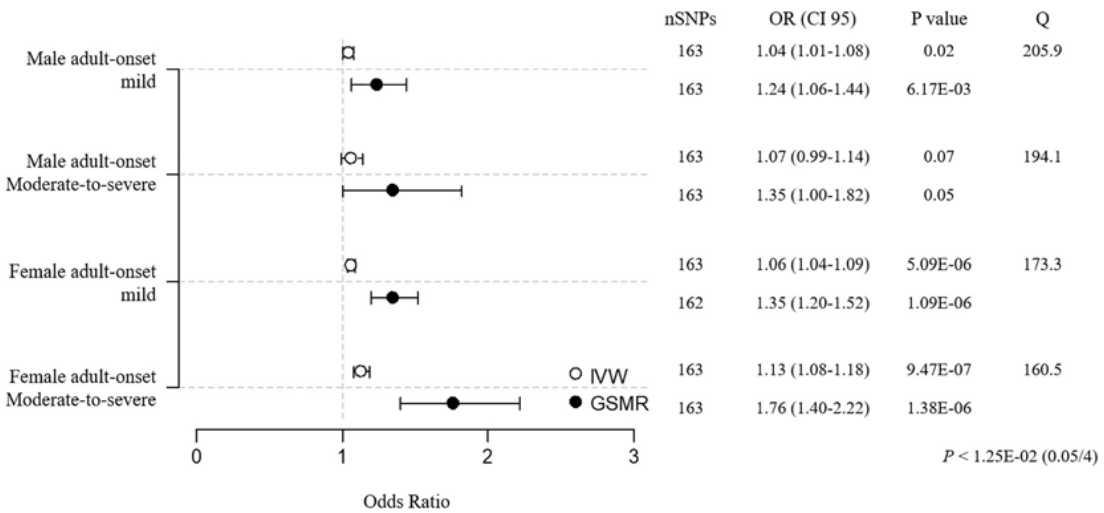

FIGURE 4 | MR estimates of associations between BMI and adult-onset asthma subtypes. The odds ratio is plotted as dots with the 95% confidence interval. White dots are the IVW method, and black dots are the GSMR method. The *P* value indicates significance when *P* < 1.25E-02 (0.05/4) by Bonferroni correction. The Q value by Cochran's Q statistic indicates residual heterogeneity in the MR estimates.

following: BMI is a causal risk factor for asthma without reverse causation; the effect of BMI on asthma is strongest in the adult-onset moderate-to-severe asthma subgroup; and finally, female subtypes are more prone to asthma due to increased BMI than male subtypes in adults.

Epidemiological and genetic studies that used MR have suggested that BMI is causal factor in asthma (Skaaby et al., 2018; Xu et al., 2019; Sun et al., 2020). In addition, BMI is a risk factor for late-onset asthma (onset age >16) and atopic asthma (Zhu et al., 2020). Our study confirms that BMI is a risk factor for asthma and demonstrates that the causal effect of BMI increases significantly in individuals with child- and adult-onset asthma, exacerbating the asthma. Further, the MR result on the stronger effect of BMI in female subgroups is consistent with observational studies (Chen et al., 2002; Guerra et al., 2002; Beuther and Sutherland, 2007).

In the Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens (TENOR) study on severe asthma, approximately 57% of individuals with severe asthma were obese, implying a high prevalence of obesity in severe asthma cases compared with an obesity rate of 35% in non-asthma adults in the general United States population (Schatz et al., 2014). In our study, the obesity rate in the adult moderate-to-severe subgroup was significantly higher than in the adult mild subgroup by 7%. Another study found that lung function improved after weight loss in obese patients with asthma, suggesting that greater obesity is related to the severity of asthma (Hakala et al., 2000). There are several potential mechanisms

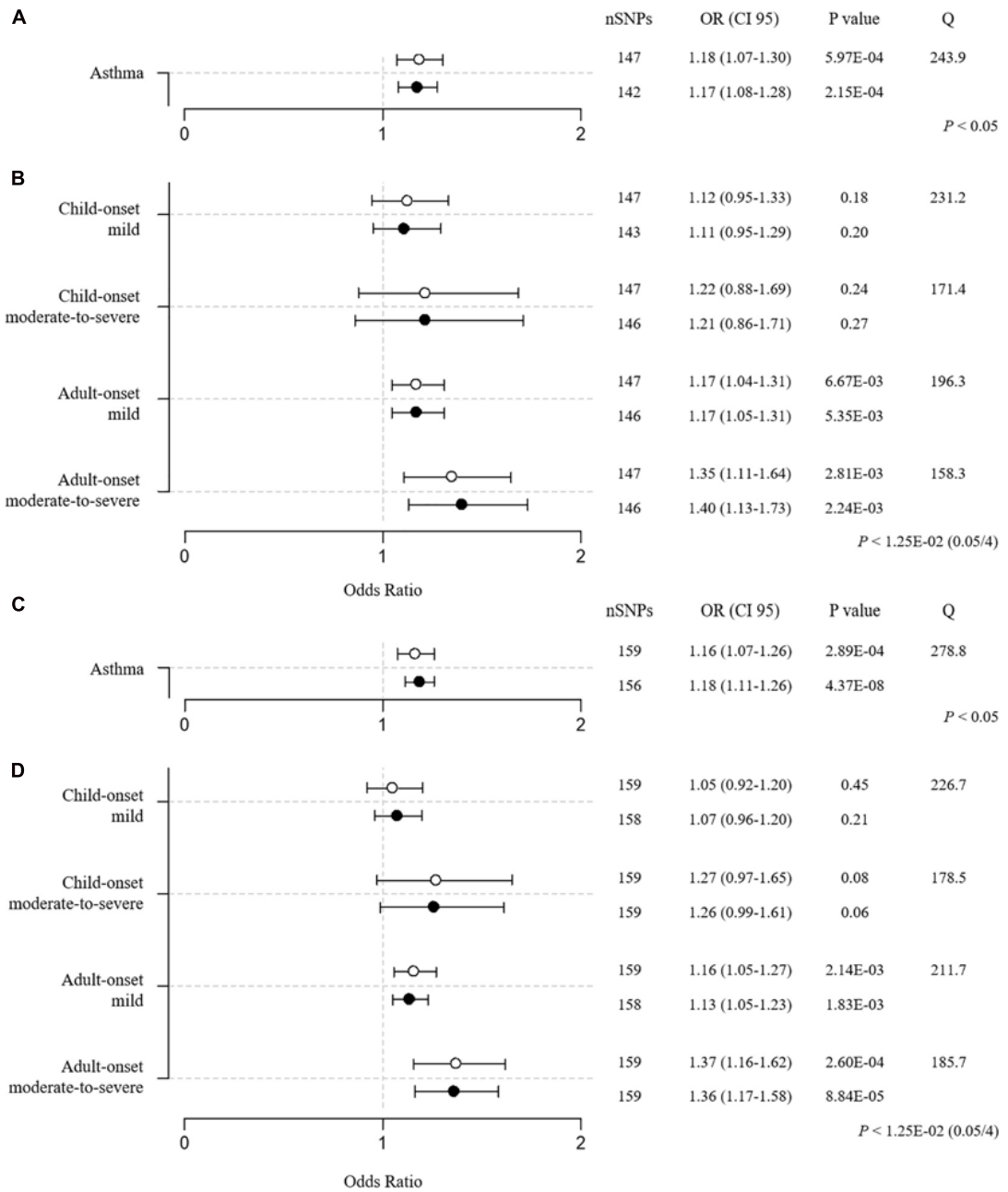

FIGURE 5 | Two-sample MR estimates of BMI → asthma **(A,C)** and BMI → 4 asthma subtypes **(B,D)**. **(A,B)** BMI SNPs were obtained from a meta-GWAS of Japanese (JBB) and European individuals (GIANT). **(C,D)** BMI SNPs were obtained from a GWAS that was performed in only controls (no asthma) from the UK Biobank. The IVW and GSMR methods are indicated by white and black dots, respectively. The Q value by Cochran's Q statistic indicates residual heterogeneity in the MR estimates.

by which BMI is linked to asthma. Obesity has been related to multiple traits of asthma, including eosinophil levels (Kim et al., 2014), lung function (Salome et al., 2010), and allergy (Luo et al., 2013). Studies have suggested that adipokines, such as leptin and adiponectin, are associated with the development and severity of asthma and mediate the exacerbation of asthma through the regulation of eosinophil survival and trafficking (Kim et al., 2014; Zhang et al., 2017; Zheng et al., 2018).

We initially aimed to identify environmental factors that cause the development of asthma. However, of 69 factors, only BMI was identified as a causal influence in asthma. We assume that our study was limited in obtaining the appropriate instruments for certain phenotypes. Environmental data from self-reported questionnaires (e.g., dietary intake, neuroticism, alcohol, smoking, sociodemographic factors, and physical activities) are prone to responder bias (Rask-Andersen et al., 2017). Thus,

the presence of an interviewer is recommended to reduce the likelihood of responder bias when obtaining self-reported questionnaire data.

Further, certain anthropometric factors (e.g., fat and non-fat mass) were measured with a less accurate method (bioelectric impedance using a Tanita BC418MA body composition analyzer; UK Biobank) than such techniques as dual-energy x-ray absorptiometry (Speed et al., 2019). Our primary results show that fat mass and percentage, but not fat free mass, are causal factors in asthma by IVW and GSMR (**Supplementary Table 4**). However, the weighted median method does not support the causal effect of fat mass and percentage. A valid association analysis using precise values can improve the statistics of MR analyses.

Our MR analyses of 69 environmental factors → asthma used only the UK Biobank data as a resource. The one-sample MR setting has several benefits: MR and epidemiological findings can be compared in the same individuals, the validity of causal inferences is unaffected by differences in population characteristics when using 2 samples, and harmonization of genetic variants across datasets is not required (Burgess et al., 2019). However, a limitation of the one-sample analysis is that if the links between genetic instrument and exposure are weak, the causal estimation might suffer from reflection of the confounded associations between exposure and outcome and inflation of false positive (type 1 error) rates (Burgess et al., 2019). Bias in a one-sample analysis with a binary disease outcome can be avoided, such that genetic associations with the exposure are estimated in the controls only; consequently, genetic associations with exposure and outcome will not be correlated (Gharahkhani et al., 2019; Hindy et al., 2019; Wade et al., 2019). We replicated the two-sample MR analyses of BMI → asthma using BMI SNPs from another source of GWAS summary statistics and BMI SNPs from a GWAS only in the control, confirming the one-sample analysis findings.

Although our study stratified asthma by age of onset and sex, concerns remain, because these stratifications were not applied when obtaining genome-wide association statistics on BMI. Genetic studies on BMI have suggested significantly positive genetic correlations between childhood and adult BMI (r_g = 0.76, *P*-value = 1.45E-112) (Vogelezang et al., 2020) and between male and female BMI (r_g = 0.879, *P*-value = 5.9E-4) (Yang et al., 2015). Thus, it is likely that depending on the instrument source, BMI might have disparate causal effects on asthma subtypes. Notably, a recent study that used the IVW method confirmed the causal impact of adult BMI on asthma, whereas the possible impact of childhood BMI on the risk of asthma was less clear, mediated predominantly by its relationship with adult BMI, implicating that children with high BMI can reduce their risk of asthma by becoming normal-weight adults. This study was limited, in that there were far fewer childhood BMI SNPs (*N* = 14 and 5) than adult BMI SNPs (*N* = 323 and 115), decreasing the power of the MR estimation (Au Yeung et al., 2021). Our two-sample MR using 25 childhood BMI SNPs (Vogelezang et al., 2020) supports a causal relationship with asthma. However, based on the previous study (Au Yeung et al., 2021) and our result, childhood BMI has a weak causal impact on asthma but not on any specific subtype.

There is much evidence that suggests gender-specific effects of BMI on asthma. Previous epidemiological reports have suggested that the incidence and symptoms of adult asthma are higher and more severe in women than in males (Chen et al., 2003; Zein and Erzurum, 2015). A recent study that performed sex-specific transcriptomics in 5 tissues from asthma patients also showed sexual dimorphism in asthma, including sex-specific dysregulation of genes and signaling pathways (Gautam et al., 2019). Moreover, the effect of BMI on asthma is greater in female than male adults (Chen et al., 2002; Guerra et al., 2002; Beuther and Sutherland, 2007). Consistent with these reports, the asthma cases and M-S asthma subgroups in our study included more female than male adults (**Table 3**). Further, FAM and FAM-S subtypes had significantly increased obesity compared with MAM and MAM-S subtypes (**Table 3**). Although we observed that female adult subtypes are more susceptible to the causal effect of BMI than the male groups, the effects did not meet our strict criteria. We speculate that the genetic correlations between male and female asthma subtypes are too high to render any distinctive causal patterns (**Supplementary Table 14**) compared with correlations between the child- and adult-onset mild and moderate-to-severe subtypes (**Supplementary Table 13**). For these reasons, it is unlikely that gender-specific BMI instruments have a causal effect on gender-specific asthma subtypes.

In conclusion, our data indicate that elevated BMI levels are causally related to the risk of adult-onset moderate-to-severe asthma. Thus, reducing body weight can help alleviate the susceptibility to moderate-to-severe asthma.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the North West Multicentre Research Ethics Committee, the National Information Governance Board for Health & Social Care, and the Community Health Index Advisory Group. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

T-WH, BO, and J-OK designed the study. T-WH analyzed the data and wrote the first draft of the manuscript. J-OK revised the manuscript. BO, JL, HK, and J-OK collected the data and provided technical support. All authors contributed to the interpretation of the results and critical revision of the

manuscript for important intellectual content and approved the final version of the manuscript.

FUNDING

This work was supported by a grant from Kyung Hee University in 2019 (KHU-20191224) and the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) [The Bio & Medical Technology Development Program of the NRF (2019M3E5D3073365) and NRF-2019R1A2C1083980].

ACKNOWLEDGMENTS

This study was conducted using the resources of UK Biobank (application 56987).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.639905/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 07 June 2021
doi: 10.3389/fgene.2021.676136

Causal Inference Between Chronic Periodontitis and Chronic Kidney Disease: A Bidirectional Mendelian Randomization Analysis in a European Population

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *04 March 2021*
Accepted: *27 April 2021*
Published: *07 June 2021*

Citation:
Yang J, Chen T, Zhu Y, Bai M and Li X (2021) Causal Inference Between Chronic Periodontitis and Chronic Kidney Disease: A Bidirectional Mendelian Randomization Analysis in a European Population. Front. Genet. 12:676136. doi: 10.3389/fgene.2021.676136

Background: Previous epidemiological studies have shown significant associations between chronic periodontitis (CP) and chronic kidney disease (CKD), but the causal relationship remains uncertain. Aiming to examine the causal relationship between these two diseases, we conducted a bidirectional two-sample Mendelian randomization (MR) analysis with multiple MR methods.

Methods: For the casual effect of CP on CKD, we selected seven single-nucleotide polymorphisms (SNPs) specific to CP as genetic instrumental variables from the genome-wide association studies (GWAS) in the GLIDE Consortium. The summary statistics of complementary kidney function measures, i.e., estimated glomerular filtration rate (eGFR) and blood urea nitrogen (BUN), were derived from the GWAS in the CKDGen Consortium. For the reversed causal inference, six SNPs associated with eGFR and nine with BUN from the CKDGen Consortium were included and the summary statistics were extracted from the CLIDE Consortium.

Results: No significant causal association between genetically determined CP and eGFR or BUN was found (all $p > 0.05$). Based on the conventional inverse variance-weighted method, one of seven instrumental variables supported genetically predicted CP being associated with a higher risk of eGFR (estimate = 0.019, 95% CI: 0.012–0.026, $p < 0.001$).

Conclusion: Evidence from our bidirectional causal inference does not support a causal relation between CP and CKD risk and therefore suggests that associations reported by previous observational studies may represent confounding.

Keywords: causal inference, chronic periodontitis, chronic kidney disease, Mendelian randomization, single nucleotide polymorphisms

INTRODUCTION

Chronic kidney disease (CKD) leading to end-stage renal disease (ESRD) and requiring dialysis or kidney transplantation is greatly associated with shortened life expectancy (Webster et al., 2017). The prevalence of CKD is around 10% worldwide, which is becoming one of the major burdens for health care in every country (GBD 2017 Risk Factor Collaborators, 2018). CKD demonstrates a series of changes in glomerular, tubular, and endocrine renal structures. As the pathogenesis of CKD is considerably complicated and yet to be uncovered, the current therapeutic options for CKD are limited to controlling its risk factors, such as blood pressure, diabetes, and chronic inflammation (Köttgen et al., 2010).

As a low-grade chronic inflammation, chronic periodontitis (CP) has been reported to be highly associated with kidney function measures, including estimated glomerular filtration rate (eGFR) and blood urea nitrogen (BUN) (Kinane et al., 2017). Meanwhile, greater deterioration of periodontal status, including poor oral hygiene and gingival, has been observed among CKD patients, especially those under dialysis treatment, than health controls (Gautam et al., 2014; Tadakamadla et al., 2014). Due to the restriction of methodological bias, it is a particular challenge to determine the causality by conventional observational study, in terms of the existing of confounding, reverse causation, and measurement error (Boyko, 2013). Therefore, investigating the causal relationships between CP and CKD through other effective approaches is of great urgency for disease prevention and treatment strategies (Nanayakkara and Zhou, 2019).

Mendelian randomization (MR) is a powerful genetic epidemiological tool used to evaluate causal effects, overcoming the limitations of conventionally observational studies (Smith and Ebrahim, 2003). Two-sample MR analysis is an extensive application of the MR approach, allowing the use of GWAS summary statistics for MR studies rather than limiting them using individual-level data within one sample (Burgess et al., 2015). Using two-sample MR analysis, the causal relationship between CP and risk factors of CKD, e.g., cardiovascular disease (Bell et al., 2020) and blood pressure (Yu et al., 2020), has been assessed. In this study, we took advantage of the recent large-scale meta-analysis of the GWAS of CP and CKD to bidirectionally perform a two-sample MR analysis for examining the causal associations between these two diseases.

MATERIALS AND METHODS

Study Design

In this bidirectional two-sample MR analysis, genetic variants were used to investigate the causal effect and direction of CP with eGFR (the primary kidney function trait) and BUN (the second kidney function trait). Briefly, the modifiable risk factor-associated single-nucleotide polymorphisms (SNPs) hired as instrumental variables (IVs) are randomly allocated obeying Mendel's law of independent assortment. The SNPs are distributed at the forming of the zygote, which always precedes the onset of disease and is less likely to be affected by confounding or reverse causation. Therefore, grouped by the naturally allocated genetic IVs, the MR approach mimics a randomized controlled trial using individual- or summary-level data from observational studies (Smith and Hemani, 2014).

To obtain reliable results, the valid IVs must satisfy three important assumptions within the MR analysis process (Smith et al., 2020): (1) the IVs are solidly related to the exposure, (2) the IVs are not correlated with any confounders influencing both exposure and outcome, and (3) the IVs affect the outcome only through their effects on the exposure and not through any other causal pathways (**Figure 1**). Details on the MR design have been described elsewhere (Burgess and Thompson, 2015; Tillmann et al., 2017). For each inference direction, the analysis included three main procedures: the selection of suitable genetic IVs for the corresponding exposure, application of multiple MR methods, and pleiotropic effect analyses, as described below.

Participants and Data Sources

For our study, summary statistics of the genome-wide association study (GWAS) for CP was derived from the Gene-Lifestyle Interactions in Dental Endpoints (GLIDE) Consortium, analyzing a total of 12,289 clinically diagnosed periodontitis cases and 22,326 controls (Shungin et al., 2019). Summary-level data of CKD concerning kidney function measures (i.e., eGFR and BUN) were extracted in currently the biggest the Chronic Kidney Disease Genetics (CKDGen) Consortium (including 41,395 cases and 4,39,303 controls) (Wuttke et al., 2019).

The participants of these two GWAS studies are mostly people with European ancestry. In both these corresponding original studies, all participants provided written informed consent. Each study included in the GLIDE and the CKDGen Consortiums was approved by a local institutional review board and an ethics committee.

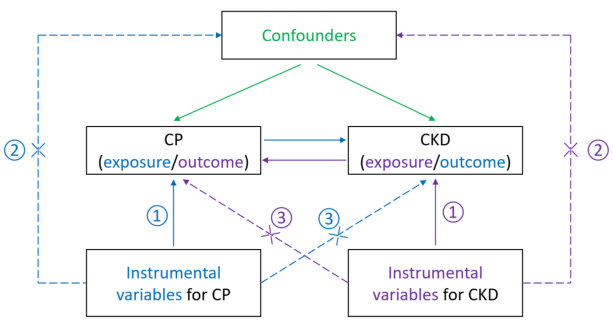

FIGURE 1 | Illustration of the current bidirectional Mendelian randomization setting with required instrumental variable assumptions. That is, each genetic variant (SNP) is ① associated with the exposure (a disease or phenotypic characteristics), ② but not associated with unmeasured confounders of the exposure–outcome association, and ③ not associated with the risk of outcome (another disease or phenotypic characteristics) conditional on the exposure and confounder. The blue items indicate the assumptions for causal inference of CP on CKD, while the purple items show the assumptions for reverse inference. The green items present confounders of the exposure–outcome association. CP, chronic periodontitis; CKD, chronic kidney disease; SNP, single nucleotide polymorphism.

Selection of Genetic Instrumental Variables

All genetic instrumental variables for the current bidirectional MR analysis were filtered to fit the three basic MR assumptions as described above.

For the first assumption, the genetic instruments for estimating the causal effect of CP on the risk of CKD were obtained from a GWAS analysis of the GLIDE Consortium, in which eight SNPs were suggestively ($p < 5 \times 10^{-6}$) associated with periodontitis (Shungin et al., 2019). To investigate the causal effect of kidney function on chronic periodontitis, 256 and 75 index SNPs reported significantly associated with eGFR and BUN in a meta-analysis of GWAS on kidney function ($p < 5 \times 10^{-8}$) were included as candidate genetic instruments, respectively.

The assumptions of genetic instrumental variables being independent of outcome and confounding factors were investigated for the genome-wide significant associations with corresponding outcome variables and their related confounding factors by searching in a web-based GWAS catalog[1] (Staley et al., 2016). The SNPs co-associated with outcome variable and potential confounders were removed to satisfy these two assumptions (Smith et al., 2008).

Besides, pairwise linkage disequilibrium clumping (Purcell et al., 2007) was performed for identifying the independent signals among correlated SNPs, and the removal of correlated SNPs was conducted by Steiger filtering for the exclusion of reverse causality (Hemani et al., 2017).

Statistical Analysis

We employed multiple complementary methods of MR for a comprehensive and precise causal effect investigation, including the inverse variance weighted (IVW) (Burgess and Thompson, 2015), the weighted median (WM) (Bowden et al., 2016), and the Mendelian randomization–Egger (MR-Egger) (Burgess and Thompson, 2017) methods. For the fundamental estimates of the causal effect of the exposure on the risk of the outcome, we performed the IVW method which is conventionally used in two-sample MR studies (Burgess and Thompson, 2015). The IVW method uses the associations (beta-coefficients and standard errors) combined with risk factors and the results of regressing each genetic variant in turn, using summarized data from all the genetics variants to estimate causality (Rees et al., 2019). The WM method could give consistency analyses by calculating a single weighted median estimator for combining data on multiple genetic instruments (Bowden et al., 2016). Compared to the IVW method which only provides consistent results when all genetic variants in the analysis are valid IVs, the WM method could give a consistent estimator even if some of the genetic instruments in the reference are not valid instrument variables (Hartwig et al., 2017). MR–Egger methods provide assess potential asymmetry for bias from the pleiotropic effect of the multiple genetic variants and give estimates of the causal effect (Verbanck et al., 2018). MR–Egger has the advantages to assess the directional pleiotropy under the weaker assumption (Burgess and Thompson, 2017), e.g., the Instrument Strength Independent of Direct Effect (InSIDE) assumption.

The power analysis was conducted by a web-based application[2] (Brion et al., 2013) to evaluate the minimum detectable magnitude of association for outcomes in bidirectional causal inferences between CP and CKD. All results are presented as an estimate or odds ratio (OR) with a 95% confidence interval (CI) of the outcomes OR or per predicted increase/decrease. All statistical tests were two-sided, and the evidence of association was cutoff at a prespecified *p*-value below 0.05. All analyses were performed in R version 4.0.3 (R Project for Statistical Computing, Vienna, Austria), with packages *MendelianRandomization* (0.5.0) (Yavorska and Burgess, 2017) and *forestplot* (1.10)[3].

RESULTS

Selection of Instrumental Variables for CP and Kidney Function

After the removal of SNPs associated with potential confounders in the online GWAS database, the pairwise linkage disequilibrium clumping and matching of coding alleles between the summary statistics of the exposure and those of the outcome, and the exclusion of correlated SNPs by Steiger filtering, the valid instrumental variables were selected to fit the three basic MR assumptions above. Seven SNPs suggestively associated with CP were selected as genetic instruments for the MR analysis of CP causally associated with CKD. In the reversed MR analysis, six SNPs and nine SNPs significantly associated with two commentary kidney function measures (eGFR and BUN), respectively, were included. The corresponding summary statistics for these SNPs for MR analyses were retrieved from the reported summary GWAS results of CKD and CP, respectively (**Tables 1**, **2**).

Causal Association of CP and CKD by Conventional MR Method

We estimated the association between CP-related SNPs and risk of CKD, and between CKD-related SNPs and risk of CP, using the IVW method. The results are presented in **Figure 2**. This conventional estimate showed no convincing evidence to support the causal relation between CP and CKD in either of two reversed directions (CP-related SNPs on risk of eGFR, effect = 0.003, 95% confidence interval [CI]: −0.003–0.008, $p = 0.317$; CP-related SNPs on risk of BUN, effect = 0.002, 95% CI: −0.004–0.009, $p = 0.472$; eGFR-related SNPs on risk of CP, effect = −0.333, 95% CI: −3.124–2.459, $p = 0.815$; BUN = −related SNPs on risk of CP, effect = −0.021, 95% CI: −1.447–1.405, $p = 0.977$). For the single genetic instrument, only one of seven SNPs used as genetic instruments in the IVW method demonstrated that CP was causally associated with eGFR ($p < 0.001$ for rs2976950), which changed little of the overall IVW estimate of all CP-related SNPs on the risk of eGFR ($p = 0.317$) (Burgess and Thompson, 2015).

[1] http://www.phenoscanner.medschl.cam.ac.uk

[2] https://shiny.cnsgenomics.com/mRnd/

[3] http://gforge.se/packages/

TABLE 1 | Summary statistics for Mendelian randomization analysis of the potential causal effect of chronic periodontitis on kidney function.

SNP	Chr	EA	OA	EAF	Exposure: chronic periodontitis			Primary outcome: eGFR			Secondary outcome: BUN		
					Beta	SE	*p*	Beta	SE	*p*	Beta	SE	*p*
rs13005050	2	C	T	0.14	0.1432	0.0310	3.76E-06	0.0009	0.0006	1.03E-01	−0.0027	0.0014	6.29E-02
rs4956201	4	C	A	0.89	0.2406	0.0474	3.89E-07	−0.0006	0.0008	4.42E-01	0.0001	0.0020	9.61E-01
rs6816769	4	C	T	0.89	0.1348	0.0294	4.57E-06	0.0004	0.0006	4.81E-01	0.0006	0.0014	6.53E-01
rs78422482	4	A	G	0.01	0.2425	0.0510	2.02E-06	−0.0006	0.0010	5.15E-01	0.0024	0.0024	3.10E-01
rs73155039	7	A	G	0.99	0.8316	0.1757	2.22E-06	−0.0004	0.0019	8.35E-01	0.0034	0.0052	5.23E-01
rs2976950	8	A	G	0.60	0.0963	0.0195	7.99E-07	0.0018	0.0004	4.52E-07	0.0007	0.0010	4.37E-01
rs151226594	11	G	T	0.01	0.3671	0.0768	1.75E-06	0.0012	0.0014	3.90E-01	0.0030	0.0034	3.67E-01

These SNPs are associated with chronic periodontitis at the genome-wide significance level ($p < 5 \times 10^{-6}$) in a meta-analysis with up to 34,615 individuals of European ancestry (Shungin et al., 2019). SNP, single nucleotide polymorphism id; Chr, chromosome; EA, effect allele; OA, other allele; EAF, effect allele frequency; Beta, SNP effect size; SE, standard error; p, p-value; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen.

TABLE 2 | Summary statistics for Mendelian randomization analysis of the potential causal effect of kidney function on chronic periodontitis.

SNP	Chr	EA	OA	EAF	Primary exposure: eGFR			Secondary exposure: BUN			Outcome: chronic periodontitis		
					Beta	SE	*p*	Beta	SE	*p*	Beta	SE	*p*
rs11694902	2	A	G	0.76	0.0050	0.0004	3.28E-34				0.0047	0.0257	8.56E-01
rs17462630	2	C	G	0.14	0.0041	0.0005	2.14E-16				0.0026	0.0189	8.91E-01
rs9868185	3	G	A	0.31	0.0055	0.0004	4.04E-37				0.0012	0.0182	9.47E-01
rs12920176	16	A	C	0.79	0.0026	0.0004	1.01E-09				0.0002	0.0186	9.92E-01
rs113445505	19	T	C	0.20	0.0096	0.0005	1.21E-99				−0.0089	0.0184	6.26E-01
rs6127099	20	T	A	0.34	0.0034	0.0004	1.53E-20				0.0001	0.0211	9.94E-01
rs10874312	1	A	G	0.66				0.0070	0.0009	1.73E-14	0.0035	0.0187	8.51E-01
rs760077	1	A	T	0.41				0.0134	0.0010	2.10E-44	−0.0124	0.0184	4.99E-01
rs34773350	2	C	T	0.86				0.0083	0.0012	2.90E-11	0.0034	0.0257	8.94E-01
rs9849724	3	G	T	0.46				0.0047	0.0009	4.09E-08	0.0043	0.0180	8.13E-01
rs4976646	5	C	T	0.34				0.0073	0.0009	2.91E-15	0.0033	0.0196	8.67E-01
rs13230625	7	A	G	0.70				0.0134	0.0013	1.08E-26	0.0077	0.0262	7.71E-01
rs6597862	10	C	A	0.76				0.0058	0.0010	8.33E-09	−0.0011	0.0210	9.59E-01
rs3925584	11	T	C	0.55				0.0096	0.0009	9.85E-29	0.0078	0.0183	6.69E-01
rs4886755	15	G	A	0.51				0.0095	0.0009	1.73E-28	−0.0047	0.0175	7.89E-01

These SNPs are associated with chronic periodontitis at the genome-wide significance level ($p < 5 \times 10^{-8}$) in a meta-analysis with up to 480,698 individuals of European ancestry (Wuttke et al., 2019). SNP, single-nucleotide polymorphism id; Chr, chromosome; EA, effect allele; OA, other allele; EAF, effect allele frequency; Beta, SNP effect size; SE, standard error; p, p-value; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen.

Causal Association of CP and CKD by Different MR Approaches

The bidirectional MR estimates between CP and CKD by multiple methods are presented in **Figure 3**. The associations of CP with CKD biomarkers were consistent in the sensitivity analysis that used the WM but not in the MR–Egger method. The intercept test of MR–Egger suggested a potential directional pleiotropy ($p = 0.003$ for CP on eGFR, using all seven SNPs). This was also indicated in the scatter plots in terms of the results from the IVW method (**Figure 2**).

We assessed the statistical power of this current bidirectional MR study. Based on the variance in CP, eGFR, and BUN according to corresponding seven, six, and nine SNPs, respectively, and the sample sizes of 12,289 cases and 22,326 controls in cohorts from the GLIDE Consortium for CK and 41,395 cases and 4,39,303 controls in cohorts from the CKDGen Consortium for CKD, our study could have over 99% power at an alpha rate of 5% to detect a statistically significant causal effect.

DISCUSSION

In the present study, we investigated the potential causal roles of CP in the development of CKD and the reverse causal relation of kidney function with the progress of CP, by conducting multiple complementary MR approaches. Using genetic variants as proxies for CP and kidney function measures, including eGFR and BUN, our study did not observe strong evidence to support that genetically predicted CP was associated with decreased eGFR or increased BUN and vice versa.

Previous observational studies based on cross-sectional or case–control design can only describe a connection between CP and CKD because of the absence of chronological sequence

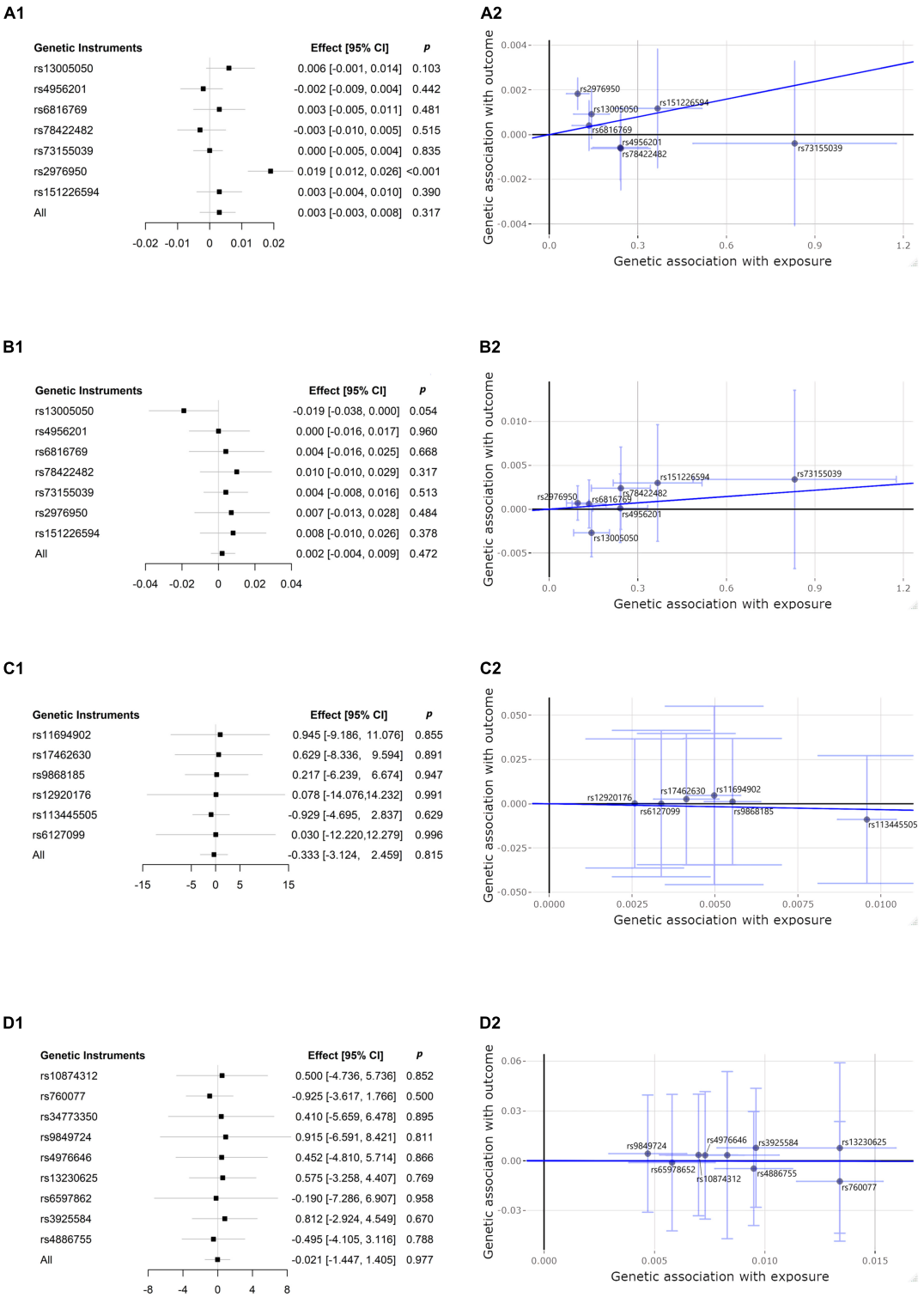

FIGURE 2 | Forest plots and scatter plots of associations between chronic periodontitis (CP) and chronic kidney disease (CKD), dedicated by kidney function measures of estimated glomerular filtration rate (eGFR) and blood urea nitrogen (BUN). CP-related single-nucleotide polymorphism (SNP) and risk of eGFR: **(A1,A2)**; CP-related SNP and risk of BUN: **(B1,B2)**; eGFR-related SNP and risk of CP: **(C1,C2)**; BUN-related SNP and risk of CP: **(D1,D2)**. Forest plots **(A1,B1,C1,D1)** present the estimates with a horizontal line representing 95% confidence intervals (CIs) for the exposure-related SNP allele for outcome risk. Scatter plots **(A2,B2,C2,D2)** present the per-allele association with outcome risk plotted against the per-allele association with one standard deviation of exposure (with vertical and horizontal purple lines showing the 95% CI for each SNP). The slope of the navy solid line in the scatter plots corresponds to each Mendelian randomization (MR) estimate. *p*, *p*-value.

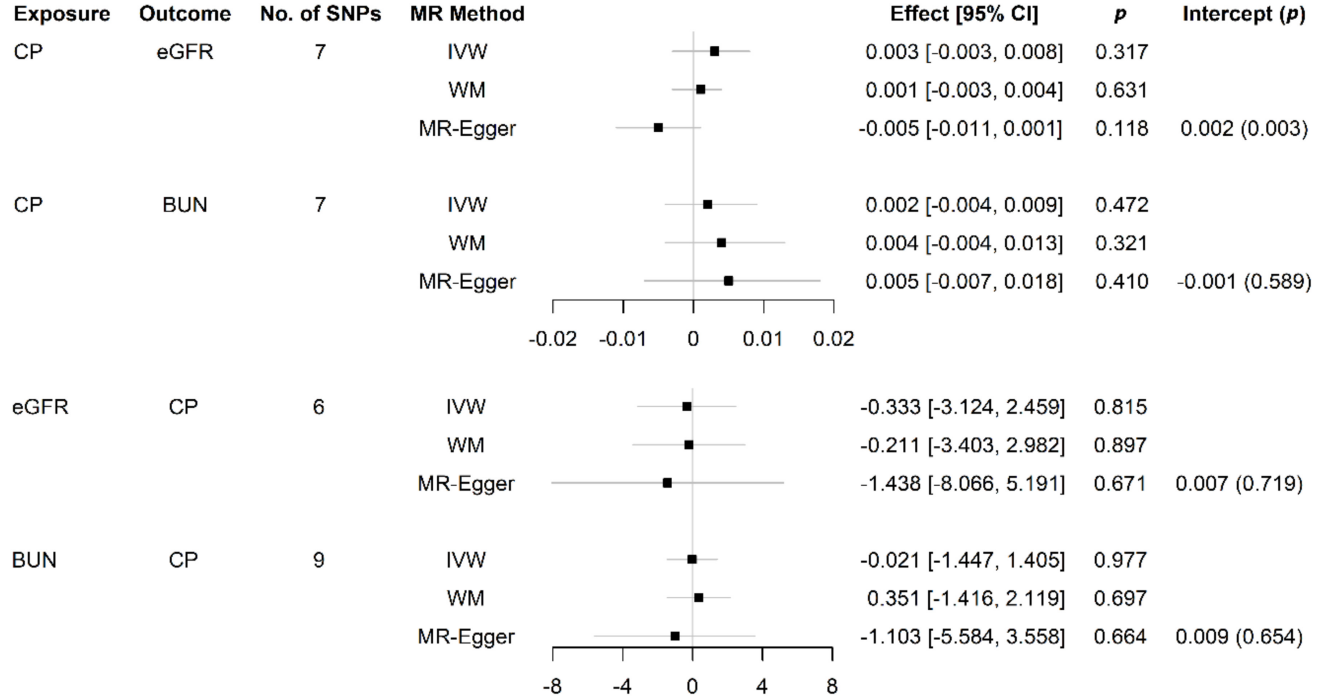

FIGURE 3 | Causal effects between CP and kidney function measures using different MR approaches. SNP: single-nucleotide polymorphism; No.: number; MR: Mendelian randomization; CI: confidence interval; *p*, *p*-value; CP: chronic periodontitis; eGFR: estimated glomerular filtration rate; BUN: blood urea nitrogen; IVW: inverse variance weighted; WM: weighted median; MR–Egger: the Mendelian randomization–Egger method.

(Chen et al., 2006; Kshirsagar et al., 2007; de Souza et al., 2014). In further population-based studies with cohort design, the onset of the exposure can be observed to happen before or after the outcome of interest, but the causal relationship between the traits is yet difficult to be assessed according to the affection by reverse causation or confounding effects (Grubbs et al., 2015, 2016). In the present MR study, we combined the summary statistics of CP and measures of kidney function from large-scale cohorts with European ancestry, to investigate the causal effect on these two traits. To the best of our knowledge, our study performed the first MR analysis on the causal effect between CP and CKD. Also, unlike previous studies based on smaller sample sizes, our two-sample MR study is sufficiently powered to assess a causal relationship between CP and CKD.

Our result is consistent with the conclusion from the newest systematic review and meta-analysis of observational studies on CP and CKD, including seven case–control studies, 38 cross-sectional studies, and two retrospective cohort studies (Zhao et al., 2018). Despite the lack of evidence to support CP as causal factors for CKD from our study, it does not hint that treatment for periodontitis in hemolysis patients with CKD or kidney transplantation patients with ESRD is unnecessary. Improved early treatment and dental care for the prevention of periodontitis could assist in the relief of the overall inflammatory status in the period of hemolysis treatment (Schmalz et al., 2016). Furthermore, for immunosuppressive therapy on patients with kidney transplantation, care of oral and periodontal condition is important for preventing complications and improvement of survival (Kitamura et al., 2019).

The current study has several strengths. First, this study investigated the largest GWAS datasets of CP included in the GLIDE Consortium, analyzing a total of 12,289 clinically diagnosed periodontitis cases and 22,326 controls, and of CKD included in the CKDGen Consortium (41,395 cases and 4,39,303 controls). The participants recruited in these two independent Consortiums are mostly of European descent, which minimizes the influence of population stratification (Burgess and CRP CHD Genetics Collaboration, 2013). Second, our two-sample design estimating the association between the genetic variant exposure and genetic variant outcome was from two independent comparable populations to gain a larger statistical power (Burgess et al., 2015). The bidirectional analysis guarantees the inference of causality between CP and CKD in both directions (Cao et al., 2019). Third, to control the pleiotropic effect from a certain single genetic variable, we used as much as multiple variants robustly associated with exposure variables as genetic instruments for assessing their effect on the outcome variables (Palmer et al., 2012).

Although our study used the newest data available, this study has some potential limitations. MR uses an average risk effect of genetic variants on a specific trait in participants' lifetime; in such case, it could not answer whether an exposure within a certain period of life has any effect on the risk of an outcome. We used the most recent GWAS of CP and CKD in the population of European ancestry to gain sufficient statistical power to test

the potential causal relation between CP and CKD; however, it might be limited to explore a tiny effect between these pairs of traits based on the weak instrument bias. The presented beta and standard error values for all instrumental variables show their effect size; all seven SNPs used as candidate genetic instruments for CP were weakly associated with CP, with a threshold of $p < 5 \times 10^{-6}$ instead of 5×10^{-8}. In addition, the functional mechanisms for most of these SNPs related to periodontitis remain unclear. The weak instruments tend to shift the MR estimate toward the null in two-sample MR (Davies et al., 2018), which may therefore result in the uncertain causality between CP and CKD in our study. Future high-quality GWAS are warranted to further examine the potential etiological role of CP in various diseases.

In conclusion, using CP-associated SNPs as genetic instruments retrieved from the GWAS results within large populations with European ancestry, our MR study does not find sufficient evidence to support a causal effect of CP as an exposure on the development of CKD as an outcome. Similarly, in the reverse inference, limited evidence was obtained in support of a causal role of CKD on CP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JY and TC contributed to the protocol development, data collection, and analysis. JY, TC, and YZ drafted the manuscript. MB and XL supervised the method and visualized the results. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This work was supported by the Beijing Jishuitan Hospital Research Grant (QN-201922).

ACKNOWLEDGMENTS

We thank the reviewers of this publication for their constructive comments. We gratefully acknowledge the GLIDE (Shungin et al., 2019) and the CKDGen (Wuttke et al., 2019) projects for providing available GWAS summarized data. All GLIDE and CKDGen authors appearing in the main author by-line are listed in Supplementary Material.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.676136/full#supplementary-material

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Conflict of Interest: YZ was employed by the company Beijing Lucidus Bioinformation Technologies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 12 July 2021
doi: 10.3389/fgene.2021.634394

A Novel Method for Mendelian Randomization Analyses With Pleiotropy and Linkage Disequilibrium in Genetic Variants From Individual Data

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Mendelian randomization makes use of genetic variants as instrumental variables to eliminate the influence induced by unknown confounders on causal estimation in epidemiology studies. However, with the soaring genetic variants identified in genome-wide association studies, the pleiotropy, and linkage disequilibrium in genetic variants are unavoidable and may produce severe bias in causal inference. In this study, by modeling the pleiotropic effect as a normally distributed random effect, we propose a novel mixed-effects regression model-based method PLDMR, pleiotropy and linkage disequilibrium adaptive Mendelian randomization, which takes linkage disequilibrium into account and also corrects for the pleiotropic effect in causal effect estimation and statistical inference. We conduct voluminous simulation studies to evaluate the performance of the proposed and existing methods. Simulation results illustrate the validity and advantage of the novel method, especially in the case of linkage disequilibrium and directional pleiotropic effects, compared with other methods. In addition, by applying this novel method to the data on Atherosclerosis Risk in Communications Study, we conclude that body mass index has a significant causal effect on and thus might be a potential risk factor of systolic blood pressure. The novel method is implemented in R and the corresponding R code is provided for free download.

Keywords: causal effect, individual data, linkage disequilibrium, Mendelian randomization, mixed-effects model, pleiotropy

OPEN ACCESS

Edited by:
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Soochow University, China

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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *27 November 2020*
Accepted: *15 June 2021*
Published: *12 July 2021*

Citation:
Wang Y, Li T, Fu L, Yang S and Hu Y-Q (2021) A Novel Method for Mendelian Randomization Analyses With Pleiotropy and Linkage Disequilibrium in Genetic Variants From Individual Data. Front. Genet. 12:634394. doi: 10.3389/fgene.2021.634394

1. INTRODUCTION

Conventional epidemiology has made enormous contributions to identifying certain significant exposures associated with common diseases, like fine particle air pollution was found to increase the risk of lung cancer mortality (Knowler et al., 2002; Pope et al., 2002). However, some epidemiological findings have later been revealed to be misleading by randomized controlled trials (RCTs) (Smith and Ebrahim, 2005). Furthermore, even if RCTs can correct the bias, despite the high cost of RCTs, the randomization of some potential confounders like nutrition and physical activity may be unfeasible (Smith and Ebrahim, 2003), thus some statistical methods were developed and employed to infer the causal relationship of interested exposures and diseases in epidemiology studies.

Mendelian randomization (MR) applies the method of instrumental variables (IVs) to estimate the causal effect of a non-genetic exposure on a disease outcome (Lawlor et al., 2008). MR exceeds conventional observational epidemiology in many aspects. Just as the role that IVs play in econometrics, setting genetic variants, e.g., single-nucleotide polymorphisms (SNPs), as instrumental variables, MR is capable of excluding the unknown confounders which often interfere with the conventional epidemiological analyses. What is more, not like RCTs spending large amounts of time and money in designing experiments and measuring physiological indexes, MR is practical and economical in the sense of using statistical methods. Methodological studies on MR in recent years have facilitated the reuse of results from genome-wide association studies (GWASs) (Burgess et al., 2013; Bowden et al., 2015, 2016). The GWAS is able to detect association between genetic variants and traits (Visscher et al., 2017). Immense results of GWASs are available through various online databases, such as Gene ATLAS and GWAS Catalog (Buniello et al., 2018; Canela-Xandri et al., 2018), from where we can get summary statistics like effects of SNPs on exposures and outcomes. To discover the causal relationships between exposure-outcome pairs, these statistics are necessary for MR methods. There are also some methods developed to infer causal relationships in individual-level data (Kang et al., 2016; Windmeijer et al., 2019), in addition to the general two-sample MR methods, which can be easily conducted and only require one-sample individual-level data.

Selecting a genetic variant as an IV, we must follow several critical assumptions (Angrist et al., 1996), among which the exclusion restriction assumption implies any effect of an IV on the outcome must be via an effect of the IV on the exposure (i.e., no pleiotropy; Angrist et al., 1996). However, it is possible that pleiotropy occurs in MR studies when taking multiple genetic variants as IVs, as numerous conclusions from GWASs have suggested (Soranzo et al., 2009; Lauc et al., 2013; Hu et al., 2018; Parker et al., 2019; Watanabe et al., 2019). To correct the bias in causal effect estimation produced by the latent pleiotropy of IVs, MR-Egger was proposed and widely employed in MR analyses, which viewed individual IV estimates as separate study results in meta-analysis and applied Egger's regression for interpreting pleiotropy in causal inference (Bowden et al., 2015; Yavorska and Burgess, 2017; Zhan and Fang, 2019). The latest version of the package MendelianRandomization (Yavorska and Burgess, 2017) allows MR-Egger to adjust for the bias brought by the linkage disequilibrium (LD) between genetic variants. However, MR-Egger (Bowden et al., 2015) only considers correcting the average pleiotropic effect, ignoring the potential variance of pleiotropic effects for invalid IVs, which may also influence causal inference. Thus, whether MR-Egger is able to handle LD and random pleiotropic effects simultaneously needs to be verified. LDA MR-Egger (Barfield et al., 2018) improves the performances of MR-Egger when LD exists between genetic variants but still has problems when the variance of pleiotropic effect is considerable. Other two-sample MR methods such as MR-LDP (Cheng et al., 2020) and RAPS (Zhao et al., 2020) are unable to correct the directional pleiotropic effect.

TABLE 1 | Causal inference of BMI on SBP and GLU, respectively, in analyzing ARIC dataset.

Method	SBP			GLU		
	β	Standard error	*p*-value	β	Standard error	*p*-value
MR-LDP	0.0080	0.0051	0.1162	0.0055	0.0036	0.1349
RAPS	0.0104	0.0036	0.0042	0.0053	0.0025	0.0344
MR-Egger	0.0149	0.0098	0.1301	−0.0001	0.0066	0.9826
LDA MR-Egger	0.0136	0.0110	0.2280	−0.0012	0.0091	0.8919
LDMR	0.0143	0.0091	0.1330	−0.0019	0.0078	0.8108
$PLDMR_a$	0.0163	0.0067	0.0244	−0.0007	0.0062	0.9146
PLDMR	0.0163	0.0067	0.0248	−0.0007	0.0062	0.9139

The threshold of p-value for selecting SNPs is 5×10^{-8}. The total number of SNPs is 21.

In this paper, we first introduce the mixed-effects regression model inherited from MR-Egger (Bowden et al., 2015) and briefly review MR-Egger method. Then we propose our novel method, pleiotropy and linkage disequilibrium adaptive Mendelian randomization (PLDMR), which models and corrects both the mean and variance of pleiotropic effects, as well as LD between genetic variants in causal effect estimation and statistical testing. We also derive two approximations of PLDMR, i.e., LDMR when the variance of pleiotropic effect is about zero and $PLDMR_a$ when the sample size is sufficiently large. We further compare the statistical properties of PLDMR against MR-Egger as well as several two-sample summary-level data methods developed in recent years, such as MR-LDP (Cheng et al., 2020), RAPS (Zhao et al., 2020), and LDA MR-Egger (Barfield et al., 2018), in terms of estimation and statistical testing in various simulation scenarios. Furthermore, we apply PLDMR, LDMR, and $PLDMR_a$ to the data of Atherosclerosis Risk in Communications Study (ARIC) and identify the significant causal effect of body mass index (BMI) on systolic blood pressure (SBP). We conclude that incorporating the variance of the pleiotropic effects and LD into MR analyses can efficiently estimate the causal effect and make more credible causal inference.

2. MATERIALS AND METHODS

2.1. Mendelian Randomization and Regression Models

Let us first recall the regression models used in MR-Egger (Bowden et al., 2015). For n individuals, let the matrix $\boldsymbol{G} = (G_{ij})_{n\times m}$ denote their centralized measurement of the m genetic variants, where G_{ij} is the genotype of individual i at the jth variant, $1 \leq i \leq n, 1 \leq j \leq m$. $\boldsymbol{X} = (X_1, X_2, ..., X_n)^T$ and $\boldsymbol{Y} = (Y_1, Y_2, ..., Y_n)^T$ are centralized measurements of the exposure and outcome of the n individuals, respectively. The exposure $\boldsymbol{X}$ is the linear combination of m genotypes and an error term $\boldsymbol{\varepsilon_X} = (\varepsilon_{X_1}, \varepsilon_{X_2}, ..., \varepsilon_{X_n})^T$, and the outcome $\boldsymbol{Y}$ is the linear combination of m genotypes, the exposure and an error term $\boldsymbol{\varepsilon_Y} = (\varepsilon_{Y_1}, \varepsilon_{Y_2}, ..., \varepsilon_{Y_n})^T$. To simplify the model, we reflect the influence of unknown confounders on $\boldsymbol{X}$ and $\boldsymbol{Y}$ in

the correlatedness of the error terms $\boldsymbol{\varepsilon_X}$ and $\boldsymbol{\varepsilon_Y}$. The causal effect of the exposure on the outcome is β in the model, which is of interest. The coefficients $\boldsymbol{\gamma} = (\gamma_1, \gamma_2, ..., \gamma_m)^T$ represent the effect of m genetic variants on the exposure, and $\boldsymbol{\alpha} = (\alpha_1, \alpha_2, ..., \alpha_m)^T$ is the pleiotropic effect of m genetic variants on the outcome. Specifically,

$$\boldsymbol{X} = \boldsymbol{G\gamma} + \boldsymbol{\varepsilon_X},$$

$$\boldsymbol{Y} = \boldsymbol{G\alpha} + \boldsymbol{X}\beta + \boldsymbol{\varepsilon_Y},$$

$$\begin{pmatrix} \boldsymbol{\varepsilon_X} \\ \boldsymbol{\varepsilon_Y} \end{pmatrix} \sim N\left(\begin{pmatrix} \boldsymbol{0} \\ \boldsymbol{0} \end{pmatrix}, \begin{pmatrix} \sigma_X^2 & \rho\sigma_X\sigma_Y \\ \rho\sigma_X\sigma_Y & \sigma_Y^2 \end{pmatrix} \otimes \boldsymbol{I_n} \right),$$

where $\sigma_X \in (0, \infty), \sigma_Y \in (0, \infty), \rho \in (-1, 1)$, $\boldsymbol{\alpha} \sim N(\mu_\alpha \boldsymbol{1}, \sigma_\alpha^2 \boldsymbol{I_m})$ is the random pleiotropic effect independent of $\boldsymbol{G}, \boldsymbol{\varepsilon_X}$, and $\boldsymbol{\varepsilon_Y}$ (Zhao et al., 2020), $\boldsymbol{I}$ is the identity matrix, $\otimes$ is the Kronecker product, and $\boldsymbol{1}$ is all 1's vector of length m. To take genetic variants as valid IVs in the conventional MR studies, the following assumptions should be satisfied (Angrist et al., 1996): (i) The genetic variants are randomly assigned, thus independent of unknown confounders; (ii) The genetic variants should be associated with the exposure; (iii) Any effect of the genetic variants on the outcome must be via an effect of that on the exposure. Equivalently speaking, (i) assumes $\boldsymbol{G}$ is independent of $\boldsymbol{\varepsilon_X}$ and $\boldsymbol{\varepsilon_Y}$; (ii) requires $\gamma_j \neq 0$ for each genetic variant j, which can be met by selecting genetic variants with methods like linear regression; (iii) implies no pleiotropic effect, i.e., $\boldsymbol{\alpha} = \boldsymbol{0}$. Our aim is to estimate the causal effect β and then make the statistical inference on it. To this end, we employ the mixed-effects model as described above to relax the requirement in (iii).

2.2. Revisit Egger Regression and MR-Egger

Let $\tilde{\Gamma}_j$ and $\tilde{\gamma}_j$ denote the coefficient estimates of the simple linear regression of the outcome $\boldsymbol{Y}$ and the exposure $\boldsymbol{X}$ on the genotype $\boldsymbol{G_{\cdot j}} = (G_{1j}, G_{2j}, ..., G_{nj})^T$ at variant j, respectively, and $SE(\tilde{\Gamma}_j)$ denote the standard error of $\tilde{\Gamma}_j, 1 \leq j \leq m$. An adaption of Egger regression was proposed (Bowden et al., 2015) as follows to estimate the causal effect,

$$\tilde{\boldsymbol{\Gamma}} = \beta_{0E}\boldsymbol{1} + \beta_E\tilde{\boldsymbol{\gamma}} + \boldsymbol{e}_{\tilde{\boldsymbol{\Gamma}}},\ \boldsymbol{e}_{\tilde{\boldsymbol{\Gamma}}} \sim N(\boldsymbol{0}, \sigma^2 \mathrm{diag}(SE^2(\tilde{\Gamma}_1), SE^2(\tilde{\Gamma}_2), ..., SE^2(\tilde{\Gamma}_m)),$$

where $\tilde{\boldsymbol{\Gamma}} = (\tilde{\Gamma}_1, \tilde{\Gamma}_2, ..., \tilde{\Gamma}_m)^T, \tilde{\boldsymbol{\gamma}} = (\tilde{\gamma}_1, \tilde{\gamma}_2, ..., \tilde{\gamma}_m)^T$.

Imposing the constraint of $\beta_{0E} = 0$ on the above regression model yields the inverse-variance weighted (IVW) estimate of the causal effect (Burgess et al., 2013), which is also commonly used in the meta-analysis. Notice that both MR-Egger and IVW are applicable to the summary data that are accessible in most GWASs.

2.3. PLDMR Adjusted for Pleiotropy and Linkage Disequilibrium

With the rapidly increasing number of genetic variants involved in Mendelian randomization studies, it is necessary to take the correlation among variants into account in estimating the causal effect of exposure on the outcome. Instead of the marginal regression of exposure/outcome on the genotype, multiple linear regression of $\boldsymbol{Y}$ on $\boldsymbol{G}$, and $\boldsymbol{X}$ on $\boldsymbol{G}$ are employed to derive the coefficient estimates $\hat{\boldsymbol{\Gamma}}$ and $\hat{\boldsymbol{\gamma}}$, respectively. To be precise, $\hat{\boldsymbol{\Gamma}} = (\boldsymbol{G}^T\boldsymbol{G})^{-1}\boldsymbol{G}^T\boldsymbol{Y}$ and $\hat{\boldsymbol{\gamma}} = (\boldsymbol{G}^T\boldsymbol{G})^{-1}\boldsymbol{G}^T\boldsymbol{X}$. Further, we have

$$\begin{aligned} \hat{\boldsymbol{\Gamma}} &= (\boldsymbol{G}^T\boldsymbol{G})^{-1}\boldsymbol{G}^T(\boldsymbol{G\alpha} + \boldsymbol{X}\beta + \boldsymbol{\varepsilon_Y}) \\ &= \boldsymbol{\alpha} + \beta\hat{\boldsymbol{\gamma}} + (\boldsymbol{G}^T\boldsymbol{G})^{-1}\boldsymbol{G}^T\boldsymbol{\varepsilon_Y} \\ &= \mu_\alpha\boldsymbol{1} + \beta\hat{\boldsymbol{\gamma}} + (\boldsymbol{\alpha} - \mu_\alpha\boldsymbol{1}) + (\boldsymbol{G}^T\boldsymbol{G})^{-1}\boldsymbol{G}^T\boldsymbol{\varepsilon_Y}. \end{aligned}$$

Based on the independence of $\boldsymbol{\alpha}$ and $\boldsymbol{\varepsilon_Y}$ and also their normality, we have the following regression model

$$\hat{\boldsymbol{\Gamma}} = \mu_\alpha\boldsymbol{1} + \beta\hat{\boldsymbol{\gamma}} + \boldsymbol{\varepsilon}_{\hat{\boldsymbol{\Gamma}}},\ \boldsymbol{\varepsilon}_{\hat{\boldsymbol{\Gamma}}} \sim N(0, \boldsymbol{W}^{-1}),$$

where $\boldsymbol{W} = [\sigma_\alpha^2\boldsymbol{I_m} + \sigma_Y^2(\boldsymbol{G}^T\boldsymbol{G})^{-1}]^{-1}$. The corresponding likelihood function is

$$\begin{aligned} &L(\mu_\alpha, \beta, \sigma_\alpha^2, \sigma_Y^2; \hat{\boldsymbol{\Gamma}}, \hat{\boldsymbol{\gamma}}) \\ &= (2\pi)^{-\frac{m}{2}}|\boldsymbol{W}|^{\frac{1}{2}}\exp\left[-\frac{1}{2}(\hat{\boldsymbol{\Gamma}} - \mu_\alpha\boldsymbol{1} - \beta\hat{\boldsymbol{\gamma}})^T\boldsymbol{W}(\hat{\boldsymbol{\Gamma}} - \mu_\alpha\boldsymbol{1} - \beta\hat{\boldsymbol{\gamma}})\right]. \end{aligned}$$

Notice that both unknown parameters σ_α^2 and σ_Y^2 are involved in $\boldsymbol{W}$, which renders difficulty in the calculation of the maximum likelihood estimates (MLEs). For the positive definite matrix $(\boldsymbol{G}^T\boldsymbol{G})^{-1}$, there exists an $m \times m$ orthogonal matrix $\boldsymbol{Q}$ and an $m \times m$ diagonal matrix $\boldsymbol{\Lambda}$ such that $(\boldsymbol{G}^T\boldsymbol{G})^{-1} = \boldsymbol{Q\Lambda Q}^T$. Let $r^2 = \sigma_\alpha^2/\sigma_Y^2$, we then express $\boldsymbol{W}^{-1}$ as $\sigma_Y^2(r^2\boldsymbol{I_m} + \boldsymbol{Q\Lambda Q}^T)$ and further diagonalize $\boldsymbol{Q}^T\boldsymbol{W}^{-1}\boldsymbol{Q}$ as $\sigma_Y^2(r^2\boldsymbol{I_m} + \boldsymbol{\Lambda})$. So the likelihood function is transformed to

$$\begin{aligned} &L(\mu_\alpha, \beta, r^2, \sigma_Y^2; \hat{\boldsymbol{\Gamma}}, \hat{\boldsymbol{\gamma}}) = (2\pi\sigma_Y^2)^{-\frac{m}{2}}|r^2\boldsymbol{I_m} + \boldsymbol{\Lambda}|^{-\frac{1}{2}} \cdot \\ &\exp\left[-\frac{1}{2\sigma_Y^2}(\boldsymbol{Q}^T\hat{\boldsymbol{\Gamma}} - \mu_\alpha\boldsymbol{Q}^T\boldsymbol{1} - \beta\boldsymbol{Q}^T\hat{\boldsymbol{\gamma}})^T(r^2\boldsymbol{I_m} + \boldsymbol{\Lambda})^{-1}(\boldsymbol{Q}^T\hat{\boldsymbol{\Gamma}}\right. \\ &\left.-\mu_\alpha\boldsymbol{Q}^T\boldsymbol{1} - \beta\boldsymbol{Q}^T\hat{\boldsymbol{\gamma}})\right]. \end{aligned}$$

We call the R package BB (Varadhan and Gilbert, 2009) implementing the spectral projected gradient algorithm (Varadhan and Roland, 2008) to get the MLEs $\hat{\mu}_\alpha, \hat{\beta}$, and $\hat{r}^2$. As

$$\begin{aligned} \hat{\beta} &= \frac{\hat{\boldsymbol{\gamma}}^T\boldsymbol{W}^{\frac{1}{2}}\left(\boldsymbol{I_m} - \boldsymbol{P}_{\boldsymbol{W}^{\frac{1}{2}}\boldsymbol{1}}\right)\boldsymbol{W}^{\frac{1}{2}}\hat{\boldsymbol{\Gamma}}}{\hat{\boldsymbol{\gamma}}^T\boldsymbol{W}^{\frac{1}{2}}\left(\boldsymbol{I_m} - \boldsymbol{P}_{\boldsymbol{W}^{\frac{1}{2}}\boldsymbol{1}}\right)\boldsymbol{W}^{\frac{1}{2}}\hat{\boldsymbol{\gamma}}} \\ &\sim N\left(\beta, \frac{1}{\hat{\boldsymbol{\gamma}}^T\boldsymbol{W}^{\frac{1}{2}}(\boldsymbol{I_m} - \boldsymbol{P}_{\boldsymbol{W}^{\frac{1}{2}}\boldsymbol{1}})\boldsymbol{W}^{\frac{1}{2}}\hat{\boldsymbol{\gamma}}}\right), \end{aligned}$$

where $\boldsymbol{P}_{\boldsymbol{W}^{\frac{1}{2}}\boldsymbol{1}} = \frac{\boldsymbol{W}^{\frac{1}{2}}\boldsymbol{1}\boldsymbol{1}^T\boldsymbol{W}^{\frac{1}{2}}}{\boldsymbol{1}^T\boldsymbol{W}\boldsymbol{1}}$ is the orthogonal projection onto $\boldsymbol{W}^{\frac{1}{2}}\boldsymbol{1}$. The plug-in method is invoked to get $\widehat{Var}(\hat{\beta})$, the

estimate of the variance of $\hat{\beta}$. Based on these estimates, we have approximately

$$\frac{\hat{\beta}-\beta}{\sqrt{\widehat{Var}(\hat{\beta})}} \sim t(m-2),$$

which can easily yield the confidence interval of the causal effect β or the p-value in testing the statistical hypothesis $H_0 : \beta = \beta_0$, where $t(m-2)$ represents the t-distribution with $m-2$ degrees of freedom. We use PLDMR for the statistical inference of the causal effect in the presence of pleiotropy and multiple SNPs in LD in Mendelian randomization analyses.

Considering the sample size n is usually an order of tens of thousands, we have $\boldsymbol{G}^T\boldsymbol{G} = O(n)$ and further $\boldsymbol{W} \approx \sigma_\alpha^{-2}\boldsymbol{I_m}$. As an approximation in the situation of big n, the estimate of the causal effect β and its variance are easily derived from classical simple linear regression. We denote this approximation as PLDMR$_a$. The accuracy of this approximation is illustrated in the simulation study for varied sample sizes from several hundreds to several tens of thousands and varied σ_α^2.

Another special case of our interest is $\sigma_\alpha^2 = 0$, i.e., $\boldsymbol{\alpha} = \mu_\alpha \mathbf{1}$, or $\sigma_\alpha^2 \approx 0$. We have $\boldsymbol{W} \approx \sigma_Y^{-2}(\boldsymbol{G}^T\boldsymbol{G})$ and then the estimates of the causal effect and its variance can be derived approximately from the following simple linear regression

$$\hat{\boldsymbol{\Gamma}} = \mu_\alpha \mathbf{1} + \beta\hat{\boldsymbol{\gamma}} + \boldsymbol{\varepsilon}_{\hat{\boldsymbol{\Gamma}}}, \boldsymbol{\varepsilon}_{\hat{\boldsymbol{\Gamma}}} \sim N(0, \sigma_Y^2(\boldsymbol{G}^T\boldsymbol{G})^{-1}).$$

So regressing $\hat{\boldsymbol{\Gamma}}$ on $\hat{\boldsymbol{\gamma}}$ yields

$$\hat{\beta}_{LDMR} = \frac{\hat{\boldsymbol{\gamma}}^T\left(\boldsymbol{G}^T\boldsymbol{G} - \frac{\boldsymbol{G}^T\boldsymbol{G}\boldsymbol{J}\boldsymbol{G}^T\boldsymbol{G}}{\mathbf{1}^T\boldsymbol{G}^T\boldsymbol{G}\mathbf{1}}\right)\hat{\boldsymbol{\Gamma}}}{\hat{\boldsymbol{\gamma}}^T\left(\boldsymbol{G}^T\boldsymbol{G} - \frac{\boldsymbol{G}^T\boldsymbol{G}\boldsymbol{J}\boldsymbol{G}^T\boldsymbol{G}}{\mathbf{1}^T\boldsymbol{G}^T\boldsymbol{G}\mathbf{1}}\right)\hat{\boldsymbol{\gamma}}},$$

where $\boldsymbol{J}$ is all 1's $m \times m$ matrix, and further

$$\hat{\beta}_{LDMR} \sim N\left(\beta, \frac{1}{\hat{\boldsymbol{\gamma}}^T(\boldsymbol{G}^T\boldsymbol{G})^{\frac{1}{2}}(\boldsymbol{I_m} - \boldsymbol{P}_{(\boldsymbol{G}^T\boldsymbol{G})^{\frac{1}{2}}\mathbf{1}})(\boldsymbol{G}^T\boldsymbol{G})^{\frac{1}{2}}\hat{\boldsymbol{\gamma}}}\right).$$

Again, we can use this normality to construct the confidence interval of β or test the statistical hypothesis of β when the variance of pleiotropic effect is about zero. We refer to this method as LDMR. In contrast to PLDMR, the estimators of LDMR and PLDMR$_a$ have closed forms and thus have no computational burden.

2.4. The Design of Simulation Studies

To evaluate the proposed methods, a series of scenarios of different parameter settings are designed to conduct the simulation studies. We explore and compare the estimation results and statistical properties of MR-LDP, RAPS, MR-Egger, and LDA MR-Egger with PLDMR in nine combinations of three patterns of pleiotropy (balanced, negative and positive) and three magnitudes of linkage disequilibrium (no, low, and high). We also vary n, the sample size, and σ_α^2, the variance of pleiotropic effect, to illustrate whether PLDMR can be approximated by LDMR and PLDMR$_a$ in the two situations, i.e., $\sigma_\alpha^2 \approx 0$ and large n, respectively. Additionally, we generate genotype data in LD for every individual i as the steps listed below:

(i) Construct a Toeplitz $m \times m$ matrix $\boldsymbol{\Sigma_g}$, i.e., the (j_1, j_2) cell element is $\rho_g^{|j_1-j_2|}, 1 \le j_1, j_2 \le m$;
(ii) Randomly draw $\boldsymbol{z_i} = (z_{i1}, z_{i2}, ..., z_{im})^T$ from $MVN(0, \boldsymbol{\Sigma_g})$ and calculate $\Phi(z_{ij})$, where Φ denotes the cumulative distribution function of $N(0,1)$, $1 \le i \le n$, $1 \le j \le m$;
(iii) For the given minor allele frequency MAF$_j$ at the jth locus, assign genotype G_{ij} as the $\Phi(z_{ij})$ quantile of Binomial$(2, \text{MAF}_j)$, $1 \le j \le m$.

The Toeplitz matrix used in (i) is able to weaken the correlation of genotypes at two loci j_1, j_2 with respect to their "relative distance" $|j_1 - j_2|$. Also, we can control the relative strength of linkage disequilibrium by tuning the magnitude of ρ_g.

3. RESULTS

3.1. Comparison of Statistical Properties Between PLDMR and Existing Methods

All of the methods are implemented using R software (version 3.6.0). To evaluate the proposed methods comprehensively, we choose MAF$_j \sim$ Uniform$([0.2, 0.4])$, $\gamma_j \sim$ Uniform$([0.5, 4])$, $1 \le j \le m$, and fix $\sigma_X = \sigma_Y = 2$, $\rho = 0.5$. We further set $\rho_g = 0, 0.3,$ and 0.6 to represent no LD, low LD, and high LD; $\mu_\alpha = 0, +0.1,$ and -0.1 to represent balanced pleiotropy, positive pleiotropy, and negative pleiotropy; $\sigma_\alpha = 0.01, 0.1$ and 0.2 to represent different strengths of the pleiotropic effect; $n =$ 1,000, 5,000, 10,000, and 20,000 to represent a wide range of sample sizes. The nominal significance level is 0.05 and the replications are 10,000 for each scenario. For brevity, results of the simulation study with $\sigma_\alpha = 0.1$ and $m = 25$ are shown in **Figures 1**, **2**, and the remainders are shown in **Supplementary Material**.

Four existing two-sample summary-level data methods, i.e., MR-LDP (Cheng et al., 2020), RAPS (Zhao et al., 2020), MR-Egger (Bowden et al., 2015), and LDA MR-Egger (Barfield et al., 2018), are also included in the comparison. Summary level data is obtained by splitting the one-sample individual data into two halves and then conducting simple linear regression in each part. The reference LD correlation matrix needed for these methods is generated from the genotypes of an additional independent 5,000 individuals. We use the R packages MendelianRandomization (version 0.4.2) (Yavorska and Burgess, 2017), MR.LDP (version 1.0), mr.raps (version 0.3.1) to implement the above methods. The code of LDA MR-Egger (Barfield et al., 2018) is downloaded from the github homepage of the author. In addition to PLDMR, LDMR, and PLDMR$_a$, we also add PLDMR$_t$, which represents the PLDMR method evaluated at the true values of σ_α^2 and σ_Y^2 instead of the estimated ones in weighted linear regression.

Now let us look at the performances of the eight methods mentioned above in terms of estimation and testing when the true value of β is 0. As is shown in **Figure 1**, MR-LDP controls

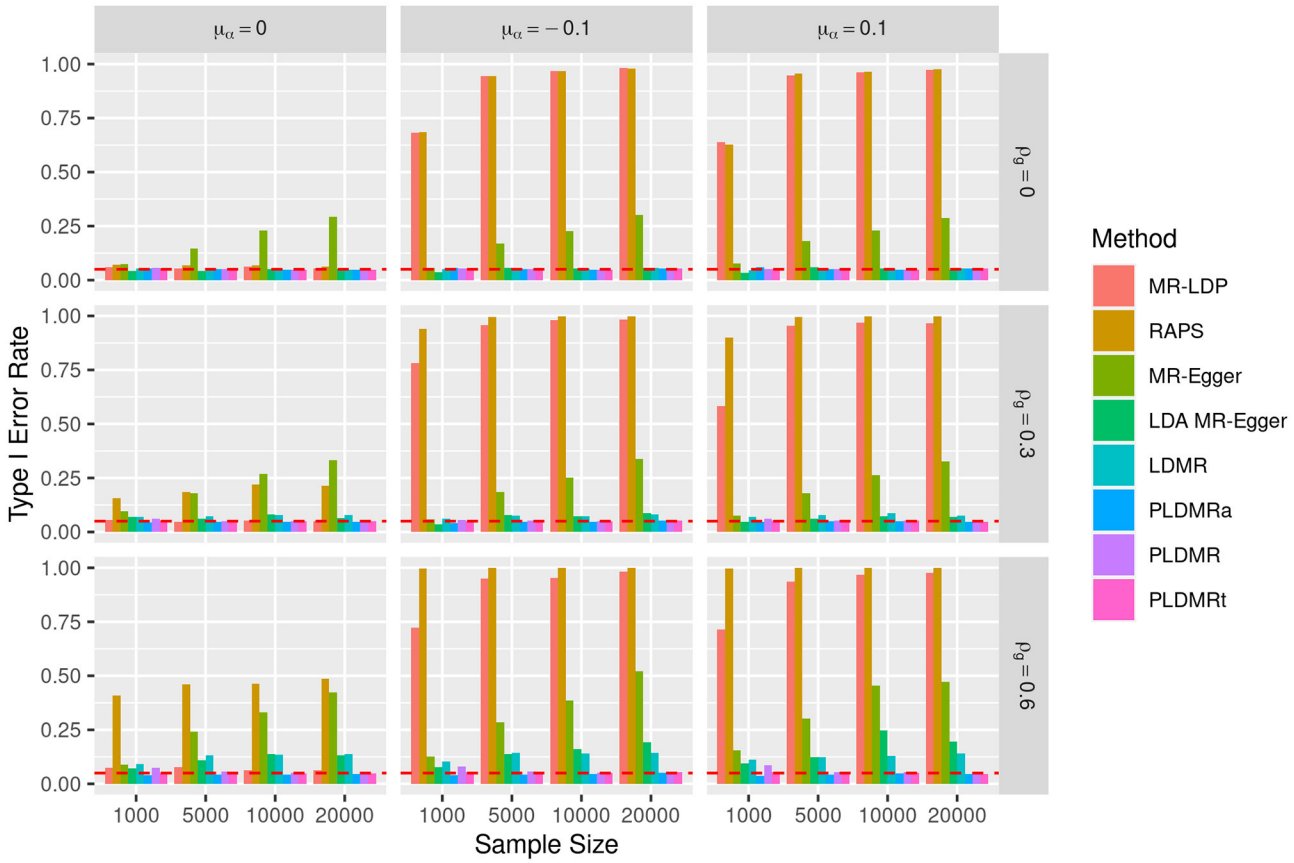

FIGURE 1 | Bar plot of the type I error rates of all methods under the null hypothesis of $H_0 : \beta = 0$. Sample size (n = 1,000, 5,000, 10,000, 20,000), the number of genetic variants $m = 25$, and $\sigma_\alpha = 0.1$. $\mu_\alpha = 0, -0.1, 0.1$ represents the mean of pleiotropic effect and $\rho_g = 0, 0.3, 0.6$ stands for the relative strength of LD between the genetic variants. The red dashed horizontal line indicates the nominal significance of 0.05.

type I error rate well in the scenarios of balanced pleiotropy (left panel), but fails in the scenarios of directional pleiotropy (mid and right panels). RAPS fails to control type I error rate when LD or directional pleiotropy exists and only controls the type I error rate in the top-left figure. The type I error rate of MR-Egger method inflates as the sample size increases in each scenario. The type I error rates of LDA MR-Egger and LDMR behave similarly in each scenario, albeit there exists some inflation in the scenarios of high LD (bottom panel). No obvious inflation can be observed from the type I error rates of PLDMR, PLDMR_a and PLDMR_t, although PLDMR_a shows some conservativeness in the scenarios of high LD. **Supplementary Figure 1** shows the estimation performance of all methods. MR-LDP and RAPS are biased in the scenarios of directional pleiotropy (mid and right panels). MR-Egger and LDA MR-Egger behave similarly in each scenario, as they are both severely biased in the scenarios of directional pleiotropy and high LD (bottom-mid and bottom-right figures). LDMR, PLDMR, and PLDMR_a are unbiased in each scenario. However, the standard errors of MR-LDP and RAPS are apparently smaller than those of other methods in the scenarios of balanced pleiotropy (left panel).

Figure 2 depicts the power of detecting the causal effect when $\beta = 0.05$. The powers of MR-LDP are higher than LDMR, PLDMR, PLDMR_a, and PLDMR_t in the scenarios of balanced pleiotropy (left panel), but are invalid in the scenarios of directional pleiotropy due to its failure in controlling type I error rates. RAPS is the most powerful method for detecting the causal effect in the scenario of balanced pleiotropy and no LD (top-left figure), but is doubtful in other cases. MR-Egger can control type I error rates only when sample size is small and the correlation between SNPs is low ($\rho_g = 0, 0.3$), in which cases its power is lower than LDMR, PLDMR, PLDMR_a, and PLDMR_t. LDA MR-Egger performs better than LDMR, PLDMR, PLDMR_a, and PLDMR_t when sample size is large and the correlation between SNPs is low. **Supplementary Figure 2** shows the performances of estimations when $\beta = 0.05$, which exhibits a similar pattern as when $\beta = 0$.

Figures 3, **4** show the performances of the eight methods using different numbers of IVs. We fix sample size n at 5,000 in this simulation and the variance of the pleiotropic effect is $\sigma_\alpha = 0.01$. MR-LDP still fails to control the type I error rate in the scenarios of directional pleiotropy and RAPS is unable to control type I error rate either when LD exists or directional pleiotropy

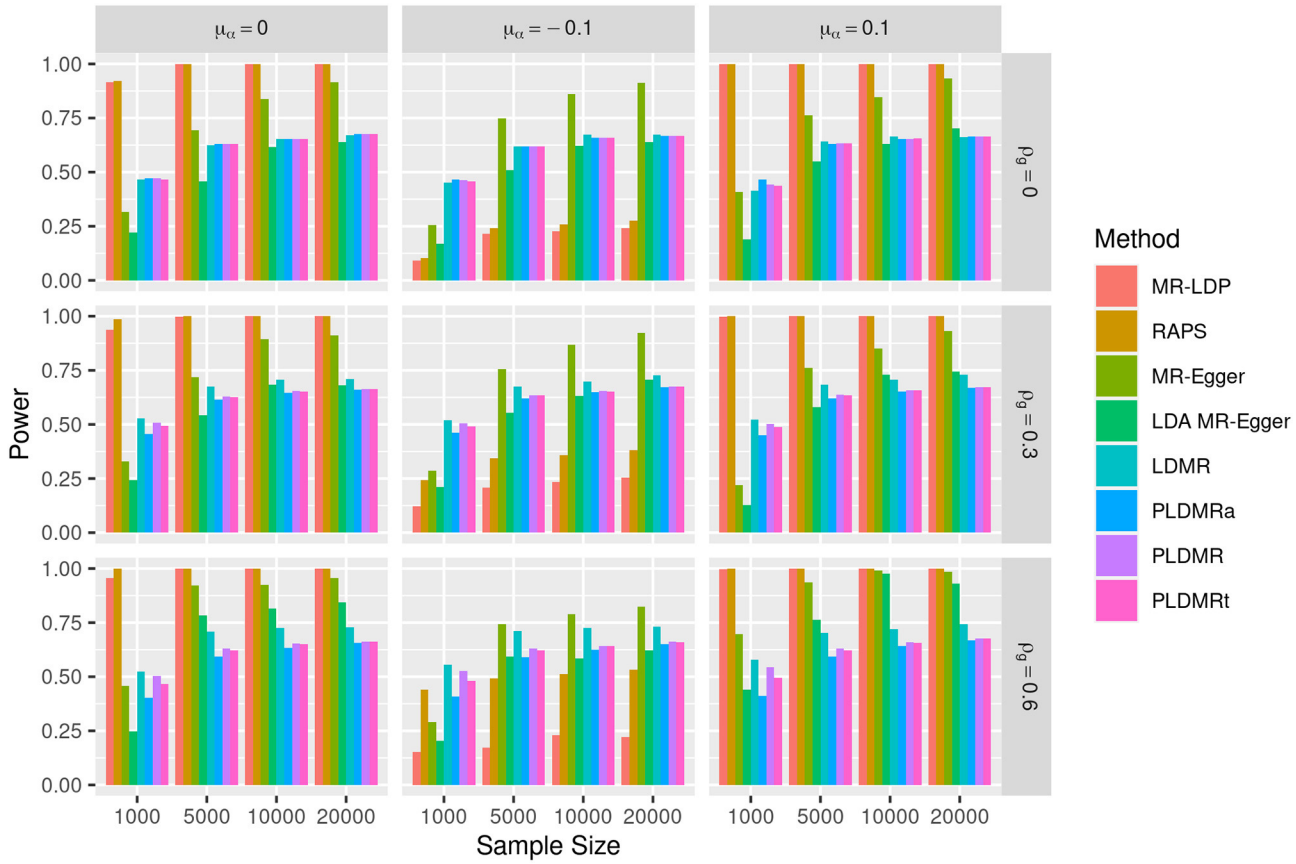

FIGURE 2 | Bar plot of the powers of all methods under the alternative hypothesis of $H_1 : \beta = 0.05$. Sample size n = 1,000, 5,000, 10,000, 20,000, the number of genetic variants $m = 25$, and $\sigma_\alpha = 0.1$. $\mu_\alpha = 0, -0.1, 0.1$ represents the mean of pleiotropic effect and $\rho_g = 0, 0.3, 0.6$ stands for the relative strength of LD between the genetic variants.

exists. It can be obviously observed that the type I error rates of MR-Egger inflates when directional pleiotropic effect exists, whereas LDA MR-Egger fails to control type I error rate in the scenarios of directional pleiotropic effect and strong LD. LDMR, PLDMR, PLDMR_a, and PLDMR_t control type I error rates at 0.05 and show no noticeable changes as m increases. In **Figure 4**, the standard errors of all methods decrease with respect to the number of IVs m, except for MR-LDP and RAPS in the scenarios of directional pleiotropic effect.

In addition, we compare the type I error rates of all methods under different settings of σ_α in **Supplementary Figures 3, 5**. In **Supplementary Figure 3** with $\sigma_\alpha = 0.01$, MR-Egger can control type I error rate at 0.05 in situations of balanced pleiotropy and no LD but still fails in situations of directional pleiotropy, low and high LD groups. LDMR, PLDMR, and PLDMR_t control type I error rates well at around 0.05, whereas PLDMR_a obviously is conservative, especially in high LD situations. Except for the conservativeness showed by MR-LDP when sample size is small in the scenarios of balanced pleiotropy, the behaviors of MR-LDP and RAPS are almost the same as those when $\sigma_\alpha = 0.1$. When $\sigma_\alpha = 0.2$, the conclusion is similar to that when $\sigma_\alpha = 0.1$ (**Supplementary Figure 5**). Furthermore, the powers of all methods when $\sigma_\alpha = 0.01$ and 0.2 are also shown in **Supplementary Figures 7, 9**, from where we can conclude that the increasing magnitude of the powers of LDMR, PLDMR, and PLDMR_a with respect to sample size under large σ_α is much slower than that with smaller σ_α. The behaviors of estimations of all methods are shown by **Supplementary Figures 4, 6, 8, 10**.

3.2. Briefing and Preprocessing of ARIC Data

Nowadays obesity has become a key issue of global concern (Xu and Lam, 2018). In studying obesity, we usually use BMI to define overweight and obesity. So it is an important factor to use BMI in the relevant research. In order to investigate the causal effect of BMI on SBP and glucose (GLU), we use data on 15,792 individuals from ARIC study. The ARIC study is one of the largest multi-ethnic sampling frame studies in the United States. Nearly 70% of the participants were European Americans, and the rest were African Americans. ARIC includes 909,622 SNPs and more than 450 phenotypes.

Regarding BMI as an exposure and choosing SNPs significantly associated with BMI (p-value $< 5 \times 10^{-8}$) with reference to GWAS Catalog database (We also choose

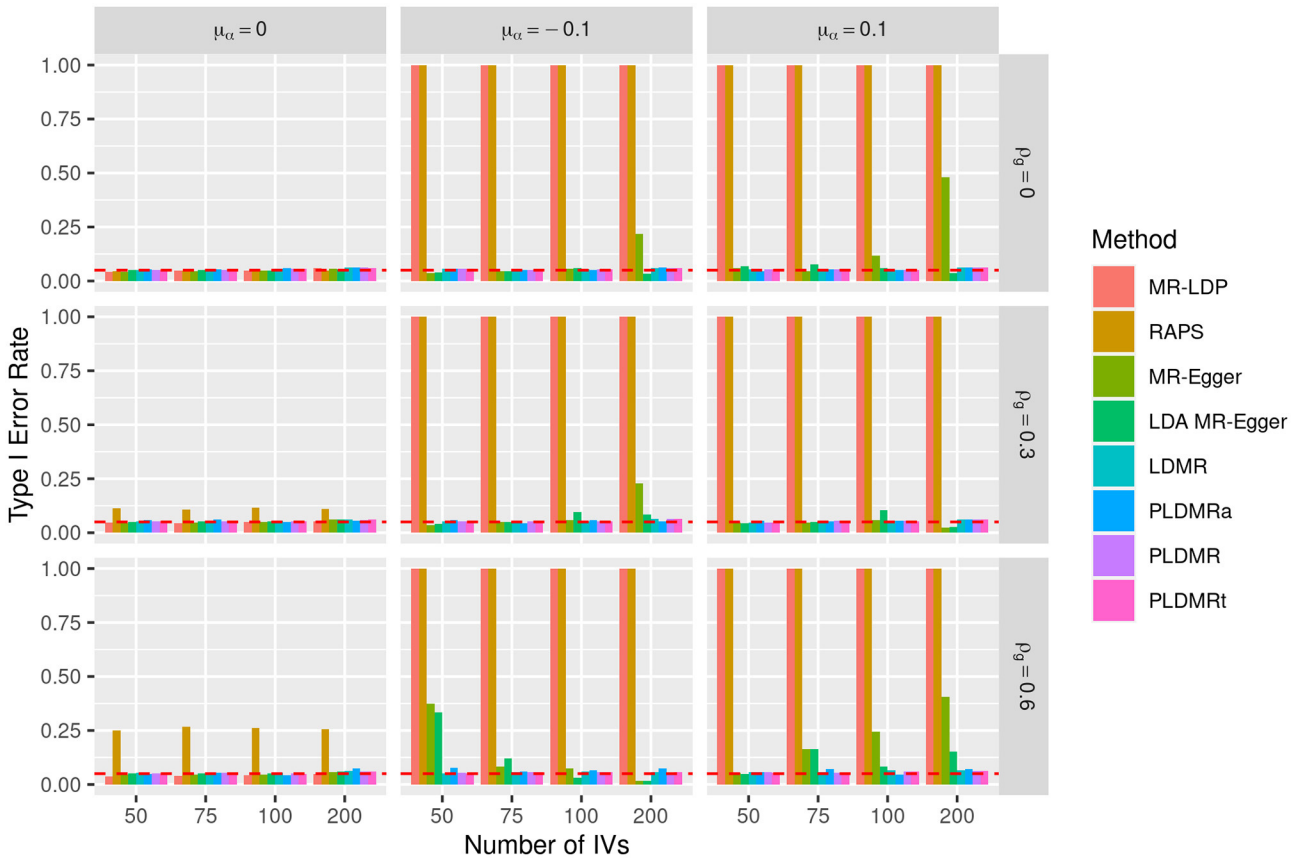

FIGURE 3 | Bar plot of the type I error rates of all methods under the null hypothesis of $H_0 : \beta = 0$. Sample size is $n = 5{,}000$, $\sigma_\alpha = 0.01$, and the number of genetic variants $m = 50, 75, 100, 200$. $\mu_\alpha = 0, -0.1, 0.1$ represents the mean of pleiotropic effect and $\rho_g = 0, 0.3, 0.6$ stands for the relative strength of LD between the genetic variants. The red dashed horizontal line indicates the nominal significance of 0.05.

$p < 1 \times 10^{-4}$ as another threshold and the corresponding results are shown in **Supplementary Table 2**), we identify 616 significant SNPs as instrumental variables from ARIC dataset for MR analysis. We only consider individuals of white origin in the following analysis for avoiding the population stratification problem. After model checking, BMI follows normal distribution and it is necessary to log-transform SBP and GLU. We adjust for covariates including sex, age, and age^2 by regressing BMI, SBP, GLU on these covariates, respectively, and then use the corresponding regression residuals as the adjusted BMI, adjusted SBP and adjusted GLU for the subsequent analysis. After pruning out SNPs with missing value proportion >20% and testing for Hardy-Weinberg equilibrium of the candidate SNPs, multiple linear regression is employed to select genetic variants positively associated with the exposure BMI. Finally, 21 SNPs (see details in **Supplementary Table 1**) and 6,782 individuals are included in this study after the preliminary processing of data.

3.3. Causal Inference of BMI on SBP

The results of $\hat{\boldsymbol{\Gamma}}$ and $\hat{\boldsymbol{\gamma}}$ of 21 SNPs are depicted in **Figure 5**, and the estimated causal effects, standard errors, and p-values are listed in **Table 1**. The estimate of r^2 is about 0.015, which implies that pleiotropy may exist for those 21 SNPs. The point estimate of the causal effect is 0.0162 with the standard error 0.00677. The result of PLDMR$_a$ is similar to that of PLDMR, with estimator 0.0163 with standard error 0.00666 for causal effect of BMI on SBP, while the MR-Egger and LDMR methods give point estimates of 0.0149 (with standard error 0.00985) and 0.0143 (with standard error 0.00911) for causal effect, respectively. Most importantly, PLDMR$_a$ and PLDMR imply a significant causal effect of BMI on SBP with p-values 0.0244 and 0.0272, while MR-Egger and LDMR show no significance in the causal relationship of BMI and SBP (p-value = 0.130 and 0.133, respectively). In addition, we conduct MR-LDP, LDA MR-Egger and RAPS methods by randomly selecting 1,000 individuals from the whole dataset to estimate reference LD correlation matrix and splitting the remained 5,782 individuals into two halves to obtain summary statistics. The estimates of the causal effect given by MR-LDP and LDA MR-Egger are 0.00802 and 0.0136, respectively (with standard errors 0.00510 and 0.0109, respectively), which show no significance in the causal relationship between BMI and SBP (p-value = 0.116 and

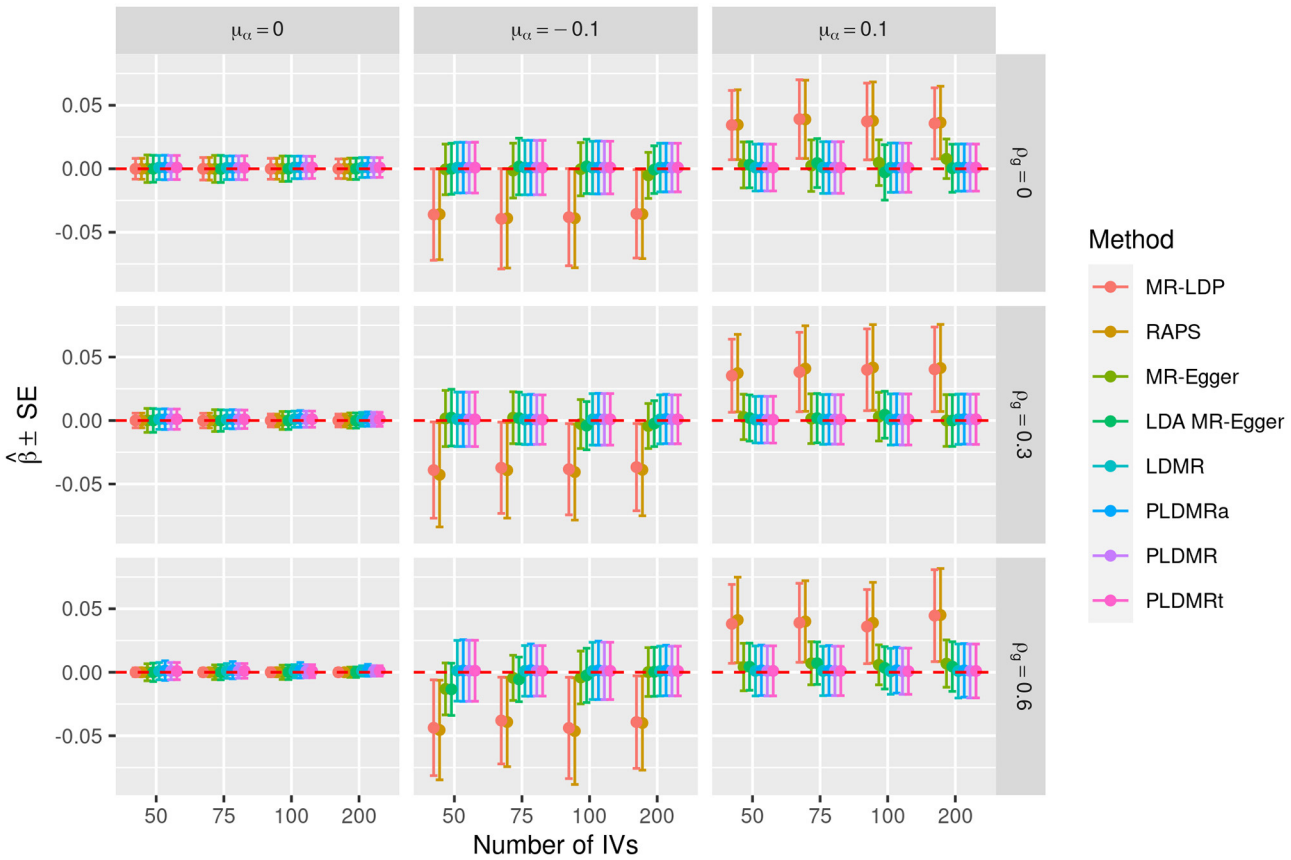

FIGURE 4 | Plot of the performances of all eight estimating methods when $\beta = 0$. Sample size is $n = 5,000$, $\sigma_\alpha = 0.01$, and the number of genetic variants $m = 50, 75, 100, 200$. $\mu_\alpha = 0, -0.1, 0.1$ represents the mean of pleiotropic effect and $\rho_g = 0, 0.3, 0.6$ stands for the relative strength of LD between the genetic variants. The solid circles are the mean values of estimators, the upper and lower bars are the means plus and minus one standard error in 10,000 replications. The red dashed line indicates the true value of β.

0.228, respectively). RAPS estimates the causal effect as 0.0104 (with standard error 0.00362) and implies a significant causal relationship between BMI and SBP (p-value = 0.00419).

Existing studies have already shown that there is a relationship between BMI and blood pressure or hypertension (Feng et al., 2012; Shihab et al., 2012; Hall et al., 2015). Recently, a population-based cohort study from UK Biobank including 120,000 individuals identified the association between BMI and hypertension, SBP and DBP, where Mendelian randomization was used to show significant positive association between BMI and SBP with p-value 2×10^{-4} (Lyall et al., 2017). In addition, a MR analysis is conducted by studying a total of 19,502 people from 36 study populations of European descents, confirming that BMI has causal relationship with SBP with p-value 6.7×10^{-76} (Fall et al., 2013). These results all support the conclusion inferred from our method. So when pleiotropy exists and can not be ignored, our method PLDMR is recommended.

3.4. Causal Inference of BMI on GLU

We also investigate the relationship between BMI and GLU (**Supplementary Figure 11**). The estimate of r^2 is 0.000406, which means the pleiotropic effect is relatively small. Only RAPS shows a significant causal association between BMI and GLU ($\hat{\beta}$ = 0.00527 with standard error 0.00249 and p-value 0.0344). A large-scale MR study investigating a European population (34,538 people) concluded no significant association of BMI with glucose deterioration with p-value 0.787 (Wang et al., 2018). No statistical significance between BMI and fasting glucose was reported in another study (Xu et al., 2020) (p-value 0.546). The results of PLDMR are consistent with the findings in the literature.

4. DISCUSSION

4.1. Relation Between PLDMR and Other Existing Methods

Many methods have been proposed to detect the invalid instrumental variables involved in Mendelian randomization analysis and then to correct the estimate of causal effect. For example, the Q test employs Cochran's Q statistic, which follows χ^2 distribution with one degree of freedom, to detect outliers and exclude them out in further analysis of the summary level data (Bowden et al., 2018). They also proposed a scale

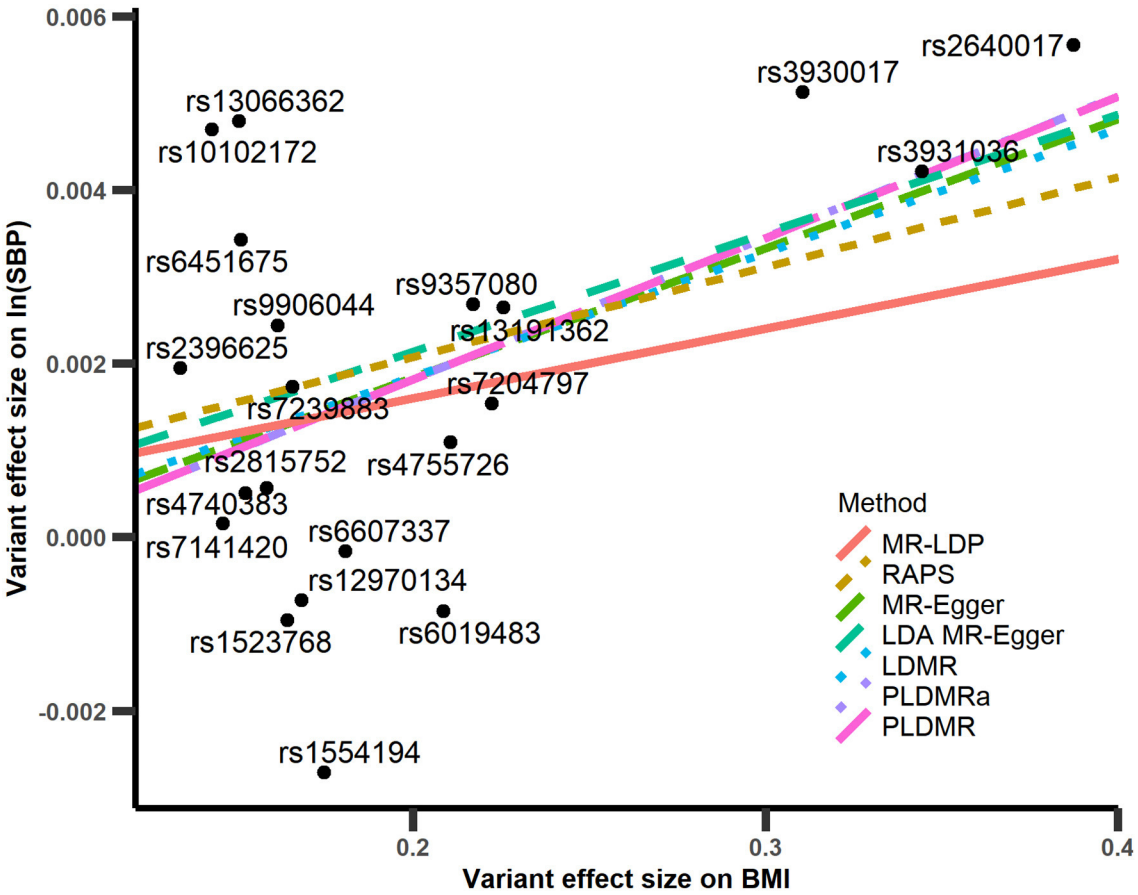

FIGURE 5 | Scatter plot of $\hat{\Gamma}$ with respect to $\hat{\gamma}$ in the analyses of BMI-SBP. The red line is the regression line of MR-LDP method, the brown dashed line is the regression line of RAPS, the yellow-green dashed line is the regression line of MR-Egger method, the green dashed line is the regression line of LDA MR-Egger method, the blue short dashed line is the regression line of LDMR method, the purple short dashed line is the regression line of PLDMR$_a$, and the magenta long dashed line is the regression line of PLDMR.

factor ϕ, which is associated with the squared ratio $r^2 = \sigma_\alpha^2/\sigma_Y^2$, to quantify the degree of heterogeneity in the *Q*-test (Bowden et al., 2018). Similar to the *Q*-test method, MR-PRESSO (Verbanck et al., 2018) first conducts a global test to detect the overall pleiotropic effect in a MR study, and then applies outlier test to rule out invalid genetic variants in follow-up study. Unlike the *Q*-test and MR-PRESSO methods, PLDMR contains all of the genetic variants associated with the exposure in a MR study and corrects the causal effect estimate with the mean and variance of pleiotropic effect.

Another strategy for adjusting the pleiotropy in MR studies is to additionally assume that the number of invalid genetic variants is less than half of the total number of variants, like the weighted median estimator and sisVIVE (Bowden et al., 2016; Kang et al., 2016). Adaptive lasso (Windmeijer et al., 2019) has been applied to select valid IVs and propose consistent estimate for causal effect by combining weighted median method with sisVIVE for individual level data. With these additional conditions on pleiotropic effect, it has been proved that $\boldsymbol{\alpha}$ is estimable (Kang et al., 2016) and identification of the true set of invalid genetic variants is consistent (Windmeijer et al., 2019). However, when these conditions are not met (for example, the fraction of invalid genetic variants is >50%), these methods fail to give a proper estimate of causal effect.

TWMR (Porcu et al., 2019) is similar to multivariable MR (Burgess and Thompson, 2015), which takes multiple expression quantitative trait loci as exposures to control the pleiotropic effects mediated by expression loci to the trait. However, any other pleiotropic effects mediated by environmental factors such as diet and education can still be potential confounders which affect the performances of these two methods. Moreover, we have conducted simulation studies to verify the performance of TWMR. Because we only consider one exposure in this study, the TWMR is unable to identify the pleiotropic effects in this case and thus the results cannot meet expectations. Furthermore, GSMR (Zhu et al., 2018) can also be applied to two-sample summary-level data. It solves the pleiotropy and LD problems by

excluding the SNPs which have pleiotropic effect and/or strong LD correlations between each other (Zhu et al., 2018). We also conduct simulation studies to compare our method and GSMR. As all the SNPs have pleiotropic effects and most of them are correlated with each other in our simulation study, the number of remaining SNPs after HEIDI test and LD pruning procedures may be <10, which would cause the instability warning in executing GSMR. In addition, it is observed from Figures 2A,C (Cheng et al., 2020) in the comparison of MR-LDP and GSMR that GSMR is not able to control type I error rate well when h_α^2 is not zero, which is equivalent to the parameter setting of $\mu_\alpha = 0$ and $\sigma_\alpha^2 > 0$ in our simulation, thus we have excluded GSMR from the comparison.

PLDMR takes a similar strategy to RAPS (Zhao et al., 2020), but PLDMR also borrows the idea from MR-Egger. To be precise, RAPS only models the variance of pleiotropic effects to correct for causal effect, while PLDMR models both the mean and variance of pleiotropic effects. What is more, RAPS assumes the selected genetic variants are in linkage equilibrium but PLDMR allows the existence of LD in all IVs. Similar to RAPS, MR-LDP (Cheng et al., 2020) also models the variance of pleiotropic effects, which regards pleiotropic effects as latent variables and uses expectation-maximization (EM) algorithm to estimate the causal effect. LDA MR-Egger (Barfield et al., 2018) improves MR-Egger when LD exists among the selected SNPs. The estimator derived from LDA MR-Egger is actually quite similar to that of LDMR, despite of a little difference in weight matrices.

4.2. Limitations and Forecast of PLDMR

We have shown in **Figure 1** that the small sample size n and high LD may cause type I error rate inflation, although very slight, for PLDMR method. This may mainly be caused by the relatively large variance of PLDMR estimator when the sample size n is small, since the term $(\boldsymbol{G}^T\boldsymbol{G})^{-1}$ in the variance is associated with n and the diagonal elements of this matrix decrease at rate $\frac{1}{n}$. On the other hand, the slow growth of PLDMR's power under large variance of pleiotropic effect (**Supplementary Figure 9**) may be interpreted as "the strong pleiotropy in MR studies can dominate the power growth benefit from the increase in sample size."

Furthermore, although we propose a measurement $r^2 = \sigma_\alpha^2/\sigma_Y^2$ to describe the relative strength of pleiotropy, we have not developed a method to test for the potential pleiotropy in genetic variants. The test for pleiotropic effect is important as it adds the interpretability of MR analyses when PLDMR returns a different result from the traditional MR methods which do not take pleiotropy into account. MR-Egger models pleiotropy in the intercept term of the Egger's regression, thus the test for pleiotropy is equivalent to test whether the intercept in regression is zero (Bowden et al., 2015), while the Q-test in fact focuses on the regression residuals (Bowden et al., 2018). When testing pleiotropic effect with PLDMR, it is important to notice that we must test two parameters μ_α and r^2 simultaneously, which is similar to the random-effects model in meta analysis (Han and Eskin, 2011) and may be conducted by the likelihood ratio test with a mixture of χ^2 distributions.

PLDMR also has restrictions on the data involved. Because of the requirement of matrix $\boldsymbol{G}^T\boldsymbol{G}$ in calculating multiple regression coefficients $\hat{\Gamma}, \hat{\gamma}$ and weight matrix $\boldsymbol{W}^{\frac{1}{2}}$, individual data is needed for PLDMR method, whereas two-sample MR methods like MR-Egger (Bowden et al., 2015) only need summary level data and thus can be easily implemented using results from online database like GWAS Catalog. To extend the application of PLDMR in summary level data, similar to most MR methods which consider LD in summary level data analyses (Zhu et al., 2018; Porcu et al., 2019), we can approximately substitute the matrix $\boldsymbol{G}^T\boldsymbol{G}$ with the reference LD panels such as 1000Genomes or even ARIC dataset itself. This is work left for future study.

Ultimately, we conclude that although MR-Egger allows correction for LD, the type I error of testing the null hypothesis of $H_0 : \beta = 0$ still inflates when directional pleiotropy and LD simultaneously exist between genetic variants, and LDA MR-Egger also fails to control type I error rate when there exists strong LD between genetic variants. PLDMR method controls type I error rate well and stays consistent with true value plug-in method PLDMR_t, especially when MR-LDP and RAPS are unable to control type I error rates in the cases of directional pleiotropic effects. We further conclude that LDMR and PLDMR_a are effective approximation of PLDMR method when the variation of pleiotropy is small and the sample size is large, respectively.

DATA AVAILABILITY STATEMENT

The datasets ARIC for this study can be applied from the dbGaP Study https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000090.v4.p1. GWAS Catelog is available at https://www.ebi.ac.uk/gwas/. The R package MendelianRandomization is available at https://cran.r-project.org/web/packages/MendelianRandomization/index.html. The R package BB is available at https://cran.r-project.org/web/packages/BB/index.html. The R package MR.LDP is available at https://github.com/QingCheng0218/MR.LDP. The R package mr.raps is available at https://github.com/qingyuanzhao/mr.raps. The R code for LDA MR-Egger is available at https://rbarfield.github.io/Barfield_website/pages/Rcode.html.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YW and TL developed the proposed method, designed the simulation study, and wrote the initial draft of manuscript. Y-QH contributed to the development of the method and to drafting the manuscript. LF preprocessed the data and guided data analysis. SY reviewed and approved the final manuscript.

All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by the National Natural Science Foundation of China (No. 11971117;11571082).

ACKNOWLEDGMENTS

We wish to thank the editor and reviewers for constructive suggestions on an earlier version of this paper. This study makes use of data from dbGaP (Accession number: phs000090.v4.p1).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.634394/full#supplementary-material

The source code for generating the simulation results and PLDMR method can be found in https://github.com/YQHuFD/PLDMR.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

REVIEW
published: 12 August 2021
doi: 10.3389/fgene.2021.683082

Mendelian Randomization in Stroke: A Powerful Approach to Causal Inference and Drug Target Validation

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Stroke is a leading cause of death and disability worldwide. However, our understanding of its underlying biology and the number of available treatment options remain limited. Mendelian randomization (MR) offers a powerful approach to identify novel biological pathways and therapeutic targets for this disease. Around ~100 MR studies have been conducted so far to explore, confirm, and quantify causal relationships between several exposures and risk of stroke. In this review, we summarize the current evidence arising from these studies, including those investigating ischemic stroke, hemorrhagic stroke, or both. We highlight the different types of exposures that are currently under study, ranging from well-known cardiovascular risk factors to less established inflammation-related mechanisms. Finally, we provide an overview of future avenues of research and novel approaches, including drug target validation MR, which is poised to have a substantial impact on drug development and drug repurposing.

Keywords: stroke, genetics, Mendelian randomization, polygenic risk scores, drug target validation

OPEN ACCESS

Edited by:
Lei Zhang,
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Reviewed by:
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Lang Wu,
University of Hawaii at Manoa, United States

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Specialty section:
This article was submitted to Statistical Genetics and Methodology, a section of the journal Frontiers in Genetics

Received: *19 March 2021*
Accepted: *28 June 2021*
Published: *12 August 2021*

Citation:
Acosta JN, Szejko N and Falcone GJ (2021) Mendelian Randomization in Stroke: A Powerful Approach to Causal Inference and Drug Target Validation. Front. Genet. 12:683082. doi: 10.3389/fgene.2021.683082

INTRODUCTION

Stroke is a leading cause of mortality and disability worldwide. While the overall incidence of stroke is decreasing, the global burden of this disease remains high because the absolute number of disability-adjusted life years lost due to stroke is increasing as the population grows and ages (Johnson et al., 2019). Thus, there is an urgent need for novel preventive, therapeutic, and rehabilitation strategies. Among these three, new acute treatments are particularly needed, as the few alternatives available thus far include thrombolytic therapy and mechanical thrombectomy for ischemic stroke (Powers et al., 2019), targeted blood pressure reduction for intracerebral hemorrhage (ICH; Hemphill et al., 2015), and early securing of the aneurysm in subarachnoid hemorrhage (Connolly et al., 2012).

In this setting, the field of stroke research can greatly benefit from the tools that population genetics has to offer. Mendelian randomization (MR) is a statistical method aimed at determining and quantifying causal relationships between genetically determined exposures and outcomes of interest (Davies et al., 2018). Importantly, in contrast to randomized clinical trials (the most

frequently used tool to evaluate causality), MR can be performed using already available open-access data from different sources, allowing the evaluation of larger numbers of possible mechanisms and accelerating the speed of the translational cycle.

In this review, we introduced the concept of MR and provide an overview of the existing MR studies focused on stroke. To identify these studies, we conducted a systematic search of the medical literature using PubMed and the keywords "Mendelian randomization stroke," "Mendelian randomization intracerebral hemorrhage," and "Mendelian randomization subarachnoid hemorrhage" in the MEDLINE database. We also explored some analytical possibilities beyond classical MR, including the use of multiomics data and drug target validation MR.

MENDELIAN RANDOMIZATION: A PRIMER

MR constitutes a special case of instrumental variable analysis, a widely used analytical framework for causal inference. When the MR assumptions are met, it is possible to identify and quantify causal relationships between exposures and outcomes of interest (**Figure 1**). MR is best explained using an example. For this section, we will use the link between cholesterol levels and systolic blood pressure as our starting point. In randomized clinical trials, investigators randomly assign study participants to an intervention or placebo to study causal relationships between the intervention and the outcome of interest. In our example, researchers would randomly assign participants to receive a cholesterol-lowering drug or placebo and then measure systolic blood pressure levels in both groups. In MR analysis, we would use genetic variants strongly associated with the exposure of interest as the instrument. In this case, we would choose genetic variants strongly associated with cholesterol levels. As these variants are randomly segregated before birth, one could then separate groups according to the number of risk alleles, and the resulting analyses would not be confounded by environmental exposures happening after birth. Thus, we can measure systolic blood pressure levels in those groups and implement the necessary comparisons.

The implementation of MR analyses requires important assumptions (**Figure 2**). First, the genetic variants used as instruments must be strongly associated with the exposure or risk factor of interest. This is generally easily accomplished, since these variants are often derived from large-scale genome-wide association studies (GWAS) of the exposure of interest. Second, there should be no confounders affecting the association between genetic variants and the outcome of interest. This assumption is not trivial, and, at the moment, there is no method to definitively confirm this assumption. Although not always performed, a practical way to tackle this problem is to test the genetic variants used as instruments against an array of different other potential covariates that could lead to bias. Third, the genetic variants used as instruments affect the outcome of interest only *via* the exposure of interest, with no

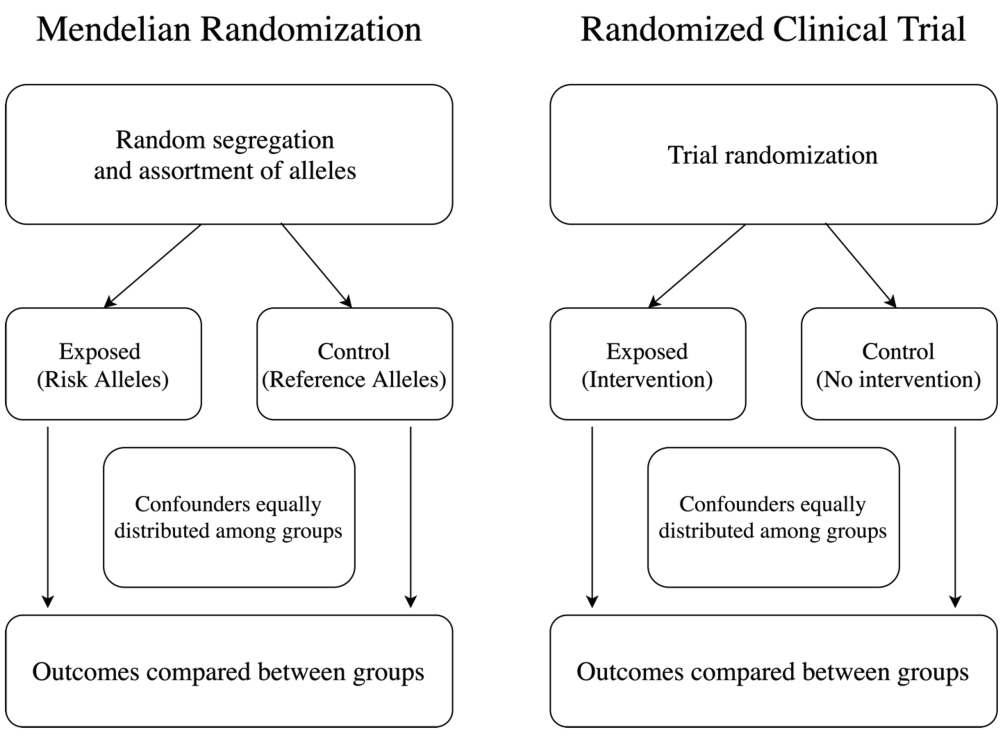

FIGURE 1 | Mendelian randomization (MR) as instrument variable analysis. MR is a type of instrumental variable analysis, similar to randomized clinical trials. Exposed individuals in MR are those carrying risk alleles for determined genetic variants known to associate with an exposure of interest.

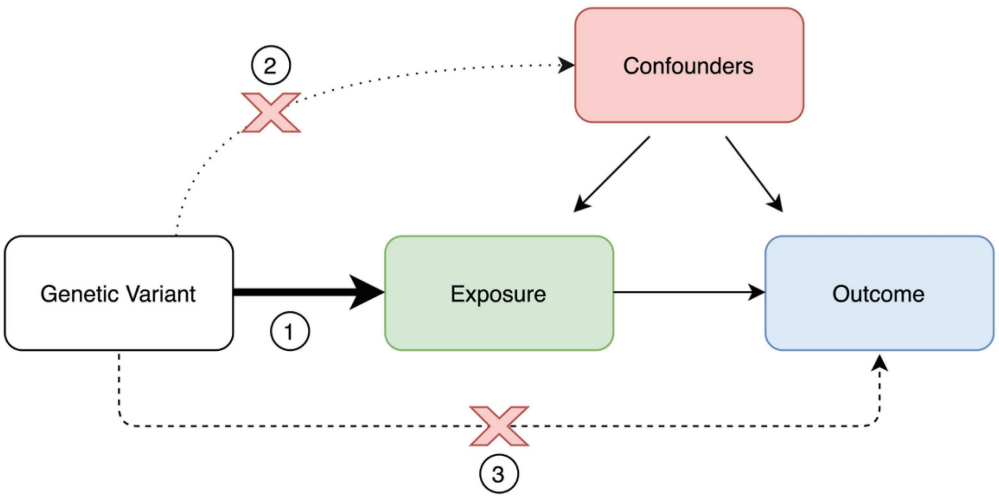

FIGURE 2 | Mendelian randomization paradigm and assumptions. Mendelian randomization assumptions: (1) valid instrument, (2) no association with confounders, and (3) no horizontal pleiotropy.

other alternative pathways coming into play. These alternative pathways are part of what is called horizontal pleiotropy. There are several statistical methods to test and correct for horizontal pleiotropy, including the Mendelian randomization–Egger (MR–Egger) intercept, MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO), and Causal Analysis Using Summary Effect Estimates (CAUSE; Bowden et al., 2015; Verbanck et al., 2018; Morrison et al., 2020).

Crucial to MR studies is the online availability of open-access resources, including tools to rapidly test hypotheses such as MR-Base (Hemani et al., 2018); large biobanks such as the UK Biobank (Bycroft et al., 2018), the China Kadoorie Biobank (CKB; Chen et al., 2011), Biobank Japan (Nagai et al., 2017), and the All of Us Research Program (Denny et al., 2019); and repositories of summary statistics from large GWAS like the GWAS catalog (Buniello et al., 2019), the GWAS atlas (Tian et al., 2020), and specifically for stroke, the Cerebrovascular Disease Knowledge Portal (Crawford et al., 2018). These and other helpful resources are summarized in **Table 1**.

TRADITIONAL CARDIOVASCULAR RISK FACTORS

Cardiovascular risk factors represent the most important determinants of risk of stroke. While several observational studies have shown associations between traditional vascular risk factors and risk of different types of stroke, deriving causality from observational data is problematic due to the possibility of bias. In the last 5 years, several MR studies have been conducted to explore, confirm, and quantify causality behind these already described associations (**Table 2**).

Blood Pressure

Robust existing evidence indicates that high blood pressure is one of the main risk factors for stroke. However, several questions remain, including how early and sustained changes in blood pressure affect outcomes later in life and whether specific pharmacological interventions provide additional benefits over others. Georgakis et al. (2020b) utilized MR analyses to assess the relationship between genetically proxied blood pressure levels and the risk of any stroke as well as its subtypes. They demonstrated that blood pressure levels were causally associated with the risk of any stroke and most subtypes, with the exception of lobar ICH. The effect of blood pressure appeared stronger in large artery stroke (LAS) and small vessel stroke (SVS). Additionally, the authors explored genetic proxies for two different antihypertensive drugs classes, calcium channel blockers and beta-blockers, finding that a 10 mmHg reduction in systolic blood pressure using genetic proxies for calcium channel blockers was protective for any stroke and its subtypes, especially for SVS (40% reduction). Furthermore, the same reduction in blood pressure through calcium channel blockers variants was also associated with lower white matter hyperintensity (WMH) volumes, a well-established neuroimaging biomarker for cerebral small vessel disease. A study from the same group also evaluated whether blood pressure pulsatility (i.e., pulse pressure) affects stroke risk independently of the mean arterial pressure. Using multivariable MR within the UK Biobank, they found that pulse pressure was independently associated with ischemic stroke risk (but not ICH) in participants older than 55 years, being particularly important for LAS (Georgakis et al., 2020a). Beyond ischemic stroke, MR analyses have confirmed observational evidence that indicate that high blood pressure is associated with higher risks of intracranial aneurysms and subarachnoid hemorrhage (Bakker et al., 2020).

Lipids

The role of lipids in cardiovascular disease (CVD) has been extensively studied. However, the effect of circulating lipid levels on stroke appear to be heterogeneous and depend on the specific subtypes being evaluated. Hindy et al. (2018) investigated the effect of blood lipids on the risk of ischemic stroke and its

TABLE 1 | Online resources available for Mendelian Randomization studies.

References	Name	Website	Description
Crawford et al., 2018	Cerebrovascular Disease Knowledge Portal	http://www.cerebrovascularportal.org/	A platform that allows for searching, visualizing, and analyzing variations related to cerebrovascular disease.
Buniello et al., 2019	GWAS Catalog	https://www.ebi.ac.uk/gwas/	Catalog of GWAS results
Tian et al., 2020	GWAS atlas	https://atlas.ctglab.nl/	Catalog of GWAS results
Lambert et al., 2019	PGS Catalog	http://www.pgscatalog.org/	Catalog of Polygenic Risk Scores
Hemani et al., 2018	MR-base	https://www.mrbase.org/	A database and analytical platform for Mendelian Randomization
Bycroft et al., 2018	UK Biobank	https://www.ukbiobank.ac.uk/	Observational study, enrolling >500,000 participants, open-access
Gaziano et al., 2016	Million Veteran Program	https://www.research.va.gov/mvp/	Observational study, enrolling 1 million participants, restricted access
Denny et al., 2019	All of Us	https://allofus.nih.gov/	Observational study, aiming to enroll 1 million participants, open-access
Chen et al., 2011	China Kadoorie Biobank	https://www.ckbiobank.org/	Observational study, 500,000 participants, restricted access
Nagai et al., 2017	Biobank Japan	https://biobankjp.org/english/index.html	Observational study, ~200,000 participants, restricted access
Wong et al., 2017	dbGAP	https://www.ncbi.nlm.nih.gov/gap/	Repository, open-access
Lappalainen et al., 2015	EGA	https://ega-archive.org/	Repository, open-access

subtypes using summary statistics from the GWAS completed by the Stroke Genetics Network (SiGN), which included 17,000 ischemic stroke cases and more than 32,000 controls of European ancestry. They found that low-density lipoprotein cholesterol (LDL-C) levels were associated with risk of ischemic stroke, especially LAS. HDL-C levels were inversely associated with the risk of SVS. There were no consistent associations for triglycerides. More recently, Allara et al. (2019) conducted a two-sample MR study to identify relationships between lipid levels and the risk of several cardiovascular outcomes, including stroke. In their study, higher HDL-C levels were associated with lower risk of ICH, while higher LDL-C levels were associated with higher risk of ischemic cerebrovascular disease. Interestingly, they also found an inverse association between triglycerides and the risk of ICH. A rigorous study performed by Valdes-Marquez et al. (2019) investigated the effect of LDL-C on the risk of ischemic stroke and its subtypes and did not find any significant associations with these conditions. Georgakis et al. (2020c) also looked at the association between lipid fractions and small vessel disease, a broad phenotype that includes SVS, white matter disease, and ICH. They found that higher HDL-C was associated with lower risk of SVS and lower WMH volume. Interestingly, they also found a relationship between higher HDL-C and higher risk of ICH. Along this lines, Sun et al. (2019) found a strong positive association between LDL-C and ischemic stroke and a strong inverse association with ICH in participants of the China Kadoorie Biobank. This inverse association between genetically determined LDL-C levels and the risk of ICH was confirmed in cohorts of European descent by Falcone et al. (2020). The latter two studies confirmed previous observational results that suggested that very low LDL-C levels increase the risk of ICH (Wang et al., 2013; Saliba et al., 2018; Ma et al., 2019), pointing to novel pathways in ICH.

Another study recently explored association pertains to lipoprotein(a) [Lp(a)] levels and ischemic stroke. Using summary statistics from MEGASTROKE and the International Genomics of Alzheimer's Project (IGAP), Pan et al. (2019) found a causal positive association between Lp(a) levels and LAS and, remarkably, an inverse association with SVS and Alzheimer disease (AD). Using individual-level data from the UK Biobank, Larsson et al. (2020b) confirmed a positive association with any ischemic stroke but could not replicate the protective association with AD. The latter result, however, could have been due to lack of statistical power, as the investigators did find a protective association with parental AD or dementia.

Type 2 DM

Extensive data point to a deleterious effect of diabetes on ischemic stroke, but there is no such evidence for hemorrhagic stroke. Larsson et al. (2017) used MR analyses to confirm a causal relationship between type 2 diabetes mellitus (T2DM) and risk of ischemic stroke, especially LAS, but found null associations between other metabolic markers such as glucose, insulin levels, and body mass index (BMI). Gan et al. (2019) replicated this causal association between T2DM and ischemic stroke in the CKB, and Liu et al. (2018) found an association between T2DM and lacunar stroke. Similarly, using a combined exposure comprising phenotypes related to insulin resistance (fasting insulin adjusted for BMI, HLD-C and triglycerides, and insulin sensitivity), Chen et al. (2020) found a causal relationship between insulin resistance and ischemic stroke, particularly small vessel stroke. On the other hand, Yeung et al. (2018) did not find an association between glycated hemoglobin (HbA1c) and ischemic stroke in an MR study within the UK Biobank. More recently, Georgakis et al. (2021) investigated the effects of T2DM, hyperglycemia, insulin resistance, and β-cell dysfunction on the risk of stroke and related traits, finding that type 2 diabetes and higher HbA1c levels are associated with higher risk stroke, and particularly of LAS and SVS, with similar associations found for insulin resistance and β-cell dysfunction. β-Cell dysfunction was also associated with the risk of ICH. Furthermore, a study focused on neuroimaging

TABLE 2 | Summary of MR studies looking at traditional risk factors for stroke.

References	Exposure	Outcomes	Findings
Georgakis et al., 2020b	Blood pressure levels	Any stroke, ischemic stroke, LAS, CES, and SVS	Ten mmHg increase in SBP was associated with approximately 40% increase in the risk of any stroke or ischemic stroke. It was also associated with the risk of stroke subtypes, except for lobar ICH. Similar findings were presented for DBP. Decreases in SBP through calcium channel blockers but not through beta-blockers was associated with decrease in ischemic stroke risk.
Georgakis et al., 2020a	PP	Ischemic stroke and subtypes	Pulse pressure was independently associated with stroke risk in participants older than 55 years.
Hindy et al., 2018	LDL-C	Ischemic stroke and subtypes	Higher LDL-C was associated with higher risk of ischemic stroke and LAS.
Allara et al., 2019	Lipid fractions levels	CVD outcomes	Higher LDL-C was associated with higher risk of ischemic stroke. Higher HDL-C levels were associated with lower risk of ICH. Higher triglycerides were associated with lower risk of ICH.
Valdes-Marquez et al., 2019	LDL-C	CHD, Ischemic stroke and its subtypes	LDL-C levels were not associated with the risk of ischemic stroke or its subtypes.
Georgakis et al., 2020c	Lipid fractions levels	SVS, WMH volume, and ICH	Higher HDL-C levels were associated with lower risk of SVS and lower WMH volumes. Higher HDL-C levels were associated with higher risk of ICH.
Sun et al., 2019	Lipid fractions levels	Ischemic stroke and ICH	Higher LDL-C levels were associated with higher risk of ischemic stroke and lower risk of ICH.
Falcone et al., 2020	Lipid fractions levels	ICH	Higher LDL-C levels were associated with lower risk of ICH.
Pan et al., 2019	Lp(a)	Ischemic stroke, its subtypes, AD	Lp(a) levels were associated with higher risk of LAS, but lower risk of SVS and Alzheimer's disease.
Larsson et al., 2020b	Lp(a)	Ischemic stroke, AD, parental AD or dementia	Lp(a) levels were associated with the risk of ischemic stroke. There is an inverse relationship between Lp(a) and Parental AD or dementia.
Larsson et al., 2017	T2DM	Ischemic stroke and its subtypes	T2DM was associated with the risk of ischemic stroke, specially LAS.
Gan et al., 2019	T2DM	Ischemic stroke	T2DM was associated with ischemic stroke.
Liu et al., 2018	T2DM	Small vessel disease phenotypes	T2DM was associated with MRI-confirmed lacunar stroke.
Chen et al., 2020	Insulin resistance	Ischemic stroke and subtypes	Insulin resistance was associated with ischemic stroke, particularly SVS.
Yeung et al., 2018	HbA1c	Ischemic stroke	No association.
Georgakis et al., 2021	Type 2 diabetes, HbA1c, insulin resistance, and β-cell dysfunction	Ischemic stroke, ischemic stroke subtypes, intracerebral hemorrhage, related phenotypes	T2DM and higher HbA1c levels were associated with higher risk of ischemic stroke, especially LAS and SVS. Insulin resistance and β-cell dysfunction show similar associations, with the latter also associated with intracerebral hemorrhage. T2DM was also associated with lower white matter integrity (fractional anisotropy). T2DM, HbA1c, and β-cell dysfunction were associated with lower grey matter volume and total brain volume.
Qian et al., 2019	Smoking	Ischemic stroke	Smoking was associated with any ischemic stroke and LAS.
Larsson et al., 2019a	Smoking initiation	Ischemic stroke, subtypes, and ICH	Smoking initiation was associated with ischemic stroke, LAS, and SVS, but not with CES or ICH.
Larsson et al., 2020c	Smoking	14 CVDs	Smoking was associated with a broad range of CVDs including coronary artery disease, heart failure, abdominal aortic aneurysm, ischemic stroke, transient ischemic attack, peripheral arterial disease, and arterial hypertension.
Acosta et al., 2021	Smoking initiation	SAH	Smoking initiation was associated with the risk of nontraumatic SAH.
Dale et al., 2017	General adiposity and central adiposity	Stroke	Central adiposity but not general adiposity was associated with stroke risk.
Marini et al., 2020	BMI and WHR	Ischemic stroke, subtypes, ICH, WMH volume	Higher WHR but not higher BMI was associated with all-cause ischemic stroke, LAS, SVS, non-lobar ICH and WMH volume.
Zhuang et al., 2020	Physical activity	Stroke	No association.
Hou et al., 2020	Atrial fibrillation	CES	Bidirectional association between atrial fibrillation and CES.

CVD, cardiovascular disease; ICH, intracerebral hemorrhage; LAS, large artery stroke; SVS, small vessel stroke; CES, cardioembolic stroke; WMH, white matter hyperintensity; SAH, subarachnoid hemorrhage; PP, pulse pressure; HDL-C, high-density lipoproteins cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); T2DM, type 2 diabetes mellitus; HbA1c, glycosylated hemoglobin; BMI, body mass index; and WHR, waist-to-hip ratio.

markers of cerebrovascular disease found that type 2 diabetes was also positively associated with fractional anisotropy (a measure of white matter integrity) and inversely associated with gray matter volume and total brain volume, with similar inverse associations seen for HbA1c and β-cell dysfunction.

Smoking

A large body of literature indicate that smoking is associated with both ischemic stroke and aneurysmal subarachnoid hemorrhage (Peters et al., 2013). Qian et al. (2019) reported a causal association between smoking and any ischemic stroke and LAS. In other MR studies, Larsson et al. (2019a, 2020c) confirmed the association between smoking initiation and the risk of any ischemic stroke, LAS and SVS, but did not find an association with cardioembolic stroke (CES) or ICH. In a one-sample MR study using the UK Biobank, Acosta et al. (2021) confirmed an association between genetic propensity to smoke and risk of nontraumatic subarachnoid hemorrhage. Similarly, in a large GWAS of intracranial aneurysms and subarachnoid hemorrhage, smoking was shown to be a powerful risk factor for this disease (Bakker et al., 2020).

Obesity

Observational evidence suggests that obesity is a risk factor for stroke. However, MR studies have yielded heterogeneous results. One study indicated that while both general and central adiposity had causal effects on coronary heart disease, only central adiposity appeared to be associated with ischemic stroke (Dale et al., 2017). Further evidence supporting this hypothesis was provided by a more recent MR study by Marini et al. (2020) which found that higher waist-to-hip ratio, but not higher BMI, was causally associated with all-cause ischemic stroke, LAS, SVS, non-lobar ICH, and WMH volume.

Lack of Physical Activity

Physical activity, especially moderate-to-vigorous physical activity, has been associated with lower risk of stroke in several observational studies. To date, only one MR has investigated this relationship, finding null results for this association (Zhuang et al., 2020). Therefore, further research is warranted in order to fully understand these conflicting results.

Atrial Fibrillation

Atrial fibrillation (AF) is considered a major risk factor of cardioembolic ischemic stroke. Remarkably, patients with stroke are also at a higher risk of developing AF (Sposato et al., 2015, 2018). Only one MR study has looked at this relationship and, as expected given the overwhelming amount of observational evidence, found causal evidence to support this link using bidirectional MR (Hou et al., 2020).

NONTRADITIONAL RISK FACTORS

A number of studies have explored nontraditional risk factors that had mixed or inconclusive evidence when evaluated in nongenetic, observational studies. Prominent among these are studies that focused on inflammation, coagulation factors, sleep health, and nutrition. MR studies have evaluated several of these factors in search of causal associations (**Table 3**).

Inflammatory Biomarkers

Observational studies have demonstrated that patients with underlying inflammation have a higher risk of stroke (Anrather and Iadecola, 2016), although this association could be the result of confounding. Therefore, genetically determined inflammatory biomarkers have been targeted by many studies investigating the risk of stroke. Zhang et al. (2020) examined whether genetically raised plasma C-reactive protein (CRP) concentration levels were associated with ischemic stroke without finding a significant association. In line with these findings, Lin et al. (2020) evaluated numerous inflammatory biomarkers and found no association between genetically elevated levels of these biomarkers and ischemic stroke. Yuan et al. (2020b) analyzed genetically determined circulating interleukins in relation to coronary artery disease (CAD), atrial fibrillation, and ischemic stroke, and its subtypes. There was a suggestive ($p < 0.05$ but not statistically significant after correction for multiple testing) positive association between interleukin-1 receptor antagonist and cardioembolic stroke and a suggestive inverse association between interleukin-6 and ischemic stroke, CES, and SVS, and of interleukin-16 with CAD. Georgakis et al. (2020d) reported the results of an MR study focused on interleukin-6 signaling effects on ischemic stroke and other cardiovascular outcomes and demonstrated that genetically downregulated interleukin-6 signaling was associated with lower risk of ischemic stroke. The same group published a study investigating genetically determined levels of circulating cytokines and risk of stroke (Georgakis et al., 2019). They showed that genetic predisposition to elevated circulating levels of monocyte chemoattractant protein-1 was associated with higher risk of stroke, in particular with LAS and CES. Another study by Yuan et al. (2020a) investigated causal associations of increased tumor necrosis factor levels and several highly prevalent CVDs. Genetically elevated tumor necrosis factor levels were positively associated with both CAD and ischemic stroke. In addition, Wang et al. (2020) investigated the impact of growth differentiation factor 15 on the risk of CVDs using an MR approach. They found evidence for a relationship between circulating of growth differentiation factor 15 levels and increased risk of CES, atrial fibrillation, CAD, and myocardial infarction. Finally, Song et al. (2020) published results of their study investigating association of genetically determined T-cell immunoglobulin and mucin domain 1 with incidence of stroke, which found a causal effect of TIM-1 on any stroke and ischemic stroke.

Hematological Traits

The role of hematological traits and pathways in the occurrence of stroke has also been extensively evaluated using MR. Gill et al. (2018b) investigated genetically determined platelet count and risk of different CVDs, finding that higher genetically determined platelet count is associated with higher risk of ischemic stroke. The same group released results of an MR

TABLE 3 | Summary of MR studies looking at nontraditional risk factors for stroke.

References	Exposure	Outcomes	Findings
Larsson et al., 2020a	Alcohol	CVDs	Causal relationship between higher alcohol consumption and increased risk of stroke and peripheral artery disease.
Yuan et al., 2020c	PUFA	IS	High-level plasma ALA was protective for IS, but AA was the opposite. LA, EPA, DHA, and DPA had no effects on IS.
Au Yeung and Schooling, 2020	Urinary sodium	CVDs	Higher log-transformed urinary sodium was associated with higher risk of stroke.
Larsson et al., 2019b	Serum magnesium and calcium	IS	Genetically higher serum magnesium concentrations were associated with a reduced risk of cardioembolic stroke but found no significant association of genetically higher serum calcium concentrations with any IS subtype.
Wang et al., 2021	Tea	IS	Genetically predicted an extra daily cup of tea consumption was causally associated with a reduced risk of small vessel stroke.
Qian et al., 2020	Coffee	IS and ICH	Coffee consumption was not causally associated with risk of stroke or its subtypes.
Choi et al., 2020	Serum bilirubin levels	IS an ICH	Causal associations between serum bilirubin levels and decreased stroke risk.
Huang et al., 2019	25(OH)D	CVDs	No evidence to support that genetically increased 25(OH)D was associated with a lower risk of IS, ICH, and SAH.
Larsson et al., 2018b	25(OH)D	IS	No evidence that genetically determined higher S-25OHD concentrations were causally associated with any ischemic stroke subtype.
Larsson et al., 2018a	$VitK_1$	IS	Genetic predisposition to higher circulating vitamin K_1 levels was associated with an increased risk of large artery atherosclerotic stroke.
Schooling et al., 2018	Testosterone	CVDs	Genetically determined testosterone was associated with IS.
Zhang et al., 2020	CRP	IS	No clear support that genetically determined elevated CRP concentration was causally associated with the risk of IS.
Yuan et al., 2020b	Circulating ILs	CVDs	Positive association of IL-1 with cardioembolic stroke and suggestive inverse associations of IL-6 with any IS, cardioembolic stroke, and small vessel stroke, and of IL-16 with CAD.
Georgakis et al., 2020d	Genetic proxies for IL-6R-mediated downregulation of IL-6 signaling	IS and other CVDs	Genetically downregulated IL-6 signaling to be associated with lower risks of IS.
Yuan et al., 2020a	TNF	CVDs	Genetically predicted TNF levels were positively associated with coronary artery disease and IS.
Wang et al., 2020	GDF-15	CVDs	Causal relationship of circulating GDF-15 levels with the increased risk of cardioembolic stroke, atrial fibrillation, coronary artery disease and myocardial infarction, but not any IS, large-artery atherosclerotic stroke, small vessel stroke, heart failure, and nonischemic cardiomyopathy.
Song et al., 2020	TIM-1	Stroke	Causal effect of TIM-1 on stroke.
Georgakis et al., 2019	Cytokines	Stroke	Genetic predisposition to elevated circulating levels of MCP-1 was associated with higher risk of stroke, in particular with large-artery stroke and cardioembolic stroke.
Lin et al., 2020	Inflammatory biomarkers	IS	No convincing evidence to support that inflammatory biomarkers like IL-1Ra, sIL-6R, and CRP were causally associated with the risk of IS or its subtypes.
Gill et al., 2018c	Iron	Stroke	MR evidence that higher iron status was associated with increased stroke risk and, in particular, CES.
Titova et al., 2020	Sleep duration	Stroke	No clear support that a genetically determined short or long sleep duration has influence on the risk of total stroke or stroke types.
Lu et al., 2020	Sleep duration	Stroke	Sleep duration was not causally associated with risk of stroke and its subtypes.
Cai et al., 2020	Sleep traits	IS	Potential causal role of short sleep duration and insomnia symptoms in LAS.
Larsson and Markus, 2019	Insomnia	CAD and stroke	Causal link between insomnia and ischemic stroke and its subtypes.
Marouli et al., 2020	Thyroid hormones	Stroke or CAD	A 1-SD increase in TSH was associated with a 5% decrease in the risk of stroke.
Cai et al., 2019	MDD	SVS	Possible causal effect of MDD on increased risk of SVS.
Gill et al., 2019b	Depression	IS and functional outcome after IS	No evidence of genetically determined risk of depression affecting IS risk but consistent MR evidence suggestive of a possible effect on functional outcome after IS.
Gill et al., 2018b	Platelet count	CVDs	Higher genetically determined platelet count was causally associated with higher risk of IS.
Gill et al., 2018a	FXI	IS, ICH, MI	Causal effect of higher, genetically determined FXI levels on risk of any IS.

(Continued)

TABLE 3 | Continued

References	Exposure	Outcomes	Findings
Harshfield et al., 2020	Hematological traits	IS and its subtypes	Several factors on the intrinsic clotting pathway were significantly associated with CES and LAS, but not with SVS. On the common pathway, increased gamma (γ') fibrinogen was significantly associated with AIS/CES. Furthermore, elevated plateletcrit was significantly associated with AIS/CES, eosinophil percentage of white cells with LAS, and thrombin-activatable fibrinolysis inhibitor activation peptide antigen with AIS. Follow-up analysis in UK Biobank showed that among individuals with atrial fibrillation, those with genetically lower levels of factor XI are at reduced risk of AIS compared to those with normal levels of factor XI.
Jia et al., 2019	Gut microbiota dependent metabolites (betaine, carnitine, choline, and trimethylamine N-oxide)	Stroke	No association.

CVDs, cardiovascular disorders; ICH, intracerebral hemorrhage; IS, ischemic stroke; PUFA, polyunsaturated fatty acids; AA, arachidonic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; SAH, subarachnoid hemorrhage; FAs, circulating fatty acids; CRP, C-reactive protein; ILs, interleukins; IL-1, interleukin 1; IL-6, interleukin 6; IL-16, interleukin 16; TNF, tumor necrosis factor; GDF-15, growth differentiation factor 15; TIM-1, T-cell immunoglobulin and mucin-1; MCP-1, monocyte chemoattractant protein-1; LAS, large artery stroke; CAD, coronary artery disease; TSH, thyroid stimulating hormone; MDD, major depressive disorder; SVS, small vessel stroke; MR, Mendelian randomization; FXI, factor XI; MI, myocardial infarction; CES, cardioembolic stroke; and AIS, arterial ischemic stroke.

study focused on genetically determined factor XI levels that found causal evidence supporting factor XI as a possible target to reduce the risk of cardioembolic stroke (Gill et al., 2018a). Harshfield et al. (2020) reported a study that, rather than focusing on a single target, evaluated several different hematological traits in connection to risk of ischemic stroke and its subtypes. Several factors in the intrinsic coagulation pathway were significantly associated with CES and LAS but not with SVS. Specifically, gamma fibrinogen, a component of the common pathway, was associated with CES, plateletcrit was associated with CES, eosinophil percentage of white cells was associated with LAS, and thrombin-activatable fibrinolysis inhibitor activation peptide antigen was associated with any ischemic stroke. Follow-up analyses in the UK Biobank showed that among individuals with atrial fibrillation, those with genetically lower versus normal levels of factor XI have a reduced risk of ischemic stroke. Finally, one group investigated circulating vitamin K_1 levels in connection to cerebrovascular disease and found that genetic predisposition to higher circulating vitamin K_1 levels was associated with an increased risk of LAS (Larsson et al., 2018a).

Nutritional Factors

A number of MR studies also investigated the role of nutritional factors in the occurrence of stroke, another research avenue extensively explored from an observational perspective. Larsson et al. (2020a) examined whether genetically determined predisposition to alcohol consumption had influence on risk of CVD. This study provided evidence for a causal relationship between higher alcohol consumption and increased risk of any stroke and peripheral artery disease.

Beyond alcohol, several other nutritional factors have been investigated. One research group examined the role of plasma phospholipid fatty acids in risk of 15 CVD-related phenotypes. Genetically higher plasma α-linolenic, linoleic, and oleic acid levels were inversely associated with LAS and venous thromboembolism, whereas arachidonic and stearic acid levels are positively associated with these two CVD-related outcomess (Yuan et al., 2019).

Iron metabolism, including anemia and polycythemia, have long been postulated to play a role in cerebrovascular disease. Gill et al. (2018c) published results of an MR study investigating this question, finding that higher iron levels were associated with increased risk of stroke and, in particular, CES.

Two groups reported on genetically predicted levels of vitamin D and the risk of stroke. Huang et al. investigated whether vitamin D played a role in risk and mortality of several vascular diseases by conducting an MR study that included both Asian and European participants (Huang et al., 2019). They found no evidence to support that genetically increased vitamin D was associated with a lower risk of ischemic stroke, ICH, SAH, and lipid levels in neither Chinese nor European. Similarly, Larsson et al. (2018b) reported the results of a study on genetically determined vitamin D concentrations and ischemic stroke and its subtypes that failed to find significant associations.

Other Risk Factors

Among other investigated risk factors, it is worth mentioning two studies investigating endocrine changes. Schooling et al. (2018) analyzed genetic predictors of testosterone and their associations with different CVD phenotypes. They confirmed prior results from observational studies showing a significant association between genetically proxied testosterone and risk of ischemic stroke. Another study by Marouli et al. (2020) used MR analyses to analyze whether thyroid function affects the risk of stroke *via* atrial fibrillation, finding that a 1 SD increase in TSH was associated with a 5% decrease in the risk of ischemic stroke.

Finally, another four studies are also worth mentioning. Larsson et al. (2019b) examined serum magnesium and calcium levels in relation to ischemic stroke using MR. They found that genetically higher serum magnesium concentrations were associated with a reduced risk of CES but not with other stroke subtypes. Au Yeung and Schooling (2020) investigated the impact of urinary sodium on CVD. Higher genetically determined log-transformed urinary sodium was associated

with higher risk of stroke. Choi et al. (2020) used MR tools to describe a causal association between serum bilirubin levels and decreased stroke risk. Lastly, Jia et al. (2019) investigated the role of gut microbiota-dependent metabolites and risk of several CVDs, finding no evidence of a causal link between these metabolites (betaine, carnitine, choline, and trimethylamine N-oxide) and the risk of stroke.

MENDELIAN RANDOMIZATION FOR DRUG TARGET VALIDATION AND DRUG REPURPOSING

Beyond hypothesis-driven MR studies, population genetics offers powerful tools to accelerate the discovery of novel biological pathways by agnostically evaluating several biological targets and/or reevaluating targets for drug repurposing (Nelson et al., 2015; Schmidt et al., 2020). This approach is significantly potentiated by the growing culture of open-access research and the increasing availability of high-throughput genomic and proteomic technologies.

Drug target MR studies use genetic variants that lie within or near the genes coding for these targets (the latter called cis-variants) as instruments, which either have an effect on the actual serum levels of the target protein or other endpoint, such as an intermediate biomarker, gene expression levels, or metabolite levels. This distinction is important because drug target MR aims to answer a different question than conventional MR. While conventional MR establishes causal relationships between biomarkers or traits and an outcome, drug target MR addresses whether modifications of a specific drug target or protein will have an effect on the outcome (Gill et al., 2021).

Drug target MR overcomes problems related to pleiotropy, which are especially relevant when looking at possible targets for intervention. In addition, by testing these drug targets phenome-wide, investigators can also pinpoint possible adverse effects. While drug target MR is a robust methodology, it also has limitations. While new high-throughput technologies have increased the amount of proteome-wide data available, publicly available summary statistics and proteomic studies with adequate sample sizes are still limited. This relative paucity of proteomic data leads to the utilization of cis-variants associated with a biomarker in the causal pathway, which could in turn lead to some problems. For example, circulating levels of a biomarker could not represent accurately its cellular concentration, which is often the value of interest, and this could limit the ability to detect causality. Additionally, the biomarker of interest could be relevant only in certain physiological or disease states, or during a critical period of time, which could also limit results. Lastly, not all proteins are druggable (i.e., able to be pharmacologically manipulated), which of course would defeat the purpose of doing drug target MR (Mokry et al., 2015).

Gill et al. (2019a) compared the results of drug target MR and clinical trials for three antihypertensive drug classes (angiotensin-converting-enzyme inhibitors, β-blockers, and calcium channel blockers) in coronary heart disease and stroke, finding comparable estimates. Along these lines, Chong et al. (2019) conducted a proteome-wide MR study to investigate potential therapeutic targets in ischemic stroke. The authors analyzed 653 circulating proteins as possible causal factors for the three main subtypes of ischemic strokes (LAS, SVS, and CES) and hemorrhagic stroke. In their analyses, they found eight biomarker–stroke associations encompassing seven unique targets. Of these biomarkers, five had already been associated with CVDs, including the coagulation factor 11, Lp(a), ABO, CD40 (a member of the tumor necrosis factor superfamily), and MMP12 (a member of the matrix metalloproteinase family, implicated in vascular remodeling). Novel biomarkers included SCARA5, a protein with a role in iron homeostasis, and TNFS12, a pleiotropic tumor necrosis factor-like cytokine linked to atrial fibrillation, a potential mediating mechanism.

Drug repurposing is another potentially useful approach, as previously approved drugs can be more easily brought to clinical practice if a beneficial effect is found. There are no clear examples of MR studies specifically focused on drug repurposing for stroke. However, we bring the reader's attention to two such studies that explored the potential of repurposing medications to treat Alzheimer's disease, unfortunately with null results (Walker et al., 2020; Wu et al., 2021). These studies provide an appropriate example and analytical framework for future studies applying this approach to stroke and cerebrovascular diseases.

CONCLUSION

Stroke constitutes an increasingly prevalent condition worldwide and remains one of the leading causes of death and disability. MR has proved to be a powerful methodology to confirm or refute associations described by observational studies and identify novel therapeutic targets for stroke. The ability of MR studies to add valuable scientific evidence to the field of cerebrovascular disease research will be greatly increased by the utilization of novel tools, including proteome-wide and drug target validation analyses.

AUTHOR CONTRIBUTIONS

JA, NS, and GF reviewed the literature, drafted the manuscript, and revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

GF was supported by the National Institute on Aging (K76AG59992), the National Institute of Neurological Disorders and Stroke (R03NS112859), the American Heart Association (18IDDG34280056 and 817874), the Yale Pepper Scholar Award (P30AG021342), and the Neurocritical Care Society Research Fellowship. JNA is supported by the American Heart Association's Bugher Fellowship in Hemorrhagic Stroke Research.

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