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No causal effects of plasma homocysteine levels on the risk of coronary heart disease or acute myocardial infarction: A Mendelian randomization study

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Abstract

Background: Although many observational studies have shown an association between plasma homocysteine levels and cardiovascular diseases, controversy remains. In this study, we estimated the role of increased plasma homocysteine levels on the etiology of coronary heart disease and acute myocardial infarction.

Methods: A two-sample Mendelian randomization study on disease was conducted, i.e. "coronary heart disease" ($n = 184{,}305$) and "acute myocardial infarction" ($n = 181{,}875$). Nine single nucleotide polymorphisms, which were genome-wide significantly associated with plasma homocysteine levels in 57,644 subjects from the Coronary ARtery DIsease Genome wide Replication and Meta-analysis (CARDIoGRAM) plus The Coronary Artery Disease (C4D) Genetics (CARDIoGRAMplusC4D) consortium genome-wide association study and were known to be associated at $p < 5 \times 10^{-8}$, were used as an instrumental variable.

Results: None of the nine single nucleotide polymorphisms were associated with coronary heart disease or acute myocardial infarction ($p > 0.05$ for all). Mendelian randomization analysis revealed no causal effects of plasma homocysteine levels, either on coronary heart disease (inverse variance weighted; odds ratio = 1.015, 95% confidence interval = 0.923–1.106, $p = 0.752$) or on acute myocardial infarction (inverse variance weighted; odds ratio = 1.037, 95% confidence interval = 0.932–1.142, $p = 0.499$). The results were consistent in sensitivity analyses using the weighted median and Mendelian randomization-Egger methods, and no directional pleiotropy ($p = 0.213$ for coronary heart disease and $p = 0.343$ for acute myocardial infarction) was observed. Sensitivity analyses confirmed that plasma homocysteine levels were not significantly associated with coronary heart disease or acute myocardial infarction.

Conclusions: The findings from this Mendelian randomization study indicate no causal relationship between plasma homocysteine levels and coronary heart disease or acute myocardial infarction. Conflicting findings from observational studies might have resulted from residual confounding or reverse causation.

Keywords

Two-sample mendelian randomization, genome-wide association study, plasma homocysteine levels, coronary heart disease, acute myocardial infarction, causation

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Introduction

With the improvement of people's standard of living, the change in dietary structure and the increase in

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population ageing, the numbers of patients with coronary heart disease (CHD) and acute myocardial infarction (AMI) are increasing. Many risk factors contribute to cardiovascular disease (CVD), including age, sex, smoking, obesity, hypertension, hyperlipidemia, diabetes, family genetic history and other abnormalities.[1–4] In addition, homocysteine (Hcy), as a newly discovered risk factor, has gradually become a research hotspot.[5] Not only can it affect blood pressure levels, serum glucose levels, and lipid and lipoprotein metabolism, but it could also promote the development of inflammation and result in CVD.[6] An epidemiological study suggests that the incidence of high homocysteine (HHcy) levels in Chinese populations is greater than that in European and American populations. Due to the influence of daily life habits, diet structure, genetics, and the environment, the incidence of HHcy levels in China has gradually increased.[7] Such epidemiological trends have also gradually attracted widespread attention.

Traditional observational epidemiology has met many challenges in discovering the cause of disease and causal inference. When researchers resort to the design of randomized controlled trials (RCTs) to find evidence of a direct association between exposure factor X and disease outcome Y, there are limitations due to human medical ethics and the inherent characteristics of many experimental designs.[8] These trials are difficult to carry out. In recent years, the Mendelian randomization (MR) design has introduced the concept of an instrumental variable (IV) from econometrics, treating genetic variation as a tool variable of exposure factors to be studied, which provides an effective solution to the above problems.[9]

Two-sample Mendelian randomization (TSMR) analysis is a commonly used method with several advantages.[10] First, with the advent of genome-wide association studies (GWASs), large amounts of data from GWASs have been published. Second, using the association established by observational studies to conduct two-cohort studies is equivalent to enlarging the sample size of the study, which can improve the effectiveness of the test. In addition, the sample size of published GWASs is usually large, and the number of IVs is extremely large, which increases the genetic interpretation of IVs with regard to exposure and is more conducive to accurate and reliable results.[11] In this study, we verified the assumption that CHD and AMI are caused by HHcy levels. Next, we estimated the causal effect of Hcy levels on CHD and AMI by the TSMR method.

Methods

Data sources

We selected genetic variants associated with plasma Hcy levels and then extracted the corresponding effect sizes for CHD and AMI using the largest GWAS summary-level dataset.[12] No ethics approval was needed for our study due to this being a re-analysis of previously collected and published data. Plasma Hcy data ($n = 57{,}644$) were extracted from the UK Biobank imputed genotype data (http://www.ebi.ac.uk/efo/EFO_0004578).[13,14] Additionally, CHD was confirmed in more than one of the three major coronary arteries or their major branches (branched diameter ≥ 2 mm) ($\geq 50\%$), and AMI was diagnosed based on common standards including (a) symptoms of persistent ischemic chest pain; (b) ischemic changes on electrocardiogram with dynamic evolution; and (c) increases in the levels cardiac biomarkers according to the published guidelines. The dataset was acquired from the Coronary ARtery DIsease Genome wide Replication and Meta-analysis (CARDIoGRAM) plus The Coronary Artery Disease (C4D) Genetics (CARDIoGRAMplusC4D) consortium ($n = 184{,}305$ for CHD, and $n = 171{,}875$ for AMI).[12] All of the datasets were from European populations included in RCTs and population-based studies. Genomic control was applied to each sample to correct for inflated test statistics due to potential population stratification in our datasets.

Study design

In our TSMR analysis, the genetic variants that were included as IVs satisfied the following three assumptions and are shown in Figure 1: (a) IVs must be strongly associated with the outcomes, including CHD and AMI; (b) IVs should be independent of any known confounders; and (c) the selected IVs should be conditionally independent of the outcomes (CHD or AMI), exposure (plasma Hcy levels) and confounders. Satisfaction of the second and third assumptions serves as a definition of independence from pleiotropy.

Selection and validation of IVs

IVs had to be associated with the exposure (plasma Hcy levels). To ensure a close relationship between IVs and plasma Hcy levels, the selected p value had to be less than 5×10^{-8} in the corresponding GWAS summary-level dataset. In addition, we used PLINK 1.90[15] to calculate the pairwise-linkage disequilibrium (LD) to ensure independence among the selected IVs. When $r^2 > 0.001$, the single nucleotide polymorphisms (SNPs) were removed from our analysis.

The selected IVs were conditionally independent of CHD or AMI, given the traits related to plasma Hcy levels, and independent of any known confounders. This ensures that the IVs influence CHD or AMI only through plasma Hcy levels rather than another

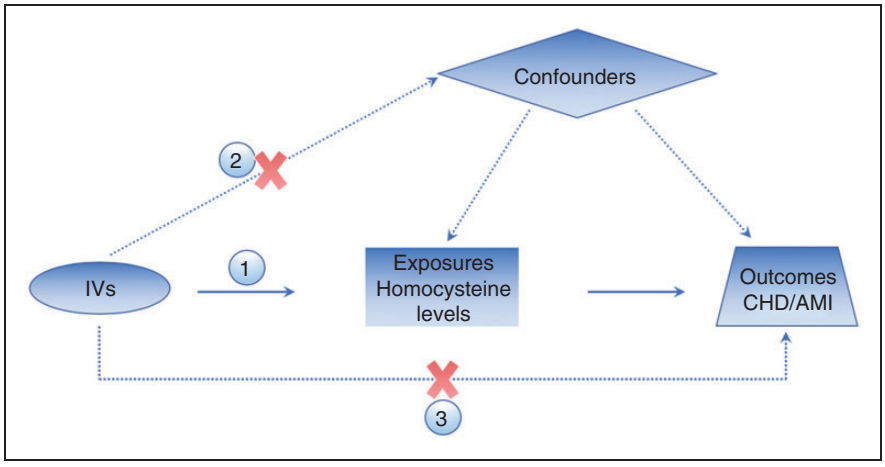

Figure 1. Schematic representation of two-sample Mendelian randomization (TSMR) analysis. Three assumptions of Mendelian randomization (MR) analysis are as follows: (1) instrumental variables (IVs) must be associated with plasma homocysteine levels, (2) IVs must not be associated with confounders, and (3) IVs must influence coronary heart disease (CHD)/acute myocardial infarction (AMI) only through plasma homocysteine levels.

pathway or confounders. This is consistent with the previous two assumptions.[16] First, we obtained the corresponding estimates of the effects of these variables on CHD or AMI. If the selected SNPs were not correlated with CHD or AMI, we chose the proxy SNPs that were highly correlated ($r^2>0.8$) based on the SNP Annotation and Proxy (SNAP) search system for substitution.[17] Then, we employed MR-Egger regression to evaluate the horizontal pleiotropic pathway.[18] Subsequently, we removed any palindromic SNPs with minor allele frequencies above 0.3 to ensure that the effects of the SNPs on the exposure (plasma Hcy levels) corresponded to the same allele as did their effects on CHD or AMI.[19] Next, we used the GWAS Catalog (https://www.ebi.ac.uk/gwas/) to check for the associations between selected IVs and to adjust for potential confounding factors. Additionally, we calculated the *F* statistic with a Web application (https://sb452.shinyapps.io/overlap/) to detect the association of selected IVs with the exposure.[20]

Pleiotropy assessment

We used MR-Egger regression to evaluate the horizontal pleiotropic pathway between IVs and CHD or AMI, independent of plasma Hcy levels.[18] MR-Egger regression, as an effective way to examine the publication bias in meta-analysis, was developed from Egger regression. This approach is expressed as $\alpha_i = \beta_{\gamma i} + \beta_0$. In this equation, α_i is the effect between IVs and CHD or AMI; γ_i is the estimated effect between IVs and plasma Hcy levels; slope β is the estimated causal effect of plasma Hcy levels on CHD or AMI; and intercept β_0 is the estimated average value of the horizontal pleiotropic pathway. If the intercept has $p>0.05$, then that indicates no horizontal pleiotropic pathway exists. In addition, the slope also gave us the estimated pleiotropy-corrected causal effect. However, this estimate may be underpowered if the selected SNPs collectively fail to explain a large proportion of the variance in the exposure.[18]

TSMR analysis

We employed the inverse variance-weighted (IVW) method to evaluate the causal effect between plasma Hcy levels and CHD or AMI in the TSMR analysis in this study.[21] The causal effect β was estimated and is shown as w_i (α_i/γ_i). In this equation, i refers to the IVs, α_i represents the association effect of IVs on CHD or AMI, γ_i defines the association effect of IVs on plasma Hcy levels, and w_i represents the weights of the causal effect of plasma Hcy levels on CHD or AMI.

TSMR sensitivity analysis

The weighted median, simple median, maximum likelihood and penalized weighted median methods were employed to analyze the follow-up sensitivity in our current study.[22] Compared with the IVW, the weighted median, simple median, maximum likelihood and penalized weighted median methods are more robust for individual genes with strongly outlying causal estimates and generate a consistent estimate of the causal effect when valid IVs exceed 50%.[16,23] Subsequently, we used a leave-one-out sensitivity analysis to determine whether the influence of a single SNP disproportionately affected the association. Then, we performed TSMR analysis again leaving out each SNP in turn, and the overall analysis including all SNPs was shown

for comparison.[24] All of the analyses were implemented by the "TwoSampleMR" package in the R software environment.

Results

IV selection and validation

In total, we obtained nine IVs for CHD and nine IVs for LD-independent AMI ($r^2<0.001$). These IVs achieved genome-wide significance ($p < 5\times10^{-8}$) in plasma Hcy level datasets, but not all of the SNPs were directly found in the CHD or AMI datasets. The details of all independent IVs in this TSMR analysis are shown in Table 1. Subsequently, we used the intercept term to estimate the exposures from MR-Egger regression and found that no horizontal pleiotropic pathway existed in our TSMR analysis (Table 2). We analyzed the *F* statistics to identify the strength of the relationship between IVs and exposures. If the *F* statistics were greater than 10, this was considered strong enough to mitigate any bias from the causal IV estimate. The *F* statistics for our selected IVs were 1280 for CHD and 2561 for AMI, which were strong enough to mitigate any bias from the causal IV estimate.

Analyzed by TSMR and sensitivity analysis

According to the IVW analysis results, the odds ratio (OR) and 95% confidence interval (CI) per unit increase in plasma Hcy level within CHD were 1.015 (0.923–1.106), $p=0.752$, and 1.037 (0.932–1.142), $p=0.499$. These results suggest that genetically predicted plasma Hcy levels were not associated with CHD or AMI (Figure 2). The overall estimates, calculated by IVW or MR-Egger, did not reveal associations between plasma Hcy levels and CHD or AMI (Figure 3). Sensitivity analyses using the leave-one-out associations approach also confirmed the lack of associations (Figure 4).

Discussion

The concentration of plasma Hcy increases with increasing age and is positively correlated with dietary methionine intake but negatively correlated with plasma folic acid and vitamin B_6 and B_{12} levels. Smoking, drinking, consuming coffee and overweight status can increase the plasma Hcy concentration, and their combined effect is greater than the single effect.[25] It is generally believed that a fasting plasma Hcy>10 μmol/l is defined as a HHcy level. Moreover, many studies have suggested that a HHcy level is an independent risk factor for CVD and stroke. A direct association between plasma Hcy levels and CVD has been found in observational population

Table 2. Mendelian randomization (MR)-Egger regression intercepts.

Exposure	Outcome	Intercepts (95% CI)	*p*-Value
Homocysteine levels	CHD	0.010 (–0.004–0.024)	0.213
Homocysteine levels	AMI	0.009 (–0.008–0.025)	0.343

AMI: acute myocardial infarction; CHD: coronary heart disease; CI: confidence interval.
The significant result ($p > 0.05$) indicates that the *y*-intercept of the MR-Egger regression line is not significantly different from zero and thus no pleiotropy exists.

Table 1. Genome-wide significant single nucleotide polymorphisms (SNPs) for homocysteine (Hcy) levels and their association with coronary heart disease (CHD) and acute myocardial infarction (AMI).

				Hcy			CHD			AMI		
SNP	Gene	E/O allele	Eaf	Beta	SE	*p*	Beta	SE	*p*	Beta	SE	*p*
rs12780845	CUBN	A/G	0.65	0.0529	0.009184	8.00×10^{-10}	–0.004668	0.010052	0.642386	–0.001224	0.011201	0.912996
rs154657	DPEP1	A/G	0.47	0.0963	0.006888	2.00×10^{-43}	0.015338	0.010241	0.134226	0.026263	0.01144	0.021692
rs1801133	MTHFR	A/G	0.34	0.1583	0.007653	4.00×10^{-104}	–0.014295	0.010761	0.18403	–0.012974	0.011941	0.277272
rs1801222	CUBN	A/G	0.34	0.0453	0.006888	8.00×10^{-10}	0.007535	0.01048	0.472165	0.005501	0.011641	0.636532
rs2851391	CBS	T/C	0.47	0.056	0.008163	2.00×10^{-12}	0.010318	0.009466	0.275702	0.004664	0.010479	0.656272
rs4660306	MMACHC	T/C	0.33	0.0435	0.006888	2.00×10^{-9}	0.000713	0.010004	0.943179	–0.006438	0.0111	0.561914
rs548987	SLC17A3	C/G	0.13	0.0597	0.009949	1.00×10^{-8}	0.005065	0.01424	0.722074	0.002453	0.016096	0.878883
rs838133	FUT2	A/G	0.45	0.0422	0.007143	7.00×10^{-9}	–0.000168	0.011573	0.988418	0.003258	0.01273	0.797976
rs9369898	MUT	A/G	0.62	0.0449	0.007143	2.00×10^{-10}	0.006616	0.009389	0.481011	0.01372	0.010397	0.186951

Eaf: allele frequency; E/O allele: effect allele/other allele; SE: standard error.

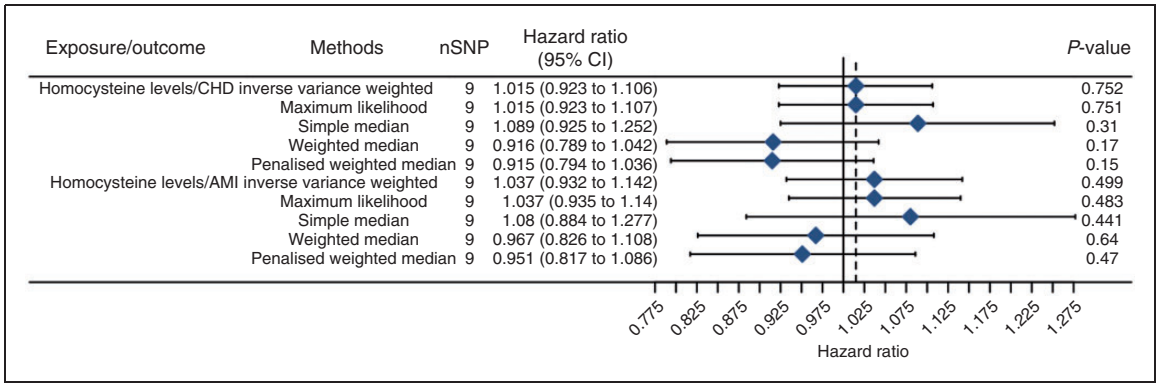

Figure 2. Two-sample Mendelian randomization of plasma homocysteine levels and the risk of coronary heart disease (CHD) or acute myocardial infarction (AMI). We used the unit increase in plasma homocysteine levels genome-wide association study (GWAS) summary-level statistics. The results are standardized to a one-unit increase in exposure. CI: confidence interval; SNP: single nucleotide polymorphism.

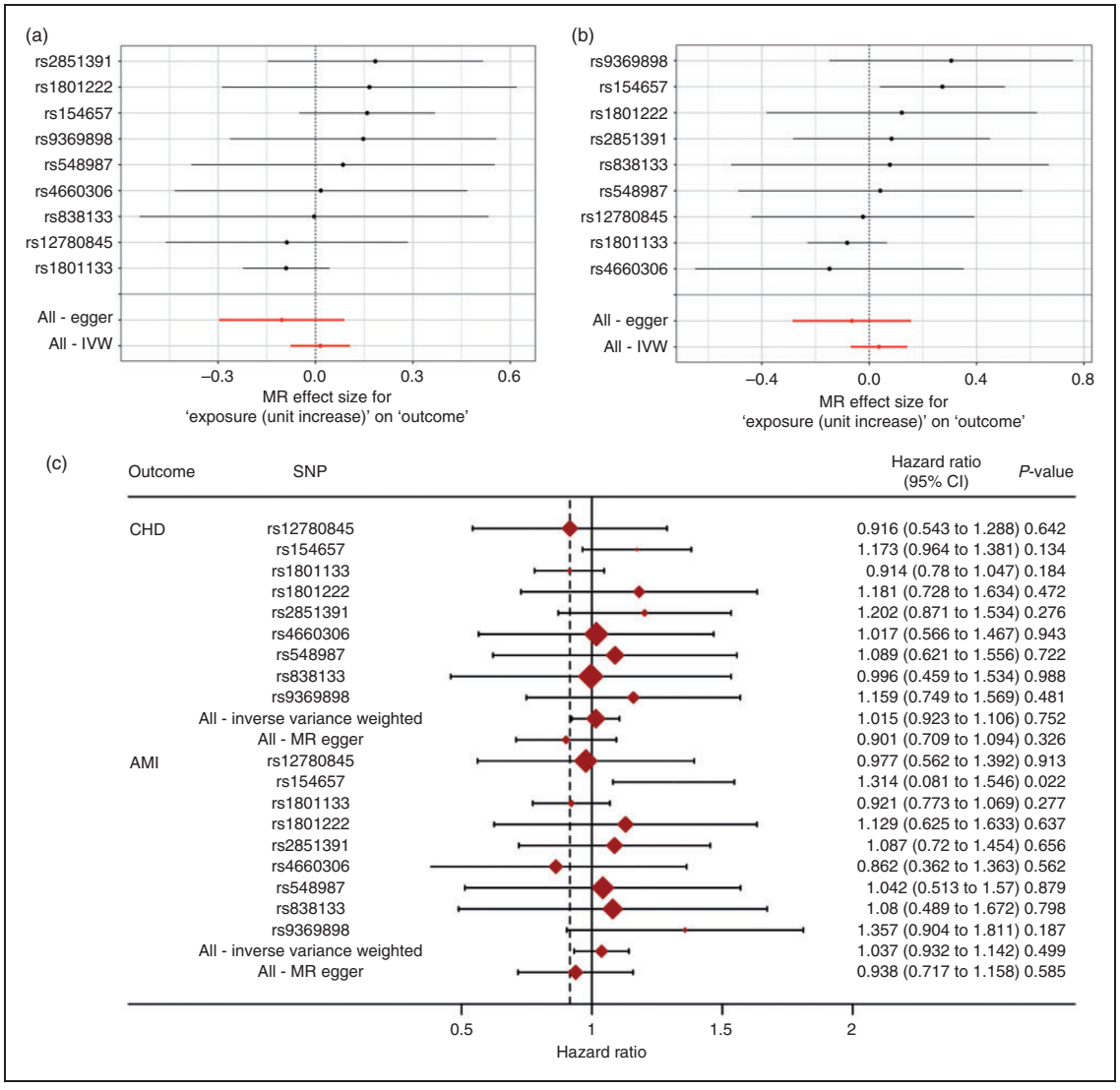

Figure 3. Results of the single- and multi-single nucleotide polymorphism (SNP) analyses for the SNP effect of plasma homocysteine level on outcomes. (a) Coronary heart disease (CHD); (b) acute myocardial infarction (AMI); and (c) the details about the results. CI: confidence interval; IVW: inverse variance-weighted; MR: Mendelian randomization.

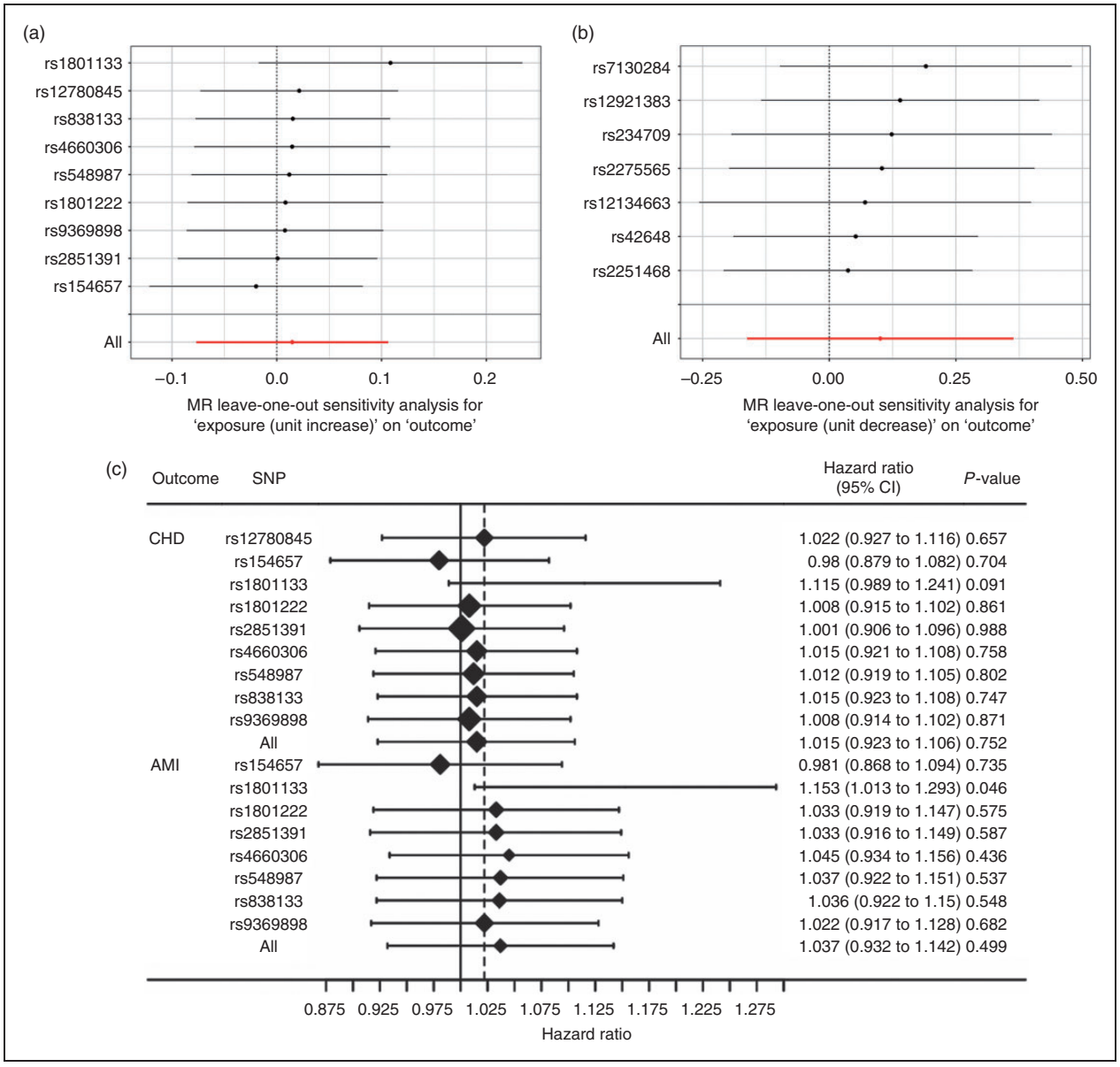

Figure 4. Sensitivity analyses using the leave-one-out approach for the association of plasma homocysteine level with outcomes. (a) Coronary heart disease (CHD); (b) acute myocardial infarction (AMI); and (c) the details about the results. CI: confidence interval; MR: Mendelian randomization; SNP: single nucleotide polymorphism.

epidemiological studies, such as the discovery of HHcy in the general healthy population. High cysteine levels increase the risk of CVD.[26] Recently, Olsen et al.[27] showed a potential interaction between plasma total Hcy and serum Vitamin A (Vit-A, retinol) in relation to incident AMI. Plasma total Hcy was higher among AMI patients in the upper versus lower Vit-A tertile, and was associated with AMI only in the upper Vit-A tertile. These findings may shed light on the hitherto unclear relationship between Hcy and CVD. A meta-analysis of prospective studies found that reduced Hcy levels were associated with a reduced risk of CHD and stroke.[28] Hcy may cause atherosclerosis, leading to CHD and AMI, through the following five pathways: (a) vascular endothelial cell damage and dysfunction; (b) dyslipidemia; (c) stimulating vascular smooth muscle cell proliferation; (d) enhancing coagulation function and inducing thrombosis; and (e) promoting the expression of inflammatory factors.[29–31]

However, other scholars have questioned the conclusion that HHcy is not an independent risk factor for CVD and stroke. The latest meta-analysis from Cochrane, Oxford Evidence-based Medicine, in 2017 showed that HHcy can increase the stroke risk and

that HHcy intervention was limited to patients with hypertension and genetic mutations in China, suggesting that HHcy could not be regarded as an independent risk factor, like hypertension, hypercholesterolemia, smoking, and diabetes.[32] Cohort studies have found that some inflammatory biological factors, such as C-reactive protein and Hcy levels, can be used as biomarkers to improve the predictive ability of CVD prediction models constructed with traditional risk factors, especially for low- and medium-risk groups, such as the Framingham, the Multi-Ethnic Study of Atherosclerosis (MESA), and the National Health and Nutrition Examination Survey III (NHANES III) models.[33] However, in the MESA and NHANES III models, it was observed that when Hcy was added to the traditional risk factors used for prediction, the diagnostic accuracy of the working characteristic curve increased by only 1.6% and 2.5%, respectively, and the predictive ability of the model only slightly improved. Such a small change is of little significance to patient management, as is the case from the perspective of public health.[34] The American Academy of Cardiology (ACC)/American Heart Association (AHA) guidelines for cardiovascular risk assessment in 2013[35] and the European guidelines for clinical practice in CVD in 2012[36] did not regard HHcy as a risk factor for CVD.

RCTs are the most powerful method of demonstrating the hypothesis of etiology in epidemiological research. However, RCTs require more rigorous research design and cost more. Therefore, it is difficult to implement RCTs. The application of MR in traditional epidemiology can ingeniously remedy the shortcomings of traditional epidemiological research in identifying the etiology, such as confounding factors and unclear causal sequence, and provide new ideas and methods for epidemiological research with regard to etiology.[37] Since the genotype of the offspring is also inherited randomly from their parents, it is a very reliable method to use the SNP as a genetic variable tool to infer a causal relationship between two factors. In recent years, MR research has been described by some researchers as the best alternative to RCTs.[38] In our study, we analyzed the correlation between plasma Hcy levels and CHD or AMI with the aid of a large-scale GWAS. It was found that an increase in Hcy levels did not directly lead to the occurrence of CHD or AMI. More recently, Chen et al.[39] also assessed the association between serum Hcy levels and ST-segment elevation myocardial infarction (STEMI), and showed that Hcy was not elevated in STEMI patients regardless of Killip severity. These findings suggest that Hcy as a lone risk factor in AMI patients is small and that the association with outcome is quite fragile. Hcy may be a bystander instead of a causative factor.

Study limitations

There were several limitations in our studies. First, because we only used summary statistics and had no access to the original individual clinical outcome measures, we could not conduct analyses stratified by subtypes of CHD or AMI. Second, different standards of quality control in individual-level GWASs may affect our results. Therefore, the results cannot be easily generalized. Finally, we only reveal the relationship between homocysteine and CHD or AMI from a genetic point of view, without involving other environmental factors.

Conclusions

Using a genetic approach, we found that plasma Hcy levels are not causally associated with CHD or AMI risk. However, additional human and animal studies are still needed to further confirm our TSMR results.

Author contribution

LM and G-XD conceived the study, participated in the design, performed the statistical analyses, and drafted the manuscript. R-XY conceived the study, participated in the design and helped to draft the manuscript. R-JN and SY drafted the paper. YW and HL revised the paper. All authors gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Declaration of conflicting interests

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