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Genetically predicted lipid traits mediate the association between folic acid and atherosclerosis

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Atherosclerosis (AS) is one of the most common causes of death from cardiovascular disease, and low folic acid (FA) levels have been reported to be strongly associated with an increased risk of AS. We aimed to obtain causal estimates of the association between FA and AS and to quantify the mediating role of known modifiable risk factors. Based on the largest genome-wide association study (GWAS) from the IEU Open GWAS Project for all human studies, we conducted a two-sample Mendelian randomization (MR) study of genetically predicted FA and AS. A two-step MR design was then used to assess the causal mediating effect of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) on the relationship between FA and AS. This MR analysis showed that genetically determined FA levels [IVW: Odds Ratio (OR) = 0.623, 95% CI 0.421–0.924, *P* = 0.018] were associated with a reduced risk of AS. Inverse variance weighted (IVW) MR analysis also showed that genetically predicted FA was positively correlated with HDL-C levels (OR = 1.358, 95% CI 1.029–1.792, *P* = 0.031) and negatively correlated with LDL-C (OR = 0.956, 95% CI 0.920–0.994, *P* = 0.023) and TG levels (OR = 0.929, 95% CI 0.886–0.974, *P* = 0.003). LDL-C, HDL-C, and TG mediate 3.00%, 6.80%, and 4.40%, respectively, of the total impact of FA on AS. The combined effect of these three factors accounts for 13.04% of the total effect. Sensitivity analysis verifies the stability and reliability of the results. These results support a potential causal protective effect of FA on AS, with considerable mediation through many modifiable risk factors. Thus, interventions on levels of LDL-C, HDL-C, and TG have the potential to substantially reduce the burden of AS caused by low FA.

Keywords Folic acid, Low-density lipoprotein cholesterol, High-density lipoprotein cholesterol, Triglycerides, Atherosclerosis, Mendelian randomization

Atherosclerosis (AS) is a progressive disease characterized by the pathological accumulation of plaque in the arterial wall. Endothelial cell dysfunction is thought to be the key process that promotes atherosclerosis. AS is a potential cause of approximately 50% of deaths due to its frequent development into ischaemic heart disease, acute myocardial infarction, and peripheral vascular disease[1–3]. AS is thought to be caused by a variety of factors, including genetic and environmental factors. Risk factors include hyperhomocysteinemia (HHcy), hyperlipidemia (HL), and aberrant epigenetic mechanisms associated with gene-environment interactions[4,5]. In an in vivo and in vitro study, the authors noted that folic acid (FA) supplementation attenuated the increase in atherosclerosis induced by HL and hhcy[6].

FA is an essential B vitamin for cardiovascular health. Previous studies have found that low folate levels are associated with atherosclerotic events[7–9]. More precisely, low FA levels are strongly associated with an increased risk of vascular disease in humans, including atherosclerosis and stroke, in humans. Conversely, high circulating FA concentrations reduce the risk of primary coronary events[10]. Patrick et al.[11] found that in HHcy, FA supplementation may control cholesterol content by lowering plasma homocysteine levels. In addition, FA has been shown to stabilize arterial plaque, and supplementation with low doses of FA is also effective at lowering total cholesterol (TC) and LDL-C[12]. Meanwhile, Omid et al.[13] pointed out in a study that supplementing folic acid can reduce serum triglyceride (TG) and TC concentrations. Therefore, we hypothesize that the mediating role of LDL-C, high-density lipoprotein cholesterol (HDL-C), or TG may at least partially explain the association between FA and AS.

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However, it is worth noting that traditional observational studies are limited in their ability to identify causal effects and explain potential confounding factors. Mendelian randomization (MR)-mediated analysis can be used to address these unmet research needs. Since MR has been developed to assess causality by using genetic variation as an instrumental variable for the exposure of interest, it can effectively avoid the limitations imposed by observational studies. The advantages of this method also apply to mediated analyses; for example, mediated analyses do not require any unmeasured confounding between exposure, mediator, and outcome, which is difficult to achieve in traditional observational methods. In this study, we aimed to investigate the effect of FA on the risk of AS and to establish the role of LDL-C, HDL-C, and TG as mediators by means of a two-step two-sample MR.

Data and methods

Study design overview

In this study, a two-step two-sample MR design was used to explore the causal mediating effect of LDL-C, HDL-C and TG (mediator) on the relationship between FA (exposure) and AS (outcome) based on publicly available large-scale GWAS data of the IEU Open GWAS Project (https://gwas.mrcieu.ac.uk/). First, we tested the effect of FA on AS, and then tested the effect of potential mediating using a two-step MR. Our first step was to examine FA's causal effect on potential mediators, while our second step was to examine potential mediators causal effect on AS. Indirect effect (mediated effect) was estimated by subtracting the direct effects from the total effects. Supplementary Fig. 1 illustrates the flow diagram of our MR study.

Data sources

Genetic association estimates for FA were obtained from the human GWAS database comprising of an analysis from 64,979 participants of European ancestry, which included 9,851,867 autosomal single nucleotide polymorphisms provided by the MRC-IEU. The LDL-C GWAS dataset consists of 440,546 samples and 12,321,875 SNPs. The GWAS dataset associated with HDL-C consists of 9796 samples and 23,170,761 SNPs. The GWAS dataset for TG consists of 441,016 samples and 12,321,875 SNPs. Finally, genetic variants in AS were identified from GWAS including 213,140 participants and 16,380,428 SNPs. To avoid bias due to overlapping exposure and outcome samples, we obtained exposure and outcome samples from different databases. In addition, all participants were of European origin and there was no ethnic discrimination.

Our study used only published or publicly available GWAS summary data that has been approved by the applicable ethics and institutional review committee, and therefore does not require ethical approval. In this study, detailed information on all GWAS data is presented in Supplementary Table 1.

Instrumental variables (IVs)

We extracted independent SNPs that were strongly associated with exposure as IVs, filtered by a criterion of $P < 5 \times 10^{-5}$ in a 5000 kb window around leader SNPs that were less associated with other SNPs in the region (LD, $r^2 < 0.01$)[14]. Last but not least, in order to determine the statistical of the selected SNPs, the F statistic was calculated based on the formula $F = R^2 (N\text{-}k\text{-}1)/[(1 - R^2) k]$, where R^2 is the proportion of variability explained by each SNP, N is the GWAs sample size, and k is the number of SNPs. A F-statistic is < 10 indicates that the IV is a weak instrument and is therefore not used.

Proportion of mediation effects

The overall effect of exposure on outcomes can be divided into indirect and direct effects[15]. After adjusting for LDL-C, HDL-C, and TG, MR revealed a direct effect of FA on the risk of AS. The indirect effects of LDL-C, HDL-C and TG were calculated using the product method by multiplying the effects of AS on LDL-C, HDL-C and TG and the effects of LDL-C, HDL-C and TG on AS. The following equation is used to calculate the proportion of mediating effect[16]:

$$E(\%) = \frac{\sum_{k=1}^{k} \beta_1 \times \beta_{2\ldots k}}{\sum_{k=1}^{k} \beta_3 + \beta_1 \times \beta_{2\ldots k}}$$

In the formula, β_1 represents the MR effect of FA on the mediator k via, β_2 represents the MR effects of mediator k on AS risk, and β_3 represents the MR effects of FA on AS risk.

Mendelian randomization analyses

We performed all analyses in R software version 4.2.0 with the TwoSampleMR package version 0.5.6. For the statistical study, the "MR-PRESSO" and "MR-RAPS" procedures were used. Different MR methods have different degrees of explanatory and application contexts and vary in statistical efficiency, so we use many MR methods to estimate causal effects. We use estimates of causal effects from inverse variance weighted (IVW) models as the main result. To verify the robustness of the MR results, Cochran's Q-statistic was performed to detect heterogeneity between IVs[17]. In addition, a series of sensitivity analyses were carried out by weighted median (WM)[18], MR-Egger regression[19], MR-Robust Adjustment Profile Score (MR-RAPS)[20], radial regression of MR (Radial MR)[21], MR-Pleiotropy Residuals sum and Outliers (MR-PRESSO)[22] analysis and leave-one-out analysis. MR-PRESSO detects outliers with horizontal multiplicativity ($P < 0.05$) and returns corrected causal estimates after removing them[22]. Leave-one-out analyses were conducted to determine whether SNPs strongly affected the stability of causal estimates[23]. A nominally significant result was determined at the $P < 0.05$ threshold. In addition, we performed Bonferroni corrections for multiple testing of the two-step MR test to determine statistically significant results. The significant p-value threshold was therefore set at $P < 0.017$ (0.05/MR test parameter). Finally,

to facilitate the reader's assessment of our results, we combined the MR (STROBE-MR) Reporting Guidelines for Enhanced Epidemiologic Observational Studies (Supplementary Table 2).

Results

Selection of IVs

At the start of our analysis, we excluded SNPs that did not meet the genome-wide significance criteria ($P < 5 \times 10^{-5}$) and those that were clustered in a state of linkage disequilibrium ($r^2 < 0.01$.) Then, we excluded SNPs that had palindromic structures. We additionally exclude SNPs related to confounding factors by searching for pleiotropic associations between the SNPs used and other traits in PhenoScanner (www.phenoscanner.medschl.cam.ac.uk), and did not find any such associations. Supplementary Tables 3–9 provide details of all SNPs involved in the current study. All SNPs complied for further analysis had an F-statistic value greater than 10.

Association of genetically predicted FA with genetically predicted risk of AS

In the primary IVW MR analysis, Fig. 1 showed that an increase in genetically predicted FA was correlated with a reduced risk of genetically predicted AS, with an odds ratio (OR) of 0.623 [95% confidence interval (CI) = 0.421–0.924, $P = 0.018$].

Association of genetically predicted FA with LDL-C, HDL-C, and TG

According to our preliminary IVW analysis data showed a significant positive correlation between genetically predicted FA and HDL-C (OR = 1.358, 95% CI = 1.029–1.792, $P = 0.031$) (Fig. 2). In addition, the results of the preliminary analysis (IVW) showed a significant negative correlation between FA and LDL-C (OR = 0.956, 95% CI 0.920–0.994, $P = 0.023$) and TG (OR = 0.929, 95% CI = 0.886–0.974, $P = 0.003$). After Bonferroni correction adjusted for multiple testing ($P < 0.017$), only a negative causal relationship was found between FA and TG.

Association of genetically predicted LDL-C, HDL-C, and TG with genetically predicted AS risk

We found that genetic prediction of LDL-C (IVW: OR = 1.388, 95% CI 1.094–1.762, $P = 0.007$), TG (IVW: OR = 1.343, 95% CI 1.078–1.674, $P = 0.008$), and HDL-C (IVW: OR = 0.893, 95% CI = 0.806–0.900, $P = 0.031$) and AS were both determined at the significance level for causality ($P < 0.05$) (Fig. 3). Using the Bonferroni-corrected threshold of $P < 0.017$, the causal relationship between HDL-C and AS did not reach statistical significance.

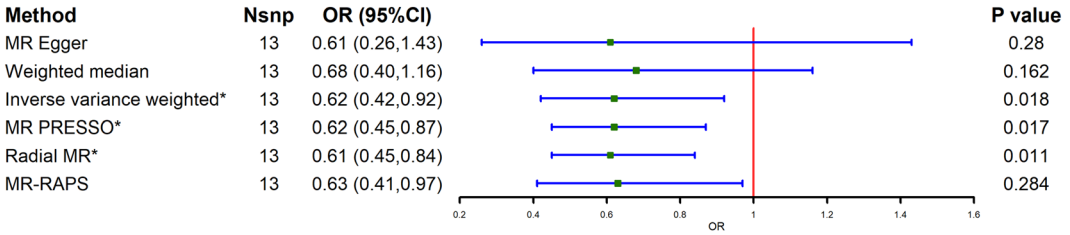

Figure 1. The causal effect of FA on AS. The green point means the effect (OR). FA, Folic acid; AS, atherosclerosis.

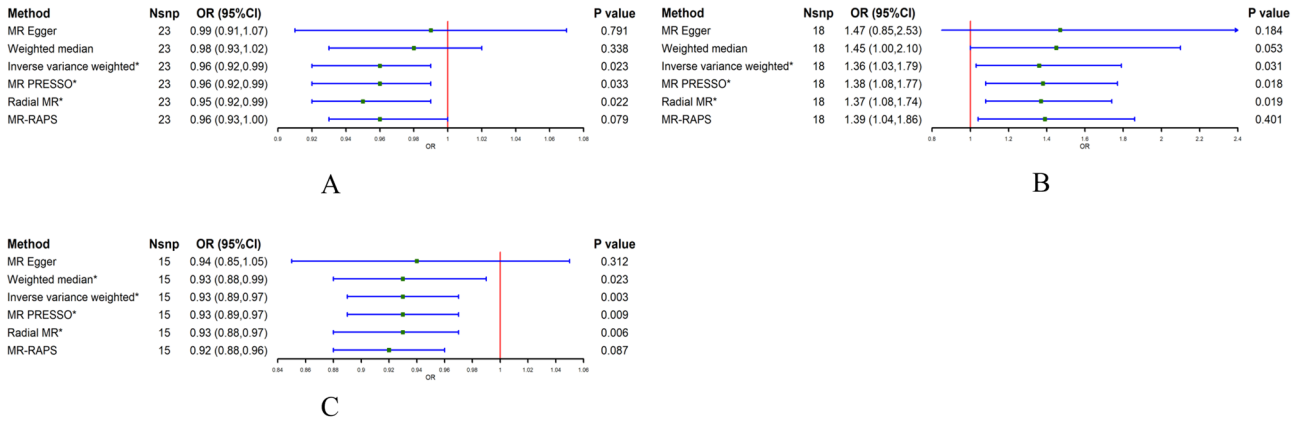

Figure 2. The causal effect of FA on Mediators. The green point means the effect (OR). (**A**) Causal effect of FA on Low-density lipoprotein cholesterol; (**B**) Causal effect of FA on High-density lipoprotein cholesterol; (**C**) Causal effect of FA on triglyceride; FA, Folic acid.

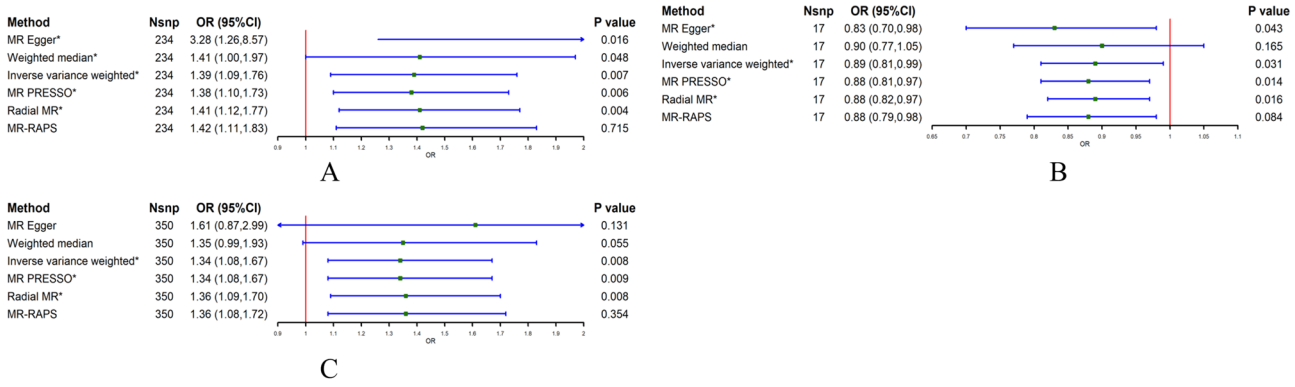

Figure 3. The causal effect of Mediators on AS. The green point means the effect (OR). (**A**) The causal effect of Low-density lipoprotein cholesterol on AS; (**B**) The causal effect of High-density lipoprotein cholesterol on AS; (**C**) The causal effect of triglyceride on AS; AS, Atherosclerosis.

Mediation of genetically predicted LDL-C, HDL-C, and TG

After the analysis revealed no reverse causality between exposure and mediators, we used LDL-C, HDL-C and TG for the mediation analysis (Supplementary Fig. 2). Table 1 shows the proportion of FA effects on each indicator-mediated AS included in the mediation analysis. For the causal effect of FA on AS, the percentages mediated by LDL-C, HDL-C and TG were 3.00%, 6.80% and 4.40%, respectively. The combined effect of these three factors accounts for 13.04% of the total effect.

Sensitivity analysis

A series of sensitivity analyses were performed to assess heterogeneity and potential horizontal pleiotropy. Cochran's Q-test showed no evidence of heterogeneity between the SNPs in terms of their causal relationships (Table 2). We further screened for possible horizontal pleiotropy in all associations by using the MR-Egger intercept term and the global test of MR-PRESSO. As there was no statistically significant difference between MR-Egger's egger-intercept term and zero ($P > 0.05$), we can infer that there was no horizontal pleiotropy in these IVs (Table 2). MR-PRESSO analyses also showed that our MR analyses were not significantly affected by

Trait	Total effect	Direct effect	Mediation effect	Proportion mediated (%)
Estimated association of FA with AS	−0.544	−0.473	−0.071	13.04
Estimated association of mediators with AS				
LDL-C	−0.487	−0.473	−0.015	3.00
HDL-C	−0.507	−0.473	−0.035	6.80
TG	−0.495	−0.473	−0.022	4.40

Table 1. MR analysis of the correlation between genetically predicted FA and genetically predicted AS risk and the mediating role of risk factor explanations. FA, Folic acid; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, triglyceride; AS, atherosclerosis.

		Pleiotropy test			Heterogeneity test					
		MR-Egger			MR-Egger			IVW		
Exposure	Outcome	Intercept	SE	*P*	Q	Q-df	Q-*P*val	Q	Q-df	Q-*P*val
FA	AS	0.001	0.017	0.963	8.683	11	0.651	8.686	12	0.729
FA	LDL-C	−0.001	0.002	0.369	27.237	21	0.163	28.328	22	0.165
FA	HDL-C	−0.004	0.012	0.742	12.874	16	0.682	12.987	17	0.737
FA	TG	−0.001	0.002	0.761	20.019	13	0.095	20.168	14	0.125
LDL-C	AS	−0.011	0.006	0.071	212.642	232	0.814	215.932	233	0.782
HDL-C	AS	0.013	0.011	0.274	14.166	15	0.513	15.454	16	0.492
TG	AS	−0.003	0.005	0.538	382.435	348	0.099	382.853	349	0.103

Table 2. Heterogeneity and pleiotropy analysis. MR, Mendelian randomization analysis; IVW, Inverse variance weighted; FA, Folic acid; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, triglyceride; AS, atherosclerosis.

pleiotropy (Table 3). The results of the leave-one-out sensitivity analysis showed that after removing each SNP in turn, the results of the IVW analysis for the remaining SNPs were the same as those for all SNPs, and no SNPs were found that significantly affected the causal association estimates (Supplementary Figs. 3–9).

Discussion

Using summary statistics from the MRC-IEU GWAS dataset, we explored the causal relationship between FA and AS by conducting a comprehensive genetic analysis. We found that FA intake was associated with a reduced risk of AS. At the same time, this study identified LDL-C, HDL-C, and TG as potential mediators between FA and AS associations through mediation analysis. Most Importantly, for this study, in order to ensure the robustness of the results, we conducted sensitivity analysis through a variety of methods, and the results showed that there was no multiple validity and heterogeneity, while leave-one-out analysis also showed that no single SNP could significantly affect causal estimation. Therefore, all SNPs used in our analyses and the research results are reliable.

The findings of the genetic correlation analysis were in line with observational study, which found that FA may be more positively effective in reducing cholesterol, improving atherosclerosis, and increasing plaque stability[12]. A prospective cohort study showed that low maternal FA concentration in early pregnancy seemed to be associated with lower carotid distensibility in school-age children, which was an early sign of atherosclerosis[24–26]. In vitro experiments have shown that FA protects against oxidized LDL-C induced endothelial dysfunction by reducing reactive oxygen species clusters[27]. Yang et al.[6] found that FA attenuates the atherogenic function of HL and HHcy-induced increases in intermediate monocytes through DNA methylation pathways in in vivo and in vitro experiments. Another study also demonstrated that FA deficiency exacerbated atherosclerotic lesions in rats fed a high-fat diet for 12 weeks and FA had a hypolipidemic effect in atherogenic rats, thus reducing serum TC and LDL-C levels[7].

To add new perspectives to previous studies, our findings also suggest that FA may reduce the risk of AS through other important mediators (LDL-C, HDL-C, and TG). According to the literature, Olszewski and McCully et al.[28] studied the correlation between lipid metabolism and homocysteine concentrations in patients with AS and showed that patients with elevated homocysteine concentrations also had higher LDL-C cholesterol concentrations. In addition, a 2009 study showed a significant negative correlation between homocysteine and HDL-C cholesterol concentrations[29]. At the same time, Momin et al.[30] showed that the increase in serum homocysteine concentration was proportional to serum triglyceride levels. Interestingly, contemporary studies have shown that the greatest effect on reduced homocysteine concentrations can be attributed to FA[31,32]. Meanwhile, in one study it was found that after 4 weeks of continuous drinking of a beverage containing FA, the subjects had a 31.6% decrease in mean homocysteine concentration and a significant decrease in LDL-C cholesterol along with an increase in HDL-C lipoprotein cholesterol, which significantly improved the atherosclerotic index[33].

In conclusion, our findings suggest that cholesterol levels may mediate the role of FA in AS. The exact mechanism of action of this association remains to be determined, but there are still some reports that can be used to explain: Firstly, mature vascular smooth muscle cells (VSMC) retain phenotypic plasticity, and VSMC is a major component of atherosclerotic plaques[34]. Prevention of the phenotypic transition of VSMC has been shown to slow the progression of atherosclerosis[35]. It has been found that FA could ameliorate the progression of atherosclerosis in LDL-C receptor-deficient mice by inhibiting VSMC dedifferentiation through inhibition of the mTOR/p70S6K signaling pathway[36]. Secondly, FA has a protective impact on the stability of arterial plaque, with its anti-inflammatory properties possibly playing a key role in the process. FA improves arterial endothelial function, decreases inflammation, and decreases blood cholesterol levels while also lowering blood pressure and Hcy, TNF-α, and MMP-9, according to the research of Li et al.[12]. Finally, lipid metabolism is disturbed, increasing the excessive production of free radicals and disrupting the antioxidant state, thus causing endothelial cell damage; this represents the initial step in the formation of atherosclerosis[37]. Previous studies have found that folic acid supplementation to reduce Hcy may affect lipids, and Hcy exacerbates the process of LDL-C aggregate trapping by narrowing the lumen of blood vessels through endothelial dysfunction, thereby impeding the passage of LDL-C aggregates[34,38].

To further investigate mediators for FA in AS, our study has several advantages compared to previous studies: To our knowledge, this is the first MR study to assess the causal relationship between FA and AS in an European

Exposure	Outcome	RAW estimates			Outlines corrected estimates		
		N	Bate	*P* value	N	Bate	*P* value
FA	AS	13	−0.473	0.017	NA	NA	NA
FA	LDL-C	23	−0.045	0.033	NA	NA	NA
FA	HDL-C	18	0.326	0.018	NA	NA	NA
FA	TG	15	−0.074	0.009	NA	NA	NA
LDL-C	AS	234	0.323	0.006	NA	NA	NA
HDL-C	AS	17	−0.123	0.014	NA	NA	NA
TG	AS	350	0.295	0.008	NA	NA	NA

Table 3. MR-PRESSO estimates between exposures and outcomes. FA, Folic acid; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, triglyceride; AS, atherosclerosis; NA, not available.

population. In contrast to clinical observational studies, MR analysis also avoids the effects of reverse causality and confounding factors. We also carried out extensive sensitivity analysis to test the assumptions of the MR model.

Nevertheless, there are some limitations to this study that are worth noting: (1) Due to the GWAS dataset used for our analyses, our research population is solely Europeans, not people from around the world, so our research results cannot be applied globally. (2) Additional subgroup analysis was not possible due to the use of public databases and the absence of demographic data (such as gender and ethnicity) in the original European study. (3) Our analysis was based on publicly available GWAS data; however, the FA data sources we selected did not clearly define folic acid doses, so our current conclusions do not specify folic acid levels.

Conclusion

In conclusion, these results support a potential causal protective effect of FA on AS, substantially mediated by the modifiable risk factors LDL-C, HDL-C and TG. Based on our two-step, two-sample MR analyses with multiple sensitivity testing, we believe that FA can be used as a primary prevention for AS and patients with risk factors for AS.

Data availability

The authors applied a broad consent to allow research participants to query and download a wide range of their data because the data for this study was obtained via a database at https://gwas.mrcieu.ac.uk/.

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Author contributions

All authors have substantially contributed to the design, performance, analysis, and reporting of the work. W.H. Designed and performed the study. H.W. collected and analyzed the data. H.C. interpreted the results. J.Q. wrote the paper. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

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Competing interests

The authors declare no competing interests.

Additional information

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