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ORIGINAL RESEARCH

Associations Between Genetically Predicted Iron Status and Cardiovascular Disease Risk: A Mendelian Randomization Study

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BACKGROUND: Mendelian randomization (MR) studies suggest a causal effect of iron status on cardiovascular disease (CVD) risk, but it is unknown if these associations are confounded by pleiotropic effects of the instrumental variables on CVD risk factors. We aimed to investigate the effect of iron status on CVD risk controlling for CVD risk factors.

METHODS AND RESULTS: Iron biomarker instrumental variables (total iron-binding capacity [n=208 422], transferrin saturation [n=198 516], serum iron [n=236 612], ferritin [n=257 953]) were selected from a European genome-wide association study meta-analysis. We performed 2-sample univariate MR of each iron trait on CVD outcomes (all-cause ischemic stroke, cardioembolic ischemic stroke, large-artery ischemic stroke, small-vessel ischemic stroke, and coronary heart disease) from MEGASTROKE (n=440 328) and CARDIoGRAMplusC4D (Coronary Artery Disease Genome Wide Replication and Meta-Analysis Plus the Coronary Artery Disease Genetics) (n=183 305). We then implemented multivariate MR conditioning on 7 CVD risk factors from independent European samples to evaluate their potential confounding or mediating effects on the observed iron–CVD associations. With univariate MR analyses, we found higher genetically predicted iron status to be associated with a greater risk of cardioembolic ischemic stroke (transferrin saturation: odds ratio, 1.17 [95% CI, 1.03–1.33]; serum iron: odds ratio, 1.21 [95% CI, 1.02–1.44]; total iron-binding capacity: odds ratio, 0.81 [95% CI, 0.69–0.94]). The detrimental effects of iron status on cardioembolic ischemic stroke risk remained unaffected when adjusting for CVD risk factors (all $P<0.05$). Additionally, we found diastolic blood pressure to mediate between 7.1 and 8.8% of the total effect of iron status on cardioembolic ischemic stroke incidence. Univariate MR initially suggested a protective effect of iron status on large-artery stroke and coronary heart disease, but controlling for CVD factors using multivariate MR substantially diminished these associations (all $P>0.05$).

CONCLUSIONS: Higher iron status was associated with a greater risk of cardioembolic ischemic stroke independent of CVD risk factors, and this effect was partly mediated by diastolic blood pressure. These findings support a role of iron status as a modifiable risk factor for cardioembolic ischemic stroke.

Key Words: biomarkers ■ blood pressure ■ genome-wide association study ■ iron ■ ischemic stroke ■ Mendelian randomization analysis ■ risk factors

Iron is an essential nutrient needed to support many biological processes. Both extremes of iron status have been associated with adverse cardiovascular outcomes. Iron deficiency is the most prevalent micronutrient deficiency worldwide and is associated with significant comorbidities affecting 70% of patients with heart failure,[1] whereas iron overload is implicated in numerous cardiometabolic diseases.[2] When circulating iron exceeds the transport carrying capacity of transferrin, iron begins to circulate freely, generating a toxic

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CLINICAL PERSPECTIVE

What Is New?

- We leveraged a Mendelian randomization approach to explore the link between iron status on cardiovascular disease while accounting for potential confounders and conducted mediation analyses to understand how elevated iron levels contribute to cardiovascular disease risk.
- Our analyses revealed a significant relationship between higher iron status and an increased risk of cardioembolic stroke that is independent of traditional cardiovascular disease risk factors, with iron's adverse effects on diastolic blood pressure partially mediating this association.

What Are the Clinical Implications?

- Our findings underscore the potential of iron status as a novel modifiable risk factor for cardioembolic stroke prevention.
- Targeted interventions should prioritize individuals and populations predisposed to accumulating excess iron over their lifetimes.

Nonstandard Abbreviations and Acronyms

AFGen	Atrial Fibrillation Genetics Consortium
CARDIoGRAMplusC4D	Coronary Artery Disease Genome Wide Replication and Meta-Analysis Plus the Coronary Artery Disease Genetics
CES	cardioembolic stroke
DBDS	Danish Blood Donor Study
HUNT	Trøndelag Health Study
IV	instrumental variable
IVW	inverse variance weighted
LAS	large-artery stroke
MGI	Michigan Genomics Initiative
MR	Mendelian randomization
MR-PRESSO	Mendelian randomization pleiotropy residual sum and outlier test
MVMR	multivariate Mendelian randomization
TC	total cholesterol
TSAT	transferrin saturation
UVMR	univariate Mendelian randomization
WM	weighted median

iron species known as non–transferrin-bound iron. Cardiac, pancreatic, and hepatic cells all internalize non–transferrin-bound iron via different mechanisms.[3,4] Cellular uptake of non–transferrin-bound iron increases the intracellular labile iron pool, resulting in generation of reactive oxygen species and subsequent oxidative tissue damage.[5] Excess iron in cardiomyocytes has been shown to induce ferroptosis, a form of regulated cell death driven by iron-dependent lipid peroxidation that is linked to cardiovascular disease (CVD).[6]

Although a link between iron overload and CVD risk was proposed >40 years ago,[7] epidemiological data to date have shown conflicting results. Clinical studies have reported associations between atherosclerosis and increased serum iron[8] or serum ferritin[9–13] concentrations. Consistent with these observations, some studies demonstrated an association between iron depletion, either by iron chelation therapy[14] or blood donation,[15–17] and a decreased risk of CVD. Conversely, other cross-sectional[18,19] and longitudinal[20] studies have reported a lack of an association. Most studies evaluating these relationships to date have been conducted in populations with a relatively high prevalence of chronic diseases. As a result, it remains uncertain if the observed variability in iron status contributes to the onset of these diseases or is a consequence of the diseases themselves, particularly since many commonly used iron status biomarkers (eg, ferritin) can be elevated in response to inflammation.

Mendelian randomization (MR) is a statistical tool that uses genetic variation to explore causal effects of a risk factor on a health outcome. By leveraging random allocation of genetic variants at birth, MR can overcome common confounding biases typically seen in observational studies. It enables researchers to infer causal associations between an exposure, typically a modifiable one, and an outcome, even in the presence of unknown confounders. Of note, unlike a randomized controlled trial, MR reflects the lifetime effect of the exposure on the outcome and may result in greater effect estimates than those observed in a trial setting. MR operates under 3 key assumptions: the genetic instruments selected as instrumental variables (IVs) must (1) reliably predict the exposure, (2) only be associated with the outcome through its association with the exposure, and (3) not be associated with confounders of the exposure–outcome association (Figure S1). To date, few

MR studies evaluating the effects of iron status on CVD risk have been published, and these have found higher genetically predicted iron status to be associated with a decreased risk of atherosclerotic disease[21–23] and hypercholesteremia[24] and an increased risk of ischemic stroke (IS)[25] and venous thromboembolism.[22] These MR studies used 3 genetic variants in iron-regulatory genes as IVs selected from a European genome-wide association study (GWAS) of iron biomarkers in <50 000 individuals.[26] However, the latest GWAS of iron status biomarkers has reached a sample size of up to 257 953 and increased the number of genome-wide significant loci from 11 to 123.[27] Additionally, the IVs used in published studies do not satisfy all MR assumptions, as these have been reported to have genome-wide significant associations with known confounders, including cholesterol, blood pressure, and body mass index (BMI). Consequently, it is unknown if the reported associations are confounded by pleiotropic effects of the IVs on CVD risk factors.

In-depth analyses using the recently discovered genome-wide significant loci of iron status as IVs are needed to obtain reliable effect estimates of iron status on CVD risk. Our aim was to investigate the associations between genetically predicted iron status and risk of IS and coronary heart disease (CHD) by employing an MR framework that controls for CVD risk factors. A secondary aim was to investigate the pathways by which iron status influences CVD risk using mediation analyses.

METHODS

This study was conducted following the "Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian Randomization (STROBE-MR)" recommendations (Table S1). The work presented was performed using publicly available summary-level data from published GWAS. Data source details including IEU OpenGWAS (https://gwas.mrcieu.ac.uk/) IDs and direct links to download the data are shown in Table S2. All analyses were conducted using R version 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria). The MR analysis code may be obtained from the corresponding author upon reasonable request. Approval from an institutional review board was not required given that analyses were based on publicly available summary statistics and no patients were involved in the design of the study.

Exposure Data Source

Summary-level data were obtained from the largest iron GWAS available to date, which consisted of a meta-analysis of GWAS of 6 European populations (DeCODE, INTERNAL, SardiNIA, DBDS [Danish Blood Donor Study], HUNT [Trøndelag Health Study], and MGI [Michigan Genomics Initiative]), for 4 iron status biomarkers (serum iron, transferrin saturation [TSAT], serum ferritin, and total iron-binding capacity [TIBC])[27] (Table 1). Descriptive characteristics of the populations studied, the biomarker quantification methods used, and the data sources are outlined in Table S2. The physiological significance of the iron status indicators is described in Table S3.

Outcome Data Sources

Detailed information on the outcome data sources used in this study are shown in Table 1 and Table S4.

CVD Risk Factors

Known CVD risk factors were selected to (1) investigate the causal effect of iron on the risk factor using 2-sample univariate MR and (2) to investigate the confounding or mediating effects of the significant risk factors on the associations of genetically predicted iron status and CVD using multivariate MR (MVMR). The risk factors selected were blood lipids (high-density lipoprotein cholesterol, low-density lipoprotein cholesterol [LDL-C], total cholesterol [TC], triglycerides, apolipoprotein A, and apolipoprotein B), blood pressure (diastolic blood pressure [DBP] and systolic blood pressure [SBP]), BMI, and an inflammatory marker (interleukin-6). Summary statistics for blood lipids, blood pressure outcomes, and BMI were obtained from the UK Biobank, and GWAS data for interleukin-6 were from a meta-analysis of 11 independent European cohorts.[28]

Cardiovascular Diseases

CHD, all-cause IS, and IS subtypes were selected as primary CVD outcomes. CHD summary statistics data were obtained from the CARDIoGRAMplusC4D (Coronary Artery Disease Genome Wide Replication and Meta-AnalysisPlus the Coronary Artery Disease Genetics) 1000-Genomes GWAS meta-analysis,[29] which consisted of a GWAS meta-analysis of CHD in 48 multiethnic populations. Of the total sample size, 76% of the participants included were of European descent. Summary-level data for IS and IS subtypes (cardioembolic [CES], large-artery stroke [LAS], small-vessel) were obtained from a GWAS meta-analysis in 17 European populations led by the MEGASTROKE consortium.[30] We were unable to rule out potential sample overlap between the MEGASTROKE and the exposure data because both GWAS meta-analyses involved individuals from DeCODE. To mitigate the risk of type I error,[31] we tested an additional outcome closely related to IS, atrial fibrillation (AF), to validate our findings. Summary-level GWAS data for AF were obtained from a GWAS meta-analysis of European consortiums

Table 1. Summary of Exposure and Outcome GWAS Datasets

	Data source	Source ID	Consortium	Variable type	Sample size/ cases/controls	Population	Sex	PMID	Year
Iron status									
TSAT	NTNU Open Research Data	10.18710/S9TJEL	HUNT, MGI, DeCODE, INTERVAL	Continuous	198516	European	M/F	35710628	2022
Serum iron	NTNU Open Research Data	10.18710/S9TJEL	HUNT, MGI, DeCODE, INTERVAL, SardiNIA	Continuous	236612	European	M/F	35710628	2022
TIBC	NTNU Open Research Data	10.18710/S9TJEL	HUNT, MGI, DeCODE, INTERVAL, SardiNIA	Continuous	208422	European	M/F	35710628	2022
Ferritin	NTNU Open Research Data	10.18710/S9TJEL	HUNT, MGI, DeCODE, INTERVAL, DBDS	Continuous	257953	European	M/F	35710628	2022
Cardiovascular disease									
CHD	IEU OpenGWAS	ieu-a-7	CARDIoGRAMplusCD4	Binary	60801/123504	Mixed (76% European)	M/F	26343387	2015
CES	IEU OpenGWAS	ebi-a-GCST006910	MEGASTROKE	Binary	7193 / 406111	European	M/F	29531354	2018
LAS	IEU OpenGWAS	ebi-a-GCST006907	MEGASTROKE	Binary	4373 / 406111	European	M/F	29531354	2018
SVS	IEU OpenGWAS	ebi-a-GCST006909	MEGASTROKE	Binary	5386/406111	European	M/F	29531354	2018
IS	IEU OpenGWAS	ebi-a-GCST006908	MEGASTROKE	Binary	34217/ 406111	European	M/F	29531354	2018
AF	IEU OpenGWAS	ebi-a-GCST006061	AFGen, Broad AF, and UKBB	Binary	55114/ 482295	European	M/F	29892015	2018
Blood lipids									
Triglycerides	Neale Lab (R2)	30870_irnt	UKBB	Continuous	343992	European	M/F	NA	2018
TC	Neale Lab (R2)	30690_irnt	UKBB	Continuous	344278	European	M/F	NA	2018
LDL-C	Neale Lab (R2)	30780_irnt	UKBB	Continuous	343621	European	M/F	NA	2018
HDL-C	Neale Lab (R2)	30760_irnt	UKBB	Continuous	315133	European	M/F	NA	2018
Apolipoprotein B	Neale Lab (R2)	30640_irnt	UKBB	Continuous	342590	European	M/F	NA	2018
Apolipoprotein A	Neale Lab (R2)	30630_irnt	UKBB	Continuous	313387	European	M/F	NA	2018
Blood pressure									
DBP	Neale Lab (R2)	4079_irnt	UKBB	Continuous	340162	European	M/F	NA	NA
SBP	Neale Lab (R2)	4080_irnt	UKBB	Continuous	340159	European	M/F	NA	NA
Anthropometric measure									
BMI	Neale Lab (R2)	21001_irnt	UKBB	Continuous	359983	European	M/F	NA	NA
Inflammatory marker									
Interleukin-6	IEU OpenGWAS	ebi-a-GCST90012005	11 European cohorts	Continuous	21758	European	M/F	33067605	2020

AF, indicates atrial fibrillation; BMI, body mass index; CARDIoGRAMplusC4D, Coronary Artery Disease Genome Wide Replication and Meta-Analysis Plus the Coronary Artery Disease Genetics; CES, cardioembolic stroke; CHD, coronary heart disease; DBDS, Danish Blood Donor Study; DBP, diastolic blood pressure; GWAS, genome-wide association study; HDL-C, high-density lipoprotein cholesterol; HUNT, Trøndelag Health Study; IS, ischemic stroke (all-types); LAS, large-artery stroke; LDL-C, low-density lipoprotein cholesterol; MGI, Michigan Genomics Initiative; SBP, systolic blood pressure; SVS, small-vessel stroke; TC, total cholesterol; TIBC, total iron-binding capacity; TSAT, transferrin saturation; and UKBB, UK Biobank.

including AFGen (Atrial Fibrillation Genetics Consortium) and the Broad AF study.[32]

Instrumental Variable Selection

Independent single-nucleotides (SNPs) (r^2<0.001) associated with each iron trait at genome-wide significance level (P<5×10^{-8}) were extracted from the iron GWAS meta-analysis.[27] Rare SNPs with a minor allele frequency <1% in Europeans were excluded. SNPs defined as being ambiguous with intermediate allele frequencies were removed. Additionally, to further minimize the potential for pleiotropy, SNPs that had direction of effects that were not consistent with systemic iron status were removed (ie, higher iron status results in increased TSAT, serum iron, and ferritin, and decreased TIBC). Finally, SNPs that were not genotyped in the outcome data set were replaced by proxy

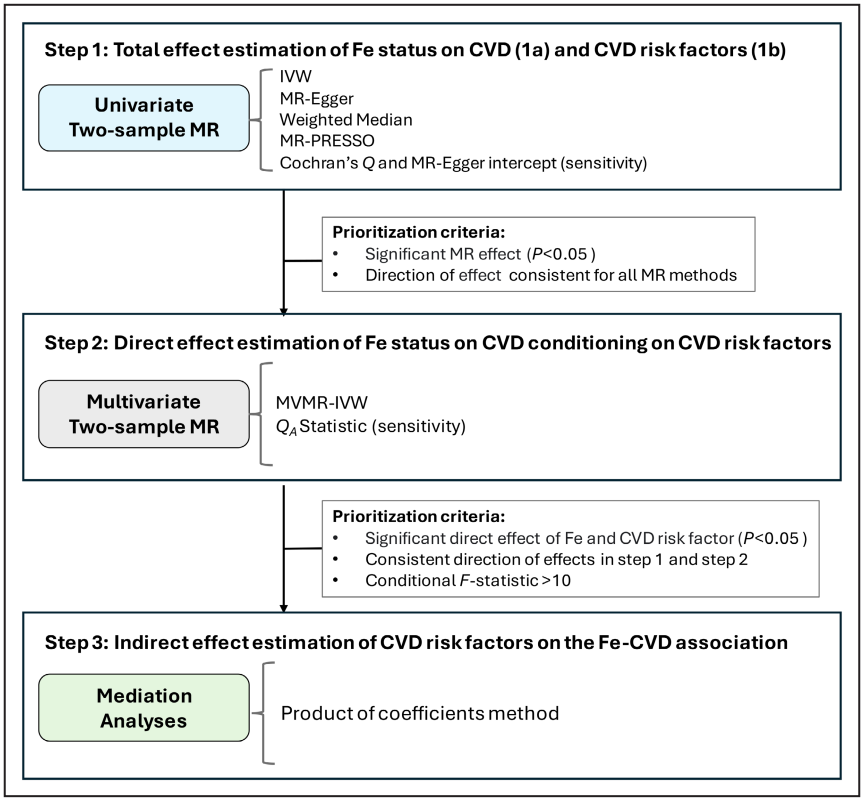

Figure 1. Study design and workflow.
CVD indicates cardiovascular disease; Fe, iron; IVW, inverse variance weighted; MR, Mendelian randomization; MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier test; and MVMR, multivariate Mendelian randomization.

SNPs if available or removed if no proxy SNP was found. Proxy SNPs were defined as a SNP in linkage disequilibrium (r^2>0.8) with the genetic instrument in a European reference population (Table S5).

The strength and validity of the genetic instruments were evaluated by calculating the variance in the iron trait explained by an SNP (R^2) and the *F*-statistic. The R^2 for each SNP was calculated using the equation $R^2=2\beta^2$ MAF (1-MAF), where β is the regression coefficient from the SNP–iron trait association from the GWAS, and MAF is the minor allele frequency for that SNP. The *F*-statistic was calculated using the equation $F\text{-statistic}=(n\text{-}k\text{-}1/k)$ ($R^2_{\text{instrument}}$ / 1- $R^2_{\text{instrument}}$), where n is the population sample size, k is the number of SNPs in the instrument, and $R^2_{\text{instrument}}$ is the sum of the R^2 for each SNP included in the instrument.[33] We considered the MR standard *F*-statistic >10 to indicate adequate instrument strength.[33] The SNPs selected as instrumental variables and their respective R^2 and *F*-statistic are shown in Table S5. The $R^2_{\text{instrument}}$ and the *F*-statistic of the instruments were calculated after data harmonization for all 4 iron biomarkers for each outcome evaluated as shown in Table S6.

Mendelian Randomization

We developed a workflow combining variations of the MR method (Figure 1). Initial analyses consisted of univariate MR analyses to identify major CVD risk factors that are influenced by iron traits (Figure 1; step 1a) and to evaluate the total effect of iron traits on CVD outcomes (Figure 1; step 1b). Variables meeting the selection criteria were carried over to step 2, which consisted of MVMR analyses to determine the direct effects of iron traits on CVD outcomes conditioning on major CVD risk factors (Figure 1; step 2). Finally, CVD risk factors satisfying the selection criteria were carried over to step 3, where these were evaluated as mediators of the iron trait–CVD associations and the indirect effects of iron traits on CVD were calculated (Figure 1; step 3). The detailed MR models implemented and the variables tested at each step are presented in Figure S2.

Univariate MR

We performed 3 main MR estimations for each exposure–outcome test, which included the inverse variance weighted (IVW) method under multiplicative

random effects,[34] the MR-Egger method, and the weighted median (WM) method. The IVW method requires either all SNPs used as IVs to be valid instruments or that there is balanced horizontal pleiotropy, whereas the MR-Egger and WM are more robust to bias introduced by weak IVs; the MR-Egger is less susceptible to bias introduced by horizontal pleiotropy,[35] and the WM provides an unbiased estimate if <50% of the SNPs used as IVs present evidence of pleiotropy.[36] When significant evidence of pleiotropy was detected (described below under Sensitivity Analysis), the WM and MR-Egger estimates were prioritized for interpretation of results. Univariate MR (UVMR) analyses were performed using the "TwoSampleMR" R package.[37] MR estimates are presented as the β and SE per 1-SD unit change in the iron status biomarker for continuous outcomes, and as odds ratio and 95% CI per 1-SD unit change in the iron status biomarker for dichotomous outcomes.

Multivariate MR

MVMR is an extension of the 2-sample UVMR method that can be used to estimate the effect of an exposure on an outcome while controlling for confounders.[38] We performed MVMR on CVD outcomes meeting our prioritization criteria conditioning on 1 CVD risk factor at a time. MVMR estimates were calculated using the IVW method and all MVMR analyses were performed using the "MVMR" R package,[39,40] which extend the typical MR main and sensitivity tests (IVW and Cochran's *Q*) to MVMR analyses. The conditional *F*-statistic, which accounts for the association between each SNP with other exposures included in the estimation, was calculated to assess instrument strength.

Mediation Analysis

Mediation analyses were conducted to assess the mechanisms by which iron status influences CVD outcomes. Effect estimates obtained from the UVMR (total effects of iron on CVD [Figure 1; step1a] and CVD risk factor [Figure 1; step1b]) and MVMR (direct effects of iron on CVD conditioning on a CVD risk factor [Figure 1; step 2]) were used to calculate the indirect effects of iron status on a CVD outcome using the product of coefficients method.[41] The SE and 95% CI were calculated using the Delta method.[42] The percentage of effect mediated by a CVD risk factor was calculated as the estimated indirect effect divided by the total effect from UVMR×100.[42] Mediation estimates are presented as the log odds ratio, SE, and 95% CI. Of note, mediation MR requires further assumptions in addition to those needed for 2-sample MR. Mediation analyses in this study were conducted under the assumption that there is no interaction between the exposure and the mediator, as there are currently no methods available to test for these interactions using summary-level data.[42]

Sensitivity Analysis

Pleiotropy refers to a phenomenon in which a variant has causal effects on >1 phenotype. In the context of MR, significant bias is introduced when there is evidence of pleiotropy within the genetic instruments (ie, if the MR assumption 3 is not met). We evaluated the presence of directional pleiotropy using the Cochran's *Q* statistic and the MR-Egger intercept for the UVMR analyses. Additionally, we performed the MR pleiotropy residual sum and outlier (MR-PRESSO) test using the "MRPRESSO" package in R, which identifies pleiotropic outliers.[43] When outliers were detected, the MR-PRESSO outlier-corrected effect estimates were calculated and presented. For MVMR analyses, heterogeneity was evaluated with the Q_A statistic. Finally, we evaluated potential pleiotropic effects of the SNPs used as genetic instruments using the online tools LDtrait[44] and PhenoScanner.[45,46] We searched for iron-associated SNPs or SNPs in linkage disequilibrium (r^2>0.8) with these SNPs that had genome-wide significant associations ($P<5\times10^{-8}$) with CVD risk factors (such as blood lipids, BMI, and blood pressure) or with CVD outcomes (such as CHD, IS, and AF) (Table S5). SNPs previously reported to have a genome-wide significant association with a CVD outcome were excluded in sensitivity analyses for MVMR.

Power Calculations

Statistical power for binary outcomes (ie, CVD outcomes) was calculated using the online tool for power calculations for MR[47] and for continuous outcomes (ie, CVD risk factors) using published methods and R code.[48] Based on our strongest instrumental variable with an R^2 of 0.055 at an α=0.05, our analyses were sufficiently powered (>80%) to detect an odds ratio ≤0.94 or ≥1.06 for CHD, ≤0.93 or ≥1.07 for IS, ≤0.81 or ≥1.19 for CES, ≤0.83 or ≥1.17 for LAS, ≤0.85 or ≥1.15 for small-vessel stroke, and ≤0.94 or ≥1.06 for AF using 2-sample MR. Detailed power calculations for each iron exposure–outcome combination are shown in Table S7.

RESULTS

Univariate MR

We used common independent SNPs (Table S5) explaining 5.5%, 3.9%, 3.2%, and 1.5% of the variance in TSAT, TIBC, serum iron, and ferritin, respectively. Instrument *F*-statistic for all 4 iron biomarkers ranged

from 109 to 1024 after harmonization of exposure and outcome data (Table S6).

The MR estimates for the effects of genetically predicted iron status on CVD risk factors are shown in Tables S8–S11. For the iron–blood lipids, iron–blood pressure, and iron–BMI analyses, the WM and MR-Egger estimates were prioritized for interpretation of results as the Cochran's *Q* statistic demonstrated evidence of pleiotropy between genetic instruments ($P<0.05$); little to no evidence of pleiotropy was evident from the MR-Egger intercept test ($P>0.05$). For the iron–interleukin-6 analyses, no evidence of heterogeneity was observed ($P>0.05$ for the Cochran's *Q* statistic and MR-Egger intercept); thus, the IVW MR estimates were taken into consideration.

Higher genetically predicted TSAT, indicative of higher iron status, was associated with lower LDL-C (WM: β=−0.11; SE=0.01; $P=3.9\times10^{-14}$), TC (WM: β=−0.10; SE=0.02; $P=5.3\times10^{-10}$), and apolipoprotein B (WM: β=−0.10; SE=0.01; $P=9.2\times10^{-13}$), and with higher apolipoprotein A (WM: β=0.02; SE=0.009; $P=0.007$), and triglycerides (WM: β=0.03; SE=0.009; $P=0.002$). No evidence of an effect was observed for high-density lipoprotein cholesterol (WM: $P=0.5$), BMI (WM: $P=0.1$), or interleukin-6 (IVW: $P=0.2$). With respect to blood pressure outcomes evaluated, higher genetically predicted TSAT was associated with higher DBP (WM: β=0.06; SE=0.01; $P=1.22\times10^{-9}$), but was not associated with SBP (WM: $P=0.7$). Consistent effects on all CVD risk factors except for BMI were observed when serum iron and TIBC were evaluated as exposures (Tables S8–S10). While genetically predicted TSAT and serum iron were not associated with BMI, higher TIBC (indicative of lower iron status) was associated with a higher BMI (WM: β=0.03; SE=0.009; $P=0.01$). Overall, the direction of effects for ferritin as the exposure were consistent with the other iron biomarkers, but weaker associations were observed due to the lower statistical power of its IVs (Table S11).

The MR-PRESSO analyses identified possible pleiotropic outliers for all CVD risk factors evaluated except for interleukin-6 (Tables S8–S11). For analyses of TIBC as the exposure, no change was observed with respect to significance and direction of effect after implementation of the outlier correction (Table S10), but few differences were observed for analyses of TSAT and serum iron as the exposures (Tables S8 and S9). The MR-PRESSO outlier-corrected estimates suggested a significant association of lower genetically predicted TSAT (WM: β=−0.03; SE, 0.009; $P=0.01$) and serum iron (WM: β=−0.04; SE=0.01; $P=0.01$) with a higher BMI but a null effect on the blood lipids and blood pressure (Tables S8 and S9). Caution should be taken when interpreting these estimates, as the lack of an effect may have resulted from a weak instrument and a lower statistical power after the removal of SNPs.

The MR odds ratio per 1-SD unit increase in TSAT, serum iron, TIBC, and ferritin are presented in Table 2. UVMR analyses showed evidence for a causal effect of higher iron status on CVD outcomes, but the direction of effect differed by outcome. Higher genetically predicted iron status, as evidenced by a higher TSAT, serum iron, or lower TIBC, was associated with a greater risk of CES, a lower risk of CHD, and was not associated with all-cause IS or small-vessel stroke. Lower genetically predicted TIBC, indicative of higher iron status, was associated with a lower risk of LAS. Using MR-PRESSO, outliers were detected for the associations of TSAT and TIBC with CHD. Nonetheless, outlier-corrected estimates and *P* values were in agreement with the other MR methods (Table 2). Analyses for AF appeared to have significant heterogeneity (*P* value for Cochran's *Q* statistic <0.05); thus, WM and MR-PRESSO were prioritized for interpretation. Consistent with the detrimental effects of higher iron status on CES, we found greater genetically predicted iron status, as evidenced by higher TSAT and serum iron and lower TIBC, to be associated with an increased risk of AF (Table 2).

Multivariate MR

We carried out MVMR using selected variables meeting the prioritization criteria from the UVMR analyses. Because the power of the genetic instrument is greatly reduced when conditioning on additional exposures using MVMR, we excluded ferritin from the MVMR analyses given that the genetic instruments for ferritin explain only a small proportion of its variance. For all MVMR models of TSAT, serum iron or TIBC as exposures, the conditional *F*-statistic was >10, demonstrating that weak instrumental bias is unlikely to be present (Tables S12–S14).

MVMR analyses provided evidence for a causal effect of higher iron status on CES after conditioning on CVD risk factors, and this observation was consistent across TSAT, serum iron and TIBC as shown in Figure 2. The statistically significant association of higher iron status with risk of CHD and LAS observed in the UVMR analyses disappeared after controlling for blood lipids (Figure 3). The confounding effects of BMI on the analyses were not consistent throughout the 3 Fe status exposures. The protective effect of lower TIBC and higher TSAT on CHD remained significant when conditioning for BMI. However, the presumed effects serum iron on CHD disappeared when conditioning on BMI (Figure 3).

Evaluation of heterogeneity using the Q_A statistic showed little to no heterogeneity in the analyses evaluating CES as the outcome (*P* heterogeneity >0.05; Table S12). Substantial heterogeneity was noted in the MVMR analyses for CHD and LAS (*P* heterogeneity <0.05; Tables S13

Table 2. Univariate 2-Sample Mendelian Randomization Analyses Evaluating the Associations of Genetically Predicted Iron Status and Cardiovascular Disease Outcomes

Outcome	Exposure	MR method	SNPs, n	OR (95% CI)	*P* effect	*P* pleiotropy*
CHD	TSAT	IVW	15	0.92 (0.83–1.03)	0.17	0.0003
		MR-Egger	15	0.88 (0.77–1.02)	0.11	0.33
		WM	15	0.91 (0.84–0.99)	0.02	…
		MR-PRESSO	14	0.91 (0.85–0.99)	0.04	…
	Serum iron	IVW	12	0.89 (0.81–0.98)	0.02	0.12
		MR-Egger	12	0.87 (0.75–1.00)	0.07	0.56
		WM	12	0.89 (0.82–0.98)	0.02	…
	TIBC	IVW	15	1.13 (1.03–1.24)	0.01	0.23
		MR-Egger	15	1.08 (0.96–1.20)	0.22	0.14
		WM	15	1.10 (1.00–1.21)	0.05	…
		MR-PRESSO	14	1.13 (1.03–1.24)	0.03	…
	Ferritin	IVW	35	0.92 (0.80–1.05)	0.23	0.04
		MR-Egger	35	0.79 (0.61–1.02)	0.07	0.17
		WM	35	0.84 (0.71–1.00)	0.05	…
		MR-PRESSO	33	0.90 (0.79–1.02)	0.11	…
IS	TSAT	IVW	14	1.03 (0.93–1.13)	0.63	0.003
		MR-Egger	14	1.04 (0.92–1.18)	0.56	0.71
		WM	14	1.04 (0.96–1.11)	0.35	…
		MR-PRESSO	12	1.02 (0.97–1.08)	0.45	…
	Serum iron	IVW	12	1.04 (0.91–1.18)	0.60	0.002
		MR-Egger	12	1.03 (0.86–1.25)	0.74	0.96
		WM	12	1.03 (0.94–1.14)	0.49	…
		MR-PRESSO	11	1.02 (0.93–1.13)	0.64	…
	TIBC	IVW	14	0.96 (0.89–1.03)	0.25	0.63
		MR-Egger	14	0.96 (0.88–1.05)	0.39	0.98
		WM	14	0.95 (0.88–1.04)	0.26	…
	Ferritin	IVW	34	1.01 (0.88–1.16)	0.85	0.16
		MR-Egger	34	1.01 (0.77–1.33)	0.95	0.97
		WM	34	1.06 (0.87–1.28)	0.57	…
CES	TSAT	IVW	14	1.17 (1.03–1.33)	0.02	0.36
		MR-Egger	14	1.12 (0.95–1.32)	0.19	0.42
		WM	14	1.14 (0.99–1.31)	0.07	…
	Serum iron	IVW	12	1.21 (1.02–1.44)	0.03	0.23
		MR-Egger	12	1.13 (0.89–1.45)	0.34	0.44
		WM	12	1.17 (0.97–1.41)	0.09	…
	TIBC	IVW	14	0.81 (0.69–0.94)	0.006	0.48
		MR-Egger	14	0.84 (0.70–1.01)	0.09	0.43
		WM	14	0.83 (0.70–0.98)	0.03	…
	Ferritin	IVW	34	1.05 (0.79–1.40)	0.72	0.04
		MR-Egger	34	1.47 (0.84–2.57)	0.18	0.18
		WM	34	1.38 (0.97–1.97)	0.08	…
SVS	TSAT	IVW	14	1.03 (0.85–1.24)	0.77	0.06
		MR-Egger	14	1.15 (0.92–1.43)	0.25	0.13
		WM	14	1.10 (0.92–1.31)	0.30	…
	Serum iron	IVW	12	1.01 (0.78–1.32)	0.92	0.02
		MR-Egger	12	1.18 (0.84–1.66)	0.37	0.23
		WM	12	1.03 (0.81–1.31)	0.80	…

(*Continued*)

Table 2. Continued

Outcome	Exposure	MR method	SNPs, n	OR (95% CI)	*P* effect	*P* pleiotropy*
	TIBC	IVW	14	0.88 (0.73–1.05)	0.15	0.38
		MR-Egger	14	0.83 (0.67–1.03)	0.12	0.40
		WM	14	0.87 (0.72–1.04)	0.12	…
	Ferritin	IVW	34	1.05 (0.80–1.36)	0.75	0.66
		MR-Egger	34	1.08 (0.62–1.87)	0.79	0.89
		WM	34	1.18 (0.80–1.73)	0.41	…
LAS	TSAT	IVW	14	0.86 (0.67–1.10)	0.22	0.006
		MR-Egger	14	0.86 (0.62–1.18)	0.36	0.99
		WM	14	0.84 (0.70–1.00)	0.05	…
		MR-PRESSO	13	0.87 (0.70–1.08)	0.23	…
	Serum iron	IVW	12	0.82 (0.61–1.12)	0.21	0.009
		MR-Egger	12	0.86 (0.55–1.33)	0.51	0.79
		WM	12	0.82 (0.65–1.04)	0.10	…
	TIBC	IVW	14	1.23 (1.07–1.40)	0.003	0.96
		MR-Egger	14	1.16 (0.91–1.48)	0.24	0.45
		WM	14	1.19 (0.95–1.50)	0.12	…
	Ferritin	IVW	34	0.83 (0.60–1.16)	0.29	0.20
		MR-Egger	34	0.74 (0.38–1.46)	0.39	0.70
		WM	34	0.75 (0.48–1.18)	0.21	…
AF	TSAT	IVW	11	1.07 (0.92–1.24)	0.37	2.85×10^{-17}
		MR-Egger	11	1.00 (0.83–1.19)	0.96	0.23
		WM	11	1.07 (1.00–1.13)	0.04	…
		MR-PRESSO	8	1.09 (1.01–1.17)	0.047	…
	Serum iron	IVW	11	1.09 (0.90–1.31)	0.39	1.46×10^{-17}
		MR-Egger	11	1.00 (0.75–1.21)	0.72	0.16
		WM	11	1.05 (0.96–1.15)	0.30	…
		MR-PRESSO	8	1.14 (1.02–1.27)	0.046	…
	TIBC	IVW	13	0.92 (0.85–1.00)	0.05	0.01
		MR-Egger	13	0.92 (0.83–1.02)	0.12	0.87
		WM	13	0.93 (0.87–0.99)	0.02	…
	Ferritin	IVW	33	1.00 (0.88–1.13)	0.99	0.0005
		MR-Egger	33	1.08 (0.84–1.40)	0.54	0.48
		WM	33	1.01 (0.88–1.16)	0.90	…

MR-PRESSO outlier-corrected estimates are presented when outliers were detected.

AF indicates atrial fibrillation; CES, cardioembolic stroke; CHD, coronary heart disease; IS, ischemic stroke (all-cause); IVW, inverse-variance weighted method under multiplicative random effects; LAS, large-artery stroke; MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier test; SVS, small-vessel stroke; TIBC, total iron-binding capacity; TSAT, transferrin saturation; and WM, weighted median.

**P* pleiotropy value for IVW method represents the Cochran's *Q* test and for the MR-Egger method represents the Egger-intercept test.

and S14). From our LDlink and PhenoScanner searches we identified 2 SNPs that have been shown to be associated at genome-wide significance level with coronary artery disease and 1 SNP with venous thromboembolism (Table S5). Additional sensitivity analyses excluding these 3 SNPs did not affect the results, thus demonstrating robustness of findings (Table S15).

Mediation Analysis

We conducted mediation analyses to evaluate the indirect effect of TSAT, serum iron, and TIBC on CES via selected mediators (DBP, BMI, LDL-C, TC, triglycerides, apolipoprotein B, apolipoprotein A) using effect estimates derived from UVMR and MVMR analyses. Significant mediation of the iron status-CES relationship via DBP was observed consistently across the 3 iron status exposures (Table 3). The estimated percentage of the total effect of Fe status on CES mediated by DBP was 7.1%, 8.0%, and 8.8% for serum iron, TIBC, and TSAT, respectively. The magnitude of mediation observed for BMI and blood lipid fractions evaluated was substantially lower (0%–2%) and was not statistically significant on the basis of the wider 95% CI that crossed the null. Detailed calculations are shown in Table S16.

DISCUSSION

To our knowledge, this is the first study to implement MVMR to evaluate the effects of iron status on CVD outcomes while adjusting for potential confounding variables. Our findings revealed a noteworthy association between higher genetically predicted iron status and an increased risk of CES that is independent of major CVD risk factors, including blood lipids, DBP, and BMI. Further employing mediation analyses, we found that DBP partially mediates the effect of iron status on CES. Interestingly, UVMR initially suggested a protective effect of higher iron status on CHD and LAS, but these results were substantially diminished when MVMR analyses factoring CVD risk factors were implemented.

Our analyses using univariate 2-sample MR to evaluate the effects of iron status on CVD outcomes were consistent with previous MR studies based on smaller-scale GWAS of iron traits.[21–25] First, we replicated the observed protective effect of higher iron levels on atherosclerotic CVD outcomes, including CHD and LAS. We also replicated the analyses pertaining to CES indicating an adverse effect of higher iron status on this outcome. Finally, consistent with a previous MR study,[49] we found an association between higher genetically predicted iron status with an increased risk of AF. This finding holds particular relevance, as AF is the most significant risk factor for CES,[50] aligning consistently with the observed effects of iron levels on CES.

We further evaluated the causal effects of Fe status on major CVD risk factors using UVMR. We found, for

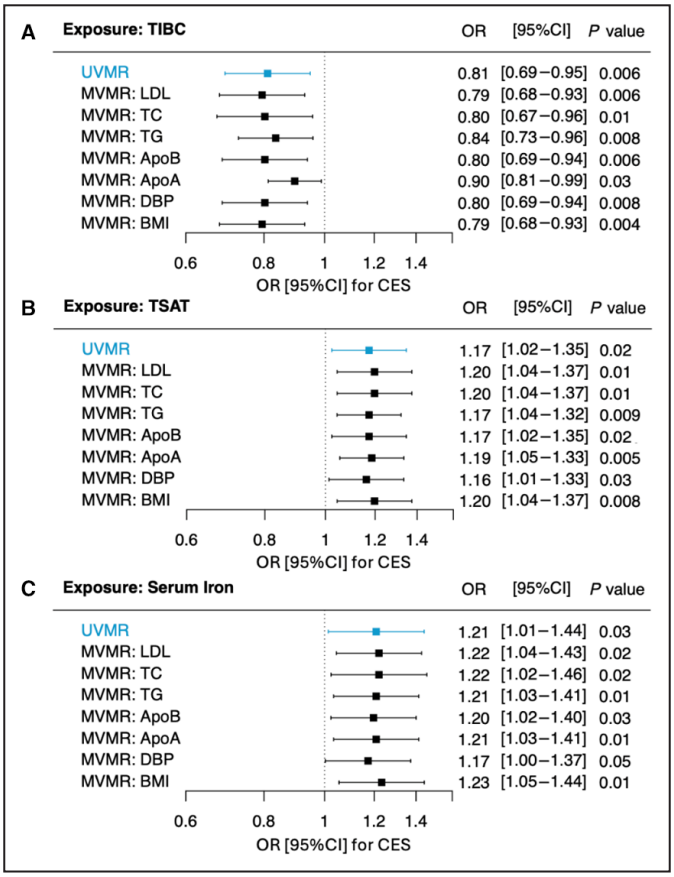

Figure 2. Associations between genetically predicted iron status and cardioembolic ischemic stroke using univariate and multivariate Mendelian randomization.
A, TIBC as the exposure with CES as the outcome. **B**, TSAT as the exposure with CES as the outcome. **C**, Serum iron as the exposure with CES as the outcome. MR estimates presented as OR and 95% CIs per 1-SD unit increase in the iron exposure. ApoA indicates apolipoprotein A; ApoB, apolipoprotein B; BMI, body mass index; CES, cardioembolic stroke; DBP, diastolic blood pressure; LDL, low-density lipoprotein; MVMR, multivariate Mendelian randomization; OR, odds ratio; TC, total cholesterol; TG, triglycerides; TIBC, total iron binding capacity; TSAT, transferrin saturation; and UVMR, univariate Mendelian randomization.

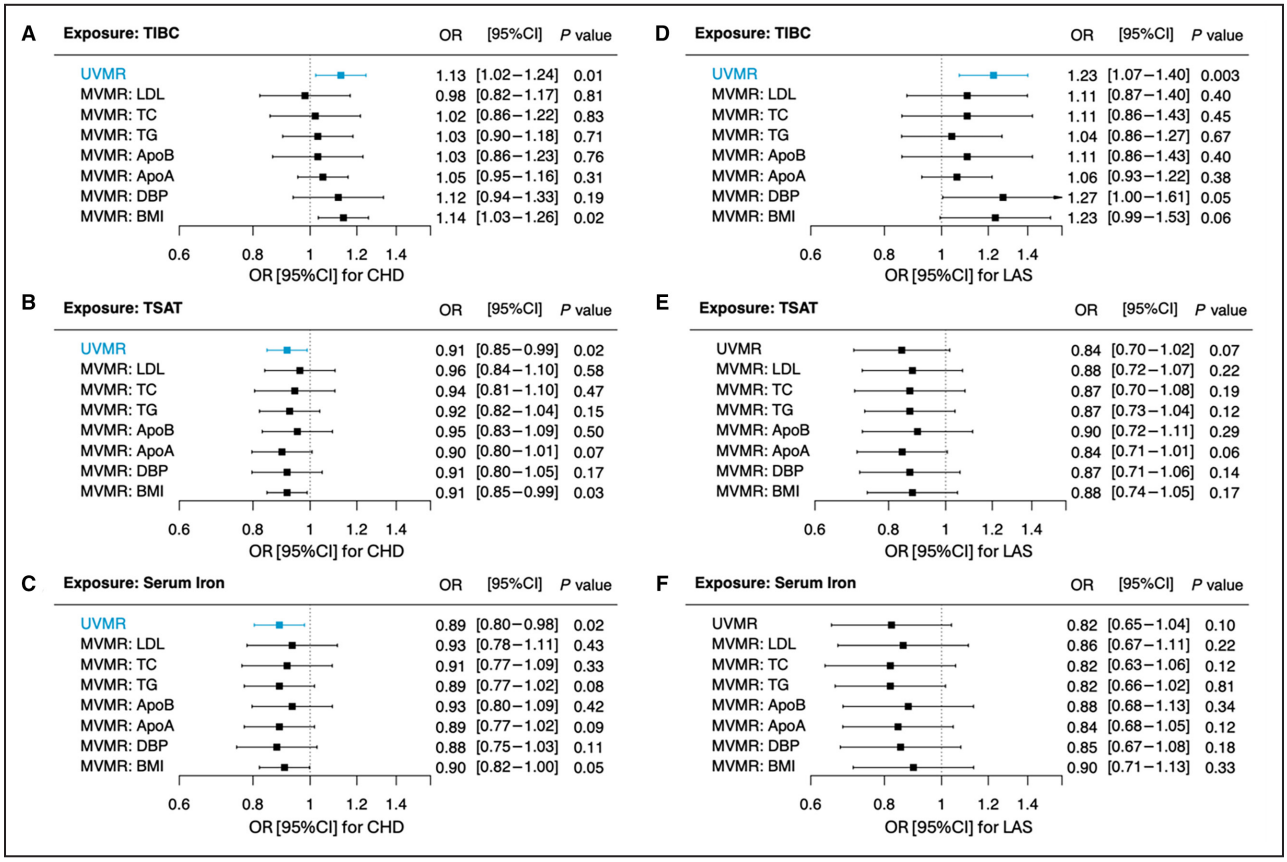

Figure 3. Associations between genetically predicted iron status and atherosclerotic cardiovascular disease outcomes using univariate and multivariate Mendelian randomization.

A, TIBC as the exposure with CHD as the outcome. **B**, TSAT as the exposure with CHD as the outcome. **C**, Serum iron as the exposure with CHD as the outcome. **D**, TIBC as the exposure with LAS as the outcome. **E**, TSAT as the exposure with LAS as the outcome. **F**, Serum iron as the exposure with LAS as the outcome. MR estimates presented as OR and 95% CIs per 1-SD unit increase in the iron exposure. ApoA indicates apolipoprotein A; ApoB, apolipoprotein B; BMI, body mass index; DBP, diastolic blood pressure; CHD, coronary heart disease; LAS, large-artery stroke; LDL, low-density lipoprotein; MVMR, multivariate Mendelian randomization; OR, odds ratio; TC, total cholesterol; TG, triglycerides; TIBC, total iron-binding capacity; TSAT, transferrin saturation; and UVMR, univariate Mendelian randomization.

the first time using causal inference methods, an association between higher genetically predicted iron status and higher DBP. In agreement, several studies have shown an association between higher iron status and a greater risk of hypertension.[51–53] Interestingly, we did not find a significant association between genetically predicted iron status and SBP, which may be explained, in part, by insufficient statistical power, as evidenced by our power calculations. Nonetheless, our findings are in line with observational studies showing a positive association between iron status and DBP but no association with SBP.[52–55] Although the mechanistic origins of these differences are currently unknown, recent findings from an MR study showed differences in the pathophysiology of SBP and DBP and their effects on CVD.[56] Additional research is needed to further understand the mechanisms through which iron exerts differential effects on DBP and SBP.

We then evaluated the causal effects of iron status on BMI and found lower iron status, as evidenced by higher TIBC, to be associated with a higher BMI. This is consistent with observational studies reporting a relationship between obesity and iron deficiency, which is believed to stem from an interplay between iron regulation and adiposity.[57] With respect to blood lipids, higher genetically predicted iron status was associated with better lipid profiles as evidenced by lower LDL-C, TC, and apolipoprotein B and higher apolipoprotein A concentrations. While these results are in agreement with previous MR studies showing an association between genetically predicted higher serum iron and lower LDL-C and TC levels, and a decreased risk of hypercholesteremia and hyperlipidemia,[24,58] we found evidence suggestive of substantial pleiotropy in the analyses. The observed heterogeneity likely results from the significant overlap between iron and lipid IVs, as evidenced by our sensitivity analyses showing a lack of an association after removal of pleiotropic outliers. Finally, genetically predicted iron status was not associated with interleukin-6 in our analyses.

Table 3. Indirect Effects Reported as Log Odds Ratio of Iron Status on Cardioembolic Stroke via Selected Mediators

E→M→O	Indirect effect	SE	95% CI	% of total effect mediated
TSAT-DBP-CES	0.014	0.006	0.004 to 0.028	8.8*
TSAT-BMI-CES	−0.002	0.002	−0.006 to 0.002	1.3
TSAT-LDL-CES	0.001	0.008	−0.014 to 0.016	0.6
TSAT-TC-CES	0.001	0.007	−0.013 to 0.015	0.6
TSAT–triglycerides –CES	0	0.002	−0.003 to 0.004	0
TSAT–apolipoprotein B–CES	0.001	0.006	−0.011 to 0.013	0.6
TSAT–apolipoprotein A–CES	−0.002	0.002	−0.006 to 0	1.3
SI-DBP-CES	0.014	0.007	0.003 to 0.029	7.1*
SI-BMI-CES	0	0.002	−0.006 to 0.004	0
SI-LDL-CES	0.001	0.008	−0.018 to 0.016	0.5
SI-TC-CES	0.001	0.008	−0.015 to 0.018	0.5
SI–triglycerides –CES	0	0.002	−0.004 to 0.004	0
SI-ApoB-CES	0.002	0.007	−0.013 to 0.016	1.0
SI-ApoA-CES	−0.003	0.002	−0.009 to 0	1.5
TIBC-DBP-CES	−0.015	0.006	−0.028 to −0.005	8.*
TIBC-BMI-CES	0.003	0.002	0.000 to 0.007	1.6
TIBC-LDL-CES	0	0.008	−0.015 to 0.015	0
TIBC-TC-CES	−0.001	0.007	−0.015 to 0.013	0.5
TIBC–triglycerides–CES	0	0.001	−0.003 to 0.002	0
TIBC–apolipoprotein B–CES	0	0.007	−0.013 to 0.013	0
TIBC– apolipoprotein A–CES	0.002	0.001	0 to 0.005	1.1

The indirect effect is reported as the log odds ratio.
The SE and 95% CI for the for the indirect effect was calculated using the delta method. The SE was calculated as: $\sqrt{EM\,\beta^2 \times MO\,SE^2 + MO\,\beta^2 \times EM\,SE^2}$.
The percentage of the total effect mediated was calculated as the estimated indirect effect divided by the total effect from univariate MR × 100.
BMI indicates body mass index; CES, cardioembolic stroke; DBP, diastolic blood pressure; E→M→O, exposure → mediator → outcome; LDL, low-density lipoprotein; SI, serum iron; TC, total cholesterol; TIBC; total iron-binding capacity; and TSAT, transferrin saturation.
*indicates statistical significance based on the 95% CI not crossing the null.

Nonetheless, future MR studies should evaluate this relationship in the opposite direction (ie, the effects of interleukin-6 on iron status), as it is well established that inflammation, particularly via interleukin-6, is a negative regulator of iron status.[59]

Although the results from UVMR analyses carry important implications, a major limitation is the potential bias introduced by pleiotropic effects of the genetic instruments, as several Fe-associated SNPs are strongly associated with major CVD risk factors. To overcome this limitation, we performed MVMR analyses conditioning on selected CVD risk factors. Most notably, with these analyses we found that the effects of iron status on CES were unaffected by adjustment for DBP, BMI, or blood lipids (LDL-C, TC, triglycerides, apolipoprotein B, apolipoprotein A). This provides evidence in support of a causal effect of higher iron status on CES incidence that is independent of major CVD risk factors. Available observational evidence evaluating the associations between iron status and risk of stroke is inconclusive as reviewed.[25] However, these contrasting observations may stem from differences between IS subtypes as evidenced by our opposing results when evaluating IS by subtype. This highlights the importance of evaluating IS with stratification by subtype.

We hypothesized that iron's detrimental effect on CES is at least partially mediated by CVD risk factors evaluated in this study. Using mediation analyses, we identified DBP as a potential pathway through which elevated iron levels contribute to an increased risk of CES. Accumulation of excess iron, such that it increases the labile iron pool, results in the production of highly reactive species, consequently inducing oxidative stress. It is known that oxidative stress contributes to the pathogenesis of hypertension,[60] a common vascular risk factor for CES. Thus, iron-induced oxidative stress may be a mechanism by which higher iron levels influence CES development directly and indirectly via its effects on blood pressure, particularly DBP. Finally, blood lipids did not appear to play a mediating role in the relationship between iron status and CES, suggesting that the effect of higher iron status on CES risk is unlikely to operate via the blood lipid fractions evaluated.

In contrast to the robust evidence observed for CES, the protective effect of higher iron status on CHD and LAS was diminished after adjustment for CVD risk

factors using MVMR, suggesting a potential lack of a direct effect of iron status on CHD and LAS when accounting for major CVD risk factors. Of note, significant heterogeneity was observed in these analyses and a lack of an association may have resulted from a lower statistical power as it is expected when implementing MVMR methods. Thus, more investigations are needed to characterize the complex interplay between iron, atherosclerosis, and subsequent CVD outcomes.

Our study has limitations that warrant attention. First, due to our reliance on summary- rather than individual-level data, we were unable to explore potential sex-based differences in the relationships between iron status and CVD risk factors. This is particularly relevant to iron and CVD, as both iron status markers and risk of CVD differ significantly between men and women. To our knowledge, there are no sex-stratified summary-level GWAS data from either iron status or CVD that are publicly available and adequately powered to address these questions. However, it remains crucial to replicate these analyses in men and women separately. Furthermore, while we selected the best available outcome data sets that closely matched the population in the exposure data set and that adjusted for similar covariates, the possibility of population stratification cannot be ruled out. This is particularly relevant for CHD, as 24% of the study population was non-European. Additionally, since our study was based in mostly European populations, we are unable to extrapolate our results to non-European populations. Evaluating this question in other populations is of pressing need, as risk of excess body iron accumulation differs between populations, with individuals of East Asian ancestry presenting the greatest body iron burden.[61]

Results obtained through MR methods rely on the premise that all necessary assumptions are met; therefore, interpretation should be framed within the context of such assumptions (shown in Figure S1). Because MR methods estimate the lifetime effect of iron status, the magnitude of the MR estimates may be greater in our study than what would be observed clinically. This characteristic of MR holds implications for our study as iron accumulation differs throughout life stages. For instance, risk of iron overload in women escalates after menopause due to the cessation of menses and the effect size of elevated iron on disease development may differ accordingly. Moreover, all MR approaches implemented in this study assume linear associations, but there is a possibility that the effect of iron on some of the exposures evaluated is not linear.

In conclusion, our findings underscore the significance of iron status as a novel modifiable risk factor for CES. When controlling for CVD risk factors, the presumed protective effect of higher iron status on atherosclerotic heart disease outcomes was diminished. Future studies are needed to evaluate possible sex and population differences in the effects of iron status on CVD risk. Additionally, observational studies are warranted to validate the clinical utility of iron status biomarkers in predicting risk of CES. Finally, intervention trials will be essential to evaluate whether lowering iron levels in individuals predisposed to iron overload could effectively mitigate the risk of experiencing a CES event.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Data S1. MEGASTROKE Consortium Members
Tables S1–S16
Figures S1–S2
References [62,63]

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