The effect of iron status on risk of coronary artery disease: a Mendelian randomization study

Authors: Dipender Gill¹, ² MD, Fabiola Del Greco M³ PhD, Ann P Walker⁴ PhD, Surjit KS Srai⁴ PhD, Michael A Laffan¹, ² MD, PhD, Cosetta Minelli² MD, PhD

Institutional affiliations:
1. Imperial College Healthcare NHS Trust, London, United Kingdom
2. Imperial College London, London, United Kingdom
3. Institute for Biomedicine, Eurac Research, Bolzano, Italy
4. University College London, London, United Kingdom

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Corresponding author details:
Dr Dipender Gill
Academic Clinical Fellow in Clinical Pharmacology and Therapeutics, Imperial College Healthcare NHS Trust
Honorary Clinical Lecturer, Imperial College London
Postal address: Postgraduate Centre, St. Mary’s Hospital, London, W2 1NY, United Kingdom
Telephone: +44 (0) 7904843810
E-mail: dipender.gill@imperial.ac.uk

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Abstract

Objective: Iron status is a modifiable trait that has been implicated in cardiovascular disease. This study uses the Mendelian randomization (MR) technique to investigate whether there is any causal effect of iron status on risk of coronary artery disease (CAD).

Approach and Results: A two-sample MR approach is used to estimate the effect of iron status on CAD risk. Three loci (rs1800562 and rs1799945 in the HFE gene, and rs855791 in TMPRSS6) that are each associated with serum iron, transferrin saturation, ferritin and transferrin in a pattern suggestive of an association with systemic iron status are used as instruments. SNP-iron status association estimates are based on a GWAS meta-analysis of 48,972 individuals. SNP-CAD estimates are derived by combining the results of a GWAS meta-analysis of 60,801 CAD cases and 123,504 controls with those of a meta-analysis of 63,746 CAD cases and 130,681 controls obtained from Metabochip and GWAS studies. Combined MR estimates are obtained for each marker by pooling results across the three instruments. We find evidence of a protective effect of higher iron status on CAD risk (iron OR 0.94 per SD unit increase, 95%CI 0.88 to 1.00, $p=0.039$; transferrin saturation OR 0.95 per SD unit increase, 95%CI 0.91 to 0.99, $p=0.027$; log-transformed ferritin OR 0.85 per SD unit increase, 95%CI 0.73 to 0.98, $p=0.024$; transferrin OR 1.08 per SD unit increase, 95%CI 1.01 to 1.16, $p=0.034$).

Conclusion: This MR study supports the hypothesis that higher iron status reduces CAD risk. These findings may highlight a therapeutic target.
ABBREVIATIONS

CAD: coronary artery disease
CHD: coronary heart disease
CI: confidence interval
GWAS: genome-wide association study
IVW: inverse-variance weighted
MR: Mendelian randomization
RBC: red blood cell
SNP: single-nucleotide polymorphism
INTRODUCTION

Iron serves in a number of fundamental processes including erythropoiesis and cellular metabolism (1). Although iron status has been implicated in cardiovascular disease (1), the evidence for this is mixed. In support of a detrimental effect of higher iron status on cardiovascular risk, a reduced incidence of heart disease in premenopausal women as compared to men and postmenopausal women has been attributed to lower levels of stored iron (2). Higher iron stores have also been positively associated with risk factors for cardiovascular disease such as type 2 diabetes (3). Furthermore, genetic mutations resulting in hereditary haemochromatosis are associated with an increased incidence of cardiovascular morbidity (4), and chelation of heavy metals using disodium EDTA in patients that have experienced a recent myocardial infarction reduced adverse cardiovascular outcomes (5). However, these findings contrast with the results of a meta-analysis of observational studies that suggests a protective effect of higher iron status on the risk of coronary heart disease (CHD) (6). In addition, iron deficiency has been associated with increased mortality in patients with heart failure (7).

It can be difficult to disentangle causal effects from spurious associations attributable to confounding and reverse causation in observational study. The Mendelian randomization (MR) approach can overcome these issues by using genetic variants such as single nucleotide polymorphisms (SNPs) as proxies or instruments for a phenotype or exposure of interest (8). It is because genetic variants are allocated randomly at the time of conception that this approach is not typically confounded by environmental, lifestyle factors or reverse causation. If the underlying assumptions of MR analysis are met (8), SNPs associated with iron status can be used as instruments in an investigation of the causal effect of iron status on risk of coronary artery disease (CAD). This principle has previously been adopted to explore the causal effect of iron status on atherosclerosis (9), and a similar approach has also been taken to show that red blood cell (RBC) traits are associated with risk of CHD (10).

The instruments used in an MR study must influence the intermediate phenotype of interest (8, 11), which in this case is systemic iron status. Various correlated markers of iron status are available, including serum iron, transferrin saturation, ferritin and transferrin (1, 12-15). Genetic instruments for iron status that are used in an MR study should have a concordant association with each of these markers, and specifically SNPs that are deemed to increase systemic iron status should be associated with increased levels of serum iron, transferrin saturation and ferritin, and decreased levels of transferrin (9, 11, 16). Another potential limitation of the MR approach concerns pleiotropy, where genetic variants affect the outcome (CAD risk) through pathways that are independent of the intermediate phenotype of interest.
(iron status), thus violating a fundamental assumption of MR to bias the causal effect estimates generated (8, 17). In this study, we select instruments for systemic iron status and perform an MR study investigating its causal effect on CAD risk. Furthermore, we explore the possibility that any pleiotropic effects of the instruments may be biasing the estimates generated.

MATERIALS AND METHODS
Materials and Methods are available in the online-only Data Supplement. The overall study design is demonstrated graphically in Figure 1.

RESULTS
The three instruments have F statistics for the four iron status markers ranging from 47 to 2,127 (Table 1). Individual SNP-iron marker estimates are given in Table 1, while Table 2 reports the SNP-CAD estimates from the meta-analysis of CARDIoGRAMplusC4D 1000G and CARDIoGRAMplusC4D Metabochip.

Individual and pooled MR estimates for the effect of the four markers of iron status on risk of CAD are reported in Figure 2. The results, expressed as odds ratios (OR) for CAD per standard deviation unit increase in the iron status marker, demonstrate a protective effect on CAD risk for iron (OR 0.94, 95%CI 0.88 to 1.00, p=0.039), transferrin saturation (OR 0.95, 95%CI 0.91 to 0.99, p=0.027) and (log-transformed) ferritin (OR 0.85, 95%CI 0.73 to 0.98, p=0.024). The effect estimate for transferrin (OR 1.08, 95%CI 1.01 to 1.16, p=0.034) is also in keeping with the other results to suggest that higher iron status is protective of CAD, as higher transferrin levels reflect lower iron status.

Search of an online database of SNP-phenotype associations demonstrated that all three instruments are also associated with RBC traits (18, 19). Furthermore, the iron status raising allele at rs1800562 in the HFE gene is associated with lower low density lipoprotein levels and the iron status raising allele at rs1799945 in the HFE gene is associated with higher systolic and diastolic blood pressures (20, 21).

DISCUSSION
This work suggests a protective effect of higher iron status on the risk of CAD. The pooled MR estimates for serum iron, transferrin saturation, ferritin and transferrin all suggest that higher iron status lowers the risk of CAD. The objective of this study is to explore whether CAD risk is affected by iron status and instruments were selected to reflect this. The finding that all the considered iron status makers give similar causal estimates is consistent with the
effect of CAD risk being mediated by iron status rather than any individual marker. The small differences in estimates and confidence interval widths for the causal effects of the four markers might be explained by chance and possibly differential measurement error across markers, rather than indicating distinct causal pathways. In addition, the variation in magnitude of the MR estimate confidence intervals across the three SNPs for each iron status marker might also reflect the strengths of the SNP-iron status marker associations (as evaluated by the F statistics given in Table 1). In our current MR study, we demonstrate a causal effect of iron status on CAD risk using only the three SNPs associated with all four iron status markers at genome-wide significance. Given our interest in systemic iron status, we only include genetic variants that have shown genome-wide significant association with all four iron status markers in a pattern concordant with an effect on systemic iron status (i.e. increased levels of serum iron, transferrin saturation and ferritin, and decreased levels of transferrin) to minimise risk of including invalid instruments. For example, rs8177240 in the TF gene has genome-wide significant associations with serum iron and transferrin saturation, but in opposite directions (Supplementary Table I). As any effect on systemic iron status should have a concordant direction of effect on both serum iron and transferrin saturation, this genetic variant is unlikely to be a valid instrument. While our approach has the advantage of minimising risk of incorporating invalid instruments, it pays the price of sacrificing the additional power that might be afforded by considering as instruments all genetic variants associated with any iron status marker at genome-wide significance (22).

A potential source of bias with the MR approach relates to the issue of pleiotropy (8, 17). While the availability of many instruments allows for implementation of statistical methods to detect and adjust for pleiotropy in sensitivity analyses, such techniques are not applicable when few instruments are available, such as in our study (23-27). Despite this, we have investigated the possibility of pleiotropy by searching for secondary phenotypes which have shown association with the three instruments. The association of the three iron status instruments with RBC traits may be expected given the well-established relationship between iron status and anaemia (12), but this would not bias the MR analysis if any effect of RBC traits on CAD risk was acting downstream of iron status, rather than independently of it (8, 17). The association of the iron status raising allele at rs1800562 (HFE gene) with lower low density lipoprotein (LDL) levels and the iron status raising allele at rs1799945 (HFE gene) with higher systolic and diastolic blood pressures are likely to be affecting CAD risk independently of iron status, and would therefore be expected to bias the MR estimates (20, 21). Lower LDL levels and higher blood pressure are known to reduce and increase CAD risk respectively (20, 21). Consistent with the hypothesis of some bias attributable to pleiotropy, rs1800562 and rs1799945 give MR estimates for all markers that tend to
respectively over-estimate and under-estimate the effect of iron status on CAD risk as compared to rs855791 (TMPRSS6 gene), although the confidence intervals for the three SNPs largely overlap for all markers (Figure 2). Moreover, the pooled MR estimate across the three SNPs is comparable to that of rs855791 alone, which has no known pleiotropic associations. Thus, the overall conclusions of this work are unlikely to be severely affected by these pleiotropic effects.

Early work attributed the observed association of heart disease with disorders of iron storage, older age in men and post-menopausal status in women to the effect of higher systemic iron status (2). However, consequent observational studies did not support this (28). A randomised controlled trial has demonstrated a protective effect of heavy metal chelation induced by disodium EDTA on heart disease, but it is unclear how generalizable this finding is, and the observed effect might be specific to patients that have suffered a recent myocardial infarction or attributable to effects independent of systemic iron status and overall body iron stores (5). By contrast, the conclusions of our MR study are in-keeping with a systematic review and meta-analysis of prospective observational studies investigating the association of body iron status and CHD risk (6). All except one of the 17 studies included in this meta-analysis adjusted for smoking and major cardiovascular risk factors such as blood pressure and lipid profile, with some studies also adjusting for social class and chronic disease (6). The risk ratio of CHD for individuals with levels of the iron status marker in the top third compared with individuals in the bottom third was 0.80 (95%CI 0.73 to 0.87) for iron, 0.82 (95%CI 0.75 to 0.89) for transferrin saturation, 1.03 (95%CI 0.87 to 1.23) for ferritin, and 0.99 (95%CI 0.86 to 1.13) for transferrin (6). The non-significant results for ferritin and transferrin might be attributable to confounding caused by inflammation, which would act to increase serum levels of ferritin and decrease those of transferrin (29), while increasing the risk of CHD (30), thus potentially biasing the ferritin-CHD and transferrin-CHD associations to mask a true protective effect of higher iron status on CHD. The authors concluded that while their overall results may suggest a protective effect of higher body iron stores on risk CHD, it is difficult to infer causality due to the possibility of residual confounding and reverse causality bias (6). For example, increased iron status has also been associated with risk of diabetes mellitus, which is an established risk factor for cardiovascular disease (3, 31). In our MR study, we have used genetic variants as instrumental variables for iron status to overcome these limitations of observational research and strengthen the evidence for a protective effect of iron status on CAD risk.

Iron deficiency is a treatable condition that affects up to 2 billion people worldwide (1). The suggestion here that low iron status may have a causal effect on cardiovascular disease therefore has potentially significant clinical and public health implications. However, it is
important to interpret the findings of our MR study in context. While it is unlikely that pleiotropy is wholly responsible for the pattern of our results, we cannot completely exclude this. Compensatory developmental processes, referred to as canalization, can buffer the effects of genetic variation and may have impacted on our MR estimates, although this would be expected to bias results towards the null (32). We note that the Wald-type estimator has been shown to induce bias in the MR analysis of binary outcomes, although of small magnitude (e.g. < 10%) in typical MR analyses (33). Use of the same combined discovery and replication results from the GWAS meta-analysis to both identify the instruments and estimate their associations with iron status markers may also result in over-estimation of the SNP-iron status associations (the Beavis effect or Winner’s Curse) (11, 34, 35), in turn leading to underestimation of the true causal effect of iron status on CAD risk (bias towards the null), which in our two-sample MR analysis is estimated as the SNP-CAD association divided by the SNP-iron status association. Our use of instruments that have strong associations with all four markers of iron status should however minimise any effect of such bias (36). Finally, the conclusions of our work relate to patterns of iron status observed in the population-based studies contributing to the GIS consortium and therefore reflect effects in the general population. Further research is needed to investigate the causal effects of iron status on CAD risk in subjects with severe iron overload or deficiency. Similarly, our study does not offer insight into whether the estimates are equally applicable to both men and women. Despite these limitations, the results of this work show consistent and biologically plausible effects. Iron status may be affecting CAD risk via effects on RBCs (10, 12). Iron deficiency is also known to impact cellular metabolism (37), and may increase CAD risk by this mechanism.

In conclusion, this work is suggestive of a protective effect of higher iron status on risk of cardiovascular disease. This warrants further investigation, as these findings may highlight possible therapeutic targets and risk reduction strategies.
ACKNOWLEDGEMENTS

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SOURCES OF FUNDING

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DISCLOSURES

None.
REFERENCES
HIGHLIGHTS

- Systemic iron status is a modifiable trait that has been implicated in cardiovascular disease.
- Serum iron, transferrin saturation, ferritin and transferrin are all markers of systemic iron status.
- By using genetic variants associated with these four markers as surrogates for systemic iron status, this study implements the Mendelian randomization approach to demonstrate a causal effect of systemic iron status on risk of coronary artery disease.
- These findings may highlight a possible therapeutic target for the prevention and treatment of coronary artery disease.
TABLES AND FIGURES

Table 1. Results for the SNP-iron status associations (12). EA: effect allele; EAF: effect allele frequency, as reported by the Genetics of Iron Status consortium (12); R²: percentage of the iron marker variation explained by the SNP (38); F: F statistic (39); GX: the per-allele effect on standard deviation units of the iron marker; GX SE: standard error of GX.

Table 2. SNP-CAD associations obtained by meta-analysis of CARDIoGRAMplusC4D 1000G (60,801 CAD cases and 123,504 controls) and CARDIoGRAMplusC4D Metabochip (63,746 CAD cases and 130,681 controls) using a summary data method that accounts for participant overlap between the two studies (34,997 cases and 49,512 controls) (40-42). EA: effect allele; GY: the per-allele effect on CAD, log(OR); GY SE: standard error of GY; p: p value.

Figure 1. Graphical representation of the two-sample MR study design. Three SNPs that each have genome-wide significant associations with increased serum iron, transferrin saturation and ferritin, as well as decreased transferrin levels are used as instruments for systemic iron status. By using genetic variants associated with the four iron status markers as surrogates, the Mendelian randomization (MR) approach is used to estimate the causal effect of systemic iron status on risk of coronary artery disease (CAD).

Figure 2. Forest plot of the SNP-specific and pooled MR estimates for the causal effect of each iron status marker on CAD risk (odds ratio, OR). The size of the black squares reflects the precision of the MR estimates and the horizontal lines indicate their 95% confidence intervals (95% CI). The pooled MR estimate is depicted by the centre of the diamond, with the corner edges on either side indicating the 95% CI.
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Figure 1

rs1800562 in the HFE gene
rs1799945 in the HFE gene
rs855791 in the TMPRSS6 gene

Instruments

SNP-Iron status association

Systemic Iron status
- ↑serum iron
- ↑transferrin saturation
- ↑ferritin
- ↓transferrin

MR estimates

Coronary artery disease risk
Figure 2
GENETIC ASSOCIATIONS

As markers of iron status, we consider serum iron (μmol/l), transferrin saturation (%), log10-transformed ferritin (μg/l) and transferrin (g/l) (1). The SNP-iron status association estimates are obtained from combined discovery and replication cohorts in a published genome-wide association study (GWAS) meta-analysis of 48,972 subjects of European descent performed by the Genetics of Iron Status (GIS) consortium, where adjustments were made for age, population stratification (ancestry principal components) and other study specific covariates (1).

For the SNP-CAD association estimates, we use publicly available results from the CARDIoGRAMplusC4D 1000 Genomes-based GWAS (referred to here as CARDIoGRAMplusC4D 1000G) and CARDIoGRAMplusC4D Metabochip (2, 3). CARDIoGRAMplusC4D 1000G is a GWAS meta-analysis of 60,801 CAD cases and 123,504 controls that adjusts for population stratification (genomic control method) (2). Participants are of European, east Asian, south Asian, Hispanic and African American ancestry (2). The diagnosis of CAD varies between studies; cases included subjects with documented acute coronary syndrome, coronary artery bypass grafting, percutaneous coronary revascularization, stenosis of greater than 50% in one or more of the coronary vessels, and cardiac angina (2). CARDIoGRAMplusC4D Metabochip is a meta-analysis of 63,746 CAD cases and 130,681 controls genotyped with either the Metabochip array or GWAS data imputed using HapMap (3). Participants are of European and south Asian descent (3). The study uses CAD definitions similar to CARDIoGRAMplusC4D 1000G and corrects for population stratification, age and sex (3). Results for both CARDIoGRAMplusC4D 1000G and CARDIoGRAMplusC4D Metabochip can be downloaded from www.CARDIoGRAMplusC4D.org (2, 3). We obtain SNP-CAD association estimates by meta-analysis of results from CARDIoGRAMplusC4D 1000G and CARDIoGRAMplusC4D Metabochip using a summary data method that accounts for participant overlap between the two studies (34,997 cases and 49,512 controls) (4). The approach ‘decouples’ the results from the two studies by transforming the covariance structure of the data such that metaanalysis methods assuming independence may consequently be applied (4).
INSTRUMENT SELECTION

Increased systemic iron status is associated with increased levels of serum iron, transferrin saturation and ferritin, and decreased levels of transferrin (1, 5-8). Thus, these four correlated markers may be treated as surrogates of systemic iron status, the single intermediate phenotype of interest in our MR study. Genetic instruments for iron status should therefore be expected to have a concordant association with each of these four markers, and specifically SNPs that are deemed to increase systemic iron status should be associated with increased levels of serum iron, transferrin saturation and ferritin, and decreased levels of transferrin (9-11). The meta-analysis performed by the GIS consortium identified 12 genetic loci that differentially affect the four considered iron status markers (Supplementary Table 1) (1). Three loci (rs1800562 and rs1799945 in the HFE gene, and rs855791 in TMPRSS6) are associated with all four iron status markers at genome-wide significance (p < 5 x 10^{-8}) in a pattern consistent with an effect on systemic iron status (i.e. increased levels of serum iron, transferrin saturation and ferritin, and decreased levels of transferrin) (Supplementary Table 1) (1, 11), and only these are considered as instruments for systemic iron status in our MR analysis. The rs1800562 and rs1799945 SNPs in the HFE gene are not in linkage disequilibrium (LD r2 < 0.01).

The strength of each instrument is evaluated using its F statistic, and only SNPs with an F statistic greater than 10 are used, thus minimising any weak instrument bias (12, 13).

MENDELIAN RANDOMIZATION ESTIMATES

To derive MR estimates, a two-sample summary data approach is performed separately for each SNP using the Wald-type estimator, with standard error derived using the Delta method (14). Combined MR estimates are obtained by pooling MR estimates across SNPs using a fixed-effect inverse-variance weighted (IVW) meta-analysis.

The overall study design is demonstrated graphically in Figure 1.

PLEIOTROPY

To explore the possibility that the instruments for iron status may be exerting effects on CAD risk through pleiotropic pathways that are independent of iron status and thus biasing the results of the MR analysis (15, 16), an online database of SNP-phenotype associations
(PhenoScanner, http://www.phenoscanner.medschl.cam.ac.uk/phenoscanner) is used to search for secondary phenotypes associated with the three selected instruments at genomewide significance (p < 5 x 10^-8) (17). All analyses are performed using the statistical programme R (version 3.3.1).

REFERENCES
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### Supplementary Tables and Figures

**Supplementary Table I.** Summary results of the GWAS meta-analysis performed by the GIS consortium identifying 12 genetic loci that differentially affect the four considered iron status markers (1). Genome-wide significant associations are highlighted in bold. Three loci (rs1800562 and rs1799945 in the *HFE* gene, and rs855791 in *TMPPRSS6*) are associated with all four iron status markers at genome-wide significance (*p* < 5 × 10^-8) in a pattern consistent with an effect on systemic iron status (i.e. increased levels of serum iron, transferrin saturation and ferritin, and decreased levels of transferrin) (1, 2), and only these are considered as instruments for systemic iron status in our MR analysis. EA: effect allele; EAF: effect allele frequency; GX: the per-allele effect on standard deviation units of the iron marker; GX SE: standard error of GX; p: p value.

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### References