Genetic support of a causal relationship between iron status and type 2 diabetes: a Mendelian randomization study

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Abbreviations used: BMI, body mass index; BMP, bone morphogenetic protein; DIAGRAM, the DIAbetes Genetics Replication And Meta-analysis; GIS, the Genetics of Iron Status; GWAS, genome-wide association study; HFE, hemochromatosis protein; IVW, inverse variance weighted; MR, Mendelian randomization; MR-BMA, MR based on Bayesian model averaging; MRMix, MR analysis using mixture-model; SMAD, mothers against decapentaplegic homolog; SNP, single-nucleotide polymorphism; TMPRSS6, transmembrane serine protease 6; T2D, type 2 diabetes.
ABSTRACT

Context: Iron overload is a known risk factor for type 2 diabetes (T2D); however, both iron overload and iron deficiency have been associated with metabolic disorders in observational studies.

Objective: Using Mendelian randomization (MR), we assessed how genetically predicted systemic iron status affected T2D risk.

Design and Methods: A two-sample MR analysis was used to obtain a causal estimate. We selected genetic variants strongly associated ($P < 5 \times 10^{-8}$) with four biomarkers of systemic iron status from a study involving 48,972 subjects performed by the Genetics of Iron Status consortium and applied these biomarkers to the T2D case-control study (74,124 cases and 824,006 controls) performed by the Diabetes Genetics Replication and Meta-analysis consortium. The simple median, weighted median, MR-Egger, MR analysis using mixture-model, weighted allele scores, and MR based on Bayesian model averaging approaches were used for the sensitivity analysis.

Results: Genetically instrumented serum iron (OR: 1.07; 95% CI: 1.02–1.12), ferritin (OR: 1.19; 95% CI: 1.08–1.32), and transferrin saturation (OR: 1.06; 95% CI: 1.02–1.09) were positively associated with T2D. In contrast, genetically instrumented transferrin, a marker of reduced iron status, was inversely associated with T2D (OR: 0.91; 95% CI: 0.87–0.96).

Conclusions: Genetic evidence supports a causal link between increased systemic iron status and increased T2D risk. Further studies involving various ethnic backgrounds based on individual-level data and studies regarding the underlying mechanism are warranted for reducing the risk of T2D.

Keywords: iron, ferritin, transferrin, Mendelian randomization, type 2 diabetes
INTRODUCTION

The essential element iron plays a crucial role in many fundamental biological processes, including energy metabolism, redox balance, oxygen delivery, and inflammation. However, iron is also potentially toxic, as excess free iron contributes to the generation of reactive oxygen species and is related to a wide variety of chronic diseases. Indeed, precisely controlled body iron level is essential for maintaining metabolic homeostasis.

The hormone hepcidin (encoded by the \textit{HAMP} gene) plays a central role in regulating systemic iron and is controlled primarily through the BMP/SMAD (bone morphogenetic protein/mothers against decapentaplegic homolog) signaling pathway in response to iron stimulation. Genetic differences that affect the hepcidin-ferroportin axis are a principal cause of both iron overload and iron deficiency (1). For example, the \textit{HFE} and \textit{TMPRSS6} genes (which encode the hemochromatosis protein HFE and transmembrane serine protease 6, respectively) contain the three loci selected in this study and were identified via either monogenic diseases or functional studies. Studies to date have shown that the HFE and TMPRSS6 proteins regulate \textit{HAMP} transcription primarily via the BMP/SMAD signaling pathway (2). Mutations in the \textit{HFE} gene cause late-onset (i.e., type 1) hemochromatosis (OMIM #235200) (3), whereas mutations in the \textit{TMPRSS6} gene cause iron-refractory iron deficiency anemia (OMIM #206200) (4, 5). Genome-wide association studies (GWASs) revealed that the iron overload–related single-nucleotide polymorphisms (SNPs) rs1800562 (C282Y) and rs1799945 (H63D) in the \textit{HFE} gene are associated with an increased risk of diabetes (6), whereas the iron deficiency–related SNPs rs855791 (A736V) and rs4820268 (D521) in the \textit{TMPRSS6} gene are associated with a decreased risk of diabetes (7, 8).

A case-control study have found a direct correlation between iron stores and the prevalence of type 2 diabetes (T2D, non-insulin dependent diabetes mellitus), with a lower
ratio between the soluble fragment of the transferrin receptor and ferritin being associated with an increased risk of T2D (OR: 2.4; 95% CI: 1.03-5.5) (9). Previous observational studies have also found that higher iron status increases the risk of T2D. In addition, several meta-analyses examined the putative link between systemic iron status and the risk of T2D, suggesting that several iron indices—including ferritin, transferrin saturation, and heme iron intake—are correlated with the risk of diabetes (10-13). We also conducted a meta-analysis previously included 17 studies from 15 publications to evaluate the association between circulating ferritin level (per 100 µg/L) and T2D risk, which also found a positive correlation (RR: 1.22; 95% CI: 1.14-1.31), and a greater effect in women (RR: 1.53; 95% CI: 1.29-1.82) than those in men (RR: 1.21; 95% CI: 1.15-1.27) (14). In contrast, other studies found an association between iron deficiency and obesity. For example, as far back as the early 1960s epidemiology studies suggested a link between obesity and iron deficiency (15, 16). Comprehensive reviews and meta-analyses have since confirmed that obese/overweight participants generally have lower serum iron levels, higher hemoglobin concentration, higher ferritin levels, and lower levels of transferrin saturation (17, 18). Indeed, our previous meta-analysis has revealed the overweight/obese participants had an increased risk of iron deficiency (OR: 1.31; 95% CI: 1.01-1.68) (18). A major limitation with respect to observational studies is the difficulty distinguishing between bona fide causal relationships and spurious associations due to confounding and reverse causation (19, 20). As genetic variants are determined at conception and have effects that are potentially lifelong, Mendelian randomization (MR) studies are less vulnerable to key confounders (21). By using UK Biobank datasets, previous studies have found p.C282Y homozygotes associated with diabetes risk in men (22), however, their following MR analyses have not identified the causal link between genetically instrumented iron status and the risk of T2D (22, 23). Here, we conducted a MR analysis using the summary datasets with a larger sample size from the
DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) consortium, to elucidate the causal effect of systemic iron status on T2D risk.

METHODS

Study design

In this two sample MR study, we used data from two different studies—one for the exposures and one for the outcome—in order to estimate the effects of exposure on outcome. Essentially, we applied genetic predictors of iron status to extensively genotyped case-control studies of T2D, thereby obtaining estimates of the putative association between iron and T2D risk. The overall study design is depicted graphically in Figure 1.

Genetic associations with systemic iron status

A meta-analysis of genome-wide studies was conducted previously by the Genetics of Iron Status (GIS) consortium in order to obtain association estimates between SNPs and biomarkers of iron status (24). Data from 11 discovery cohorts and 8 replication cohorts were used in the meta-analysis, which combined data obtained from 48,972 European subjects. The descriptive and statistics of the included cohorts in GIS consortium study are shown in Supplemental Table 1 (25). Adjustments were made for age and principal component scores, with analyses performed separately for males and females before combining estimates.

The first-stage regression F statistic was used to assess the strength of the instruments and was calculated using the following equation: $F = (R^2/k)/([1−R^2]/[n−k−1])$, where $R^2$ is the proportion of the circulating trace element status variability accounted for by the SNP, $k$ is the number of instruments used in the model, and $n$ is the sample size (26).
Genetic associations with T2D

The summary-level GWAS statistics of T2D were obtained from the DIAGRAM study, the aim of which is to characterize genetic information for T2D primarily in samples obtained from participants of European descent. The aggregated GWAS results were included for 32 studies involving 74,124 T2D cases and 824,006 controls of European ancestry. Considering that body mass index (BMI) may mediate the associations between iron status and T2D, we used the estimates for T2D without BMI adjustment in our primary analysis, and then performed a sensitivity analysis by using BMI-adjusted estimates. The descriptive and statistics of the included cohorts in DIAGRAM study are shown in Supplemental Table 2. Imputation was performed using the Haplotype Reference Consortium reference panel for all of the included studies, with the exception of the deCODE GWAS, which was imputed using a population-specific reference panel (27). The summary-level data are publicly available at http://diagram-consortium.org/.

MR estimates

An inverse variance weighted (IVW) meta-analysis of MR estimates derived using the ratio method was used to generate the main MR estimates for the association between each biomarker of iron status and the risk of T2D (28). The fixed-effect model was employed when using the three SNPs associated with all four iron status biomarkers. Standard errors were calculated using the Delta method (29). A threshold of P < 0.05 was used to determine statistical significance.
Sensitivity analysis

To examine whether the instruments for iron status exert effects on T2D risk through pleiotropic pathways that are independent of iron status, thus potentially biasing the results of the MR analysis (30, 31), we used the PhenoScanner database for SNP-phenotype associations (PhenoScanner, http://www.phenoscanner.medschl.cam.ac.uk/phenoscanner) to search for secondary phenotypes associated with the three selected instruments at genome-wide significance ($P < 5 \times 10^{-8}$) (32).

To account for potential bias due to unknown pleiotropy, we conducted sensitivity analyses using the simple median, weighted median (33), and MR-Egger methods (34), which are more robust to the inclusion of pleiotropic instruments. The simple median and weighted median methods provide consistent estimates even when up to 50% of the information is derived from invalid SNPs (33). MR-Egger regression additionally provides an estimate of the true causal effect that is consistent even if all genetic variants are invalid (34); however, MR-Egger can be imprecise, particularly if the estimates are similar or if the number of genetic instruments is low. MR-Egger intercept tests were conducted in order to assess the validity of the instrumental variable assumptions, with a non-null intercept indicating that the IVW estimate is biased (34). To verify our main findings in consideration of the horizontal pleiotropy, we employed another MR analysis using mixture-model (MRMix) (35). Given the relatively low statistical power of these approaches compared with the main analysis, they were used solely to confirm a consistent effect estimate compared to that seen in the main IVW estimate, rather than to ascertain statistical significance itself via a $P$-value threshold. Heterogeneity was estimated using the Cochran’s $Q$ statistic (36, 37).

Since the relatively rare variant rs1800562 (effect allele frequency is 0.067) may produce false positive findings, we further conducted MR sensitivity analyses using allele
scores weighted by various parameters (38), including minor allele frequency (MAF), variance of SNP (Var(SNP)), and the proportion of variance in the risk factor explained by the SNP ($R^2$).

The separately selected SNPs by their genome-wide significant association with each iron status biomarker were used for assessing the consistency in our MR analyses. BMI-adjusted estimates from the DIAGRAM consortium were also used for sensitivity analysis.

Furthermore, to avoid the limitations of logistic regression methods and prioritize the most causally related risk factors, we further conducted a novel analysis based on Bayesian model averaging approach (39). Briefly, all possible combinations of the four biomarkers of iron status were considered and posterior probability (PP) for each specific model was generated. Then, a marginal inclusion probability (MIP) for each iron biomarker was computed, where MIP refers to the sum of the PP over all possible models where the iron biomarker is presented. Furthermore, the model-averaged causal estimate (MACE) for each iron biomarker by ranking all the iron biomarkers according to the corresponding MIP was computed. Finally, the best models by the PP values (with a PP threshold of 0.02) of the individual models were prioritized. Invalid instruments were detected as outliers with respect to the fit of the linear model using the $Q$ statistic (40). The Cook’s distance ($Cd$) was used for quantifying influential observations (41).

Data were analyzed using the “TwoSampleMR” package (version 0.4.23) in the statistical program R (version 3.6.1; the R Foundation for Statistical Computing).
RESULTS

Genetic instruments for systemic iron status

Three SNPs including rs1800562 and rs1799945 in the *HFE* gene and rs855791 in the *TMPRSS6* gene were employed for our main analysis. The F statistics for these three SNPs ranged from 47 for 2127 among the four biomarkers of iron status (Supplemental Table 3), as described previously (42, 43), making significant bias from use of weak instruments unlikely (26). The individual SNP-iron marker estimates are listed in Supplemental Table 3. The SNP-T2D estimates from the summary data without or with BMI adjustment are listed in Supplemental Table 4 and 5, respectively.

Causal relationship between systemic iron status and T2D risk

The results of our MR analysis, which are reported as the odds ratio (OR) of T2D per SD unit increase in each iron status biomarker, revealed an association between increased iron status and the risk of T2D, with serum iron (OR: 1.07; 95% CI: 1.02-1.12; \( P < 0.01 \)), ferritin (OR: 1.19; 95% CI: 1.08-1.32; \( P < 0.01 \)), and transferrin saturation (OR: 1.06; 95% CI: 1.02-1.09; \( P < 0.01 \)) having a significant effect. In addition, higher transferrin levels, which are indicative of reduced iron status, were associated with a decreased risk of T2D (OR: 0.91; 95% CI: 0.87-0.96; \( P < 0.01 \)). The relationship between each biomarker of iron status and the risk of T2D is shown graphically in Figure 2.

Sensitivity analysis provided no indication of unknown pleiotropy

We further examined the biological pleiotropy of these instruments to evaluate the possible biases using the PhenoScanner database (32). As expected, all of these three SNPs were also associated with red blood cell traits due to the altered iron status. A potentially protective effect on T2D risk may be contributed by the iron status increasing allele of...
rs1800562 with reduced low-density lipoprotein cholesterol and total cholesterol levels (44). Other protective effects on T2D risk may be contributed by all of these three SNPs due to their associations with reduced glycated hemoglobin (HbA1c) level (45, 46). On the contrary, the two SNPs rs1800562 and rs1799945 in HFE region are positively associated with diastolic blood pressure (47-49).

The simple median, weighted median, and MR-Egger estimates produced directionally consistent effects as the IVW estimates, albeit with wider CIs (Table 1). The MR-Egger intercepts for the four biomarkers did not differ significantly from null ($P = 0.30, 0.51, 0.41,$ and 0.99 for serum iron, ferritin, transferrin saturation, and transferrin, respectively), thus providing no statistical indication of pleiotropy for T2D (Table 1). The directions of the estimates of causal effects generated by MRMix approach ($\theta$) are consistent with our previous findings, and the proportions of valid instrumental variables ($\pi_0$) are 1 for all biomarkers (Table 1). Given the relatively low statistical power of these approaches compared with the main analysis, they were used only to confirm an effect estimate directionally consistent to that seen in the main IVW MR, rather than to ascertain statistical significance itself via any given $P$-value threshold. Lastly, for all four iron biomarkers, Cochran’s $Q$ statistics showed low heterogeneities ($P = 0.35, 0.37, 0.44,$ and 0.56 for serum iron, ferritin, transferrin saturation, and transferrin, respectively) (Table 1).

Investigation of BMI-unadjusted T2D risk using the separately selected SNPs by their genome-wide significant association with each iron status biomarker also produced directionally consistent results as shown in Figure 3 and Table 2. Similarly, the MR-Egger intercepts using the separately selected SNPs did not differ significantly from null ($P = 0.21, 0.16, 0.69,$ and 0.28 for serum iron, ferritin, transferrin saturation, and transferrin, respectively), which providing no statistical indication of pleiotropy for T2D (Table 2). And using the MRMix approach also derived directionally consistent results with high $\pi_0$ values.
(1, 0.647, 1, and 0.552 for iron, Ferritin, Transferrin saturation, and Transferrin, respectively) (Table 2). The Cochran’s $Q$ statistics using the separately selected SNPs showed low heterogeneities for serum iron ($P = 0.21$) and transferrin saturation ($P = 0.52$), however, showed significant heterogeneities ($P < 0.01$) for ferritin and transferrin (Table 2). The MR analysis based on T2D summary data with adjustment for BMI derived consistent results as shown in Supplemental Figure 1-2 and Supplemental Table 6-7.

To evaluate the causal effects of the iron biomarkers on T2D risk in consideration of the measured pleiotropy, we conducted MR based on Bayesian model averaging (MR-BMA) analyses using the SNPs associated with at least one of the biomarkers of iron status. Totally, 12 SNPs (Including rs1800562, rs1799945, rs855791, rs8177240, rs7385804, rs744653, rs651007, rs411988, rs9990333, rs4921915, rs6486121, rs174577), 9 SNPs (Excluding invalid instruments rs651007, rs174577, rs4921915 with $q$ statistic > 10), or 8 SNPs (Further excluding influential instrument rs1800562 with $Cd$ exceed the threshold in all four best models) were employed for the MR-BMA analyses (Supplemental Table 8-9). The PPs of the best specific models and the MIPs of the risk factors were consistent with the results derived from the logistic regression approaches (Table 3). All the risk factors were then ranked by their MIPs, where the best models were prioritized and ranked by their PPs (Table 3).

Furthermore, when using MAF, Var(SNP), and $R^2$ weighted allele scores for MR sensitivity analyses, the $R^2$ weighted analysis still derived statistically consistent results ($P < 0.05$), whereas the results of MAF and Var(SNP) weighted analyses only showed directionally consistent (Supplemental Table 10).
DISCUSSION

Here, we report the first evidence that several genetically determined markers of systemic iron status are associated with the risk of T2D. Our assumption in MR is that the instruments (SNPs) should be associated with the outcome of interest (T2D) only via the exposure (systemic iron status as reflected by the four iron biomarkers). We used a two-sample study design that can cost-effectively produce unbiased estimates in order to determine the effects of systemic iron status on T2D risk. In addition, our use of samples generally pertaining to European subjects with appropriate genomic control (27) reduced the likelihood of bias due to concealed genetic associations. Our main MR analysis using the SNPs associated with all four iron biomarkers revealed that higher systemic iron status is associated with an increased risk of T2D based on IVW (with fixed effects, given that we included only three SNPs). Finally, simple median, weighted median, MR-Egger, MRMix, weighted allele scores, and MR-BMA approaches yielded directionally consistent results as IVW.

Both iron overload and iron deficiency are associated with metabolic disorders, and perturbations in iron homeostasis can have a plethora of effects on T2D (50). For example, iron overload is a potent risk factor for diabetes, whereas iron deficiency is associated with obesity and insulin resistance (51). Although the pathogenic mechanism still needs further investigation, various tissues may reflect the deleterious effects of iron overload on glycemic control. First, the elevated iron level could impair the function of pancreatic β-cells in both human (52) and high-fat diet-fed mice (53). Second, dietary iron overload could elevate AMPK activity and impair insulin signaling in skeletal muscle and liver in mice (54). Third, elevated iron status could decrease adiponectin secretion and insulin sensitivity of adipocytes in human and high-iron diet-fed mice (55). Finally, breakdown of heme into carbon monoxide, biliverdin, and free iron by the enzyme heme oxygenase-1 (HO-1) could
also promote chronic metabolic inflammation and insulin resistance in hepatocytes and macrophages (56).

To examine the causal relationship between iron status and T2D, we used a Mendelian randomization approach and found that higher systemic iron is associated with an increased risk of T2D. Increased systemic iron status has been associated with increased levels of serum iron, transferrin saturation, and ferritin, as well as decreased levels of transferrin (57). Thus, genetic variants were selected as instrumental variables based on their genome-wide significant association with these four biomarkers of iron status (i.e., increased serum iron, increased ferritin, increased transferrin saturation, and decreased transferrin levels) (21). The aforementioned GIS consortium study identified 11 loci related to these biomarkers of iron status with genome-wide significance ($P < 5 \times 10^{-8}$) (24). Of these 11 loci, three (rs1800562 and rs1799945 in the HFE gene, and rs855791 in the TMPRSS6 gene) were associated with all four iron status biomarkers at genome-wide significance ($P < 5 \times 10^{-8}$), with low linkage disequilibrium between the rs1800562 and rs1799945 SNPs in the HFE gene (linkage disequilibrium: $r^2 < 0.01$) (24).

Nutritional factors are causally associated to many chronic diseases (58, 59), however, such effects would be difficult to study in observational studies, as these factors are correlated and often co-occur. In this respect, MR is valuable for identifying risk factors that could serve as potential targets for clinical and/or behavioral interventions (60, 61). Our use of genetically instrumented serum iron, ferritin, transferrin saturation, and transferrin enabled us to avoid potential confounding, therefore distinguishing the effects of iron status. Given that most genetic variants explain only a small portion of the variation in a risk factor, a relatively large sample size is required for an MR study with sufficient power (62). Using cross-trait meta-analysis of GWAS on a certain disease or health status could identify novel genetic loci (63). Several consortia containing large numbers of participants, including the
GIS consortium for studying iron status (24) and the DIAGRAM consortium for studying T2D (27), have published data and have publicly available information regarding the association between genetic variants and either risk factors or disease status, thus providing precise estimates of genetic associations and enabling us to obtain causal estimates based on a well-powered MR study using a cost-effective approach (28). Previous MR studies have revealed that increased iron levels are causally associated with increased risk of stroke (particularly cardioembolic stroke) (43), and decreased risk of coronary artery disease (42) and Parkinson’s disease (64). No causal relationship has been identified previously between instrumented iron status and diabetes using the MR approach (22, 23). Thus, we suggested a causal link between systemic iron status and T2D for the first time.

A previous study using UK Biobank datasets has found that male p.C282Y homozygotes had a higher prevalence of diagnosed diabetes mellitus (predominantly type 2 but including type 1; OR: 1.53; 95% CI: 1.16 to 1.98), compared with no p.C282Y mutations (irrespective of H63D status) (22). However, the following MR analysis showed no, but directionally consistent, association ($\beta$: 0.006; $P = 0.06$) between genetically instrumented transferrin saturation and diabetes in men (22). Another MR study evaluated the causal links between 25 predominantly metabolic traits and liver iron content and found a causative effect of central obesity, as measured by higher waist-to-hip ratio (adjusted for BMI), on elevated liver iron content ($\beta$: 0.162; $P = 0.003$) (65). However, type 2 diabetes showed no causative effect on liver iron content ($\beta$: -0.021; $P = 0.393$) (65). Interestingly, a MR–phenome-wide association study (MR–PheWAS) using data from the UK Biobank also found directionally consistent trend of the association ($\beta$: 0.06; $P = 0.11$) between the 3 SNPs (rs1800562, rs1799945, and rs855791) instrumented serum iron and T2D risk (23). Actually, there are 32 cohorts (including UK Biobank) used in the DIAGRAM study in our analysis. Our findings provide the first evidence that all four genetically determined biomarkers (serum iron, ferritin,
transferrin saturation, and transferrin) of systemic iron status are significantly associated with the risk of T2D.

There are several limitations to this study. Firstly, our analyses were conducted at the summary level, which made it impossible to conduct stratified analysis. Secondly, although the MR-Egger intercepts and MRMix results suggested no statistical evidence of horizontal pleiotropy, the PhenoScanner database showed that the instrumental variables used in our MR analyses were truly associated with total cholesterol, low-density lipoprotein-cholesterol, HbA1c, and diastolic blood pressure, which may introduce some pleiotropy bias. Finally, the datasets used in our analyses were mainly derived from Europeans, which may hamper their translational relevance to other racials.

The findings of this study provided evidence that higher systemic iron status plays a causal role in the pathogenesis of T2D, which are consistent with previous recognized association between iron and T2D based on observational studies. We have conducted meta-analyses previously and found that the disturbances of iron homeostasis are associated obesity (18) and T2D (14). Indeed, a positive correlation has been established between heme iron intake and the risk of T2D (11).
Conclusions

Using MR approach, we examined the putative hypothesis that systemic iron status has a causal effect on T2D risk. Specifically, we performed a two-sample MR study based on iron status data measured in 48,972 individuals in the general population and T2D data obtained from 74,124 T2D cases and 824,006 controls. We then used three SNPs as instruments in order to increase statistical power by combining their MR estimates and to investigate the possible presence of pleiotropy. In summary, our results provide the first evidence that systemic iron status could be a causal factor in T2D development. Future studies should focus on examining this causal relationship in various populations with different ethnic backgrounds based on individual-level data, as well as the possible underlying mechanism, thereby providing new insights into potential strategies designed to prevent T2D.
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Additional Information

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Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.
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**TABLE 1** Associations between genetically instrumented systemic iron status and T2D without BMI adjustment using the three SNPs associated with all four iron biomarkers

| Exposure          | IVW-fixed  | IVW-random | Simple median | Weighted median | MR-Egger | MRMix[^3] |
|-------------------|------------|------------|---------------|-----------------|----------|-----------|
|                   | OR 95% CI  | OR 95% CI  | OR 95% CI     | OR 95% CI       | Q statistic (P) Intercept (P) | θ  | π₀  | σ² |
| Iron              | 1.07 1.02, 1.12 | 1.07 1.00, 1.17 | 1.07 1.01, 1.14 | 1.29 1.07, 1.56 | 0.88 (0.35) -0.04 (0.30) | 0.06 | 1 | 2.64e-04 |
| Ferritin          | 1.19 1.08, 1.32 | 1.19 1.08, 1.31 | 1.23 1.06, 1.43 | 1.29 1.07, 1.55 | 0.81 (0.37) -0.01 (0.51) | 0.125 | 1 | 1.71e-04 |
| Transferrin saturation | 1.06 1.02, 1.10 | 1.06 1.02, 1.10 | 1.07 1.03, 1.11 | 1.10 1.03, 1.19 | 0.59 (0.44) -0.01 (0.41) | 0.04 | 1 | 2.25e-04 |
| Transferrin       | 0.91 0.87, 0.96 | 0.91 0.90, 0.93 | 0.92 0.83, 1.01 | 0.91 0.86, 0.97 | 0.33 (0.56) 0.00 (0.99) | -0.138 | 1 | 1.42e-04 |

[^1]: Data source and sample size: T2D case-control (n = 74,124 and 824,006, respectively) study based on DIAGRAM consortium; genetic instruments were selected based on GIS consortium study (n = 48,972). 95% CI, 95% confidence interval; BMI, body mass index; DIAGRAM, the DIAbetes Genetics Replication And Meta-analysis; GIS, the Genetics of Iron Status; IVW, inverse variance weighted; MR, Mendelian randomization; MRMix, MR analysis using mixture-model; OR, odds ratio; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.

[^2]: SNPs rs1800562, rs1799945, and rs855791 associated with all four iron status biomarkers at genome-wide significance (P < 5 × 10⁻⁸) were used as genetic predictors for systemic iron status.

[^3]: θ, the estimates of causal effects generated by MRMix approach; π₀, the proportion of valid instrumental variables; and σ², the unknown variance parameter associated with the invalid instrumental variables.
| Exposure          | OR  | 95% CI       | OR  | 95% CI       | OR  | 95% CI       | OR  | 95% CI       | Q statistic (P) | Intercept (P) | θ  | π₀ | σ² |
|-------------------|-----|--------------|-----|--------------|-----|--------------|-----|--------------|----------------|---------------|----|----|----|
| Iron              | 1.06| 1.01, 1.11   | 1.06| 0.99, 1.13   | 1.05| 0.97, 1.14   | 1.14| 1.02, 1.26   | 4.49 (0.21)    | -0.01 (0.21)  | 0.035 | 1 | 1.14e-04 |
| Ferritin          | 1.06| 0.98, 1.15   | 1.06| 0.83, 1.36   | 1.08| 0.95, 1.24   | 1.17| 1.05, 1.31   | 25.16 (<0.01)  | -0.03 (0.16)  | 0.13  | 0.647 | 1.23e-03 |
| Transferrin saturation | 1.06| 1.03, 1.09   | 1.06| 1.03, 1.09   | 1.07| 1.01, 1.12   | 1.07| 1.02, 1.13   | 2.28 (0.52)    | -0.002 (0.69) | 0.03  | 1 | 4.95e-05 |
| Transferrin       | 0.95| 0.92, 0.97   | 0.95| 0.89, 1.00   | 0.88| 0.79, 0.97   | 0.97| 0.93, 1.00   | 30.42 (<0.01)  | -0.008 (0.28) | -0.28 | 0.552 | 4.72e-03 |

1 Data source and sample size: T2D case-control (n = 74,124 and 824,006, respectively) study based on DIAGRAM consortium; genetic instruments were selected based on GIS consortium study (n = 48,972). 95% CI, 95% confidence interval; BMI, body mass index; DIAGRAM, the DIAbetes Genetics Replication And Meta-analysis; GIS, the Genetics of Iron Status; IVW, inverse variance weighted; MR, Mendelian randomization; MRMix, MR analysis using mixture-model; OR, odds ratio; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.

2 SNPs associated with serum iron (rs1800562, rs1799945, rs855791, rs8177240, and rs7385804), Ferritin (rs1800562, rs1799945, rs855791, rs744653, rs651007, and rs411988), Transferrin saturation (rs1800562, rs1799945, rs855791, rs8177240, and rs7385804), and Transferrin (rs1800562, rs1799945, rs855791, rs744653, rs8177240, rs9990333, rs4921915, rs6486121, and rs1745777) at genome-wide significance (P < 5 × 10⁻⁸) were used as genetic predictors for each iron biomarker.

3 θ, the estimates of causal effects generated by MRMix approach; π₀, the proportion of valid instrumental variables; and σ², the unknown variance parameter associated with the invalid instrumental variables.
TABLE 3 Ranking of risk factors and models (sets of risk factors) for T2D

| Risk factor or model | Ranking by MIP | MIP | θMACE | Ranking by PP | PP | θλ |
|---------------------|---------------|-----|--------|---------------|----|-----|
| Model averaging using 12 SNPs |               |     |        |               |    |     |
| (Including rs1800562, rs1799945, rs855791, rs8177240, rs7385804, rs744653, rs651007, rs411988, rs9990333, rs4921915, rs6486121, rs174577) | |     |        |               |    |     |
| Iron                | 4             | 0.177 | 0.008  | 4             | 0.17 | 0.05 |
| Ferritin            | 1             | 0.305 | 0.023  | 1             | 0.293| 0.082|
| Transferrin saturation | 3         | 0.266 | 0.017  | 3             | 0.254| 0.061|
| Transferrin         | 2             | 0.27  | -0.015 | 2             | 0.264| -0.056|
| Model averaging using 9 SNPs |               |     |        |               |    |     |
| (Excluding invalid instruments rs651007, rs174577, rs4921915 with q statistic > 10) | |     |        |               |    |     |
| Iron                | 4             | 0.089 | 0.004  | 4             | 0.08 | 0.052|
| Ferritin            | 1             | 0.532 | 0.074  | 1             | 0.519| 0.14 |
| Transferrin saturation | 2         | 0.268 | 0.015  | 2             | 0.257| 0.055|
| Transferrin         | 3             | 0.131 | -0.006 | 3             | 0.125| -0.043|
| Model averaging using 8 SNPs |               |     |        |               |    |     |
| (Excluding influential instrument rs1800562 with Cook’s distance exceed the threshold) | |     |        |               |    |     |
| Iron                | 4             | 0.15  | 0.002  | 4             | 0.143| 0.017|
| Ferritin            | 1             | 0.488 | 0.04   | 1             | 0.477| 0.083|
| Transferrin saturation | 2         | 0.214 | 0.008  | 2             | 0.206| 0.035|
| Transferrin         | 3             | 0.164 | -0.004 | 3             | 0.158| -0.026|

Results were generated using the MR-BMA approach. Totally, four genetically instrumented biomarkers of systemic iron status were assessed as risk factors. All of the risk factors and the best individual models with a PP value > 0.02 were presented. A negative causal estimate (θMACE or θλ) indicates a protective effect as suggested by the model, whereas a positive value indicates a risk factor. θλ is the causal effect estimate for a specific model and θMACE is the model averaged causal effect of a risk factor. MIP, marginal inclusion probability; MR, Mendelian randomization; MR-BMA, MR based on Bayesian model averaging; PP, posterior probability; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.
FIGURE LEGENDS

**FIGURE 1** Graphical overview of the 2-sample MR study design. Three SNPs, each of which has a genome-wide significant association with increased serum iron, increased ferritin, increased transferrin saturation, and decreased transferrin levels, were used as instruments for systemic iron status. By using genetic instruments associated with these four iron status biomarkers, the MR approach can be used to estimate the causal effect of systemic iron status on the risk of T2D. MR, Mendelian randomization; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.

**FIGURE 2** Forest plots summarizing the SNP-specific and overall MR estimates for the causal effects (fixed-effect IVW) on T2D without BMI adjustment using the SNPs associated with all four iron biomarkers. The causal effects of serum (A) iron, (B) ferritin, (C) transferrin saturation, and (D) transferrin on T2D risk (OR) are estimated. The solid black diamonds represent the estimates of the causal effects for the genetic instruments and the horizontal lines indicate the 95% CIs. The overall MR estimate is indicated by the center of the gray diamond, with the width of the diamond indicating the 95% CI. 95% CI, 95% confidence interval; BMI, body mass index; IVW, inverse variance weighted; MR, Mendelian randomization; OR, odds ratio; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.

**FIGURE 3** Forest plots summarizing the SNP-specific and overall MR estimates for the causal effects (random-effect IVW) on T2D without BMI adjustment using the separately selected SNPs associated with each iron status biomarker. The causal
effects of serum (A) iron, (B) ferritin, (C) transferrin saturation, and (D) transferrin on T2D risk (OR) are estimated. The solid black diamonds represent the estimates of the causal effects for the genetic instruments and the horizontal lines indicate the 95% CIs. The overall MR estimate is indicated by the center of the gray diamond, with the width of the diamond indicating the 95% CI. 95% CI, 95% confidence interval; BMI, body mass index; IVW, inverse variance weighted; MR, Mendelian randomization; OR, odds ratio; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.
Figure 1

Instruments: rs1800362 in the HFE gene, rs1799945 in the HFE gene, rs55791 in the TMPRSS6 gene

Exposure: Systemic iron status (Iron, Ferritin, Transferrin saturation, Transferrin)

MR estimates

Outcome: Risk of T2D
Figure 2

A

| SNPs | Impn-T2D | OR (95% CI) |
|------|----------|-------------|
| rs18003562 | 1.14 (1.05, 1.23) |
| rs1799945 | 1.09 (1.06, 1.12) |
| rs557981 | 1.05 (1.04, 1.08) |
| Overall estimate | 1.07 (1.02, 1.12) |

B

| SNPs | Ferritin-T2D | OR (95% CI) |
|------|--------------|-------------|
| rs18003562 | 1.23 (1.08, 1.38) |
| rs1799945 | 1.28 (1.04, 1.56) |
| rs557981 | 1.04 (0.82, 1.31) |
| Overall estimate | 1.15 (1.06, 1.26) |

C

| SNPs | Transformin-T2D | OR (95% CI) |
|------|-----------------|-------------|
| rs18003562 | 1.14 (1.03, 1.12) |
| rs1799945 | 1.12 (1.05, 1.18) |
| rs557981 | 1.03 (0.95, 1.10) |
| Overall estimate | 1.06 (1.02, 1.00) |

D

| SNPs | Transformin-T2D | OR (95% CI) |
|------|-----------------|-------------|
| rs18003562 | 0.92 (0.87, 0.97) |
| rs1799945 | 0.88 (0.74, 1.03) |
| rs557981 | 0.95 (0.71, 1.27) |
| Overall estimate | 0.91 (0.87, 0.96) |
