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Abstract

Dietary heme iron intake is linked to an increased risk of type 2 diabetes (T2D), but the underlying plasma biomarkers are not well understood. We analyzed data from 204,615 participants in three large US cohorts over up to 36 years, examining the associations between iron intake and T2D risk. We also assessed plasma metabolic biomarkers and metabolomic profiles in subsets of 37,544 and 9,024 participants, respectively. Here we show that heme iron intake, but not non-heme iron, is associated with a higher T2D risk, with a multivariable-adjusted hazard ratio of 1.26 (95% CI: 1.20, 1.33; P for trend <0.001) comparing the highest to the lowest quintiles. Heme iron accounts for significant proportions of the T2D risk linked to unprocessed red meat and specific dietary patterns. Increased heme iron intake correlates with unfavorable plasma profiles of insulinemia, lipids, inflammation, and T2D-linked metabolites. We also identify metabolites, including L-valine and uric acid, potentially mediating the heme iron-T2D relationship, highlighting their pivotal role in T2D pathogenesis.

Introduction

Excess iron stores, particularly from heme iron intake, can act as pro-oxidants, catalyzing the formation of reactive oxygen species and leading to physiological dysfunction or disease states.[1,2] For this reason, concerns have been raised about novel plant-based meat alternatives that add heme to enhance meaty flavor and apeparance.[3] Many studies,[4–7] including two from our previous research,[5,6] have found that heme iron intake is associated with a higher risk of type 2 diabetes (T2D). Given its high abundance in red meat, heme iron is proposed as one potential mechanism explaining the adverse associations of red meat consumption and several dietary patterns with T2D risk.[8,9] However, few studies have investigated the extent to which these associations could be attributed to heme iron intake.

Additionally, the relationship between iron intake and circulating metabolic biomarkers remains largely unexplored, representing a significant gap in our understanding that could illuminate the potential biological effects of heme iron consumption on T2D risk. Furthermore, recent advancements in high-throughput metabolomics profiling technologies have enabled nutritional epidemiological research to investigate the complex interrelationships between diet, metabolic processes, and disease outcomes.[10] Unravelling the metabolic biomarkers and metabolites that interplay with the heme iron-T2D association may provide new insights into the underlying biological mechanisms and potentially lead to the development of strategies for early prevention.

Leveraging data from three large prospective US adult cohorts, we updated our previous analyses of the associations between various types of iron intake (total, heme, nonheme, dietary, and supplemental) and T2D risk,[5,6] extending the follow-up by nearly 20 years. We further assessed the proportion of T2D risk associated with red meat consumption and several dietary patterns that could be attributed to heme iron intake. Additionally, we evaluated the relationships between iron intake and ten established plasma metabolic biomarkers involved in the domains of insulinemia, glycemia, lipids, and inflammation, as well as two biomarkers of iron metabolism that were previously associated with T2D risk in our research.[11] Finally, we integrated plasma metabolomics data to uncover the metabolite profile underlying the association between heme iron and T2D risk.

Results

Characteristics of the study participants

Our primary analysis of iron intake and T2D risk included data from 204,615 participants from the Nurses' Health Study (NHS), NHS2, and the Health Professionals Follow-up Study (HPFS) (**Figure 1a**). Baseline characteristics of the participants, according to quintiles of heme iron intake, are presented in **Table 1** and **Supplementary Table 1**. Participants with higher total iron and nonheme iron intakes were more physically active, more likely to use multivitamins, and less likely to smoke compared to those with lower intakes. They also had higher intakes of cereal fiber, magnesium, fruits, vegetables, and whole grains. In contrast, those with a greater heme iron intake were generally less physically active, more likely to smoke, and had lower intakes of cereal fiber, magnesium, fruits, and whole grains, while consuming more red meat, poultry,

and fish. Analyses of plasma metabolic biomarkers and metabolomic profiles included subsets of 37,544 and 9,024 participants, respectively (**Figure 1a**). These participants were several years older and had a higher prevalence of hypertension and high cholesterol than the baseline population (**Figure 1b** and **Supplementary Table 2**). However, their BMI, physical activity levels, and dietary iron intake were generally similar to those of the larger cohort.

Iron intake and risk of T2D

We first updated our previous analyses of the associations between various types of iron intake (total, heme, nonheme, dietary, and supplemental) and T2D risk. During up to 36 years of follow-up, we documented 20,705 incident T2D cases (8,666 in NHS, 7,985 in NHS2, and 4,054 in HPFS). Intakes of total iron, nonheme iron, dietary iron, and supplemental iron were inversely associated with the risk of T2D in the age-adjusted model (**Table 2**). However, these exposures were unrelated to T2D risk in the multivariable-adjusted model. In contrast, heme iron intake was associated with a higher risk of T2D, and the multivariable-adjusted HR comparing extreme quintiles of intake was 1.26 (95% CI: 1.20, 1.33; P for trend<0.001). Results were consistent across three cohorts (**Supplementary Table 3**).

Dose-response analyses indicated a generally linear association between heme iron intake and T2D risk, with a HR of 1.28 (95% CI: 1.22, 1.34) for every 1 mg/d increment (approximate to the difference between the median values of the highest and lowest quintiles). However, at high intake levels, we observed a flattened slope in T2D risk (P for nonlinearity=0.003, **Figure 2a**). Subgroup analysis revealed that the associations between heme iron intake and T2D risk persisted across different strata of age, sex, BMI, waist-hip ratio, race, postmenopausal status, and phytate intake (**Figure 2b**). However, a stronger association was observed among participants with a lower BMI (P for interaction=0.01). In sensitivity analyses, the results remained robust even after additional adjustment for waist circumference, or animal fat and animal protein, or whole grains, fruits, and vegetables, or neighborhood socioeconomic status (**Supplementary Table 4**). Heme iron intake was also associated with a higher T2D risk when we restricted the analysis to participants with available conventional metabolic biomarkers or metabolomics data (**Supplementary Table 4**).

Contribution of heme iron intake in diet-T2D associations

Heme iron intake was positively correlated with the intake of red meat, especially unprocessed red meat, as well as the Western dietary pattern score, which is rich in red meat (**Figure 2c**). In contrast, heme iron had a negative correlation with dietary patterns that limit red meat consumption, such as the prudent diet, hPDI, AHEI, AMED, and DASH. We assessed what proportion of the association between these dietary factors and T2D risk could be statistically explained by heme iron intake, using mediation approach. Our results showed that 65.6% (95% CI: 39.7, 84.6; P<0.001) of the association between unprocessed red meat and T2D risk was accounted for by heme iron intake (**Figure 2d**). However, heme iron only explained a minor proportion (2.0%; 95% CI: 1.3, 3.0; P<0.001) of the association between processed red meat and T2D.

For dietary patterns mentioned earlier, the proportion of association attributed to heme iron intake was between 8.7% to 26.2% (all P<0.001).

Iron intake and conventional metabolic biomarkers

Next, we examined associations of iron intake with a panel of widely accepted plasma metabolic biomarkers. A 1 mg/d increment in heme iron intake was significantly associated with unfavorable profiles of several biomarkers after adjusting for BMI and other covariates: 6.3% (95% CI: 3.1, 9.7) higher level of C-peptide, 3.7% (95% CI: 1.9, 5.4) lower HDL-C, 6.0% (95% CI: 2.9, 9.3) higher TAG, 10.1% (95% CI: 4.4, 16.0) higher TAG/HDL-C ratio, 8.5% (95% CI: 3.3, 14.0) higher CRP, 5.0% (95% CI: 2.6, 7.4) lower adiponectin, 10.1% (95% CI: 5.7, 14.7) higher leptin, 28.1% (95% CI: 18.8, 38.1) higher ferritin, and 23.1% (95% CI: 13.3, 31.8) lower TfR/ferritin ratio, respectively (**Figure 3a**). For other types of iron intake, the only significant association was between total iron and a higher plasma ferritin level; no other significant associations were observed. Results for heme iron generally remained the same when restricting the analysis to participants who were selected as controls in previous substudies (**Figure 3b**), who provided fasting blood samples, or who reported no history of hypertension or hypercholesterolemia at blood collection (**Supplementary Table 5**). Further adjusting for neighborhood socioeconomic status also did not change the results.

Plasma metabolomic biomarkers for heme iron-T2D association

To integrate plasma metabolomic data, we constructed a multi-metabolite profile score to predict the risk of T2D (n=851 cases). Metabolites contributing positively to the score, including L-alanine, L-tyrosine, L-valine, uric acid, C5 carnitine, and several glycerolipids, showed positive associations with T2D-related biomarkers such as C-peptide, TAG, TAG/HDL-C ratio, CRP, and leptin. They were also negatively associated with beneficial biomarkers like HDL-C and adiponectin (**Figure 4a**). Conversely, metabolites with a negative weight in the score, such as betaine, glycine, L-glutamine, and L-asparagine, demonstrated associations in the opposite direction. As expected, a higher metabolomic score was associated with a higher risk of T2D, with a HR of 2.12 (95% CI: 1.81, 2.48; P for linearity<0.001) per 1-SD increment (**Figure 4b**). Subsequent analysis revealed that every 1 mg/d increment of heme iron intake was associated with 0.16 (95% CI: 0.10, 0.21; P <0.001) higher metabolomic score (**Figure 4c**). As comparison, we did not observe a significant association between nonheme iron intake and the T2D metabolomic score (P=0.37).

Following the four predefined criteria, we identified 17 potential intermediate metabolites that might play a role underlying the heme iron-T2D association (**Extended Data Figure 1**). Of these, 13 metabolites, including L-valine, L-lysine, L-targinine, uric acid, and several glycerolipids (di- and triacylglycerols), were positively associated with T2D risk (**Figure 4d** and **Supplementary Table 6**), heme iron intake (**Figure 4e** and **Supplementary Table 6**), and an unfavorable metabolic biomarker profile (**Figure 4f**). The remaining four metabolites—C18:2 cholesterol ester (CE), C18:2 lysophosphatidylcholine (LPC), C38:2 phosphatidylethanolamine (PE) and C18:3 CE—had opposite associations. The associations between heme iron intake and T2D risk were all attenuated after adjusting for these 17 intermediate metabolites (**Extended**

Data Figure 1b). Sensitivity analysis restricted to control participants yielded similar results compared to those obtained using all participants (**Extended Data Figure 2**).

Discussion

In this large prospective cohort study, we found that among various types of iron intake, only a higher intake of heme iron was associated with higher risk of T2D. Notably, heme iron accounted for a large proportion of the association between unprocessed red meat intake and T2D risk, as well as a modest to moderate proportion for several dietary patterns. Additionally, we observed that higher heme iron intake was associated with unfavorable profiles of plasma biomarkers in the domains of insulinemia, lipids, inflammation, iron stores, and metabolites correlated with T2D. We also identified several potential intermediate metabolites (L-valine, L-lysine, uric acid, and several lipid metabolites) linking heme iron intake to T2D risk.

In line with previous studies,[4] we found no significant associations between the intake of total, nonheme, dietary, or supplemental iron and T2D risk. Heme and nonheme are the two forms of iron found in foods. Nonheme iron, which constitutes the major fraction of total and dietary iron intake, is typically sourced from healthy plant foods such as whole grains, fruits, and some vegetables.[12] This might explain the null association for these iron types. Furthermore, the absorption rate of nonheme iron, ranging from 2% to 20%, is lower than that of heme iron (15% to 35%).[13] This difference is corroborated by our analysis of iron store biomarkers, which demonstrated that heme iron, rather than nonheme iron, was correlate with higher iron stores, indicated by higher ferritin levels and a lower TfR/ferritin ratio. Higher iron stores have been linked to a higher T2D risk in both epidemiological[11] and Mendelian Randomization studies.[14] Our findings also suggested that iron supplementation is generally safe in terms of T2D risk.

The higher risk of T2D associated with heme iron intake was consistent with a recent meta-analysis of prospective cohort studies.[4] We observed a stronger unfavorable association for heme iron intake among participants with a lower BMI compared to those with a higher BMI. One explanation is that obesity may affect iron metabolism by impairing absorption and reducing the body's ability to store iron,[13] thereby leading to a weaker unfavorable association with T2D risk. In addition to assessing the direct association between heme iron intake and T2D risk, we also examined the extent to which heme iron intake could account for the association of red meat and various T2D-related dietary patterns, a hypotheses extensively suggested in prior research.[8,15] Our analyses revealed that heme iron intake contributed more than half of the association between unprocessed red meat and T2D risk, yet it explained only a small proportion of the association for processed red meat. This finding is expected given the strong correlation between heme iron intake and unprocessed red meat. The harmful association of processed red meat and T2D risk might be attributed to the high content of other compounds, such as nitrates and nitrites.[16,17] The attributable proportion for dietary patterns was moderate. Their associations with T2D risk could also be partly due to other dietary factors, such as the high added sugar in the western diet and high fiber and phytochemicals in healthy dietary patterns.

The biological mechanisms linking heme iron intake to T2D risk are complex. Our analysis of metabolic biomarkers and metabolomics data has suggested the involvement of insulin resistance, inflammation, and the metabolism of several metabolites. We found that heme iron intake was associated with higher C-peptide levels (an indicator of insulin production), a higher TAG/HDL-C ratio (a marker of insulin resistance), and a proinflammatory profile, evidenced by higher CRP and leptin levels and lower adiponectin levels, even after adjusting for BMI and other T2D risk factors. Our findings align with animal studies that show iron overload can disrupt glucose metabolism through insulin resistance.[18,19] Moreover, heme iron, acting as a potent pro-oxidant, could exacerbate the accumulation of reactive oxygen species, promoting oxidative damage and inflammation—key factors in the diabetes pathophysiology.[20] Given that these metabolic biomarkers are also risk factors for cardiovascular diseases, it is not unexpected that heme iron is associated with a higher risk of cardiovascular diseases as well.[21] Moving beyond conventional metabolic biomarkers, we incorporated metabolomics data and developed a multi-metabolite profile score as a new biomarker for assessing T2D risk. Unsurprisingly, we observed that heme iron intake was significantly associated with a higher T2D metabolite score. In addition, we identified several metabolites, including L-valine, L-lysine, L-targinine, uric acid, and several lipids, that may play a role in the association between heme iron intake and T2D risk. Higher levels of these three amino acids and uric acid have been associated with increased T2D risk,[22–24] and previous studies have reported positive associations between iron stores and both valine and uric acid,[25,26] suggesting that heme iron may impact the metabolism of these metabolites. We found that the intermediate lipid metabolites were strongly associated with biomarkers of insulin resistance, indicating they could result from metabolic disruptions related to insulin resistance. It remains uncertain how other identified intermediate metabolites are involved, necessitating further research.

The main strength of our study was the integration of conventional clinical biomarkers and cutting-edge metabolomics data, advancing our understanding beyond previous studies that relied solely on epidemiological data. Such integration not only allowed us to delve into the potential biological mechanisms involved but also could help in developing strategies for early prevention of T2D and in guiding more personalized nutritional recommendations tailored to individual metabolic profiles. Adopting an integrative research approach that combines several complementary methods represents a new philosophy of preventive nutrition to improve nutritional recommendations.[27] Other strengths included the large sample size and long follow-up, permitting a wide array of subgroup analyses. Due to the detailed data available, we were also able to adjust for numerous possible confounders, particularly saturated fat and trans fat, which are associated with higher T2D risk and are found in red meat alongside heme iron. Additionally, our repeated dietary assessments ensured a reliable measure of long-term dietary intake over the study periods. Despite the strengths, our study had several limitations as well. Firstly, although our FFQs have been validated against the gold standard of dietary assessment—7-day weighed dietary records, measurement error is inevitable when using FFQs to collect dietary intake data. Secondly, we were unable to identify other prospective cohorts that have derived heme

iron intake to replicate our findings. Moreover, the sample size for analyses examining the associations of conventional metabolic biomarkers and metabolites was small. Finally, due to the observational nature of our study design, we cannot rule out the possibility of residual confounding factors. However, the consistent results from our sensitivity analyses and analyses across multiple data types—including epidemiological, metabolic biomarker, and metabolomics—bolster our confidence in the robustness of our findings.

In conclusion, heme iron intake was associated with a higher risk of T2D, and it accounted for around half of the T2D risk associated with unprocessed red meat and a moderate proportion of the risk for several T2D-related dietary patterns. Our results on plasma metabolic and metabolomic biomarkers further support the heme iron-T2D association. These findings have important public health implications in shaping guidelines to prevent diabetes by limiting the daily consumption of foods rich in heme iron, particularly red meat. They also raise concerns about adding plant heme to plant-based meat alternatives.

Methods

Study population

The NHS was initiated in 1976 and enrolled 121,700 registered female nurses aged 30-55 years. The NHS2 was established in 1989 and recruited 116,429 younger female nurses aged 25-42 years. The HPFS started in 1986, enrolling 51,529 male health professionals aged 40-75 years. Participants in each cohort were followed every 2 to 4 years via mailed questionnaires that were designed to collect information on dietary habits, lifestyle factors, and medical history. Blood samples were collected from a subset of participants in NHS (n=32,826) between 1989 and 1990, in NHS2 (n=29,611) between 1996 and 1999, and in HPFS (n=18,159) between 1993 and 1995. The study protocols for each cohort were approved by the Institutional Review Boards (IRB) of the Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital. Because the cohorts were initiated many years ago, explicit informed consent was not obtained from participants at initiation. The IRBs allowed participants' completion of questionnaires as implied consent.

In the present analysis, we excluded participants who self-reported a history of diabetes, cardiovascular disease, or cancer at baseline (1984 for NHS, 1991 for NHS2, and 1986 for HPFS). We also excluded participants without dietary data at baseline or those who reported unusual total energy intakes (<500 or >3500 kcal/d for women and <800 or >4200 kcal/d for men). After these exclusions, 71,268 participants from NHS, 91,010 from NHS2, and 42,337 from HPFS remained for our main analyses (**Figure 1a** and **Extended Data Figure 3**).

Dietary assessment

Dietary data were collected via semiquantitative food frequency questionnaires (FFQs) every four years, starting from 1984 and 1986 in NHS, 1991 in NHS2, and 1986 in HPFS. More than 95% of the participants completed at least two FFQs during follow-up (**Supplementary Table 7)**. These FFQs have been validated against dietary records,

and their reproducibility is well-documented.[28,29] Nutrient values were calculated using the Harvard University Food Composition Table, which is primarily derived from US Department of Agriculture data, supplemented with manufactures' data, and is updated regularly. Participants reported the use of multivitamin supplements and any specific iron supplements including the dosage. We calculated the total iron intake by summing iron from all dietary and supplemental sources. The Spearman correlation coefficient for total iron intake estimated from FFQs and two 7-day dietary records in our validation study was 0.58 in women and 0.60 in men.[28,29] Additionally, we estimated the intake of heme iron—solely present in animal products—and calculated the nonheme iron intake by subtracting heme iron from the total intake. The primary food sources contributing to different types of iron intake were shown in **Supplementary Table 8**. Iron intake was further adjusted for total energy intake using the nutrient residual method.[30] Dietary scores previously associated with T2D, including the western diet, prudent diet, healthful plant-based diet (hPDI), Alternate Healthy Eating Index (AHEI), Alternate Mediterranean Diet Score (AMED), and Dietary Approaches to Stop Hypertension (DASH), were derived as described previously.[15,31]

Ascertainment of type 2 diabetes

Participants who self-reported physician-diagnosed diabetes on their biennial questionnaires were sent an additional validated questionnaire to gather further details on symptoms, diagnostic tests, and medication use to confirm the diagnosis.[32,33] Only confirmed T2D cases that met the National Diabetes Data Group criteria through 1997 or the American Diabetes Association criteria from 1998 onward were included.[34] The threshold for fasting blood glucose in the diagnostic criteria was changed starting from 1998;[35] HbA1c was further incorporated into the criteria beginning in 2010.[36]

Assessment of covariates

We collected information on race, body weight, physical activity, smoking status, multivitamin use, aspirin use, family history of T2D, as well as history of hypertension and hypercholesterolemia from self-reported biennial questionnaires. Body mass index (BMI) was calculated using height reported at cohort baseline and body weight reported during the follow-up. We also ascertained menopausal status and postmenopausal hormone use among NHS and NHS2 participants. Fasting status was collected through questionnaires completed at blood collection.

Conventional metabolic biomarker measurement

Plasma concentrations of C-peptide, HbA1c, total cholesterol, high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), triacylglycerol (TAG), C-reactive protein (CRP), adiponectin, leptin, ferritin, and transferrin receptor (TfR) were measured in multiple prior substudies (**Supplementary Table 9**) using standard methods.[37,38] Data from these substudies were combined and corrected for batch effects using an average batch correction method.[24] After excluding participants having a history of diabetes, cardiovascular disease, or cancer at blood collection, we included 37,544 participants (n=2,586 to 14,639 for individual biomarkers) in the biomarker analyses (**Figure 1a** and **Extended Data Figure 3**). All participants were free of the disease under study at the time of blood collection.

Metabolomics measurement

Plasma metabolomics profiling was performed in several nested case-control studies (**Supplementary Table 9**), using high-throughput liquid chromatography-mass spectrometry (LC-MS) techniques at the Broad Institute of MIT and Harvard (Cambridge, MA). A detailed description of the metabolomics profiling method can be found elsewhere.[40,41] Polar metabolites were separated via hydrophilic interaction liquid chromatography with positive ionization MS detection (HILIC-pos). Lipid profiling was conducted using C8 chromatography paired with positive ionization detection (C8-pos). For each method, pooled plasma reference samples were analyzed every 20 participant samples to standardize temporal drift in instrument response over time and between batches. Additionally, quality control samples, to which the laboratory was blinded, were randomly distributed among study samples, and were also profiled.

Of the 453 named metabolites identified in the metabolomics profiling, we excluded metabolites with an intraclass correlation coefficient <0.3 across blinded quality control replicates (n=10) or with ≥25% missing data (n=166), resulting in 277 metabolites for final analyses. Metabolites with skewed distribution (absolute skewness >2) were log-transformed,[42] and all metabolites were standardized to z-scores within each substudy. Missing data for metabolites were imputed using the random forest imputation method, as previously recommended for metabolomics analyses.[43] The final subset for metabolomic analyses included 9,024 participants (**Figure 1a** and **Extended Data Figure 3**). Similarly, all participants were free of the disease under study at the time of blood collection.

Statistical analysis

We calculated person-time of follow-up for each participant from the age at which the baseline questionnaire was returned to the age of diagnosis of T2D, death, or the end of follow-up (June 2020 for NHS, June 2019 for NHS2, and January 2020 for HPFS), whichever came first. To better capture long-term habitual dietary intake and minimize within-person variation, we used the updated cumulative average of dietary intakes from baseline to the censoring event. Cox regression models were conducted to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) of higher versus the lowest quintiles of iron intake with incident T2D risk. We used the median value of iron intake within each quintile and treated it as a continuous variable to test for a linear trend. All analyses were stratified by age and calendar year. For multivariable models, we additionally adjusted for BMI, race, family history of diabetes, menopausal status and postmenopausal hormone use (NHS and NHS2 only), multivitamin use (only for analyses of heme iron and dietary iron), smoking status, alcohol drinking, physical activity, baseline history of hypertension and hypercholesterolemia, intakes of total energy, magnesium, trans fat, and cereal fiber, ratio of polyunsaturated fat intake to saturated fat intake, and glycemic load, with covariate status updated every 2-4 years. Analyses were performed separately within each cohort, and the results were pooled using a fixed-effect meta-analysis.

To evaluate the dose-response relationship between heme iron intake and the risk of T2D, we pooled individual-level data from the three cohorts and performed restricted cubic spline analyses, with additional stratification by cohort. Heme iron intake was analysed as a continuous variable as its distribution is generally normal (**Figure 2a**), although normality was not formally tested. In subgroup analyses, we assessed whether the association between heme iron intake and T2D risk varied by age(<55 or ≥55 years), sex (male or female), BMI (<25, 25-30, or ≥30 kg/m^2), waist-hip ratio (high or low), race (white or non-white), postmenopausal status (pre or postmenopausal, among women only), and phytate intake (above or below the median intake). P values for interaction were determined by comparing models with and without multiplicative interaction terms for heme iron intake and the aforementioned factors using a likelihood ratio test. We conducted several sensitivity analyses to test the robustness of our results regarding heme iron intake: 1) additionally adjusting for waist circumference; 2) additionally adjusting for animal fat and animal protein; 3) additionally adjusting for whole grains, fruits, and vegetables; 4) additionally adjusting for neighborhood socioeconomic status;[44] and 5) restricting the analyses to participants with available conventional metabolic biomarker or metabolomics data.

Intake of red meat and several dietary patterns that either emphasize or limit red meat consumption, such as the Western diet, prudent diet, hPDI, AHEI, AMED, and DASH, have been associated with the risk of T2D.[15,31] To estimate the proportions of T2D risk associated with red meat consumption and these dietary patterns that can be attributed to heme iron intake, we conducted mediation analyses using the publicly available "mediate" SAS macro (https://ysph.yale.edu/cmips/research/software/mediate_340185_284_47911_v2.pdf), following the approach used in previous studies.[45,46] This macro compares a full model, which includes the exposure, an intermediate variable, and any covariates, with a partial model that leaves out the intermediate variable, and calculates the proportion.

In the sub dataset for metabolic biomarkers, multivariable linear regressions were conducted to examine the relationship between different types of iron intake and 12 plasma metabolic biomarkers. All biomarkers were natural log-transformed to improve normality, and thus results were presented as percentage differences: [exp (β-coefficient) −1] × 100%. We used the average iron intake from two FFQs administered close to blood collection (1986 and 1990 for NHS, 1995 and 1999 for NHS2, and 1990 and 1994 for HPFS). Multivariable models were adjusted for age at blood draw, sex, case-control status in the original substudies, fasting status, BMI, race, menopausal status and postmenopausal hormone use (women only), multivitamin use (only for analyses of heme iron and dietary iron), aspirin use, smoking status, alcohol drinking, physical activity, baseline history of hypertension and hypercholesterolemia, intakes of total energy, magnesium, trans fat, and cereal fiber, ratio of polyunsaturated fat intake to saturated fat intake, and glycemic load. We conducted sensitivity analyses, focusing specifically on participants who were selected as controls in the original substudies, who provided fasting blood samples, or who reported no history of hypertension or hypercholesterolemia at blood collection. We additionally checked the results after further adjusting for neighborhood socioeconomic status.

In the metabolomic sub dataset, to evaluate whether heme iron intake is associated with plasma metabolomic biomarkers of T2D, we developed a multi-metabolite profile score to predict T2D risk, using elastic net penalized Cox regression within a 1000-fold computing framework.[47] For each computing round, the elastic net model was trained on 999 folds and then applied to the remaining fold to calculate the metabolite profile score. After 1000 such iterations, scores were derived for all participants. The score was calculated as the weighted sum of the selected metabolites with weights equal to the elastic net regression coefficients. We then evaluated the association between metabolites selected in the score and 12 conventional metabolic biomarkers by multivariable linear regression, as well as the association between the metabolite profile score and T2D risk via multivariable Cox regression. Subsequently, we examined the association between heme iron intake and the T2D metabolite score using multivariable linear regression. We further identified potential mediating metabolites of the association between heme iron intake and T2D risk according to four predefined criteria (**Extended Data Figure 1a**):[48] (1) existence of an association between heme iron intake and T2D; (2) existence of an association between heme iron intake and the potential mediating metabolite; (3) existence of an association between the potential mediating metabolite and T2D in the same direction as with heme iron intake; and (4) attenuation of the heme iron-T2D association after adjustment for the potential mediating metabolite. The associations of potential mediating metabolites with metabolic biomarkers were also assessed.

All statistical tests were two sided, with a significance threshold of 0.05 for T2D analyses. P values for different metabolic biomarkers and metabolites were corrected for multiple testing using the Bonferroni method. We used SAS version 9.4 (SAS institute) for the T2D analysis, and R version 4.2 for the metabolic biomarker and metabolomics analyses.

Data availability: Because of participant confidentiality and privacy concerns, data are available upon written request. According to standard controlled access procedure, applications to use NHS/NHSII/HPFS resources will be reviewed by our External Collaborators Committee for scientific aims, evaluation of the fit of the data for the proposed methodology, and verification that the proposed use meets the guidelines of the Ethics and Governance Framework and the consent that was provided by the participants. Investigators wishing to use NHS/NHSII/HPFS data are asked to submit a brief description of the proposed project (contact: nhsaccess@channing.harvard.edu). Investigators can expect initial responses within 4 weeks of request submission. Details are available on https://www.nurseshealthstudy.org/researchers and https://sites.sph.harvard.edu/hpfs/for-collaborators/.

Code availability: The main analysis code used in this study is available on GitHub at https://github.com/fengleiwang/heme_t2d.

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Author contributions: FW and FBH designed the research. FW, AJG, and AJT conducted analyses. DEH, DKT, AHE, JEM, CC, EBR, and QS participated in acquisition of data. KHL and LL provided statistical expertise. FW wrote the first draft of the paper. ZM, MGF, DDW and WCW contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content. All authors approved the final version of the manuscript.

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Table 1. Age-standardized baseline characteristics by quintiles of heme iron intake

	NHS (1984)			NHS2 (1991)			HPFS (1986)		
	Quintile 1	**Quintile 3**	**Quintile 5**	**Quintile 1**	**Quintile 3**	**Quintile 5**	**Quintile 1**	**Quintile 3**	**Quintile 5**
No. of participants	14,252	14,252	14,254	18,199	18,207	18,201	8469	8469	8468
Age (years)	50.7 (7.3)	49.8 (7.2)	50.0 (7.0)	35.8 (4.7)	36.0 (4.7)	36.6 (4.6)	53.5 (9.9)	52.8 (9.4)	52.9 (9.2)
White (%)	98	98	97	97	97	95	95	96	94
Body mass index (kg/m^2)	24.1 (4.2)	25.0 (4.7)	25.5 (4.9)	23.4 (4.6)	24.5 (5.1)	25.8 (5.8)	24.7 (3.0)	25.5 (3.1)	26.2 (3.6)
Physical activity (MET-h/w)	15.2 (23.4)	13.6 (19.4)	12.6 (17.6)	24.3 (32.3)	20.1 (25.1)	19.3 (25.8)	25.2 (29.2)	20.3 (23.6)	17.3 (21.7)
Current smoker (%)	21	24	28	10	12	15	6	10	12
Alcohol consumption (g/d)	6.9 (11.9)	7.2 (11.3)	6.3 (10.3)	3.2 (6.5)	3.1 (6.0)	2.8 (5.5)	10.9 (16.3)	12.1 (15.8)	10.2 (13.7)
Premenopausal (%)	43	43	41	98	97	96	/	/	/
Multivitamin supplement use (%)	40	37	34	49	44	40	47	42	38
Family history of diabetes (%)	26	28	31	31	34	37	20	20	21
Prevalent hypertension (%)	18	20	21	5	6	8	17	20	21
Prevalent high cholesterol (%)	8	8	7	12	14	17	12	10	9
Dietary intake									
Total calories (kcal/d)	1745 (536)	1784 (545)	1687 (503)	1795 (567)	1803 (539)	1728 (542)	1996 (626)	2034 (635)	1936 (609)
Glycemic load	123 (46)	113 (42)	92 (36)	137 (51)	122 (43)	101 (39)	142 (53)	126 (46)	103 (39)
Ratio of polyunsaturated fat to saturated fat	0.6 (0.2)	0.6 (0.2)	0.5 (0.1)	0.6 (0.2)	0.5 (0.1)	0.5 (0.1)	0.7 (0.3)	0.6 (0.2)	0.5 (0.2)
Cereal fiber (g/d)	5.3 (3.2)	4.7 (2.7)	3.6 (2.3)	6.6 (4.2)	5.6 (3.1)	4.5 (2.7)	7.3 (5.7)	5.8 (3.5)	4.4 (3.2)
Magnesium (mg/d)	318 (125)	310 (107)	286 (95)	328 (120)	311 (103)	294 (105)	380 (141)	351 (114)	317 (109)
Unprocessed red meat (serving/w)	2.5 (1.4)	5.6 (2.4)	9.9 (4.1)	2.4 (1.8)	4.9 (2.7)	7.8 (4.7)	2.1 (1.6)	5.3 (2.9)	9.6 (5.2)
Processed red meat (serving/w)	1.6 (1.9)	2.2 (2.2)	2.5 (2.5)	1.1 (1.5)	1.7 (1.9)	1.8 (2.1)	1.5 (2.2)	2.7 (3.1)	3.2 (3.3)
Poultry (serving/w)	2.7 (2.1)	3.7 (2.7)	4.0 (3.7)	2.7 (2.0)	4.9 (2.6)	6.7 (4.4)	2.8 (2.2)	4.2 (2.8)	4.8 (4.6)
Fish (serving/w)	1.7 (1.5)	2.3 (1.8)	2.3 (2.1)	1.3 (1.2)	1.9 (1.5)	2.5 (2.4)	2.4 (2.1)	2.8 (2.3)	2.8 (2.7)
Fruits (serving/d)	1.6 (1.3)	1.4 (1.0)	1.2 (0.9)	1.4 (1.1)	1.2 (0.9)	1.0 (0.8)	2.0 (1.7)	1.6 (1.2)	1.2 (1.0)
Vegetables (serving/d)	3.0 (1.8)	3.1 (1.6)	3.0 (1.6)	2.8 (1.9)	2.9 (1.7)	3.0 (1.8)	3.2 (2.1)	3.1 (1.7)	3.0 (1.7)
Whole grains (serving/d)	1.3 (1.3)	1.1 (1.1)	0.8 (0.9)	1.5 (1.3)	1.2 (1.1)	1.0 (0.9)	2.1 (1.9)	1.5 (1.3)	1.1 (1.1)

Data were expressed as mean (SD) unless otherwise specified. All variables except age are age-standardized. Prevalent hypertension and high cholesterol were self-reported on the biennial questionnaire. HPFS, Health Professionals Follow-up Study; MET, metabolic equivalent; NHS, Nurses' Health Study.

Table 2. Pooled hazard ratios of type 2 diabetes according to quintiles of iron intake

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P for trend
Total iron						
Median (mg/d)	10.7	13.2	16.2	21.2	33.7	
Cases/PYs	4656/1048252	4464/1050949	4214/1052343	3878/1053010	3493/1051516	
Age-adjusted	Ref	0.95 (0.91, 0.99)	0.90 (0.86, 0.94)	0.83 (0.80, 0.87)	0.77 (0.73, 0.80)	$3.0x10^{-38}$
Age-adjusted + BMI	Ref	0.95 (0.91, 0.99)	0.93 (0.89, 0.97)	0.89 (0.86, 0.93)	0.86 (0.82, 0.90)	$3.3x10^{-11}$
Multivariable-adjusted	Ref	1.01 (0.97, 1.05)	1.03 (0.98, 1.07)	1.01 (0.96, 1.05)	0.97 (0.93, 1.02)	0.08
Heme iron						
Median (mg/d)	0.7	0.9	1.0	1.2	1.5	
Cases/PYs	2554/1052982	3367/1052863	4059/1051772	4844/1051001	5881/1047447	
Age-adjusted	Ref	1.32 (1.25, 1.39)	1.58 (1.50, 1.66)	1.87 (1.79, 1.97)	2.25 (2.15, 2.36)	0.0[a]
Age-adjusted + BMI	Ref	1.09 (1.04, 1.15)	1.18 (1.13, 1.24)	1.28 (1.22, 1.35)	1.38 (1.32, 1.45)	$1.2x10^{-54}$
Multivariable-adjusted	Ref	1.07 (1.02, 1.13)	1.15 (1.09, 1.21)	1.22 (1.16, 1.28)	1.26 (1.20, 1.33)	$3.3x10^{-23}$
Nonheme iron						
Median (mg/d)	9.6	12.1	15.1	20.2	32.7	
Cases/PYs	4806/1047906	4441/1051208	4176/1052275	3816/1053077	3466/1051601	
Age-adjusted	Ref	0.92 (0.88, 0.96)	0.86 (0.83, 0.90)	0.79 (0.76, 0.83)	0.74 (0.70, 0.77)	$5.4x10^{-46}$
Age-adjusted + BMI	Ref	0.94 (0.90, 0.98)	0.92 (0.88, 0.96)	0.88 (0.84, 0.92)	0.85 (0.82, 0.89)	$2.0x10^{-12}$
Multivariable-adjusted	Ref	1.00 (0.96, 1.05)	1.02 (0.98, 1.07)	0.99 (0.95, 1.04)	0.97 (0.92, 1.02)	0.06
Dietary iron						
Median (mg/d)	10.1	11.7	13.0	14.6	17.8	
Cases/PYs	4391/1049053	4394/1051398	4377/1051668	4156/1052156	3387/1051792	
Age-adjusted	Ref	0.99 (0.95, 1.04)	0.99 (0.95, 1.03)	0.94 (0.90, 0.98)	0.77 (0.73, 0.80)	$3.7x10^{-37}$
Age-adjusted + BMI	Ref	0.96 (0.92, 1.00)	0.96 (0.92, 1.00)	0.94 (0.90, 0.98)	0.84 (0.81, 0.88)	$3.1x10^{-13}$
Multivariable-adjusted	Ref	1.01 (0.97, 1.06)	1.05 (1.00, 1.10)	1.07 (1.02, 1.12)	1.02 (0.97, 1.07)	0.31
Supplemental iron						
Median (mg/d)	0.0096	0.0102	0.7	6.3	18.6	
Cases/PYs	4698/1047636	4196/1050167	4074/1053056	4124/1053712	3613/1051497	
Age-adjusted	Ref	0.89 (0.85, 0.92)	0.86 (0.82, 0.89)	0.88 (0.84, 0.91)	0.79 (0.75, 0.82)	$3.2x10^{-17}$
Age-adjusted + BMI	Ref	0.93 (0.89, 0.97)	0.91 (0.88, 0.95)	0.94 (0.91, 0.98)	0.88 (0.85, 0.92)	$7.7x10^{-05}$
Multivariable-adjusted	Ref	0.99 (0.94, 1.03)	0.99 (0.94, 1.03)	1.02 (0.98, 1.07)	0.96 (0.91, 1.00)	0.09

Multivariable-adjusted models were stratified by age and calendar year, and adjusted for BMI (<23, 23-25, 25-30, or 30+ kg/m^2), race (White or Nonwhite), family history of diabetes (yes or no), menopausal status and postmenopausal hormone use (premenopausal, postmenopausal [never use, past use, or current use], NHS and NHS2 only), multivitamin use (yes or no, only for analyses of heme iron and dietary iron), smoking status (never, past, or current), alcohol drinking (0, 0-5, 5-15, or 15+g/d), physical activity (<3, 3-9, 9-28, 18-27, or 27+ MET-h/w), baseline history of hypertension (yes or no), baseline history of hypercholesterolemia (yes or no), intakes of total energy (quintiles), magnesium (quintiles), trans fat (quintiles), and cereal fiber (quintiles), ratio of polyunsaturated fat intake to saturated fat intake (quintiles), and glycemic load (quintiles). All statistical tests were two sided. BMI, body mass index. MET, metabolic equivalent; NHS, Nurses' Health Study; PY, person-year.

[a] P value was too small to report.

Figure legends for main text figures

Figure 1. Study overview. Panel **(a)** illustrates the three sections of statistical analyses designed for the present study. We first examined the association between iron intake and risk of incident type 2 diabetes from three large prospective cohorts (n=204,615). Then, we assessed the relationship between iron intake and 12 metabolic biomarkers in a subset of 37,544 participants. Finally, we integrated metabolomics data to investigate the interplay between heme iron intake, metabolites, and type 2 diabetes risk in a subset of 9,024 participants. Panel **(b)** shows the distribution of age, BMI, and sex across these three populations. Centre lines in the boxes of age represent medians, box heights represent the interquartile range, dots represent outliers and whiskers represent ranges (excluding outliers). BMI, body mass index. HPFS, Health Professionals Follow-up Study; NHS, Nurses' Health Study. Created with BioRender.com.

Figure 2. Association between heme iron intake and type 2 diabetes and its attributable proportion in diet-T2D associations. Panel **(a)** shows the dose-response relationship for type 2 diabetes, evaluated by the restricted cubic spline analysis. Reference levels were set to the median heme iron values of the first quintile. Vertical dotted lines indicate median values of each heme iron quintile. Solid lines indicate HRs, and dashed lines depict 95% CI. P for linearity is $6.5x10^{-24}$. Panel **(b)** presents the subgroup analysis for type 2 diabetes. Data are HRs (central points) and 95% CIs (error bars) for T2D per 1 mg/d heme iron intake, estimated by multivariable-adjusted Cox regression. High waist-hip ratio was defined as >0.90 for men, and >0.85 for women according to World Health Organization. All results were stratified by age and calendar year, and adjusted for body mass index, race, family history of diabetes, menopausal status and postmenopausal hormone use (NHS and NHS2 only), multivitamin use, smoking status, alcohol drinking, physical activity, baseline history of hypertension, baseline history of hypercholesterolemia, intakes of total energy, magnesium, trans fat, and cereal fiber, ratio of polyunsaturated fat intake to saturated fat intake, and glycemic load. Covariates, including subgrouping variables, were updated every 2-4 years. Panel **(c)** shows the Pearson correlation coefficients for heme iron intake and red meat and dietary patterns. Panel **(d)** presents the associations of red meat and dietary patterns with type 2 diabetes with and without adjusting for heme iron intake (evaluated by multivariable-adjusted Cox regression), as well as the proportion explained by heme iron intake underlying these associations (estimated by mediation analysis). We pooled data from three cohorts for the mediation analysis. All results were stratified by age, calendar year, and cohort, and adjusted for body mass index, race, family history of diabetes, current postmenopausal hormone use, multivitamin use, smoking status, alcohol drinking, physical activity, baseline history of hypertension, baseline history of hypercholesterolemia, and intakes of total energy. Analyses in all panels from (a) to (d) were conducted in the epidemiological dataset (n=204,615). All statistical tests were two sided. AHEI, Alternate Healthy Eating Index; AMED, Alternate Mediterranean Diet Score; BMI, body mass index; DASH, Dietary Approaches to Stop Hypertension; hPDI, healthful plant-based diet; NHS, Nurses' Health Study.

Figure 3. Percentage of differences in metabolic biomarker concentrations associated with iron intake. Panel **(a)** shows results for different types of iron intake (n=37,544), adjusted for age at blood draw, sex, case-control status, fasting status, body mass index, race, menopausal status and postmenopausal hormone use (NHS and NHS2 only), multivitamin use (only for analyses of heme iron and dietary iron), aspirin use, smoking status, alcohol drinking, physical activity, baseline history of hypertension, baseline history of hypercholesterolemia, intakes of total energy, magnesium, trans fat, and cereal fiber, ratio of polyunsaturated fat intake to saturated fat intake, and glycemic load. Data are percentage differences (bars) and 95% CIs (error bars) derived from multivariable-adjusted linear regression coefficients. Panel **(b)** compares associations for heme iron intake and biomarkers among all participants (n=37,544) versus those among controls only (n=23,873), with the same covariates adjustments as in panel **(a)**. The units for iron intake in the analyses approximate to the

difference between the median values of the highest and lowest quintiles. All statistical tests were two sided, and Bonferroni correction was applied for biomarkers. CRP, C-reactive protein; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TAG, triacylglycerol; TfR, transferrin receptor.

Figure 4. Plasma metabolomic biomarkers underlying the association between heme iron intake and type 2 diabetes. Panel **(a)** presents the associations of 50 metabolites selected in the T2D metabolomic score (present in at least 800 out of 1000 iterations) with 12 metabolic biomarkers (n=6,915) from multivariable-adjusted linear regression. Panel **(b)** presents the association between the T2D metabolomic score and risk of developing T2D (n=9,024). Solid lines indicate log HRs estimated by multivariable-adjusted Cox regression, and dashed lines depict 95% CI. P for linearity is $4.7x10^{-71}$. Panel **(c)** shows the association between heme iron intake and T2D metabolomic score (n=9,024). Data are multivariable-adjusted linear regression coefficients (central points) and 95% CIs (error bars) for standardized T2D metabolomic score comparing higher quintiles of heme iron intake to the lowest. P trend is $6.8x10^{-8}$. Panel **(d)** shows the association between 17 potential mediating metabolites and T2D risk from multivariable-adjusted Cox regression (n=9,024). Panel **(e)** shows the association between the potential mediating metabolites and heme iron intake from multivariable-adjusted linear regression (n=9,024). The height of bars in panel (**d**) and (**e**) indicates the size of beta coefficients. Panel **(f)** presents the associations of mediating metabolites and 12 metabolic markers from multivariable-adjusted linear regression (n=6,915). Analyses for 12 metabolic biomarkers and for heme iron-metabolite were adjusted for the same covariates as those in Figure 2; analyses for metabolite-T2D were adjusted for the same covariates as those in Table 2. All statistical tests were two sided, and Bonferroni correction was applied for metabolites. CRP, C-reactive protein; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TAG, triacylglycerol; TfR, transferrin receptor.

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