The Association of Multiple Biomarkers of Iron Metabolism and Type 2 Diabetes - the EPIC-InterAct Study

Short running title: Iron Metabolism and Type 2 Diabetes Incidence

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Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; EPIC: European Prospective Investigation into Cancer and Nutrition; GGT: gamma-glutamyl transpeptidase; HHC: hereditary hemochromatosis; HR: hazard ratio; hsCRP: high sensitivity C-reactive protein; NAFLD: non-alcoholic fatty liver disease; SD: standard deviation; TSAT: transferrin saturation; T2D: type 2 diabetes; 95% CI: 95% confidence intervals

Abstract

Objective

Observational studies show an association between ferritin and type 2 diabetes (T2D), suggesting a role of high iron stores for T2D development. However, ferritin is influenced by factors other than iron stores, which is less the case for other biomarkers of iron metabolism. We investigate associations of ferritin, transferrin saturation (TSAT), serum iron and transferrin with T2D incidence, to clarify the role of iron in the pathogenesis of T2D.

Research and Design Methods

The EPIC-InterAct study includes 12,403 incident T2D cases and a representative sub-cohort of 16,154 individuals from a European cohort with 3.99 million person-years of follow-up. We studied the prospective association of ferritin, TSAT, serum iron and transferrin with incident T2D in 11,052 cases and a random sub-cohort of 15,182 individuals and assessed whether these associations differed by subgroups of the population.

Results

Higher levels of ferritin and transferrin were associated with a higher risk of T2D [HR in men and women, respectively: 1.07 (95% CI: 1.01; 1.12) and 1.12 (1.05; 1.19) per 100 µg/L higher ferritin level; 1.11 (1.00; 1.24) and 1.22 (1.12; 1.33) per 0.5 g/L higher transferrin level] after adjustment for age, centre, BMI, physical activity, smoking status, education, hsCRP, ALT and GGT. Elevated TSAT (≥45% versus <45%) was associated with a lower risk of T2D in women [0.68 (0.54; 0.86)] but was not statistically significantly associated in men [0.90 (0.75; 1.08)]. Serum iron was not associated with T2D. The association of ferritin with T2D was stronger among leaner individuals ($p_{interaction}$<0.01).

Conclusions

The pattern of association of TSAT and transferrin with T2D suggests that the underlying relationship between iron stores and T2D is more complex than the simple link suggested by the association of ferritin with T2D.

Hereditary hemochromatosis (HHC), a genetic disorder characterized by systemic iron overload, is reported to be associated with diabetes mellitus (1). Similarly, an overrepresentation of diabetes mellitus cases has also been described among individuals with conditions of acquired iron overload, such as thalassemia major (2). This raises the question whether high levels of body iron is a risk factor for type 2 diabetes in the general population, as this would have implications for the prevention and treatment of type 2 diabetes. Cross-sectional and prospective population studies report a positive association between ferritin and type 2 diabetes (3,4). However, although ferritin is considered a marker of iron stores in healthy individuals (5–7), it is also an acute phase reactant and is influenced by inflammation, liver disease and insulin resistance, which are also associated with type 2 diabetes (8–11).

The use of other commonly measured biomarkers of iron metabolism may provide additional information on the role of iron in the pathogenesis of type 2 diabetes, because they reflect different aspects of iron metabolism and are less influenced by the above mentioned conditions. Transferrin is the iron binding protein in circulation and its levels rise with increasing iron requirements. Serum iron is difficult to interpret in isolation as it has a diurnal variation and hence varies significantly without changes in total body iron (12). Transferrin saturation (TSAT) is the proportion of transferrin bound to serum iron and is in part a marker of iron absorption, as it reflects the proportion of circulating iron in the context of iron requirements. TSAT is elevated in the presence of non-transferrin bound iron, which in turn is responsible for iron-related oxidative damage (13,14).

We investigated the association of ferritin, TSAT, serum iron and transferrin with incident type 2 diabetes in a large prospective European case-cohort study. We also assessed whether these associations have a threshold effect or differ by subgroups of the population, such as individuals not presenting signs of conditions commonly associated with hyperferritinemia.

RESEARCH DESIGN AND METHODS

The EPIC-InterAct study

Participants and study design

The InterAct study is a large case-cohort study of incident type 2 diabetes nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) study, the design and population characteristics of which have been published previously (15). In brief, a total of 12,403 incident cases of type 2 diabetes were ascertained and verified during 3.99 million person-years of follow-up (mean follow-up of 11.7 years) of 340,234 eligible EPIC participants (men and women aged 20-80 years at baseline, with a stored blood sample and reported diabetes mellitus status). The subcohort (*n*=16,154), which was a representative sample of the original cohort, was identified by randomly selecting individuals from each center. We excluded individuals who had prevalent clinically diagnosed diabetes at baseline. By design there are individuals with incident diabetes that were also randomly selected into the subcohort (*n*=778) and these are included as cases in case-cohort analyses (15). A detailed breakdown of participants with data on the iron biomarkers and covariates are detailed in the results. Participants gave written informed consent and the study was approved by the local ethics committee in the participating countries and the Internal Review Board of the International Agency for Research on Cancer.

Measurements

Standardized information was collected by questionnaire and physical examination at recruitment as part of EPIC. Participants were asked about their level of education, smoking status and alcohol consumption (which was subsequently converted into mean g/day). Diet and physical activity were assessed using questionnaires (15,16). Presence of a family history of type 2 diabetes, defined as type 2 diabetes in a first degree relative, was asked in most cohorts except those in Italy, Spain, Oxford and Heidelberg. Menopausal status was defined as menopausal (post-menopausal or post-ovariectomy) and non-menopausal (pre- or peri-menopausal). A blood sample was taken at varying times of the day and stored frozen for future measurements (15). Follow-up data on mortality and disease status was ascertained via registries, clinical records and other sources of clinical information (15).

Type 2 diabetes case ascertainment and verification

Incident type 2 diabetes cases were identified using multiple sources of evidence including self-report, linkage to primary-care registers, secondary-care registers, medication use, hospital admissions and mortality data. Cases were considered verified if confirmed by at least two independent sources. Cases in Denmark and Sweden were identified via local and national diabetes and pharmaceutical registers and hence all ascertained cases were considered to be verified (15). Follow-up was censored at date of diagnosis, 31st December 2007 or date of death, whichever occurred first.

Laboratory measurements

Serum samples were used to measure the biomarkers in all centers, except Umea (*n*=1,845) where only plasma samples were available and only ferritin could be measured. All measurements were done at the Stichting Huisartsen Laboratorium, Etten-Leur, the Netherlands. Cobas® assays were used to measure ferritin (electrochemiluminescence immunoassay sandwich principle), iron (colorimetric assay) and transferrin (immunoturbidimetric assay) on a Roche Hitachi Modular P analyzer. The assay range for serum iron was 0.9-179 µmol/l, that for transferrin was 1.26-63 µmol/l and that for ferritin was 0.5-2000 µg/l. Results below the lower detection limit for each assay were considered missing (2 results for serum iron only). TSAT was calculated as [iron (µmol/L) x 100)] / [transferrin (g/L) x 22.75]. Cobas® assays on the same analyzer were also used to measure hsCRP (particle-enhanced immunoturbidimetric assay), ALT and AST (UV test) and GGT (enzymatic calorimetric assay). Quality control was based on the Westgard rules (17).

Statistical analysis

Baseline characteristics of individuals were compared across sex-specific quartiles of the ferritin distribution in the subcohort. Distributions of ferritin levels were compared by sex, as well as BMI and waist circumference categories in the subcohort. After log-transformation of variables with skewed distributions (ferritin, hsCRP, GGT and alcohol consumption), a multivariable regression model adjusted for age, center and sex and unadjusted Pearson correlation coefficients were used to describe the relationships between each biomarker of iron metabolism and each other and with possible confounders.

We estimated associations of differences (defined in **Table 3**) in ferritin, iron and transferrin in natural units with the risk of type 2 diabetes using Prentice weighted Cox regression models with age as the underlying timescale, fitted separately within each country, with estimates combined across countries using random effects meta-analysis. Prentice weighted Cox regression is used to analyze a case-cohort study to take account of the enrichment of incident cases occurring outside of the random subcohort. We used hazard ratios as estimates of risk. We used a cut-off of TSAT≥45% as this is the threshold recommended by clinical guidelines to rule out genetic causes of hyperferritinemia (18) and also the threshold at which substantial levels of non-transferrin bound iron appear (14). We fitted three different models with increasing levels of adjustment for key potential confounders, namely, age, study center, BMI, physical activity, smoking status, level of education, hsCRP, ALT and GGT. AST and ALT were highly correlated (r=0.75) and as AST is less specific for liver disease than ALT, so we only included ALT in the model. We included participants who had data available for the relevant biomarker and all these potential confounders, unless stated otherwise. In order to compare results with pooled estimates from a recent meta-analysis (3), results were also reported for the top quintile compared to the lowest quintile of ferritin (sex-specific quintiles defined in the subcohort). Because the distribution of ferritin is substantially different in men and women in the general population, we also reported results for one sex-specific standard deviation of ferritin. We also presented hazard ratios for various cut-offs of TSAT and for a 5% higher level of TSAT. Adjusted and unadjusted cubic splines were generated for the association of each biomarker with type 2 diabetes in men and women. The splines were calculated between the 1st and 99th percentile of the

relevant biomarker with knots at the 5th, 25th, 75th and 95th percentiles and the median as the reference.

The association of ferritin with type 2 diabetes was also estimated in a restricted sample of individuals who did not present signs of common correlates of hyperferritinemia, namely inflammation, liver disease, high alcohol consumption and obesity (*n*=10,958). These were defined as individuals with hsCRP <10 mg/L, ALT and AST ≤40 U/L, GGT ≤60 U/L (men), ≤40 U/L (women) and a low to moderate self-reported alcohol consumption (<30 g/day in men and <20 g/day in women, as suggested by the European Association for the Study of Liver) (19). The same association was also estimated after excluding individuals with ferritin levels higher than 1000 µg/L (*n*=125), in an attempt to exclude individuals with conditions of extreme iron overload, such as HHC.

For biomarkers which showed a significant association with type 2 diabetes in men and women, *p*-values for interaction between the biomarker and variables related to iron metabolism were estimated by including a parameter representing the interaction between the biomarker (continuous) and the variable of interest (categorical) in Prentice-weighted Cox regression models adjusted for age, sex and center fitted within each country, with estimates combined using random effects meta-analysis. Hazard ratios of type 2 diabetes for each biomarker were then estimated within strata for each variable of interest. Waist circumference was categorized according to sex-specific cut-offs (20) and BMI according to the World Health Organization classification (defined in **Figure 2**).

Sensitivity analyses were carried out for the association of ferritin and type 2 diabetes, as it is the one where confounding is most likely, adjusting additionally for menopausal status, alcohol consumption and red meat consumption. Information on waist circumference and family history of type 2 diabetes were missing in respectively 7.3% and 50.4% of the study population, mainly because it had not been assessed in certain centers. Therefore, these variables were not included as covariates in the main models, but sensitivity analyses were carried out among individuals with information on waist circumference (*n*=23,122) and family history (*n*=11,565). All analyses were performed using Stata 13.

RESULTS

Of all 27,779 InterAct participants (12,403 incident type 2 diabetes cases), between 23,554 (10,371 cases) and 25,113 individuals (11,052 cases) had data available for the relevant biomarkers and all the covariates for the main models and were included in this analysis. The median (interquartile range) of ferritin in the subcohort was 144 (80-241) µg/l in men and 58 (29-107) µg/l in women. 8.31% of men and 4.78% of women in the subcohort had a TSAT level ≥45%. Summary statistics of biomarkers and baseline characteristics of participants by quartiles of ferritin in the subcohort are detailed in **Tables 1 and 2.** Individuals in the highest quartile of ferritin were older, consumed more alcohol, had lower levels of transferrin and higher levels of TSAT and liver enzymes compared to individuals in the rest of the subcohort. Leaner individuals had smaller SDs of ferritin (**Supplementary Table 1**)**.** In linear regression analyses adjusting for age, sex and center (**Supplementary Table S2**), ferritin was associated with each of the other iron markers and with all of the possible confounding factors with the exception of estimated dietary iron intake for which the relationship was weak. TSAT was strongly correlated with serum iron (r=0.91) and inversely correlated with hsCRP (r=-0.15). Estimated dietary iron intake was only weakly associated with ferritin and not with the other iron biomarkers.

Hazard ratios (HR) of type 2 diabetes for each biomarker are summarized in **Table 3** and **Supplementary Figure S1** and the adjusted and unadjusted associations estimated from spline regression are displayed in **Figure 1 and Supplementary Figure S2, respectively**. A 100 µg/L higher ferritin level was associated with a higher risk (95% CI) of type 2 diabetes in men, 1.07 (1.01; 1.12) and women, 1.12

(1.05; 1.19), after adjusting for age, center, BMI, physical activity, smoking status, level of education, hsCRP, ALT and GGT. Hazard ratios per sex-specific standard deviation of ferritin were similar in men and women (**Supplementary Table S3**). The spline analyses showed that the strengths of the associations were weakened by the adjustment (particularly for ferritin), while the shapes of the associations remained generally similar. A TSAT≥45% versus <45% was associated with a significantly lower risk of type 2 diabetes in women only. Using cut offs of 50% and 55% for TSAT or estimating hazard ratios per 5% higher level of TSAT did not substantially affect the results, but the association was no longer statistically significant in women using cut-offs of 50% or 55% (**Supplementary Table S4**). A higher serum iron level was not associated with type 2 diabetes. A higher transferrin level was associated with a higher risk of type 2 diabetes in men, 1.11 (1.00; 1.24), and women, 1.22 (1.12; 1.33). The associations of ferritin and transferrin with type 2 diabetes were most attenuated after adjusting for BMI and ALT (data not shown). Sensitivity analyses excluding individuals who developed diabetes within the first two years of follow-up did not change the results (data not shown).

Restricting the analyses to individuals not presenting any sign of overt inflammation, liver disease, high alcohol consumption or obesity, moderately weakened the association of ferritin with type 2 diabetes in men to a HR (95% CI) of 1.04 (0.96; 1.12), while the association remained similar in women with a HR of 1.12 (1.02; 1.24) per 100 µg/L higher level of ferritin, adjusted for age, center, type 2 diabetes risk factors, hsCRP and liver enzymes. Among individuals with ferritin levels lower than 1000 µg/L, the association of ferritin with type 2 diabetes was similar in men with a HR of 1.09 (1.02; 1.15), but a higher HR in women of 1.26 (1.15; 1.38).

In age and center adjusted analyses, associations for ferritin, TSAT, serum iron and transferrin were stronger in women, compared to men, although differences did not reach conventional levels of statistical significance for transferrin ($p_{interaction}$=0.004, <0.001, 0.01, and 0.47 respectively). There was a stronger association of ferritin with type 2 diabetes among leaner individuals (**Figure 2**), with a significant interaction with waist circumference ($p_{interaction}$=0.004) and BMI ($p_{interaction}$<0.001). Transferrin showed a stronger association with type 2 diabetes among individuals at extremes of waist circumference ($p_{interaction}$=0.034). There was no interaction of either ferritin or transferrin with menopausal status, estimated dietary iron or alcohol consumption and no interaction of transferrin with BMI.

Adjusting for menopausal status, alcohol consumption, red meat intake, family history of type 2 diabetes or waist circumference did not substantially modify the association of ferritin in men or women (**Supplementary Table S5**).

CONCLUSIONS

This study, which was conducted in a large European population, showed that higher ferritin and transferrin levels were associated with an increased risk of type 2 diabetes in men and women. Even among individuals showing no signs of overt inflammation, liver disease, high alcohol consumption or obesity, ferritin was associated with type 2 diabetes in women, but to a lesser extent in men. An elevated TSAT was associated with a lower risk of type 2 diabetes in women when a cut-off of 45% was used and serum iron was not associated with type 2 diabetes. The associations of all four iron biomarkers with type 2 diabetes were stronger in women than in men. This likely reflects physiological differences in iron metabolism and biomarker distributions between men and women, causing the relative risk of absolute biomarker differences to be greater in women.

The association of ferritin with type 2 diabetes has previously been reported, but the association from the latest meta-analysis of prospective studies was stronger and less precise than that found in this present study, with HR (95% CI) of 1.73 (1.35; 2.22) for the top quintile compared to the lower quintile in the meta-analysis (3). This difference may be explained by the much larger number of cases in the present study (11,052 versus 3,391), and the lack of adjustment for liver enzymes in many of the studies included in the meta-analysis. The stronger association in women than in men using natural units was no longer apparent when using standardized units, suggesting that the stronger association is a reflection of the different distributions of ferritin in men and women in the population. Contrary to suggestions from previous studies (21,22) we did not observe a threshold effect of ferritin with incident type 2

diabetes in the InterAct study, but rather a linear association, with an increased risk even at levels of ferritin considered within the reference range. For the first time, we demonstrated that ferritin showed a relatively stronger association among leaner individuals. This may be due to the fact that leaner individuals have a lower absolute risk of type 2 diabetes and that the standard deviation of ferritin is smaller among leaner individuals, hence the relative risk of ferritin is larger in leaner than in overweight or obese individuals. The association of higher transferrin with type 2 diabetes has previously been reported in a small prospective study, which also showed the absence of association of serum iron with type 2 diabetes (23).

Nevertheless, our study found a more complex relationship between TSAT and diabetes. Results from existing prospective studies of TSAT with type 2 diabetes are conflicting. A study from NHANES did not find any association between TSAT and type 2 diabetes using different cut-offs for TSAT (24). In contrast, a meta-analysis of three Danish studies found that TSAT≥50% was associated with a higher risk of type 2 diabetes (25). However, these were relatively small studies with fewer than 1,500 cases in each study. This is the first prospective study to show that an elevated TSAT is associated with a lower risk of type 2 diabetes, which was statistically significant in women only. Recent cross-sectional studies have shown a similar association of high ferritin and low TSAT among individuals with 'pre-diabetes' (26–28). TSAT is a useful biomarker of iron metabolism in addition to ferritin (26), as TSAT levels are less affected by inflammation than ferritin (29) and are thought to reflect levels of non-transferrin bound iron (13,14). In patients with HHC, which is characterized by high iron absorption, TSAT is elevated first, followed by ferritin once tissue iron accumulation has occurred (1). Non-transferrin bound iron is thought to

be an important source of organ iron deposition and toxicity as it is avidly taken up by tissues, independently of the transferrin receptor (14) and levels have been shown to be higher in patients with type 2 diabetes compared to controls (30). However, the direction of association between TSAT and type 2 diabetes observed in this study does not support a simple association between increased iron absorption or higher non-transferrin bound iron and type 2 diabetes. This may be because not all cases of iron overload are characterized by an elevated TSAT. For example, the insulin resistance-associated hepatic iron overload syndrome is characterized by mild to moderate hepatic iron overload on liver biopsy, generally with an elevated ferritin but a normal TSAT (31–33). Alternatively, a higher TSAT could reflect more successful scavenging of non-transferrin bound iron and therefore be protective for type 2 diabetes. Finally, as TSAT is inversely associated with inflammation, negative confounding by inflammation may mask an association of TSAT with type 2 diabetes.

High levels of ferritin and transferrin are markers of high and low iron stores, respectively and were strongly inversely correlated. However, they were both positively associated with type 2 diabetes in this study. Participants with low ferritin levels had a lower risk of developing type 2 diabetes compared to the median, suggesting that low iron stores *per se* are not associated with a higher risk of type 2 diabetes. While a cross-talk between iron and insulin resistance is likely, the initiating factor of the vicious circle remains unclear (34). Cross-sectional studies showed that ferritin was correlated with 2-hour glucose concentration and inversely with insulin sensitivity in individuals without type 2 diabetes (8), as well as inversely with adiponectin (35,36). A recent study showed that ferritin and transferrin were

prospectively associated with indices of hepatic, muscular and adipocyte insulin resistance (37). Some experimental studies report an upregulation of transferrin expression by insulin in human hepatocytes (38,39), while other studies suggest an antagonist effect of transferrin on insulin action, leading to insulin resistance (40). We suggest that the association of both ferritin and transferrin with incident type 2 diabetes could be explained, at least in part, by insulin resistance. This is supported by the fact that in the present study, the strength of the associations of ferritin and transferrin with type 2 diabetes was most strongly attenuated after adjustment for BMI and ALT, which are both associated with insulin resistance (41,42).

Taken together, these observed associations of TSAT and transferrin with type 2 diabetes do not support the clear role of iron in the pathogenesis of type 2 diabetes that might have been suggested by the association of ferritin. Case series in the 20th century reported a relatively high prevalence of diabetes among patients with HHC, which formed an important basis for the hypothesized role of iron in the pathogenesis of type 2 diabetes. However, the Hemochromatosis and Iron Overload Screening Study found the sex- and age-adjusted prevalence of self-reported diabetes similar in C282Y homozygotes and in participants without *HFE* C282Y and H63D mutations (43–46). Also, genetic studies to date show that C282Y is not associated with type 2 diabetes, while H63D is modestly associated (47,48). HHC was historically defined in the 19th century as the co-occurrence of cirrhosis, diabetes and skin pigmentation and this triad became the *sine qua non* of HHC until the end of the 20th century. Because diabetes was part of the triad used to define HHC, clinicians would look for type 2 diabetes in people they suspect of having HHC. This creates an ascertainment bias, which is less likely to occur now that genetic testing

is the gold standard for the diagnosis of HHC. An alternative explanation to the associations observed in this study is that disorders of iron overload caused by different mechanisms show different associations with type 2 diabetes, but that these differences may not be captured by the use of biomarkers. However, the use of more invasive measures of iron stores necessary to distinguish these disorders is unlikely to be feasible on a large scale.

This is the first prospective study to comprehensively report the association of four commonly used clinical measures of iron stores and type 2 diabetes. It is limited by the fact that we had a single measure of TSAT for each individual and that most samples were non-fasting, which may have affected the dichotomous categorization of TSAT. However, this applied to all participants irrespective of their diabetes status, and the consequence would be non-differential error which would under-estimate the strength of the association between TSAT and type 2 diabetes. This could contribute to the lack of observed association in men, but does not explain the association in women. Also, we were unable to exclude participants with clinically diagnosed HHC. However, although *HFE* mutations are common, the clinical penetrance of the disease is extremely low (49), therefore this is unlikely to have substantially affected our results. As for all observational studies, we cannot exclude reverse causality or residual confounding as potential explanations for our findings. However, there was no relationship between the iron biomarkers and HbA1c at baseline in the subcohort and sensitivity analyses excluding individuals who developed diabetes within the first two years of follow-up did not change the results. These observations lessen the likelihood of reverse causality.

In conclusion, the observed pattern of association of these biomarkers of iron metabolism and type 2 diabetes suggests a more complex relationship than simply high iron stores being a risk factor for type 2 diabetes. It remains to be clarified whether the associations of higher ferritin and transferrin with type 2 diabetes aredue to a causal role of iron in the pathogenesis of type 2 diabetes, or whether it simply reflects the underlying progression of insulin resistance. The genetics of iron metabolism in general and specifically of different disorders of iron metabolism based on their mechanisms may be useful in addressing these questions which are difficult to answer using traditional observational designs.

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Table 1. Baseline characteristics by quartiles of ferritin in men in the subcohort (*n*=5,697)

	Ferritin quartiles (Range of ferritin, µg/L)				*p* for difference across quartiles*	Overall subcohort
	Q1 (4-80)	**Q2 (81-144)**	**Q3 (145-241)**	**Q4 (242-2283)**		
Age (years)	52.2 (8.9)	52.1 (9.1)	52.8 (9.1)	53.5 (8.3)	<0.001	52.9 (8.9)
BMI (kg/m2)	26.2 (3.5)	26.4 (3.5)	26.6 (3.5)	27.4 (3.6)	<0.001	26.6 (3.6)
Education (%)					0.0001	
Low	7.9	6.1	4.9	4.4		5.6
Primary	37.0	35.0	31.5	32.0		34.1
Vocational	21.4	21.4	22.7	25.3		22.8
Secondary	13.2	13.3	13.7	12.3		13.3
Higher	20.5	24.1	27.2	26.1		24.2
Physical Activity (%)					0.006	
Inactive	17.5	17.8	19.7	18.9		18.7
Moderately inactive	28.6	29.9	30.7	34.0		30.9
Moderately active	25.9	27.1	24.2	24.8		25.5
Active	28.0	25.1	25.2	22.3		24.9
Smoking (%)					0.7	
Never	34.5	32.8	30.6	29.2		31.7
Former	32.5	32.5	37.7	42.6		36.7
Smoker	33.0	34.7	31.6	28.2		31.6
Family history of T2D (%)	12.1	16.2	12.8	19.3	<0.001	15.5
Alcohol intake (g/day) †	10.5 (2.2;24.6)	12.3 (3.4;28.3)	15.2 (5.4;36.2)	19.3 (7.1;40.2)	<0.001	13.5 (4.0;32.4)
Dietary iron intake (mg/day)	15.3 (4.9)	15.4 (5.0)	15.2 (5.0)	15.3 (5.0)	0.9	15.3 (5.0)
Biomarkers (mean, SD)						
TSAT (%)	26.8 (9.9)	29.7 (9.8)	30.8 (9.2)	33.8 (12.5)	<0.001	30.3 (10.7)
Iron (µmol/L)	17.3 (6.0)	17.8 (5.7)	18.2 (5.5)	19.2 (6.6)	<0.001	18.1 (6.0)
Transferrin (g/L)	2.9 (0.4)	2.7 (0.4)	2.6 (0.3)	2.5 (0.4)	<0.001	2.7 (0.4)
Glucose (mmol/L)	5.0 (1.6)	5.1 (1.3)	5.2 (1.5)	5.6 (1.5)	<0.001	5.2 (1.5)
HbA1c (mmol/mol)	36.4 (4.8)	36.3 (4.5)	36.4 (5.4)	36.3 (6.4)	0.97	36.4 (5.3)
hsCRP (mg/L) †	0.9 (0.5;1.9)	1.0(0.5;2.1)	1.1 (0.6;2.5)	1.4 (0.7;2.9)	<0.001	1.1 (0.5;2.3)
ALT (U/L)	22.6 (10.4)	24.4 (11.8)	26.6 (13.6)	32.9 (20.0)	<0.001	26.6 (14.9)
AST (U/L)	29.3 (7.6)	30.0 (8.4)	30.9 (9.5)	35.0 (15.3)	<0.001	31.3 (10.9)
GGT (U/L) †	24.0 (18.0;34.0)	26.0 (19.0;40.0)	29.5 (21.0;46.0)	36.0 (25.0;60.0)	<0.001	28.0 (20.0;44.0)

Data shown are mean (standard deviation) or median (interquartile range) for skewed variables marked †

* ANOVA for normally distributed continuous variables, Kruskal-Wallis test for continuous variables with skewed distribution (†) and Chi^2 for categorical variables

Table 2. Baseline characteristics by quartiles of ferritin in women in the subcohort (*n*=9,485)

	Ferritin quartiles (Range of ferritin, µg/L)				*p* for difference across quartiles*	Overall subcohort
	Q1 (1-29)	**Q2 (30-58)**	**Q3 (59-107)**	**Q4 (108-3017)**		
Age (years)	47.2 (8.0)	50.0 (9.4)	54.1 (8.7)	57.0 (7.8)	<0.001	52.1 (9.3)
BMI (kg/m2)	25.5 (4.4)	25.4 (4.6)	25.4 (4.4)	26.4 (4.6)	<0.001	25.7 (4.5)
Education (%)					0.002	
Low	12.4	10.2	7.3	6.3		8.9
Primary	33.1	30.5	32.6	34.2		32.8
Vocational	20.3	22.0	24.4	26.6		23.4
Secondary	15.6	16.6	18.0	17.0		16.6
Higher	18.6	20.7	17.8	15.9		18.3
Physical Activity (%)					0.0008	
Inactive	30.1	26.4	26.8	24.5		27.0
Moderately inactive	34.2	35.4	34.8	36.2		35.1
Moderately active	20.3	20.9	20.4	21.1		20.9
Active	15.4	17.3	18.0	18.2		17.1
Smoking (%)					0.0006	
Never	59.7	54.6	54.1	56.2		56.0
Former	20.2	21.3	21.7	22.4		21.4
Smoker	20.1	24.1	24.3	21.4		22.6
Family history of T2D (%)	19.7	18.7	19.5	23.5	0.1	20.5
Alcohol intake (g/day) †	1.7 (0;7.2)	2.7 (0.2;10.6)	3.6 (0.4;12.0)	5.1 (0.6;13.5)	<0.001	3.0 (0.2;11.1)
Estimated dietary iron intake (mg/day)	12.3 (3.6)	12.4 (3.7)	12.3 (3.6)	12.0 (3.4)	0.009	12.2 (3.6)
Biomarkers (mean, SD)						
TSAT (%)	20.8 (10.5)	27.2 (9.7)	28.5 (9.0)	30.5 (10.3)	<0.001	26.7 (10.5)
Iron (µmol/L)	14.3 (6.7)	17.1 (6.0)	17.3 (5.4)	17.8 (5.8)	<0.001	16.6 (6.2)
Transferrin (g/L)	3.1 (0.5)	2.8 (0.4)	2.7 (0.4)	2.6 (0.4)	<0.001	2.8 (0.4)
Glucose (mmol/L)	4.7 (1.3)	4.7 (1.0)	4.8 (1.2)	5.0 (1.3)	<0.001	4.8 (1.2)
HbA1c (mmol/mol)	35.6 (4.6)	35.5 (4.3)	36.4 (4.7)	36.9 (5.8)	<0.001	36.1 (4.9)
hsCRP (mg/L) †	0.9 (0.4;1.9)	1.0 (0.5;2.2)	1.1 (0.6;2.5)	1.4 (0.7;3.1)	<0.001	1.1 (0.5;2.4)
ALT (U/L)	17.0 (8.7)	17.7 (9.0)	19.5 (12.4)	21.9 (15.1)	<0.001	19.0 (11.7)
AST (U/L)	25.7 (9.9)	26.1 (10.1)	27.3 (7.9)	29.1 (13.7)	<0.001	27.1 (10.7)
GGT (U/L) †	14.0 (11.0;18.0)	16.0 (12.0; 22.0)	18.0 (14.0;26.0)	20.0 (15.0;31.0)	<0.001	17.0 (13.0;24.0)

Data shown are mean (standard deviation) or median (interquartile range) for skewed variables marked †

* ANOVA for normally distributed continuous variables, Kruskal-Wallis test for continuous variables with skewed distribution (†) and Chi^2 for categorical variables

Table 3. Hazard ratio (95% confidence intervals) of Type 2 Diabetes for the higher biomarker level as stated, by sex and meta-analyzed across countries.

Sex	Biomarker	Model	HR (95% CI)	*p* value	Heterogeneity I^2 (%)
Men					
	Ferritin (per 100 µg/L)	1	1.18 (1.12; 1.25)	<0.001	
		2	1.13 (1.08; 1.19)	<0.001	
		3	1.06 (1.01; 1.12)	0.021	72.20
	TSAT≥45%	1	0.99 (0.81; 1.20)	0.885	
		2	1.06 (0.86; 1.32)	0.579	
		3	0.90 (0.75; 1.08)	0.259	0.0
	Serum Iron (per 5 µmol/L)	1	1.03 (0.98; 1.08)	0.293	
		2	1.04 (0.98; 1.11)	0.166	
		3	1.00 (0.94; 1.07)	0.976	49.40
	Transferrin (per 0.5 g/L)	1	1.20 (1.12; 1.30)	<0.001	
		2	1.16 (1.05; 1.29)	0.003	
		3	1.11 (1.00; 1.23)	0.061	54.50
Women					
	Ferritin (per 100 µg/L)	1	1.31 (1.22; 1.41)	<0.001	
		2	1.22 (1.14; 1.31)	<0.001	
		3	1.12 (1.05; 1.19)	0.001	53.50
	TSAT≥45%	1	0.54 (0.44; 0.67)	<0.001	
		2	0.73 (0.59; 0.91)	0.004	
		3	0.68 (0.54; 0.86)	0.002	0.0
	Serum Iron (per 5 µmol/L)	1	0.92 (0.89; 0.95)	<0.001	
		2	1.02 (0.97; 1.07)	0.403	
		3	1.00 (0.95; 1.05)	0.869	38.00
	Transferrin (per 0.5 g/L)	1	1.30 (1.21; 1.41)	<0.001	
		2	1.24 (1.15; 1.34)	<0.001	
		3	1.22 (1.12; 1.33)	<0.001	55.30
Men and Women					
	Ferritin (upper vs. lower quintile)†	1*	2.46 (2.05; 2.96)	<0.001	
		2*	1.77 (1.57; 2.00)	<0.001	
		3*	1.36 (1.20; 1.54)	<0.001	5.3

Model 1: age and center adjusted
Model 2: further adjustment for BMI, physical activity, smoking status and level of education
Model 3: further adjustment for hsCRP, ALT and GGT
*additional adjustment for sex

†Ferritin quintile cut-points:
Men (µg/L): ≤68, >68-117, >117-177, >177-270, >270
Women (µg/L): ≤24, >24-45, >45-73, >73-121, >121

Legends for Figures and Supplementary Tables and Figures.

Figure 1. Adjusted Hazard ratios for Type 2 Diabetes (T2D) by Ferritin, TSAT, Serum Iron and Transferrin levels in men and women.

Figure 2. Hazard ratios of type 2 diabetes per 100 µg/L of ferritin and 0.5 g/L of transferrin in men and women by strata adjusting for age, sex and center and meta-analyzed across countries.

Supplementary Figure S1. Hazard ratios (95% confidence intervals) of Type 2 Diabetes for the higher biomarker level as stated, by sex and meta-analyzed across countries.

Supplementary Figure S2. Unadjusted Hazard ratios for Type 2 Diabetes (T2D) by Ferritin, TSAT, Serum Iron and Transferrin levels in men and women.

Supplementary Table S1. Means (SD) and median (interquartile range) of Ferritin (µg/L) by BMI and waist circumference categories in men and women in the subcohort.

Supplementary Table S2. Multivariable linear regressions (age, sex and center adjusted) and correlations (unadjusted) of each biomarker with other variables in the subcohort.

Supplementary Table S3. Hazard ratios (95% confidence intervals) of Type 2 Diabetes per sex-specific standard deviation of Ferritin in men and women.

Supplementary Table S4. Hazard ratios (95% confidence intervals) of Type 2 Diabetes for Transferrin Saturation using varying cut-off points and per 5% higher level of TSAT in men and women.

Supplementary Table S5. Hazard ratio (95% confidence intervals) of Type 2 Diabetes for 100 µg/L higher level of ferritin by sex in specific subgroups, adjusting for age, center, BMI, physical activity, smoking status, level of education, hsCRP, ALT and GGT and specified additional covariates.