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Biomarkers of body iron stores and risk of developing type 2 diabetes

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Aim: Iron may contribute to the pathogenesis of type 2 diabetes mellitus (DM) by inducing oxidative stress and interfering with insulin secretion. Elevated ferritin levels are associated with increased DM risk among healthy individuals. However, it is yet unknown if ferritin predicts DM incidence among high-risk individuals with impaired glucose tolerance (IGT). Furthermore, the association between soluble transferrin receptors (sTfR), a novel marker of iron status, and DM risk has not yet been prospectively investigated in these individuals. We conducted this study to evaluate the association between baseline levels of ferritin and sTfR and the risk of developing DM among overweight and obese individuals at high risk of DM.

Methods: This nested case–control study (280 cases and 280 matched controls) was conducted within the placebo arm of the Diabetes Prevention Program, is a clinical trial conducted among overweight/obese individuals with IGT. Ferritin and sTfR levels were measured by immunoturbidimetric assays. Incident DM was ascertained by annual 75-g oral glucose tolerance test and semi-annual fasting glucose.

Results: Compared with controls, cases had higher sTfR levels (3.50 ± 0.07 vs. 3.30 ± 0.06 mg/l; $p = 0.03$), but ferritin levels were not statistically different. The multivariable odds ratios (OR) and 95% confidence intervals (95% CI) for DM incidence comparing highest with the lowest quartiles of sTfR was 2.26 (1.37–4.01) (p-trend: 0.008).

Conclusions: Modestly elevated sTfR levels are associated with increased DM risk among overweight and obese individuals with IGT. Future studies should evaluate factors determining sTfR levels and examine if interventions that lower body iron stores reduce DM incidence.

Keywords: ferritin, transferrin receptors, type 2 diabetes
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Introduction

Iron is a strong pro-oxidant that catalyses several cellular reactions that yield reactive oxygen species [1]. It has been proposed that increased iron-induced oxidative stress is associated with elevated body iron levels may play a pathogenic role in chronic diseases including certain cancers [2,3], coronary artery disease [4–7] and type 2 diabetes mellitus (DM) [8,9]. Several studies suggest a possible link between elevated levels of circulating ferritin, a marker of body iron stores, and levels of circulating insulin and glucose [10–14], hypertension [10,15], dyslipidaemia [10,16,17], metabolic syndrome [11,13,18,19], polycystic ovarian disease [20,21] and

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obesity [10,22]. In addition, individuals with DM have higher circulating ferritin levels compared with non-diabetic individuals [23–25].

The limited number of epidemiological studies that have investigated the association between circulating ferritin levels and the risk of developing DM have reported that elevated ferritin levels (at concentrations significantly lower than those seen in patients with haemochromatosis) may increase the risk of developing DM among apparently healthy individuals [26–29]. However, most of these studies relied on self-report of DM and were conducted in apparently healthy populations. The role of elevated body iron stores as a predictor of DM incidence among high-risk individuals with impaired glucose tolerance (IGT) has not been evaluated. IGT is associated with higher body iron stores compared with normal glucose tolerance (NGT) [30,31], although it is possible that the association between ferritin and risk of DM may be different among those with IGT compared with healthy individuals. Furthermore, the association between circulating levels of soluble transferrin receptors (sTfR), a novel marker of body iron status that is less affected by systemic inflammation [32,33], and DM risk has not yet been prospectively investigated in this high-risk group. Therefore, we conducted a nested case–control study within the Diabetes Prevention Program (DPP) cohort to evaluate the association between baseline levels of ferritin and sTfR, and the risk of developing DM among overweight and obese individuals with IGT.

Methods

The DPP was a randomized clinical trial testing interventions to prevent or delay the development of DM among high-risk individuals [34,35]. The 27 clinical centres in the USA recruited 3,234 participants of both sexes, approximately 50% of whom were members of ethnic or racial minority groups and 20% of whom were $\geq$60 years old. The eligibility criteria included $\geq$25 years of age, body mass index (BMI) $\geq$24 kg/m^2 ($\geq$22 kg/m^2 in Asian Americans) and fasting plasma glucose levels between 95 and 125 mg/dl in addition to IGT (2-h 75-g postload glucose of 140–199 mg/dl). Major exclusions included a recent myocardial infarction, symptoms of coronary heart disease, major illness, prior diagnosis of DM, use of medications known to impair glucose tolerance and a triglyceride level $\geq$600 mg/dl. Eligible participants received standard advice on healthy diet and physical activity and were randomly assigned to an intensive lifestyle intervention, metformin or placebo. The case–control study reported here was nested within the placebo arm of the DPP cohort (n = 1,082).

Case Definition and Control Selection

During the average follow-up time of 2.8 years, there were 301 documented cases of incident DM in the placebo arm of the DPP. The diagnosis of DM was determined by an annual 75-g oral glucose tolerance test (OGTT) or by a semi-annual fasting plasma glucose level with confirmation by a second test, using the standardized criteria of the American Diabetes Association [36] and the World Health Organization [37]. We excluded 21 cases because of inadequate blood sample or lack of participant consent for this substudy. Therefore, a total of 280 cases were included in this study.

Control selection was based on sampling techniques described by Prentice and Breslow ('risk-set' sampling) [38]. That is, for each incident case of DM identified during the follow up, one control was selected at random (and with replacement) from a list of individuals who were 'at risk' of DM at the time of diagnosis of the case. In addition, the controls were matched to the cases based on sex, race/ethnicity and age within 5 years.

Assessment of Covariate Data

Body weight was measured to the nearest 0.1 kg semi-annually. Waist circumference, assessed in standing position, was measured midway between the highest point of the iliac crest and the lowest point of the costal margin of the midaxillary line. Self-reported levels of leisure time physical activity were assessed annually with the Modifiable Activity Questionnaire [39]. Blood pressure measurements were conducted by certified clinic staff and calculated as the average of two readings, both taken with the participant in the sitting position. Data on age, family history of DM and race/ethnicity were based on self-reports. Fasting plasma glucose, insulin, lipid profile, glycated haemoglobin (HbA_{1c}), and C-reactive protein (CRP) were measured in the Central Biochemical Laboratory (University of Washington, Seattle, WA, USA) [35]. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as [insulin (mU/ml) $\times$ glucose (mmol/l)]/22.5.

Measurement of Body Iron Stores

Assays for ferritin and sTfR were conducted using stored fasting plasma samples collected among DPP participants at baseline. These assays were performed at the Children's Hospital (Boston, MA, USA). Both ferritin and sTfR were measured by a particle-enhanced immunoturbidimetric assay using the Hitachi 911 analyzer (Ferritin 1661400 and sTfR 2148315 assay kits, Roche Diagnostics, Indianapolis, IN, USA). Each case and control pair was

assayed in the same analytical batch to eliminate bias because of interassay variability. The laboratory personnel were blinded to the case and control status. The coefficients of variation for both these markers were $\leq 5\%$.

Statistical Analyses

We compared means (continuous variables) or proportions (categorical variables) of baseline characteristics by case and control status. Furthermore, we used conditional logistic regression to evaluate if ferritin and sTfR are associated with risk of incident DM [38]. The main exposure variables were modeled as quartiles to avoid assuming linearity and to assess dose–response relationship. These quartiles were derived based on the distribution among the control population. The odds ratios (ORs) and the 95% confidence intervals (95% CIs) were estimated using the lowest quartile as the reference category. In the multivariable analysis, we considered the following covariates as potential confounders: BMI, family history of DM, physical activity and HbA_{1c}. Furthermore, we evaluated the effect of additional adjustment for CRP and HOMA-IR. Tests of linear trend across quartiles were conducted by assigning the median value for each category and fitting this as a continuous variable in the model. All statistical analyses were performed using SAS® software version 9.1 (SAS Institute, Cary, NC, USA). All statistical tests conducted were two-sided and $p < 0.05$ were considered statistically significant.

Results

Table 1 shows the comparison between cases and controls with respect to several known risk factors for DM at baseline. Compared with controls, incident cases of DM had higher BMI and waist circumference, lower levels of physical activity and higher levels of triglycerides, CRP, HOMA-IR, fasting glucose, insulin and HbA_{1c}. STfR levels were higher in cases compared with controls (3.50 ± 0.07 vs. 3.29 ± 0.06 mg/l; $p < 0.03$), while ferritin levels were similar in the two groups (116.0 ± 7.0 vs. 104.3 ± 6.3 ng/ml; $p = 0.16$).

Table 2 provides the partial Spearman correlation coefficients adjusted for age, sex and ethnicity/race for ferritin and sTfR with several anthropometric and metabolic parameters, by case and control status. Among controls, both ferritin and sTfR had similar positive correlations with BMI; however, sTfR showed a stronger correlation with waist circumference compared with ferritin ($r = 0.20$; $p = 0.0004$ vs. 0.11; $p = 0.07$). Among cases, sTfR levels correlated positively with BMI and waist circumference, but ferritin did not correlate with either of these two anthropometric measures. Among both cases and controls, ferritin and sTfR showed an

Table 1 Comparison of baseline characteristics by case and control status

	Cases (n = 280)	Controls (n = 280)	p value*
Age (years)	50.4 ± 0.6	50.2 ± 0.6	Matched
% Caucasians	54.0	54.0	Matched
% Females	63.6	63.6	Matched
% Premenopausal women	35.0	35.4	0.26
Body mass index (kg/m^2)	35.1 ± 0.4	33.6 ± 0.4	0.004
Waist circumference (cm)	108.7 ± 0.9	104.1 ± 0.8	0.0001
Physical activity (METs/week)**	50.0 ± 3.4	55.5 ± 5.5	0.03
Percentage with family history of diabetes	67.0	67.5	0.93
Systolic blood pressure (mmHg)	125.5 ± 0.9	124.2 ± 0.8	0.27
Diastolic blood pressure (mmHg)	78.6 ± 0.6	79.5 ± 0.6	0.24
Total cholesterol (mg/dl)	202.3 ± 2.0	203.8 ± 2.3	0.60
LDL cholesterol (mg/dl)	123.8 ± 1.9	126.2 ± 2.1	0.37
HDL cholesterol (mg/dl)	43.2 ± 0.7	44.8 ± 0.7	0.08
Triglycerides (mg/dl)	178.3 ± 6.2	161.3 ± 5.4	0.04
CRP (mg/l)	0.7 ± 0.06	0.5 ± 0.03	0.01
Glycated haemoglobin (%)	6.1 ± 0.03	5.8 ± 0.02	<0.0001
Fasting plasma insulin (μU/dl)	29.8 ± 0.9	25.3 ± 0.8	0.0001
Fasting plasma glucose (mg/dl)	112.4 ± 0.6	105.1 ± 0.4	<0.0001
HOMA-IR	8.3 ± 0.3	6.6 ± 0.2	<0.0001
Ferritin (ng/ml)	116.4 ± 7.0	104.3 ± 6.3	0.16
Soluble transferrin receptor (mg/l)	3.50 ± 0.07	3.29 ± 0.06	0.03

CRP, C-reactive protein; HOMA-IR, Homeostasis model assessment for insulin resistance.Data for continuous variables presented as mean ± s.e.
*p value derived from a paired t-test for difference in means of continuous variables and McNemars test for categorical variables.
**Metabolic equivalent units (METs).

Table 2 Partial spearman correlation coefficients (p value if statistically significant) adjusted for age, race and sex between body iron stores and several factors among cases and controls

	Controls (n = 280)		Cases (n = 280)	
	sTfR	Ferritin	sTfR	Ferritin
Ferritin	−0.13*	—	−0.13*	
Body mass index	0.21**	0.17**	0.12*	−0.03
Waist circumference	0.20**	0.11	0.13*	−0.03
Physical activity	−0.08	−0.03	0.03	0.03
Systolic blood pressure	0.12*	0.007	−0.04	−0.05
Diastolic blood pressure	0.10	0.06	−0.04	−0.02
Total cholesterol	−0.05	−0.03	0.05	0.06
HDL cholesterol	−0.02	−0.07	−0.03	−0.02
Triglycerides	−0.01	0.12*	0.02	0.03
CRP	0.05	0.27**	0.05	0.10
Glycated haemoglobin	−0.12*	−0.11*	−0.12*	−0.12
Fasting plasma insulin	0.10	0.12*	0.03	−0.01
Fasting plasma glucose	−0.06	0.010	−0.003	−0.06
HOMA-IR	0.09	0.12*	0.04	−0.02

CRP, C-reactive protein; HOMA-IR, homeostasis model assessment for insulin resistance; sTFR, soluble transferrin receptors.
*$p < 0.05$.
**$p < 0.001$.

unexpected inverse correlation with HbA_{1c} in contrast to earlier studies [23,31]. Among the controls, we found a positive correlation between levels of ferritin and CRP, similar to that observed in earlier reports [31,40,41]. sTfR levels, however, were not correlated with CRP. Similar to CRP, triglyceride levels were positively correlated with ferritin but were not correlated with sTfR levels among the controls. In addition, as reported previously [31], ferritin and sTfR were inversely correlated in both groups.

In the conditional logistic regression model adjusted for BMI (table 3), the OR for the association of sTfR with DM, comparing quartile 4 to quartile 1, was 1.55 (95% CI: 0.93–2.57; p-trend: 0.13). However, when family history of DM, physical activity, HbA_{1c} and ferritin were included in the model, there was a trend of increasing risk with increasing sTfR (OR for highest vs. lowest quartile: 2.26; 95% CI: 1.37–4.01; p-trend: 0.008). The association between sTfR levels and DM incidence was strengthened when CRP was included in the multivariable model; the OR comparing extreme quartiles of sTfR was 2.39 (95% CI: 1.34–4.28; p-trend: 0.005). This OR did not substantially change when HOMA-IR was also included in this model (OR: 2.23; 95% CI: 1.22, 4.06; p-trend: 0.02). In our study, the multivariable OR for DM comparing extreme quartiles of ferritin adjusted for BMI and other diabetes risk factors was 1.65 (95% CI: 0.90–3.02; p-trend: 0.02), but the risk of DM was not elevated in middle two quartiles. However, when we compared the highest quartile with the first three quartiles together, the OR was 1.82 (95% CI: 1.14–2.90), suggesting a potential threshold effect for this association. This OR did not substantially change with additional adjustment for CRP and HOMA-IR (OR: 1.77; 95% CI: 1.09–2.89).

In all conditional logistic regression models, additional adjustment for HDL or triglycerides did not affect the results (data not shown). In addition, for both ferritin and sTfR, there was no evidence of effect modification by sex, race, BMI, waist circumference, insulin levels, HbA_{1c} or CRP. In secondary analyses, when we modelled a ratio between sTfR and ferritin as the main exposure, the results were not significant. The OR for DM comparing quartile 1 to quartile 4 of this ratio was 0.76 (95% CI: 0.41–1.22; p-trend: 0.23).

Discussion

In this prospective nested case–control study conducted among adult men and women at high risk for DM, we confirmed an association between ferritin and DM risk with a possible threshold effect. In addition, we found a positive association between sTfR levels at baseline and the risk of developing DM, independent of established risk factors, CRP and ferritin levels.

The human body exhibits an extraordinary capacity to accumulate iron, as observed in hereditary haemochromatosis [42]. Patients with this genetic disorder develop secondary DM possibly because of direct deposition of iron in the liver and pancreatic β-cells [43,44]. Haemochromatosis is usually associated with ferritin levels in the range of 1000–10 000 ng/ml [45,46]. However, less extreme elevations in ferritin levels (<200 g/ml) have also been linked to insulin resistance and DM [8,11,12,21,47,48]. It has been previously reported that people with DM have higher ferritin levels compared with non-diabetic individuals [23–25]. Elevated circulating ferritin may be a component of the metabolic syndrome [49] and lowering of ferritin levels has beneficial effects on the components of the metabolic syndrome [50–52]. Several cross-sectional and retrospective case–control studies have linked elevated ferritin levels with DM [23,53–55]. Furthermore, a few prospective studies have also reported that relatively high levels of ferritin are associated with an increased of risk of developing DM in apparently healthy individuals [26–29]. Several potential mechanisms may explain the role of iron in the development of DM. In addition to the induction of oxidative stress, iron may also impede insulin extraction in the liver and thus interfere with its suppressive

Table 3 Odds ratio and 95% confidence intervals for the risk of diabetes mellitus by quartiles of ferritin and sTfR derived from conditional logistic regression models

	Quartiles*				
	Q1	**Q2**	**Q3**	**Q4**	**p-trend****
sTfR					
Median (mg/l)	2.3	2.9	3.5	4.4	
Number of cases	57	71	65	87	
Model 1	1	1.25 (0.77–2.04)	1.19 (0.72–1.97)	1.58 (0.96–2.60)	0.09
Model 2	1	1.29 (0.79–2.13)	1.17 (0.70–1.96)	1.55 (0.93–2.57)	0.13
Model 3	1	1.54 (0.89–2.66)	1.67 (0.95–2.96)	2.26 (1.27–4.01)	0.008
Model 3 + CRP	1	1.62 (0.93–2.83)	1.72 (0.97–3.05)	2.39 (1.34–4.28)	0.005
Model 3 + CRP + HOMA-IR	1	1.59 (0.90–2.81)	1.62 (0.90–2.93)	2.23 (1.22–4.06)	0.02
Ferritin					
Median (ng/ml)	20.1	53.1	93.5	203.7	
Number of cases	83	47	63	87	
Model 1	1	0.59 (0.36–0.98)	0.79 (0.48–1.30)	1.12 (0.66–1.88)	0.18
Model 2	1	0.58 (0.35–0.97)	0.78 (0.47. 1.29)	1.02 (0.60–1.74)	0.36
Model 3	1	0.65 (0.37–1.14)	1.05 (0.60–1.84)	1.65 (0.90–3.02)	0.02
Model 3 + CRP	1	0.60 (0.33–1.07)	0.94 (0.53–1.68)	1.53 (0.83–2.82)	0.03
Model 3 + CRP + HOMA-IR	1	0.64 (0.35–1.16)	1.03 (0.57–1.86)	1.61 (0.85–3.02)	0.03

CRP, C-reactive protein; HOMA-IR, homeostasis model assessment for insulin resistance; sTFR, soluble transferrin receptors.Model 1 = accounting for matching factors (age, sex and race).Model 2 = model 1 + body mass index.Model 3 = mode1 2 + family history of diabetes, physical activity, glycated haemoglobin and ferritin (or sTfR).

*Quartiles of ferritin and sTfR were derived based on distribution among the controls.

**p-trend was calculated using medians for each quartile as a continuous variable.

effect on hepatic glucose production [8,56–58]. Furthermore, elevated body iron levels may also impair pancreatic insulin secretion [59] and interfere with insulin action and glucose uptake in adipocytes [60].

The majority of observational studies have assessed the relation between iron and DM by measuring circulating ferritin levels. Although ferritin is a sensitive clinical tool commonly used to assess body iron stores, it is also an acute phase reactant that is affected by other factors, including systemic inflammation [17,61,62]. In fact, it has been suggested that increased ferritin levels in patients with DM may reflect systemic inflammation rather than iron overload [9]. Circulating sTfR has been proposed as a novel marker of iron status that is less affected by the presence of inflammation. When the iron–transferrin complex binds to its cellular receptor (TfR), iron enters cells by endocytosis, accompanied by a proteolytic cleavage of the soluble extracellular domain of TfR into the circulation [63,64]. Serum levels of this soluble form (sTfR) are therefore directly proportional to the tissue TfR concentration. The circulating level of sTfR correlates inversely with body iron stores and its clinical utility as a marker of body iron status is currently being explored [65,66]. Some investigators have suggested that the sTfR-to-ferritin ratio may be a better marker of body iron status than ferritin alone [67,68].

Two prospective studies that evaluated the ferritin–DM association also measured sTfR levels [26,29] and reported that not only high ferritin level but also a low sTfR-to-ferritin ratio was significantly associated with an increased risk of DM. However, the independent association between sTfR levels *per se* and DM risk was not reported. In the overweight/obese population in the DPP, we did not find an association between the sTfR-to-ferritin ratio and DM risk. However, we did observe a significant positive association between sTfR levels and DM risk, independent of established DM risk factors. Given that ferritin is positively associated with DM risk and inversely correlates with sTfR, the positive association between sTfR and DM risk might be unexpected. In our study, we found that the correlations of ferritin and sTfR with obesity and metabolic variables were generally similar. In addition, the sTfR–DM association was statistically significant even with additional adjustment for ferritin and CRP. It is therefore possible that sTfR levels may be associated with increased DM risk through mechanism unrelated to iron overload. It is possible that sTfR levels may increase as a compensatory mechanism for a reduction in free iron levels that may occur secondary to oxidative stress. Animal and human data also support the above possibilities. *In vitro* data, for example, suggest that insulin is capable of redistributing TfR to the cell surface leading to

increased cellular iron uptake in peripheral tissues and the liver [69]. In addition, experiments in animal models indicate that acute insulin administration leads to an increase in sTfR concentrations [70]. In a recent study among 221 Caucasian men (mean age 54.4 years), sTfR correlated inversely with insulin sensitivity ($r = -0.30$; $p = 0.02$) and those with IGT had significantly higher serum sTfR levels compared with those with NGT (9.4 ± 4.4 vs. 8.2 ± 2.6 mg/l; $p = 0.02$) [31]. These data point to the possibility that hyperinsulinaemia in states of insulin resistance may contribute to high-circulating sTfR levels. However, in our study, the results for the association between sTfR levels and DM risk did not change significantly when we additionally adjusted for insulin levels or HOMA-IR, suggesting that this association was independent of hyperinsulinaemia. Overall, the relationship between sTfR and DM is complex and not well understood but the findings of the current study suggest that it may play a role in the pathogenesis of DM; alternatively, sTfR may be a biomarker of some other factor that is causally related to DM.

The strengths of our study include representation of both sexes and several ethnic groups, use of OGTT for the diagnosis of DM, complete ascertainment for DM in all participants and adjustment for several potential confounders in the analysis. However, our study has several potential limitations. High sTfR levels may be a marker of iron deficiency. However, iron deficiency is probably uncommon in our generally healthy study population. Furthermore, the results were similar when we excluded participants most likely to be iron deficient (premenopausal women) and those with low ferritin levels (men: <12 ng/ml and women: <10 ng/ml) from our analysis. Another potential concern is the residual confounding by adiposity. However, in secondary analyses, when we included waist circumference in addition to BMI in multivariable models, the results were similar. Finally, although sTfR levels predicted the development of DM in this observational study, we cannot determine if it plays a causative role and it is possible that high sTfR levels may be primarily a marker of peripheral hyperinsulinaemia.

In summary, among adults at high risk for DM, higher levels of ferritin and sTfR are associated with elevated risk of DM. Future studies need to concurrently assess a broader range of iron markers (e.g. free iron) to more accurately assess body iron status. In addition, evaluating the allelic variants related to iron metabolism with regard to DM risk may also be useful because these markers are less susceptible to confounding by other metabolic factors. Furthermore, it is important to evaluate factors that determine sTfR levels in adults and the role of sTfR in metabolic processes related to diabetes risk. Finally, future studies should explore whether interventions that affect body iron status may modulate DM risk.

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