Iron Metabolism Is Associated With Adipocyte Insulin Resistance and Plasma Adiponectin

The Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study

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OBJECTIVE—Adipocyte insulin resistance (IR) is a key feature early in the pathogenesis of type 2 diabetes mellitus (T2DM), and although scarce, data in the literature suggest a direct role for iron and iron metabolism–related factors in adipose tissue function and metabolism. Serum ferritin and transferrin were shown to be associated with muscle insulin resistance (IR) and T2DM, but little is known about the role of iron metabolism on adipose tissue. We therefore investigated whether markers of iron metabolism were associated with adipocyte IR and plasma adiponectin.

RESEARCH DESIGN AND METHODS—Serum ferritin, transferrin, total iron, non–transferrin-bound iron (NTBI), transferrin saturation, and plasma adiponectin were determined in 492 individuals. Adipocyte IR was defined by the product of fasting insulin and nonesterified fatty acids (NEFAs). Using linear regression analyses, we investigated the difference in adipocyte IR or adiponectin (in %) according to differences in iron metabolism markers.

RESULTS—Serum ferritin (β = 1.00% increase in adipocyte IR per 10 μg/L [95% CI 0.66–1.34]), transferrin (4.18% per 0.1 g/L [2.88–5.50]), total iron (1.36% per μmol/L [0.61–2.12]), and NTBI (5.14% per μmol/L [1.88–8.52]) were associated with adipocyte IR after adjustment for several covariates, including inflammatory markers. All markers of iron metabolism were also associated with NEFAs (all $P < 0.01$). In addition, ferritin and transferrin were inversely associated with adiponectin (both $P < 0.01$).

CONCLUSIONS—The observed associations of several markers of iron metabolism with adipocyte IR and adiponectin suggest that factors related to iron and iron metabolism may contribute to adipocyte IR early in the pathogenesis of T2DM.

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Iron is an important catalyst in the formation of highly reactive hydroxyl radicals (1,2), and intracellular reactive oxygen species have been shown to play an important causal role in the induction of insulin resistance (IR) (3). Consequently, massive iron overload, as present in hereditary hemochromatosis, frequently leads to diabetes (4). In addition, recent data have shown that modest iron overload (i.e., iron markers within the normal range) may also be involved in the pathogenesis of type 2 diabetes mellitus (T2DM) in general (5–7). Prospective studies have linked serum ferritin, the most reliable marker of body iron stores, and dietary heme iron intake to incident T2DM (5–7). Several cross-sectional and longitudinal studies have shown that serum ferritin is associated with muscle IR measured by homeostasis model assessment (HOMA-IR) or by hyperinsulinemic-euglycemic clamp (1,8–12), but not with β-cell function (8). These results suggest a contribution of iron to the pathogenesis of T2DM that is primarily related to the induction of IR.

Little is known, however, about the possible sites (e.g., muscle, liver, adipose tissue) where iron may induce IR, because most studies have only evaluated HOMA-IR or whole-body glucose disposal. Some studies have suggested that adipose tissue may be a primary target organ for the metabolic effects of iron. Incubation of rat adipocytes with iron decreased insulin-stimulated glucose transport and increased lipolysis in adipocytes (13,14). In addition, an iron-enriched diet led to iron accumulation and IR in visceral adipose tissue in mice (15), whereas an iron-restricted diet led to lower free fatty acids and triglycerides (16). One epidemiological study found the association between ferritin and incident T2DM was attenuated after adjustment for serum adiponectin (7), which suggests mediation by adipocyte IR. However, the effect of iron on adipose tissue is yet far from clear, and in

vivo data on the effect of iron on adipocyte IR are not available, except for correlations of serum ferritin with adiponectin (17).

For this reason, we investigated the associations of several markers of iron metabolism with adipocyte IR and related traits in the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study, a cohort study of subjects with or at an increased risk of T2DM and cardiovascular diseases (CVDs) who underwent extensive metabolic characterization. In a secondary analysis, we also investigated the potential role of oxidative stress in these associations.

RESEARCH DESIGN AND METHODS

Subjects and study design

CODAM participants were selected from a large cohort in the general population, as described in detail elsewhere (18–20). Briefly, subjects were selected if they were of Caucasian ethnicity, aged >40 years, and had at least one of the following inclusion criteria: positive family history of T2DM (first-degree relatives), history of gestational diabetes, BMI ≥25 kg/m^2, use of antihypertensive drugs, postprandial glucose ≥6.0 mmol/L, or glycosuria. In total, 574 individuals were included, and their lifestyle and cardiovascular and metabolic profile was extensively characterized during two visits to the University's Metabolic Research Unit.

We performed the current cross-sectional analyses in the baseline evaluation of this cohort and excluded 13 participants with insulin therapy and 7 with a self-reported history of liver disease. To exclude possible hereditary hemochromatosis, we excluded 21 subjects with elevated serum ferritin (>150 μg/L for premenopausal women, >400 μg/L for men and postmenopausal women) and elevated transferrin saturation (>45%), according to Dutch guidelines (21). Subjects with missing data on iron markers (n = 9), glucose or insulin (n = 6), adiponectin (n = 7), or important covariates (n = 19) were also excluded. Hence, the current study reports on 492 subjects.

The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre, and all subjects gave written informed consent.

Markers of iron stores and iron metabolism

Participants were asked to stop their lipid-lowering drugs 14 days before the visit and to stop all other medication the day before the visit. After an overnight fast, venous blood samples were collected for assessment of several biomarkers, and an oral glucose tolerance test (OGTT) was performed. Serum was allowed to clot at room temperature for 45 min. After centrifugation at 3,000 rpm for 15 min, serum aliquots were stored at −20°C, and plasma (EDTA) aliquots were stored at −80°C until use. Samples were thawed only once before measurements.

Ferritin, transferrin, total iron, and unsaturated iron-binding capacity (UIBC) were determined in serum on a Hitachi 912 autoanalyzer using assays #1661400, #1931628, #1876996, and #1030600, respectively (Roche Diagnostics, Almere, The Netherlands). Non–transferrin-bound iron (NTBI) levels were measured in serum by a method using iron-sensitive fluorescence-labeled apotransferrin, as described in detail previously (22). All interassay coefficients of variation were <5%. Transferrin saturation was calculated as [total serum iron/(total serum iron + UIBC)].

IR and adiponectin

Plasma glucose levels were measured in NaF/KOx plasma with a hexokinase glucose-6-phosphate dehydrogenase method (ABX Diagnostics). Insulin concentrations were determined in EDTA plasma using a two-sided immunoradiometric test using paired monoclonal antibodies (Medgenix Diagnostics). Nonesterified free fatty acids (NEFAs) were measured in EDTA plasma using an enzymatic calorimetric NEFA C method (Wako Diagnostics, Richmond, VA).

The adipocyte IR index was calculated as the product of fasting insulin and fasting NEFA concentrations, as described before (23–25). This index provides a valid marker of the sensitivity of adipocytes to the antilipolytic effect of insulin, because NEFA concentrations and fatty acid turnover in clamp studies are highly correlated and insulin suppresses lipolysis already at low (i.e., fasting) concentrations (10–20 mU/L) (23,26). HOMA2-IR, which has correlated well with glucose disposal in clamp studies (27), was computed using the HOMA calculator (http://www.dtu.ox.ac.uk/homacalculator/index.php). Total adiponectin was measured in EDTA plasma with a competitive ELISA kit (BioVendor Laboratory Medicine Inc., Brno, Czech Republic).

Other covariates

BMI was calculated as weight in kilograms/height in meters squared. Waist circumference was measured in centimeters with a tape at the level midway between the lateral lower rib margin and the anterior superior iliac spine. Smoking behavior and family history of T2DM (first-degree relatives) were assessed by questionnaire. Dietary calorie intake and mean alcohol consumption were estimated by a validated food frequency questionnaire (20). Physical activity was derived from a validated Short Physical Activity Questionnaire (SQUASH) (28).

Malondialdehyde (MDA) was measured in EDTA with a reagent kit for high-performance liquid chromatography analyses (Chromsystems Instruments and Chemicals, Munich, Germany; interassay coefficient of variation, 4–11%), and total antioxidative status (TAS) was measured in serum with an enzymatic kit (Randox Diagnostics, County Antrim, U.K.; interassay coefficient of variation 2.5%). Serum interleukin-6, interleukin-8, tumor necrosis factor-α, high-sensitivity C-reactive protein (hs-CRP), serum amyloid A, and soluble intercellular adhesion molecule-1 were determined on a multiarray detection system based on electrochemiluminescence technology (SECTOR Imager 2400, Meso Scale Discovery, Gaithersburg, MD) (29). Creatinine levels were determined in EDTA plasma with a Jaffé diagnostic test (Roche Diagnostics), and estimated glomerular filtration rate (eGFR) was calculated using the short Modification of Diet in Renal Disease equation (30). Glucose metabolism status (i.e., normal, impaired, or T2DM) was diagnosed according to the World Health Organization 1999 criteria, as previously described (18–20). Prior CVD was defined by self-reported history of CVD, signs of myocardial infarction or ischemia on electrocardiogram, or an ankle-arm index <0.9 in either leg, as previously described in detail (18,19).

Statistical analysis

Variables with a skewed distribution (i.e., ferritin, adipocyte IR, HOMA2-IR, adiponectin, all inflammatory markers) were $\log_e$-transformed before further analyses. Because NTBI levels (i.e., <0.01 μmol/L; coded as 0) were undetectable in 102 subjects and transformation did not improve its distribution, this variable was not transformed for further analyses. A low-grade inflammation (LGI)

score was calculated by averaging the *Z* scores ([individuals' observed values − population mean)/SD]) of the six ($\log_e$-transformed) inflammatory markers (interleukin-6, interleukin-8, tumor necrosis factor-α, hs-CRP, serum amyloid A, and soluble intercellular adhesion molecule-1) (29). General characteristics of the study population were compared across categories of glucose metabolism status using ANOVA for continuous variables and χ^2 test for discrete variables.

Associations between markers of iron metabolism (ferritin, transferrin, serum iron, NTBI, and transferrin saturation) and metabolic variables (adipocyte IR, NEFAs, adiponectin, HOMA2-IR) were examined with the use of multiple linear regression analyses. For $\log_e$-transformed outcome variables (i.e., adipocyte IR, adiponectin, and HOMA2-IR), regression coefficients were converted into percentage change in the original variable (e.g., percentage increase in adipocyte IR per 10 μg/L increase in ferritin). All analyses were adjusted for age, sex, glucose metabolism status, prior CVD, eGFR, smoking status, alcohol consumption, dietary energy intake, physical activity, family history of T2DM, use of medication, waist circumference, and the LGI score. In addition, analyses with serum ferritin and transferrin were mutually adjusted for each other to account for negative confounding because they are both independently associated with IR despite their negative reciprocal correlation (11,12). Appreciation of normal probability plots of residuals confirmed that in all models (including those with NTBI as determinant), the assumption of normality was not violated.

To examine whether oxidative stress could explain (part of) the associations, if present, between iron markers and IR, we examined whether MDA or TAS significantly mediated associations between iron markers and IR. MDA or TAS was added to the fully adjusted regression models, and the "mediated effect" was quantified by the absolute attenuation of the regression coefficient. The CIs around this "mediated effect" were derived from bootstrapping (with 5,000 generated datasets), as previously described in detail (19).

We also investigated whether the associations differed between sexes by adding interaction terms between iron parameters and sex to the fully adjusted models. We found no effect-modification by sex, and therefore, all results are presented for men and women combined. A two-sided $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL).

RESULTS

Study population

General characteristics of the study population are reported in Table 1. Adipocyte IR increased and plasma adiponectin decreased across the spectrum of normal glucose metabolism (NGM), impaired glucose metabolism (IGM), and T2DM (both $P < 0.001$). In addition, median serum ferritin and mean serum transferrin increased from NGM to IGM and T2DM ($P < 0.001$ and $P = 0.050$, respectively), along with TAS ($P < 0.001$). No significant differences were observed for serum iron, NTBI, transferrin saturation, or MDA.

Associations of iron metabolism with adipocyte IR, NEFAs, and adiponectin

After adjustment for age, sex, glucose metabolism status, and each other, serum ferritin and transferrin were significantly associated with higher adipocyte IR: β = 1.38% (95% CI 1.01–1.76) increase in adipocyte IR per 10 μg/L increase in ferritin and 5.26% (3.77–6.78) per 0.1 g/L increase in transferrin (model 2; Table 2). These associations were slightly attenuated but remained significant after adjustment for covariates such as prior CVD, eGFR, smoking status, alcohol consumption, dietary energy intake, physical activity, family history of T2DM, use of medication (model 3), waist circumference (model 4), and LGI score (model 5): 1.00% (0.66–1.34) and 4.18% (2.88–5.50), respectively. Serum iron and NTBI were initially not associated with adipocyte IR (models 1–3; Table 2). However, after adjustments for potential negative confounding variables (i.e., waist circumference and the LGI score in model 5), both were significantly associated with higher adipocyte IR: 1.36% (0.61–2.12) per μmol/L increase in serum iron, and 5.14% (1.88–8.52) per μmol/L increase in NTBI. Transferrin saturation was inversely associated with adipocyte IR in analyses adjusted for age, sex, and glucose metabolism status, but this was completely attenuated after adjustment for covariates (Supplementary Table 1).

To underscore that the observed associations with adipocyte IR indeed reflect associations with NEFAs (and thus potentially reduced suppression of the release of NEFAs from the adipose tissue) and not just hyperinsulinemia, associations of iron metabolism markers with fasting plasma NEFAs are also reported in Table 2. After adjustment for covariates, ferritin, transferrin, serum iron, and NTBI were all associated with higher NEFAs: β = 1.94 μmol/L (95% CI 0.71–3.16) increase in NEFAs per 10 μg/L increase in ferritin, 6.07 μmol/L (1.46–10.7) increase per 0.1 g/L increase in transferrin, 3.99 μmol/L (1.36–6.61) increase per μmol/L increase in serum iron, and 15.7 μmol/L (4.60–26.8) increase per μmol/L increase in NTBI. Moreover, additional adjustment for insulin did not materially affect these associations: 2.10 (0.83–3.37) for ferritin, 6.61 (1.87–11.4) for transferrin, 3.99 (1.36–6.63) for serum iron, and 15.7 (4.57–26.8) for NTBI.

Finally, ferritin and transferrin were inversely associated with plasma adiponectin in fully adjusted models: β = −0.63% (95% CI −0.92 to −0.33) decrease in adiponectin per 10 μg/L increase in ferritin and −1.62% (−2.72 to −0.50) decrease per 0.1 g/L increase in transferrin (model 5; Table 2). In contrast with ferritin and transferrin, serum iron and NTBI were not significantly associated with lower plasma adiponectin.

Associations of iron metabolism with HOMA2-IR

Serum ferritin and transferrin were both significantly associated with higher HOMA2-IR after adjustment for age, sex, glucose metabolism status, and each other: β = 0.95% (95% CI 0.66–1.25) increase in HOMA2-IR per 10 μg/L increase in ferritin, and 3.76% (2.60–4.94) per 0.1 g/L increase in transferrin (model 2; Table 2). After adjustment for covariates, ferritin (0.73% [0.49–0.97]) and transferrin (2.94% [2.01–3.88]) remained significantly associated with HOMA2-IR. In fully adjusted models, serum iron and NTBI were also positively associated with HOMA2-IR, although these were not statistically significant (model 5; Table 2). Finally, transferrin saturation was inversely associated with HOMA2-IR (models 1 and 2; Supplementary Table 1), but this association was attenuated and no longer significant after adjustments for waist circumference and LGI (models 4 and 5).

Mediation by markers of oxidative stress

We also examined whether MDA or TAS mediated the observed associations of

Table 1—*General characteristics of the study population* (n = 492)

	Glucose metabolism status			
	NGM (*n* = 268)	IGM (*n* = 114)	T2DM (*n* = 110)	*P*
Age (years)	58.8 ± 7.3	60.0 ± 6.4	60.8 ± 6.4	0.025
Male sex (%)	59	58	64	0.608
Prior CVD (%)	23	30	40	0.003
Current smoker (%)	21	18	20	0.815
Family history of T2DM (%)	35	45	52	0.007
Energy intake (MJ/day)	9.48 ± 2.7	9.07 ± 2.9	8.62 ± 2.6	0.020
Alcohol consumers (%)	93	91	86	0.165
Alcohol (g/day) in consumers	10.1 (2.7–25.2)	9.5 (1.4–28.6)	10.1 (2.9–27.9)	0.804
Physical activity (10^3 · METs/week)	7.37 ± 4.5	6.17 ± 4.3	6.60 ± 4.1	0.036
Use of medication				
Antihypertensive (%)	27	42	58	<0.001
Lipid-lowering (%)	16	18	26	0.084
Glucose-lowering (%)	0	3	49	<0.001
BMI (kg/m^2)	27.6 ± 3.9	28.9 ± 4.3	30.2 ± 4.6	<0.001
Waist circumference (cm)	96.1 ± 10.9	100.8 ± 11.8	104.8 ± 11.6	<0.001
Fasting glucose (mmol/L)	5.3 (5.0–5.5)	6.0 (5.5–6.3)	7.4 (6.9–8.6)	<0.001
Fasting insulin (pmol/L)	52 (41–68)	67 (45–99)	84 (60–121)	<0.001
NEFAs (μmol/L)	481 ± 157	553 ± 196	583 ± 196	<0.001
Adipocyte IR index	24 (17–33)	34 (22–61)	46 (33–71)	<0.001
HOMA2-IR	0.98 (0.78–1.28)	1.28 (0.88–1.91)	1.71 (1.22–2.47)	<0.001
Adiponectin (μg/mL)	8.1 (6.2–10.9)	6.9 (5.6–9.7)	5.7 (4.2–8.0)	<0.001
Ferritin (μg/L)	129 (70–227)	162 (80–256)	216 (127–340)	<0.001
Transferrin (g/L)	2.50 ± 0.34	2.58 ± 0.34	2.57 ± 0.37	0.050
Serum iron (μmol/L)	18.8 ± 6.2	19.4 ± 5.2	18.7 ± 6.2	0.628
NTBI (μmol/L)	1.11 (0.22–2.28)	1.21 (0.23–1.90)	1.12 (0.12–2.11)	0.463
Transferrin saturation (%)	35 ± 12	35 ± 11	33 ± 11	0.283
MDA (μmol/L)	0.18 ± 0.05	0.18 ± 0.05	0.17 ± 0.04	0.221
TAS (mmol/L)	1.06 ± 0.10	1.08 ± 0.10	1.13 ± 0.10	<0.001
Interleukin 6 (pg/mL)	1.51 (1.00–2.25)	1.54 (1.14–2.34)	1.67 (1.24–2.39)	0.072
Interleukin 8 (pg/mL)	4.10 (3.40–5.00)	4.30 (3.56–5.50)	4.99 (4.09–6.34)	<0.001
Tumor necrosis factor-α (pg/mL)	6.15 (5.21–7.53)	6.11 (5.29–7.61)	6.30 (5.40–7.56)	0.757
hs-CRP(mg/L)	1.71 (0.86–3.21)	2.09 (1.00–4.38)	3.06 (1.42–6.42)	<0.001
Serum amyloid A (mg/L)	1.29 (0.78–2.09)	1.35 (0.89–2.61)	1.64 (1.02–3.06)	0.013
sICAM-1 (μg/L)	203 (175–237)	212 (188–249)	231 (196–266)	<0.001
LGI score	−0.12 ± 0.61	0.03 ± 0.59	0.25 ± 0.64	<0.001
eGFR (mL/min/1.73 m^2)	84 ± 16	85 ± 16	90 ± 21	0.005

Data are expressed as mean ± SD, median (interquartile range), or percentages. sICAM-1, soluble intercellular adhesion molecule-1.

ferritin, transferrin, serum iron, and NTBI with adipocyte IR and HOMA2-IR. Fully adjusted associations (model 5; Table 2) of ferritin (β = 1.00% [95% CI 0.66–1.34]) and transferrin (4.18% [2.88–5.50]) with adipocyte IR were significantly mediated by TAS, although only to a limited extent. The effect size of the association between ferritin and adipocyte IR was attenuated by 0.06 (0.00–0.16) when TAS was included in the regression model, and attenuation by TAS was 0.21 (0.01–0.53) for transferrin attenuation by TAS. Likewise, fully adjusted associations (model 5; Table 2) of ferritin (0.73% [0.49–0.97]) and transferrin (2.94%, [2.01–3.88]) with HOMA2-IR were significantly mediated by TAS, with mediated portions of 0.05 (0.01–0.13) for ferritin and 0.18 (0.03–0.45) for transferrin. No significant mediation by TAS was found for associations with iron or NTBI (data not shown). In addition, there was no significant mediation by MDA in any of the associations (data not shown).

Additional analyses

All analyses, as presented above, excluded subjects with possible hemochromatosis, defined by the combination of elevated transferrin saturation (>45%) and elevated ferritin. Excluding all subjects with elevated transferrin saturation (*n* = 68) did not appreciably change the associations between ferritin, transferrin, serum iron, and NTBI with metabolic outcomes, however (data not shown).

To account for the possibility of markers of iron metabolism being affected by the presence of infectious or inflammatory diseases at the time of blood sampling, we also repeated all the analyses excluding 41 subjects with serum hs-CRP >10 mg/L. This did not materially change the associations of ferritin, transferrin, serum iron, and NTBI with metabolic outcomes (data not shown). In addition, similar associations were observed after stringent exclusion of 173 subjects with self-reported history of pulmonary, renal, gastrointestinal, thyroid, rheumatic disease, or cancer (data not shown).

CONCLUSIONS—The current study has four novel findings. First, we showed

Table 2—*Associations markers of iron metabolism with adipocyte IR and serum adiponectin*

		Ferritin (per 10 μg/L)		Transferrin (per 0.1 g/L)		Serum iron (per μmol/L)		NTBI (per μmol/L)	
Outcome	Model	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Adipocyte IR									
(% change)	1	1.26	0.88–1.64‡	4.58	2.99–6.19‡	−0.01	−0.93 to 0.93	1.08	−2.92 to 5.26
	2	1.38	1.01–1.76‡	5.26	3.77–6.78‡	0.08	−0.78 to 0.95	2.06	−1.68 to 5.94
	3	1.29	0.92–1.67‡	5.23	3.78–6.71‡	0.53	−0.32 to 1.39	2.77	−0.86 to 6.53
	4	1.03	0.69–1.37‡	4.08	2.78–5.42‡	1.08	0.32–1.84†	4.01	0.74–7.38*
	5	1.00	0.66–1.34‡	4.18	2.88–5.50‡	1.36	0.61–2.12‡	5.14	1.88–8.52†
NEFAs									
(μmol/L change)	1	1.95	0.83–3.06†	6.65	2.12–11.2†	2.32	−0.36 to 4.99	9.77	−1.87 to 21.4
	2	2.72	1.52–3.91‡	7.28	2.70–11.9†	3.35	0.75–5.96*	14.6	3.33–25.9*
	3	2.06	0.85–3.27†	6.26	1.70–10.8†	3.18	0.59–5.78*	13.1	2.12–24.1*
	4	1.98	0.76–3.20†	5.92	1.29–10.5*	3.49	0.89–6.10†	13.8	2.75–24.8*
	5	1.94	0.71–3.16†	6.07	1.46–10.7†	3.99	1.36–6.61†	15.7	4.60–26.8†
Adiponectin									
(% change)	1	−1.19	−1.48 to −0.90‡	−0.94	−2.19 to 0.33	−0.55	−1.29 to 0.19	−2.95	−6.05 to 0.24
	2	−0.66	−0.95 to −0.37‡	−1.63	−2.72 to −0.52†	0.23	−0.41 to 0.87	−0.55	−3.26 to 2.24
	3	−0.65	−0.94 to −0.35‡	−1.66	−2.75 to −0.56†	−0.03	−0.66 to 0.62	−1.05	−3.71 to 1.68
	4	−0.63	−0.93 to −0.33‡	−1.59	−2.70 to −0.48†	−0.09	−0.73 to 0.56	−1.20	−3.85 to 1.53
	5	−0.63	−0.92 to −0.33‡	−1.62	−2.72 to −0.50†	−0.15	−0.80 to 0.50	−1.46	−4.13 to 1.29
HOMA2-IR									
(% change)	1	1.04	0.75–1.34‡	3.19	1.95–4.44‡	−0.48	−1.21 to 0.24	−0.76	−3.86 to 2.43
	2	0.95	0.66–1.25‡	3.76	2.60–4.94‡	−0.62	−1.28 to 0.05	−0.78	−3.62 to 2.15
	3	0.99	0.71–1.27‡	3.93	2.83–5.03‡	−0.18	−0.82 to 0.47	0.10	−2.61 to 2.89
	4	0.75	0.50–0.99‡	2.87	1.93–3.83‡	0.30	−0.25 to 0.85	1.18	−1.14 to 3.54
	5	0.73	0.49–0.97‡	2.94	2.01–3.88 ‡	0.48	−0.07 to 1.03	1.89	−0.42 to 4.26

β Values are unstandardized regression coefficients and represent the change in adipocyte IR, adiponectin, and HOMA2-IR according to increases in ferritin, transferrin, serum iron, and NTBI, respectively. Model 1: crude associations. Model 2: Model 1 + adjusted for age, sex, and glucose metabolism status, and ferritin and transferrin adjusted for each other. Model 3: Model 2 + adjusted for prior CVD, eGFR, smoking status, alcohol consumption, dietary energy intake, physical activity, family history of T2DM, and use of medication. Model 4: Model 3 + adjusted for waist circumference. Model 5: Model 4 + adjusted for low-grade inflammation score. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

that iron metabolism was robustly associated with adipocyte IR. Ferritin, transferrin, serum iron, as well as NTBI were associated with adipocyte IR, also after taking a broad range of covariates into account. Second, these findings were further corroborated by associations of these markers with plasma NEFAs and by inverse associations between serum ferritin and transferrin with adiponectin. Third, adjustment for inflammatory markers or exclusion of subjects with elevated hs-CRP did not attenuate these associations, but in some cases, even increased the magnitude of the associations. Finally, TAS significantly mediated associations of ferritin and transferrin with adipocyte IR and HOMA2-IR. Taken together, these results suggest that stores of body iron and/or iron metabolism may be involved in the development of IR not only in liver or muscle but also in adipocytes.

The observed associations of iron parameters with adipocyte IR and adiponectin may be important because adipocyte IR or adipocyte dysfunction is thought to be one of the first events in the pathogenesis of T2DM (31). The results of our and other epidemiological studies are supported by several in vitro and animal studies. Incubation of adipocytes with iron or transferrin resulted in increased lipolysis and decreased insulin-stimulated glucose transport (13,14). Moreover in mice, an iron-enriched diet caused iron accumulation in visceral fat together with IR (15), and an iron-restricted diet led to lower free fatty acids and triglycerides (16).

Previous epidemiological studies have shown associations of ferritin and transferrin with HOMA-IR, serum triglycerides, HDL, or the triglycerides-to-HDL ratio, in agreement with these findings (1,9–12). However, HOMA-IR and these lipid variables do not necessarily reflect adipocyte IR, and these studies did not adjust for potential confounding variables such as smoking, diet, physical activity, and family history of T2DM. The current study confirms and extends previous in vitro data and epidemiological studies by showing firstly that ferritin and transferrin were both associated with HOMA-IR, independently of each other and several potential metabolic confounders, and secondly, by showing that ferritin, transferrin, iron, and NTBI were independently associated with an adipocyte IR index. Although adipocyte IR and HOMA2-IR were both derived from fasting insulin levels, ferritin, transferrin, iron, and NTBI were also independently associated with fasting NEFAs, even after adjustment for insulin. This suggests that, although highly correlated, the adipocyte IR and HOMA2-IR indices do not represent the same site of IR and that our findings indeed concern adipocyte lipolysis.

In addition, ferritin and transferrin were inversely associated with plasma adiponectin, in agreement with previous studies (7,17). Some authors have suggested that the association of ferritin with adiponectin may be explained by abdominal fat (7), but adjustment for obesity and related LGI did not affect the observed

associations in our and one previous study (17). The potential inhibitory role of iron on adiponectin production and secretion is yet unclear, but may be interesting. Low adiponectin levels are associated with the development of T2DM and have been suggested to be a specific marker of adipose tissue dysfunction (7,32). Therefore, the observed associations of ferritin and transferrin with both adipocyte IR and low adiponectin levels support the hypothesis that iron metabolism may influence adipose tissue function.

In contrast to serum ferritin, published data on serum total iron and NTBI levels in obesity or T2DM are scarce. One study showed that NTBI levels were significantly higher in patients with T2DM than in healthy controls (33). We did not observe this difference in our study, but we note that our population as a whole had an increased metabolic risk and that median NTBI levels were relatively high, even in the NGM group. Of interest, the associations of both total serum iron and NTBI with adipocyte IR emerged only after adjustment for waist circumference and the LGI score. The increases in magnitude of associations between serum iron and NTBI with adipocyte IR or HOMA-IR across models 1–5 may be attributed to negative confounding of obesity and LGI on these associations. Obesity and its related LGI may increase hepcidin production (34), which in turn lowers circulating iron levels by decreased intestinal absorption and decreased release from macrophages (35). In regression analyses that are not adjusted for obesity and LGI, the "true" positive association between iron/NTBI levels and adipocyte IR is confounded toward the null (models 1–3). After these negative confounding variables had been taken into account, the "true" associations between iron levels and adipocyte IR became apparent. Associations of adiponectin and HOMA2-IR with serum iron and NTBI were not significant, even after taking into account the negative confounding, as described above. Still, a trend was observed for HOMA-IR. One explanation could be that serum iron and NTBI are characterized by more random error (due to circadian fluctuations and day-to-day variations) than ferritin and transferrin, which may decrease the magnitude of its associations.

A possible mechanism through which iron may induce IR is the formation of toxic free radicals. Addition of a free-radical scavenger completely blocked the effect of iron or transferrin on in vitro adipocyte lipolysis (13). Serum ferritin has also been associated with circulating oxidized lipoproteins and advanced oxidation protein products (1), suggesting that iron may cause IR through induction of oxidative stress. In addition, we observed significant mediation by TAS in the associations of ferritin and transferrin with adipocyte IR and HOMA2-IR. The small magnitude of the mediated effect may be because TAS is a systemic measure and does not efficiently reflect local or intracellular oxidative stress. Nevertheless, the significant mediation in our study supports the hypotheses that the association between iron and IR does involve induction of oxidative stress. The association of NTBI levels with adipocyte IR also suggests the involvement of oxidative stress: NTBI is believed to be a more redox-active form of iron because it is not so tightly bound to carrier proteins such as transferrin (33,36).

In the evaluation of iron metabolism in obesity and T2DM, the concurrent LGI has regularly been implicated as an important confounder because several markers of iron metabolism (ferritin, transferrin, hepcidin) are also acute-phase proteins (21,35). Remarkably, associations remained unchanged after adjustment for LGI or after exclusion of subjects with elevated hs-CRP, indicating that (systemic) LGI did not have a confounding role in the associations between iron parameters and IR. Finally, serum ferritin has been shown to correlate well with the amount of iron removed by phlebotomy, even in subjects with obesity-related LGI (37). Therefore, we conclude that the above associations truly reflect involvement of iron metabolism and not just LGI.

The current study showed consistent associations of several markers of iron metabolism with systemic markers of IR in subjects who were carefully metabolically phenotyped.

The main limitation of our study is its cross-sectional design, which does not allow us to draw definite conclusions on causality. In a reverse-causation scenario, our results could be explained by actions of insulin on iron metabolism. Indeed, insulin has been shown to upregulate transferrin receptor expression and transferrin production, and therefore, iron uptake and ferritin levels (38,39). The reciprocal relation between insulin and iron metabolism is probably very complex and cannot simply be dissected in epidemiological studies. However, in our study, markers of iron metabolism were also associated with NEFAs, which do not have a known reciprocal effect on iron metabolism. In addition, small trials in patients with T2DM or nonalcoholic fatty liver disease have shown that iron depletion by phlebotomy reduces HOMA2-IR, supporting our concept (37,40). Whether this also holds true for adipocyte IR remains to be investigated. Finally, our study was conducted in middle-aged and older Caucasian subjects who were selected on the basis of an increased risk for metabolic and CVD, and extrapolation to other study populations or other ethnicities should be done with caution.

In conclusion, we have shown that several markers of iron metabolism are associated not only with HOMA2-IR but also with adipocyte IR in humans. These findings suggest that body iron stores and/or iron metabolism–related factors may contribute to the induction of IR early in the pathogenesis of T2DM. Of note, body iron stores can easily be influenced by low-cost interventions such as phlebotomies or dietary interventions. Therefore, iron metabolism, and particularly effects of iron on adipose tissue, represents an interesting feature of the metabolic syndrome that deserves further investigation.

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