Insulin Resistance Modulates Iron-Related Proteins in Adipose Tissue

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PATHOPHYSIOLOGY/COMPLICATIONS

OBJECTIVE

Circulating markers of iron overload are associated with insulin resistance. Less is known about the impact of iron overload on adipose tissue (AT). We hypothesized that gene expression markers of iron metabolism in AT could be associated with insulin action.

RESEARCH DESIGN AND METHODS

The AT expression of ferroportin (*SLC40A1*), transferrin (*TF*), *TF* receptor (*TFRC*), ferritin (FT) heavy polypeptide 1 (*FTH1*), and FT light polypeptide (*FTL*) was analyzed cross-sectionally in three independent cohorts and also after weight loss–induced changes in insulin sensitivity (clamp *M* value) in an independent fourth cohort.

RESULTS

In human AT, *TF* mRNA and protein levels were decreased with obesity and insulin resistance in the three cohorts and were positively associated with adipogenic mRNAs and insulin action. Otherwise, *FTL* mRNA and protein and *SLC40A1* transcripts were positively associated with BMI and negatively linked to adipogenic genes and insulin action. Bariatric surgery–induced weight loss led to increased *TF* and decreased *TFRC, FTH1, FTL*, and *SLC40A1* in subcutaneous AT in parallel to improved insulin action.

CONCLUSIONS

These results suggest that iron overload impacts on AT in association with insulin resistance.

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In the last two decades, different epidemiologic studies have demonstrated that iron stores in the general population predict the development of the metabolic syndrome, glucose intolerance, type 2 diabetes (T2D) (1–6), and gestational diabetes mellitus (7,8). Dandona et al. (9) showed for the first time in patients with β-thalassemia that insulin resistance is associated with iron overload before sufficient β-cell damage occurs to result in diabetes. Insulin is known to stimulate iron uptake by adipocytes and hepatocytes (10). Reciprocally, iron interferes with insulin action in the liver. Frequent blood donations, leading to decreased iron

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stores, have been demonstrated to reduce postprandial hyperinsulinemia in healthy volunteers (11), to improve insulin sensitivity (12), and to constitute a protective factor for the development of T2D (13–15).

The link between iron and T2D has been reviewed and updated in several excellent manuscripts (16,17). Three systematic reviews and meta-analysis have confirmed the association of iron overload and increased T2D risk (18–20).

Circulating ferritin (FT) was also positively associated with visceral and subcutaneous fat area and with the degree of insulin resistance (21). Very recently, serum FT was found to be linked to adipocyte insulin resistance, defined as the product of fasting insulin and nonesterified fatty acids (22,23).

Despite these clinical associations, little attention has been paid to the molecular mechanisms regulating iron homeostasis in adipocytes. In the 3T3-L1 mouse cell line, two important iron-related genes (heavy FT [*FTH*] and iron regulatory protein [*IRP1*]) were found to be upregulated during adipocyte differentiation (24). Of note, adipocyte iron overload led to decreased adiponectin gene expression in association with insulin resistance in mice (23).

Despite these intriguing results, we found no studies dealing with human adipose tissue (AT). The few studies that have explored iron-related genes in AT were performed in rodent models. For instance, *FTH* and FT light chains (*FTL*) were increased in AT from obese rats, whereas transferrin expression (*TF*) was decreased (25).

To briefly summarize the most important factors involved in iron metabolism, it is important to recognize that ferroportin (FP or solute carrier family 40 [iron-regulated transporter], member 1 [SLC40A1]) plays a fundamental role in the release of iron from tissues into the bloodstream (26). The FP-mediated efflux of Fe^{2+} is negatively regulated by hepcidin (*HAMP*), a liver-derived peptide hormone that binds to FP and promotes its phosphorylation, internalization, and lysosomal degradation (27). Exported iron is scavenged by TF, which maintains Fe^{3+} in a redox-inert state and delivers it into tissues through TF receptor (TFR). Interestingly, *FP, HAMP*, and *TFRC* gene expression is consistently expressed in AT from mice (23,28). FT is essential for the cell to store and detoxify excess intracellular iron in the cytosol. FT is a conserved protein consisting of 24 H (heavy) and L (light) subunits, encoded by FTH1 and FTL genes (26). FTH1 carries the ferroxidase activity that is necessary for iron deposition in nanocage, while FTL facilitates iron nucleation and increases the turnover of the ferroxidase site.

While circulating markers of iron overload are well-known to be associated with insulin resistance, less is known about the potential impact of iron overload on AT physiology. We hypothesized that iron overload has its effect on tissue expression and protein levels of different factors involved in iron metabolism (*SLC40A1*, *HAMP, TF, TFRC, FTH1,* and *FTL*) in association with systemic insulin action. After the initial results found in cross-sectional studies, we tested whether weight loss changes in insulin sensitivity lead to changes in the AT expression of these factors.

RESEARCH DESIGN AND METHODS

Subjects' Recruitments for AT Samples

AT samples were obtained from four independent cohorts. In the first and second cohort, a group of 174 (89 visceral AT [VAT] and 85 subcutaneous AT [SAT]) (cohort 1) and 71 VAT samples (cohort 2) from participants with normal body weight and different degrees of obesity, with BMI within 20–68 kg/m^2, recruited at the Endocrinology Service of the Hospital of Girona Dr. Josep Trueta (cohort 1) and the Endocrinology Department of the Universidad de Navarra (cohort 2) were analyzed. In a third cohort of nondiabetic morbidly obese (BMI $>$35 kg/m^2) subjects with different degrees of insulin action (measured using hyperinsulinemic-euglycemic clamp), 32 paired SAT and VAT samples (cohort 3) were studied. These subjects were recruited at the Endocrinology Service of the Hospital of Girona Dr. Josep Trueta (Cohort 3).

All subjects were of Caucasian origin and reported that their body weight had been stable for at least 3 months before the study. Subjects were studied in the postabsorptive state. BMI was calculated as weight in kilograms divided by the square of height in meters. They had no systemic disease other than obesity, and all were free of any infections in the previous month before the study. Liver diseases (specifically, tumoral disease and hepatitis C virus infection) and thyroid dysfunction were specifically excluded by biochemical workup. All subjects gave written informed consent, validated and approved by the ethics committee of the Hospital of Girona Dr. Josep Trueta and the Universidad de Navarra after the purpose of the study was explained to them.

AT samples were obtained from SAT and VAT depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia, and gastric bypass surgery). Samples of AT were immediately transported to the laboratory (5–10 min). The handling of tissue was carried out under strictly aseptic conditions. AT samples were washed in PBS, cut off with forceps and scalpel into small pieces (100 mg), and immediately flash frozen in liquid nitrogen before storage at -80°C.

Study of the Effects of Fat Mass Reduction Induced by Bariatric Surgery

In a fourth cohort, 25 Caucasian obese (mean $\pm$ SD BMI 43.7 $\pm$ 4.6 kg/m^2, age 47 $\pm$ 9 years) subjects, who underwent bariatric surgery thought Roux-en-Y gastric bypass in the Hospital of Girona Dr. Josep Trueta, were part of an ongoing study (29). Inclusion criteria were age between 30 and 60 years, BMI $\geq$35 kg/m^2, and ability to understand the study protocol. Exclusion criteria were use of medications that could interfere with insulin action and history of a chronic systemic disease. AT samples from the SAT depot were obtained during bariatric surgery. Postoperative samples of SAT were obtained by subcutaneous biopsy at the mesogastric level after 2 years from surgery. Fasting blood samples were obtained on the same day of the biopsy. All subjects gave written informed consent, validated and approved by the ethics committee of the Hospital of Girona Dr. Josep Trueta, after the purpose of the study was explained to them.

Hyperinsulinemic-Euglycemic Clamp

Insulin action was determined by hyperinsulinemic-euglycemic clamp (30). After an overnight fast, two catheters were inserted into an antecubital vein, one for each arm, used to administer constant infusions of glucose and insulin and to obtain arterialized venous blood samples. A 2-h hyperinsulinemic-euglycemic clamp was initiated by a two-step primed infusion of insulin (80 mU/m^2/min for 5 min, 60 mU/m^2/min for 5 min) immediately followed by a continuous infusion of insulin at a rate of 40 mU/m^2/min (regular insulin [Actrapid; Novo Nordisk, Plainsboro, NJ]). Glucose infusion began at minute 4 at an initial perfusion rate of 2 mg/kg/min being then adjusted to maintain plasma glucose concentration at 88.3–99.1 mg/dL. Blood samples were collected every 5 min for determination of plasma glucose and insulin. Insulin sensitivity was assessed as the mean glucose infusion rate during the last 40 min. In the stationary equilibrium, the amount of glucose administered (*M*) equals the glucose taken by the body tissues and is a measure of overall insulin sensitivity.

Analytical Methods

Serum glucose concentrations were measured in duplicate by the glucose oxidase method using a Beckman glucose analyzer II (Beckman Instruments, Brea, California). Glycosylated hemoglobin (HbA_{1c}) was measured by the high-performance liquid chromatography method (Bio-Rad, Muenchen, Germany, and autoanalyzer Jokoh HS-10, respectively). Intra- and interassay coefficients of variation were <4% for all these tests. Serum insulin was measured in duplicate by RIA (Medgenix Diagnostics, Fleunes, Belgium). The intra-assay coefficient of variation was 5.2% at a concentration of 10 mU/L and 3.4% at 130 mU/L. The interassay coefficients of variation were 6.9 and 4.5% at 14 and 89 mU/L, respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: [insulin (mU/L) × glucose mmol/L]/22.5. Serum C-reactive protein (ultrasensitive assay; Beckman, Fullerton, CA) was determined by a routine laboratory test, with intra- and interassay coefficients of variation <4%. The lower limit of detection was 0.02 mg/L.

RNA Expression

RNA purification and gene expression procedures and analyses were performed as previously described (30). Briefly, RNA purification was performed using an RNeasy Lipid Tissue Mini kit (QIAgen, Izasa S.A., Barcelona, Spain), and the integrity was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression was assessed by real time PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Barcelona, Spain), using TaqMan and SYBRgreen technology suitable for relative genetic expression quantification. Primer/probe sets used are detailed in Supplementary Table 1.

Protein Preparation

Proteins were extracted from AT by using a Polytron PT-1200C homogenizer (Kinematica AG, Lucerne, Switzerland) directly in radioimmunoprecipitation assay buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mmol/L NaCl, and 50 mmol/L Tris-HCl, pH 8.0), supplemented with protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 13,000 rpm for 60 min at 4°C, recovering the soluble fraction below the fat supernatant and avoiding the nonhomogenized material at the bottom of the centrifuge tube. Protein concentration was determined using the RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

TF and FT Protein Level Measurements

TF and FT levels in AT were measured by ELISA (ET3105-1 and EF2003-1, respectively; Assaypro LLC, Saint Charles, MO) following the manufacturer's instructions. For ET3105-1 (Human Transferrin ELISA kit), intra-assay and interassay coefficients of variation were 4.7% and 7.2% respectively, and for EF2003-1 (Human Ferritin ELISA kit) intra-assay and interassay coefficients of variation were 4.9% and 7.1%, respectively.

Statistical Analyses

Statistical analyses were performed using the SPSS 12.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean ± SD for Gaussian variables or median (interquartile range) for non-Gaussian variables. Parameters that did not fulfill normal distribution were mathematically transformed to improve symmetry for subsequent analyses. The relation between variables was analyzed by simple correlation (using Spearman and Pearson tests) and by multiple linear regression models. ANOVA and unpaired *t* tests were used to compare clinical variables and iron metabolism–related gene expression relative to obesity and T2D. Levels of statistical significance were set at $P < 0.05$.

RESULTS

Iron-Related Gene Expression in Human AT

Anthropometrical and clinical parameters of cohort 1 and 2 are shown in Table 1. Most of the studied genes were expressed at substantial levels in human AT. It is well-known that iron stores increase after menopause. In cohort 1, circulating FT concentration was significantly increased, whereas circulating TF concentration was decreased in postmenopausal women. Of note, VAT *FTL* gene expression was significantly increased in postmenopausal women and men compared with premenopausal women (24.2 ± 15.8 vs. 14.77 ± 5.2 relative gene expression units [R.U.], $P = 0.02$). In SAT, *FTL* gene expression also tended to be increased in postmenopausal women and men (28.5 ± 12.3 vs. 21.9 ± 10.7 R.U., $P = 0.09$). However, no significant differences in the expression of the other iron-related genes were found according to menopausal status or sex.

HAMP gene expression also was analyzed, but in comparison with the other iron-related genes studied *HAMP* mRNA levels were found at negligible levels (even undetectable in some samples). In both SAT and VAT, *TF* gene expression was significantly decreased in obese participants, mainly in those with T2D (Table 1), being significantly and negatively correlated with obesity measures and HOMA-IR (Tables 2 and 3). On the contrary, *SLC40A1* and *FTL* were significantly increased in obese participants and positively correlated with obesity measures and HOMA-IR

Table 1—Anthropometric and clinical parameters together with AT gene expression data of study subjects from cohorts 1 and 2

	Cohort 1				
	Lean	Overweight	Obese	Obese plus T2D	*P*
Participants (*n*)	10	19	37	23	
Sex (men/women)	2/8	6/13	9/28	8/15	
Age (years)	48.9 ± 9.6	54.9 ± 15.2	44.6 ± 10.2#	45.1 ± 11.3#	**0.01**
BMI (kg/m^2)	22.6 ± 1.5	27.4 ± 1.6*	44.5 ± 7.9*#	44.4 ± 4.3*#	**<0.0001**
Fat mass (%)	28.01 ± 1.5	32.56 ± 6.1	55.81 ± 11.1*#	55.24 ± 9.5*#	**<0.0001**
Fasting glucose (mg/dL)	92.2 ± 8.9	91.2 ± 19.8	95.4 ± 13.5	129.4 ± 48.7*#†	**0.001**
Fasting insulin (mU/L)	5.2 ± 2.7	7.4 ± 3.1	10.9 ± 4.4 (*n* = 10)	26.9 ± 12.7 (*n* = 7)#	**0.01**
HOMA-IR	1.21 ± 0.7	1.78 ± 0.9	2.74 ± 1.44 (*n* = 10)	7.52 ± 5.1 (*n* = 7)	0.2
HbA_{1c} (%)	5.12 ± 0.09	4.99 ± 0.42	4.95 ± 0.53	5.78 ± 1.72†	**0.04**
HbA_{1c} (mmol/mol)	32 ± 1	31 ± 3.2	31 ± 3.5	40 ± 14.6†	**0.04**
VAT gene expression (R.U.)					
TF	0.022 ± 0.015	0.018 ± 0.015	0.0069 ± 0.0061*#	0.0047 ± 0.0030*#	**<0.0001**
SLC40A1	0.24 ± 0.07	0.27 ± 0.09	0.37 ± 0.15*#	0.34 ± 0.10*	**0.003**
FTL	9.41 ± 3.9	9.1 ± 3.5	13.22 ± 5.56*#	11.59 ± 3.91	**0.01**
TFR	0.036 ± 0.02	0.037 ± 0.03	0.031 ± 0.030	0.027 ± 0.019	0.6
FTH	0.011 ± 0.006	0.011 ± 0.005	0.0142 ± 0.0074	0.011 ± 0.0064	0.4
SAT gene expression (R.U.)					
TF	0.024 ± 0.018	0.030 ± 0.03	0.019 ± 0.018	0.011 ± 0.0065*#†	**0.03**
SLC40A1	0.16 ± 0.07	0.13 ± 0.05	0.21 ± 0.11#	0.22 ± 0.11#	**0.02**
FTL	13.86 ± 4.1	15.65 ± 12.16	19.11 ± 6.49	18.28 ± 6.12	0.1
TFR	0.024 ± 0.009	0.021 ± 0.008	0.023 ± 0.012	0.026 ± 0.019	0.8
FTH	0.017 ± 0.007	0.016 ± 0.008	0.013 ± 0.0072	0.0135 ± 0.0093	0.1

	Cohort 2			
	Lean	Obese	Obese plus T2D	*P*
Participants (*n*)	10	31	30	
Sex (men/women)	4/6	6/25	8/22	
Age (years)	46.6 ± 15.2	39.4 ± 14.1	40.3 ± 11.3	0.3
BMI (kg/m^2)	22.18 ± 2.5	42.6 ± 4.1*	46.3 ± 7.8*	**<0.0001**
Fat mass (%)	27.32 ± 6.8	53.1 ± 4.7*	52.13 ± 7.8*	**<0.0001**
Fasting glucose (mg/dL)	93.6 ± 17.2	90.3 ± 10.7	111.9 ± 23.2*†	**<0.0001**
Fasting insulin (mU/L)	6.6 ± 1.3	18.01 ± 16.4	20.9 ± 12.2	0.2
HOMA-IR	1.4 ± 0.25	4.05 ± 3.8	5.8 ± 4.3	0.08
HbA_{1c} (%)	5.3 ± 0.2	5.7 ± 0.3	7.4 ± 1.4*†	**0.02**
HbA_{1c} (mmol/mol)	34 ± 0.9	39 ± 1.4	57 ± 8*†	**0.02**
VAT gene expression (R.U.)				
TF	0.015 (0.01–0.03)	0.019 (0.01–0.05)	0.012 (0.003–0.02)†	**0.04**
SLC40A1	0.09 (0.05–0.13)	0.30 (0.14–1.22)*	0.23 (0.18–0.36)*†	**0.01**
FTL	4.7 (3.4–11.1)	16.7 (9.8–26.1)*	21.9 (9.7–49)*†	**0.002**
FTH	0.032 (0.01–0.04)	0.078 (0.06–0.13)*	0.12 (0.08–0.19)*†	**<0.0001**

Data are means ± SD or median (interquartile range) unless otherwise indicated. Boldface indicates statistically significant values. #$P < 0.05$ compared with overweight participants, performing Bonferroni post hoc test. *$P < 0.05$ compared with lean participants, performing Bonferroni post hoc test. †$P < 0.05$ compared with obese participants, performing Bonferroni post hoc test.

(Tables 2 and 3). No significant differences were found in *TFRC* and *FTH1* gene expression as regards obesity status (Table 1).

In both SAT and VAT, *TF* gene expression was positively associated with adipogenic (such as *PPARγ* and *FASN*) and insulin-related pathway genes (*IRS1* and *GLUT4*) (Tables 2 and 3). These associations remained significant after controlling for age and sex. VAT *TF* gene expression was also negatively associated with inflammatory gene markers (such as *TNFα*) (Table 2). Otherwise, SAT and VAT *FTL* and *SLC40A1* gene expression was positively associated with proinflammatory (in VAT) and negatively with adipogenic genes (Tables 2 and 3). In addition, in both SAT and VAT, *FTL, FTH1*, and *TFRC* gene expression was significantly associated with that of the oxidative stress marker cytochrome b-245, α polypeptide ($p22^{phox}$ subunit or CYBA gene) (Tables 2 and 3). CYBA has been suggested as a marker of AT iron storage–induced oxidative stress via NADPH oxidase, being that *CYBA* gene expression is strongly associated with FT levels (31).

Table 2—Bivariate correlation among VAT iron-related genes and anthropometric, clinical parameters as well as AT gene expression in subjects from cohorts 1, 2, and 3

	Cohort 1 (*n* = 89)									
	TF (R.U.)		*SLC40A1* (R.U.)		*FTL* (R.U.)		*FTH* (R.U.)		*TFR* (R.U.)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Age (years)	0.28	**0.006**	−0.12	0.2	0.01	0.9	−0.06	0.5	0.08	0.4
BMI (kg/m^2)	−0.54	**<0.0001**	0.38	**<0.0001**	0.28	**0.007**	0.027	0.8	−0.17	0.11
Fat mass (%)	−0.27	**0.02**	0.23	0.05	0.21	0.08	−0.08	0.5	−0.09	0.4
Fasting glucose (mg/dL)	−0.18	0.09	0.12	0.2	−0.10	0.4	−0.16	0.14	−0.10	0.36
Fasting insulin (mU/L)	−0.37	**0.02**	0.30	0.06	0.20	0.3	−0.22	0.2	−0.19	0.2
HOMA-IR	−0.42	**0.01**	0.35	**0.04**	0.29	0.1	−0.28	0.1	−0.15	0.4
HbA_{1c}	0.03	0.8	−0.16	0.2	−0.15	0.2	−0.18	0.1	−0.03	0.8
PPARγ expression (R.U.)	0.61	**<0.0001**	−0.59	**<0.0001**	0.18	0.1	0.11	0.38	0.15	0.2
IRS1 expression (R.U.)	0.63	**<0.0001**	−0.26	**0.01**	−0.32	**0.008**	−0.026	0.8	−0.11	0.4
GLUT4 expression (R.U.)	0.69	**<0.0001**	−0.45	**<0.0001**	−0.16	0.1	0.08	0.5	0.22	0.05
FASN expression (R.U.)	0.43	**<0.0001**	−0.46	**<0.0001**	−0.24	**0.002**	0.01	0.9	0.07	0.5
TNFα expression (R.U.)	−0.36	**0.004**	0.29	**0.008**	0.40	**<0.0001**	0.35	**0.002**	0.18	0.1
CYBA expression (R.U.)	−0.02	0.85	0.14	0.2	0.62	**<0.0001**	0.20	0.06	0.28	**0.008**
	Cohort 2 (*n* = 71)									
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*		
Age (years)	0.18	0.1	−0.06	0.6	0.03	0.7	0.03	0.7		
BMI (kg/m^2)	−0.08	0.5	0.33	**0.006**	0.38	**0.001**	0.26	**0.03**		
Fat mass (%)	0.17	0.1	0.26	**0.03**	0.22	0.07	0.25	**0.04**		
Fasting glucose (mg/dL)	−0.05	0.6	−0.18	0.15	0.24	0.06	0.34	**0.006**		
Fasting insulin (mU/L)	−0.33	**0.01**	−0.07	0.6	0.03	0.8	0.10	0.4		
HOMA-IR	−0.30	**0.02**	−0.11	0.4	0.05	0.6	0.15	0.2		
HbA_{1c}	0.04	0.8	−0.07	0.6	0.12	0.3	0.14	0.2		
	Cohort 3 (*n* = 32)									
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Age (years)	0.06	0.7	−0.30	0.05	0.26	0.1	−0.1	0.5	−0.1	0.5
BMI (kg/m^2)	−0.05	0.7	0.32	**0.04**	−0.15	0.4	0.18	0.2	0.15	0.3
Fat mass (%)	0.11	0.5	0.21	0.2	−0.16	0.3	0.19	0.2	0.08	0.6
Fasting glucose (mg/dL)	0.04	0.8	0.14	0.4	−0.05	0.7	0.04	0.8	−0.08	0.6
HbA_{1c}	−0.21	0.2	0.01	0.9	0.14	0.4	0.08	0.6	0.04	0.8
Hyperinsulinemic-euglycemic clamp (mg/kg · min)	0.52	**0.002**	−0.31	0.05	−0.49	**0.004**	0.21	0.2	0.26	0.1
PPARγ expression (R.U.)	−0.11	0.5	−0.51	**0.001**	0.12	0.4	0.2	0.2	0.37	**0.02**
ADIPOQ expression (R.U.)	0.34	**0.03**	−0.44	**0.006**	0.27	0.1	0.14	0.4	0.26	0.1
IRS1 expression (R.U.)	0.19	0.2	0.43	**0.007**	−0.33	**0.04**	0.26	0.1	0.28	0.08
GLUT4 expression (R.U.)	0.40	**0.01**	−0.07	0.7	0.22	0.2	−0.1	0.5	−0.16	0.3
TNFα expression (R.U.)	−0.03	0.8	0.49	**0.003**	0.37	**0.03**	−0.12	0.4	−0.29	0.09
CYBA expression (R.U.)	−0.35	**0.04**	0.19	0.28	0.64	**<0.0001**	−0.07	0.7	−0.15	0.4

Bivariate correlation was performed using nonparametric (Spearman) or parametric (Pearson) tests. Boldface indicates statistically significant values.

In cohort 2, similar associations with clinical parameters were found, being that *TF* gene expression significantly decreased in obese subjects with T2D and *SLC40A1*, *FTL*, and *FTH1* gene expression significantly increased in obese subjects (Fig. 1*A* and Tables 1 and 2).

We also explored a third cohort (cohort 3) of nondiabetic morbidly obese participants (5 men and 27 women) in whom a euglycemic clamp procedure was performed. Anthropometrical and clinical parameters of this cohort include age (48.29 ± 9.1 years), BMI (43.8 ± 6.9 kg/m^2), percent fat mass (56.1 ± 10.1%), fasting glucose (96.03 ± 12.01 mg/dL), HbA_{1c} (5.4 ± 0.35% [mmol/mol] [36 ± 1.4 mmol/mol]), and hyperinsulinemic-euglycemic clamp (4.25 ± 2.4 mg/kg · min). *TF* gene expression was also positively associated with some adipogenic genes, whereas *SLC40A1* and *FTL* gene expressions tended to be negatively associated with these genes (Tables 2 and 3). Of note, the strong relationship between *FTL* and *CYBA* was maintained

Table 3—Bivariate correlation among SAT iron-related genes and anthropometric, clinical parameters as well as AT gene expression in subjects from cohorts 1 and 3

	TF (R.U.)		*SLC40A1* (R.U.)		*FTL* (R.U.)		*FTH* (R.U.)		*TFR* (R.U.)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Cohort 1 (*n* = 85)										
Age (years)	0.19	0.06	−0.049	0.65	−0.33	**0.004**	−0.09	0.4	−0.11	0.3
BMI (kg/m^2)	−0.34	**0.002**	0.23	**0.03**	0.29	**0.005**	−0.21	0.05	0.05	0.6
Fat mass (%)	−0.25	**0.02**	0.03	0.8	−0.08	0.5	−0.18	0.08	0.04	0.7
Fasting glucose (mg/dL)	−0.18	0.09	0.24	**0.025**	0.07	0.5	0.04	0.7	0.16	0.1
Fasting insulin (mU/L)	−0.31	0.06	0.05	0.7	0.27	0.1	0.03	0.8	0.31	0.06
HOMA-IR	−0.37	**0.03**	0.04	0.8	0.36	**0.04**	0.10	0.5	0.35	**0.04**
HbA_{1c}	0.11	0.4	0.01	0.9	−0.13	0.3	0.05	0.7	0.18	0.07
PPARγ expression (R.U.)	0.42	**0.001**	−0.44	**0.001**	−0.25	**0.03**	0.28	**0.01**	0.13	0.3
IRS1 expression (R.U.)	0.38	**0.001**	0.03	0.8	−0.19	0.1	0.29	**0.01**	0.05	0.7
GLUT4 expression (R.U.)	0.50	**<0.0001**	−0.30	**0.01**	−0.58	**<0.0001**	−0.02	0.8	−0.37	**0.002**
FASN expression (R.U.)	0.38	**0.001**	−0.29	**0.01**	−0.39	**0.001**	0.16	0.2	−0.25	**0.04**
TNFα expression (R.U.)	−0.03	0.8	0.1	0.4	0.02	0.8	0.28	**0.01**	0.08	0.5
CYBA expression (R.U.)	−0.06	0.6	0.26	**0.01**	0.49	**<0.0001**	0.46	**<0.0001**	0.45	**<0.0001**
Cohort 3 (*n* = 32)										
Age (years)	0.25	0.1	0.02	0.9	0.09	0.5	−0.21	0.2	0.14	0.4
BMI (kg/m^2)	−0.12	0.4	0.09	0.6	0.14	0.4	0.39	**0.01**	0.08	0.6
Fat mass (%)	0.10	0.5	0.10	0.5	0.04	0.8	0.28	0.08	0.13	0.4
Fasting glucose (mg/dL)	0.10	0.5	0.30	0.05	0.13	0.4	0.26	0.1	0.21	0.2
HbA_{1c}	−0.10	0.5	0.21	0.2	0.21	0.2	0.47	**0.003**	0.38	**0.01**
Hyperinsulinemic-euglycemic clamp (mg/kg · min)	0.42	**0.01**	−0.26	0.1	−0.32	0.06	−0.23	0.2	0.03	0.8
PPARγ expression (R.U.)	0.23	0.17	−0.34	**0.04**	−0.20	0.2	0.20	0.2	0.24	0.1
ADIPOQ expression (R.U.)	0.27	0.09	−0.24	0.15	−0.15	0.3	0.06	0.7	0.28	0.08
IRS1 expression (R.U.)	0.16	0.3	0.26	0.1	−0.40	**0.01**	−0.01	0.9	−0.03	0.8
GLUT4 expression (R.U.)	0.60	**<0.0001**	0.08	0.6	0.07	0.7	−0.49	**0.002**	−0.38	**0.02**
TNFα expression (R.U.)	0.16	0.3	0.19	0.25	0.32	0.06	0.09	0.6	−0.03	0.8
CYBA expression (R.U.)	−0.06	0.7	0.25	0.1	0.66	**<0.0001**	0.08	0.6	−0.35	**0.04**

Bivariate correlation was performed using nonparametric (Spearman) or parametric (Pearson) tests. Boldface indicates statistically significant values.

in this third cohort. *TF* and *FTL* gene expression were reciprocally associated with hyperinsulinemic-euglycemic clamp *M* value in both SAT and VAT (Tables 2 and 3 and Fig. 1*B*). All these associations remained significant after controlling for sex.

Interestingly, in the high–FT level group (defined as higher than the median: 51 ng/mL) in cohort 3, SAT *SLC40A1* gene expression was significantly increased (0.16 ± 0.08 vs. 0.11 ± 0.06 R.U., $P = 0.04$), whereas VAT *TFRC* were decreased (0.017 ± 0.01 vs. 0.037 ± 0.03 R.U., $P = 0.03$).

TF and FT Protein Levels in Human AT

In a subgroup of 37 (5 nonobese and 32 obese) consecutive participants from cohort 1, TF and FT levels were measured by ELISA in AT. Both SAT and VAT TF and FT protein levels were strongly correlated with SAT and VAT *TF* and *FTL* gene expression, respectively (for TF, $r = 0.65$, $P = 0.03$, and $n = 11$ in SAT and $r = 0.60$, $P = 0.01$, and $n = 17$ in VAT; for FT, $r = 0.76$, $P = 0.006$, and $n = 11$ in SAT and $r = 0.65$, $P = 0.007$, and $n = 17$ in VAT). Surprisingly, SAT and VAT FT protein levels were not associated with *FTH1* gene expression ($r = -0.01$, $P = 0.9$, and $n = 11$ in SAT and $r = 0.15$, $P = 0.4$, and $n = 17$ in VAT). Of note, in both SAT and VAT, TF levels were significantly reduced in obese participants (7.57 ± 0.33 vs. 8.19 ± 0.25 ng/mg of SAT, $P = 0.001$, and 7.89 ± 0.34 vs. 8.58 ± 0.18 ng/mg of VAT, $P < 0.0001$), whereas FT levels were significantly increased (3.35 ± 1.71 vs. 2.05 ± 0.23 ng/mg of SAT, $P = 0.004$, and 7.29 ± 4.17 vs. 2.50 ± 0.64 ng/mg of VAT, $P < 0.0001$). SAT and VAT TF and FT protein levels were reciprocally correlated with BMI ($r = -0.46$, $P = 0.004$, and $r = 0.43$, $P = 0.009$, respectively). In addition, TF protein levels were positively associated with lipogenic (*FASN* [$r = 0.35$, $P = 0.04$] and *ACC1* [$r = 0.47$, $P = 0.005$]) and insulin-related (*IRS1* [$r = 0.34$, $P = 0.06$]) gene expression, with ACC1 protein levels ($r = 0.73$, $P = 0.01$, and $n = 10$) in VAT, whereas in this fat depot, FT levels were inversely associated with *IRS1* gene expression ($r = -0.41$, $P = 0.01$). In SAT, TF levels tended to be correlated with *FASN* ($r = 0.34$, $P = 0.06$) and *IRS1* ($r = 0.32$, $P = 0.08$) gene expression. Interestingly, circulating TF concentration was significantly correlated with SAT *TF* gene expression ($r = 0.56$, $P = 0.03$, and $n = 14$).

Effects of Fat Mass Reduction (Bariatric Surgery–Induced Weight Loss)

Next, we hypothesized that bariatric surgery–induced weight loss in obese subjects would lead to release pressure on the mechanisms promoting AT expansion and improve insulin sensitivity and that these changes would have a reflection in iron-related gene expression in AT. Accordingly, in cohort 4, bariatric surgery–induced weight loss led to increased AT *TF* and decreased *FTL, FTH1, SLC40A1*, and *TFR* transcripts mirroring improved AT function and insulin action, increasing gene expression of adipogenic genes (typically downregulated in insulin resistant states), and decreasing

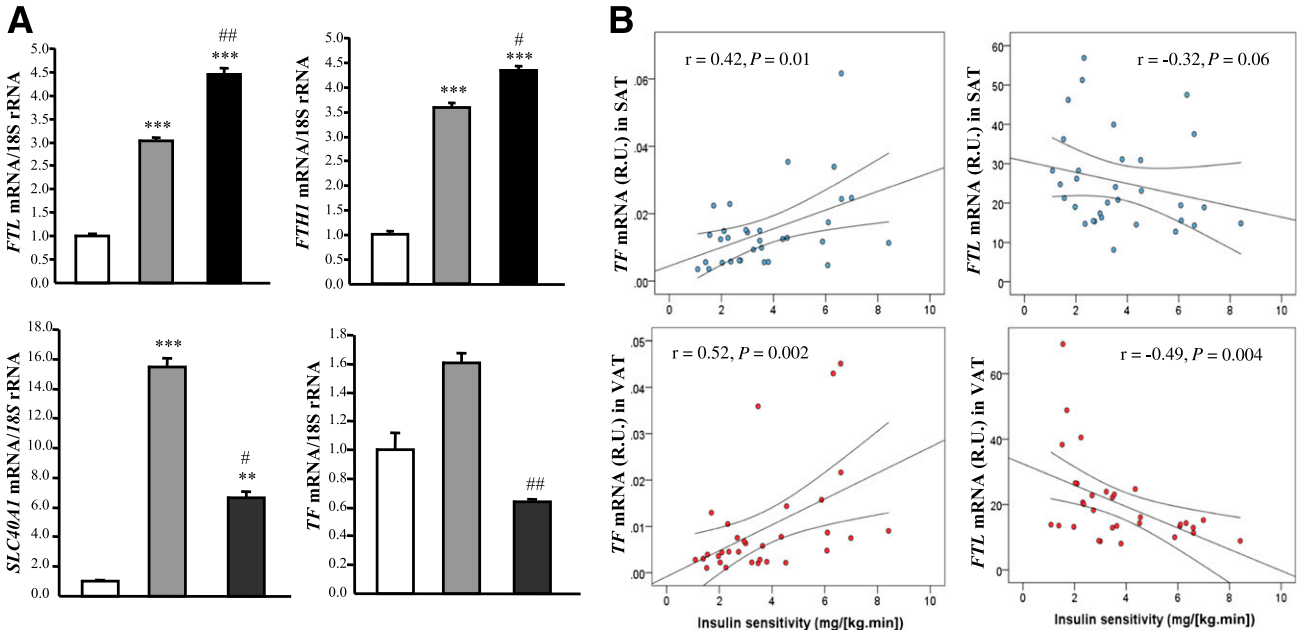

Figure 1—*A*: *FTL, FTH, SLC40A1*, and *TF* gene expression in VAT as regards obesity and T2D in cohort 2. These data are expressed as mean ± SEM. White bars, lean participants; gray bars, normal glucose tolerant obese participants; black bars, impaired glucose tolerant and T2D obese participants. $^{**}P < 0.005$ compared with lean subjects. $^{***}P < 0.001$ compared with obese subjects. $\#P < 0.05$ compared with obese subjects. $\#\#P < 0.005$ compared with obese subjects. *B*: Correlation between *TF* and *FTL* gene expression and insulin sensitivity (measured using euglycemic clamp) in SAT and VAT in cohort 3.

inflammatory genes (Supplementary Table 2). In addition, the percent change in weight loss positively correlated with *ADIPOQ* ($r = 0.56$, $P = 0.003$) and *TF* ($r = 0.45$, $P = 0.02$) and negatively with *LEP* ($r = -0.64$, $P = 0.001$), *TNFα* ($r = -0.46$, $P = 0.02$), *FTL* ($r = -0.42$, $P = 0.03$), *FTH1* ($r = -0.42$, $P = 0.03$), *TFRC* ($r = -0.62$, $P = 0.001$), and *CYBA* ($r = -0.59$, $P = 0.002$) gene expression. Of note, the percent of change of *TF* was significantly correlated with the percent change of *ADIPOQ* ($r = 0.45$, $P = 0.04$). The percent change of serum FT or TF did not correlate with the change of AT *TF, FTL, FTH1, SLC40A1*, or *TFRC* gene expression. It is well-known that bariatric surgery, especially Roux-en-Y gastric bypass, leads to iron deficiency that could interfere with the changes induced in gene expression after weight loss. However, we found no significant differences in serum FT 2 years after bariatric surgery (median 20.5 [interquartile range 8.25–52.5] vs. 37 [16.7–101.2] ng/mL, $P = 0.3$). In fact, the changes in the expression of iron-related genes in AT were linked to weight reduction independently of iron stores.

CONCLUSIONS

T2D patients are known to lose less weight after dieting than nondiabetic obese subjects (32,33). Thus, it is important to explore the factors that are linked to limited AT renewal and resistance to weight loss. Iron could be one of these factors.

According to current findings, iron seems to accumulate in human AT with increased body fatness and impaired insulin action. In three independent cohorts, *FTL* (a marker of intracellular iron accumulation) and *SLC40A1* (an iron export mediator) were raised in obese and insulin-resistant subjects, while *TF* gene expression, known to be directly associated with iron uptake, was decreased. Interestingly, SAT *SLC40A1* gene expression was significantly increased, whereas VAT *TFRC* was decreased in association with high circulating FT concentration. In agreement with these results in humans, Dongiovanni et al. (34) recently described in mice that an iron-enriched diet led to iron accumulation in VAT in parallel to impaired insulin action in this tissue.

Healthy AT expansion is defined as an enlargement of AT through effective recruitment of adipogenic precursor cells to the adipogenic program, along with an adequate angiogenic response and appropriate remodeling of the extracellular matrix (35–37). This "healthy" process is disturbed with obesity-associated metabolic disturbances (38–40). In this sense, the strong association between *TF* and adipogenic genes hints at TF as an important adipogenic component in association with AT expandability, possibly impacting on systemic glucose metabolism. On the other hand, the negative association of *FTL* and *SLC40A1* with adipogenic genes and their positive association with inflammatory and oxidative stress markers (*TNFα* and *CYBA*) pose iron accumulation as an important contributor to obesity-induced AT dysfunction and inflammation (38–40). Importantly, weight loss–induced improvement of insulin sensitivity led to parallel changes of iron-related gene expression. For instance, the increase of *TF* correlated positively with the increase of *ADIPOQ* gene expression. In agreement with these results, Tajima et al. (31) showed in mice that reduction of iron levels by deferoxamine, an iron chelator, inhibited the development of adipocyte hypertrophy through reduction of macrophage infiltration into fat tissue. These authors observed a parallel

reduction in oxidative stress and inflammatory cytokine production, leading to an improvement of glucose metabolism through improved insulin signaling in fat and skeletal muscle. The relationship described in our current study between FTL and CYBA (also named p22 [phox] or superoxide-generating NADPH oxidase light chain subunit) mRNA levels was previously reported by these authors in mice (31). It is well established that iron overload generates highly toxic hydroxyl radicals through Fenton chemistry (41). This oxidative damage might underlie the negative effects of iron overload on AT. Supporting this hypothesis, in a recent study iron and copper administration in standard diet–fed rats led to increased adipocyte hypertrophy, macrophage infiltration, and AT oxidative stress (42). Furthermore, supporting our findings, Gabrielsen et al. (23) showed in mice AT and 3T3-L1 cells that iron administration led to intracellular iron accumulation in adipocyte, decreasing *ADIPOQ* gene and protein expression and insulin action. These negative effects were reversed by iron restriction. This study also described functionally the positive role of SLC40A4 (as a cellular iron exporter) in AT, avoiding intracellular iron accumulation. Furthermore, they found that phlebotomy increases serum adiponectin levels in patients with high FT levels, suggesting an improvement in AT functionality (23).

Future studies should delineate how AT expression of these genes influence iron availability in the body. Until now, only tissue iron in the liver has been identified to be associated with systemic insulin action (43,44). AT iron should be evaluated in association with the expression of the different genes involved in iron metabolism. It is possible that this expression changes concomitantly with raised iron stores and impaired insulin action. In fact, we have found that AT FT, an indicator of AT iron, was positively associated with obesity and negatively with IRS1 mRNA levels. Further research is needed to explore this bidirectional cross-talk among insulin action, tissue iron, and the expression of iron-related genes.

To sum up, all these data emphasize that different markers of iron metabolism in AT are associated with insulin action. Once insulin action is modified through weight loss, these markers change concomitantly and in the expected direction. More studies on how iron overload impacts on AT functionality are required.

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