ORIGINAL ARTICLE

Circulating Retinol-Binding Protein-4 Concentration Might Reflect Insulin Resistance–Associated Iron Overload

José Manuel Fernández-Real, José María Moreno, and Wifredo Ricart

OBJECTIVES—The mechanisms behind the association between retinol-binding protein-4 (RBP4) and insulin resistance are not well understood. An interaction between iron and vitamin A status, of which RBP4 is a surrogate, has long been recognized. We hypothesized that iron-associated insulin resistance could be behind the impaired insulin action caused by RBP4.

RESEARCH DESIGN AND METHODS—Serum ferritin and RBP4 concentration and insulin resistance were evaluated in a sample of middle-aged men (n = 132) and in a replication independent study. Serum RBP4 was also studied before and after iron depletion in patients with type 2 diabetes. Finally, the effect of iron on RBP4 release was evaluated in vitro in adipose tissue.

RESULTS—A positive correlation between circulating RBP4 and log serum ferritin (r = 0.35 and r = 0.61, respectively; $P <$ 0.0001) was observed in both independent studies. Serum RBP4 concentration was higher in men than women in parallel to increased ferritin levels. On multiple regression analyses to predict serum RBP4, log serum ferritin contributed significantly to RBP4 variance after controlling for BMI, age, and homeostasis model assessment value. Serum RBP4 concentration decreased after iron depletion in type 2 diabetic patients (percent mean difference −13.7 [95% CI −25.4 to −2.04]; P = 0.024). The iron donor lactoferrin led to increased dose-dependent adipose tissue release of RBP4 (2.4-fold, P = 0.005) and increased RBP4 expression, while apotransferrin and deferoxamine led to decreased RBP4 release.

CONCLUSIONS—The relationship between circulating RBP4 and iron stores, both cross-sectional and after iron depletion, and in vitro findings suggest that iron could play a role in the RBP4–insulin resistance relationship. ***Diabetes*** **57:1918–1925, 2008**

Adipose tissue is increasingly viewed as an endocrine organ that secretes many types of adipokines (such as leptin, tumor necrosis factor-α, interleukin 6, and adiponectin) that modulate the action of insulin in other tissues. Retinol-binding protein-4 (RBP4), a new fat-derived adipokine that specifically binds to retinol, has recently been reported to provide a link between obesity and insulin resistance (1,2). Circulating RBP4 levels and adipose tissue RBP4 expression were raised in several different mouse models of obesity and insulin resistance. In these animal models, increasing the circulating levels of RBP4 leads to glucose intolerance, augmented hepatic gluconeogenesis, and attenuated insulin signaling in skeletal muscle, whereas knock-out of the RBP4 gene increases insulin sensitivity.

In humans, different authors have reported increased serum RBP4 concentration in subjects with obesity, insulin resistance, or type 2 diabetes compared with lean subjects (2–6), although not all studies are concordant. At least two recent studies (7,8) did not observe a relationship between RBP4 and insulin resistance in women. Although some problems exist with serum RBP4 measurements (9), RBP4 mRNA was in fact downregulated in subcutaneous abdominal adipose tissue in postmenopausal women (7). Furthermore, the authors did not see a relationship between adipose tissue RBP4 expression and serum RBP4 levels (7).

The mechanisms by which RBP4 induces insulin resistance are not well understood. Treatment of mice with fenretinide (which facilitates the excretion of RBP4 into urine) decreased insulin resistance induced by a high-fat diet (1). Sex and fasting plasma glucose levels seem to be independent determinants of plasma RBP4 concentration. Many adipokines have been found to be sexually dimorphic. Both leptin and adiponectin are increased in serum of women compared with men. This observation has been explained on the basis of different fat amounts and the influences of sex hormones (10,11). Plasma RBP4 concentrations, however, exhibit an opposite pattern. The median (range) for RBP4 in plasma was 21.0 μg/ml (10.7–48.5) for men and 18.1 μg/ml (9.3–34.6) for women (P = 0.001) (3). Since data regarding menopausal status were not available, the authors arbitrarily subdivided sex groups at 50 years of age. Plasma RBP4 levels in women aged >50 years were found to be significantly higher than those in women aged <50 years. However, no such age-associated difference in RBP4 plasma levels was observed in men. Thus, the pattern of serum RBP4 concentrations in serum resembles that of iron stores: higher in men than women, in whom iron stores increase after menopause. Furthermore, treatment with fenretinide induces a dose-dependent peripheral anemia evidenced by erythrocytopenia and decreased hemoglobin concentration and packed cell volume in addition to excretion of RBP4 into urine (12).

On the other hand, RBP4 is an indicator of vitamin A (retinol) intake (13,14). The interaction between vitamin A and iron status is well known. For instance, vitamin A deficiency may impair iron metabolism and aggravate

From the Diabetes, Endocrinology, and Nutrition Unit, Dr. Josep Trueta Hospital, Girona Institute for Biomedical Research, and CIBEROBN Fisiopatología de la Obesidad y Nutrición, Girona, Spain.
Corresponding author: José Manuel Fernández-Real, uden.jmfernandezreal@htrueta.scs.es.
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anemia (15–17). In fact, iron deficiency anemia and vitamin A deficiency often coexist (15–17).

In the last years, increased iron intake and raised iron stores have been recognized as significant, independent contributors to insulin resistance in the general population and in patients with type 2 diabetes (rev. in 18,19). On the other hand, iron supplementation significantly increased plasma retinol and RBP4 (20). Given the interactions between vitamin A and iron, we hypothesized that raised iron stores could be behind the association between increased serum RBP4 concentration and insulin resistance. We found that serum ferritin concentration was positively associated with serum RBP4 concentration in two independent studies. Given these cross-sectional observations, we further aimed to explore these interactions in vitro and to evaluate serum RBP4 concentration after iron depletion in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Study of RBP4 in apparently healthy men. We studied 132 nondiabetic men who were consecutively enrolled in a prospective study of cardiovascular risk factors (21). All subjects reported that their body weight had been stable for at least 3 months before the study. A food-frequency questionnaire was obtained from all subjects. None of the subjects were taking any medication or had any evidence of metabolic disease other than obesity. Inclusion criteria were *1*) BMI <40 kg/m^2, *2*) absence of any systemic disease, and *3*) absence of clinical symptoms and signs of infection in the previous month by structured questionnaire to the patient. Informed consent was obtained from all subjects. The hospital ethics committee approved the study.

BMI was calculated as weight (in kilograms) divided by square of height (in meters). The subjects' waist was measured with a soft tape midway between the lowest rib and the iliac crest. The hip circumference was measured at the widest part of the gluteus region. The waist-to-hip ratio was then calculated. Blood pressure was measured in the supine position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used, and the first and fifth phases were recorded. Values used in the analysis are the average of three readings taken at 5-min intervals. Patients were requested to withhold from alcohol and caffeine during at least 12 h before the different tests. All subjects had fasting plasma glucose <7.0 mmol/l. Type 2 diabetes was ruled out by an oral glucose tolerance test, according to criteria from the American Diabetes Association.

Replication study. All subjects, recruited among hospital and lab staff, were of Caucasian origin and reported that their body weight had been stable for at least 3 months before the study. They had no systemic disease and all were free of any infections in the previous month before the study. Liver and renal diseases were specifically excluded by biochemical work-up.

Study of RBP4 release in adipose tissue explants. To gain further insight of the RBP4-iron relationship, we hypothesized that iron donors (holotransferrin and lactoferrin) and iron buffers (apotransferrin and deferoxamine) could influence RBP4 release from adipose tissue. To this end, adipose tissue was obtained from 14 obese subjects (10 visceral and 4 subcutaneous fat depots) undergoing open abdominal surgery (gastrointestinal bypass) under general anesthesia after an overnight fast. The mean age was 45 years (range 41–55) and the BMI 44.9 ± 12.4 kg/m^2. Medical histories, physical examinations, electrocardiogram, and blood screening showed that all patients were in good health. None of the subjects had a history of hepatic or renal disorders. The study had the approval of the ethical committee, and all patients gave informed written consent.

Samples of adipose tissue were immediately transported to the laboratory (5–10 min). The handling of tissue was carried out under strictly aseptic conditions. The tissue was cut with scissors into small pieces (5–10 mg) and incubated in buffer plus albumin (3 ml/g of tissue) for ~5–30 min. After incubation, the tissue explants were centrifuged for 30 s at 400*g*. Then 100 mg of minced tissue was placed into 1 ml M199 (Life Technologies, Invitrogen) containing 10% fetal bovine serum (Hyclone, Thermo Fisher Scientifi), 100 unit/ml penicillin (Life Technologies, Invitrogen), and 100 μg/ml streptomycin (Life Technologies, Invitrogen) and incubated for 48 h in suspension culture under aseptic conditions (18,19).

We performed the follow treatments: vehicle, 10 μmol/l human transferrin (T0665; Sigma-Aldrich), 10 μmol/l human apolipoprotein-transferrin (T1147; Sigma-Aldrich), human lactoferrin (L4894; Sigma-Aldrich), and 10 μmol/l metformin (D5035; Sigma-Aldrich). The effects of 20 μmol/l deferoxamine mesylate (D9533; Sigma-Aldrich) were also tested in six explants. In independent experiments, we evaluated the dose-dependent effects of lactoferrin (1, 10, and 100 μmol/l) and the time course using 10 μmol/l of lactoferrin at times 0, 1, 3, 6, 12, 24, and 48 h. The supernatants were collected, aliquoted, and stored at −80°C until assay. Circulating RBP4 in supernatants was analyzed by competitive enzyme-linked immunosorbent assay (AdipoGen, Seoul, Korea) according to the manufacturer's instructions. Sensitivity of the method was 1 ng/ml. The intra- and interassay coefficients of variation were between 2 and 10%. Circulating adiponectin in supernatants was analyzed by enzyme-linked immunosorbent assay (Linco Research, St. Charles, MO), according to the manufacturer's instructions. Sensitivity of the method was 0.78 ng/ml. The intra- and interassay coefficients of variation were between 2 and 10%. To evaluate cell integrity, lactate deshidrogenase (LDH) activity was analyzed by a cytotoxicity detection kit (LDH) (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions.

RBP4 expression analysis in adipose tissue explants. Total RNA from adipose tissue was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen Science, Gaithersburg, MD) according to the manufacturer's protocol. Quantity and integrity of RNA were measured using the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), assuring that the values measuring integrity were >8.5 in all samples. First-strand cDNA was synthesized from 1 μg total RNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time TaqMan PCR was performed with commercial inventoried primers and 6-carboxyfluorescein dye–labeled TaqMan minor groove binder probes (Applied Biosystems) for RBP4 and cyclophilin A (used as endogenous control) and 2× Universal PCR Master Mix (Applied Biosystems). Each TaqMan reaction contained 4 μl of cDNA, corresponding to 100 ng of total RNA, in a total reaction volume of 25 μl. The relative expression was determined by the comparative threshold method, as described in the *ABI Prism 7700 User Bulletin* (P/N 4303859) from Applied Biosystems.

Study of changes in circulating RBP4 after iron depletion in type 2 diabetes. The study subjects (n = 13 male patients; [means ± SD] age 55.8 ± 8.4 years; BMI 29.1 ± 3.48 kg/m^2; A1C 6.1 ± 0.8%) are part of a well-characterized sample of high-ferritin type 2 diabetic patients subjected to blood letting and previously reported by us (22,23). In the original cohort, patients with elevated serum ferritin concentrations were randomized to either iron depletion (intervention group; n = 13) or to observation (n = 15) according to a randomization table that included age, BMI, and A1C. The two groups were also matched for pharmacological treatment and chronic diabetes complications. The iron depletion intervention consisted of three blood extractions (500 ml each) at 2-week intervals. The patients were studied at baseline and at 4 and 12 months after the last phlebotomy. In the present study, available serum samples were used for measuring RBP4 in 6 patients in the intervention group and 12 patients in the observation group at baseline and 12 months after iron depletion or follow-up. The clinical and biochemical characteristics of these subjects were comparable with those of the initial cohort (22). Subjects were studied in the postabsorptive state.

Study of insulin sensitivity. The experimental protocol started between 8:00 and 9:30 A.M. after an overnight fast. All of the subjects were on a weight-maintaining diet before the test. A bolus of human actrapid insulin (0.1 unit/kg; Novo Nordisk, Copenhagen, Denmark) was administered into an antecubital vein, and blood was sampled from a vein on the dorsum of the same hand. To arterialize the venous blood, the hand was placed on a hot box at a constant temperature of 40°C for 20 min before the start of the study and kept there until the end of the test. Sampling was carried out every minute until 15 min after the injection of insulin. Insulin sensitivity was indicated by the first-order rate constant for disappearance rate of glucose estimated from the slope of the regression line of the logarithm of blood glucose against time during the first 3–15 min.

Analytical methods. Serum glucose concentrations were measured in duplicate by the glucose oxidase method with the use of a Beckman Glucose Analyser II (Beckman Instruments, Brea, CA). The coefficient of variation was 1.9%. Total serum cholesterol was measured through the reaction of cholesterol esterase/cholesterol oxidase/peroxidase. HDL cholesterol was quantified after precipitation with polyethylene glycol at room temperature. Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase. Serum insulin levels during the frequently sampled intravenous glucose tolerance test were measured in duplicate by monoclonal immunoradiometric assay (IRMA; Medgenix Diagnostics, Fleunes, Belgium). Intra- and interassay coefficients of variation were <6%. Serum transferrin and ferritin were measured as previously reported (22). Whole-blood hemoglobin and hematocrit were determined by routine laboratory tests (Coulter Electronics, Hialeah, FL).

Serum RBP4 concentrations in the clinical study were measured by nephelometry (Dade Behring, Marburg, Germany). Sensitivity of the method is 0.01 mg/dl. The intra- and interassay coefficients of variation were 3.1% and 2.2%, respectively. The change in serum RBP4 after iron depletion was

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TABLE 1
Clinical and laboratory variables of study subjects according to serum RBP4 quartiles

	RBP4 quartiles				ANOVA P value
	Q1	Q2	Q3	Q4	
n	33	33	33	33	
RBP4 (mg/dl) (95% CI for mean)	2.44–2.79	3.40–3.54	3.92–4.06	4.85–5.39	0.00001
Age (years)	51.5 ± 12.4	50 ± 9.4	52 ± 10.8	49.6 ± 10.9	0.8
BMI (kg/m^2)	27.2 ± 3.4	28.2 ± 4	27.3 ± 3.5	27.8 ± 2.9	0.7
Waist-to-hip ratio	0.93 ± 0.07	0.94 ± 0.06	0.93 ± 0.06	0.93 ± 0.06	0.9
Systolic blood pressure (mmHg)	129.7 ± 12.7	125.4 ± 15	122.7 ± 17.2	128.4 ± 15	0.3
Diastolic blood pressure (mmHg)	82.3 ± 8.1	81.1 ± 9.7	77.8 ± 13.4	81.8 ± 10.9	0.4
Fasting glucose (mg/dl)	97 ± 12	99.1 ± 12.1	97 ± 8	97.7 ± 11.5	0.9
Fasting insulin (mU/l)	7.17 ± 3.47	7.54 ± 3.31	9.34 ± 5.07	9.34 ± 5.84	0.4
HOMA	1.62 ± 0.81	1.67 ± 0.73	2.2 ± 1.3*	2.32 ± 1.6*	0.2
Cholesterol (mg/dl)	200.7 ± 38.3	210.8 ± 51.7	207.2 ± 35.7	210 ± 29.8	0.8
HDL cholesterol (mg/dl)	49.6 ± 8.8	50.4 ± 13.9	50.3 ± 11.1	53.3 ± 11.5	0.6
Log fasting triglycerides	1.94 ± 0.19	2.03 ± 0.25	1.92 ± 0.2	1.93 ± 0.25	0.3
Log serum ferritin	1.95 ± 0.43	2.03 ± 0.38	2.09 ± 0.31†	2.21 ± 0.37†‡	0.10
Transferrin (mg/dl)	238.3 ± 40	266.7 ± 43	256 ± 47.4	272.8 ± 34.1§	0.07

Data are means ± SD, unless otherwise indicated. *P = 0.026 when both upper quartiles were compared with the remaining subjects. †P = 0.03 when both upper quartiles were compared with the remaining subjects. ‡P = 0.03 when compared with remaining subjects. §P = 0.04 when compared with remaining subjects.

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analyzed using the same enzyme-linked immunosorbent assay described above in the in vitro study.

Statistical methods. Descriptive results of continuous variables are expressed as means ± SD. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test, and variables were given a log transformation if necessary. These parameters (RBP4, homeostasis model assessment [HOMA], and triglycerides) were analyzed on a log scale and tested for significance on that scale. The anti–log-transformed values of the means are reported in the Tables. The study also had a 67% power to detect significant differences of at least 1 SD in serum RBP4 concentration between obese and nonobese subjects. The relationships between variables were tested using Pearson's test and multiple linear regression analysis (stepwise). We used χ^2 test for comparisons of proportions and unpaired or paired t tests for comparisons of quantitative variables. The analyses were performed using the program SPSS (version 11.0).

RESULTS

RBP4 and serum ferritin in apparently healthy subjects. Main characteristics of study subjects classified according to RBP4 quartiles are shown on Table 1. HOMA value was significantly associated with log serum ferritin (r = 0.22, P = 0.01) but not with serum RBP4 concentration (r = 0.07, P = 0.4). This could be due to the narrow range of HOMA index in this population. However, when lean subjects (BMI <25 kg/m^2, n = 50) were excluded from the analyses, the HOMA value was significantly associated with both log serum ferritin (r = 0.32, P = 0.003) and serum RBP4 (r = 0.23, P = 0.04) (n = 82). This suggests a threshold effect (i.e., that adipose tissue mass needs to exceed a certain threshold for RBP4 release to associate with HOMA). Accordingly, we explored HOMA value and log serum ferritin according to RBP4 quartiles. Subjects with serum RBP4 concentration in the two upper quartiles showed significantly higher HOMA index in parallel to log serum ferritin concentrations (Fig. 1). In addition, those subjects in the highest quartile also had significantly higher serum transferrin (Table 1). A significant and positive correlation between circulating RBP4 and log serum ferritin (r = 0.35, P < 0.0001) was observed (Fig. 2). On multiple regression analyses to predict serum RBP4, log serum ferritin independently contributed to 5% of RBP4 variance (β = 0.22, P = 0.02) after controlling for BMI (P = 0.3), age (P = 0.3), and HOMA value (P = 0.7).

Replication study. In this independent replication study, we evaluated 20 men and 13 women similar in age (34.7 ± 4 vs. 33.7 ± 4.8 years, P = 0.5), BMI (26.5 ± 3.4 vs. 26.4 ± 5.5 kg/m^2, P = 0.9), fasting glucose (5.36 ± 0.83 vs. 5.26 ± 0.96 mmol/l, P = 0.7), and fasting insulin (8.4 ± 4.9 vs. 8.6 ± 4.5 mU/l, P = 0.9). Log serum ferritin correlated positively with serum RBP4 concentration (r = 0.61, P < 0.0001). Serum RBP4 concentration was higher in men than women (P = 0.001) in parallel to increased ferritin levels (P < 0.0001) (Fig. 3).

Study of changes in circulating RBP4 after iron depletion. The two groups of subjects (all men) were similar in age, glycated hemoglobin, and baseline serum ferritin concentrations. Serum RBP4 concentration tended to increase over time in type 2 diabetic patients at follow-up (no intervention) (mean +6.8 ± 3%), while RBP4 decreased after iron depletion (mean −6.8 ± 5% [percent mean differences and 95% CI for the difference: −13.7 {−25.4 to −2.04}], P = 0.024) (Fig. 4).

Insulin sensitivity was essentially unchanged (1.95 ± 0.86 vs. 1.98 ± 0.79 mg · dl^{-1} · min^{-1}, P = 0.9) in the control group, while this parameter tended to increase after iron depletion (1.03 ± 0.65 vs. 2.46 ± 0.8 mg · dl^{-1} · min^{-1}, P = 0.06). Interestingly, the lower the serum RBP4 concentration at follow-up the higher the increase in insulin sensitivity (r = −0.83, P = 0.02).

Study of RBP4 release in adipose tissue explants. RBP4 was spontaneously secreted by adipose tissue explants (median [interquartile range] 216.9 ng/ml [63.1–427]). After metformin treatment, RBP4 did not change significantly (191.4 ng/ml [61.4–508]). Lactoferrin (10 μmol/l) led to significantly increased (2.4-fold) RBP4 release into the media (450 ng/ml [177.2–809.9], P = 0.005) (Fig. 5*A*). After 48 h of incubation, lactoferrin treatment led to a 1.32-fold higher RBP4 expression (P = 0.01) (Fig. 5*B*). There were no significant differences between fat depots. Lactoferrin increased RBP4 release dose dependently (Fig. 6*A*). In the time-course experiments, the increase in expression and secretion of RBP4 was observed in the first hour after lactoferrin treatment and

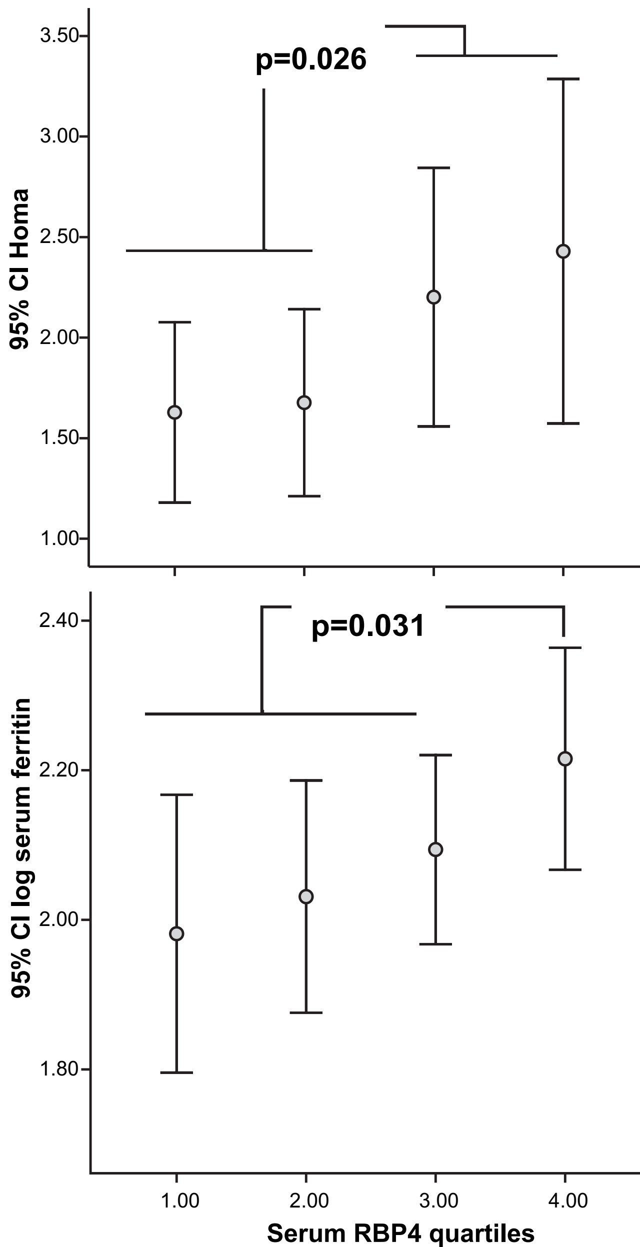

FIG. 1. Ninety-five percent CI (95% CI) for the mean of the HOMA index and log serum ferritin according to RBP4 quartiles.

remained essentially unchanged throughout the experiments (Fig. 6*B*).

Incubation with human apolipoprotein–transferrin (10 μmol/l) led to decreased RBP4 concentration in the media in comparison with vehicle (169.2 ng/ml [interquartile range 54.5–351], $P = 0.04$) (Fig. 5*A*). Human transferrin (10 μmol/l) treatment did not lead to significant changes in RBP4 concentration (227.82 ng/ml [62.7–449.3]) (Fig. 5*A*). Incubation with deferoxamine (20 μmol/l) led to significantly decreased RBP4 release into the media (139 ng/ml [130.9–154.7] vs. 168 ng/ml [141.4–220], deferoxamine versus vehicle, respectively; $P = 0.02$) (Fig. 5*C*). RBP4 expression after deferoxamine treatment was decreased by 30% ($P = 0.03$) (Fig. 5*D*).

Adiponectin concentration in the supernatants (112.9 ng/ml [interquartile range 85.8–126.9]) did not change significantly after the different treatments were evaluated. LDH activity was similar after all treatments tested (0.39

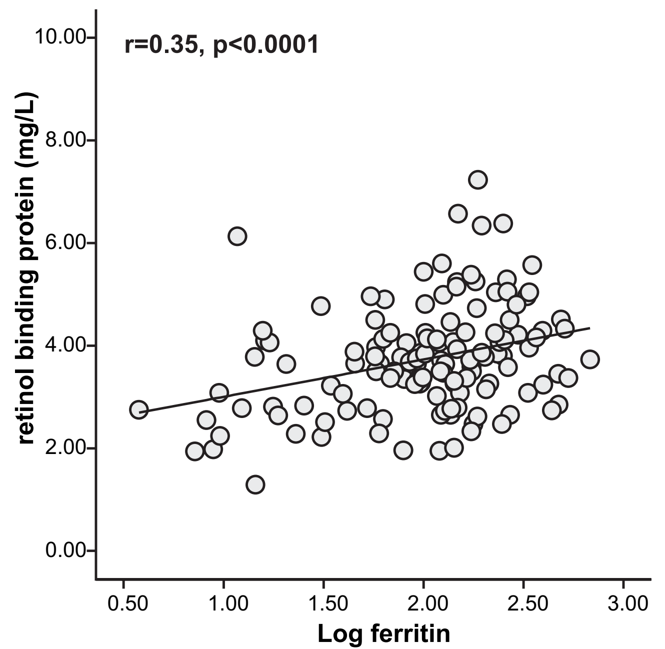

FIG. 2. Linear relationship between serum RBP4 and log serum ferritin.

[0.33–0.48] units of absorbance at 492 nm) and did not change significantly during the time-course experiments (Fig. 6*C*).

DISCUSSION

The lipocalins (such as RBP4) are a large, diverse, but relatively poorly understood family of small extracellular proteins that are characterized by the ability to bind small hydrophobic molecules, such as retinol (RBP4), and by their binding to specific cell surface receptors. These general properties suggest that such proteins are appropriate transporters for transferring biologically hazardous molecules in a safe and controlled manner between cells.

In this study, we report that circulating RBP4 concentration was associated with iron stores in middle-aged men

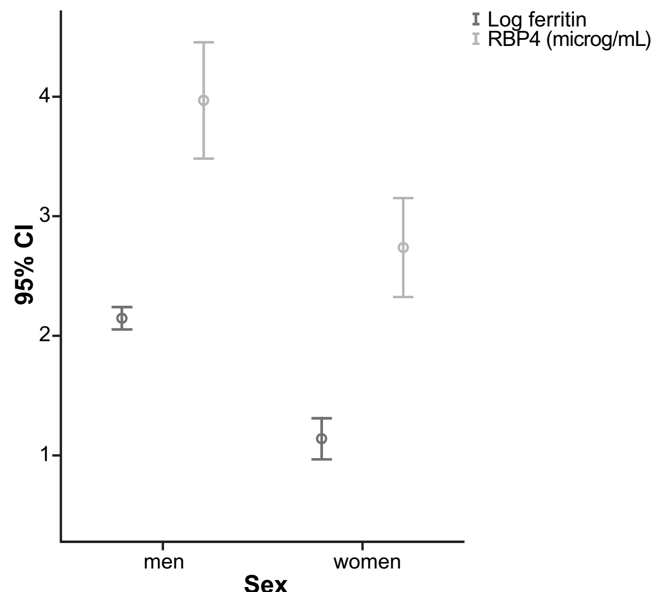

FIG. 3. Ninety-five percent CI (95% CI) for the mean of serum RBP4 concentration and log serum ferritin according to sex.

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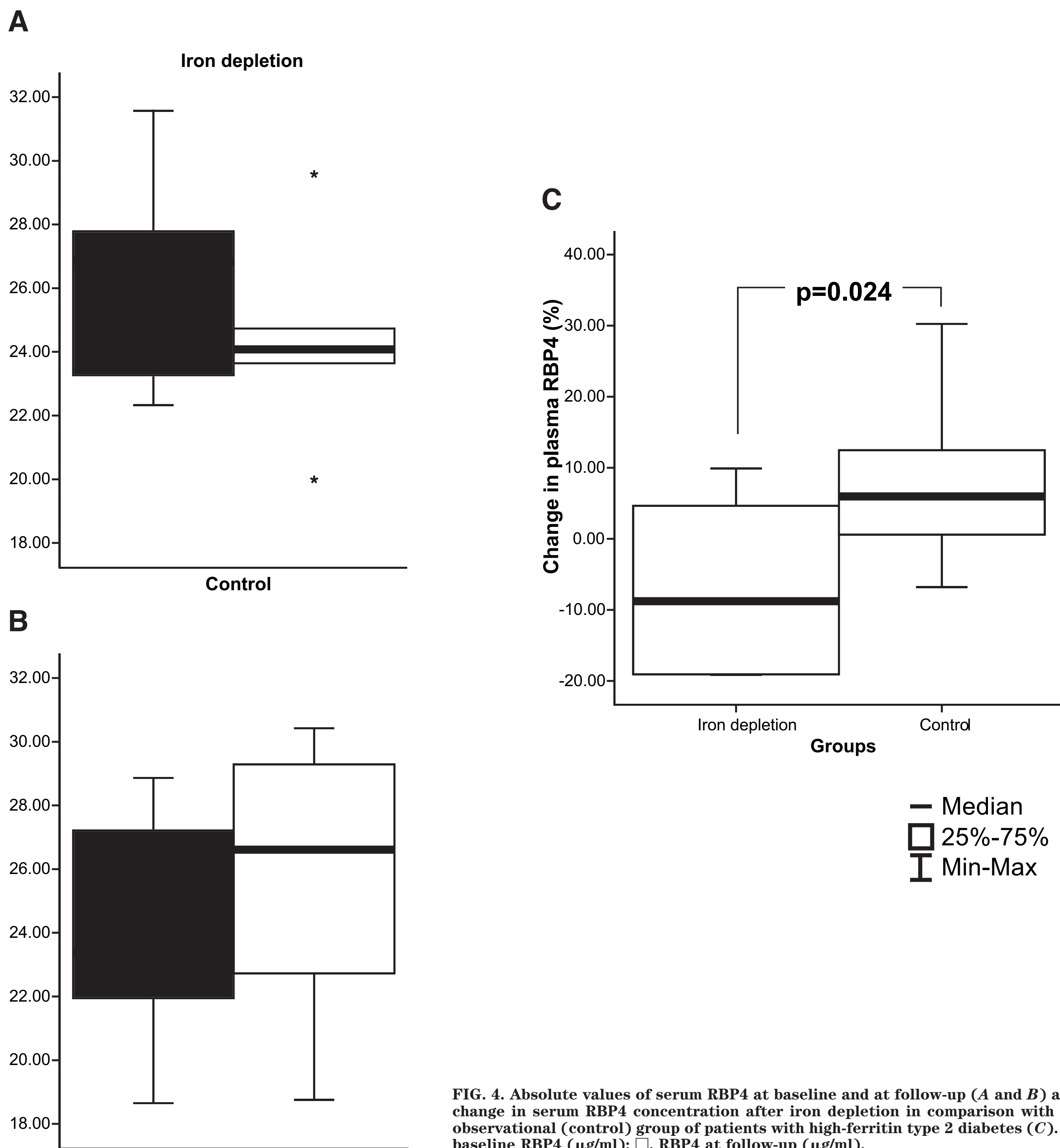

FIG. 4. Absolute values of serum RBP4 at baseline and at follow-up (*A* and *B*) and change in serum RBP4 concentration after iron depletion in comparison with an observational (control) group of patients with high-ferritin type 2 diabetes (*C*). ■, baseline RBP4 (μg/ml); □, RBP4 at follow-up (μg/ml).

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and in an independent sample of men and women. Contrary to other adipokines (10,11), serum RBP4 concentration was higher in men than women (as previously described [3]). These findings were in parallel to increased serum ferritin of men. Iron depletion led to significantly decreased serum RBP4 concentration in comparison with the observational group. Interestingly, the lower the RBP4 concentration, the higher the change in insulin sensitivity after iron depletion. These changes resembled those induced by fenretide. In addition to increased urinary excretion of RBP4, fenretide also leads to decreased total hemoglobin and anemia (13), hinting at iron-induced depletion as another potential mechanism through which fenretide could improve insulin sensitivity. Furthermore, lactoferrin, a well-known iron transport protein and iron donor (24), led to significant RBP4 release in a dose-dependent manner and to increased RBP4 expression in adipose tissue explants. Our findings imply that iron supplementation leads to enhanced RBP4 release from cells due to enhanced transcription. We also performed a study of the time-course effects of lactoferrin, together with appropriate controls for cell integrity (LDH release) (Fig. 6) and specificity (adiponectin). Consistent with these observations, RBP4 was significantly associated

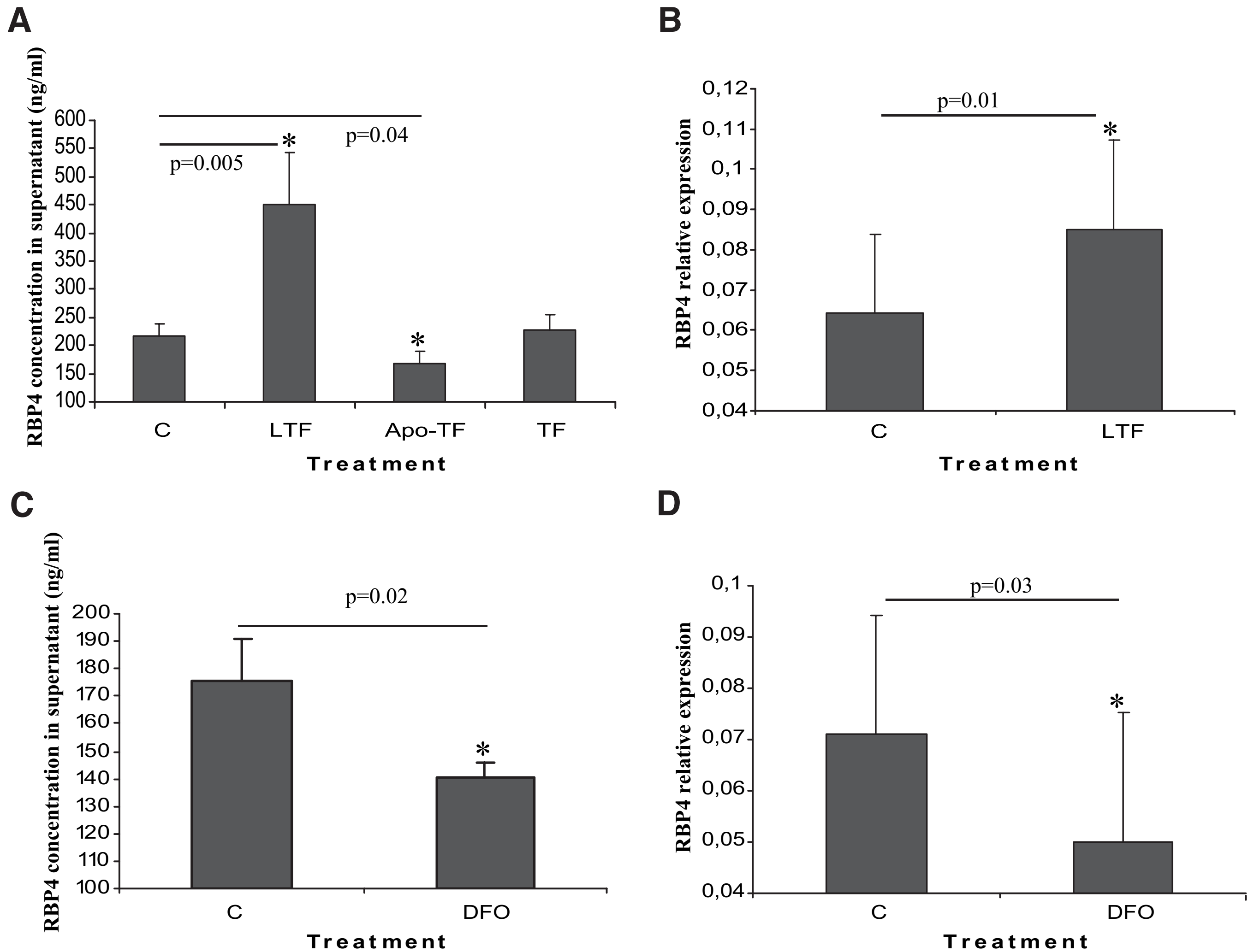

FIG. 5. ***A*: Effects of lactoferrin (LTF), apolipoprotein-transferrin (Apo-TF), and holotransferrin (TF) on RBP4 release from adipose tissue explants compared with vehicle (C). *B*: Effects of lactoferrin (10 μmol/l) on RBP4 expression. Effects of deferoxamine (20 μmol/l) (DFO) on RBP4 release (*C*) and RBP4 expression in adipose tissue explants (*D*).**

with circulating lactoferrin in obese subjects (r = 0.38, P = 0.004, n = 56; data not shown). Apotransferrin (a well-known iron buffer) led to significant decrease in RBP4 release. It could be argued that apotransferrin does not bind to transferrin receptors but would bind extracellular free iron, forming holo-transferrin, which would then be capable of binding to the transferrin receptor and internalize and actually increase cellular iron stores. However, holo-transferrin did not show significant effects on RBP4 release. Furthermore, deferoxamine (another iron buffer) also led to significantly decreased RBP4 release and expression.

Our findings imply that iron stores could independently affect RBP4 levels and insulin sensitivity. However, we cannot exclude that iron-dependent insulin resistance is influenced by concomitant changes in RBP4. To our knowledge, no study has previously suggested this association.

The lack of effect of holo-transferrin was relatively unexpected. However, the stage-specific capture of different iron transporters is well recognized (25). Iron delivery to cells is not only mediated by different proteins but also has different functions at different cellular stages and is dependent on cell type and cell stage (25). Transferrin transfers iron from circulation, while lactoferrin has a very acidic pH sensitivity and traffics late endosomes into a clearly different intracelluar route (26).

RBP4 has been used clinically as a rapid turnover protein for assessing the short-term fluctuation of nutritional states. RBP4 is the only specific transport protein for retinol (vitamin A) in circulation (27). It is produced by hepatocytes, which are believed to contribute to a large part of circulating RBP4, although adipocytes have the second-highest expression level (28). Elevated RBP4 levels have been recently reported in subjects with insulin resistance and type 2 diabetes (2–8). Serum ferritin is also known to be increased in insulin resistance and type 2 diabetes (18,19).

A positive relationship between ferritin and RBP and alterations of RBP in diabetes have been described in earlier studies (15,20,29,30). An interaction between iron and vitamin A intake has also been well recognized in the past (15–17). Rosales et al. (31) showed that the distribution of retinol through various body compartments is dependent on iron status, with higher concentrations of vitamin A in the livers of food-restricted control rats than in those of iron-deficient rats. These authors also demonstrated decreased plasma RBP concentration in iron-deficient rats (31). Iron deficiency lowers serum retinol,

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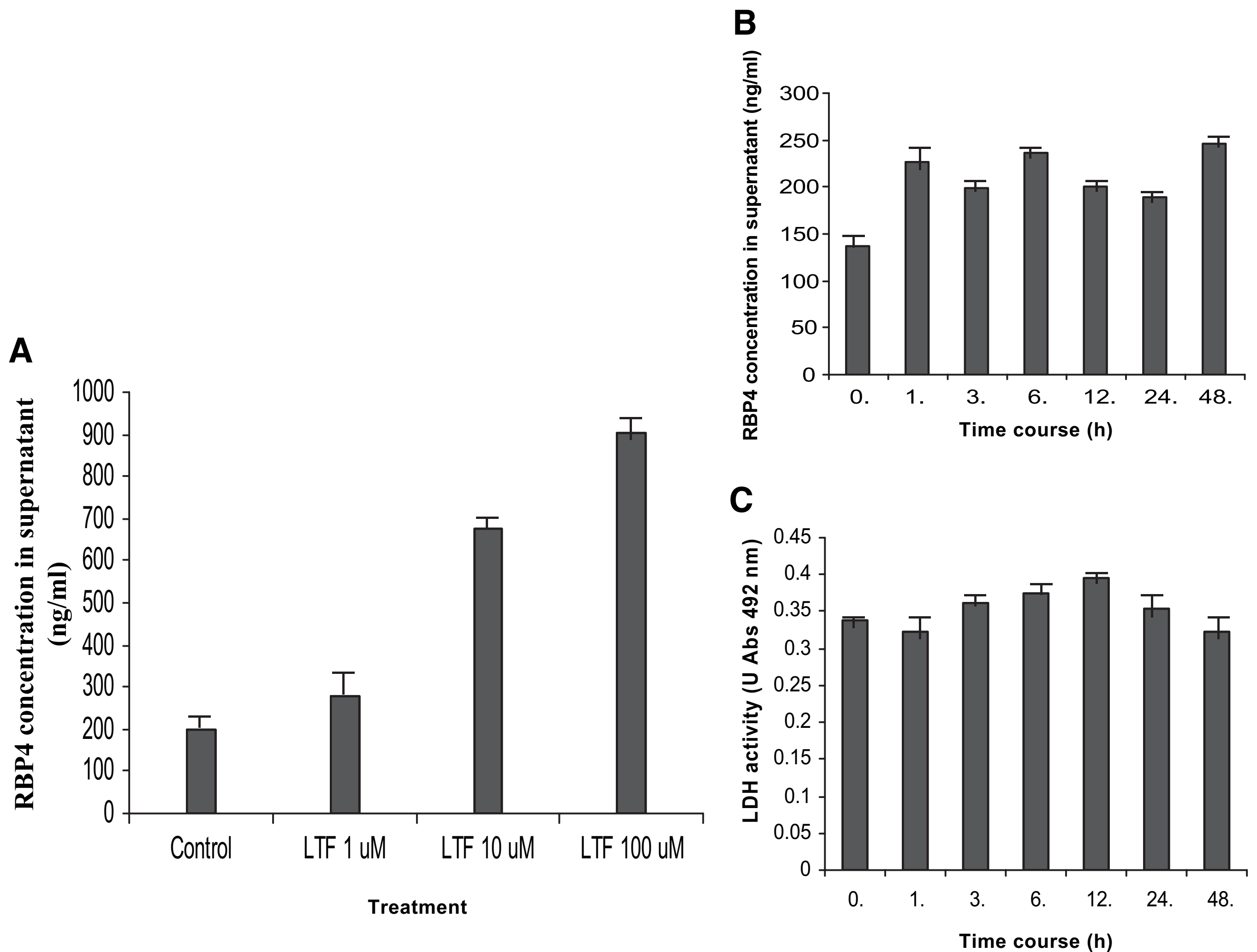

FIG. 6. a) Effects of lactoferrin on RBP4 secretion from adipose tissue explants in a dose-dependent manner (*A*) and effects of lactoferrin (10 μmol/l) during time course experiments (*B*). *C*: LDH activity during these latter experiments. The results show that an iron donor (lactoferrin) led to significant RBP4 release dose and time dependently.

and vitamin A accumulates in the liver as retinyl esters, probably due to impaired activity of hepatic acid retinyl ester hydrolase, an iron-dependent enzyme (31). Consistent with the data from these animal studies, long-term supplementation with iron caused large increases in serum retinol in children (20). Supplements containing iron alone increased plasma retinol in iron-deficient children (plasma ferritin <12 μg/l) (20). It has been suggested that low plasma retinol concentrations were in fact caused by iron deficiency rather than vitamin A deficiency (20). Deficiency and supplementation of iron have different effects on the complicated intrahepatic balance between cellular uptake and mobilization of vitamin A and, hence, RBP4 (31–33).

On the other hand, vitamin A supplementation improves indicators of iron nutritional status, such as serum iron, transferrin, transferrin saturation, hematocrit, and hemoglobin, suggesting that vitamin A affects iron metabolism (34–36). In children, plasma hemoglobin and ferritin concentrations were correlated with plasma retinol and RBP (35).

Frequent blood donations, leading to decreasing iron stores, have been demonstrated to constitute a protective factor for the development of diabetes (37). Iron stores also appear to predict an increased incidence of type 2 diabetes (38). We previously described a statistically significant increase in insulin sensitivity after iron depletion (from 2.30 ± 1.81 to 3.08 ± 2.55 mg $\cdot$ dl^{-1} $\cdot$ min^{-1} at 4 months to 3.16 ± 1.85 mg $\cdot$ dl^{-1} $\cdot$ min^{-1} at 12 months; $P =$ 0,045) in contrast to patients subjected to observation in whom insulin sensitivity did not significantly change (from 3.24 ± 1.9 to 3.26 ± 2.05 mg $\cdot$ dl^{-1} $\cdot$ min^{-1} at 4 months to 2.31 ± 1.35 mg $\cdot$ dl^{-1} $\cdot$ min^{-1} at 12 months) (22). The mechanisms by which iron depletion leads to improved insulin sensitivity are not well understood. Decreased oxidative stress, transition metal–catalyzed reactions, and reactive oxygen species have been hypothesized as potentially involved. In addition, decreased RBP4 could also play a role.

According to the findings described in the present article and those present in the literature, a scenario can be envisioned in which the intake of vitamin A and iron lead to reciprocal changes in nutritional indicators of their intake (RBP4 and ferritin). Given the well-known interaction between iron and vitamin A status, increased RBP4 concentration could be a marker of insulin resistance–associated iron overload (18,19).

In summary, the relationship between circulating RBP4, RBP4 release after iron modulation in adipose tissue explants, and decreased serum RBP4 after iron depletion

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suggest that iron could play a role in the RBP4–insulin resistance relationship.

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