Circulating Visfatin Is Associated With Parameters of Iron Metabolism in Subjects With Altered Glucose Tolerance

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OBJECTIVE — Visfatin is a novel adipokine that is predominantly secreted by visceral adipose tissue. Accumulation of visceral adipose tissue is also associated with iron metabolism. Despite the coincidence of visfatin expression in iron-rich tissues, no study has investigated the possible interaction of visfatin with parameters of iron metabolism.

RESEARCH DESIGN AND METHODS — We evaluated insulin sensitivity and parameters of iron metabolism in 95 men with normal glucose tolerance (NGT) and 43 men with altered glucose tolerance.

RESULTS — Men with newly diagnosed type 2 diabetes had significantly increased serum visfatin in parallel with increased serum prohepcidin and serum ferritin compared with the other groups. In all subjects as a whole, circulating visfatin was not found to be significantly linked to insulin sensitivity ($r = 0.07$, $P = 0.4$) but was significantly associated with serum prohepcidin concentration ($r = 0.40$, $P < 0.0001$). Obesity status and glucose tolerance status influenced the relationships among visfatin, insulin sensitivity, and parameters of iron metabolism. Among men with altered glucose tolerance, serum visfatin was strongly associated with serum prohepcidin ($r = 0.61$, $P < 0.0001$) and serum soluble transferrin receptor (sTfR) ($r = -0.51$, $P < 0.0001$). In nonobese subjects, sTfR ($P = 0.02$) and prohepcidin ($P = 0.04$) contributed independently to visfatin variance after controlling for age and BMI. When insulin sensitivity was added to the model, only the latter ($P = 0.006$) contributed to 17% of visfatin variance. In obese men, however, only sTfR ($P = 0.04$) contributed independently to visfatin variance in this latter model.

CONCLUSIONS — Serum visfatin concentration is significantly associated with parameters of iron metabolism, especially in subjects with altered glucose tolerance.

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Visfatin, also known as pre-β-cell colony–enhancing factor (1), is a novel adipokine that is predominantly secreted by visceral adipose tissue (2), although controversy exists over the contribution of this fat depot to serum visfatin in humans (2,3). The protein exerts adipogenic effects in vitro and therefore is a good candidate to explain the accumulation of visceral adipose tissue that is associated with insulin resistance (2). Accumulation of visceral adipose tissue is also associated with iron metabolism. Serum ferritin is linked to centrally distributed body fatness. Studies in Norwegian (4) and Mexican-American men (5) demonstrated independent associations between serum ferritin concentrations and the waist-to-thigh ratio (4) or the waist-to-hip ratio (5). Serum ferritin concentration was also significantly associated with the computed tomography–estimated visceral fat area, hepatic fat content, and the degree of insulin resistance (6).

The maximum level of visfatin mRNA was found in the liver tissue and the next highest amount was found in muscle tissue (1). These tissues are classically insulin sensitive but are also characterized as central in iron metabolism. High expression of visfatin was also found in bone marrow, the source of circulating iron (1). Despite the coincidence of visfatin expression in iron-rich tissues, no study has investigated the possible interaction of visfatin with iron metabolism.

On a daily basis, iron uptake from the proximal cells of the duodenum seems to increase until sufficient iron stores for erythropoiesis are attained (7). To prevent iron overload, it is essential that this uptake process is tightly regulated because mammals lack a means of excreting iron (7). The peptide hepcidin appears to exert these functions. The human gene for hepcidin encodes a prepropeptide of 84 amino acids that is expressed in hepatocytes. Cleavage of the 24–amino acid signal peptide produces a 60–amino acid residue prohormone, which is detectable in serum and urine with the use of antibodies that target the proregion (prohepcidin). Additional processing of the 34–amino acid proregion results in 25–, 22–, and 20–amino acid peptides that are also detectable in serum and urine (hepcidin) (8,9). Loss of functioning hepcidin genes in mice was associated with raised serum iron, decreased reticuloendothelial iron stores, and increased intestinal iron absorption (8,9).

Our previous work (10) has shown that iron influences glucose metabolism, even in the absence of significant iron overload.

Similar to serum ferritin (10,11), plasma visfatin has been found to be increased in human type 2 diabetes (12). Different studies (3,13,14) to date have failed to demonstrate an association of the circulating protein with insulin sensitivity. Given the associations of visceral fat and serum ferritin (4–6) and circulating visfatin (3), we hypothesized an interaction between iron metabolism and visfa-

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Abbreviations: NGT, normal glucose tolerance; sTfR, soluble transferrin receptor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—*Clinical and biochemical variables of study subjects*

Variable	NGT	Altered glucose tolerance		*P*
Men (*n*)	95	43		—
Age (years)	49.5 ± 11.7	55.3 ± 10		0.001
BMI (kg/m^2)	27.2 ± 4.5	29 ± 3.9		0.006
Waist-to-hip ratio	0.92 ± 0.07	0.96 ± 0.07		0.002
Systolic blood pressure (mmHg)	124.5 ± 15.2	130.9 ± 15.3		0.007
Diastolic blood pressure (mmHg)	79.4 ± 10	81.3 ± 8.8		NS
Cholesterol (mg/dl)	206.6 ± 37.2	210.2 ± 40.9		NS
LDL cholesterol (mg/dl)	128.8 ± 39.8	138.2 ± 78		NS
HDL cholesterol (mg/dl)	54 ± 13.3	50.9 ± 10.5		NS
Triglycerides (mg/dl)*	86 (60–122)	113.5 (79.5–180.2)		0.001
Fasting glucose (mg/dl)	93.1 ± 7.8	124.6 ± 50		<0.0001
Fasting insulin (mmol/l)	8.6 ± 4.5	12.2 ± 6.1		0.03
Blood hemoglobin (mg/dl)	14.5 ± 1	14.8 ± 3.6		NS
Blood hematocrit (%)	42.5 ± 3.8	42.5 ± 2.4		NS
Insulin sensitivity (min $^{-1}$ · 10^{-4} · mmol/l)	2.5 (1.7–4.1)	1.32 (0.7–2.22)		<0.0001
Serum ferritin (ng/ml)	135.1 (87–231.5)	146 (88–194)†	173 (90–369)*	0.023§
Serum soluble transferrin receptor (μg/ml)	8.2 ± 2.6	9.5 ± 2.65	7.5 ± 3	0.054§
Serum prohepcidin (ng/ml)	120.5 ± 49.5	117 ± 44.6	182.2 ± 66.5‖	<0.0001§
Log serum visfatin (ng/ml)	1.15 ± 0.12	1.15 ± 0.13	1.24 ± 0.14¶	0.007§

Data are means ± SD or median (interquartile range). *P = 0.021 after Sheffe's test compared with NGT group. †Impaired fasting glucose/glucose intolerance (n = 25). ‡Type 2 diabetes (n = 18). §P value by ANOVA. ‖P < 0.0001 after Sheffe's test compared with NGT and impaired fasting glucose/glucose intolerance groups. ¶P = 0.008 after Sheffe's test compared with NGT group, and P = 0.025 compared with impaired fasting glucose/glucose intolerance group.

tin. For that reason, we aimed to study the association among different parameters of iron metabolism, serum prohepcidin concentration, circulating visfatin, and insulin resistance.

RESEARCH DESIGN AND METHODS

One hundred and thirty-eight consecutive men fulfilling inclusion criteria and enrolled in a cross-sectional, population-based study on cardiovascular risk factors in healthy subjects in northern Spain were studied. All subjects were of Caucasian origin and reported that their body weight had been stable for at least 3 months before the study. None of the patients were taking any medication or had any evidence of metabolic disease other than obesity. Of these subjects, 95 had strictly NGT, and 43 showed altered glucose tolerance (i.e., impaired fasting glucose or glucose intolerance [n = 25] and previously unknown type 2 diabetes [n = 18] during an oral glucose tolerance test).

Inclusion criteria for all subjects were *1*) BMI <40 kg/m^2, *2*) absence of any systemic disease, and *3*) alcohol intake <40 g/day. Exclusion criteria were *1*) serum liver enzyme (aspartate aminotransferase, alanine aminotransferase) activity over the upper limit of normal; *2*) an elevated serum creatinine concentration; *3*) previous acute major cardiovascular event; *4*) acute illnesses and current evidence of acute or chronic inflammatory or infectious diseases; *5*) transfusion history and/or iron or vitamin therapies in the previous 5 years; *6*) history of disturbances in iron balance (e.g., hemosiderosis from any cause, atransferrinemia, paroxysmal nocturnal hemoglobinuria, iron deficiency); and *7*) mental illness rendering the subjects unable to understand the nature, scope, and possible consequences of the study. Informed written consent was obtained after the purpose, nature, and potential risks were explained to the subjects. The experimental protocol was approved by the hospital ethics committee.

Measurements

Each subject was studied in the research laboratory in the postabsorptive state. BMI, waist-to-hip ratio, and blood pressure were measured as previously reported (11,15). Patients were requested to withhold alcohol and caffeine for at least 12 h before the different tests.

Insulin sensitivity

Insulin sensitivity was measured using the frequently sampled intravenous glucose tolerance test, as previously described (15).

Analytical determinations

The serum glucose concentrations were measured in duplicate by the glucose oxidase method with the use of a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA). Total serum cholesterol was measured through the reaction of cholesterol esterase/cholesterol oxidase/peroxidase, using a BM/Hitachi 747. 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 ferritin was determined by Microparticle Enzyme ImmunoAssay (AXSYMTM; Abbot Laboratories, Abbott Park, IL) with an intra- and interassay coefficient of variation (CV) <6%. Whole blood hemoglobin level and hematocrit levels (EDTA sample; Coulter Electronics, Hialeah, FL) were determined by routine laboratory tests.

Serum visfatin concentrations were measured by an enzyme immunoassay (Phoenix Peptides, Karlsruhe, Germany). Sensitivity of the method is 0.1 ng/ml. The intra- and interassay CV was <6%. Serum soluble transferrin receptor (sTfR) concentrations were measured by an enzyme immunoassay (DRG, Heidelber, Germany). The intra- and interassay CV was <8%. Serum prohepcidin concentrations were also measured by a commercially available enzyme-linked immunosorbent assay kit by using antibodies specific for peptides 28–47 of the proregion of the molecule (DRG, Heidel-

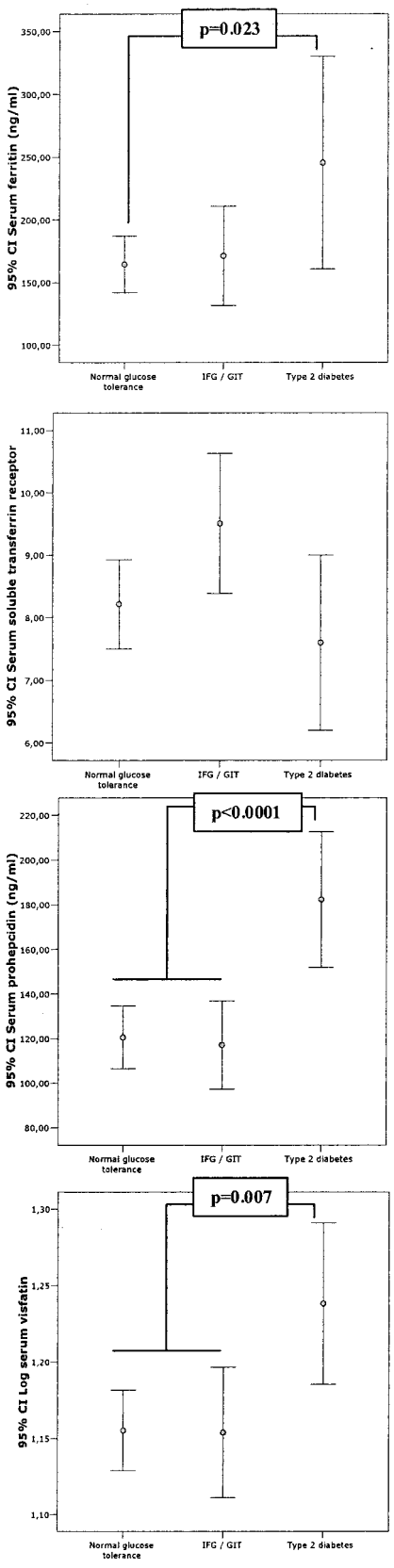

Figure 1—*CI 95% for the mean of parameters of iron metabolism across categories of glucose tolerance. IFG, impaired fasting glucose; GIT, glucose intolerance.*

berg, Germany). The detection limit was 4 ng/ml. The intra- and interassay CV was <7%.

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 then variables were given a log-transformation if necessary. These parameters (triglycerides, insulin sensitivity, and serum visfatin) were analyzed on a log scale and tested for significance on that scale. Relation between variables were tested using Pearson's test and stepwise multiple linear regression analysis. Normal versus altered glucose tolerance was entered as 1,2. We used χ^2 test for comparisons of proportions and ANOVA test with post hoc Sheffe's test for comparisons of quantitative variables across categories of glucose tolerance. For a given value of $P = 0.05$, the study had a 99% power to detect significant correlations between parameters in the whole sample of subjects in a bilateral test ($n = 138$); a 98% power in subjects with NGT ($n = 95$); and a 79% power in subjects with altered glucose tolerance ($n = 43$) (Pearson coefficient of at least 0.4). The analysis were performed using the program SPSS (version 11.0).

RESULTS — Anthropometric and biochemical characteristics of the men included in the study are shown in Table 1. Those subjects with newly diagnosed type 2 diabetes had significantly increased serum visfatin in parallel to increased serum prohepcidin and serum ferritin compared with the other groups (Table 1 and Fig. 1).

In all subjects as a whole, circulating visfatin was not found to be significantly linked to BMI ($r = 0.03$, $P = 0.6$) or insulin sensitivity ($r = 0.07$, $P = 0.4$). However, in nonobese (BMI <30 kg/m^2) subjects with NGT, the association with insulin sensitivity was significant ($r = 0.31$, $P = 0.01$, $n = 58$). In all subjects as a whole, serum visfatin concentration was significantly associated with parameters of iron metabolism such as serum prohepcidin concentration ($r = 0.40$, $P < 0.0001$). This relationship was especially significant in nonobese subjects ($r = 0.41$, $P < 0.001$, $n = 84$). Serum visfatin concentration was not found to be significantly associated with sTfR ($r = 0.11$, $P = 0.3$), blood hemoglobin, or serum ferritin ($r < 0.12$, $P > 0.3$). Serum ferritin was significantly associated with insulin sensitivity ($r = -0.27$, $P = 0.009$) (Fig. 2*A*).

When the analysis was performed separately in men with NGT, serum visfatin was not linked to any parameter of iron metabolism (r coefficients <0.15). Among men with altered glucose tolerance, however, serum visfatin was significantly and positively associated with serum prohepcidin ($r = 0.61$, $P < 0.0001$) (Fig. 2*B*) and negatively with serum sTfR ($r = -0.51$, $P < 0.0001$) (Fig. 2*C*). Given these correlation coefficients and the sample size ($n = 43$), the statistical power of these associations was 98 and 87%, respectively. The association between visfatin and prohepcidin was similarly significant in nonobese ($r = 0.62$, $P = 0.001$, $n = 26$) and in obese ($r = 0.60$, $P = 0.01$, $n = 17$) subjects with altered glucose tolerance. In these men, serum visfatin was not associated with serum ferritin or blood hemoglobin ($r < 0.11$, $P > 0.3$). Serum prohepcidin also correlated negatively with serum sTfR in these subjects ($r = -0.46$, $P = 0.002$).

Given the dual behavior of the relationship between visfatin and insulin sensitivity, we performed the linear regression analyses separately in obese and nonobese subjects. In nonobese subjects, sTfR ($P = 0.02$) and prohepcidin ($P = 0.04$) contributed independently to visfatin variance after controlling for age and BMI (Table 2). When insulin sensitivity was added to the model, only the latter ($P = 0.006$) contributed to 17% of visfatin variance (Table 2). The result persisted unchanged after adding normal versus altered glucose tolerance status. In obese men, however, only sTfR ($P = 0.04$) contributed independently to visfatin variance (Table 2).

CONCLUSIONS — We here describe how worsening of glucose tolerance is associated with increased serum ferritin in association with prohepcidin and circulating visfatin. Altered glucose tolerance and type 2 diabetes are paralleled by some degree of iron overload that is directly associated with serum prohepcidin and visfatin concentrations. In this sense, it is remarkable that the maximum visfatin mRNA expression was found in tissues that are central in iron regulation (liver, muscle, and bone marrow) (1). We here also report that serum visfatin was associated with different parameters of iron metabolism such as serum prohepcidin and serum sTfR. To our knowledge,

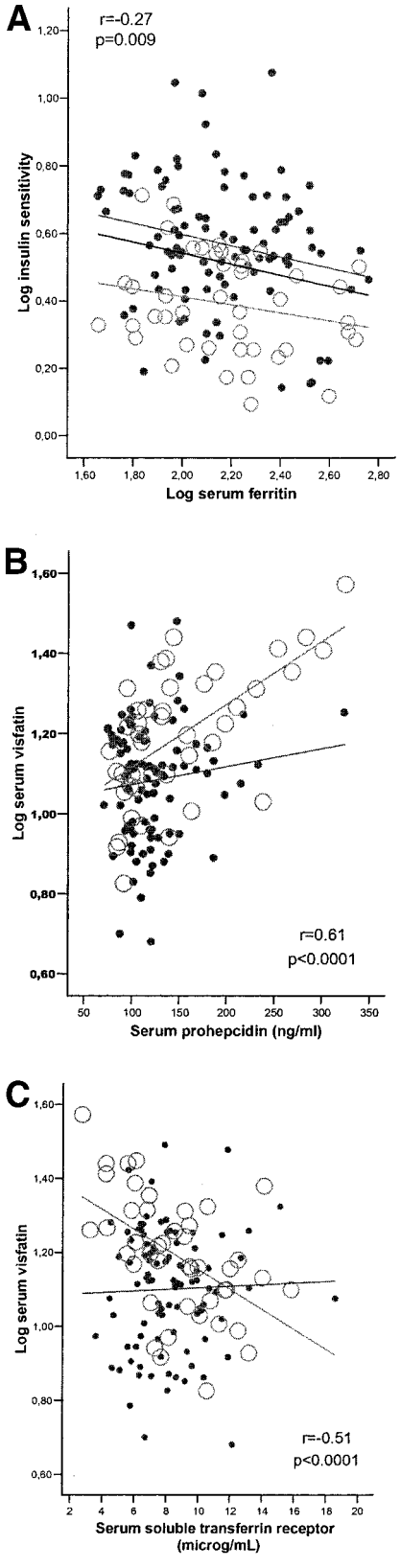

Figure 2—A*: Linear correlation association between log-transformed insulin sensitivity and log serum ferritin. The correlation coefficient refers to the whole group of subjects. In men with NGT (●) the correlation coefficient was* r = *−0.23,* P = *0.02; in men with altered glucose tolerance (○), the correlation coefficient was* r = *−0.22,* P = *0.1. These two coefficients did not differ significantly* (P = *0.95).* B*: Linear correlation association between circulating log-transformed serum visfatin and serum prohepcidin concentration in men with normal (●) and altered glucose tolerance (○). The correlation coefficient refers to this last group of subjects. In men with NGT, the correlation coefficient was* r = *0.11,* P = *0.3. These two coefficients differed significantly* (P = *0.02).* C*: Linear correlation association between log-transformed serum visfatin and serum concentration of sTfR in men with normal (●) and altered glucose tolerance (○). The correlation coefficient refers to this last group of subjects. In men with NGT, the correlation coefficient was* r = *0.049,* P = *0.7. These two coefficients differed significantly* (P = *0.015).*

this would be the first study of serum visfatin in relation with parameters of iron metabolism. This would be also the first study on circulating prohepcidin in subjects with type 2 diabetes or altered glucose tolerance.

These associations differed according to obesity status and glucose tolerance status. In nonobese subjects, serum sTfR concentration and prohepcidin contributed independently to visfatin variance after controlling for age and BMI. Insulin sensitivity, however, was the only factor contributing to serum visfatin when it was accounted for. In obese subjects, only sTfR contributed to visfatin variance when BMI, age, prohepcidin, insulin sensitivity, and glucose tolerance status were controlled for (Table 2). These findings suggest that insulin sensitivity would be the main factor influencing serum visfatin concentration across the range of insulin action observed in nonobese subjects. When insulin resistance develops (obese subjects), sTfR would turn into one of the factors influencing serum visfatin concentration. This is further discussed below.

Insulin is known to cause a rapid and marked stimulation of iron uptake by fat cells and hepatocytes, redistributing transferrin receptors from an intracellular membrane compartment to the cell surface (16). The regulation of iron uptake by insulin seems to parallel its effects on glucose transport (17).

Iron stores are significantly associated with insulin resistance as shown in this article and elsewhere (10,11). However, the mechanisms behind iron-induced insulin resistance are not well understood (18–20).

Serum sTfR concentration is closely related to cellular iron demands and, in consequence, the higher the ferritin levels, the lower the sTfR concentration. Serum sTfR concentration is also a measure of erythropoiesis and of the total mass of erythroid precursors (21–24). Iron deficiency leads to increased serum sTfR concentration, and iron excess results in decreased circulating sTfR levels (24).

Serum visfatin correlated negatively with serum sTfR concentration. Subjects with altered glucose tolerance and the lowest sTfR concentration showed the highest circulating visfatin. This suggests that increased iron stores would lead to increasing visfatin synthesis. Perhaps a threshold of iron overload (expressed as circulating sTfR) is needed to impact serum visfatin given the absence of relationship in subjects with NGT. In this sense, we observed that the relationships of visfatin with both sTfR and prohepcidin were significant in subjects with sTfR below the median of sTfR (7.8 μg/ml) ($r = -0.39$, $P = 0.006$ and $r = 0.41$, $P = 0.004$, respectively) but not in subjects with sTfR above the median ($r = -0.12$ and 0.26, both nonsignificant).

Serum prohepcidin is an indicator of endogenous hepcidin levels (8,9). Hepcidin is synthesized in response to iron overload, reducing duodenal iron absorption and iron export from monocytes/macrophages (8,9). In a recent study (25) in premenopausal women, serum prohepcidin concentration was found to be relatively stable within subjects and correlated positively with serum ferritin. Hadley et al. (25) did not find association between serum prohepcidin and iron absorption. As suggested by the authors, "the more narrow range of ferritin and associated body iron stores of healthy premenopausal women than of subject groups that also include men. . . is a possible limitation for detecting an association between serum prohepcidin and iron absorption" (25). In men with altered glucose tolerance and type 2 diabetes, serum prohepcidin showed a strong positive association with serum visfatin. This finding led us to speculate that circulating visfatin is perhaps upregulated with increasing iron stores. As visfatin, prohepcidin is a liver-derived protein (8,9). Two liver-synthesized proteins (visfatin and prohepcidin) appear to be significantly associated in direct proportion with iron stores, as suggested by their negative correlations with serum sTfR. In fact, hepatic hepcidin expression was greater in patients with positive rather than with negative iron staining in one study (26). A

Table 2—*Multiple linear regression analyses with circulating visfatin as the dependent variable*

	Circulating visfatin (dependent variable)					
	Nonobese subjects*				Obese subjects†	
Independent variables	β	*P*	β	*P*	β	*P*
Age	−0.064	0.63	0.07	0.62	−0.009	0.97
BMI (kg/m^2)	0.066	0.59	0.04	0.8	−0.003	0.99
Serum sTfR	−0.29	0.022	0.02	0.88	−0.58	0.047
Serum prohepcidin	0.25	0.047	0.20	0.16	−0.11	0.68
Log insulin sensitivity			0.41	0.006	0.25	0.35
Normal vs. altered glucose tolerance					0.19	0.48

*Adjusted R^2 0.15. †Adjusted R^2 0.27.

failure to increase significantly prohepcidin (to decrease iron absorption) or visfatin could contribute to iron-induced insulin resistance. Our data are similar to the associations between hepcidin and iron status found in mouse models and in patients with iron-deficiency anemia in whom hepcidin concentrations were low (27,28).

It could be argued that the association of visfatin and prohepcidin is due to inflammatory mechanisms. However, interleukin-6 leads to decreased visfatin (29) and increased hepcidin synthesis (30). We have not found significant associations between circulating interleukin-6 and serum prohepcidin or visfatin in a subsample of these subjects (data not shown). On the other hand, serum prohepcidin increases in renal insufficiency (31). We have included subjects with strictly normal renal function in whom serum creatinine did not correlate with serum prohepcidin concentrations ($r = -0.02$, $P = 0.8$).

It should also be remembered that iron is intimately linked to oxidative stress. Iron participates, through the Fenton reaction, in the formation of highly toxic free radicals, such as hydroxil radicals (OH, from Fe and H_2O_2), which are capable of inducing lipid peroxidation (32). Other free radicals such as $O_2^{\cdot}$ are formed by NADPH oxidase and mitochondrial electron transfer. In this sense, visfatin is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis (33). Superoxide dismutase converts $O_2^{\cdot}$ to H_2O_2, which participates in the Fenton reaction.

Oxidative stress induces both insulin resistance, by decreasing internalization of insulin (34), and increased ferritin synthesis. Factors related to hyperglycemia or oxidative stress can mediate in part the association between visfatin and parameters of iron metabolism (sTfR and prohepcidin) in subjects with altered glucose tolerance.

The strengths of this study lie in the methodology of evaluating insulin sensitivity and the evaluation of iron metabolism through different parameters. However, this is a cross-sectional case-control study that needs to be replicated in prospective observations.

In summary, circulating visfatin appears to be linked to parameters of iron metabolism, mainly in subjects with altered glucose tolerance. The regulation of visfatin and prohepcidin with increasing iron stores in subjects with altered glucose tolerance should be investigated further.

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