CLINICAL STUDY

Visceral adipose tissue zinc finger protein 36 mRNA levels are correlated with insulin, insulin resistance index, and adiponectinemia in women

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Abstract

Introduction: Adipose tissue is now recognized as an endocrine organ and secretes numerous molecules and proteins potentially involved in the physiopathology of the metabolic syndrome. Recently, we have determined the transcriptome of omental adipose tissue, leading to the identification of a new candidate gene for obesity-related metabolic complications, zinc finger protein 36 (*ZFP36*), which is known to down-regulate tumor necrosis factor-α (*TNF-α*) expression.

Objective: The objective of this study was to further examine the relationship between *ZFP36* gene expression levels, obesity-related phenotypes, and adipokines.

Methods: Abdominal subcutaneous and omental adipose tissue samples were obtained from 46 women undergoing elective gynecological surgery. Adipose tissue *ZFP36* mRNA abundance was assessed by quantitative real-time PCR. Body fat accumulation and distribution were measured by dual X-ray absorptiometry and computed tomography. Fasting blood levels of glucose, insulin, and lipids, and circulating TNF-α, interleukin-6 (IL-6), resistin, and adiponectin were also measured.

Results: No correlation was observed between s.c. *ZFP36* mRNA levels and any of the phenotypes tested. However, although omental *ZFP36* mRNA levels were not correlated with measures of body fatness and lipid profile, they were negatively correlated with fasting insulin levels ($R = -0.31$; $P = 0.05$), the insulin resistance index (HOMA-IR; $R = -0.31$; $P = 0.05$), and 2-h post-glucose insulinemia ($R = -0.32$; $P = 0.05$). Omental *ZFP36* mRNA abundance was also positively correlated with adiponectinemia ($R = 0.35$; $P = 0.03$) but not with circulating TNF-α, IL-6, and resistin concentrations.

Conclusion: These results suggest that *ZFP36* gene expression in omental adipose tissue, but not in abdominal s.c. fat, may offer partial protection against the development of insulin resistance and diabetes.

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Introduction

Obesity is associated with several risk factors for type 2 diabetes and cardiovascular disease, two conditions associated with high morbidity and mortality rates (1). These risk factors, which define the metabolic syndrome, include hyperglycemia, dyslipidemia, hypertension, abdominal/visceral obesity, and a prothrombotic/proinflammatory state (2). Adipose tissue is now recognized as an important endocrine organ secreting numerous molecules and proteins potentially involved in the physiopathology of the metabolic syndrome (3). Indeed, the identification and characterization of novel adipose tissue-expressed genes and proteins are likely to provide further insight into its endocrine function and its relationship with the development of the metabolic syndrome.

Recently, the transcriptome of abdominal visceral (omental) adipose tissue in obese men with and without the metabolic syndrome was established to propose new metabolic syndrome candidate genes. This strategy led to the identification of zinc finger protein 36 (*ZFP36*) as a candidate gene related to obesity complications (4). The *ZFP36* gene was found to be 4.6-fold overexpressed in obese men without the metabolic syndrome, suggesting that it may be related to a protective effect against the development of obesity-related metabolic complications (4). Furthermore, two *ZFP36* polymorphisms were associated with body weight, body mass index (BMI), as well as glucose and LDL-cholesterol levels in a cohort of 709 severely obese men and women. Some of these associations were found only in men (glucose) and some only in women (body weight and BMI), suggesting that *ZFP36*

may have sex-specific effects. Moreover, these two polymorphisms accounted for 10.1% of the variance in *ZFP36* mRNA levels in omental adipose tissue of non-diabetic premenopausal women (4). The ZFP36 protein has been discovered when searching for genes that were activated by insulin (5) and has been associated, in knockout animals, with a complex inflammatory syndrome due to increased circulating levels of the inflammatory response mediator tumor necrosis factor-α (TNF-α) (6, 7). Given that the metabolic syndrome is associated with increased circulating TNF-α and a chronic inflammation state (8), our previous results on the *ZFP36* gene and its physiological role suggest that it may be involved in the physiopathology of obesity-related metabolic complications.

Therefore, the present study was aimed at investigating, in women covering a wide range of adiposity, the potential relationship between *ZFP36* gene expression levels in abdominal s.c. or omental adipose tissues and body fat accumulation and distribution, fasting blood glucose, insulin, lipids, and a number of circulating inflammatory adipokines (TNF-α, interleukin-6 (IL-6), resistin, and adiponectin) concentrations. Given the postulated protective role of ZFP36 against the development of obesity-related metabolic complications, we tested the hypothesis that low adipose tissue *ZFP36* mRNA abundance would be correlated with an altered metabolic profile including hyperglycemia, dyslipidemia, and a proinflammatory state.

Research methods and procedures

Subjects

A total of 46 women undergoing elective surgery were recruited through the Gynecology Unit of the Laval University Medical Center. These women elected for total ($n=45$) or subtotal ($n=1$) abdominal hysterectomies, some with salpingo-oophorectomy of one ($n=8$) or two ($n=17$) ovaries for one or more of the following reasons: menorrhagia/menometrorrhagia ($n=24$), myoma/fibroids ($n=32$), incapacitating dysmenorrhea ($n=8$), pelvic pain ($n=1$), benign cyst ($n=10$), endometriosis ($n=7$), adenomyosis ($n=1$), pelvic adhesions ($n=4$), endometrial hyperplasia ($n=3$), or polyp ($n=2$) and ovarian thecoma ($n=1$). Apart from their gynecological condition, participants were in general good health, with normal kidney and liver function. All patients were free from autoimmune diseases or long-term diabetes complications. One patient was diagnosed with type 2 diabetes at the time of study. Patients were not taking medication possibly affecting insulin sensitivity. Five women reported the use of synthroid. Menopausal status was established using menstrual history questionnaire,

medical files, and plasma follicle-stimulating hormone levels. A total of 23 women were premenopausal, 7 were perimenopausal, and 9 were menopausal. The medical ethics committees of Laval University and Laval University Medical Center approved this study. Written informed consent was obtained from all subjects before their inclusion in the study.

Body weight, height, and resting blood pressure (systolic and diastolic) were measured preoperatively using standardized procedures as previously described (9). Body fat mass and body fat distribution were determined on the morning of, or a few days before or after the surgery. Total body fat mass, fat percentage, and fat-free mass were determined by dual energy X-ray absorptiometry using a Hologic QDR-2000 densitometer and the enhanced array whole-body software V5.73A (Hologic Inc., Bedford, MA, USA). L4–L5 s.c. and visceral adipose tissue areas were determined by computed tomography as previously described (10) using a GE Light Speed 1.1 CT scanner (General Electric Medical Systems, Milwaukee, WI, USA). The ImageJ 1.33μ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify visceral adipose tissue areas by delineating the intra-abdominal cavity at the internal-most aspect of the abdominal and oblique muscle walls surrounding the cavity and the posterior aspect of the vertebral body. Adipose tissue was highlighted and computed using an attenuation range of -190 to -30 Hounsfield units. The coefficients of variation between two measures from the same observer ($n=10$) were 0.0, 0.2, and 0.5%, for total, s.c., and visceral adipose tissue areas respectively.

On the morning of surgery, 12-h fasting blood samples were obtained to measure cholesterol and triglyceride levels in plasma and lipoprotein fractions by using a Technicon RA-analyzer (Bayer Corp Inc.), as previously described (11). Plasma VLDL were isolated by ultracentrifugation, and the HDL fraction was obtained by precipitation of the LDLs from the infranatant with heparin and $MnCl_2$ (12). The concentration of LDL-cholesterol was computed by subtraction of cholesterol levels of the infranatant measured before and after precipitation. Plasma glucose was measured using the glucose oxidase method and plasma insulin levels were measured by RIA (within-assay precision: $CV \leq 6.0\%$; Linco Research, St Charles, MO, USA). The HOMA insulin resistance index was calculated using fasting insulin (μU/ml)×fasting glucose (mmol/l)/22.5 and was used to estimate insulin sensitivity in the fasting state (13). Finally, plasma TNF-α (within-assay precision: $CV \leq 8.8\%$), IL-6 (within-assay precision: $CV \leq 7.8\%$; R&D Systems, Minneapolis, MN, USA), and resistin (within-assay precision: $CV \leq 7.0\%$; Linco Research) levels were measured by ELISA and plasma adiponectin levels were measured by RIA (within-assay precision: $CV = 6.2\%$; Linco Research).

An oral glucose tolerance test was performed a few days before (9 women) or after (33 women) the

surgery. Women were given 75 g oral glucose after a 12-h overnight fast. A blood sample was obtained from an antecubital vein at 2 h (120 min) for glucose and insulin determinations.

Adipose tissue sampling

S.c. adipose tissue was collected at the site of surgical incision (lower abdomen) and omental adipose tissue was collected from the distal portion of the greater omentum (epiploon). Samples were collected during the surgical procedure and immediately carried to the laboratory in 0.9% saline preheated at 37 °C, and were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analyses. S.c. and visceral adipose tissues were sampled from each individual, but one sample was missing from one of the fat depots for two different women. Thus, although 46 women were included in the study, only 44 adipose tissues from each depot were included in the analyses.

ZFP36 measurements

Total RNA was isolated from ∼150 mg omental or s.c. adipose tissue using RNeasy lipid tissue extraction kit (Qiagen) following the manufacturer's recommendations. On-column digestion of DNA with RNase-free DNase set (Qiagen) was used to efficiently remove traces of DNA. RNA quantity and quality were assessed using an Agilent Technologies 2100 bioanalyzer and RNA 6000 Nano LabChip kit (Agilent, Mountain View, CA, USA). cDNA was generated from 40 ng total RNA using 1.4 μg total purified RNA with Invitrogen Superscript II. RNA was denatured over 5 min at 65 °C with 350 ng random hexamers (Invitrogen) and 35 nmoles dNTP (Amersham Biosciences) in a volume of 42 μl. The solution was chilled for 5 min on ice and mixed (final volume of 70 μl) with first strand buffer, dithiothreitol and 300 units of Superscript II (Invitrogen) following the manufacturer's recommendations. After 10 min at room temperature, reaction vessels were incubated at 42 °C for 120 min. Equal amounts of cDNA were run in triplicate and amplified in a 15 μl reaction containing 7.5 μl 2X Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 10 nM Z-tailed forward primer, 100 nM reverse primer, 100 nM Amplifluor Uniprimer probe (Chemicon, Temecula, CA, USA), and 2 μl DNA target. No-template controls were used as recommended. The mixture was incubated at 50 °C for 2 min, at 95 °C for 4 min, and then cycled at 95 °C for 15 s and at 55 °C for 40 s 55 times using the Prism 7900 Sequence Detector (Applied Biosystems). Amplification efficiencies were validated and normalized to ribosomal 18S and quantity of target gene was calculated according to a standard curve. 18S is an established and accepted reference gene (14). Our own experience showed that it is comparable with other reference genes including *LRP10*, *CLN3*, and *HPCAL1*

(data not shown). Primer sequences were designed using Primer Express 2.0 (Applied Biosystems) and were the following: 5′-Ztail-GGCCAACCGTTACACCATG-3′, 5′-CGACAGGAGGCTCTCGTAGATG-3′ (ZFP36) and 5′-Ztail-TGCATGTCTAAGTACGCACGG-3′, 5′-AATGA-GCCATTCGCAGTTTCA-3′ (18S). Forward primers containing the 5′ Z sequence: ACTGAACCTGACCG-TACA were used to detect amplicons with the Amplifluor UniPrimer system. PCR data are expressed in arbitrary units (18S rRNA-normalized).

Statistical analysis

Mean *ZFP36* mRNA expression differences between s.c. and omental adipose tissue were tested using a paired *t*-test and correlations between s.c. and omental *ZFP36* mRNA levels and adiposity, lipid and glucose-related variables and adipokines were tested using (partial) Pearson and Spearman rank correlation coefficients. The contribution of abdominal fat area, metabolic syndrome, and menopausal status as covariates in correlations was tested using multiple regression analyses. Transformations were applied to non-normally distributed variables (square root: HOMA-IR, 2-h post-glucose glycemia and insulinemia; and logarithm: systolic blood pressure, glucose, resistin, IL-6, and omental *ZFP36* mRNA levels) to meet Pearson correlation statistical assumptions. To be considered as affected by the MS, women had to fulfill at least three of the risk factors described in the NCEP-ATP III (15). Since the waist girth measure was not available in the present study, a cutoff value of 100 cm^2 for visceral adipose tissue area (16) was used instead of a waist girth of 88 cm. Analyses were performed using SAS software, version 8.02 (SAS Institute Inc., Cary, NC, USA).

Results

Table 1 presents the physical characteristics of women included in this study. These women were, on average, 47.3 years old, overweight according to their mean BMI and had a normal lipid profile and glucose metabolism according to current clinical guidelines (17). However, they covered a wide range of adiposity (19.1–41.3 kg/m^2) and seven (18%) of them were characterized by the metabolic syndrome (15).

Substantial variation was found for adipose tissue *ZFP36* mRNA levels in women, and mRNA abundance in s.c. adipose tissue showed a near twofold higher level when compared with omentum (Table 1). Moreover, *ZFP36* mRNA levels were approximately twofold lower in the subgroup of women with the metabolic syndrome (13.79 ± 8.27 vs 24.94 ± 4.00; $P = 0.15$) when compared with women without the metabolic syndrome.

In s.c. adipose tissue, none of the correlations tested between *ZFP36* mRNA levels and the phenotypes under

Table 1 Physical characteristics of the study women.

	Mean ± s.d. (*n*)	Range
Age (years)	47.3 ± 5.0 (46)	39.6–61.7
BMI (kg/m^2)	27.7 ± 5.2 (46)	19.1–41.3
Body fat mass (kg)	26.5 ± 9.7 (46)	11.2–50.8
S.c. adipose tissue area (cm^2)	344 ± 149 (44)	103–759
Visceral adipose tissue area (cm^2)	102 ± 50 (44)	34–233
Fasting glucose (mM)	5.65 ± 0.55 (42)	4.80–7.80
Fasting insulin (pM)	11.66 ± 5.70 (42)	3.37–27.64
HOMA-IR	3.02 ± 1.74 (42)	0.77–8.85
2-h post-glucose glycemia (mM)	6.62 ± 1.80 (42)	4.00–14.80
2-h post-glucose insulinemia (pM)	66.03 ± 36.79 (42)	11.35–143.33
Triglycerides (mM)	1.32 ± 0.65 (43)	0.51–2.99
Total cholesterol (mM)	4.85 ± 0.60 (43)	3.55–5.95
HDL-cholesterol (mM)	1.44 ± 0.33 (43)	0.81–2.14
LDL-cholesterol (mM)	2.81 ± 0.51 (43)	1.50–3.96
Systolic blood pressure (mmHg)	129.4 ± 15.0 (46)	105.0–188.0
Diastolic blood pressure (mmHg)	75.7 ± 9.5 (46)	49.0–92.0
TNF-α (pg/ml)	1.24 ± 0.50 (41)	0.68–3.18
IL-6 (pg/ml)	2.41 ± 2.00 (42)	0.73–12.60
Resistin (ng/ml)	23.90 ± 6.40 (42)	14.02–48.34
Adiponectin (ng/ml)	47.35 ± 22.4 (42)	1.56–103.37
S.c. *ZFP36* mRNA abundance[a]	39.31 ± 28.9 (44)	2.06–119.44
Omental *ZFP36* mRNA abundance[a]	21.33 ± 20.62 (44)	1.67–117.54

n, number of women.
[a] 18S-normalized.

study were statistically significant (Table 2). However, trends for positive associations between s.c. *ZFP36* mRNA levels and plasma triglyceride and resistin concentrations were found (Table 2).

ZFP36 mRNA abundance in omental adipose tissue was negatively correlated with fasting insulin, the HOMA-IR index, and 2-h post-glucose insulinemia and positively correlated with adiponectinemia (Table 2 and Fig. 1). In other words, higher levels of *ZFP36* mRNA in omental adipose tissue were associated with a lower fasting insulin level and lower HOMA-assessed insulin resistance (or higher insulin sensitivity) and with higher plasma adiponectin concentrations. Spearman correlation coefficients were consistent with the results described above (data not shown). Moreover, abdominal visceral fat area, metabolic syndrome, and menopausal status, when included in the regression models as covariates, did not show a significant contribution ($P \geq 0.05$) to the correlation between omental *ZFP36* mRNA levels and fasting insulin, HOMA-IR, 2-h post-glucose insulinemia, and adiponectinemia (data not shown). None of the other variables tested showed significant correlations with omental *ZFP36* mRNA levels (Table 2).

The correlations between adiponectinemia and risk factors for type 2 diabetes are well known and were also tested. As expected, adiponectinemia was significantly correlated or showed a trend for significant correlations with many of the features of the metabolic syndrome such as BMI ($R = -0.290$; $P = 0.06$), fat mass ($R = -0.307$; $P = 0.05$), s.c. ($R = -0.293$; $P = 0.07$), and visceral ($R = -0.426$; $P = 0.006$) adipose tissue areas, fasting glucose ($R = -0.292$; $P = 0.06$), HOMA-IR ($R = -0.268$;

$P = 0.09$), 2-h post-glucose insulinemia ($R = -0.294$; $P = 0.07$), HDL-cholesterol ($R = 0.464$; $P = 0.002$), and LDL-cholesterol ($R = -0.438$; $P = 0.004$). Contrary to correlations with omental *ZFP36* mRNA levels,

Table 2 Pearson correlation coefficients (*R*) between adipose tissue zinc finger protein 36 (*ZFP36*) mRNA levels and adiposity and metabolism-related phenotypes and adipokines.

	s.c. AT *ZFP36* mRNA levels (*R*)	Omental AT *ZFP36* mRNA levels (*R*)
BMI	0.146	−0.119
Body fat mass	0.085	−0.110
s.c. adipose tissue area	0.174	−0.061
Visceral adipose tissue area	0.102	−0.185
Fasting glucose	0.131	−0.204
Fasting insulin	−0.061	−0.310*
HOMA-IR	−0.027	−0.314*
2-h post-glucose glycemia	0.047	−0.200
2-h post-glucose insulinemia	−0.010	−0.315*
Triglycerides	0.287[†]	−0.146
Total cholesterol	0.162	−0.064
HDL-cholesterol	−0.111	0.084
LDL-cholesterol	0.090	−0.044
Systolic blood pressure	0.220	0.019
Diastolic blood pressure	−0.060	−0.021
TNF-α	0.114	0.146
IL-6	0.197	−0.052
Resistin	0.283[†]	0.148
Adiponectin	0.064	0.350*

AT, adipose tissue; OGTT, oral glucose tolerance test; [†] $0.08 \geq P > 0.05$; and *$P \leq 0.05$.
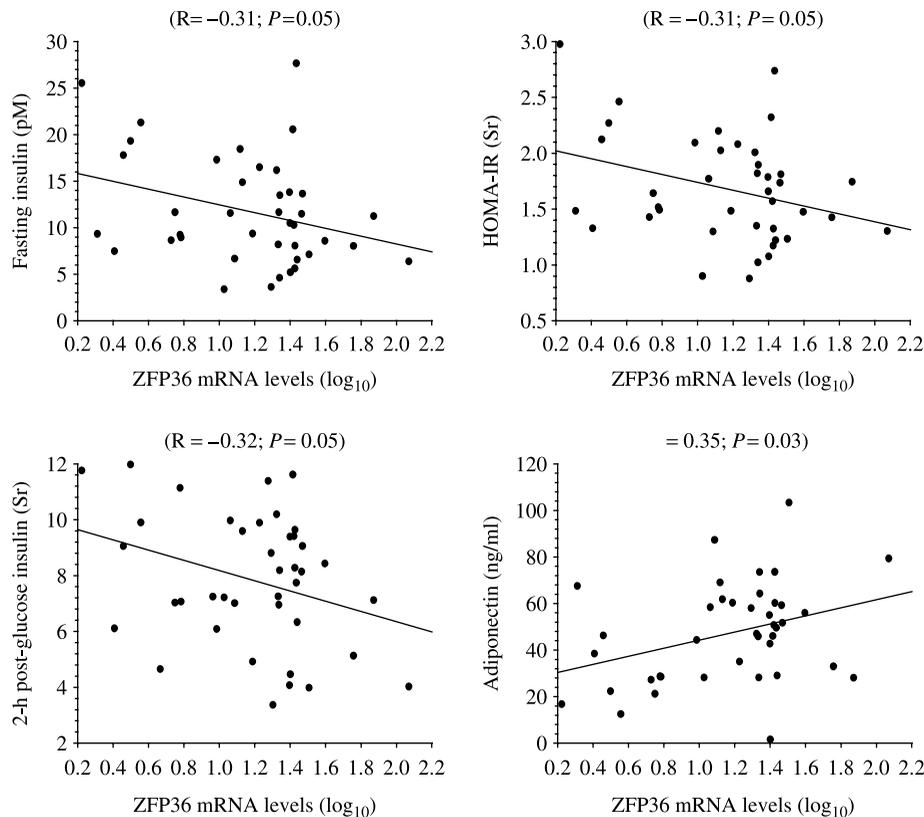
Figure 1 Pearson correlations between glucose metabolism-related phenotypes and omental adipose tissue ZFP36 mRNA levels. Sr, square root.

abdominal visceral fat area, when included in the regression models as a covariate, contributed significantly ($P \leq 0.05$) to correlations between adiponectinemia and fasting glucose, HOMA-IR, and HDL-cholesterol and LDL-cholesterol but not for fat mass, HOMA-IR, 2-h post-glucose insulinemia, and HDL-cholesterol (P values for visceral fat contribution to the models: $0.09 > P > 0.05$). Overall, only plasma HDL-cholesterol ($R = 0.408$; $P = 0.01$) and LDL-cholesterol levels ($R = -0.375$; $P = 0.02$) remained significantly correlated with adiponectinemia after the latter adjustment.

Discussion

Although expression and association studies had suggested that *ZFP36* may be a candidate gene for obesity-related metabolic complications in severely obese men and women (4), no compelling evidence existed to support its association with these conditions. Moreover, results of our original study suggested that *ZFP36* might have different effects on the cardiovascular disease risk profile in men and women (4). Here, the relationship between adipose tissue *ZFP36* mRNA levels and adiposity, metabolic variables, and adipokines was investigated in women. To our knowledge, this is the first study to examine *ZFP36*

expression in adipose tissue compartments and to link it with features of the metabolic syndrome.

ZFP36 mRNA levels in s.c. and omental adipose tissues were also previously assessed in non-diabetic, severely obese men (18). In that study, although subjects were severely obese (BMI > 40 kg/m^2), they were not diabetic and had, on average, a normal lipid profile based on the NCEP-ATPIII criteria for the metabolic syndrome (15). However, as opposed to the present study, *ZFP36* mRNA levels were increased (3.7-fold) in omental versus s.c. adipose tissue. This difference may reflect a sex-specific difference or a variable lability to cellular stimuli of *ZFP36* gene expression, depending on the level of adipose tissue insulin sensitivity. This discrepancy could also be attributed to population differences in adipose tissue cell composition.

The ZFP36 protein was discovered about 15 years ago in an attempt to identify genes that were activated by insulin (5). Its expression was found to be highly and transiently responsive to insulin in many cell types including mouse cultured fibroblasts overexpressing human insulin receptors (NIH 3T3 HIR3.5) (19), mouse 3T3-L1 adipocytes, and mouse adipose tissue (adult male BALB/c) (5). In the present study, omental *ZFP36* mRNA levels were negatively correlated with plasma insulin and markers of insulin sensitivity such as HOMA-IR, and positively correlated with circulating

concentration of adiponectin, an adipokine produced by adipocytes that is negatively regulated by insulin and proposed to act as an insulin sensitizer (20). These apparently conflicting observations may reflect species differences in *ZFP36* expression regulation, as previously suggested (5) or may reflect a certain level of insulin resistance in women with higher fasting insulin concentrations and in severely obese men with the metabolic syndrome (4). Lai *et al.* have shown that the *ZFP36* gene promoter and intron 1 harbor known and novel transcription factor-binding sites (21, 22), suggesting that *ZFP36* expression is tightly regulated. Interestingly, s.c. *ZFP36* mRNA levels were not correlated with glucose homeostasis variables, nor with its level in omental adipose tissue, which also suggests that *ZFP36* expression is highly regulated and sensitive to different stimuli in different tissues. However, although its response to insulin exposure in human and the transcription factors involved in its expression in adipocytes still remain to be established (23), it is expected that insulin modulates *ZFP36* expression.

ZFP36 gene knockout mice allowed determination of at least one of its potential physiological roles (6, 7). The results of these studies suggested that ZFP36 is involved in TNF-α post-translational regulation, through destabilization of its mRNA by binding AU-rich elements (AUUUA) located in *TNF-α* 3′ UTR (6, 7). This led to a complex inflammatory syndrome associated with an increased level of circulating TNF-α (6, 7). TNF-α is produced by many cell types including those located in adipose tissue and is now recognized to induce, directly and indirectly, insulin resistance in humans and other species (24). However, in the present study, *ZFP36* mRNA levels were not correlated with circulating TNF-α concentration as well as omental TNF-α mRNA abundance (barely detectable by real-time PCR in the few samples tested, data not shown). These results suggest that its association with glucose metabolism can be independent of *TNF-α* gene expression dysregulation and circulating levels of the cytokine.

ZFP36 is also known to decrease mRNA stability of a few other inflammatory transcripts (*GMCSF, IL-3,* and *COX-2* genes) (25). Adiponectin is not currently one of its known targets, but since *ZFP36* and adiponectin are both expressed in adipocytes (5, 26) and since adiponectin shares structural and sequence homology with TNF-α and has an anti-inflammatory function (27), it could be possible that ZFP36 interacts directly with adiponectin mRNA. However, although the adiponectin transcript harbors four AU-rich elements in its 3′ UTR, this may not necessarily be the case. *ZFP36* mRNA levels and circulating adiponectin concentrations were positively correlated in the present study, although ZFP36 supposedly would destabilize target gene mRNAs. Moreover, the correlations between *ZFP36* and adiponectin mRNA levels in omental and s.c. adipose tissue were not significant (data not shown).

Thus, whether ZFP36 modulates adiponectin expression at the post-transcriptional level still needs to be determined.

It is also important to point out that, in contrast to correlations of insulin and adiponectin with adiposity and lipid-related phenotypes, the correlations observed with omental *ZFP36* mRNA levels were independent of body fatness, suggesting that *ZFP36* is less likely than insulin and adiponectin to be 'dysregulated' in obesity *per se,* potentially offering a new therapeutic avenue for insulin resistance and diabetes. This observation is also in agreement with the results of our previous study from which *ZFP36* was proposed as new candidate gene for metabolic complications independently of obesity (4).

In summary, omental *ZFP36* mRNA level was correlated with fasting insulin, HOMA-IR, and adiponectinemia, which indirectly suggests a role for ZFP36 in glycemic control. Accordingly, increased *ZFP36* expression in omental adipose tissue, but not in abdominal s.c. fat, may offer partial protection against the development of insulin resistance and diabetes.

Acknowledgements

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