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CLINICAL RESEARCH

Subcutaneous and Visceral Adipose Tissue FTO Gene Expression and Adiposity, Insulin Action, Glucose Metabolism, and Inflammatory Adipokines in Type 2 Diabetes Mellitus and in Health

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Abstract
Background FTO gene variants are linked to obesity. We tested for site-specific differences in FTO gene expression in subcutaneous and visceral adipose tissue (SAT and VAT, respectively) from individuals with and without type 2 diabetes mellitus (T2D) and the relationships between fasting glucose, in vivo insulin action, and measures of adiposity with FTO gene expression in adipose tissue.
Methods Paired subcutaneous and visceral fat were excised at elective surgery in $n=16$ subjects (six with T2D, age-matched). Metabolic parameters were measured in fasted state; body composition by dual-energy X-ray absorptiometry; and insulin action by hyperinsulinemic euglycemic clamp. Adipose tissue mRNA gene expression was determined by quantitative RT-PCR.
Results Subjects with T2D had SAT and VAT FTO mRNA expression similar to controls. There was no depot specificity between SAT and VAT FTO mRNA expression. Insulin action did not relate to SAT or VAT FTO mRNA expression. SAT FTO mRNA expression was related to fasting glucose and waist circumference only. SAT and VAT FTO mRNA expression was not related to direct measures of total or central abdominal adiposity. SAT FTO mRNA expression was related to SAT tumor necrosis factor-alpha and nuclear factor-κB mRNA expression.
Conclusions FTO gene expression is not increased in SAT and VAT in T2D and does not relate to insulin action. The links between FTO and metabolic complications of diabetes require further elucidation.

Keywords Obesity · Diabetes mellitus · FTO · Adipose · Inflammation · Adiponectin · Leptin · Insulin resistance · Glucose · Metabolism · Obesity · Adipose tissue · Visceral fat · Fat

Abbreviations

T2D	type 2 diabetes mellitus
DEXA	dual-energy X-ray absorptiometry
RT-PCR	reverse transcription polymerase chain reaction
SAT	subcutaneous adipose tissue
VAT	visceral adipose tissue
CREBP3	cAMP response element binding protein-3
SAA	serum amyloid A
TNF-α	tumor necrosis factor-alpha
MIP	macrophage inflammatory protein-1
IL	interleukin
IL-8R	interleukin-8 receptor
RBP4	retinol binding protein 4
NF-κB	nuclear factor-κB

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Introduction

Obesity plays a major role in the development of type 2 diabetes mellitus (T2D); longitudinal studies indicate abdominal obesity predicts incident cases of T2D [1–3] as do a number of adipose tissue products such as adiponectin, complement C3, and tumor necrosis factor-alpha (TNF-α) [4–10]. Genetic factors are known to explain between 40% and 60% of the population variance in total and abdominal adiposity [11–14] and contribute to the large proportion of risk to development of T2D [15]. Recently, genome-wide association studies have identified FTO as a possible gene explaining T2D, with its major impact appearing to be on body size or adiposity [16, 17]. Adipose tissue and its products are involved in the regulation of insulin sensitivity and inflammation and are thought to contribute to the decline in β-cell function resulting to hyperglycemia.

This study examined adipose tissue expression of FTO in subjects with and without T2D. We determined whether (1) there were site-specific differences between subcutaneous and visceral depots in FTO expression; (2) whether adipose tissue expression of FTO was related to insulin action, lipids, glucose, and direct measures of adiposity; and (3) whether adipose tissue FTO expression related to the expression of other adipose tissue products implicated in the pathogenesis of T2D.

Methods

Subjects were people undergoing elective laparoscopic abdominal surgery (n=16, ten females). All subjects were free of inflammation, recent sepsis, or malignant conditions. Six subjects had diet- and exercise-controlled T2D based on history (four females, two males), confirmed by clinical record documentation of fasting glucose levels exceeding 7.0 mmol/L. At screening, glucose tolerance was considered normal if the fasting glucose was less than 5.5 mol/L; nine control subjects had fasting glucose levels in the range of 4.1–4.9 mmol/L. One control subject had a fasting glucose of 5.6 mol/L; normal glucose tolerance test was confirmed normal glucose tolerance. Six subjects were receiving antihypertensive therapy (n=3 T2D; irbesartan with hydrochlorothiazide n=2, perindopril n=2, ramipril n=1, and candesartan n=1). All medications were omitted for at least 12 h prior to surgery and venous blood sampling for clinical phenotyping.

Prior to surgery, all subjects underwent detailed clinical phenotyping after a 12-h overnight fast. Weight (in kilograms) in light clothing was measured to the nearest 0.1 kg. Barefoot height was measured using a stadiometer, to the nearest centimeter; body mass index was calculated (weight/height squared; in kilograms per square meter). Waist was measured at the narrowest point between the lowest rib and the iliac crest (in centimeters). Blood pressure was taken in the supine position after 15 min of rest. Body composition was measured using dual-energy X-ray absorptiometry (DEXA; Lunar DPXL, Madison, WI, USA) to determine total fat mass and fat free mass (FFM). Central abdominal fat mass was determined using an anatomically determined region extending between the top of second and bottom of fourth lumbar vertebrae, laterally to the inner aspect of the costal margin as we have described [11, 18]. Percentage central abdominal fat represents the ratio of fat to total tissue in the central abdominal window, as we have described previously [11, 18].

Insulin action was measured by hyperinsulinemic euglycemic clamp (n=9, three with T2D), following a 12-h overnight fast. Bilateral forearm cannulation was undertaken; one retrograde and arterialized for blood collection, the other for infusion of insulin and glucose. Insulin was infused at 50 mU/kg body mass/h, which in our hands achieves steady-state levels of 110–115 mU/L. Plasma glucose was measured at 10-min intervals with adjustment of the glucose infusion rate to maintain a plasma glucose level of 5.0 mmol/L. Whole body insulin action was measured by the glucose infusion rate at steady state, calculated from the last 40 min of the clamp and expressed per FFM from the DEXA measurement.

Insulin resistance was also estimated using the homeostasis model assessment (fasting plasma glucose × fasting insulin/22.5) [19].

Paired subcutaneous and visceral adipose tissue (SAT and VAT, respectively) samples were collected intraoperatively and immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction was undertaken.

The study protocol was approved by the St. Vincent's Hospital Research and Ethics Committee and all subjects gave written informed consent.

RNA Extraction and Multiplexed Tandem PCR

RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). DNA digest was performed by a standard method using Amplification Grade DNAse I (Invitrogen, Carlsbad, CA, USA).

Gene expression in 16 SAT samples and 16 VAT samples was measured by a two-step multiplexed tandem polymerase chain reaction (MT-PCR) method [1]. Primers for the genes of interest and "non-POU domain containing, octamer binding" (NONO; NM_007363) as reference gene were designed using the modified Primer 3 software. The following primers were used: inner forward primer, AAAATGCCATACCTGAAAGAGGAACC; inner reverse primer, TGACCTGTCCACCAGATTTTCATCA; inner amplicon length 90 bp. Outer forward primer, AACTTTG

Table 1 Fasting characteristics of subjects with T2D compared to control subjects

	Diabetes mellitus n=6 (four females)	Normal glucose tolerance n=10 (six females)	p value
Age (years)	62±3	57±4	0.4
Weight (kg)	93.0±6.2	70.6±1.7	0.0006
Body mass index (kg/m^2)	35.0±3.2	25.8±0.9	0.005
Waist (cm)	114±6	88±2	0.0009
Total fat mass (kg)	43.62±7.79	25.4±2.86	0.01
Percentage of total body fat	43.7±5.6	36.3±4.2	0.32
Central abdominal fat (kg)	4.34±0.67	2.01±0.22	0.001
Percentage of central abdominal fat[a]	46.9±3.9	34.7±10.3	0.02
FFM (kg)	53.39±2.61	44.66±3.04	0.08
Glucose (mmol/L)	7.8±0.7	4.6±0.2	0.0001
HbA1C (%)	7.48±0.7	–	–
Insulin (mU/L)	20.5±3.0	12.8±0.8	0.009
Adiponectin (µg/mL)	7.7±1.7	9.9±1.2	0.3
Insulin action (GDR)[b]	35.56±12.5	58.32±4.5	0.05
HOMA-IR	7.21±1.28	2.64±0.17	0.0004
Total cholesterol (mmol/L)	4.6±0.4	5.0±0.4	0.45
HDL cholesterol (mmol/L)	1.15±0.12	1.48±0.14	0.13
LDL cholesterol (mmol/L)	2.6±0.5	3.0±0.3	0.52
Triglycerides (mmol/L)	1.8±0.5	1.2±0.2	0.28

Data presented are mean ± SEM

HOMA-IR homeostasis model assessment

[a] Percentage of total tissue mass in the central abdominal region (see the "Methods" section)

[b] Glucose disposal rate (in micromoles per minute per kilogram of FFM) under hyperinsulinemic euglycemic conditions, adjusted for FFM

CTGAATTTCATGGATCCTC; outer reverse primer, CTGCCACCGCTGACCTGT; outer amplicon length 128 bp.

First-Round Multiplexed Amplification

RNA was added to an outer primer mixture at a final concentration of 0.2 µM of each primer, MT-PCR Step 1 MasterMix (Quantace, Finchley, UK) and Step 1 additives (Quantace) in a total volume of 20 µl. Each tube was placed in a Rotor-Gene thermal cycler (RG6000, Corbett Life Science, Sydney, Australia) and heat treated as follows: 10 s at 50°C, 2 min at 55°C (reverse transcription), 5 min at 95°C (RT denaturation), and 15 cycles of 10 s at 95°C, 20 s at 60°C, and 20 s at 72°C. This completed the multiplex PCR step.

Second-Round Quantification Amplifications

The product from the multiplexed amplification (final dilution in reaction 1:75) was added to MT-PCR Step 2 MasterMix (Quantace) and Step 2 additives (Quantace) diluted in water. An aliquot of 15 µl of PCR mixture was then added to 0.1 ml PCR tubes (Corbett Life Science) containing 5 µl of inner primer mixes (0.4 µM final concentration in reaction). The tubes were loaded into a RG6000 thermal cycler and PCR was performed for 35 cycles of 1 s at 95°C, 10 s at 60°C, and 10 s at 72°C. Fluorescence was measured at the end of each 72°C extension step. Melt was performed at 72–95°C.

Biochemical Assays

All blood samples were collected after a 12-h overnight fast. Plasma glucose was measured by the oxidase method (NOVA14, Nova Biomedical, Waltham, MA, USA). Serum-free insulin and adiponectin were measured by commercially available radioimmunoassay (Linco Research, St. Charles, MO, USA). Total cholesterol, high-density lipoprotein cholesterol, and triglycerides were measured spectrophotometrically at 490 nm using enzymatic colorimetry (Roche, Basel, Switzerland). Low-density lipoprotein cholesterol was estimated by the Friedewald equation [20].

Statistical Analyses

Data were analyzed using StatView 5 (Abacus Concepts, Berkeley, CA, USA). Data are given as the mean ± standard error of the mean (SEM). Differences between sites and between groups were analyzed using Student's t tests,

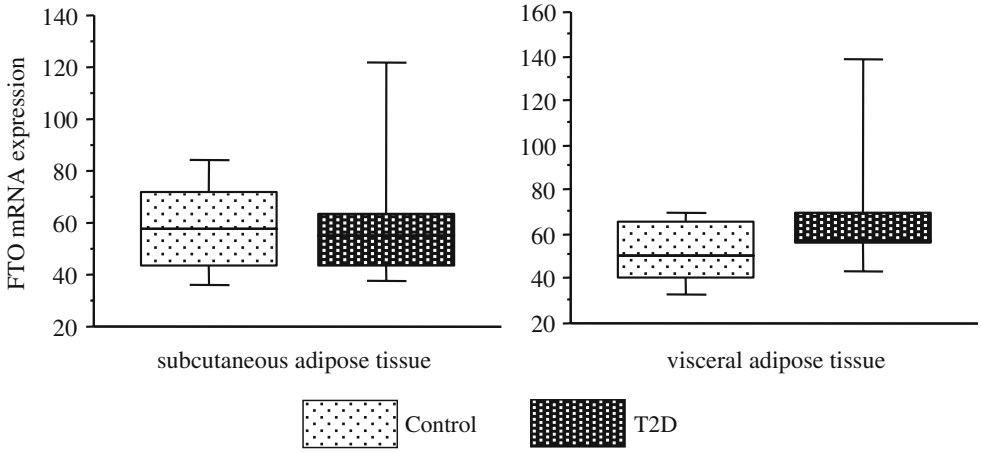

Fig. 1 FTO mRNA expression in SAT and VAT in T2D compared to age-matched control subjects

confirmed for nonparametric variables by Mann–Whitney *U* tests. Relationships between variables were examined using linear regressions, confirmed with Pearson's correlations for non-normally distributed variables; $p<0.05$ was considered significant.

Results

Subjects with T2D and controls were similar in age (Table 1). T2D subjects had greater weight, body mass index, waist, total fat mass, central abdominal fat mass, and fasting glucose (Table 1). As expected, insulin action was lower in subjects with T2D ($p=0.05$; Table 1). Adiponectin levels tended to be higher in female versus male subjects, but not significantly so (10.2±1.3 versus 7.1±1.2 μg/mL, respectively; $p=0.14$). FTO mRNA expression in SAT and VAT was not related to age (data not shown). SAT and VAT mRNA expression was similar between female and male subjects (data not shown).

Despite significantly higher fat mass, FTO mRNA expression in SAT and VAT in subjects with T2D was similar to that of controls ($p=0.71$ and $p=0.16$, respectively; Fig. 1). There were no site-specific differences in FTO mRNA expression between the SAT and VAT depots (Fig. 2).

SAT FTO mRNA expression did not relate to fasting glucose, insulin action (measured by hyperinsulinemic euglycemic clamp or by homeostasis model assessment insulin resistance [HOMA-IR]), adiponectin, total or HDL cholesterol, triglycerides, weight, body mass index, waist circumference, and total body or abdominal adiposity (data not shown).

VAT FTO mRNA expression was related to fasting glucose ($r=0.66$, $p=0.005$; Fig. 3a) and waist circumference ($r=0.52$, $p=0.03$; Fig. 3b), but not to insulin action (by hyperinsulinemic euglycemic clamp or HOMA-IR), adiponectin, total or HDL cholesterol, triglycerides, weight, body mass index, or total or abdominal adiposity (data not shown).

FTO mRNA expression in SAT and VAT was examined against the expression of adipose tissue products involved in glucose metabolism and insulin resistance in corresponding adipose tissue depots. SAT FTO mRNA expression was related to SAT TNF-α expression ($r=0.54$, $p=0.03$; Fig. 4a) and nuclear factor-κB (NF-κB) expression ($r=0.63$, $p=0.01$; Fig. 4b). No relationship was found between SAT FTO mRNA expression and SAT mRNA expression of leptin, adiponectin, macrophage inflammatory protein-1 (CCL3), IL-1β, and IL-8 (data not shown). There were no relationships found between VAT FTO mRNA expression and VAT expression of TNF-α, NF-κB, leptin, adiponectin, macrophage inflammatory protein-1, IL-1β, and IL-8 (data not shown).

Discussion

This study examined FTO gene expression in relation to insulin action and glucose metabolism in carefully phenotyped subjects with and without T2D. We found that FTO gene expression in SAT and VAT in T2D was similar to that in controls. Importantly, we found that VAT FTO gene expression was related only to fasting glucose, but not insulin action measured by hyperinsulinemic euglycemic clamp. Furthermore, there were no site-specific differences

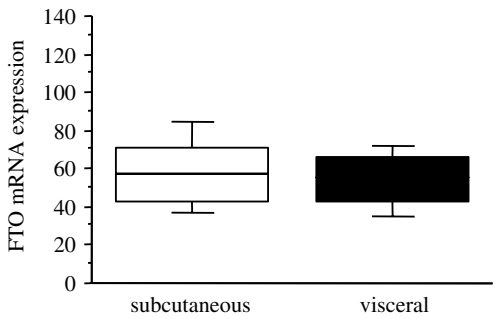

Fig. 2 Site-specific FTO mRNA expression in SAT and VAT

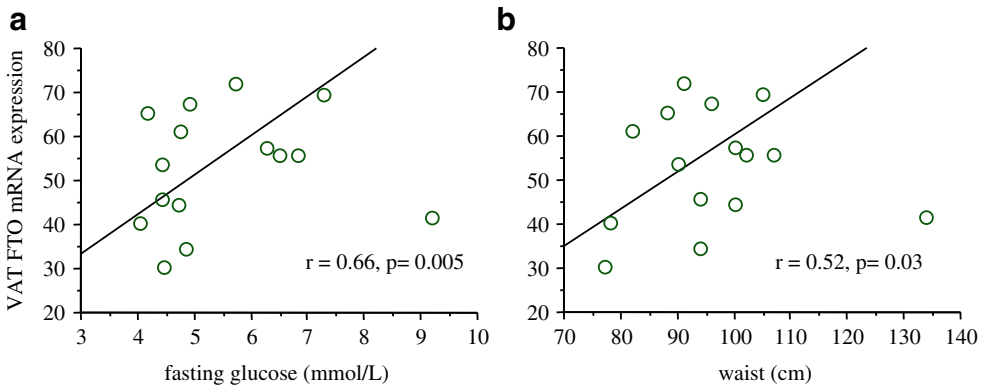

Fig. 3 VAT expression of FTO is strongly related to fasting glucose (**a**) and waist circumference (**b**)

or gender differences in FTO gene expression between the subcutaneous and visceral depots. A novel finding was the relationships between FTO mRNA expression and expression of the proinflammatory molecules TNF-α and NF-κB in SAT, the first description linking adipose tissue FTO expression and other molecules implicated in insulin resistance and diabetogenesis.

FTO is the first gene to be linked robustly to obesity in two independent genome-wide association studies [16, 17]. Importantly, the major study from Frayling et al. represents an international collaboration of over 38,000 subjects. This study identified a FTO variant strongly associated with T2D; the effect was, however, lost after adjustment for body mass index, suggestive that FTO mediated obesity, which promoted T2D [16]. A second, independent study of over 4,000 subjects confirmed the association between a FTO variant and body mass index and hip circumference [17]. A number of subsequent studies have confirmed that FTO single-nucleotide polymorphisms associate with body mass index, serum leptin levels, and waist or hip circumference [21–23]. Of interest, a gene–environment effect has been reported for FTO, suggesting that low physical activity promotes its phenotypic expression [24], supporting prior studies from the field of genetic epidemiology [18].

To date, only two studies have examined FTO gene expression in adipose tissue, with discordant results. Zabena et al. examined SAT and VAT FTO gene expression and, similar to our study, found no site-specific differences [25]. In contrast, Kloting et al. found that FTO expression was threefold higher in SAT compared to VAT [26]. Kloting et al. [26] also utilized DEXA and hyperinsulinemic euglycemic clamp to measure adiposity and insulin action. As found in our study, FTO expression did not relate to measures of adiposity or insulin action [26]. In contrast to our study which found a strong relationship between VAT FTO expression and fasting glucose, Kloting et al. found no relationship [26].

Zabena et al. also report a relationship between SAT FTO expression and SAT expression of leptin, perilipin, and visfatin [25]. In contrast, we found no association with adipose tissue leptin expression.

Strengths of our study include careful and detailed clinical phenotyping including direct measures of adiposity (rather than indirect anthropometric estimates) and measures of insulin action by hyperinsulinemic euglycemic clamp. Limitations include small numbers of subjects studied, raising the possibility of type 2 errors.

In conclusion, this study makes the novel finding that VAT FTO expression is strongly related to fasting glucose but not insulin action and that SAT expression of FTO is linked to the expression of other adipose tissue products implicated in the pathogenesis of T2D, particularly that of inflammation. The findings suggest that genes determining body size may play a deterministic role in diabetes

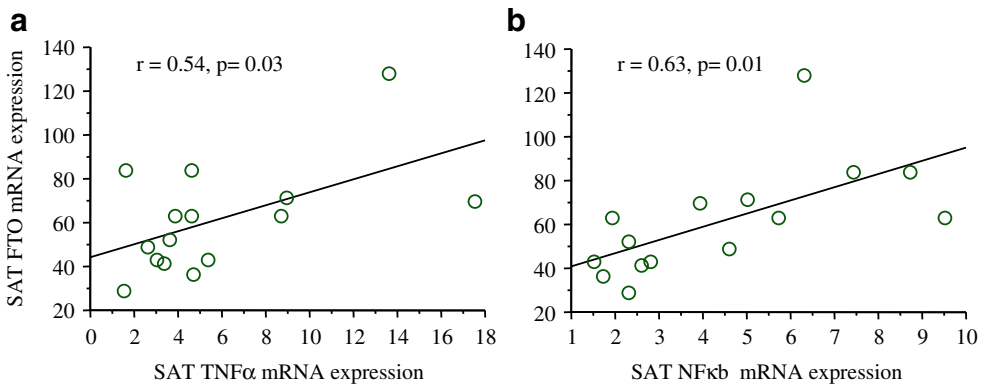

Fig. 4 SAT mRNA expression of FTO is related to mRNA expression of TNF-α (**a**) and NF-κB (**b**)

development, perhaps through inflammatory intermediaries derived from adipose tissue.

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