Subcutaneous and Visceral Adipose Tissue Gene Expression of Serum Adipokines That Predict Type 2 Diabetes

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Type 2 diabetes mellitus (T2D) is predicted by central obesity and circulating adipokines regulating inflammation. We hypothesized that visceral adipose tissue (VAT) in T2D expresses greater levels of proinflammatory molecules. Paired samples of subcutaneous (SAT) and VAT were excised at elective surgery (*n* = 16, 6 with T2D, *n* = 8 age- and gender-matched controls). Metabolic parameters were measured in the fasted state: body composition by dual-energy X-ray absorptiometry and insulin action by hyperinsulinemic–euglycemic clamp. Adipose tissue mRNA gene expression was measured by quantitative reverse transcriptase-PCR. Subjects with T2D had higher VAT expression of molecules regulating inflammation (tumor necrosis factor-α (TNFα), macrophage inflammatory protein (MIP), interleukin-8 (IL-8)). Fasting glucose related to VAT expression of TNFα, MIP, serum amyloid A (SAA), IL-1α, IL-1β, IL-8, and IL-8 receptor. Abdominal fat mass was related to VAT expression of MIP, SAA, cAMP response element–binding protein (CREBP), IL-1β, and IL-8. Insulin action related inversely to VAT complement C3 expression only. There were depot-specific differences in expression of serum T2D predictors: VAT expressed higher levels of complement C3; SAT expressed higher levels of retinol-binding protein-4 (RBP4), adiponectin, and leptin. In summary, VAT in T2D expresses higher levels of adipokines involved in inflammation. VAT expression of these molecules is related to fasting glucose and insulin action. Increased production of these proinflammatory molecules by VAT may explain the links observed between visceral obesity, insulin resistance, and diabetes risk.

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INTRODUCTION

Development of type 2 diabetes mellitus (T2D) is predicted by anthropometric estimates of total and visceral obesity (1–4). Visceral obesity and high circulating free fatty acids are implicated in the generation of lipotoxicity and impaired insulin action (5,6), contributing to the pathogenesis of T2D. Circulating markers of inflammation are increased in obesity-associated insulin resistance and T2D, associated with dysregulated adipose tissue (reviewed in refs. 7–11). Cellular mechanisms elucidated thus far indicate cross talk between cytokine-signaling and insulin-signaling pathways (reviewed in ref. 10).

Obesity-induced dysregulated adipose tissue contributes to circulating inflammation and adipokines, such that may promote diabetogenesis. Numerous studies have shown that circulating inflammatory markers and adipokines predict incident impaired glucose tolerance or T2D, including adiponectin, tumor necrosis factor-α (TNFα), complement C3 C-reactive protein, and interleukin-6 (IL-6) (refs. 11–19).

Studies of adipose tissue gene expression provide valuable information about the regulation of circulating adipokine levels as they might contribute to circulating inflammation and insulin resistance. However, many questions still remain. Are there differences in the expression of proinflammatory and prodiabetogenic adipokines in fat tissue between humans with and without T2D? As abdominal obesity predicts subsequent T2D, are there site-specific differences between visceral and subcutaneous adipose tissue (SAT) in the expression of prodiabetogenic adipokines? Does adipose tissue expression of prodiabetogenic and proinflammatory adipokines relate to reduced insulin action, higher fasting glucose or circulating adipokine levels? To examine these questions, we studied individuals with and without T2D who underwent detailed phenotypic studies of glucose metabolism, body composition, and paired subcutaneous and visceral fat biopsies. We hypothesized that, compared to controls, the adipose tissue of subjects with T2D expresses higher levels of molecules involved the regulation of inflammation.

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METHODS AND PROCEDURES

Paired subcutaneous and abdominal tissue samples were taken in 16 subjects at elective abdominal surgery for cholecystectomy or bowel surgery in cases without inflammation, recent sepsis (confirmed by normal white cell count and differential), or metastatic malignancy (10 females). Controls were age- and gender-matched and reflected the normal population for weight: healthy weight (n = 5, BMI 23.0–24.3 kg/m^2), overweight (n = 4, 26.4–29.9 kg/m^2), or obese (n = 1, BMI 31.3 kg/m^2). Six subjects had diet and exercise-controlled T2D based on history, confirmed by clinical record consultation of fasting glucose levels exceeding 7.0 mmol/l). Six subjects were receiving antihypertensive therapy (n = 3 T2D; irbesartan with hydrochlorthiazide n = 2; perindopril n = 2; ramipril n = 1, and candesartan n = 1). All medications were omitted for at least 12 h before surgery and venous blood sampling. Adipose tissue was collected by a single surgeon at the time of elective surgery. Subcutaneous fat was harvested from the periumbilical area; visceral adipose tissue (VAT) was collected from subomental fat.

All subjects underwent detailed clinical phenotyping in the fasted state (12 h, overnight). Weight (kg) was measured in light clothing to the nearest 0.1 kg. Height was measured with the subject barefoot using a stadiometer (cm); BMI was calculated (weight/height squared) (kg/m^2). Waist circumference was measured at the narrowest point between the lowest rib and the iliac crest (cm). Blood pressure was taken in the supine position after 15-min rest. Body composition was measured using dual-energy X-ray absorptiometry (Lunar DPXL, Madison, WI), to determine total body fat and fat-free mass. Central abdominal fat 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 (20,21). Visceral adiposity was also measured by computed tomography, with 4 slices taken between L2 and L4.

Insulin action was measured by euglycemic–hyperinsulinemic clamp in 9 subjects, 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 fat-free mass from the dual-energy X-ray absorptiometry measurement.

Insulin resistance was also estimated using the homeostasis model assessment (fasting plasma glucose × fasting insulin/22.5 (ref. 22). Fresh adipose tissue 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

Fresh adipose tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction was undertaken. RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN, Valencia, CA). DNA digest was performed by a standard method using Amplification Grade DNAse I (Invitrogen, Carlsbad, CA).

Multiplexed tandem PCR

Gene expression in 16 SAT samples and 16 VAT samples was measured by a two-step multiplexed tandem PCR method (23). Primers for the genes of interest and NONO (non-POU domain containing, octamer binding; NM_007363) as reference gene were designed using modified Primer 3 software (primer sequences available on request), as done previously (24,25). Given our *a priori* hypothesis that circulating inflammatory molecules that predict or are implicated in the pathogenesis of T2D are differentially expressed in adipose tissue, we selected the following genes of interest: adiponectin, TNFα, complement C3, retinol-binding protein-4 (RBP4), IL-1α, IL-1β, IL-6, IL-8, IL-8 receptor, macrophage inflammatory protein (MIP), serum amyloid A, nuclear factor-κB, cAMP response element–binding protein-3.

First round multiplexed amplification

RNA was added to an outer primer mixture at a final concentration of 0.2 μmol/l of each primer, multiplexed tandem 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 (reverse transcriptase 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 multiplexed tandem 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

Table 1 Fasting characteristics of subjects with type 2 diabetes mellitus compared to subjects with normal glucose tolerance

	Diabetes mellitus (N = 6)	Normal glucose tolerance (N = 10)	P
Age (y)	62 ± 8	57 ± 12	NS
Weight (kg)	93.0 ± 15	70.2 ± 5.8	0.001
BMI (kg/m^2)	35.0 ± 3.2	25.8 ± 0.9	0.005
Waist (cm)	114 ± 15	88 ± 8	0.001
Total body fat (kg)	42.64 ± 7.48	23.03 ± 2.53	0.008
% Total body fat	43.7 ± 12.5	37.1 ± 13.9	0.39
Central body fat (kg)	4.34 ± 0.67	2.01 ± 0.22	0.001
% Central abdominal fat	46.9 ± 3.9	34.7 ± 10.3	0.02
Fat-free mass (kg)	43.62 ± 7.79	25.40 ± 2.86	0.02
% Fat-free mass	53.37 ± 2.61	44.66 ± 3.04	NS
Glucose (mmol/l)	7.8 ± 1.8	4.6 ± 0.5	0.0002
HbA_{1c} (%)	7.48 ± 0.7	—	—
Insulin	20.5 ± 3.0	12.8 ± 0.8	0.009
Adiponectin	7.7 ± 4.2	9.9 ± 4.0	NS
Insulin action (GDR)[a]	6.4 ± 2.26	10.5 ± 0.8	0.05
Total cholesterol (mmol/l)	4.6 ± 0.4	5.0 ± 0.4	NS
HDL cholesterol (mmol/l)	1.15 ± 0.12	1.48 ± 0.14	NS
LDL cholesterol (mmol/l)	2.6 ± 0.5	3.0 ± 0.3	NS
Triglycerides (mmol/l)	1.8 ± 0.5	1.2 ± 0.2	NS
Systolic BP (mm Hg)	132 ± 3	126 ± 6	NS
Diastolic BP (mm Hg)	73 ± 4	74 ± 4	NS

BP, blood pressure; GDR, glucose disposal rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a]GDR under hyperinsulinemic–euglycemic conditions, adjusted for fat-free mass, performed in 9 subjects: 2 with diabetes mellitus, 7 controls.

Life Science) containing 5 µl of inner primer mixes (0.4 µmol/l final concentration in reaction). The tubes were loaded into an 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). 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 (26). Serum-free insulin and adiponectin were measured by commercially available radioimmunoassay (Linco Research, St Charles, MO).

Statistical analyses. Data were analyzed using StatView 5 (Abacus Concepts, Berkeley, CA). Data are given as mean ± s.e.m. Differences between sites and between groups were analyzed using Student's *t*-tests. For nonparametric variables, comparisons were made by Mann–Whitney tests. Relationships between variables were examined using linear regressions. $P < 0.05$ was considered significant.

RESULTS

Subject characteristics are shown in **Table 1**. Subjects with T2D were similar to controls for age; as expected, subjects with T2D had significantly higher weight, BMI, waist, total body fat mass, central abdominal fat mass, fat-free mass, fasting glucose ($P < 0.02$, **Table 1**). Fat mass (total and central abdominal) was approximately twofold higher in subjects with T2D compared to controls (**Table 1**). Glucose disposal rate under hyperinsulinemic–euglycemic conditions was significantly lower in T2D ($P = 0.05$, **Table 1**). Adiponectin, total-, high-density and low-density lipoprotein cholesterol, triglycerides, systolic and diastolic blood pressure were similar between subjects with T2D and controls (**Table 1**).

VAT adipokine gene expression in T2D

VAT in T2D expressed significantly higher levels of molecules involved in regulation of inflammation (TNFα, MIP, IL-8), early adipogenesis (cAMP response element–binding protein-3 (CREBP3)), and degeneration and cell death (serum amyloid A: SAA) (**Figure 1**). VAT adiponectin mRNA expression was similar between subjects with and without T2D (**Figure 1**). There was a trend for higher VAT IL-6 mRNA expression in T2D ($P = 0.09$, data not shown).

Associations between metabolic phenotypes and adipose tissue gene expression

Fasting glucose was significantly related to VAT mRNA expression of a number of molecules involved in the regulation of inflammation: TNFα ($r = 0.54$, $P = 0.04$), MIP ($r = 0.56$, $P = 0.03$), SAA ($r = 0.55$, $P = 0.04$), IL-1α ($r = 0.68$, $P = 0.03$), IL-1β ($r = 0.49$, $P = 0.05$), IL-8 receptor (IL-8R) ($r = 0.76$, $P = 0.0007$), and leptin ($r = 0.52$, $P = 0.04$) (**Figure 2**). Only SAT mRNA expression of leptin was associated with fasting glucose ($r = 0.62$, $P = 0.01$).

In multiple regression analysis, a model containing TNFα, MIP, IL-1α, and IL-8 explained 83% of the variance of fasting glucose ($P = 0.03$). The addition of total or central adiposity weakened the model (data not shown).

In a subset of subjects who had undergone hyperinsulinemic–euglycemic clamp ($n = 9$), insulin action was inversely associated with VAT mRNA expression of complement C3 ($r = -0.70$, $P = 0.05$); there was a trend toward a positive association with RBP4 expression ($r = 0.68$, $P = 0.06$). No associations were found between insulin action and VAT expression of other adipokines or proinflammatory molecules.

There was no association between fasting serum adiponectin levels and mRNA expression in SAT or VAT ($r = 0.1$ and 0.14, respectively, $P > 0.5$). Fasting insulin levels were associated with SAT expression of CREBP3 ($r = 0.55$, $P = 0.03$) and VAT expression of SAA ($r = 0.64$, $P = 0.008$) and IL-1α ($r = 0.81$, $P = 0.005$).

Total body fat mass was related to SAT expression of SAA ($r = 0.69$, $P = 0.006$) and VAT expression of C3 ($r = 0.50$, $P = 0.05$), leptin ($r = 0.59$, $P = 0.02$), MIP ($r = 0.56$, $P = 0.04$), SAA ($P = 0.81$, $P = 0.0004$), and CREBP ($r = 0.53$, $P = 0.05$). Central

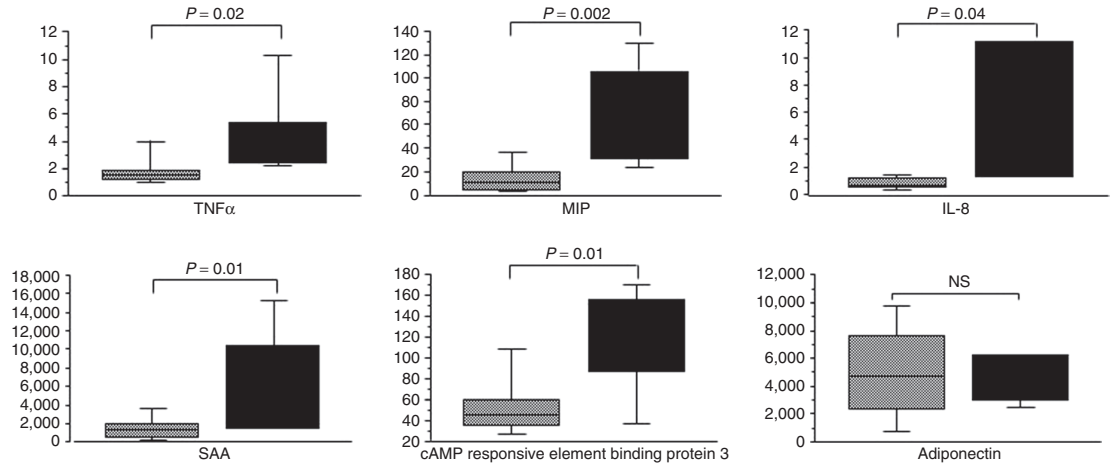
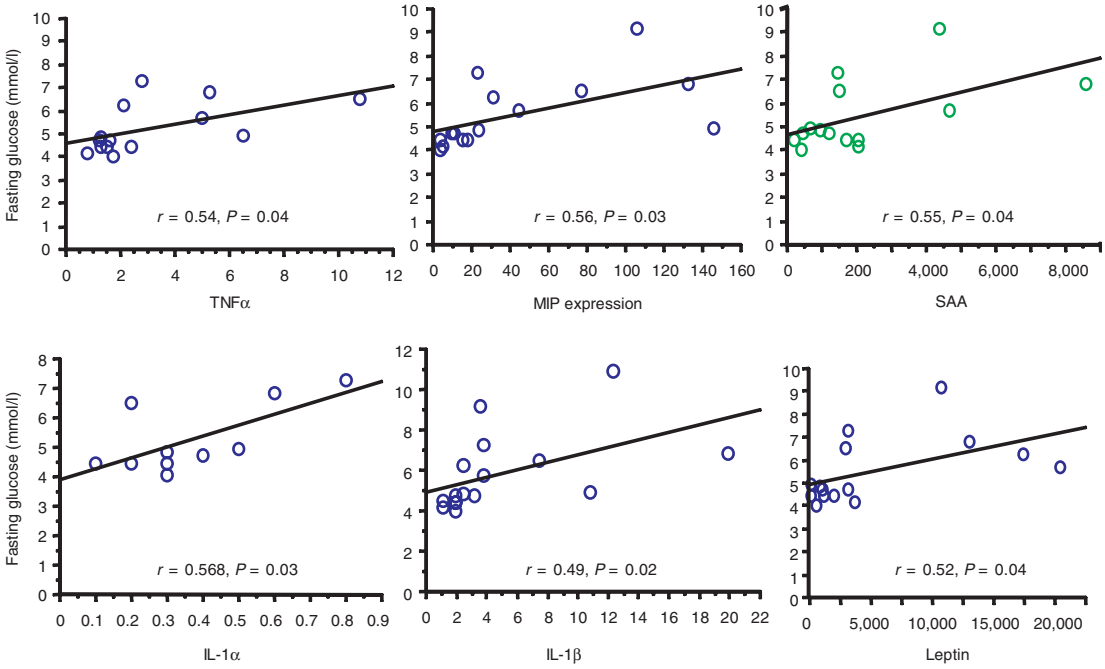


Figure 1 Visceral adipose tissue mRNA expression in subjects with type 2 diabetes mellitus compared to normal glucose tolerance. IL-8, interleukin-8; MIP, macrophage inflammatory protein; SAA, serum amyloid A; TNFα, tumor necrosis factor-α. Gray, normal glucose tolerance; black, type 2 diabetes mellitus.



[Q1]

Figure 2 Relationships between visceral adipose tissue adipokine gene expression with fasting glucose. IL, interleukin; MIP, macrophage inflammatory protein; SAA, serum amyloid A; TNFα, tumor necrosis factor-α.

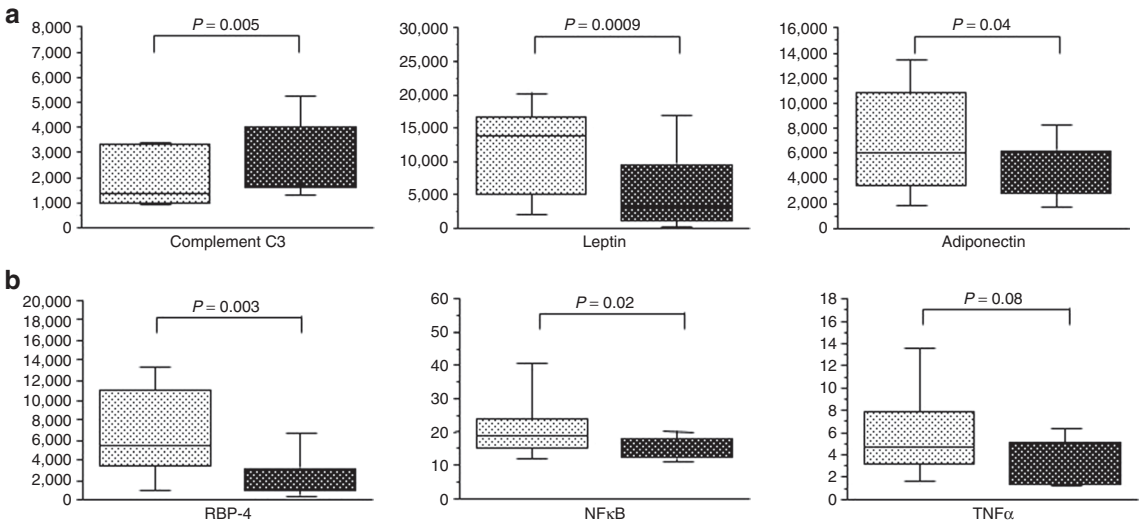


Figure 3 Site-specific differences in mRNA expression in subcutaneous and visceral adipose tissue; (**a**) known predictors of type 2 diabetes mellitus, (**b**) novel or controversial predictors of type 2 diabetes mellitus. NFκB, nuclear factor-κB; RBP4, retinol-binding protein-4; TNFα, tumor necrosis factor-α. Gray, subcutaneous; black, visceral.

abdominal fat mass was related to SAT expression of leptin (r = 0.78, P = 0.0006), SAA (r = 0.59, P = 0.02); and VAT expression of leptin (r = 0.54, P = 0.03), MIP (r = 0.57, P = 0.02), SAA (r = 0.84, P = 0.0002), CREBP (r = 0.56, P = 0.03), IL-8 (r = 0.68, P = 0.01), and IL-1β (r = 0.58, P = 0.03).

Differential adipokine gene expression between SAT and VAT

SAT, compared to VAT, had significantly higher mRNA expression for proven serum predictors of T2D (adiponectin, leptin) (**Figure 3a**) and novel or controversial serum predictors (RBP4 and nuclear factor-κB (**Figure 3b**). In contrast, VAT had higher mRNA expression of complement C3 (**Figure 3a**). Trends were seen for higher SAT mRNA expression of TNFα (**Figure 3b**, P = 0.08) and MIP (P = 0.11, data not shown). There were no site-specific differences for expression of IL-1β, IL-8, or CREBP3 (data not shown).

DISCUSSION

Circulating levels of several adipokines and proinflammatory molecules predict incident T2D in humans, including complement C3, adiponectin, TNFα, and IL-6 (refs. 11–19). In this

study, we examined the adipose tissue expression of these molecules in health and in T2D. We found that overweight-T2D subjects had higher VAT expression of molecules regulating inflammation (TNFα, MIP, IL-8), adipogenesis (CREBP3) and, possibly, cell death (SAA). We found that insulin action was strongly associated with VAT expression of complement C3. A novel finding of this study were the depot-specific differences in adipose tissue mRNA expression of circulating molecules that have been shown to predict development of T2D, with upregulation of complement C3 in VAT and adiponectin, RBP4, and nuclear factor-κB in SAT.

Few studies have examined paired SAT and VAT samples in humans to distinguish depot-specific differences. One study using a targeted approach in obese subjects reported upregulation of CCL3 (MIP) in SAT and IL-6 in VAT (27). A study of obese men found upregulated expression of proinflammatory complement pathways components in VAT compared to SAT, in particular complement C2, C3, C4, C7, and Factor D (28). The same study also found VAT complement C3 expression strongly related to fasting serum C3 (ref. 28). Prospective studies have shown fasting serum complement C3 levels predict incident T2D (16). It is plausible that greater VAT, secreting complement C3, and other proinflammatory molecules may explain (at least in part) the observational link between visceral obesity and development of T2D. The factors regulating C3 gene expression in VAT and its secretion into the circulation require further investigation.

The findings of this study suggest that subcutaneous fat depots may also contribute to the proinflammatory milieu associated with dysregulated glucose metabolism, as nuclear factor-κB and RBP4 were expressed at higher levels in SAT. Our results accord with other studies reporting higher RBP4 expression in SAT (29). In contrast, others have reported higher VAT RBP4 expression (30).

Other studies of paired VAT and SAT have reported depot-specific differences in gene expression of molecules implicated in inflammation and insulin resistance. One of the largest paired tissue sample studies to date found higher fatty acid synthase expression in VAT (31). VAT fatty acid synthase expression related to serum RBP4 and IL-6 levels and inversely with insulin action (31). A microarray study found differential expression of 347 genes in a small group of severely obese men, with higher VAT gene expression of genes involved in lipid metabolism, inflammation, and transcription (32). However, it remains unclear whether these differences are intrinsic to visceral or SAT, or responses to obesity, nutrient excess, or hyperglycemia.

There is accumulating evidence in humans of adipose tissue adaptation in a small number of intervention studies. SAT adiponectin receptor expression (R1 and R2) increased after 4 weeks of physical training (33). There was an 8–15 fold increase in SAT adiponectin gene expression over 12 months in a small severely obese cohort who lost about 50 kg after gastric bypass surgery (34). SAT adiponectin expression increased and IL-6 expression decreased after a 15-week hypocaloric intervention in obese subjects (35).

Our study considered the relationships between glucose metabolism (insulin action and fasting glucose), adipose tissue mass, and adipose tissue expression of molecules involved in systemic inflammation. Only VAT complement C3 expression related to insulin action measured by clamp. Further, SAT gene expression of a number of proinflammatory molecules related to fasting glucose. The latter finding suggests a possible contribution of SAT to the systemic inflammation that may influence β-cell function or, conversely, that elevation of blood glucose upregulates expression of proinflammatory molecules in SAT. The cross-sectional nature of our study does not allow any directional interpretation of these intriguing associations.

Strengths of this study include detailed clinical phenotyping with dual-energy X-ray absorptiometry for accurate measurement of total body and abdominal fat and measurement of insulin action by hyperinsulinemic–euglycemic clamp. A further strength is surgical tissue harvesting by a single surgeon from the same anatomical sites in all subjects, overcoming the methodological issues raised recently (36). Limitations include the cross-sectional nature of the study. The subjects with T2D were overweight or obese (as they often are) thus, our results cannot distinguish the specific associations of obesity *per se*, from those of T2D. Our study did not measure C-reactive protein and other serum inflammatory markers, which may have provided some indication of the systemic inflammation in our obese subjects that has been documented elsewhere. Nevertheless, we found no direct relationship between serum adiponectin and adipose tissue gene expression. Our sample was relatively small; however, our detailed phenotyping is not feasible for large samples.

Our study findings add to the growing literature of depot-specific adipose tissue contribution to inflammation in T2D. Further studies of adipose tissue in human intervention studies are required to establish the links between adipose tissue depot, inflammation and disturbed glucose metabolism.

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DISCLOSURE

The authors declared no conflict of interest.

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