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ORIGINAL ARTICLE

Impairments in Adipose Tissue Microcirculation in Type 2 Diabetes Mellitus Assessed by Real-Time Contrast-Enhanced Ultrasound

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BACKGROUND: In obesity and type 2 diabetes mellitus (T2D), adipose tissue expansion (because of larger adipocytes) results in reduced microvascular density which is thought to lead to adipocyte hypoxia, inflammation, and reduced nutrient delivery to the adipocyte. Adipose tissue microvascular responses in humans with T2D have not been extensively characterized. Furthermore, it has not been determined whether impaired microvascular responses in human adipose tissue are most closely associated with adiposity, inflammation, or altered metabolism.

METHODS AND RESULTS: Overnight-fasted healthy controls (n=24, 9 females/15 males) and people with T2D (n=21, 8 females/13 males) underwent a body composition scan (dual-energy X-ray absorptiometry), an oral glucose challenge (50 g glucose) and blood analysis of clinical chemistries and inflammatory markers. Abdominal subcutaneous adipose tissue microvascular responses were measured by contrast-enhanced ultrasound at baseline and 1-hour post-oral glucose challenge. Adipose tissue microvascular blood volume was significantly elevated in healthy subjects 1-hour post-oral glucose challenge; however, this effect was absent in T2D. Adipose tissue microvascular blood flow was lower in people with T2D at baseline and was significantly blunted post-oral glucose challenge compared with controls. Adipose tissue microvascular blood flow was negatively associated with truncal fat (%), glucoregulatory function, fasting triglyceride and nonesterified fatty acid levels, and positively associated with insulin sensitivity. Truncal fat (%), systolic blood pressure, and insulin sensitivity were the only correlates with microvascular blood volume. Systemic inflammation was not associated with adipose tissue microvascular responses.

CONCLUSIONS: Impaired microvascular function in adipose tissue during T2D is not conditionally linked to systemic inflammation but is associated with other characteristics of the metabolic syndrome (obesity, insulin resistance, hyperglycemia, and dyslipidemia).

Key Words: blood volume ■ diabetes mellitus, type 2 ■ insulin resistance ■ metabolism ■ microcirculation ■ obesity

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CLINICAL PERSPECTIVE

In obesity and type 2 diabetes mellitus (T2D), adipocytes become larger and there is a concomitant reduction in capillary (microvascular) density. It is thought that this reduction in microvascular density impairs the diffusion of key nutrients (eg, oxygen, glucose, and lipids) leading to adipocyte hypoxia, inflammation, and altered whole body metabolism. However, most studies in humans have focused on large vessel blood flow responses in adipose tissue and not the microcirculation, which determines nutrient exchange. As such adipose tissue microvascular responses in humans with T2D have not been extensively characterized, and more importantly, the aspects of the microcirculation (blood volume or blood flow) that are most closely associated with adiposity, inflammation, or altered metabolism remain unclear. Our findings demonstrate an impairment in both microvascular blood volume and microvascular blood flow in the adipose tissue of people with T2D. These microvascular abnormalities, in particular microvascular blood flow, were more strongly associated with the metabolic syndrome (obesity, hyperlipidemia, insulin resistance, and hyperglycemia) but not inflammation. Importantly, we found that the degree of obesity (independent of T2D) is a strong modifier of adipose tissue microvascular blood flow. Having T2D and concomitant obesity has additional detrimental effects on adipose tissue microvascular-linked dyslipidemia, hyperglycemia, and insulin sensitivity. Therefore, improving microvascular function in adipose tissue may be a novel approach to prevent obesity-related complications during T2D and the metabolic syndrome.

Adipose tissue has long been known for its capacity to store triglycerides, dispose glucose after a meal and release nonesterified fatty acids (NEFA).[1,2] More recently, it has become apparent that adipose tissue is a highly dynamic endocrine organ, capable of secreting several hormones and proinflammatory mediators that regulate appetite, energy metabolism, and insulin action.[3]

During obesity, adipose tissue expands and becomes dysfunctional. Adipose tissue expansion can occur via hypertrophy (increased adipocyte size) or via hyperplasia (increased adipocyte number), with the former being metabolically detrimental.[4] This metabolic dysfunction is partly because of increased adipocyte size without a concomitant increase in capillary number, resulting in adipose tissue hypoxia, tissue remodeling, and the recruitment and activation of macrophages.[5,6] The dysregulation of normal adipose tissue function leads to exaggerated release of NEFA's and proinflammatory cytokines into the circulation resulting in ectopic lipid deposition, low-grade chronic inflammation, and insulin resistance.[7] In addition, impaired adipose tissue blood flow may also reduce macronutrient exchange (eg, glucose and triglycerides/lipoproteins) and hormone (eg, insulin) delivery after a meal further altering the whole body metabolism.[8]

To date, most studies on human adipose tissue blood flow, such as those of Frayn et al[1,9,10] have used ^{133}Xenon washout which measures the disappearance of the isotope injected into adipose tissue where faster disappearance reflects higher blood flow in adipose tissue. Using this technique, they demonstrated that blood flow to adipose tissue increases (1) postprandially[11]; (2) in response to an oral glucose challenge (OGC)[12]; and (3) during insulin infusion (euglycemic hyperinsulinemic clamp)[13] and that these vascular responses are impaired in subjects with insulin resistance or type 2 diabetes mellitus (T2D).[8,14,15] However, given that capillaries regulate nutrient exchange, measuring vascular responses in adipose tissue at the microvascular level reveals more information about the mechanism of impaired flow.

Nutrient exchange at the microvascular level can occur via an increase in the number of microvessels (capillaries) receiving blood flow to augment the endothelial surface area, or via an increase in the rate across the microvascular bed which can increase nutrient supply. The contrast-enhanced ultrasound technique has the capacity to isolate the measurement to the microcirculation and dissect different perfusion components—in particular, microvascular blood volume (MBV, the number of capillaries being perfused), microvascular flow velocity (β, the filling rate of the capillaries being perfused), and microvascular blood flow (MBF, which is the product of MBV and β).[16,17] This technique has recently been used to demonstrate that MBV increases in adipose tissue in response to an oral glucose tolerance test and with insulin (euglycemic hyperinsulinemic clamp or intraperitoneal insulin injection).[18–21] Insulin's action to increase MBV in adipose tissue is equally blunted in both animal models and humans with T2D.[15,20] Although most studies in humans focus on the MBV,[15,18,19] the contribution of other important components of the microcirculation in adipose tissue, such as β and MBF, have been ignored. Whether adipose tissue MBV, β, and MBF are equally impaired in people with T2D or obesity has not been investigated. Finally, whether impairments in microcirculation in adipose tissue are linked to the metabolic syndrome (the degree of adiposity, inflammation, blood pressure, insulin resistance, glucotoxicity, and dyslipidemia) remains to be determined. In the present study, we sought to characterize adipose tissue microvascular responses in healthy people and those with T2D using contrast-

enhanced ultrasound, and establish whether there is an adipose tissue metabolic-linked microvascular phenotype in obesity and T2D.

METHODS

Data are stored at the Menzies Institute for Medical Research online database. Deidentified data will be made available to other researchers for purposes of reproducing the results or replicating the procedure on reasonable request to the corresponding author pending approval from the Tasmanian Health and Medical Human Research Ethics Committee.

Screening Visit

Healthy controls and people with T2D were recruited from the community (Table 1). On screening, participants were invited to the Menzies Institute for Medical Research Clinical Center to establish eligibility by using medical questionnaires. Exclusion criteria from the study were a history of smoking, current pregnancy, cardiac disease, history of severe liver disease, history of drug or alcohol abuse, or major elective surgery during the course of the study. All participants provided written informed consent. The study was performed in accordance with the Declaration of Helsinki as revised in 2008. The study protocol was approved by Tasmanian Health and Medical Human Research Ethics Committee.

Clinic Visit

After the screening visit, 24 healthy controls (9 females/15 males) and 21 subjects with T2D (8 females/13 males) were invited back after an overnight fast for testing. Participants refrained from exercise and alcohol for 48 hrs prior to testing and caffeine on the day of testing. Subjects with T2D refrained from taking their diabetes mellitus medication for 48 hours before testing. All participants were subjected to the following tests.

Body Composition

After height and weight assessment of the subjects, body composition was determined by dual-energy X-ray absorptiometry (Hologic Delphi densitometer, Hologic, Waltham, MA). Total and trunk body fat percentage were calculated using Hologic Apex System software version 4.02.

Oral Glucose Challenge

Subjects were placed in a semirecumbent position. A small polyethylene catheter was placed into the antecubital vein of 1 arm to allow blood samples to be taken. Participants rested for ≈30 minutes before undergoing a 2-hour OGC (50 g glucose) as reported previously.[22,23] Blood pressure was measured in triplicate before a fasting blood sample was taken for the measurement of serum lipids (total cholesterol, HDL [high-density lipoprotein], LDL [low-density lipoprotein], and triglycerides) and HbA1c by an accredited pathology laboratory (Royal Hobart Hospital, Tasmania, Australia). A second blood sample was taken to assess fasting blood glucose (YSI analyzer, Yellow Springs Instruments, Yellow Springs, OH), plasma insulin (ELISA, Mercodia, Sweden), and NEFA levels (Wako Pure Chemical Industries, Osaka, Japan).

Additional blood samples were collected at 15, 30, 60, 90, and 120 minutes after glucose ingestion. The blood collection tubes were immediately placed on ice for blood glucose measurement, and the remaining blood centrifuged at 2400 *g* for 10 minutes, and plasma frozen and stored at –80°C for later analysis of insulin.

The degree of insulin sensitivity was assessed using the homeostatic model assessment of insulin resistance (HOMA-IR)[24] and the quantitative insulin sensitivity check index (QUICKI)[25] using the following equations:

$$HOMA-IR = fasting\ insulin \times fasting\ glucose\ /\ 405 \quad (1)$$

$$QUICKI = 1/\begin{bmatrix} log(fasting\ insulin) \\ +log(fasting\ glucose) \end{bmatrix} \quad (2)$$

(where fasting insulin is expressed as μU/mL, and fasting glucose is expressed as mg/dL).

Elevated levels of HOMA-IR and low levels of QUICKI indicate a higher degree of insulin resistance.

Real-Time Contrast-Enhanced Ultrasound

Central (truncal) subcutaneous adipose tissue microvasculature was assessed by real-time contrast-enhanced ultrasound. A linear array transducer (L9-3) interfaced with an ultrasound system (iU22, Philips Medical Systems, Australia) was placed horizontally over the abdomen (immediately right of the umbilicus) and the beam focused on the subcutaneous adipose tissue depot. Microbubbles (Lantheus Medical Imaging, Melbourne, Australia) were diluted (1.5 mL added to 30 mL saline) and continuously infused intravenously at 2.0 to 2.6 mL/min (equating to 0.03 mL/min per kg body weight) for adipose tissue imaging. Once the systemic microbubble concentration reached steady-state (5 minutes), a high energy destructive pulse of ultrasound was transmitted to instantaneously destroy microbubbles within the volume of adipose tissue being imaged. The reflow dynamics of microbubbles into adipose tissue microvasculature was assessed in real-time at baseline and then repeated 1-hour post-OGC.

Ultrasound settings including gain settings were optimized in humans (after initial in vitro experiments) to ensure (1) a high signal-to-noise ratio and (2) to confirm imaging within the linear portion of the microbubble concentration versus acoustic intensity curve. This is achieved by quantifying tissue and microbubble acoustic intensities under a variety of settings and microbubbles infusion rates. Gain settings (90%), mechanical index (0.11 for continuous and 1.30 for flash), compression (C=30), depth and focus were identical between healthy controls and those with T2D. Background adipose tissue signal in the absence of contrast was identical between groups (healthy controls=2.1±0.3 AI; T2D=2.0±0.2 AI, mean±SD, P=0.417).

The arterial concentration of microbubbles was assessed by imaging the brachial artery at an infusion rate of 0.5 mL/min to avoid signal saturation. When the acoustic intensity was scaled up to the body weight-adjusted dose infused for adipose tissue imaging, healthy controls, and T2D had similar arterial levels (96.6±30.8 AI versus 87.4±33.2 AI, mean±SD, P=0.348).

Image Analysis

Digital image analysis was performed off-line using Qlab (Philips Medical Systems, Australia). Images were background subtracted (using the 0.5-s image) to eliminate signal from the tissue as previously reported for adipose tissue and skeletal

Table 1. Characteristics of Participants

Characteristic	Control	Type 2 Diabetes Mellitus	*P* Value
n	24	21	…
Age, y	49 (41–57)	56 (50–56)	0.157
Sex	9 females/15 males	8 females/13 males	1.000
Diabetes duration, y	…	9±5	…
Hypertension, n (%)	1 (4)	10 (48)	0.001
Hypercholesterolemia, n (%)	…	13 (62)	<0.001
Height, cm	175.8 (164.3–182.0)	172.0 (165.3–177.9)	0.460
Weight, kg	76.8 (68.6–85.6)	93.0 (81.9–104.1)	0.001
BMI, kg/m^2	25.5 (24.2–27.2)	32.6 (28.2–33.6)	<0.001
Body fat			
Total fat, %	26.9±8.6	32.1±6.2	0.030
Trunk fat, %	26.6±8.1	34.2±6.1	0.001
Fasting glucose, mmol/L	4.86 (4.44–5.27)	9.10 (7.35–11.71)	<0.001
Fasting insulin, pmol/L	42.6 (37.9–48.2)	86.7 (61.1–150.7)	<0.001
HbA1c			
%	5.35 (5.20–5.50)	7.30 (6.60–8.12)	<0.001
mmol/mol	35.0 (33.0–37.0)	56.0 (48.3–65.3)	<0.001
Insulin sensitivity indices			
HOMA-IR	1.25 (1.08–1.55)	6.17 (3.20–7.97)	<0.001
QUICKI	0.37±0.02	0.30±0.03	<0.001
Blood pressure			
SBP, mm Hg	123±11	133±14	0.006
DBP, mm Hg	76±9	85±11	0.003
Lipids			
Cholesterol, mmol/L	5.01±1.02	4.65±0.99	0.251
Triglyceride, mmol/L	0.76 (0.57–1.17)	1.76 (1.20–2.33)	<0.001
HDL, mmol/L	1.35 (1.30–1.60)	1.10 (0.98–1.33)	0.013
LDL, mmol/L	3.25±0.95	2.58±0.80	0.026
NEFA, mmol/L	0.43±0.15	0.58±0.20	0.009
Medication, n (%)			
Metformin	…	20 (95)	<0.001
Sulphonylurea	…	2 (10)	0.212
GLP-1 RA	…	2 (10)	0.212
DPP4 inhibitor	…	3 (14)	0.094
SGLT2 inhibitor	…	1 (5)	0.467
Insulin	…	2 (10)	0.212
ACEi/ARB	…	7 (33)	0.003
Diuretic	…	4 (19)	0.040
Ca^{2+} channel blocker	1 (4)	5 (24)	0.083
Statin	…	10 (48)	<0.001
Other	12 (50)	12 (57)	0.767

Data are mean±SD, or mean and interquartile range if data not normally distributed. Student *t* test (or Wilcoxon rank-sum test if data not normally distributed) was used to determine differences between continuous data. The Fisher exact test was used to compare categorical data. ACEi indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; DBP, diastolic blood pressure; DPP4, dipeptidyl peptidase 4; GLP-1 RA, glucagon-like peptide-1 receptor agonist; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; NEFA, nonesterified fatty acids; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; and SGLT2, sodium-glucose cotransporter 2.

muscle.[19,23] This time was quantified by determining the time-intensity curve using a region of interest placed inside an artery located within the subcutaneous adipose tissue.

Analysis of the data was performed identically for baseline and 1 hour after OGC. Background-subtracted acoustic intensity versus time was fitted to the function $y=A\ (1-e^{-\beta(t-tb)})$, where y is acoustic intensity at time t, tb the background time, A is plateau acoustic intensity (MBV), and β is the rate constant (a measure of microvascular refilling rate). MBF was determined by $A\times\beta$.

Inflammatory Cytokines

Plasma concentrations of TNF-α (tumor necrosis factor-α), IL (interleukin)-1β, IL-6, CRP (C-reactive protein), MCP-1 (monocyte chemoattractant protein-1), and sVCAM-1 (soluble vascular cell adhesion molecule-1) were determined using commercially available ELISA (ELISA KIT, Australia). All measurements were conducted as per manufacturer's instructions.

Statistical Methods

All statistics were performed using SigmaPlot (Systat Software, San Jose, CA). Data are presented as the means±SD after testing for normal distribution with the Kolmogorov–Smirnov test. Continuous measurements deviating from normality are expressed as a median and interquartile range. Categorical variables are reported as numbers and percentages. Student *t* test was used to compare end point measurements between controls and T2D. When data were not normally distributed, the Wilcoxon rank-sum test was performed. For categorical variables, a Fisher exact test was performed. Repeated measures 2-way ANOVA with Student–Newman–Keuls post hoc test was used to compare treatment groups over the time course of the experiment. Pearson bivariate correlations were used to evaluate associations. Spearman correlations were used to evaluate associations when data were not normally distributed. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Baseline Characteristics of Subjects

The baseline characteristics of participants are presented in Table 1. Subjects with T2D had significantly higher body weight, body mass index, total body fat (%), trunk fat (%), fasting blood glucose, fasting plasma insulin, HbA1c, HOMA-IR, systolic blood pressure, diastolic blood pressure, serum triglyceride and plasma NEFA, and lower HDL and QUICKI when compared with control participants. Interestingly, LDL was significantly lower in people with T2D compared with healthy controls and is most likely reflective of greater statin use in this group. Medications and comorbidities of participants are also shown in Table 1.

Glucose and Insulin Responses to OGC

Blood glucose levels in people with T2D were significantly higher than controls at every time point (Figure 1A). Accordingly, the 2 hour glucose area under the curve (AUC) in the T2D cohort was significantly higher ($P<0.001$) compared with controls (Figure 1B). Although fasting insulin levels were higher in the people with T2D, there were no differences in the time course of plasma insulin levels during the OGC. As such, the insulin AUC for control subjects and those with T2D were also similar (Figure 1D). This indicates that our T2D participants have some form of pancreatic dysfunction, as is the case with most people with T2D,[26] but not considered late phase T2D. Our T2D patients also do not have additional complications of T2D that are more common in late phase T2D (eg, self-reported nephropathy, retinopathy, neuropathy, or a history of heart attack or stroke).

MBV and MBF Responses to OGC

Figure 2 shows examples of adipose tissue contrast-enhanced images showing the intensity of micro-

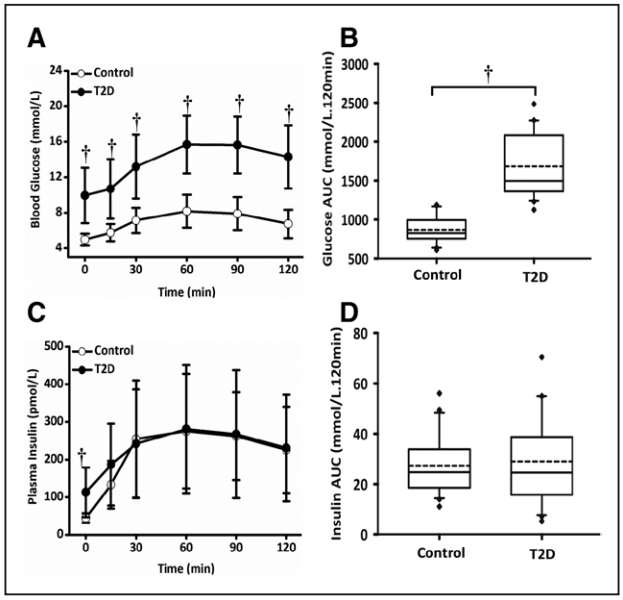

Figure 1. Blood glucose and insulin levels during the oral glucose challenge (OGC; 50 g) in control (n=24) and type 2 diabetes mellitus (T2D, n=21) people. Blood glucose (**A**) and insulin (**C**) timelines in response to an OGC, and the calculated 2-h glucose (**B**) and insulin (**D**) area under the curve (AUC) during the OGC. Data are means±SD for each group for time course data. Box plots show the 10th and 90th percentile values (whiskers), 25th and 75th percentile values (boxes), median (horizontal solid line), mean (horizontal dotted line) and outliers (circles). Repeated measures 2-way ANOVA was used to determine whether there were differences between treatment groups over the time course of the experiment, or Student *t* test (or Wilcoxon rank-sum test if data not normally distributed) was used for single point measurements. When a significant difference was found, pairwise comparisons by the Student–Newman–Keuls test was used to determine treatment differences. †$P<0.01$ vs control.

bubbles before and 1-hour post-OGC in controls (Figure 2A and 2B, respectively) and those with T2D (Figure 2D and 2E, respectively). Corresponding curve fits after a destructive pulse of ultrasound in controls (Figure 2C) and those with T2D (Figure 2F) are also presented.

Figure 3 represents the averaged adipose tissue MBV, β, and MBF values at baseline and 1 hour into a 50 g OGC in control subjects and those with T2D. There was no statistically significant difference in MBV between control and T2D subjects at baseline (Figure 3). However, adipose tissue MBV in controls was significantly elevated 1 hour after the OGC (*P*=0.020), and this response was completely absent in the people with T2D, being significantly lower than controls at the same time point (*P*=0.009; Figure 3A).

β was not significantly different between healthy and T2D at baseline or in response to the OGC (Figure 3B).

In healthy controls, MBF was not significantly elevated post-OGC. However, baseline MBF in T2D appeared lower than the control group (*P*=0.079) and was significantly lower than controls 1 hour into the OGC (*P*=0.011; Figure 3C).

Proinflammatory Cytokines

Proinflammatory cytokines measured by ELISA at baseline are shown in Figure 4. There were no statistically significant differences observed in TNF-α, IL-6, CRP, MCP-1, IL-1β, or sVCAM-1 between control subjects and those with T2D (Figure 4).

Correlates of Adipose Tissue MBV and MBF

Correlations were conducted to determine associations with adipose tissue microvascular responses (MBV and MBF) for all subjects (Table 2). These variables were classified into 4 groups: body fat composition, metabolism, blood pressure, and inflammation. Baseline MBF, but not baseline MBV, was negatively associated with truncal fat (%). However, both MBV and MBF in response to the OGC were negatively associated with truncal fat (%).

All markers of metabolism were significantly associated with baseline MBF and OGC MBF, with the exception of NEFA's which were only associated with OGC MBF. In summary, fasting blood glucose, glucose AUC during the OGC, HbA1c, fasting insulin, triglycerides, and NEFA levels were negatively associated with baseline and OGC MBF. QUICKI (a surrogate marker of insulin sensitivity) correlated positively with MBF. QUICKI was the only metabolic variable that correlated with MBV, being positively associated with the MBV response to the OGC. Fasting triglycerides and NEFA levels correlated negatively with MBF but not MBV. Systolic blood pressure correlated positively with baseline MBV. Inflammation was not associated with adipose tissue MBV or MBF at rest or in response to the OGC.

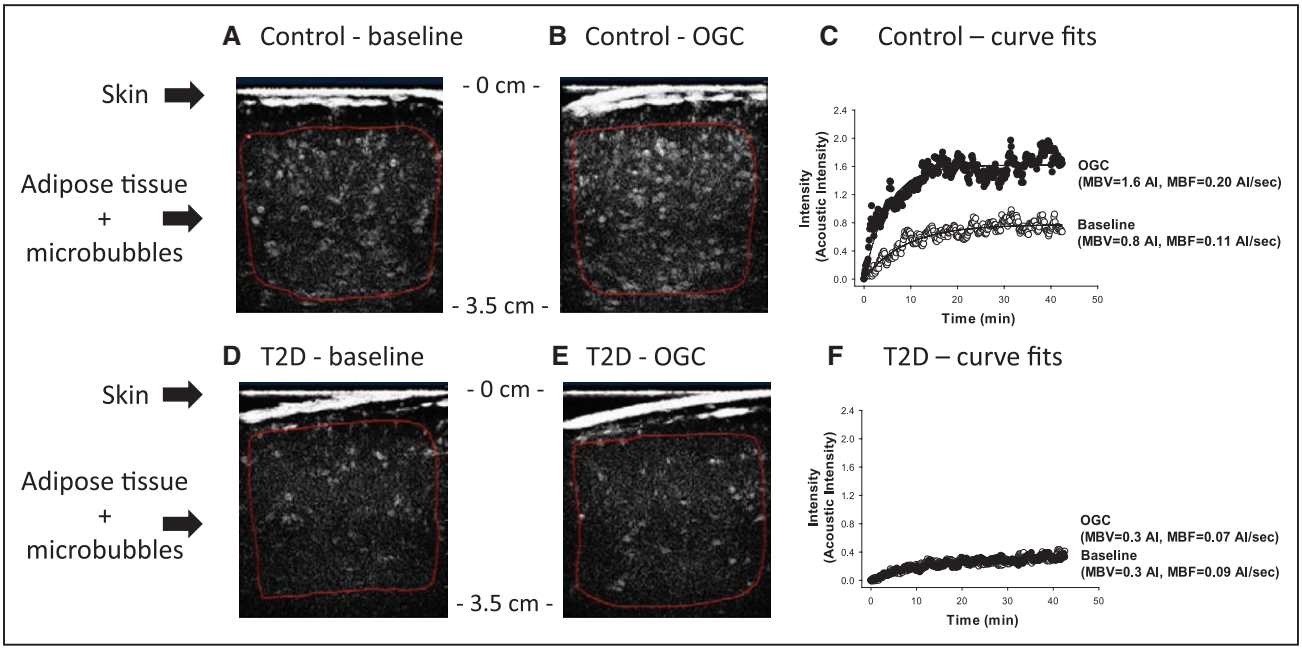

Figure 2. Examples of adipose tissue contrast-enhanced.
Images showing the intensity of microbubbles before and after a 1-h oral glucose challenge (OGC) in controls (**A** and **B**, respectively) and those with type 2 diabetes mellitus (T2D, **D** and **E**, respectively). The box in each figure represents the region of interest used for data analysis. Corresponding curve fits after a destructive pulse of ultrasound in controls (**C**) and those with T2D (**F**). MBF indicates microvascular blood flow; and MBV, microvascular blood volume.

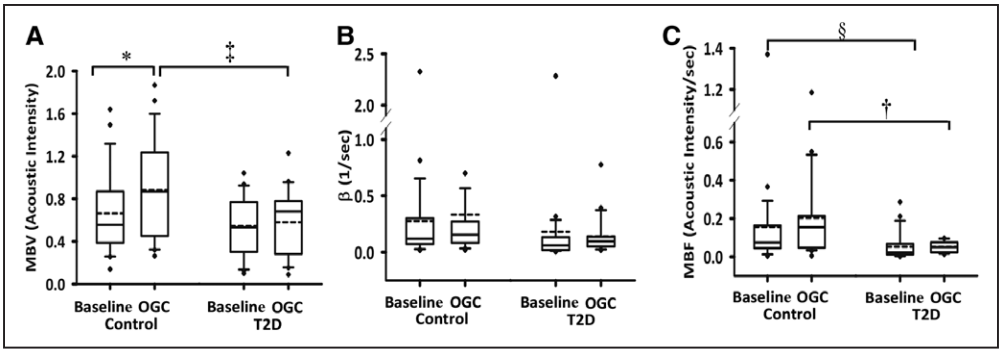

Figure 3. Adipose tissue microvascular responses to an oral glucose challenge.
Microvascular blood volume (MBV), microvascular filling rate (β), and microvascular blood flow (MBF) responses to the oral glucose challenge (OGC) in control (n=24) and type 2 diabetes mellitus (T2D, n=21) people. MBV (**A**), β (**B**), and MBF (**C**) at baseline and 1-h post-OGC. Box plots show the 10th and 90th percentile values (whiskers), 25th and 75th percentile values (boxes), median (horizontal solid line), mean (horizontal dotted line), and outliers (circles). Repeated measures 2-way ANOVA was used to determine whether there were differences between treatment groups over the time course of the experiment. When a significant difference was found, pairwise comparisons by the Student–Newman–Keuls test was used to determine treatment differences. **P*<0.05 vs control baseline; †*P*<0.05 vs control OGC; ‡*P*<0.01 vs control OGC; §*P*=0.079 vs control baseline.

When the same correlations were conducted using MBV reserve or MBF reserve (expressed as a ratio between post-OGC and baseline) on outcomes, there were 2 significant associations with MBV reserve on trunk fat (negative) and QUICKI (positive). This is not surprising given that MBV reserve and MBF reserve were not statistically different between healthy controls and T2D (*P*=0.114 and *P*=0.973, respectively). Therefore, the absolute values of MBV and MBF revealed more information about these associations.

When the correlations in the healthy cohort were investigated, most of the metabolism correlates lacked significance. Interestingly, baseline MBF remained significantly associated with total body fat (%), truncal fat (%), and fasting triglycerides levels, and the correlations were stronger in the absence of T2D (Table I in the Data Supplement).

DISCUSSION

The present study demonstrates that adipose tissue MBF at rest and in response to ingestion of a glucose load is markedly impaired with T2D. Impaired adipose MBF responses were associated with classic markers of T2D such as higher amounts of body fat, increased triglycerides and NEFA concentrations, and markers of altered metabolism such as glucose intolerance and hyperglycemia. Surprisingly, we found no difference in circulating inflammatory markers between healthy and T2D individuals. Therefore, we conclude that impaired microvascular responses in adipose tissue of people with T2D are not conditionally linked to systemic inflammation, rather, are associated with insulin resistance, hyperglycemia, and dyslipidemia.

There are very few studies investigating microvascular responses in adipose tissue in humans. Those who have assessed adipose tissue microvascular responses have focused on MBV and not MBF. Adipose MBV increases in response to insulin (euglycemic hyperinsulinemic clamp)[19,27] or a 75 g load of glucose in healthy people.[15] Adipose tissue MBV in response to a 75 g oral glucose load is impaired in T2D.[15] Our study confirms that MBV increases in response to an oral glucose load in healthy people and that this response is not apparent in people with T2D. Importantly, we have established for the first time in humans that adipose tissue MBF, rather than MBV, is more markedly impaired (by ≈70%) in T2D both basally and in response to an OGC. This is important because a growing body of literature suggests that adipose tissue is hypoxic during obesity (which is common in T2D)[6] and our data suggest, that MBF is more closely linked to adipose tissue metabolic disturbances than MBV.

Our study focused on subcutaneous adipose tissue. Whether central (visceral) adipose tissue also displays the same microvascular abnormalities is yet to be confirmed. A limitation of the study was that the T2D participants were on a variety of medications when compared with the healthy controls (Table 1). Although all diabetes mellitus-related medications were omitted for 48 hours before attending the clinic for testing, participants were still taking other medications (eg, statins and antihypertensives) which may have contributed, at least in part, to variations in blood flow responses between groups.

In the current study, people with T2D had a significantly higher amount of total body and trunk fat compared with healthy controls (Table 1). Our data indicate that the degree of adiposity, in particular truncal fat (%), was negatively associated with MBF and MBV measures, and the association was strongest with baseline MBF (Table 2). Importantly, the negative correlation

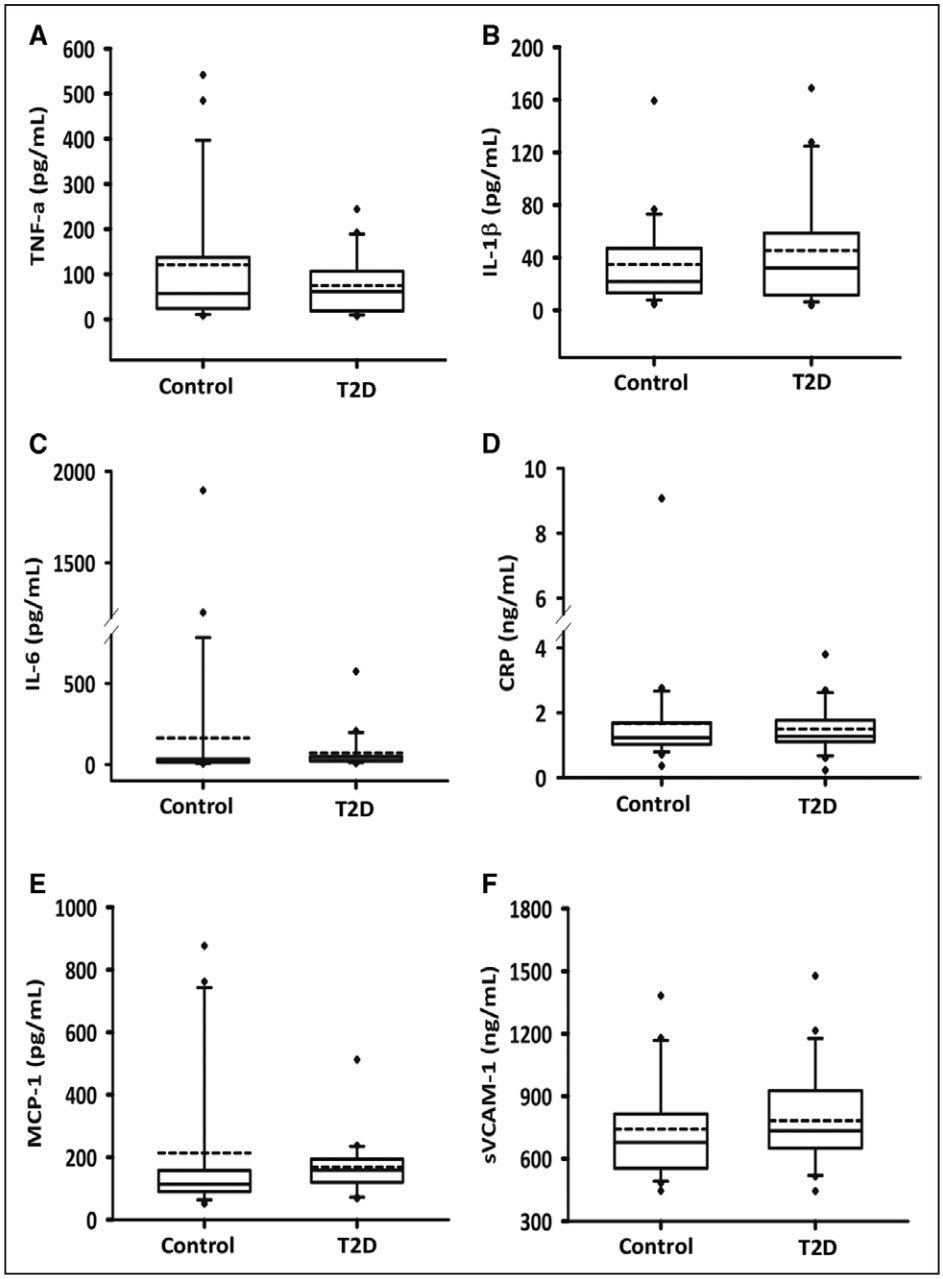

Figure 4. Circulating inflammatory markers.
Fasting plasma TNF (tumor necrosis factor)-α (**A**), IL (interleukin)-1β (**B**), IL-6 (**C**), CRP (C-reactive protein; **D**), MCP (monocyte chemoattractant protein)-1 (**E**), and sVCAM (soluble vascular cell adhesion molecule)-1 (**F**) concentrations in control (n=24) and type 2 diabetes mellitus (T2D, n=21) people. Box plots show the 10th and 90th percentile values (whiskers), 25th and 75th percentile values (boxes), median (horizontal solid line), mean (horizontal dotted line), and outliers (circles). Differences between treatment groups were assessed by Student *t* test (or Wilcoxon rank-sum test if data not normally distributed).

between body fat and MBF remained significant when assessed in the healthy group alone (Table I in the Data Supplement). Thus, it seems that the degree of obesity rather than T2D per se is significantly associated with impaired adipose tissue microvascular responses. This is not a surprising finding given that others have demonstrated a similar relationship between bulk adipose tissue blood flow and obesity[9,11,28] and it is well established that with adipocyte hypertrophy, as occurs in obesity, there is a reduction in capillary density.[29] Belcik et al[20] established that obese and insulin-resistant (*db/db*) mice have markedly larger adipocytes and impaired MBV and MBF. However, our study is the first to demonstrate a negative association between adiposity and both MBV and MBF in humans. It is intriguing that the relationship with MBF was stronger than MBV considering the reduction in capillary density might be expected to reduce MBV. We anticipated that MBV

Table 2. Correlates of Adipose Tissue MBV and MBF

	Baseline MBV		OGC MBV		MBV Reserve (Post/Baseline)		Baseline MBF		OGC MBF		MBF Reserve (Post/Baseline)	
Characteristics	r	P	r	P	r	P	r	P	r	P	r	P
Body fat												
Total fat, %	0.129	0.403	−0.221	0.149	−0.251	0.096	−0.279	0.067	−0.286	0.060	0.045	0.766
Trunk fat, %	0.193	0.209	−0.298*	0.049*	−0.369*	0.013*	−0.429*	0.004*	−0.381*	0.011*	0.092	0.546
Metabolism												
Fasting glucose, mmol/L	0.016	0.920	−0.286	0.060	−0.289	0.054	−0.362*	0.016*	−0.367*	0.015*	0.030	0.842
Glucose AUC, mmol/Lx120min	−0.092	0.551	−0.240	0.116	−0.156	0.305	−0.424*	0.004*	−0.376*	0.012*	0.084	0.583
Fasting insulin, pmol/L	0.047	0.759	−0.278	0.068	−0.243	0.107	−0.453*	0.002*	−0.374*	0.012*	0.072	0.635
HbA1c (%)	−0.041	0.792	−0.180	0.241	−0.154	0.310	−0.327*	0.031*	−0.398*	0.008*	−0.028	0.852
QUICKI	−0.037	0.809	0.328*	0.030*	0.306*	0.041*	0.498*	0.001*	0.480*	0.001*	−0.041	0.788
Fasting serum triglyceride, mmol/L	0.008	0.956	−0.279	0.067	−0.233	0.124	−0.499*	0.001*	−0.302*	0.046*	0.141	0.353
Fasting plasma NEFA, mmol/L	0.098	0.525	−0.116	0.452	−0.156	0.306	−0.226	0.139	−0.322*	0.033*	−0.120	0.430
Blood pressure												
SBP, mm Hg	0.335*	0.026*	−0.080	0.608	−0.169	0.264	0.058	0.707	−0.021	0.890	−0.070	0.646
DBP, mm Hg	0.139	0.365	−0.159	0.303	−0.030	0.842	−0.205	0.182	−0.065	0.672	0.096	0.531
Inflammation												
TNF-α, pg/mL	0.106	0.493	0.003	0.985	−0.104	0.495	−0.087	0.575	−0.120	0.436	−0.071	0.644
IL-1β, pg/mL	0.025	0.871	−0.059	0.703	−0.051	0.740	0.124	0.419	−0.109	0.478	−2.317	0.064
IL-6, pg/mL	0.136	0.378	−0.260	0.089	−0.270	0.072	−0.020	0.896	0.065	0.672	0.047	0.756
CRP, ng/mL	0.111	0.471	−0.080	0.604	−0.054	0.722	−0.065	0.675	−0.098	0.525	0.087	0.568
MCP-1, pg/mL	0.118	0.443	−0.129	0.403	−0.168	0.269	−0.209	0.171	−0.206	0.063	−0.052	0.734
sVCAM-1, ng/mL	0.006	0.969	0.219	0.153	0.156	0.305	−0.030	0.843	0.098	0.525	0.194	0.200

Data from controls and those with type 2 diabetes mellitus combined. Pearson correlation was used between normally distributed variables. Spearman correlation was used if any of the variables were not normally distributed. Reserve refers to OGC response divided by baseline response. AUC indicates area under the curve; CRP, C-reactive protein; DBP, diastolic blood pressure; IL, interleukin; MBF, microvascular blood flow; MBV, microvascular blood volume; MCP, monocyte chemoattractant protein; NEFA, nonesterified fatty acids; OGC, oral glucose challenge; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; sVCAM, soluble vascular cell adhesion molecule; and TNF, tumor necrosis factor.

*Significant correlations.

would be lower in people with T2D and were surprised to find that their baseline MBV were similar to controls. This was also similarly observed by Tobin et al.[15] We have not conducted histology to assess the capillary density and as such can only speculate that either (1) the capillary density was similar between controls and T2D or (2) there were fewer capillaries, but a greater proportion were open at baseline in T2D. Given that we found a positive association between systolic blood pressure and baseline MBV, one possibility is that the higher systolic blood pressure observed in T2D helps capillary patency at rest leaving less capillaries available for recruitment during the OGC. However, the greater reduction in MBF may have significant implications for the ability of adipose tissue to rapidly clear postprandial nutrients such as triglycerides/lipoproteins in the obese state.

It is thought that as the adipocyte undergoes hypertrophy, insufficient microvascular blood supply to the adipocyte leads to hypoxia, macrophage recruitment, and conversion of macrophages from an inactive state (M2) to an active state (M1) in which they are reported to release TNF-α, IL-6, IL-1β, and MCP-1.[30,31] Previous work has also demonstrated that activated macrophages are present in high numbers in subcutaneous adipose tissue of obese individuals and that weight loss decreases the number of activated macrophages.[32] We reasoned that the marked reduction in adipose tissue MBF in our T2D participants would be associated with a proinflammatory phenotype. However, we found no association between adipose tissue microvascular responses and inflammation. Further, we found no evidence of a systemic proinflammatory state in our T2D cohort. This was confirmed by measuring systemic levels of well-known proinflammatory mediators (TNF-α, IL-6, MCP-1, CRP, and IL-1β) and an additional marker of vascular inflammation (sVCAM). Tam et al[33] have reported that diet-induced (28-day dietary

intervention) weight gain and insulin resistance occurs in the absence of a significant inflammatory state in humans. Other work suggests that inflammation is not conditionally linked with obesity-mediated insulin resistance[34,35] and our current findings support this lack of association. However, we did not measure adipose tissue levels of proinflammatory markers, or conduct histology on adipose tissue to assess the degree of macrophage recruitment and activation, or assess adipose tissue oxygenation. We also have unpublished data in the high-fat and cafeteria-diet fed rat models (which are obese and insulin resistant) demonstrating that inflammation is not elevated (Aascha Brown, PhD, unpublished data, 2017). We may have seen inflammation in our study if we had recruited an older cohort or those with a higher amount of adiposity. Nevertheless, in our study, we found no evidence of an adipose tissue microvascular-linked proinflammatory state in people with T2D.

Given that we did not observe a microvascular-linked proinflammatory state in our T2D cohort, we next wanted to determine whether impairments in microvascular responses in adipose tissue were related to glucotoxicity and dyslipidemia, which are known to be associated with T2D. At baseline and in response to the OGC, MBF was significantly correlated with all insulin sensitivity/glucoregulatory function measures (fasting glucose, OGC AUC, fasting insulin, HbA1c, and QUICKI). In contrast, only QUICKI was associated with MBV. Belcik et al[20] demonstrated that both MBV and MBF correlated negatively with fasting blood glucose and glucose AUC after an intraperitoneal insulin challenge in obese, insulin-resistant mice. Increases in skeletal muscle MBV in response to insulin is important for muscle glucose disposal because it helps deliver glucose to the myocyte.[36–38] Here, we demonstrate that MBF, rather than MBV in adipose tissue may be more important for glucoregulatory function and insulin sensitivity. Rates of glucose uptake in adipose tissue after a meal are smaller than those of skeletal muscle, so it is uncertain whether the improvement in adipose tissue MBF in healthy people after the glucose load promotes glucose uptake or whether excess circulating glucose in the people with T2D impairs microvascular function in adipose tissue. We did not measure rates of glucose uptake in the adipose tissue bed (arteriovenous glucose difference×flow or with isotopic glucose tracers) which will be required in future experiments to help address this question.

NEFA and triglycerides are reported to be negatively associated with total adipose tissue blood flow.[8,14] Our study reports for the first time that MBF in adipose tissue has a similar negative association with NEFA and triglyceride levels. High blood viscosity because of elevated triglyceride levels (ranging from ≈0.2 to ≈10 mmol/L by intralipid infusion) has been demonstrated to affect coronary microvascular responses to hyperemia.[39] Although people with T2D in the current study had elevated triglyceride levels, this increase (1.89±0.20 mmol/L) is on the low end of the triglyceride blood viscosity range reported by Rim et al[39] and as such is unlikely to affect blood viscosity in our T2D subjects. However, direct assessment of blood viscosity would be necessary to exclude this possibility. The direction of the association between triglycerides and MBF is currently not known; however, our data has implications for impaired microvascular responses in adipose tissue in the involvement of dyslipidemia and ectopic fat accumulation.

In summary, our findings demonstrate an impairment in both MBV and MBF in adipose tissue of people with T2D. The degree of obesity (independent of T2D) is a strong modifier of adipose tissue MBF. However, there was only one association between microvascular responses and other metabolic parameters (blood glucose, insulin sensitivity, and lipid profile) in the healthy group alone suggesting that T2D with concomitant obesity has an additional detrimental impact on adipose tissue microvascular-linked lipidemia and glycemia, but not systemic inflammation. However, we cannot ascertain the direction of these associations and further experiments will help characterize the cause-and-effect. Improving microvascular function in adipose tissue may be a novel approach to prevent pathogenesis of obesity-related complications such as insulin resistance, dyslipidemia, and glucotoxicity.

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Drs Keske, Rattigan, and Richards were responsible for the conception and design of the research. D. Hu, Dr Russell, D. Remash, Dr Greenaway, Dr Premilovac, Dr Squibb, and Dr Keske performed the experiments. D. Hu and Dr Keske

analyzed the data and drafted the article. All authors contributed to writing the article. D. Hu and Dr Keske are the guarantors of this work and, as such, had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. We would like to specially thank Drs James E. Sharman Kazuaki Negishi, Faraz Pathan, and Mark Nolan from the Menzies Institute for Medical Research for their help with intravenous cannulations for the study.

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Disclosures

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Impairments in Adipose Tissue Microcirculation in Type 2 Diabetes Mellitus Assessed by Real-Time Contrast-Enhanced Ultrasound

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SUPPLEMENTAL MATERIAL

Hu et al. Impairments in Adipose Tissue Microcirculation in Type 2 Diabetes Assessed by Real-Time Contrast-Enhanced Ultrasound

Supplemental Table 1: Correlations of adipose tissue MBV and MBF in healthy controls.

Characteristics	Baseline MBV		OGC MBV		MBV Reserve (post/baseline)		Baseline MBF		OGC MBF		MBF Reserve (post/baseline)	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
Body fat												
Total fat (%)	-0.065	0.764	-0.054	0.807	-0.079	0.719	**-0.454**	**0.029**	-0.145	0.504	0.254	0.228
Trunk fat (%)	-0.029	0.894	-0.050	0.822	-0.002	0.993	**-0.504**	**0.014**	-0.140	0.518	0.295	0.158
Metabolism												
Fasting glucose (mmol/L)	0.273	0.205	-0.047	0.831	-0.104	0.637	0.200	0.357	0.258	0.231	0.064	0.762
Glucose AUC (mmol/L.120min)	-0.055	0.799	-0.088	0.689	0.094	0.669	0.132	0.542	0.146	0.501	-0.033	0.876
Fasting insulin (pmol/L)	0.054	0.802	-0.071	0.743	0.001	0.992	-0.403	0.056	-0.055	0.799	0.233	0.269
HbA1c (%)	0.117	0.588	0.232	0.288	0.100	0.650	0.129	0.551	-0.020	0.926	-0.247	0.241
QUICKI	-0.067	0.761	0.132	0.549	0.020	0.928	0.258	0.231	-0.022	0.919	-0.247	0.241

Fasting serum triglyceride (mmol/L)	-0.011	0.959	-0.132	0.542	-0.095	0.653	**-0.508**	**0.014**	-0.180	0.407	0.317	0.130
Fasting plasma NEFA (mmol/L)	0.003	0.987	0.029	0.896	0.053	0.809	-0.341	0.110	-0.347	0.103	-0.144	0.496
Blood pressure												
SBP (mmHg)	0.311	0.149	-0.120	0.584	-0.158	0.470	0.163	0.452	0.095	0.663	0.006	0.976
DBP (mmHg)	0.133	0.539	-0.118	0.592	0.076	0.730	-0.106	0.623	0.046	0.830	0.289	0.167
Inflammation												
TNF-α (pg/mL)	-0.191	0.379	-0.102	0.640	-0.079	0.709	-0.292	0.173	-0.137	0.527	0.046	0.828
IL-1β (pg/mL)	0.088	0.686	0.032	0.883	-0.064	0.762	-0.069	0.750	-0.276	0.200	-0.314	0.133
IL-6 (pg/mL)	0.116	0.595	0.058	0.788	-0.124	0.558	0.085	0.696	0.307	0.151	0.258	0.220
CRP (ng/mL)	0.382	0.071	0.019	0.930	-0.097	0.647	0.115	0.598	-0.133	0.539	-0.114	0.592
MCP-1 (pg/mL)	0.114	0.601	-0.192	0.376	-0.343	0.100	-0.166	0.444	-0.208	0.338	-0.010	0.963
sVCAM-1 (ng/mL)	0.267	0.215	0.231	0.070	0.055	0.796	0.144	0.507	0.334	0.117	0.261	0.215

Pearson's correlation was used between normally distributed variables. Spearman correlation was used if any of the variables were not normally distributed. Bold indicate significant correlations. Reserve refers to OGC response divided by baseline response.