

Insulin-mediated suppression of lipolysis in adipose tissue and skeletal muscle of obese type 2 diabetic men and men with normal glucose tolerance

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Abstract

Aims/hypothesis Impaired regulation of lipolysis and accumulation of lipid intermediates may contribute to obesity-related insulin resistance and type 2 diabetes mellitus. We investigated insulin-mediated suppression of lipolysis in abdominal subcutaneous adipose tissue (AT) and skeletal muscle (SM) of obese men with normal glucose tolerance (NGT) and obese type 2 diabetic men.

Methods Eleven NGT men and nine long-term diagnosed type 2 diabetic men (7 ± 1 years), matched for age (58 ± 2 vs 62 ± 2 years), BMI (31.4 ± 0.6 vs 30.5 ± 0.6 kg/m²) and $\dot{V}O_{2\max}$ (28.9 ± 1.5 vs 29.5 ± 2.4 ml kg⁻¹ min⁻¹) participated in this study. Interstitial glycerol concentrations in AT and SM were

assessed using microdialysis during a 1 h basal period and a 6 h stepwise hyperinsulinaemic–euglycaemic clamp (8, 20 and 40 mU m⁻² min⁻¹). AT and SM biopsies were collected to investigate underlying mechanisms.

Results Hyperinsulinaemia suppressed interstitial SM glycerol concentrations less in men with type 2 diabetes ($-7\pm 6\%$, $-13\pm 9\%$ and $-27\pm 9\%$) compared with men with NGT ($-21\pm 7\%$, $-38\pm 8\%$ and $-53\pm 8\%$) ($p=0.014$). This was accompanied by increased circulating fatty acid and glycerol concentrations, a lower glucose infusion rate (21.8 ± 3.1 vs 30.5 ± 2.0 μmol kg body weight⁻¹ min⁻¹; $p<0.05$), higher hormone-sensitive lipase (HSL) serine 660 phosphorylation, increased saturated diacylglycerol (DAG) lipid species in the muscle membrane and increased protein kinase C (PKC) activation in type 2 diabetic men vs men with NGT. No significant differences in insulin-mediated reduction in AT interstitial glycerol were observed between groups.

Conclusions/interpretation Our results suggest that a blunted insulin-mediated suppression of SM lipolysis may promote the accumulation of membrane saturated DAG, aggravating insulin resistance, at least partly mediated by PKC. This may represent an important mechanism involved in the progression of insulin resistance towards type 2 diabetes.

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Johan W. E. Jocken and Gijs H. Goossens contributed equally to this study.

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List of abbreviations

AT Adipose tissue
ATGL Adipose triacylglycerol lipase
DAG Diacylglycerol
GIR Glucose infusion rate

G0S2	G0/G1 switch regulatory protein 2
HSL	Hormone sensitive lipase
NGT	Normal glucose tolerance
PKC	Protein kinase C
PLIN1	Perilipin 1
SM	Skeletal muscle
TAG	Triacylglycerol

Introduction

Skeletal muscle (SM) lipid accumulation, and more specifically the accumulation of lipid metabolites, is strongly associated with the development of insulin resistance in the obese or obese type 2 diabetic state [1]. There are several bioactive lipid metabolites that may influence muscle insulin sensitivity, including long-chain acyl-CoA [2], ceramides [3, 4] and diacylglycerol (DAG) [5]. Although DAG is only a small fraction of the intramuscular lipid pool, it has potent biological effects and is formed during intracellular lipolysis. DAG is an important second messenger involved in intracellular signalling and may have detrimental effects on insulin action through its ability to activate conventional (α , β_1 , β_2 , and γ) and novel (δ , ϵ , θ , η) protein kinase C (PKC) isoforms in muscle [6, 7]. On activation, PKCs are redistributed within the cell, such that the membrane-associated kinase is considered to be the active form. Importantly, it seems that cellular localisation and composition of DAG rather than the absolute amount of DAG may be of major importance in insulin resistance [8].

Lipid overflow from adipose tissue (AT) is considered to play a significant role in the development of fat accumulation and insulin resistance [9]. Indeed, the obese insulin-resistant or obese type 2 diabetic state is characterised by increased circulating concentrations of NEFA and/or triacylglycerol (TAG). This increased lipid supply to SM may promote ectopic fat accumulation [10]. Insulin sensitivity of AT lipolysis is normal [11, 12] or slightly impaired in obese individuals [13, 14]. We have previously shown that in long-term diagnosed obese type 2 diabetic men with no compensatory hyperinsulinaemia, the rate of appearance of plasma NEFA is elevated compared with obese men with normal glucose tolerance (NGT) [15]. These data indicate resistance to the antilipolytic effects of insulin, which may significantly contribute to increased circulating NEFA concentrations in this population.

Nevertheless, NEFA generated by intramuscular TAG hydrolysis may also be of importance [16]. We have recently demonstrated intrinsic disturbances in SM lipolysis, including an imbalance of adipose TAG lipase (ATGL) relative to hormone-sensitive lipase (HSL) activity in the obese insulin-resistant state [17, 18], contributing to muscle lipid

accumulation and the pathogenesis of insulin resistance and type 2 diabetes [19]. The hormonal regulation of SM lipolysis is still not completely elucidated [20, 21]. For instance, findings in initial studies that measured interstitial glycerol levels in vivo suggested a lower [21] or higher [20] antilipolytic effect of insulin in SM compared with AT. However, investigations in which the fractional release of glycerol was assessed in situ showed no inhibitory effect of insulin on SM lipolytic activity [21–23].

In the present study, we investigated insulin-mediated suppression of abdominal subcutaneous AT and SM lipolysis during a three-step hyperinsulinaemic–euglycaemic clamp in patients with long-standing type 2 diabetes compared with men with NGT. Careful matching of the type 2 diabetic patients with NGT controls was performed with respect to age, body composition and physical fitness to determine the independent effect of type 2 diabetes (e.g. hyperglycaemia) on insulin-mediated inhibition of tissue lipolysis. AT and SM biopsy analyses were performed to obtain detailed information about gene expression and lipid composition in the cytosolic and muscle membrane fractions of men with type 2 diabetes and NGT.

Methods

Participants Eleven individuals with NGT and nine with type 2 diabetes matched for age (58 ± 2 vs 62 ± 2 years), BMI (31.4 ± 0.6 vs 30.5 ± 0.6 kg/m²) and $\dot{V}O_{2\max}$ (28.9 ± 1.5 vs 29.5 ± 2.4 ml kg⁻¹ min⁻¹) participated in the study. Type 2 diabetic participants had been diagnosed for at least 5 years and were not using insulin treatment. Men with NGT had a fasting plasma glucose (FPG) < 6.1 mmol/l (or < 5.6 mmol/l where there was a family history of type 2 diabetes) and a 2 h plasma glucose < 7.8 mmol/l. All participants gave written informed consent. The Medical–Ethical Committee of Maastricht University approved the study protocol.

Study design All participants were asked to refrain from alcohol and strenuous exercise for a period of 48 h before the study days. Blood-glucose and lipid-lowering medication (metformin $n=4$, simvastatin $n=3$) were withdrawn 3 days prior to the study days and participants were asked to maintain their habitual dietary intake throughout the study. Individuals came to the laboratory by car or public transport in the morning after an overnight fast and were in the supine position for the study. Insulin-mediated suppression of lipolysis in the abdominal subcutaneous AT and the gastrocnemius muscle was investigated using microdialysis, as described below. One week after the clamp, abdominal subcutaneous AT and SM biopsies were taken, as described in more detail below.

Microdialysis On arrival, two microdialysis catheters (CMA 60; CMA Microdialysis, Stockholm, Sweden) were placed in abdominal subcutaneous AT 6–8 cm to either side of the umbilicus, and two microdialysis probes were placed in the medial part of the gastrocnemius muscle of both legs after local anaesthesia (Xylocaine 2% without adrenaline [epinephrine]; AstraZeneca, Zoetermeer, the Netherlands) and under sterile conditions. Thereafter, 90 min were allowed for recovery of the tissue from the insertion trauma. After insertion, the two probes were perfused with Ringer solution (Baxter, Utrecht, the Netherlands) at a flow rate of 0.3 $\mu\text{l}/\text{min}$ (one in AT and one in SM). Microdialysate was collected from these sites in fractions every 30 min for the measurement of interstitial glycerol, glucose, lactate and pyruvate concentrations (CMA 600 Microdialysis Analyzer, Pronexus Analytical, Stockholm, Sweden). The two contralateral probes were perfused with Ringer solution supplemented with 50 mmol/l ethanol at a flow rate of 5.0 $\mu\text{l}/\text{min}$. Microdialysate was collected from these sites in fractions every 15 min to assess the ethanol outflow/inflow ratio as an indicator of local nutritive blood flow [24]. Ethanol concentrations were measured spectrophotometrically at 340 nm using a standard ethanol assay kit (176290; Boehringer, Mannheim, Germany).

Three-step hyperinsulinaemic–euglycaemic clamp After insertion of the microdialysis probes, two cannulas were inserted. Arterialised blood was obtained through a 20-gauge cannula inserted retrogradely into a superficial dorsal hand vein. The hand was warmed in a hot box (air circulating at 60°C) to achieve adequate arterialisation [25]. In the contralateral arm, a second cannula was introduced anterogradely in an antecubital vein of the forearm for the infusion of 20% (wt/vol.) glucose (IVAC560 pump, IVAC, San Diego, CA, USA) and insulin (Actrapid, Novo Nordisk Farma, Alphen aan den Rijn, the Netherlands) using a Harvard pump (Plato, Diemen, the Netherlands). A small amount of blood was drawn from the dorsal hand vein every 5 min throughout the clamp to determine glucose concentrations (EML 105, Radiometer, Copenhagen, Denmark). After the collection of three baseline samples (at –30 min, –15 min and 0 min), a three-step primed hyperinsulinaemic–euglycaemic clamp was started with infusion of 8 (0–120 min), 20 (120–240 min) and 40 $\text{mU m}^{-2} \text{min}^{-1}$ (240–360 min) insulin. The amount of glucose infused was adjusted to maintain euglycaemia at baseline glucose concentrations (5.5 ± 0.1 vs 8.6 ± 0.4 mmol/l in men with NGT and type 2 diabetes, respectively).

Plasma glucose (ABX Pentra Glucose HK CP, Radiometer, Copenhagen, Denmark), NEFA (NEFA C kit, Wako Chemicals, Neuss, Germany) and glycerol (EnzyPlus Glycerol kit, DiffChamp, Västra Frölunda, Sweden) concentrations were determined with commercially available kits using standard enzymatic methods. Plasma insulin concentrations

were measured by a specific double-antibody radioimmunoassay for human insulin (Linco Research, St Charles, Missouri, USA).

Abdominal subcutaneous AT and SM biopsies After an overnight fast an abdominal subcutaneous AT biopsy was collected 6–8 cm lateral from the umbilicus and a vastus lateralis muscle biopsy was obtained under local anaesthesia (Xylocaine) using the Bergström needle biopsy technique [26]. Tissues were snap-frozen in liquid nitrogen and immediately stored at –80°C until further analysis.

Gene-expression analysis Total RNA was extracted from frozen AT and SM specimens, as described before [17, 18]. The expression of genes involved in the lipolytic pathway (*ATGL* [also known as *PNPLA2*]; *HSL* [also known as *LIPE*]; *ABHD5*, encoding abhydrolase domain containing 5 (*ABHD5*); *G0S2*, G0/G1 switch regulatory protein 2 (*G0S2*); *PLIN1* and *PLIN2*, encoding perilipin 1 (*PLIN1*) and 2; and *AQP7*, aquaporin 7), glucose transport (*GLUT4* [also known as *SLC2A4*]), insulin signalling (*IRS1* and *IRS2*; *PDE3B*, phosphodiesterase 3B), fatty acid transport (*CD36*, fatty acid translocase), mitochondrial biogenesis (*PGC1 α* [also known as *PPARGC1A*], peroxisome proliferator-activated receptor γ , coactivator 1 α), lactate signalling (*GPR81* [also known as *HCARI*], G protein-coupled receptor 81), and glycerol re-esterification (*GK*, glycerol kinase) were investigated in both AT and SM. Gene-expression levels were normalised using the housekeeping genes 60S ribosomal protein L13a (*RPL13A*) and β_2 -microglobulin (*B2M*), as GeNorm analysis demonstrated that these housekeeping genes had the highest stability.

Antibodies and western blot analysis A rabbit polyclonal antibody raised against the phosphopeptide based on the amino-acid sequence of murine *ATGL* (400–414) pS406 LRRAQpSLPSVPLSC (corresponding to human *ATGL* pS404) was purified as described previously [27, 28]. Furthermore, commercially available primary and secondary antibodies were used as previously described [17, 18]. Muscle tissue was homogenised and resolved by SDS-PAGE as described before [17, 18, 28].

Membrane fractionation Membrane and cytosolic fractions were isolated using an ultracentrifugation protocol. Briefly, muscle biopsies were extracted 1:10 (wt/vol.) in ice-cold basic buffer (100 mmol/l EDTA, 100 mmol/l EGTA, 1.5 mol/l Tris and β -glycerolphosphate), protease (Roche Applied Science, Indianapolis, IN, USA) and phosphatase inhibitors (Sigma, St Louis, MO, USA) at 4°C. Homogenates were centrifuged at 160,000 g for 20 min, and the supernatant fraction, representing the cytosolic fraction, was removed and stored at –80°C. The pellet was resuspended in triton-supplemented

buffer and sonicated. After 1 h at 4°C with constant gentle inversion, the extract was centrifuged at 160,000 g for 20 min, and the supernatant fraction representing the membrane fraction was saved.

SM lipid composition of cytosolic and membrane fraction

Total lipids were extracted from muscle cytosolic and membrane fractions using chloroform-methanol 2:1 (vol.:vol.). The extracted lipids were separated into TAG, DAG, fatty acids and phospholipid by thin-layer chromatography as described before [18].

Statistics Data are presented as means \pm SEM. The overall effect of hyperinsulinaemia on interstitial and circulating metabolite levels over time, between groups and time \times group interaction was analysed using repeated-measures ANOVA. Post hoc testing was performed using Students' unpaired *t* test with Bonferroni adjustment for multiple testing when ANOVA showed a significant time-over-group effect. Relationships between lipid species, insulin sensitivity and insulin-mediated suppression of lipolysis were calculated using Pearson's correlation coefficient. Calculations were performed using SPSS 18.0 for Mac (Chicago, IL, USA). A value of $p < 0.05$ was considered to be statistically significant. Calculation of sample size was performed using GPower (version 3.1 for Mac, Heinrich-Heine University, Düsseldorf, Germany), indicating that nine individuals per group would be sufficient to detect a mean difference in dialysate glycerol concentration of 50 $\mu\text{mol/l}$, with a power of 0.8, α 0.05 and SD 25 $\mu\text{mol/l}$, including an anticipated drop out of 10% [21].

Results

Participants Participants' characteristics are shown in Table 1. Men with NGT and type 2 diabetes had comparable age, BMI, body fat percentage, WHR and $\dot{V}\text{O}_{2\text{max}}$. Type 2 diabetic men had been diagnosed for at least 5 years (average 7 ± 1 years) and had well-controlled glucose concentrations. As expected, fasting glucose and $\text{HbA}_{1\text{c}}$ were significantly higher in the type 2 diabetic group, whereas fasting insulin concentrations were comparable between groups. Insulin sensitivity (HOMA-insulin resistance [IR]), was significantly reduced in the type 2 diabetic group compared with the NGT group.

Circulating metabolite concentrations Plasma insulin concentrations were stable during the steady-state of the clamp and not significantly different between the groups (Fig. 1a). Blood-glucose concentrations were maintained at fasting concentrations throughout the entire clamp in both groups (Fig. 1b). Circulating NEFA and glycerol concentrations were

Table 1 Participants' characteristics

Characteristic	NGT ($n=11$)	Type 2 diabetes ($n=9$)
Age (years)	58 \pm 8	62 \pm 6
BMI (kg/m^2)	31.4 \pm 1.8	30.5 \pm 2.1
Body fat (%)	30.1 \pm 6.2	31.3 \pm 4.6
WHR	1.04 \pm 0.05	1.05 \pm 0.05
$\dot{V}\text{O}_{2\text{max}}$ ($\text{ml min}^{-1} \text{kg}^{-1}$)	28.9 \pm 4.8	29.5 \pm 6.7
Glucose (mmol/l)	5.5 \pm 0.2	8.6 \pm 1.5*
Insulin (pmol/l)	89 \pm 33	108 \pm 44
HOMA-IR	3.3 \pm 1.2	6.0 \pm 2.8*
$\text{HbA}_{1\text{c}}$ (%)	5.6 \pm 0.3	6.9 \pm 0.6
$\text{HbA}_{1\text{c}}$ (mmol/mol)	38.20 \pm 1.07	52.40 \pm 2.19*
AST ($\mu\text{kat/l}$)	0.37 \pm 0.15	0.42 \pm 0.17
ALT ($\mu\text{kat/l}$)	0.63 \pm 0.40	0.67 \pm 0.33

Values are means \pm SD

* $p < 0.05$ men with NGT vs type 2 diabetes, unpaired samples *t* test

ALT, alanine aminotransferase; AST, aspartate aminotransferase

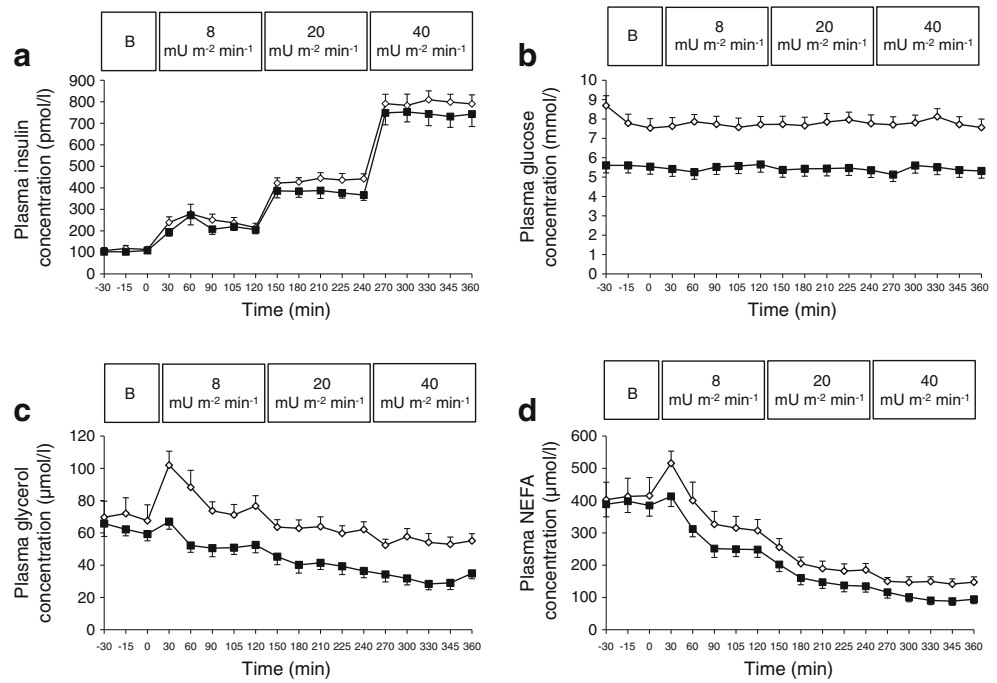
significantly elevated in type 2 diabetic men during the entire 6 h clamp period (ANOVA group $p < 0.01$; Fig. 1c, d).

Interstitial glycerol Interstitial glycerol concentrations in abdominal subcutaneous AT were comparable between groups at baseline (Fig. 2a) and decreased to the same extent during the clamp in both groups (Fig. 2a, b). At the end of step 3 (40 $\text{mU m}^{-2} \text{min}^{-1}$), values had decreased by $44 \pm 5\%$ and $47 \pm 9\%$ in the AT of men with type 2 diabetes and NGT, respectively (Fig. 2b).

Baseline SM interstitial glycerol concentrations were significantly higher in men with type 2 diabetes compared with NGT (152 ± 11 vs 119 ± 16 $\mu\text{mol/l}$; $p < 0.05$, Fig. 2c) and during the 6 h clamp (ANOVA time \times group $p = 0.043$; Fig. 2c; electronic supplementary material [ESM] Table 1). Muscle interstitial glycerol concentrations decreased by $7 \pm 6\%$, $13 \pm 9\%$ and $27 \pm 9\%$ during the subsequent hyperinsulinaemic steps in type 2 diabetic men, while this decrease was more pronounced in men with NGT ($21 \pm 7\%$, $38 \pm 8\%$ and $53 \pm 8\%$; ANOVA time \times group $p = 0.014$, Fig. 2d; ESM Table 1). In line with this, the absolute decrease from baseline in interstitial glycerol concentration during hyperinsulinaemia was blunted in the SM of type 2 diabetic men compared with men with NGT (ANOVA time \times group $p = 0.041$). Tissue nutritive blood flow, determined using the ethanol outflow/inflow ratio, was unchanged during hyperinsulinaemia in both groups, indicating changes in interstitial glycerol concentrations are not due to alterations in tissue blood flow (ESM Fig. 1).

Glucose infusion rate and interstitial glucose, lactate and pyruvate The blunted insulin-mediated suppression of SM glycerol concentration was accompanied by reduced peripheral

Fig. 1 Changes in plasma insulin (a), glucose (b), glycerol (c) and NEFA (d) during baseline and the three-step 6 h hyperinsulinaemic–euglycaemic clamp (8, 20, 40 mU m⁻² min⁻¹) in 11 obese men with NGT (black squares) compared with nine type 2 diabetic men (white squares). The response was compared using ANOVA. Curves for glucose, free glycerol and NEFA are significantly different between groups, with $p < 0.001$, $p < 0.01$ and $p < 0.01$, respectively. Values are means \pm SEM. B, baseline



glucose disposal, as indicated by the lower glucose infusion rate (GIR) at 20 mU m⁻² min⁻¹ (11.6 ± 0.7 vs 15.1 ± 1.1 $\mu\text{mol kg body weight}^{-1} \text{min}^{-1}$, $p < 0.05$) and 40 mU m⁻² min⁻¹ (21.8 ± 3.1 vs 30.5 ± 2.0 $\mu\text{mol kg body weight}^{-1} \text{min}^{-1}$, $p < 0.05$) of the clamp in type 2 diabetic men compared with men with NGT (ESM Fig. 2).

Concentrations of SM lactate, a marker of anaerobic metabolism, were significantly elevated at 40 mU m⁻² min⁻¹ during the clamp (ANOVA group $p < 0.01$; Fig. 3e) in type 2 diabetic men. Interstitial AT and SM glucose concentrations were significantly higher at baseline and during the entire clamp in type 2 diabetic men (ANOVA group $p < 0.01$; Fig. 3a, d),

Fig. 2 Interstitial glycerol concentration in subcutaneous AT (a) and SM (c) during baseline and the three-step 6 h hyperinsulinaemic–euglycaemic clamp (8, 20, 40 mU m⁻² min⁻¹) in 11 obese men with NGT (black squares) compared with nine type 2 diabetic men (white squares). Changes in interstitial glycerol concentration in subcutaneous AT (b) and SM (d) are expressed as percentage of baseline, calculated using the average from the two baseline measures (-30 and 0 min). The response was compared using ANOVA. Curves for SM (c and d) showed significant time–group interaction ($p = 0.043$ and $p = 0.014$, respectively). Values are means \pm SEM. B, baseline

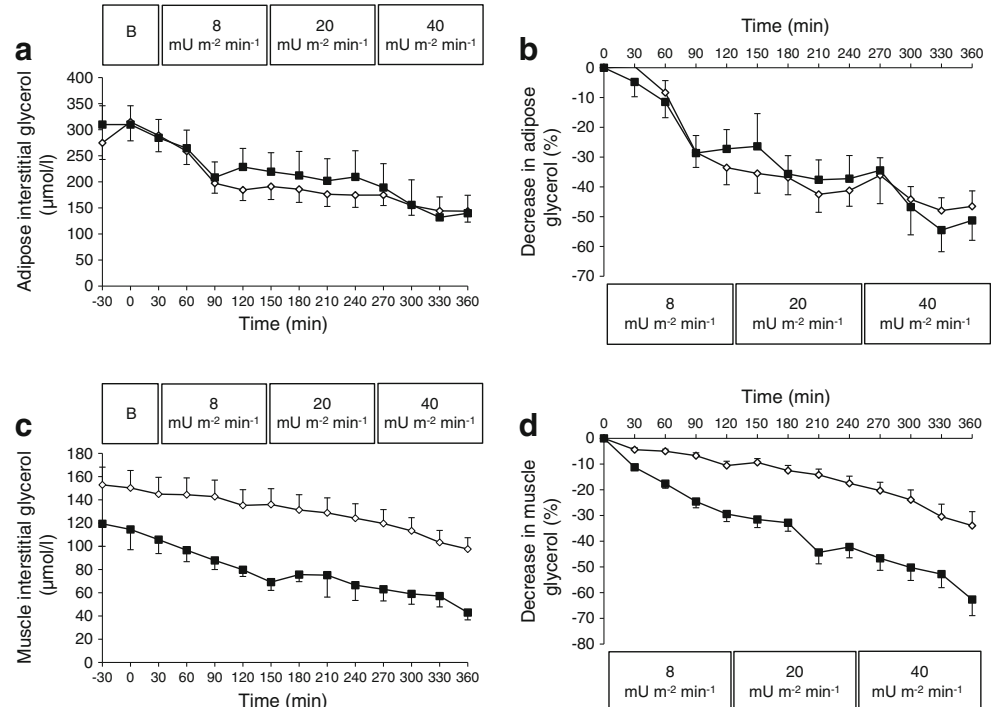
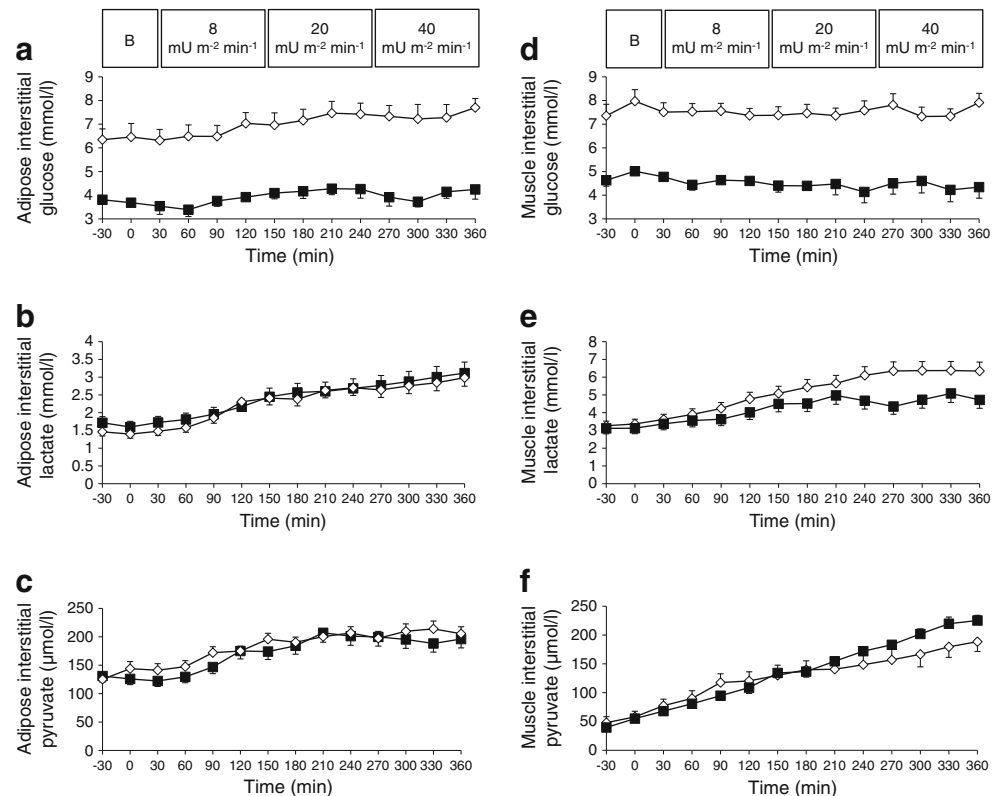


Fig. 3 Interstitial glucose (a, d), lactate (b, e) and pyruvate concentrations (c, f) in subcutaneous AT (a–c) and SM (d–f) during baseline and the three-step 6 h hyperinsulinaemic–euglycaemic clamp (8, 20, 40 $\text{mU m}^{-2} \text{min}^{-1}$) in 11 obese men with NGT (black squares) compared with nine type 2 diabetic men (white squares). The response was compared using ANOVA. AT and SM glucose (a and d) curves and SM lactate (e) curves were significantly different between groups ($p < 0.01$). Values are means \pm SEM. B, baseline



while AT and SM pyruvate concentrations were comparable between groups (Fig. 3c, f).

Gene-expression and protein data As shown in ESM Fig. 3a, *ATGL* mRNA expression was reduced in the SM of men with type 2 diabetes compared with NGT (0.70 ± 0.08 vs 1.00 ± 0.10 ; $p = 0.041$). The expression of *HSL* mRNA (0.68 ± 0.14 vs 1.00 ± 0.16 ; $p = 0.157$) and *ABHD5* mRNA (0.85 ± 0.06 vs 1.00 ± 0.07 ; $p = 0.119$) was lower in the SM of type 2 diabetic men, though the difference was not statistically significant. Besides disturbances in *ATGL* expression, reduced *GLUT4* mRNA (0.77 ± 0.06 vs 1.00 ± 0.06 ; $p = 0.014$) and a tendency for lower *PDE3B* mRNA (0.59 ± 0.09 vs 1.00 ± 0.26 ; $p = 0.072$) expression was observed in the SM of type 2 diabetic men. Finally, *PGC1 α* mRNA expression was lower in the SM of type 2 diabetic men (0.72 ± 0.09 vs 1.00 ± 0.06 ; $p = 0.032$). No differences in AT gene expression were observed between groups (ESM Fig. 3b).

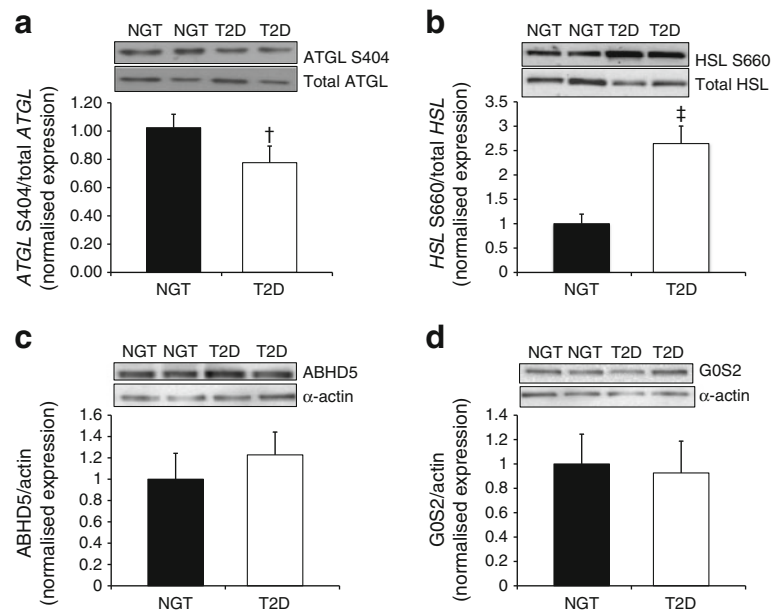
Muscle *ATGL* protein content was comparable between groups (1.00 ± 0.12 vs 0.93 ± 0.18 ; $p = 0.737$) and muscle *HSL* protein content was significantly lower in type 2 diabetic men (1.00 ± 0.31 vs 0.26 ± 0.14 ; $p = 0.037$). The ratio of phosphorylated *ATGL* at serine 404 to total *ATGL* tended to be lower in the SM of men with type 2 diabetes compared with NGT (0.78 ± 0.12 vs 1.02 ± 0.09 ; $p = 0.117$; Fig. 4a), while the ratio of phosphorylated *HSL* at serine 660 to total *HSL* was

increased in the SM of type 2 diabetic men (2.63 ± 0.36 vs 1.00 ± 0.19 ; $p = 0.011$; Fig. 4b). No differences were observed in *ABHD5* and *G0S2* protein content between groups (Fig. 4c, d). Muscle biopsies were lyophilised and dissected free of extracellular lipid, blood and connective tissue. Additionally, *PLIN1* was not detectable in any of the muscle samples, excluding significant contamination by infiltrated adipocytes.

SM lipid composition of cytosolic and membrane fraction

Enrichment of membrane (i.e. caveolin 2 [CAV] and immunoglobulin heavy chain-binding protein [BIP]) and cytosolic (i.e. glyceraldehyde-3-phosphate dehydrogenase [GAPDH], *ATGL* and *HSL*) markers in their respective subcellular compartments (ESM Fig. 4) show that the cellular fractionation methods were successful and suggest minor contamination between fractions. The cellular distribution of SM lipid species is shown in Table 2. Total SM DAG content was significantly higher in men with type 2 diabetes compared with NGT ($p = 0.022$; Table 2). Most DAG was found in the membrane fraction. Interestingly, DAG content was significantly elevated in the SM membrane fraction of type 2 diabetic men compared with men with NGT ($p = 0.029$, Table 2). Moreover, the saturated DAG species 16:0, 17:0, 18:0, 22:0 and trans18:1 were more abundant in the SM membrane fraction of men with type 2 diabetes compared with NGT (ESM Table 2). Interestingly, saturated DAG

Fig. 4 SM ATGL serine 404 phosphorylation (a), HSL serine 660 phosphorylation (b), ABHD5 (c) and G0S2 (d) protein content in men with NGT vs type 2 diabetic men. Protein levels are normalised to men with NGT and differences in loading are corrected by α -actin. PLIN1 protein was not detectable in any of the muscle samples excluding significant contamination by infiltrated adipocytes. Values are means \pm SEM and significance was tested using the unpaired *t* test. $^{\dagger}p=0.117$ and $^{\ddagger}p=0.011$. T2D, type 2 diabetes



content in the SM membrane showed a strong inverse association with insulin sensitivity (GIR: $r=-0.568$, $p<0.01$; Fig. 5a) and the decrease in interstitial muscle glycerol ($r=-0.546$, $p<0.01$; Fig. 5b). Moreover, the increase in membrane saturated DAG was positively associated with PKC activation ($r=0.376$, $p<0.01$; Fig. 5c) (ratio of PKC δ

membrane to cytosolic in men with type 2 diabetes vs NGT: 6.07 ± 1.19 vs 2.43 ± 0.69 ; $p=0.028$; ESM Fig. 5). Cytosolic DAG concentration showed no relationship with insulin sensitivity or the decrease in interstitial muscle glycerol. No differences were observed in total or subcellular TAG, phospholipid or fatty acid fractions between groups.

Table 2 Muscle lipid composition in cytosolic and membrane fractions

Lipid	TAG ($\mu\text{mol}/\text{mg}$ protein)		DAG ($\mu\text{mol}/\text{mg}$ protein)		PL ($\mu\text{mol}/\text{mg}$ protein)		FA ($\mu\text{mol}/\text{mg}$ protein)	
	NGT	Type 2 diabetes	NGT	Type 2 diabetes	NGT	Type 2 diabetes	NGT	Type 2 diabetes
SFA								
Cyto	4.91 \pm 0.84	4.67 \pm 2.91	14.17 \pm 16.26	19.35 \pm 11.05	4.68 \pm 5.91	4.96 \pm 3.35	0.56 \pm 0.38	0.95 \pm 0.81
Mem	272 \pm 157	261 \pm 169	55 \pm 31	102 \pm 51*	250 \pm 63	231 \pm 45	19.86 \pm 9.37	18.37 \pm 5.26
MUFA								
Cyto	3.39 \pm 1.78	4.54 \pm 2.87	1.45 \pm 0.96	2.29 \pm 1.92	0.99 \pm 1.18	1.12 \pm 0.61	0.19 \pm 0.16	0.31 \pm 0.16
Mem	340 \pm 270	284 \pm 181	48 \pm 20	52 \pm 20	60 \pm 16	74 \pm 31	6.88 \pm 4.65	5.83 \pm 2.78
PUFA								
Cyto	1.04 \pm 0.73	1.86 \pm 1.80	2.94 \pm 3.56	3.69 \pm 3.23	2.72 \pm 4.06	1.83 \pm 1.04	0.52 \pm 0.84	0.39 \pm 0.37
Mem	112 \pm 85	73 \pm 42	19.50 \pm 12.53	19.39 \pm 9.41	305 \pm 87	252 \pm 61	11.98 \pm 12.58	7.05 \pm 4.28
Trans								
Cyto	0.23 \pm 0.08	0.39 \pm 0.46	0.98 \pm 1.18	1.52 \pm 1.86	0.17 \pm 0.28	0.19 \pm 0.25	0.16 \pm 0.21	0.18 \pm 0.25
Mem	11.67 \pm 14.75	7.97 \pm 3.21	1.44 \pm 0.99	7.95 \pm 6.39*	4.56 \pm 0.96	4.58 \pm 2.01	0.72 \pm 0.39	0.74 \pm 1.16
Total								
Cyto	9.58 \pm 2.27	11.47 \pm 7.32	19.54 \pm 19.79	26.84 \pm 13.27	8.57 \pm 11.29	8.10 \pm 4.17	1.43 \pm 1.14	1.84 \pm 0.93
Mem	736 \pm 522	627 \pm 387	124 \pm 40	182 \pm 73*	620 \pm 163	562 \pm 117	41 \pm 26	30 \pm 8
Total	745 \pm 520	638 \pm 388	142 \pm 45	209 \pm 77*	628 \pm 164	569 \pm 116	42 \pm 26	32 \pm 8

Values are means \pm SD

* $p<0.05$ men with NGT vs type 2 diabetes, unpaired samples *t* test

Cyto, cytosolic fraction; FA, fatty acid; Mem, membrane fraction; MUFA, monounsaturated fatty acid; PL, phospholipids; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; Trans, trans fatty acid

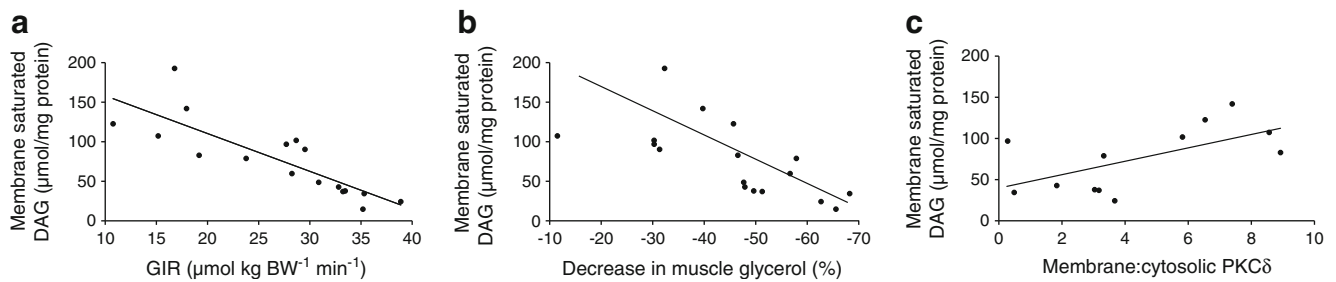


Fig. 5 Relationship between saturated DAG content in the muscle membrane and: GIR ($r=-0.568$, $p<0.01$) (a); the percentage decrease in interstitial muscle glycerol concentration ($r=-0.546$, $p<0.01$) (b) in

NGT ($n=8$) and type 2 diabetic ($n=9$) men; and the ratio of PKC δ in the membrane to PKC δ in the cytosolic muscle fraction ($r=0.376$, $p<0.01$) in men with NGT ($n=6$) and type 2 diabetes ($n=6$) (c)

Discussion

To our knowledge, the present study is the first to examine insulin resistance of abdominal subcutaneous AT and SM lipolysis in long-term-diagnosed obese type 2 diabetic men compared with men with NGT, matched for age, body composition and physical fitness. The main finding of the present study is that long-term diagnosed type 2 diabetic men demonstrated a blunted insulin-induced decrease in muscle lipolysis and higher saturated DAG content in the SM membrane fraction. Moreover, the membrane content of saturated DAG was inversely associated with insulin sensitivity and the insulin-mediated decrease in interstitial SM glycerol concentration. No differences in the insulin sensitivity of AT lipolysis were found.

Previous studies have yielded conflicting data regarding sensitivity to the antilipolytic effects of insulin in abdominal subcutaneous AT and SM. Insulin sensitivity of SM lipolysis has been suggested to be lower [21] or higher [20] compared with subcutaneous AT [20]. Moreover, it has been suggested that SM lipolysis is primarily regulated by substrate supply and is, to a lesser degree, under hormonal control [29]. This concept of differential regulation of AT and SM lipolysis is supported by the presence of different phosphodiesterase and adrenoceptor subtypes in these tissues [30, 31]. Our data suggest a clear antilipolytic effect of insulin in both the abdominal subcutaneous AT and SM of obese men with NGT. The relative decreases in AT ($\approx -47\%$) and SM ($\approx -53\%$) lipolysis were comparable in men with NGT, indicating tight insulin-mediated regulation of AT and SM lipolysis in men with NGT. This may contribute to an adequate switch between fatty acid and glucose substrate use under postprandial conditions and is of importance for the maintenance of normal glucose tolerance.

In contrast, type 2 diabetic men showed differential regulation of AT and SM lipolysis with increased fasting interstitial glycerol concentrations and a blunted decrease ($\approx -27\%$) in muscle interstitial glycerol during hyperinsulinaemia ($40 \text{ mU m}^{-2} \text{ min}^{-1}$). Additionally, muscle lactate concentrations were increased in type 2 diabetic muscle, indicative of an

increased glycolytic flux during hyperinsulinaemic conditions. This is the first study showing an impaired muscle lipolysis during hyperinsulinaemia in type 2 diabetic men, which may be associated with the accumulation of lipid metabolites and impaired insulin sensitivity, as discussed further below. AT glycerol concentrations were comparable between men with type 2 diabetes and NGT during both baseline and hyperinsulinaemic conditions. Data on the insulin sensitivity of abdominal subcutaneous AT in obese or type 2 diabetic participants are not consistent [12, 13, 22, 32–35]. This discrepancy remains to be elucidated but differences between AT depots (e.g. upper vs lower body and subcutaneous vs visceral) in the ability of insulin to suppress fatty acid release may possibly contribute to this [36]. Therefore, there is an urgent need to investigate other AT depots with respect to insulin resistance of systemic fatty acid release.

Baseline mRNA expression of *ATGL* and *HSL* and HSL protein content were reduced in type 2 diabetic muscle. Nevertheless, our data show that despite a fourfold lower total HSL protein content in muscle of type 2 diabetic men, total HSL serine 660 phosphorylation and the ratio of phosphorylated HSL serine 660 to total HSL was significantly higher at baseline in type 2 diabetic men compared with controls. This increased phosphorylation might reflect increased HSL activity, possibly explaining the reduced suppression of lipolysis at baseline in muscle of type 2 diabetic men (reflected by increased interstitial glycerol concentrations). Nevertheless, lipolytic regulation is complex and the regulation of lipolysis under hyperinsulinaemic conditions is governed by different mechanisms than under basal fasting conditions. Therefore, it remains to be determined whether increased HSL activity is responsible for the blunted insulin-mediated suppression of lipolysis in the muscle of type 2 diabetic men.

ATGL protein content was comparable between groups, while in previous studies increased ATGL protein content has been reported in type 2 diabetic muscle [18, 19]. In the present study, in contrast to previous studies, type 2 diabetic men and NGT controls were matched not only for age and BMI but also for aerobic capacity ($\dot{V}O_{2\text{max}}$). Given the fact

that muscle ATGL activity might produce lipid ligands regulating mitochondrial function via PPAR α target genes [37], matching for aerobic capacity might explain the discrepancy with previous studies [18, 19]. Furthermore, our study is the first to show that ATGL serine 404 phosphorylation tends to be lower in muscle of type 2 diabetic men. The contribution of ATGL phosphorylation to intracellular lipid accumulation and the development of insulin resistance and type 2 diabetes needs to be investigated in future research.

Given the fact that ATGL and HSL are responsible for more than 95% of the TAG hydrolase activity [38], impaired intracellular lipolysis may have major effects on the accumulation of cellular lipids, in particular DAG, which could interfere with insulin-mediated signal transduction. There are three distinct DAG isomers (*sn*-1,3; *sn*-2,3; and *sn*-1,2 DAG). To date, only *sn*-1,2 DAG has been associated with insulin signalling and PKC activation [39, 40]. However, both ATGL and HSL lack the ability to generate 1,2-DAG in vitro and it remains to be determined whether lipid droplet-derived DAG may translocate to the membrane and activate PKC [41].

At present, it is unknown whether stereoselectivity of ATGL and HSL is impaired under insulin-resistant conditions. Here, we show for the first time that blunted insulin-mediated inhibition of SM lipolysis is positively associated with membrane DAG in type 2 diabetic men. The relationship was particularly strong with the amount of saturated 16:0 and 18:0 DAG in the muscle membrane. These data strengthen previous observations from our group and others that, in particular, saturated DAG has a negative impact on insulin sensitivity [42, 43] and that increased membrane localisation may mediate this relationship [8]. Furthermore, the proportion of PKC δ in the muscle membrane was increased in type 2 diabetic men and the saturated DAG content of the membrane was positively associated with PKC activation, as shown previously [5]. Several reports in the literature suggest that saturated DAGs are related to PKC activation [42, 44]. However, several other PKC isoforms might be involved in the relationship between membrane DAG and insulin sensitivity. Together, our data indicate that a lipolytic imbalance may promote saturated DAG accumulation in the muscle membrane and worsen insulin resistance (i.e. glucose disposal), at least in part through a PKC-dependent pathway, as has been suggested recently in vitro in human primary myotubes [19].

Some limitations of our study need to be addressed. Interstitial glycerol was used as a measure of lipolysis. There are indications from our laboratory that AT and SM may take up glycerol [17, 45] and may exhibit significant glycerol kinase activity [46]. This suggests that interstitial glycerol may not reflect the overall rate of lipolysis, but may instead be the net result of TAG and glycerol metabolism in the tissue and thus reflect glycerol turnover [21]. However, this seems unlikely, as SM *GK* mRNA expression was comparable between groups (ESM Fig. 3a). A second consideration is that there is

evidence for an active transport system of insulin across the blood–tissue barrier, such that insulin concentrations in the interstitium of AT and SM are approximately 50% of those in plasma [47, 48]. Although a delayed transcapillary transport of insulin to muscle interstitial fluid has been observed in obese compared with lean men [49], this has not been observed in muscle of type 2 diabetic men compared with healthy age- and BMI-matched controls [50]. Nevertheless, we cannot exclude that the impaired insulin-mediated lipolysis in type 2 diabetic muscle is, in part, the result of reduced interstitial insulin concentrations. Third, the muscle membrane fraction was not only composed of plasma membranes but contained other membranes, including mitochondrial and ER, as indicated by the expression of the ER-marker BIP in the membrane fraction (ESM Fig. 4). As TAG is synthesised and packed in lipid droplets at the ER, this might explain the large amount of TAG in the crude membrane fraction.

Taken together, this is the first report of a blunted insulin-mediated suppression of SM lipolysis in long-term-diagnosed obese type 2 diabetic men compared with well-matched men with NGT. The impaired antilipolytic effect of insulin in skeletal muscle was accompanied by excessive accumulation of saturated DAG in the SM membrane and reduced glucose disposal. Together, our data highlight the importance of saturated DAG in the membrane as a potential mechanism involved in the progression of insulin resistance in type 2 diabetic men. Therefore, nutritional and pharmacological approaches to decrease the degree of saturation of membrane DAG composition might promote insulin sensitivity in humans.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement All clinical studies were performed and samples were obtained in the laboratory of EEB and analytical experiments were conducted at the laboratories of EEB and MJW. All authors made substantial contributions to the concept and design, acquisition of data or analysis and interpretation of data and to drafting of the article and revising it critically for intellectual content. All authors gave final approval of the version to be published.

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