

Research Article

Nobiletin exerts anti-diabetic and anti-inflammatory effects in an *in vitro* human model and *in vivo* murine model of gestational diabetes

 Caitlyn Nguyen-Ngo^{1,2},  Carlos Salomon^{3,4}, Stephanie Quak¹, Andrew Lai³, Jane C Willcox⁵ and  Martha Lappas^{1,2}

¹Obstetrics, Nutrition and Endocrinology Group, Department of Obstetrics and Gynaecology, University of Melbourne, Heidelberg, Victoria, Australia; ²Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Victoria, Australia; ³Exosome Biology Laboratory, Centre for Clinical Diagnostics, University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, University of Queensland, Brisbane, Queensland, Australia; ⁴Department of Clinical Biochemistry and Immunology, Faculty of Pharmacy, University of Concepción, Concepción 4030000, Chile; ⁵School of Allied Health, College of Science, Health and Engineering, La Trobe University, Bundoora, Victoria, Australia

Correspondence: Martha Lappas (mlappas@unimelb.edu.au)



Gestational diabetes mellitus (GDM) is a global health issue, whereby pregnant women are afflicted with carbohydrate intolerance with first onset during pregnancy. GDM is characterized by maternal peripheral insulin resistance, thought to be driven by low-grade maternal inflammation. Nobiletin, a polymethoxylated flavonoid, possesses potent glucose-sensitizing and anti-inflammatory properties; however, its effects in GDM have not been assessed. The present study aimed to determine the effects of nobiletin on glucose metabolism and inflammation associated with GDM in both *in vitro* human tissues and an *in vivo* animal model of GDM. *In vitro*, treatment with nobiletin significantly improved TNF-impaired glucose uptake in human skeletal muscle, and suppressed mRNA expression and protein secretion of pro-inflammatory cytokines and chemokines in human placenta and visceral adipose tissue (VAT). Mechanistically, nobiletin significantly inhibited Akt and Erk activation in placenta, and NF- κ B activation in VAT. *In vivo*, GDM mice treated with 50 mg/kg nobiletin daily via oral gavage from gestational day (gd) 1-17 or via i.p. injections from gd 10-17 significantly improved glucose tolerance. Pregnant GDM mice treated with nobiletin from either gd 1-17 or gd 10-17 exhibited significantly suppressed mRNA expression of pro-inflammatory cytokines and chemokines in placenta, VAT and subcutaneous adipose tissue (SAT). Using a quantitative mass spectrometry approach, we identified differentially abundant proteins associated with the effect of nobiletin *in vivo*. Together, these studies demonstrate that nobiletin improves glucose metabolism and reduces inflammation associated with GDM and may be a novel therapeutic for the prevention of GDM.

Introduction

Gestational diabetes mellitus (GDM) is a metabolic disorder occurring during pregnancy, characterized by carbohydrate intolerance of variable severity [1]. GDM affects up to 20% of global pregnancies [2–5] and leads to short-term complications, such as macrosomia, high fat adiposity, shoulder dystocia and maternal hypertension [6–8]. Of major concern, GDM is also known to detriment future offspring health, with increased risk of obesity, Type 2 diabetes and cardiovascular disease [9,10]. In addition, a lack of effective preventative therapeutics for GDM creates excessive economic burden on the healthcare system – medical care for GDM women costs 34% more than for healthy normal glucose tolerant (NGT) pregnant women, with additional ongoing healthcare costs rising by 20% within 5 years post-partum [11]. It is

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imperative that we identify safer and more effective interventions that can prevent GDM and its perinatal and long-term complications, as well as its associated economic burden.

Maternal peripheral insulin resistance [12–14] and sterile low-grade inflammation [15–19] are key features of GDM. The heightened inflammatory state induces further expression of pro-inflammatory mediators in the placenta and maternal adipose tissues [20–22]. Pro-inflammatory cytokines, such as TNF, are then thought to induce insulin resistance by impairing glucose uptake in skeletal muscle [22–27], leaving excess glucose in the maternal bloodstream for increased fetal fat adiposity [28]. TNF can also stimulate inflammation, by inducing further production of other cytokines and chemokines [24,25]. Currently, the only treatment therapies in place for GDM target only hyperglycemia, with little effect on the underlying inflammation associated with GDM. Novel therapeutics that can prevent both hyperglycemia and inflammation are crucial to securing a healthy future for affected offspring.

Polyphenols are natural bioactive compounds that may confer positive health benefits, and are thought to possess anti-obesity and anti-diabetes properties [29]. However, there is limited research investigating the impact of polyphenols on GDM. Nobiletin is a polymethoxylated flavone, most commonly found in tangerine peels [30]. Being well-tolerated and non-toxic [31,32], nobiletin has been demonstrated to hold potent anti-inflammatory [33–37] and anti-diabetic actions [37–41]. *In vitro*, nobiletin inhibited lipopolysaccharide (LPS)-induced pro-inflammatory cytokine mRNA expression in mouse macrophages [35], and improves glucose uptake in adipocytes [39]. *In vivo*, 100 mg/kg nobiletin treatment of high-fat diet (HFD) mice for 5 weeks significantly improved glucose tolerance and expression of insulin signalling proteins in visceral adipose tissue (VAT), as well as reducing pro-inflammatory cytokine and chemokine expression [37]. Similarly, in mice pre-treated with 20 mg/kg nobiletin prior to endotoxemic shock, TNF and IL6 production in serum and in kidney, lung and liver tissues were inhibited [31]. Nobiletin has been demonstrated to act through the nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) [42], Akt [43] and mitogen-activated protein kinase (MAPK) pathways [44].

We have previously shown that nobiletin suppresses the expression and production of pro-inflammatory cytokines in human placenta, fetal membranes and myometrium an *in vitro* model of preterm birth [34]. However, the *in vitro* and *in vivo* effects of nobiletin on inflammation and insulin resistance associated with GDM are not yet known. Therefore, we aimed to determine the effects of nobiletin on glucose metabolism and inflammation in *in vitro* and *in vivo* models of GDM. We also explore the signalling pathways through which nobiletin may exert its effects.

Materials and methods

Human tissue collection

The study was approved by the Mercy Hospital for Women's Research and Ethics Committee (Mercy Health, Ethics approval number R04-29), and written informed consent was obtained from all participating subjects.

Human placenta, VAT and skeletal muscle (from the rectus pyramidalis) were obtained from NGT non-obese women (body mass index, BMI <30 kg/m²) who delivered healthy, singleton infants at term (37–41 weeks of gestation) undergoing elective Caesarean in the absence of labour. Women with the following conditions were excluded: vascular/renal complication, multiple gestations, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute fetal distress and any other adverse underlying medical conditions. All the tissues were delivered to the laboratory within 10 min of delivery placed in 4°C phosphate-buffered saline (PBS) and processed immediately.

Human tissue explants

Tissue explants were used to determine the effect of nobiletin on (1) insulin-stimulated glucose uptake in human skeletal muscle, and (2) the mRNA expression and protein release of pro-inflammatory mediators in human placenta and VAT. We also test the mechanism through which nobiletin acts by examining its effect on the NF- κ B, Akt and MAPK signalling pathways.

Tissue explants for placenta [45], VAT [46] and skeletal muscle [19] were performed as previously described. Briefly, placenta and VAT were bluntly dissected to remove visible connective tissue and calcium deposits. The processed tissue were thoroughly washed in PBS and pre-incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/ml penicillin G, 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 8% O₂ (placenta) or 21% O₂ (VAT) for 1 h. After blotting dry, 100 mg wet weight sample per well was incubated in 1 ml DMEM for 20 h. Placental explants were incubated in 100 μ M nobiletin overnight (Cayman Chemical, MI, U.S.A.), skeletal muscle explants were incubated in 200 μ M nobiletin overnight and VAT explants pre-incubated in 200 μ M nobiletin for 3 h prior to overnight treatment. The optimized concentration of nobiletin was based on our previously published study [34] and a dose–response in each tissue (Supplementary Figure S1). For placenta, the 100 μ M dose of nobiletin was as effective as the higher dose, whereas for VAT, the 200 μ M dose was most effective.

The pro-inflammatory cytokine TNF is positively correlated with GDM insulin resistance [16]. Further, TNF is well-known to induce inflammation in placenta and adipose tissue, as well as inhibit the insulin signalling pathway in skeletal muscle [23,47]. Thus, in the present study, the pro-inflammatory cytokine TNF was used to generate an *in vitro* placenta, adipose and skeletal muscle model of GDM. Placenta and VAT explants were stimulated with 10 ng/ml TNF overnight, and the tissues and media collected separately to perform qRT-PCR and ELISA, respectively. Human skeletal muscle was stimulated with 10 ng/ml TNF overnight, and the tissues collected to perform radiolabelled 2DG glucose uptake assay. For mechanistic analysis, human placenta, VAT and skeletal muscle were incubated in the absence or presence of nobiletin overnight, followed by 30 min TNF incubation, and the tissues collected to perform Western blotting. Explants were performed on tissues obtained from six independent patients. To obtain optimal quality and size of VAT and skeletal muscle samples, explants were performed on tissues obtained from a total of 26 patients recruited for the study. Experiments were performed on three-six independent explants; patient characteristics and experiment details are described in Supplementary Tables S1–3.

Skeletal muscle explants and radiolabelled 2DG glucose uptake

We investigated the effects of nobiletin on insulin-stimulated glucose uptake in human skeletal muscle. Skeletal muscle explants were performed as detailed above and glucose uptake was performed as previously described [18]. After final incubation with treatment, 2-Deoxy-D-glucose (2DG) uptake was measured by adding 1 mM 2DG to Krebs buffer containing 0.5% BSA (fatty acid free) and 0.1 mM insulin for 10 min, then immediately adding 3 mCi/ml [¹⁴C]-2DG (Perkin Elmer) and incubating for a further 20 min. Tissues were then washed in ice-cold PBS and solubilized for 1 h in 0.5 ml 0.5 M NaOH at 60°C. Tissues were neutralized with 0.5 ml 0.5 M HCl and the supernatant was transferred to a vial containing 3 ml of liquid scintillation fluid. All samples were counted for radioactivity in a liquid scintillation counter. Glucose uptake was performed on tissues obtained from six patients.

Mice studies

Here, we investigated the effect of nobiletin on (1) glucose tolerance, and (2) pro-inflammatory cytokine and chemokine mRNA expression in a mouse model of GDM. Pregnant heterozygous Leptin receptor deficient *db/+* mice display identical features to human GDM, such as a mild diabetic phenotype and macrosomic pups [48,49]. In the present study, our colony of pregnant heterozygous B6.BKS(D)-*Lepr^{db/+}/J* (*db/+*) mice replicated the characteristic features of human GDM, including mild glucose intolerance [50] and increased inflammation in placenta and adipose tissue in *db/+* mice compared with WT mice (Lappas, unpublished). Therefore, pregnant *db/+* mice were used to generate an *in vivo* model of GDM.

Lepr^{db/+} (*db/+*) mice (strain B6.BKS(D)-*Lepr^{db/+}/J*; stock number 000697) were gifted by Professor Peter Thorn (University of Sydney), and obtained with approval from the Jackson Laboratory, Bar Harbor, ME, U.S.A. Mice were housed at the Austin Hospital BioResources Facility, Austin Health (Heidelberg, VIC, Australia). The mice studies were approved by the Austin Health's Animal Ethics Committee (A2014/05227). Mice were housed and maintained under standard laboratory conditions with controlled temperature (19–22°C) and 12-h light/dark cycle, under specific-pathogen-free conditions in the BioResources Facility at Austin Health (Heidelberg, VIC, Australia). All mice were fed a standard rodent diet *ad libitum*, with free access to clean drinking water throughout the duration of the present study. Male and female *db/+* mice were mated, and tail samples were taken from offspring at three weeks of age for genotyping.

Female *db/+* mice (8–12 weeks old) were time-mated with WT males, and mating was confirmed by the presence of a copulatory plug the next morning, which was designated gestation day (gd) 0. Plugged-mated females were then separated into two treatment procedures starting on either gd 1 (study group 1) or gd 10 (study group 2). From here on, pregnant *db/+* mice will be referred to as GDM mice. *Study group 1*: On gd 1, GDM mice were administered daily doses of 50 mg/kg body weight nobiletin (solubilized in DMSO and using 0.3% CMC-saline solution as a vehicle) or with DMSO in 0.3% CMC-saline solution (control) by oral gavage (total volume 300 μ l) from gd 1 to 17. The concentration of nobiletin used was based on previous animal studies [36]. A total of 26 mice were used for study group 1, including 13 GDM controls and 13 GDM mice treated with nobiletin. *Study group 2*: On gd 10, GDM mice were administered daily doses of 50 mg/kg body weight nobiletin (dissolved in DMSO) or with DMSO (control) by i.p. (total volume 50 μ l) from gd 10 to 17. A total of 12 mice were used for study group 2: 6 GDM controls, and 6 GDM mice treated with nobiletin. The experimental *N* for the animal experiments was determined based on previously published animal studies [51–55].

On gd 17, glucose tolerance was determined via an oral glucose tolerance test (OGTT) using 2 g/kg body weight glucose on 6 h fasted mice, as previously described [56]. Blood samples were collected from the tail using capillary

tubes at 0, 30, 60 and 120 min after glucose administration, and blood glucose levels were measured with a glucometer. Following OGTT, mice were allowed to recover overnight. On gd 18, mice were fasted for 5 h and then culled by cervical dislocation. The abdominal cavity was opened and the fetuses removed from the uterus. The pups were counted and weighed and the placenta and maternal tissues (VAT, subcutaneous adipose tissue (SAT), skeletal muscle and pancreas) were obtained, weighed and snap frozen in liquid nitrogen and stored at -80°C until further analysis by qRT-PCR and proteomic analysis. Maternal fat mass was determined by the combined weight of dissected fat from the intra-abdominal visceral (mesenteric, gonadal and retroperitoneal) and subcutaneous (dorsal) fat pads. RNA was extracted from each of two placentas per dam, and used for qRT-PCR analysis as detailed below.

Western blotting

Western blotting was performed using 2 μg protein as previously described [18,46]. The following probes were used: rabbit monoclonal phosphorylated (Ser473) Akt (pAkt) (#4060; Cell Signalling, Beverly, MA, U.S.A.), diluted 1:5000 in blocking buffer (5% skim milk in TBS with 0.05% Tween-20); rabbit monoclonal pan-Akt (#4685; Cell Signalling, Beverly, MA, U.S.A.), diluted 1:1000 (skeletal muscle) or 1:3000 (placenta and VAT) in blocking buffer; mouse monoclonal I κ B- α (MA5-15132; ThermoFisher), diluted 1:1000 in blocking buffer; 0.4 $\mu\text{g}/\text{ml}$ goat polyclonal α -actin (sc-1615; Santa Cruz, CA, U.S.A.); mouse monoclonal β -actin (A5316; Sigma, St Louis, Missouri), diluted 1:20000 in blocking buffer; 0.2 $\mu\text{g}/\text{ml}$ mouse monoclonal phosphorylated Erk (pErk) (sc-7383; Santa Cruz, CA, U.S.A.); 0.2 $\mu\text{g}/\text{ml}$ rabbit polyclonal Erk1/2 (sc-93, Santa Cruz, CA, U.S.A.); 0.1 $\mu\text{g}/\text{ml}$ rabbit polyclonal phosphorylated p38 (p-p38) (sc-17852-R; Santa Cruz, CA, U.S.A.); and 0.4 $\mu\text{g}/\text{ml}$ rabbit polyclonal p38 (sc-535, Santa Cruz, CA, U.S.A.). Membranes were imaged using the MP ChemiDoc system (Bio-Rad Laboratories; Hercules, CN, U.S.A.), and densitometry was performed using Image Lab 3.0 (Bio-Rad Laboratories; Hercules, CN, U.S.A.).

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues using TRI reagent according to the manufacturer's instructions (Sigma-Aldrich, Saint Louis, MO, U.S.A.). RNA concentration and purity were determined using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia). RNA was converted to cDNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CN, U.S.A.) according to the manufacturer's instructions. The qRT-PCR was performed with 100 nM of pre-designed and validated QuantiTect primers (Supplementary Table 4) (Qiagen; Chadstone Centre, Vic, Australia), as previously described [19] using a CFX384 Real-Time PCR detection system from Bio-Rad Laboratories (Gladesville, NSW, Australia). For the human *in vitro* studies, average gene C_t values were normalized against two housekeeping genes (succinate dehydrogenase complex, subunit A (SDHA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ)). For the mice *in vivo* studies, average gene C_t values were normalized against three below housekeeping genes (18S rRNA, β -2-microglobulin (B2M) and hypoxanthine-guanine phosphoribosyltransferase (HPRT)). Fold differences were determined using the comparative C_t method.

Enzyme-linked immunosorbent assay

Media from tissue explants were assayed using a sandwich ELISA to determine the protein concentrations of pro-inflammatory cytokines IL1A, IL1B and IL6, and chemokines CXCL8 and CCL2, as according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, U.S.A.; R&D Systems). The calculated interassay and intraassay coefficients of variation (CV) were all less than 10%.

Quantitative mass spectrometry

Mass spectrometry analyses were conducted within an ISO17025 accredited (National Association of Testing Authorities, Australia) research facility as previously described [57]. From each of the collected mouse tissues (i.e. skeletal muscle, placenta, pancreas, and VAT), approximately 30 mg tissue were excised from each, and placed individually in 250 μl complete radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 1% Igepal; 0.1% SDS; 0.25% sodium deoxycholate; 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4; 0.5 mM 4-(2-animoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF); 10 $\mu\text{l}/\text{ml}$ leupeptin; 1 mM activated sodium orthovanadate (Na₃VO₄); and 1 mM NaF). The tissues were homogenized for 30 s and the homogenates incubated at 4°C on a rotary shaker for 1 h. The resulting tissue homogenates were then clarified by centrifugation at 20,000 $\times g$ for 10 mins. The protein concentrations were estimated using a bicinchoninic acid assay kit according to the manufacturer's instruction (Thermo). **Filter Aided Sample Preparation:** The mouse tissue lysates were processed for mass spectrometry using the Filter Aided Sample Preparation (FASP) method [58]. In brief, 30 μg of proteins from

each mouse lysate sample were diluted with urea buffer (8 M urea, 100 mM Tris, pH 8.5). In addition, to generate the ion library required for SWATH analysis, a pooled lysate sample for each tissue (30 µg) generated by combining all lysates was also processed. Samples were transferred into a Nanosep[®] filter unit with a 30K molecular weight cut off and centrifuged for 10,000 × g for 15 min and flow-through discarded. Proteins were reduced with 200 mM dithiothreitol, wash with urea buffer and alkylated with 50 mM iodoacetamide. The samples were equilibrated with 0.05 M ammonium bicarbonate and digested using 1 µg of trypsin overnight at 37°C. **Desalting and high pH fractionation:** The tryptic peptides from individual samples were desalted using SOLAµ HRP SPE 96-well plate (Thermo Fisher Scientific) according to the manufacturer's instructions. The tryptic peptides from the pooled sample were fractionated in a similar manner by stepwise elution with increasing concentration of acetonitrile (2.5, 5, 10, 20, 50, 80% v/v) in a high pH buffer (0.1% v/v triethylamine in H₂O). **Analysis of peptides:** The fractionated peptides from the pooled sample were processed in an information dependent acquisition (IDA) manner on an AB Sciex 5600 TripleTOF mass spectrometer (Supplementary Tables S5–8). For SWATH acquisition on individual samples, the TripleTOF[®] 5600 System was configured as described by Gillet et al. [59]. **Data processing:** To generate the local ion library, a protein database search was conducted on the pooled samples using the ProteinPilot version 4.5b Software (AB SCIEX) and the Paragon[™] Algorithm. The search was performed against SwissProt Mus musculus database with a global false discovery rate (FDR) of 1% was used as the threshold for the number of proteins for import. The SWATH Acquisition Microapp 2.0 in PeakView 2.2 (SCIEX) was used to process raw SWATH data files. Processing settings for the SWATH Microapp: 2 peptides per protein, 3 transitions per peptide, peptide confidence threshold corresponding to 1% global FDR and FDR threshold of 1% was used. The resulting peak area for each protein after SWATH processing was exported to MarkerView 1.3.1 (SCIEX) for statistical analysis. The resulting data were normalized using the Total Area Sums (TAS) approach.

Ingenuity pathway analysis (IPA) and protein set enrichment analysis (PSEA)

Pathway enrichment analyses were performed with Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany). IPA was performed to identify canonical pathways, diseases and functions, and protein networks. Significantly enriched pathways for the proteins and pathways were identified with the criterion P -value < 0.05. To determine the genes associated with changes in the protein profile in skeletal muscle, placenta, pancreas and VAT, protein set enrichment analysis (PSEA) was performed and adapted from Gene Set Enrichment Analysis (GSEA, version 3.0) [60]. Normalized SWATH results from tissues obtained from mice (control and treatment) were used in the PSEA. The protein expression data were processed using the hallmark gene sets within the MSigDB database v6.2 with permutations set at 1000 and Signal2Noise metric for ranking genes. Default values were chosen for all other parameters.

Statistical analysis

Statistical analysis was performed on the normalized data unless otherwise specified. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, U.S.A.). For the *in vitro* studies, explants were performed in duplicates, and the average of the duplicates was used for final analysis. A repeated measures ANOVA was used with Fisher's Least Significant Difference (LSD) post hoc testing to discriminate among the means. For the *in vivo* mice studies, unpaired Student's t -test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann–Whitney U (unpaired) test was used for comparisons between groups (control GDM mice vs. GDM mice treated with nobiletin). For all data, statistical significance was assigned to P value < 0.05 and data were expressed as mean ± SEM.

Results

Effect of nobiletin on placenta, VAT and skeletal muscle *in vitro*

Nobiletin improves glucose uptake in skeletal muscle

The effect of nobiletin on glucose uptake in skeletal muscle was determined in the presence of TNF (Figure 1). Insulin significantly increased glucose uptake by 68.1% ($P = 0.0494$) compared with basal levels, while pre-incubation with TNF significantly impaired this increase by 43.2% ($P = 0.0059$). Co-treatment with nobiletin significantly improved insulin-stimulated glucose uptake by 63% ($P = 0.0203$), compared with TNF treatment.

Nobiletin suppresses inflammation in placenta, but not visceral fat

Human placenta was treated with TNF in the presence or absence of nobiletin (Figure 2). TNF significantly increased *Il1a*, *Il1b*, *Il6*, *Ccl2*, *Ccl3* and *Cxcl8* mRNA expression and IL6, CCL2 and CXCL8 protein concentration compared

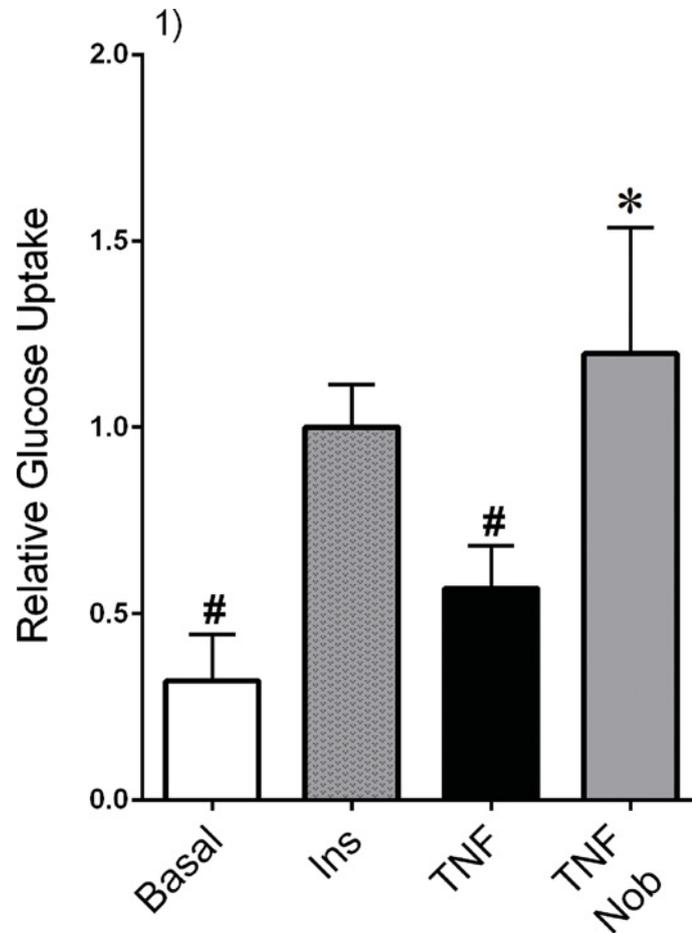


Figure 1. Effect of nobiletin on glucose uptake in human skeletal muscle tissue

Human skeletal muscle tissue was incubated with 10 ng/ml TNF for 20 h with or without 200 μ M nobiletin (Nob), followed by a 30 min incubation with 0.1 μ M insulin ($n = 5$ patients). Glucose uptake was analysed using a radiolabelled 2DG uptake assay. For all data, the fold change was calculated relative to Ins. All data are displayed as mean \pm SEM. # $P < 0.05$ vs. Ins and * $P < 0.05$ vs. TNF (repeated measures one-way ANOVA).

with basal levels. There was no effect of TNF on *Tnfa* mRNA expression or CCL3 protein concentration. Co-treatment with nobiletin significantly reduced *Il1b*, *Il6*, *Ccl2* and *Ccl3* mRNA expression, and IL6, CCL2, CCL3 and CXCL8 secretion compared with TNF treatment. There was no effect of nobiletin on *Il1a*, *Cxcl8* and *Tnfa* mRNA expression compared with TNF treatment. The effect of nobiletin on IL1A and IL1B protein secretion could not be measured as their concentrations in the incubation media were below the sensitivity of the assay.

The effect of nobiletin on pro-inflammatory mediators in TNF-stimulated VAT was also investigated and is depicted in Figure 3. TNF treatment increased *Il1a*, *Il1b*, *Ccl2*, *Cxcl1*, *Cxcl8* and *Tnfa* mRNA expression and IL6, CCL2, CXCL1 and CXCL8 release in VAT compared with basal levels. There was no effect of TNF on *Il6* mRNA expression compared with basal. Co-treatment with nobiletin significantly reduced only *Ccl2* mRNA expression and protein secretion compared with TNF treatment. Nobiletin had no effect on mRNA expression and protein secretion of any other cytokines (IL1A, IL1B, IL6 and TNF) or chemokines (CXCL1 and CXCL8).

Nobiletin acts through NF- κ B, Akt and MAPK Erk pathways in placenta and VAT

The signalling pathways that nobiletin may interact with were explored in placenta, VAT and skeletal muscle stimulated with TNF (Figure 4). Nobiletin has been shown to regulate numerous signalling pathways including NF- κ B in murine macrophages [42], Akt in intestinal Caco-2 cells [43] and MAPK pathways in human fibroblasts [44]. Therefore, it was of interest to determine if nobiletin also targets these signalling pathways in the present study. In

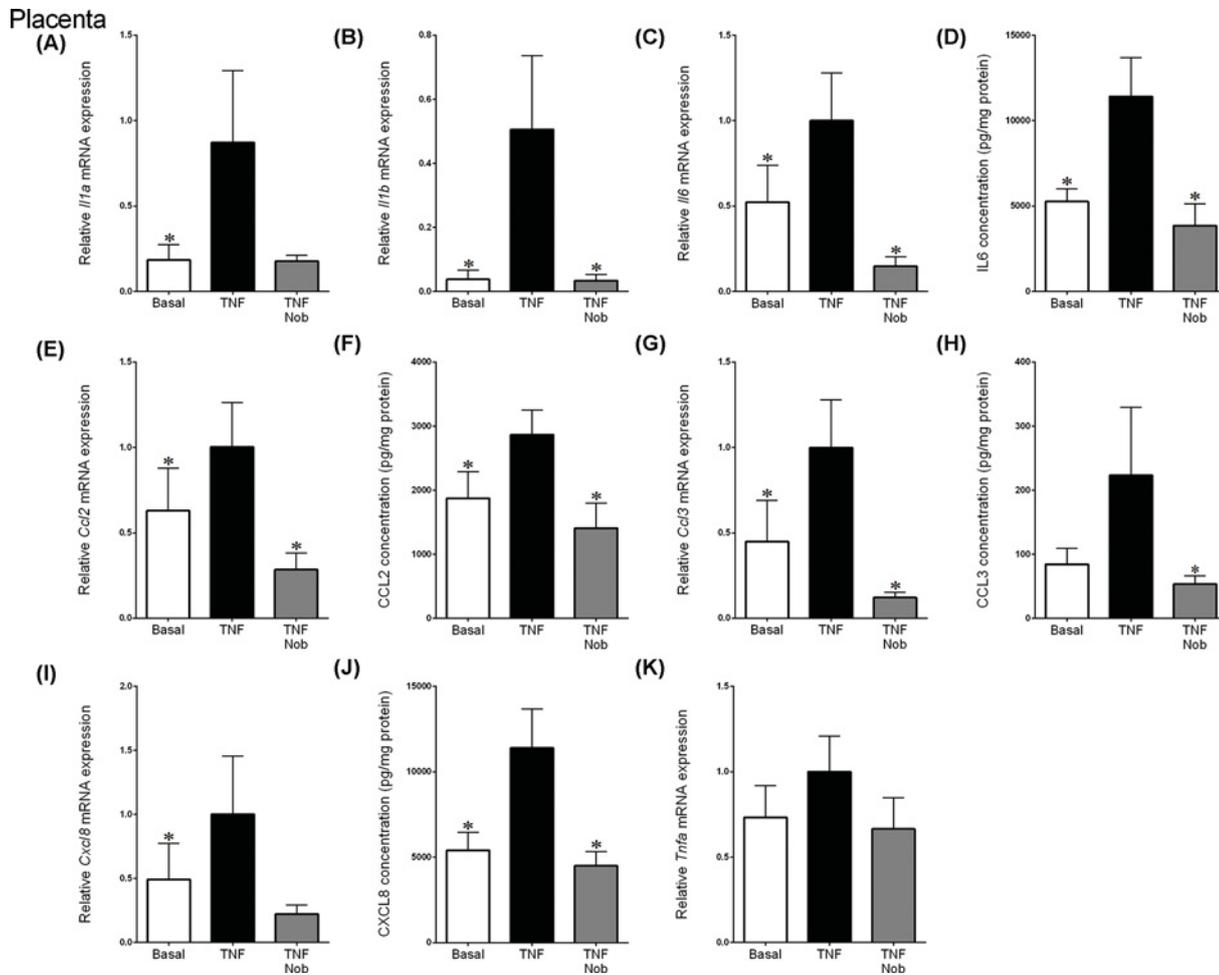


Figure 2. Effect of nobiletin on TNF-induced pro-inflammatory gene expression and protein secretion in placenta
 Human placenta was incubated with 10 ng/ml TNF in the absence or presence of 100 μ M nobiletin (Nob) for 20 h ($n=6$ patients). (A–C,E,G,I,K) *Il1a*, *Il1b*, *Il6*, *Ccl2*, *Ccl3*, *Cxcl8* and *Tnfa* mRNA expression was analysed by qRT-PCR. (D,F,H,J) IL6, CCL2, CCL3 and CXCL8 concentrations in the incubation media were analysed by ELISA. All data is displayed as mean \pm SEM. * $P < 0.05$ vs. TNF (repeated measures one-way ANOVA).

unstimulated cells, the inactive NF- κ B complex is bound to I κ B- α . Pro-inflammatory stimulation induces the phosphorylation and subsequent degradation of I κ B- α , and releases the NF- κ B complex for translocation into the nucleus [61,62]. In our study, TNF reduced total I κ B- α protein expression in all tissues. Nobiletin treatment significantly reversed this effect in VAT (Figure 4D); however, there was no effect in placenta or skeletal muscle (Figure 4A,G). Nobiletin treatment also significantly suppressed placental Akt (Figure 4B) and MAPK ERK1/2 activation (Figure 4C) compared with TNF treatment; however there was no effect on Akt or MAPK ERK1/2 activation in VAT (Figure 4E,F). There was no effect of nobiletin on any signalling pathway in skeletal muscle (Figure 4G–I).

Summary

Nobiletin treatment was found to improve TNF-impaired glucose uptake in skeletal muscle explants, and suppress TNF-induced mRNA expression and protein secretion of pro-inflammatory cytokines and chemokines in placenta but not VAT. These actions appear to be mediated through the NF- κ B/Akt/MAPK pathways in placenta and VAT.

Effect of nobiletin on GDM mice

Effect of nobiletin on maternal fasting glucose levels of GDM mice

To extend our *in vitro* findings, we determined the effect of nobiletin treatment in GDM mice. First, we investigated the effect of nobiletin on maternal fasting glucose levels in GDM dams. Pregnant mice were administered daily doses

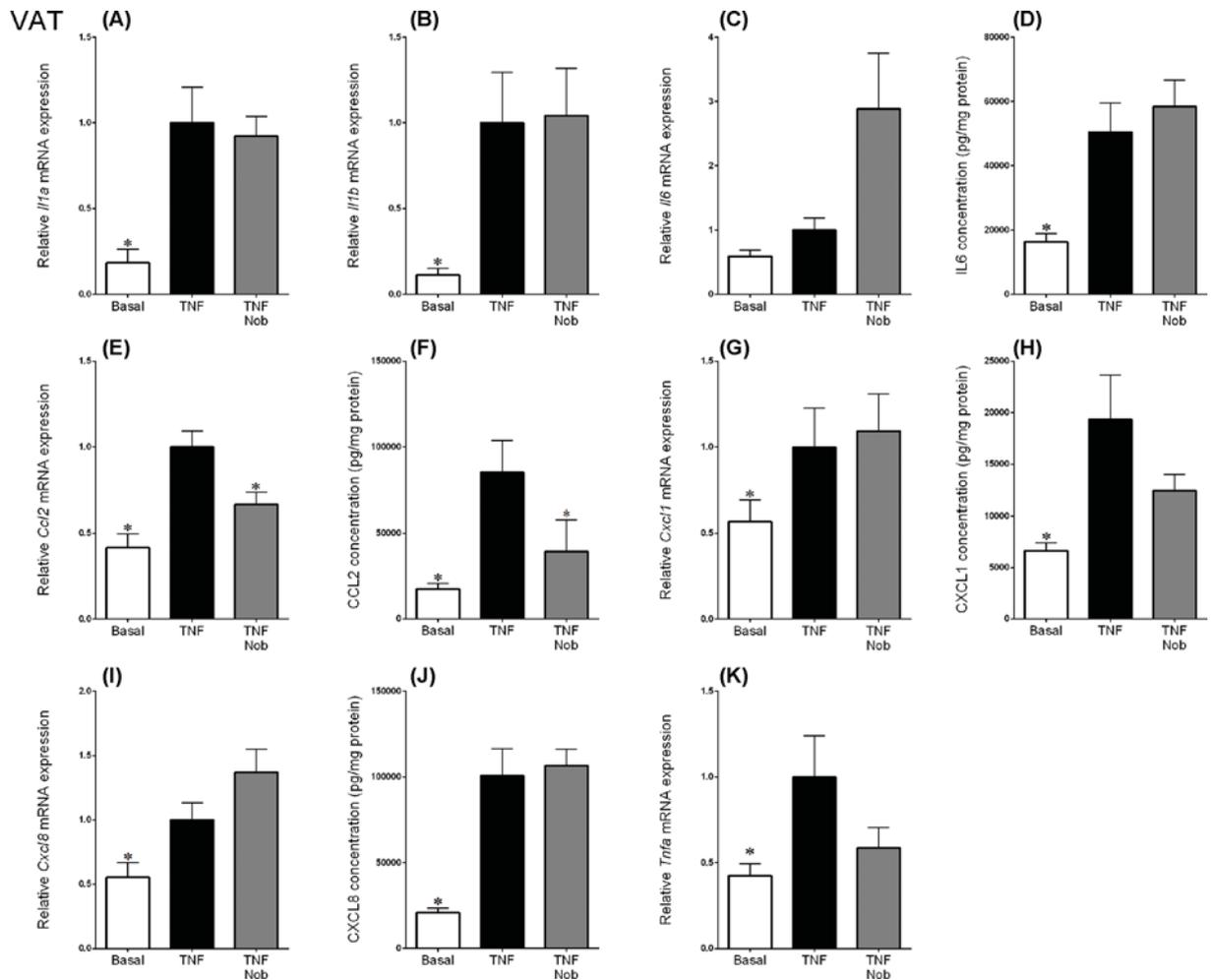


Figure 3. Effect of nobiletin on TNF-induced pro-inflammatory gene expression and protein secretion in VAT

Human VAT was incubated with 10 ng/ml TNF in the absence or presence of 200 μ M nobiletin (Nob) for 20 h ($n=6$ patients). (A–C,E,G,I,K) *Il1a*, *Il1b*, *Il6*, *Ccl2*, *Cxcl1*, *Cxcl8* and *Tnfa* mRNA expression was analysed by qRT-PCR. (D,F,H,J) IL6, CCL2, CXCL1 and CXCL8 concentrations in the incubation media were analysed by ELISA. All data are displayed as mean \pm SEM. * $P<0.05$ vs. TNF (repeated measures one-way ANOVA).

of either nobiletin (50 mg/kg) or a vehicle solution via oral gavage from gd 1 until gd 17 (gd 1–17) or via i.p. injection from gd 10 until gd 17 (gd 10–17). Maternal plasma glucose levels were measured on gd 17 following a 5 h fast and is depicted in Figure 5. Daily nobiletin treatment from either gd 1–17 or gd 10–17 significantly decreased fasting blood glucose levels by 13.3% ($P = 0.0081$) and 16.2% ($P = 0.0043$) respectively, compared with control mice. There was no effect on the area under the curve of GDM mice treated from either gd 1–17 or gd 10–17.

Effect of nobiletin on inflammation in GDM mice

Following measurement of fasting glucose levels at gd 17, mice were administered the last dose of nobiletin or vehicle control, allowed to recover and then killed the next day (gd 18). Placenta and maternal VAT and subcutaneous adipose tissue (SAT) were collected. The inflammatory profiles in each tissue are depicted in Figures 6 and 7.

GDM mice receiving daily treatments of nobiletin from either gd 1–17 or gd 10–17 had significantly reduced levels of *Cxcl1* and *Il1a* mRNA in placenta when compared with control mice. Nobiletin treatment had no significant effect on placental *Ccl2*, *Ccl3*, *Il1b* or *Tnfa* mRNA expression, irrespective of treatment length.

VAT of GDM mice treated with nobiletin from gd 1–17 or gd 10–17 had significantly lower levels of *Ccl2* and *Il1a* mRNA expression as compared with the control mice. Nobiletin treatment from gd 1–17, but not gd 10–17,

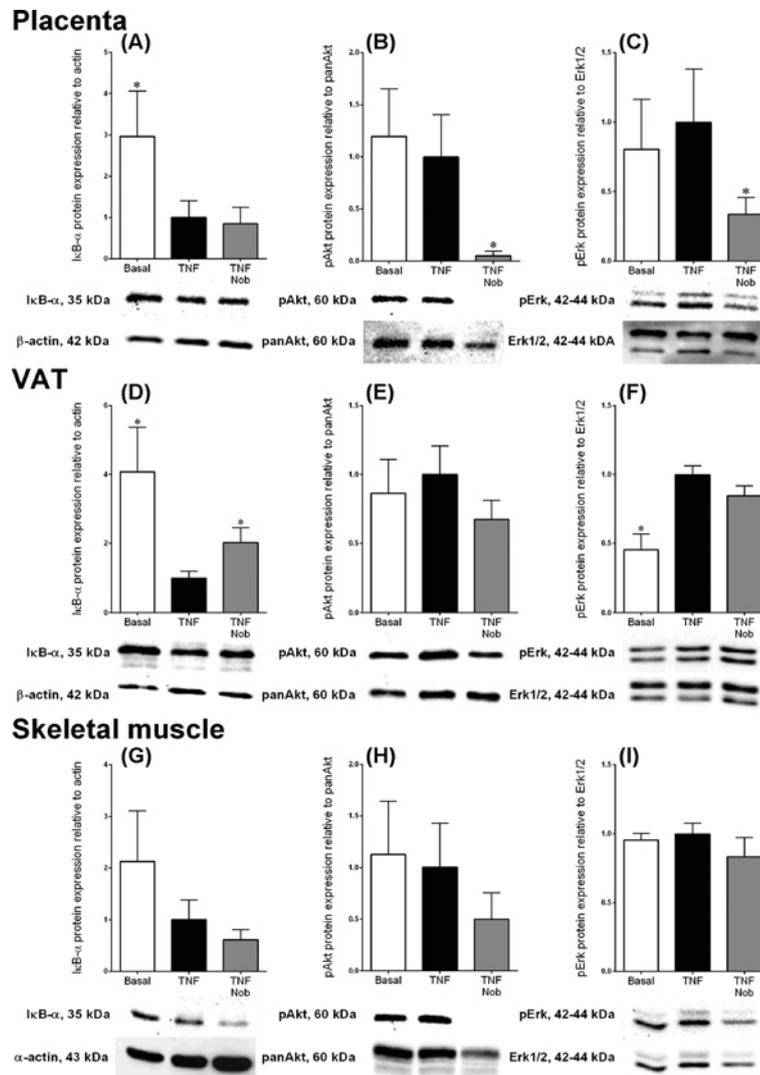


Figure 4. Effect of nobiletin on activation of NF-κB, Akt and MAPK in placenta, VAT and skeletal muscle

Human (A–C) placenta, (D–F) VAT and (G–I) skeletal muscle were incubated in the absence or presence of 200 μM nobiletin (Nob) for 20 h, before stimulation with 10 ng/ml TNF for 30 min ($n = 3–5$ patients per group). (A,D,G) Total IκB-α, (B,E,H) Akt, and (C,F,I) Erk1/2 protein expression was analysed by Western blotting. A representative Western blot from one patient is shown. For all data, the fold change was calculated relative to the TNF group and data is displayed as mean ± SEM. * $P < 0.05$ vs. TNF (repeated measures one-way ANOVA).

significantly reduced *Il1b* mRNA expression when compared with control mice. There was no significant effect of nobiletin treatment on *Ccl3*, *Cxcl1* or *Tnfa* mRNA expression, irrespective of treatment length.

SAT of GDM mice treated with nobiletin from gd 1–17 showed significantly reduced levels of *Ccl2*, *Il1a* and *Il1b* mRNA expression as compared with the control mice; however, there was no effect on *Ccl3*, *Cxcl1* or *Tnfa* mRNA expression. Of note, in mice receiving nobiletin treatment from gd 10–17, *Ccl2*, *Cxcl1*, *Il1a*, *Il1b* and *Tnfa* mRNA expression were significantly reduced compared with control mice. Like in VAT, there was no effect on *Ccl3* mRNA expression at any time point.

Effect of nobiletin on the global protein profile in GDM mice

To determine whether the changes observed in glucose metabolism and systemic inflammation in response to nobiletin were associated with changes in the proteomic content in skeletal muscle, placenta, pancreas, and VAT, a quantitative, information-independent acquisition (Sequential Windowed Acquisition of All Theoretical Mass Spectra [SWATH]) approach was used.

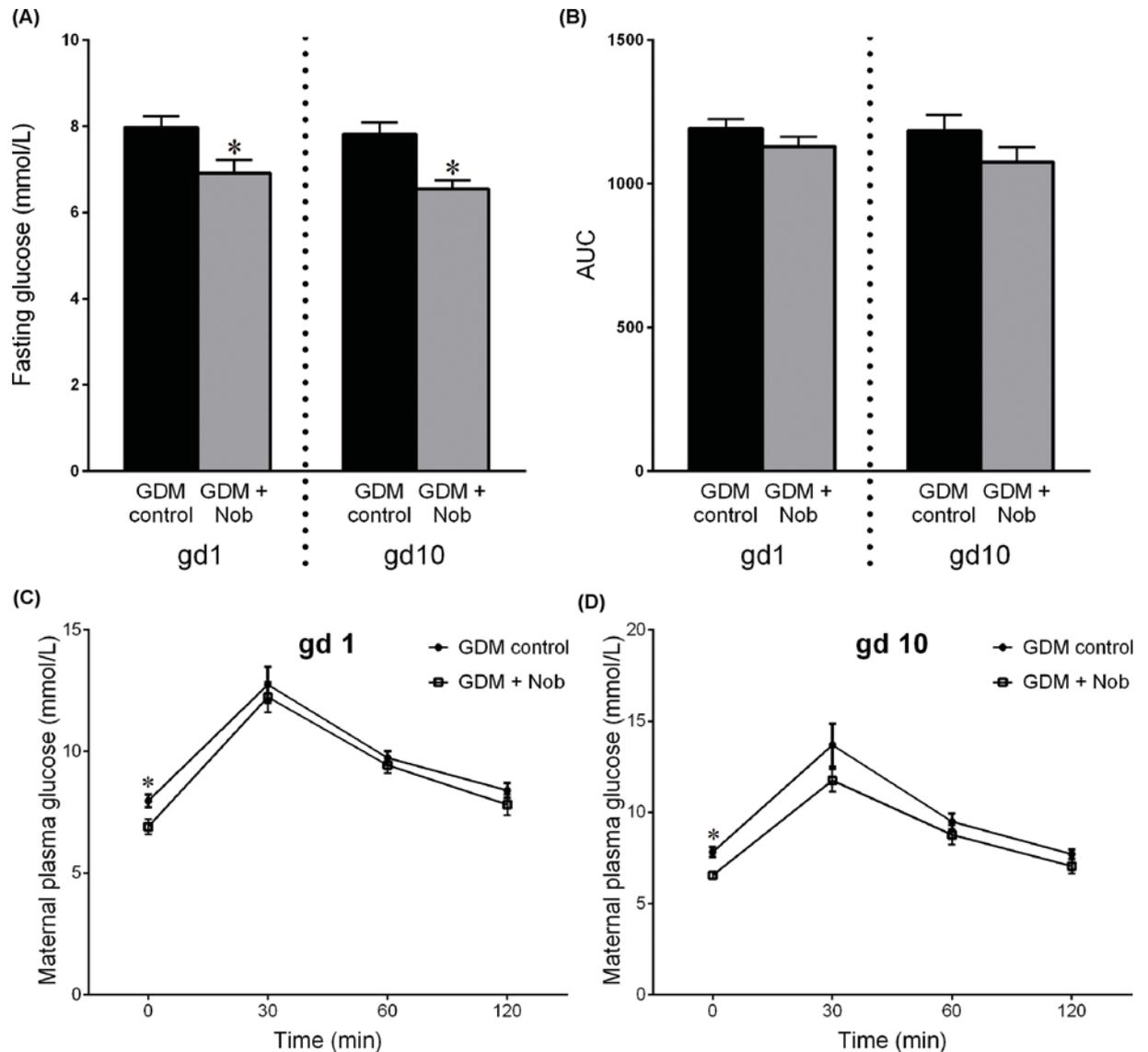


Figure 5. Effect of nobiletin on the fasting glucose levels in GDM mice

Control mice were administered daily doses of 50 mg/kg nobiletin (Nob) or a vehicle control via oral gavage commencing from gd 1–17 or via i.p. commencing from gd 10–17. **(A)** Maternal fasting glucose levels, **(B)** AUC and **(C)** and **(D)** maternal glucose levels during the 2 h oral GTT were measured via tail vein bleeding on gd 17, immediately following a 6 h fast. The data is presented as mean \pm SEM. * $P < 0.05$ vs. control (paired-sample comparison).

Information-dependent acquisition (IDA) and SWATH profile were generated from tissues using independent samples ($n = 5$) per group (i.e. tissues obtained from 10 individual GDM mice treated with or without nobiletin). The IDA library was used to identify peptide ions that were present in SWATH ion profiles. Proteins were identified and quantified by comparing SWATH-generated peptide ion profiles for each individual sample against the IDA library (PeakView). IDA of mass spectra from a pool of tissues samples was initially performed and identified a total of 1439, 2156, 1582, and 2198 proteins in skeletal muscle, placenta, pancreas, and VAT respectively (Supplementary Tables S5–8). Then, the quantification of all proteins identified for each tissue was performed in all individual samples using SWATH MS/MS. The variation in the relative abundance of proteins between controls (i.e. GDM mice) and treatment (GDM mice treated with nobiletin) was established by comparison with the SWATH profile against the IDA library and presented as volcano plots (Figure 8). In skeletal muscle, placenta, pancreas and VAT, a total of 13, 30, 36, and 43 statistically significant protein identifications (FDR 1%, and $P < 0.05$) in the relative expression of proteins in treatment compared with control were identified, respectively (Supplementary Table S9).

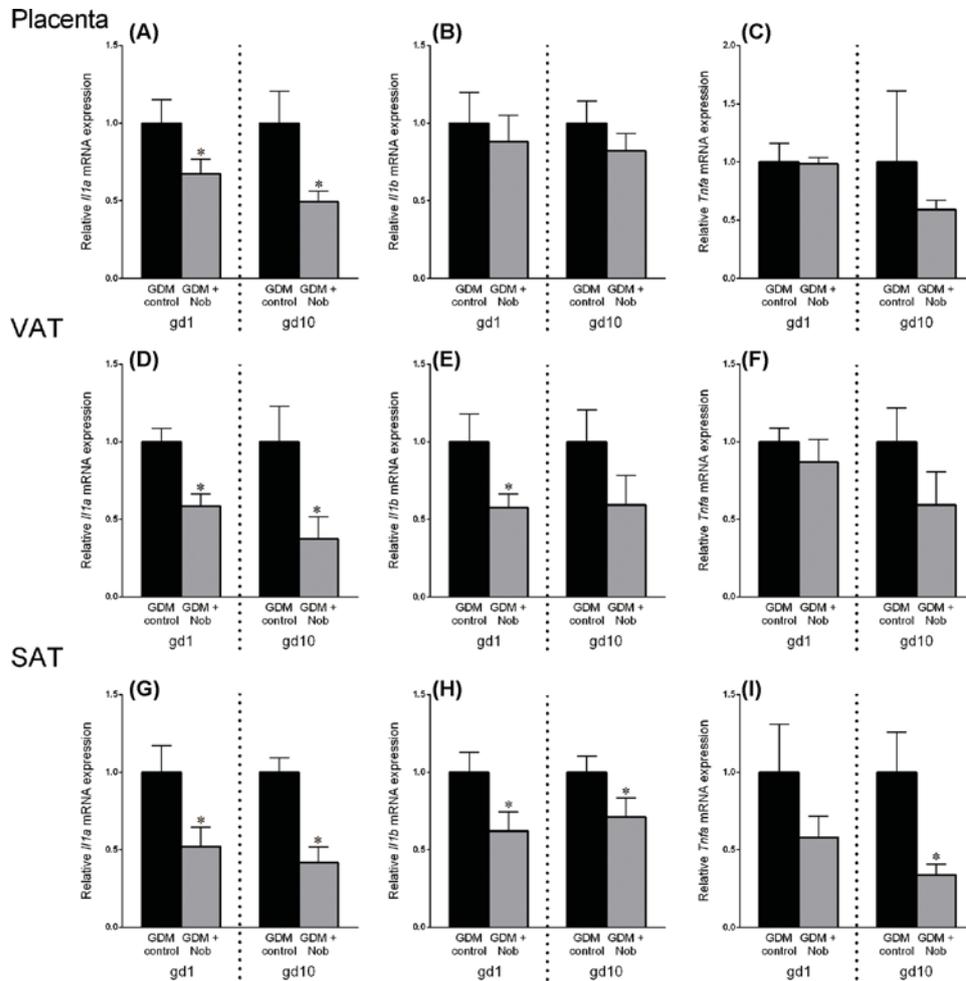


Figure 6. Effect of nobiletin on pro-inflammatory cytokines in placenta, VAT and SAT of GDM mice

GDM mice were administered daily doses of 50 mg/kg nobiletin (Nob) or a vehicle control via oral gavage commencing from gd 1-17 or via i.p. commencing from gd 10-17. On gd 18, mice were fasted for 6 h and killed. (A–C) Placenta from 3 pups, (D–F) maternal VAT and (G–I) SAT were collected. (A,D,G) *I/1a*, (B,E,H) *I/1b* and (C,F,I) *Tnfa* mRNA expression were analysed by qRT-PCR and the fold change was calculated relative to control. All data are displayed as mean \pm SEM. * $P < 0.05$ vs. control (paired-sample comparison).

The proteins significantly different (i.e. down and up-regulated proteins) in tissues from GDM mice treated with nobiletin compared with controls were subjected to ontology and pathway analysis using Ingenuity Pathways Analysis (IPA) and Protein Set Enrichment Analysis (PSEA) (Figures 8-10). The largest differences for the up-regulated compared with the down-regulated proteins in the canonical pathways identified were in skeletal muscle on mitochondrial dysfunction, oxidative phosphorylation and sirtuin (SIRT) signalling pathways; in placenta on valine degradation and xanthine and xanthosine salvage; in pancreas on LPS/IL-1 mediated inhibition of retinoid X receptor (RXR) function and serine protease inhibitor Kazal-type (SPINK)1 pancreatic cancer pathway; and in VAT on 5' AMP-activated protein kinase (AMPK) and integrin-linked kinase (ILK) pathways signalling.

Next, to investigate the potential functions of these differentially expressed proteins, IPA of the proteomic profile was performed. The main networks identified were gene expression, RNA damage and repair, and RNA post-transcriptional modification for skeletal muscle tissue; cell morphology, cell-to-cell signalling and interaction, and cellular movement for placenta tissue; cell morphology and embryonic development for the pancreas tissue, and cellular assembly and organization, and cellular function and maintenance for VAT tissue (Figure 9). The ranking of the pathways is displayed in Supplementary Table S10.

Finally, the data were analysed by PSEA and are summarized in Figure 10. In skeletal muscle, placenta, pancreas and VAT obtained from GDM mice treated with nobiletin, there was enrichment of proteins associated with MTORC1

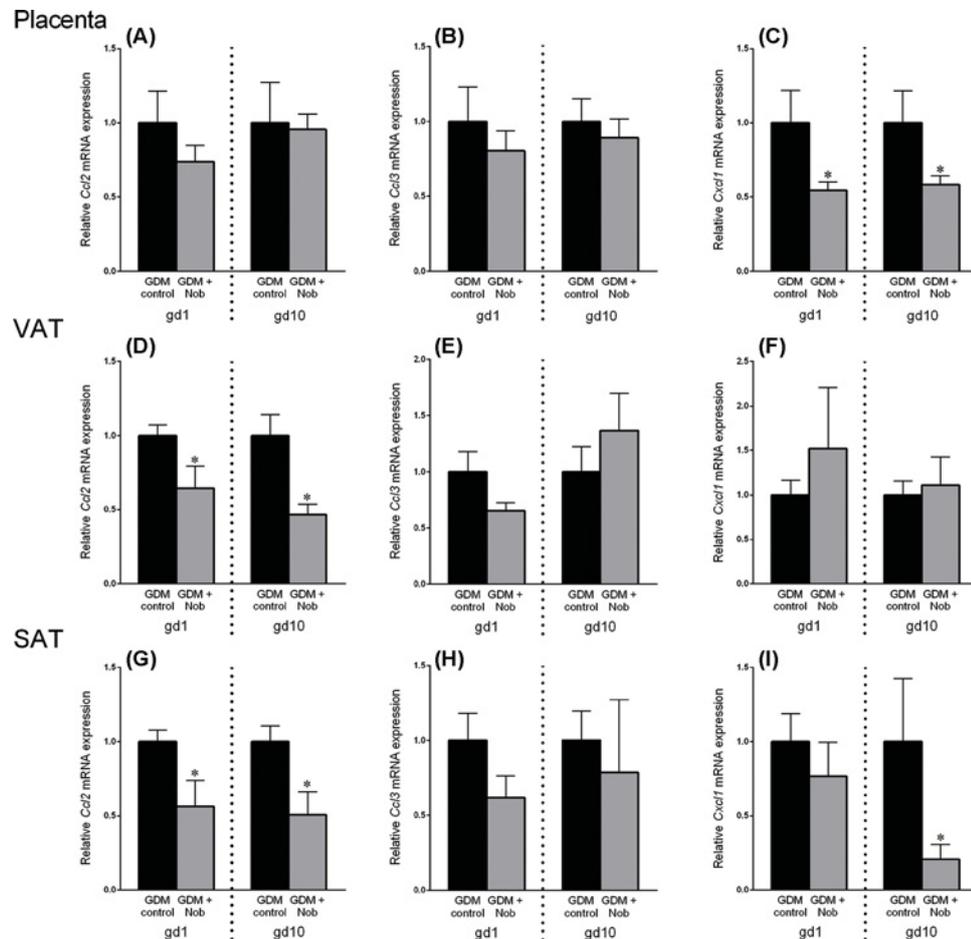


Figure 7. Effect of nobiletin on pro-inflammatory chemokines in placenta, VAT and SAT of GDM mice

GDM mice were administered daily doses of 50 mg/kg nobiletin (Nob) or a vehicle control via oral gavage commencing from gd 1–17 or via i.p. commencing from gd 10–17. On gd 18, mice were fasted for 6 h and killed. (A–C) Placenta from 3 pups, (D–F) maternal VAT and (G–I) SAT were collected. (A,D,G) *Ccl2*, (B,E,H) *Ccl3* and (C,F,I) *Cxcl1* mRNA expression were analysed by qRT-PCR and the fold change was calculated relative to control. All data are displayed as mean \pm SEM. * $P < 0.05$ vs. control (paired-sample comparison).

signalling, oxidative phosphorylation, fatty acid metabolism, xenobiotic metabolism, glycolysis and MYC target genes (Figure 10).

Summary

Nobiletin supplementation beginning from either gd 0 or gd 10 was found to improve fasting blood glucose levels of GDM mice, as well as reduce mRNA expression of pro-inflammatory cytokines and chemokines in placenta, VAT and SAT of GDM mice. Using a quantitative mass spectrometry approach, we identified several metabolic pathway targets of nobiletin in skeletal muscle, placenta, pancreas and VAT of GDM mice.

Discussion

The present study is the first to report glucose-sensitizing and anti-inflammatory properties of the citrus flavonoid nobiletin in an *in vitro* human model and *in vivo* mouse model of GDM. As previously described, TNF induces inflammation in placenta and adipose tissue, and insulin resistance in skeletal muscle [23,47]. Thus, TNF was used to generate an *in vitro* placenta, adipose and skeletal muscle model of GDM. Nobiletin treatment was found to restore TNF-impaired glucose uptake in an *in vitro* skeletal muscle model of GDM, while also reducing TNF-induced expression and secretion of pro-inflammatory cytokines and chemokines in human placenta. In concordance with

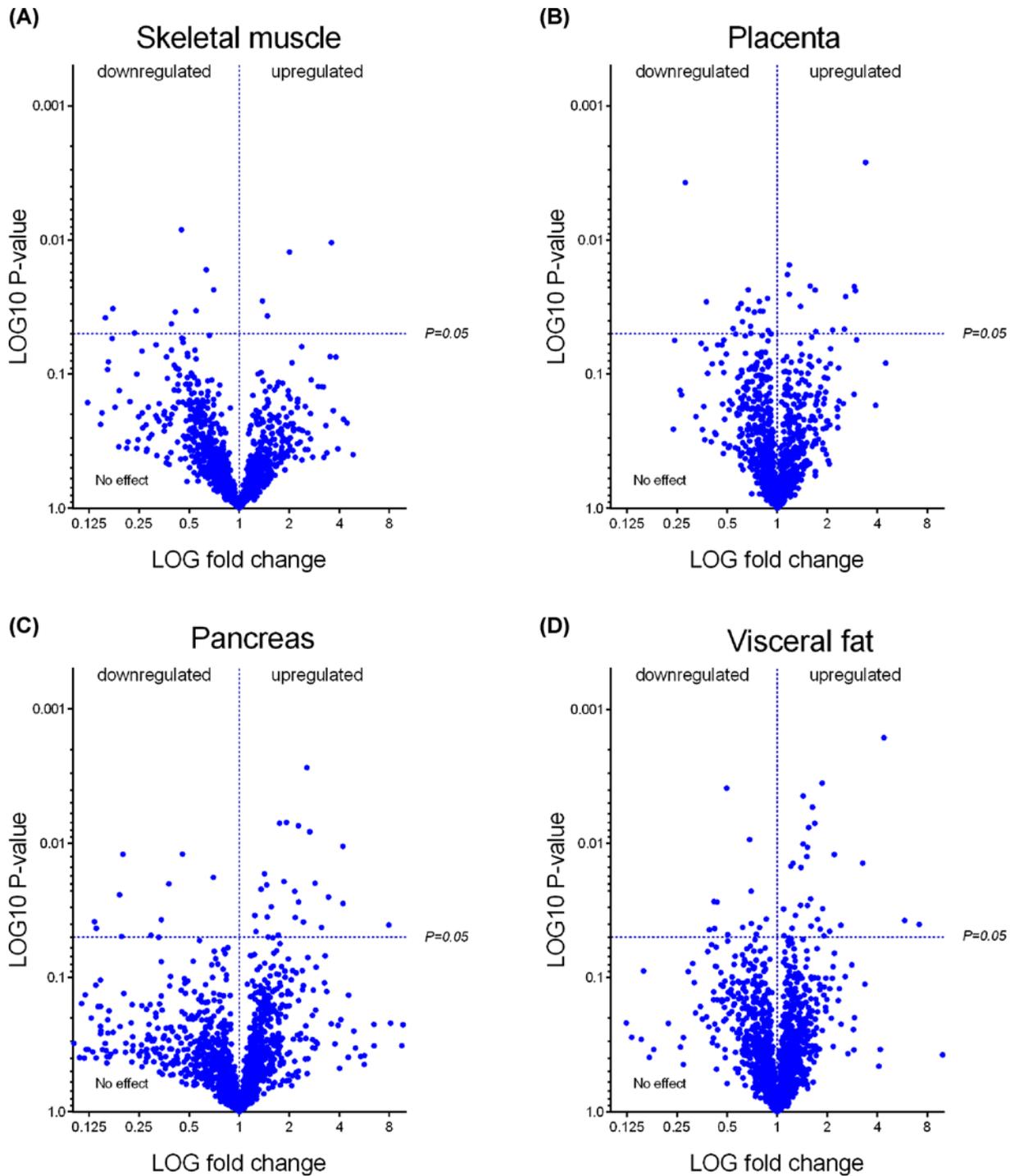


Figure 8. Effect of nobiletin on global protein profile in skeletal muscle, placenta, pancreas and VAT of GDM mice

GDM mice were administered daily doses of 50 mg/kg nobiletin (Nob) or a vehicle control via i.p. commencing from gd 10–17. On gd 18, mice were fasted for 6 h and killed. Proteins were extracted from (A) skeletal muscle, (B) placenta from 3 pups, (C) pancreas and (D) VAT and processed for SWATH and IDA analysis. In each volcano plot, the horizontal axis represents the fold change and the vertical axis, the P value, where the horizontal dotted line indicates $P=0.05$. Each point represents a protein, with those on the right above the dotted line indicating up-regulated proteins and those on the left, down-regulated proteins. Additional details for each significantly differentially regulated protein can be found in Supplementary Table S9.

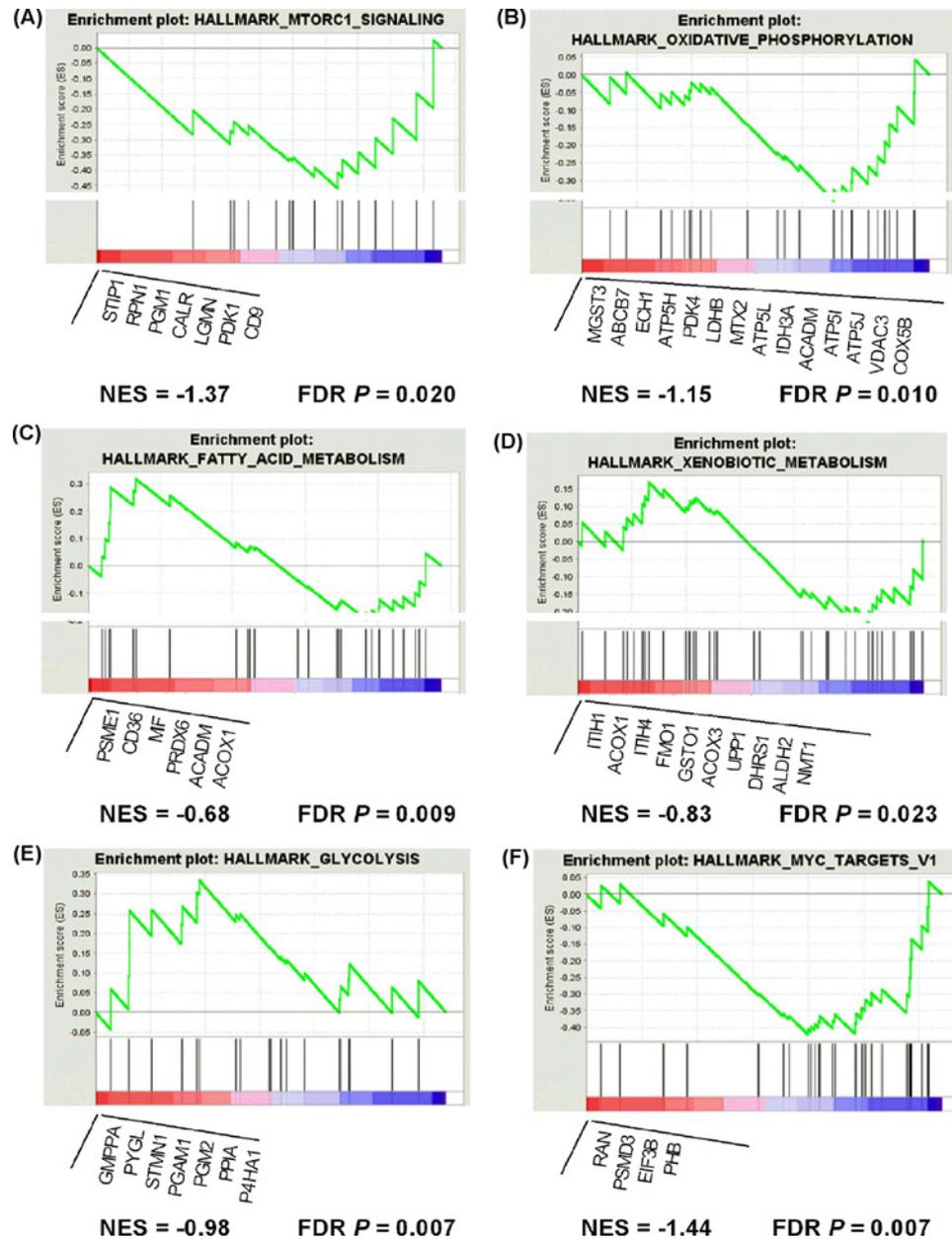
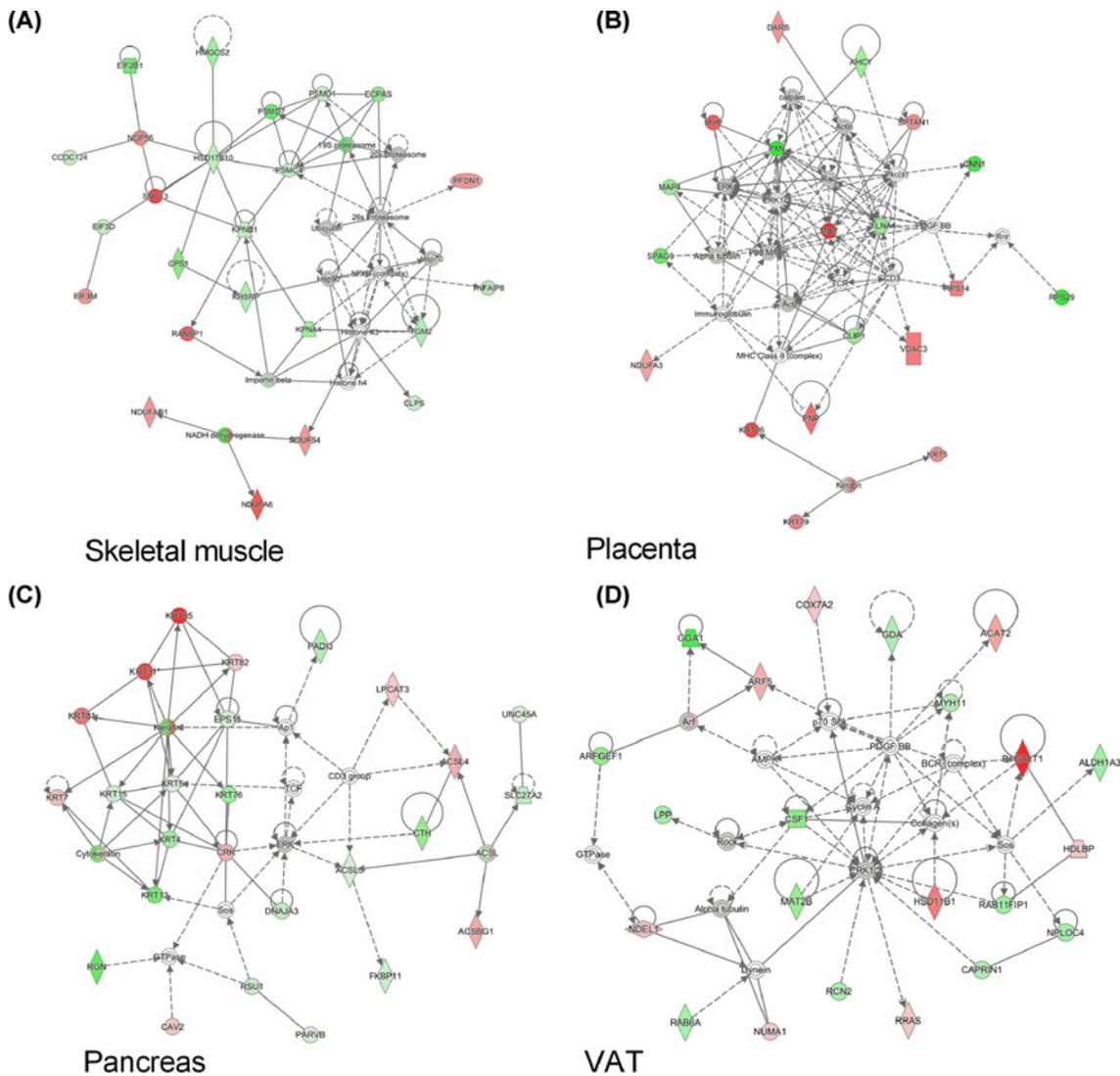


Figure 10. Protein set enrichment analysis in skeletal muscle, placenta, pancreas and VAT of GDM mice

GDM mice were administered daily doses of 50 mg/kg nobiletin (Nob) or a vehicle control via i.p. commencing from gd 10-17. On gd 18, mice were fasted for 6 h and killed. Proteins were extracted from skeletal muscle, placenta from 3 pups, pancreas and VAT and processed for SWATH and PSEA analysis. Proteins associated with (A) MTORC1 signalling, (B) oxidative phosphorylation, (C) fatty acid metabolism, (D) xenobiotic metabolism, (E) glycolysis and (F) MYC target genes were enriched in the Nob-treated GDM mice. For each signalling pathway, the Normalized Enrichment Score (NES) and False Discovery Rate P value (FDR P) are displayed.

these *in vitro* studies, nobiletin treatment of GDM mice significantly improved fasting glucose levels and decreased inflammation in placenta and maternal adipose tissue (VAT and SAT).

Maternal peripheral insulin resistance is a key feature of GDM [14]. While both VAT and skeletal muscle play key roles in insulin signalling and diabetes development, skeletal muscle contributes to 75% of glucose uptake that occurs in the body [63]. Further, insulin resistance associated with GDM is thought to be driven by TNF-induced defects in the insulin signalling pathway and glucose uptake in skeletal muscle of pregnant women *in vitro* [16,18,19]. Thus,



Tissue type	Top networks
Skeletal muscle	Gene expression RNA damage and repair RNA post-transcriptional modification
Placenta	Cell morphology Cell-to-cell signalling and interaction Cellular movement
Pancreas	Cell morphology Embryonic development Hair and skin development and function
VAT	Cellular assembly and organization Cellular function and maintenance Cellular movement

Figure 9. Effect of nobiletin on signalling networks in skeletal muscle, placenta, pancreas and VAT of GDM mice
 GDM mice were administered daily doses of 50 mg/kg nobiletin (Nob) or a vehicle control via i.p. commencing from gd 10–17. On gd 18, mice were fasted for 6 h and killed. Proteins were extracted from (A) skeletal muscle, (B) placenta from 3 pups, (C) pancreas and (D) VAT and processed for SWATH and IDA analysis. The networks represent the differentially expressed proteins associated with: (A) RNA damage and repair, and RNA post-transcriptional modification for skeletal muscle tissue; (B) cell morphology, cell-to-cell signalling and interaction, and cellular movement for placenta tissue; (C) cell morphology and embryonic development for the pancreas tissue, and (D) cellular assembly and organization, and cellular function and maintenance for VAT tissue. In each network, proteins are displayed as nodes, and the relationships between the nodes as lines. The color intensity of each node indicates the degree of up-regulation (red) or down-regulation (green) of the respective protein.

in the present study, we assessed the effect of nobiletin on glucose metabolism in skeletal muscle from pregnant women treated with TNF to induce insulin resistance. In our study, nobiletin co-treatment reversed TNF-induced reductions in insulin-stimulated glucose uptake in skeletal muscle. Interestingly, in other studies, contradictory results have been observed. In HepG2 hepatocytes, 200 μM nobiletin had no effect on basal glucose uptake, but did improve palmitate-impaired glucose uptake [40]. On the other hand, nobiletin treatment of doses ranging from 5 to 50 μM was found to increase glucose uptake in both basal and in insulin-stimulated murine adipocytes [39]. These results suggest that the effects of nobiletin on basal glucose uptake may be dose and tissue dependent. Regardless, this is the first report of nobiletin exerting insulin-sensitizing properties in the context of pregnancy and GDM and may be a novel therapeutic to improve glucose metabolism associated with GDM.

Maternal low-grade inflammation, including increased presence of circulating pro-inflammatory markers, is one of the central features of the pathophysiology underlying GDM [15–19]. Evidence suggests that human placenta, adipose tissue and skeletal muscle are critical sites in the propagation of maternal inflammation associated with GDM [20–22]. In the present study, we stimulated human placenta and adipose tissue with the pro-inflammatory cytokine TNF to generate a GDM-like model *in vitro*. Nobiletin treatment significantly reversed TNF-induced expression and secretion of pro-inflammatory cytokines and chemokines in placenta. However, in VAT, nobiletin reduced only *Ccl2* mRNA expression and protein secretion, likely suggesting nobiletin exerts tissue-specific effects. Regardless, this is the first study to report anti-inflammatory properties of nobiletin in an *in vitro* placenta and VAT model of GDM.

By examining known pro-inflammatory pathways, we sought to identify the mechanistic pathways that nobiletin may exert its properties in our *in vitro* model of GDM. Nobiletin is implicated to interfere with the NF- κB [42], Akt [43] and MAPK pathways [44]. Using targeted Western blotting *in vitro*, we found that nobiletin significantly suppressed Akt and Erk activation in placenta, and prevented I κB - α degradation in VAT. There was no effect of nobiletin treatment in skeletal muscle. Nobiletin has previously been observed to dose- and time dependently reduce Akt activation in LPS-stimulated intestinal Caco-2 cells using a similar dose and incubation period as we have reported [43]. In the same study, rats undergoing experimental colitis were supplemented with a similar dose of nobiletin as in our study for 1 week, resulting in reduced Akt activation and NF κB protein expression in rat intestinal epithelia. In contrast, prolonged oral treatment of higher dose (100 and 200 mg/kg) nobiletin to obese diabetic mice was found to increase Akt activation in VAT [37,64]. Promisingly, however, I κB - α protein expression in VAT was improved, and *Tnf*, *Il6* and *Ccl2* mRNA expression in VAT was reduced [37,64]. While it is clear nobiletin acts in a dose-, time- and tissue-dependent manner, it may be that short-term, lower dose exposure to nobiletin presents the better anti-inflammatory response in models of inflammation.

Based on the promising findings in our *in vitro* studies, we then sought to identify the effects of nobiletin on GDM mice. We assessed the effects of treatment at two time-points: one group of GDM mice were treated with nobiletin or vehicle from gd 1-17 to mimic prophylactic supplementation, while a second group of GDM mice were treated from gd 10-17 to mimic a therapeutic treatment regime following GDM diagnosis. This is applicable to human pregnancies, given extensive predictive studies that can identify risk for human GDM in the first trimester [65,66].

Given that nobiletin increased glucose uptake in *in vitro* skeletal muscle explants, we hypothesized that nobiletin treatment may lower fasting blood glucose levels *in vivo*. Similar to non-pregnant studies in HFD obese mice [37,38], our studies found that both prophylactic nobiletin treatment from gd 1-17 and therapeutic treatment from gd 10-17 reduced fasting glucose levels of GDM mice, compared with control. However, there was no significant effect of either treatment length on AUC of GDM mice, compared with control. Regardless, the finding that nobiletin reduces fasting glucose levels irrespective of treatment length is highly promising, given that fasting glucose levels during pregnancy is associated with offspring health outcomes [28]. Altogether, this may suggest that nobiletin exerts an additive insulin-like effect on glucose uptake levels, and thus may be a potent glucose-sensitizing agent.

Our *in vivo* studies also found that nobiletin treatment of GDM mice from both gd 1-17 and gd 10-17 significantly suppressed mRNA expression of pro-inflammatory cytokines and chemokines in placenta, VAT and SAT, compared with control mice. These data suggest that nobiletin is potently anti-inflammatory in GDM mice, regardless of treatment length. Interestingly, nobiletin treatment also inhibited cytokine and chemokine expression in a tissue-specific manner with most potency in SAT. Additional *in vitro* studies exploring the effect of nobiletin in SAT may provide further clarity. Nevertheless, this is the first study to report anti-inflammatory properties of nobiletin in placenta and maternal adipose tissue *in vivo*.

Finally, we used a quantitative mass spectrometry approach to identify the pathways through which nobiletin may act in skeletal muscle, placenta, pancreas and VAT of GDM mice. In skeletal muscle, nobiletin treatment differentially regulated expression of proteins associated with mitochondrial dysfunction, oxidative phosphorylation and SIRT signalling. This finding is of great importance, given the documented alterations in mitochondrial function and oxidative

phosphorylation in GDM [67,68]. Furthermore, extensive studies suggest a role for dysregulated SIRT signalling in GDM pathophysiology, making it a potential target for GDM therapeutics [69].

In placenta, nobiletin down-regulated valine degradation associated proteins, while up-regulating xanthine and xanthosine salvage associated proteins. These findings are noteworthy, given the documented increased concentrations of valine [70] and xanthine [71] in GDM pregnancies. These data suggest that nobiletin may regulate placental nutrient metabolism in GDM mice.

In pancreas, nobiletin differentially regulated expression of proteins associated with LPS/IL-1 mediated inhibition of RXR function. The RXR family of nuclear receptors are involved in several different cell processes including, but not limited to, fatty acid, lipid and xenobiotic metabolism and transport [72]. In GDM pregnancies, altered lipid levels in maternal circulation, as well as altered expression of placental nutrient transporters, suggest dysregulation of lipid metabolism and transport [73,74]. It is then of interest that enzymes involved in digestion of starches and fats, as well as cholesterol metabolism, were down-regulated, while enzymes involved in steroidogenesis were up-regulated. Proteins associated with the SPINK1 pancreatic cancer pathway were also differentially expressed. SPINK1 is an inhibitor for serine proteases, such as trypsin, where SPINK1 down-regulation has been associated with pancreatitis [75]. In the present study, we found nobiletin down-regulated serine protease 2, chymotrypsinogen and chymotrypsin-like elastase, which may indicate protective properties against pancreatitis.

Finally, in VAT, proteins associated with AMPK and ILK pathways were differentially expressed with nobiletin treatment. AMPK is a regulator of glucose and lipid metabolism and inflammation, and its dysfunction is thought to play a role in GDM pathophysiology [69]. Past evidence has shown that nobiletin may increase mRNA expression of similar lipid metabolism related genes, such as PPAR γ and fatty acid synthase, in white adipose tissue of high fat diet-fed mice for increased fatty acid storage and lipid accumulation [37,38]. Together this suggests that nobiletin may be able to target lipid uptake dysregulation in VAT; however, this requires further experimental validation. ILK signalling is involved in the connection of integrins to the cytoskeleton to maintain cell–matrix adhesion [76]. In the present study, nobiletin treatment appeared to up-regulate proteins associated with lipid metabolism, protein synthesis and cytoskeletal reorganization.

PSEA analysis of the differentially expressed proteins also found protein enrichment of distinct pathways including MTORC1, oxidative phosphorylation, fatty acid metabolism, xenobiotic metabolism, glycolysis and MYC target genes. Numerous studies highlight a role for up-regulated MTORC1 in GDM pathophysiology, suggesting that its over activation may be involved in placental nutrient transfer and metabolism, and fetal overgrowth [69]. GDM placentas have also been found to exhibit reduced fatty acid oxidation and increased triglyceride content [77]. Although nobiletin treatment enriched proteins associated with both MTORC1 and fatty acid metabolism in our study, there was no discernible effect on fetal or placental weight compared with control mice (Supplementary Figure S2). The effect of nobiletin treatment on fetal adiposity and other fetal outcomes, however, is not known and warrants further investigation, as even without increased birthweight, human GDM often results in increased fetal adiposity [6]. On the other hand, nobiletin appears to promote ATP synthesis through enrichment of proteins associated with oxidative phosphorylation and glycolysis, supporting the previously described proteomic data that nobiletin upregulates proteins associated with oxidative phosphorylation in skeletal muscle of GDM mice. Further studies are necessary to validate the proteomic and protein enrichment data presented here, as well as to better understand the role that nobiletin plays in the identified pathways.

Several limitations should be considered alongside these results.

First, our tissue explants were stimulated with TNF to generate an *in vitro* model of GDM. Several other mediators, such as IL1B, bacterial (LPS) and viral (poly(I:C)) factors, may also play roles in GDM pathophysiology. Thus, this affects the generalizability of these results, and these findings should be interpreted with some caution. Regardless, TNF is not only positively correlated with GDM insulin resistance [16] but can also induce inflammation in placenta and adipose tissue, as well as inhibit the insulin signalling pathway in skeletal muscle [23,47].

Second, to assess the effect of nobiletin on skeletal muscle glucose metabolism, we performed an *in vitro* glucose uptake assay in TNF-stimulated skeletal muscle, and an *in vivo* oral GTT in GDM mice. GDM is a metabolic disease, thus it would have been ideal to perform additional metabolic tests, such as *in vitro* glucose and lipid uptake assays in VAT or *in vivo* insulin resistance tests. However, *in vitro*, we were limited by availability and quality of VAT and skeletal muscle tissue samples. Furthermore, *in vivo*, it was not possible to perform additional insulin resistance assays on the same cohort of mice due to ethics restrictions, and time constraints surrounding breeding and mouse collection.

Third, to assess the mechanistic pathways nobiletin may target, we interrogated three major pathways *in vitro* via Western blotting, as well as using a global proteomic approach *in vivo*. For the *in vitro* investigation, we examined only total I κ B- α protein, and activation of Akt and MAPK Erk. Though ideally we would also assess the effect of

nobiletin on NF- κ B protein in cytosolic and nuclear extracts, we were limited by (1) tissue availability required for nuclear extracts for VAT and skeletal muscle; and (2) quality of antibodies for NF- κ B. Furthermore, while we would normally perform transcription factor assays; we were also unable to optimize these assays for the primary cell lines for the tissues we use in this study. For the *in vivo* investigation, we performed only IPA and PSEA analyses on skeletal muscle, placenta, pancreas and VAT from GDM mice treated with either nobiletin or vehicle control. Though insightful, the data obtained require further experimental validation to confirm the contribution of the identified signalling pathways to the effect of nobiletin.

Notwithstanding these limitations, the findings reported in this study are of great significance, given the growing evidence that inflammation is closely associated with GDM [15–19]. The combined use of *in vitro* and *in vivo* experiments provides strength to the results of our study. Results from the *in vivo* studies are further reinforced by comparing two time points of treatment administration and investigating the efficacy of nobiletin as a prophylactic versus therapeutic intervention. Given this promising data, there is room for the possible use of the nobiletin as a supplementation therapy for GDM in humans. Already, in non-pregnant humans, clinical trials have found that supplementation of either citrus flavonoids or Diabetinol[®] (of which nobiletin is the primary active ingredient) has been found to significantly improve cardiometabolic parameters, including fasting glucose and lipid profiles [78,79]. In contrast, there remains a lack of clinical information on the effect of nobiletin supplementation on pregnancy and long-term offspring outcome. Thus, future clinical trials are warranted to explore the use of nobiletin supplementation by pregnant high-risk women with GDM and its effect on fetal outcome.

In summary, we demonstrated that nobiletin can increase insulin sensitivity in human skeletal muscle impaired by inflammation and decrease inflammation-induced pro-inflammatory mediators in human placenta and adipose tissue. These effects may be elicited through the NF- κ B, Akt and MAPK Erk1/2 pathways. Of promise, our preliminary *in vivo* data show that treating GDM mice with nobiletin from either gd 1–17 or gd 10–17 significantly improved fasting glucose levels, and reduced inflammation in placenta and maternal adipose tissue, compared with control mice. These findings indicate nobiletin may be a novel therapeutic that not only improves maternal hyperglycemia but can also improve inflammation associated with GDM. Further studies are warranted to evaluate the long-term health and safety effects of nobiletin on the fetus.

Clinical perspectives

- Gestational diabetes mellitus (GDM) is a global health issue with increasing prevalence. Despite current therapies, GDM imposes significant lifelong consequences. GDM is characterized by insulin resistance and maternal inflammation. Recent evidence suggests that nobiletin is a potent glucose-sensitizing and anti-inflammatory flavonoid; however, its effects in GDM have not yet been assessed.
- Using both human and murine models of GDM, the present study demonstrates that nobiletin can improve glucose sensitivity and suppress inflammation associated with GDM. In human *in vitro* studies, nobiletin improved skeletal muscle glucose uptake, and suppressed inflammation in placenta and adipose tissue. Concordantly, in GDM mice, nobiletin supplementation lowered fasting blood glucose levels and suppressed inflammation in the placenta and adipose tissue. Using a global proteomic approach, nobiletin exerts these effects through a number of signalling pathways known to be dysregulated in GDM.
- Nobiletin may be a novel therapeutic drug to prevent the development of GDM by improving glucose metabolism and inflammation.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

M.L. designed the study and critically reviewed the manuscript. C.N.N. conducted the experiments, performed data analysis and wrote the initial draft of the manuscript. C.S. performed mass spectrometry analysis. S.Q. and A.L. provided technical assistance. J.C.W. critically reviewed the study and the manuscript.

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Abbreviations

GDM, gestational diabetes mellitus; HFD, high-fat diet; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cell; NGT, normal glucose tolerant; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

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