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**IN PREECLAMPSIA, MATERNAL THIRD TRIMESTER SUBCUTANEOUS
ADIPOCYTE LIPOLYSIS IS MORE RESISTANT TO SUPPRESSION BY INSULIN
THAN IN HEALTHY PREGNANCY**

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Short Title: Adipocyte lipolytic function in preeclampsia

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Methods

Subject recruitment and sample collection

Healthy non-labouring women at term (n=31) and non-labouring women with PE undergoing Caesarean section (n=13) were recruited from the Princess Royal Maternity Hospital, Glasgow. Age- and BMI-matched Controls (2 Controls per case) were selected from the healthy cohort. The study was approved by the Local Research Ethics Committee and all women gave written informed consent. Preeclampsia was defined according to the International Society for the Study of Hypertension in Pregnancy criteria. None of the women had a medical history of cardiovascular or metabolic disease and multiparous pregnancies were excluded. Subject characteristics were recorded at time of sampling. Delivery details were recorded from patient notes. Deprivation category (DEPCAT score), a measure of socioeconomic status, was assigned using the Scottish Area Deprivation Index for Scottish postcode sectors, 1998¹. Customised birth weight centiles were calculated using the Gestation Network Centile Calculator 5.4

(http://www.gestation.net/birthweight_centiles/centile_online.htm). Procedures were carried out according to institutional guidelines. Fasting venous blood was collected and plasma harvested at 5°C by low speed centrifugation within 30 minutes of collection and stored at -80°C. Placental biopsies were collected at delivery. Subcutaneous adipose tissue was obtained at Caesarean section from under the skin on entry into the abdomen and visceral adipose tissue was obtained from the omentum following closure of the uterus and haemostasis. Half of the biopsy was flash frozen in liquid nitrogen and stored at -80°C until analyzed. The remaining fresh material was transported immediately to the laboratory in warm buffer for adipocyte preparation.

Plasma metabolites

Plasma non esterified fatty acids (NEFA) were quantitated by colorimetric assay (Wako, Alpha Laboratories, Eastleigh, UK). Insulin (Mercodia, Sweden) and human placental lactogen (Leinco, Universal Biologicals, Cambridge, UK) were performed by ELISA according to the manufacturer's instructions. Plasma leptin, adiponectin, IL-6 and TNF α were carried out by ELISA (R&D Systems, Abingdon UK). Plasma estradiol and progesterone was estimated using the Immulite semi-automated assay system (Siemens, Erlangen, Germany).

Adipocyte preparation, sizing and DNA content

Temperature was maintained near to 37°C throughout. Adipose tissue was placed immediately in assay buffer (KRH buffer - NaCl 118mM, NaHCO₃ 5mM, KCl 4.7 mM, KH₂PO₄ 1.2mM, MgSO₄ 1.2mM, HEPES 25mM, 2.5mM CaCl₂, 20g/L bovine serum albumin, pH7.4) containing 3mM glucose. Attached connective or vascular tissue was dissected from the sample prior to digestion in 4ml of 2 mg/ml collagenase (Worthington Type 1, Lorne Laboratories, Twyford, UK) per g of tissue for 30 minutes with agitation. The digestate was then filtered (pore size 600 μ M), leaving a layer of adipocytes floating on top of the digestion buffer. The adipocytes were washed 4 times in warm KRH buffer and resuspended at approximately 90% cytocrit. An unfixed fresh cellular suspension of

adipocytes was prepared on a glass slide and a digital image captured. Digital images of fresh adipocyte preparation on glass slides were captured at x10 magnification on an Olympus BX50 microscope connected to 3-CCD colour camera (JVC). Computer visualisation of images was using Image-Pro Plus 4.0 and images were analysed with Adobe Photoshop Vs7.0.

Adipose tissue lipolysis assay

Adipocyte cell suspension (100ul) was added to 900ul of warm assay buffer and incubated in a 37°C shaking water bath at 91 cycles per minute. Appropriate reagents were added to the relevant tubes and incubated for 120 minutes. All assays were carried out in duplicate. At the end of the assay, aliquots (5ul) were obtained from the buffer layer below the cellular suspension and glycerol and non-esterified fatty acid (NEFA) concentration was quantitated by colorimetric assay using a microplate spectrophotometer (Multiscan EX, Thermo Electron Corporation) at 520nm and 550nm respectively.

Adipose tissue mRNA expression quantitation

Total RNA was isolated from adipose tissue using the ABI PRISM 6100 Nucleic Acid Prepstation following manufacturer's instructions (Applied Biosystems, Warrington, UK). cDNA was reverse transcribed from RNA using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Target gene expression was quantitated relative to a control gene² (*PPIA* Hs99999904_m1) using commercial primer probe sets (*ADRA2A* Hs00265081_s1; *ADRB1* Hs02330048_s1; *ADRB2* Hs00240532_s1; *ADRB3* Hs00609046_m1; *LIPE* Hs00193510_m1; *PNPLA2* Hs00386101_m1; *LPL* Hs00173425_m1; *INSR* Hs00961554_m1; *LEP* Hs00174877_m1; *LEPR* Hs00900240_m1; *LEPR (long)* custom primers forward LEPTRLONG_F TGTTC CGAACCCCAAGAATTGTT, reverse LEPTRLONG_R ATGTCACTGATGCTGTATGCTTGAT, probe LEPTRLONG_M 6FAM TCTGGCTTCTGAAAATT) in a final volume of 25ul on an 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Quantification analysis was carried out using SDS Version 2.3 software (Applied Biosystems), which calculated the threshold cycle (C_T) values. The expression of target assays were normalised by subtracting the C_T value of the endogenous control from the C_T value of the target assay. The fold increase relative to the control was calculated using the $2^{-\Delta C_T}$. The expression of the target assay was then expressed as a percentage relative to the endogenous control assay.

References

1. Carstairs V, Morris R. Deprivation and mortality: An alternative to social class? *Community medicine*. 1989;11:210-219.
2. Neville MJ, Collins JM, Gloyn AL, McCarthy MI, Karpe F. Comprehensive human adipose tissue mrna and microrna endogenous control selection for quantitative real-time-pcr normalization. *Obesity (Silver Spring, Md.)*. 2011;19:888-892.

Table S1. Stepwise regression models of subcutaneous and visceral adipose tissue (SAT and VAT) lipolytic function in healthy pregnancy. Stepwise regression was carried out to determine the contribution of cell size, plasma estradiol, placental lactogen, HOMA, leptin, adiponectin and TNF \pm to lipolytic function with *P*-to-enter and *P*-to-stay 0.15 in healthy pregnancy (n=31). Best models are shown with regression coefficients and *P* value given for each contributory variable and R²adjusted for the model. Fat cell insulin sensitivity index (FCISI) was calculated as the percentage inhibition of isoproterenol-stimulated lipolysis by insulin.

| | Variable | Coefficient | <i>P</i> | % r ² (adjusted) |
|-----------------------|----------------------------|-------------|----------|--------------------------------|
| SAT | | | | |
| Total basal lipolysis | TNF \pm (log pg/mL) | 0.77 | 0.020 | |
| | Estradiol (ng/mL) | -0.01 | 0.10 | 16.5 |
| Net basal lipolysis | TNF \pm (log pg/mL) | 0.64 | 0.003 | 23.5 |
| FCISI (total) | Estradiol (ng/mL) | -0.001 | 0.048 | 11.2 |
| FCISI (Net) | Cell diameter (um) | -7.7 | 0.002 | |
| | Leptin (log mg/mL) | 229 | 0.017 | |
| | TNF \pm (log pg/mL) | 223 | 0.047 | |
| | Adiponectin (ug/mL) | 10.4 | 0.074 | |
| | Estradiol (ng/mL) | -4.0 | 0.102 | 37.7 |
| VAT | | | | |
| Total basal lipolysis | HOMA (log) | 0.27 | 0.093 | |
| | Placental lactogen (ug/mL) | -0.045 | 0.108 | 13.6 |
| Net basal lipolysis | Cell diameter (um) | 0.006 | 0.035 | |
| | HOMA (log) | 0.193 | 0.032 | |
| | Placental lactogen (ug/mL) | -0.023 | 0.15 | 29.5 |
| FCISI (total) | Estradiol (ng/mL) | -0.02 | 0.001 | |
| | Placental lactogen (ug/mL) | 0.041 | 0.009 | 35.6 |
| FCISI (Net) | Leptin (log mg/mL) | 439 | <0.001 | |
| | HOMA (log) | -5.56 | <0.001 | |
| | Estradiol (ng/mL) | -7.0 | 0.004 | |
| | Placental lactogen (ug/mL) | 20.2 | 0.011 | 62.3 |

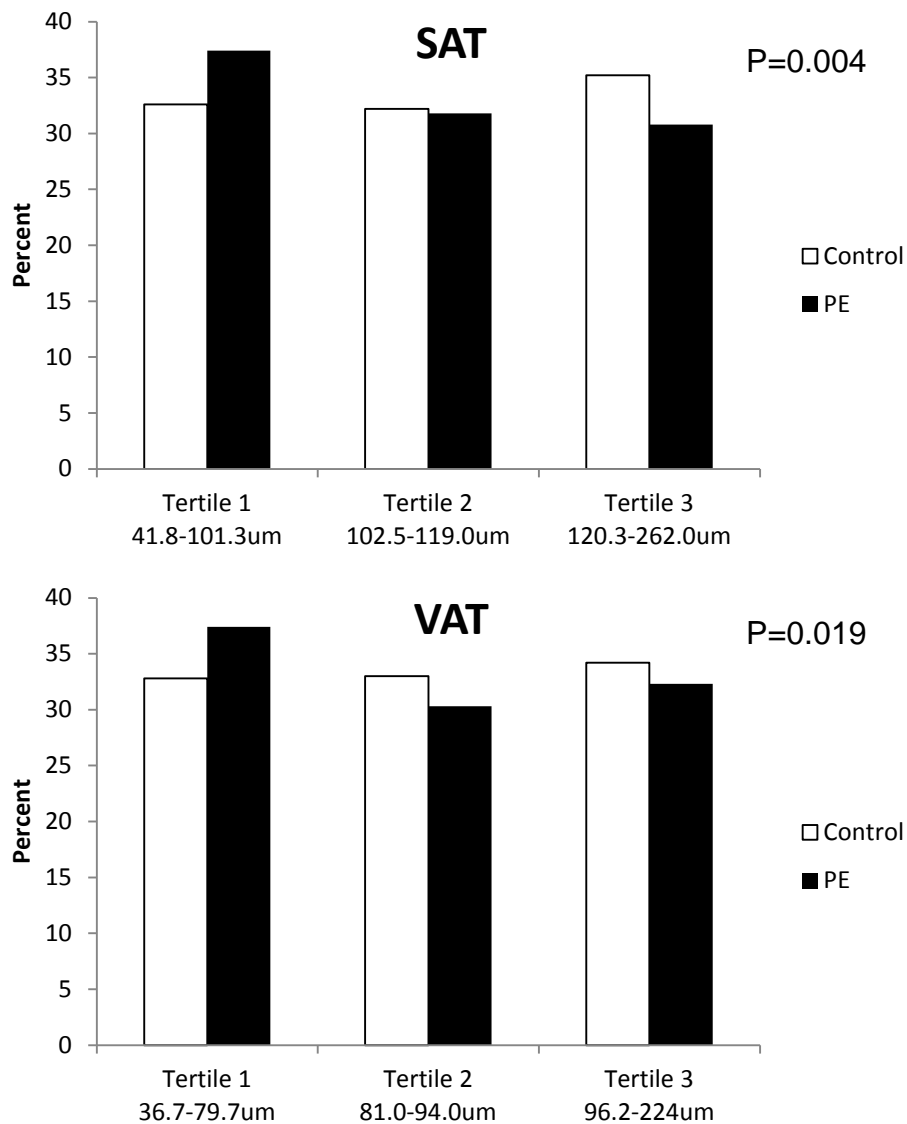


Figure S1. Subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) adipocyte diameter distribution in control and preeclamptic (PE) pregnancy. Adipocyte diameter was measured in n=100 adipocytes from each adipocyte preparation. SAT and VAT adipocyte diameters were divided into tertiles and percent adipocytes within the diameter ranges calculated for healthy and PE samples. Percentage adipocytes in each tertile for the whole control and PE groups are shown.

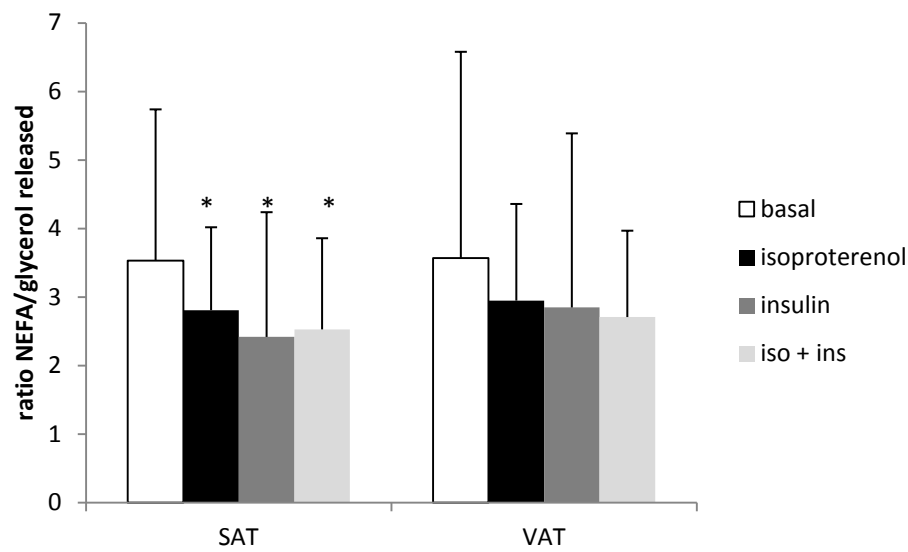


Figure S2. Maternal nonesterified fatty acid/glycerol ratio in maternal adipose tissue from the healthy cohort. Nonesterified fatty acid (NEFA) to glycerol ratios in subcutaneous adipose tissue (SAT, n=31) and visceral adipose tissue (VAT, n=31) under basal conditions or in the presences of 200nM isoproterenol, 10nM insulin or combined 200nM isoproterenol plus 10nM insulin. *Significantly different from basal, $P < 0.010$, using square root transformed data.

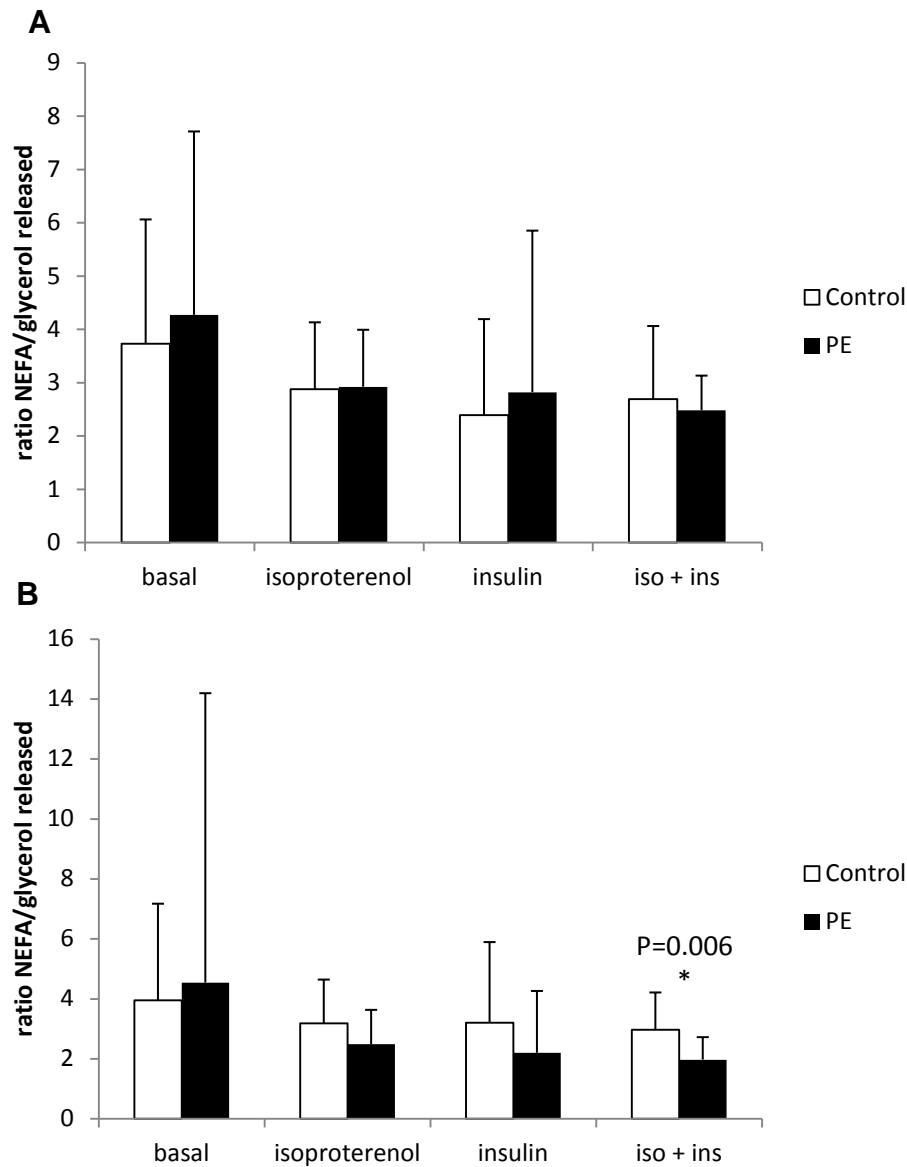


Figure S3. Maternal nonesterified fatty acid/glycerol ratio in maternal adipose tissue in women with preeclampsia and healthy age- and BMI-matched controls. Nonesterified fatty acid (NEFA) to glycerol ratios in A) subcutaneous adipose tissue (SAT) and B) visceral adipose tissue (VAT) from PE (n=13) and control (n=26) pregnancies under basal conditions or in the presences of 200nM isoproterenol, 10nM insulin or combined 200nM isoproterenol plus 10nM insulin. *Significantly different between PE and control, using square root transformed data.