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Research Article

Visceral adipose tissue activated macrophage content and inflammatory adipokine secretion is higher in pre-eclampsia than in healthy pregnancy

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Obesity increases pre-eclampsia (PE) risk. Adipose tissue inflammation may contribute to the clinical syndrome of PE. We compared adipose tissue macrophage infiltration and release of pro-inflammatory adipokines in PE and healthy pregnancy. Subcutaneous and visceral adipose tissue biopsies were collected from healthy (n=13) and PE (n=13) mothers. Basal and lipopolysaccharide (LPS) stimulated adipocyte TNFα, IL-6, CCL-2, and CRP release was measured. Adipose tissue cell densities of activated (cfms$^+$) and total (CD68$^+$) macrophages were determined. In PE only, visceral adipose tissue TNFα release was increased after LPS stimulation (57 [76] versus 81 [97] pg/ml/μg DNA, P=0.030). Basal TNFα release was negatively correlated insulin sensitivity of visceral adipocytes ($r = -0.61$, P=0.030) in PE. Visceral adipocyte IL-6 release was increased after LPS stimulation in PE only (566 [696] versus 852 [914] pg/ml/μg DNA, P=0.019). Visceral adipocyte CCL-2 basal (67 [61] versus 187 [219] pg/ml/μgDNA, P=0.049) and stimulated (46 [46] versus 224 [271] pg/ml/μg DNA, P=0.003) release was greater than in subcutaneous adipocytes in PE only. In PE, median *TNF* mRNA expression in visceral adipose tissue was higher than controls (1.94 [1.13–4.14] versus 0.8 [0.00–1.27] *TNF*/*PPIA* ratio, P=0.006). In visceral adipose tissue, *CSF1R* (a marker of activated macrophages) mRNA expression (24.8[11.0] versus 51.0[29.9] *CSF1R*/*PPIA* ratio, P=0.011) and activated (cfms+) macrophage count (6.7[2.6] versus 15.2[8.8] % cfms+/adipocyte, P=0.031) were higher in PE than in controls. In conclusion, our study demonstrates dysregulation of inflammatory pathways predominantly in visceral adipose tissue in PE. Inflammation of visceral adipose tissue may mediate many of the adverse metabolic effects associated with PE.

Introduction

Pre-eclampsia (PE) occurs in 2–4% of all pregnancies and is a leading cause of maternal and neonatal morbidity and mortality. There is considerable evidence that maternal obesity, increased insulin resistance, inflammation, and aberrant fatty acid metabolism are involved in the pathogenesis of PE [1,2]. The link between adiposity, inflammation, and insulin resistance has been increasingly acknowledged since Hotamisligil et al. [3] first demonstrated this relationship in 1993, and there has been extensive research into elucidating the mechanisms which link these conditions. White adipose tissue secretes a number of pro-inflammatory mediators which contribute significantly to the chronic inflammatory state and metabolic complications of obesity [4]. It is plausible that similar disturbances in adipocyte function could contribute to the development of the clinical syndrome of PE, a state of inflammation and insulin resistance. Indeed we have previously shown that third trimester subcutaneous adipocyte lipolysis is more resistant to insulin suppression in PE compared with healthy pregnancy [5]. Visceral

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adiposity also correlates with metabolic risk factors [6] and adverse metabolic outcomes in pregnancy including gestational diabetes mellitus, and PE [7-9].

It is now well recognized that macrophages are one of the key mediators of adipose inflammation and promoters of insulin resistance in white adipose tissue [10]. Analogous to the Th1/Th2 concept of T-cell activation, M1/M2 polarization has been described for macrophages. M1 are pro-inflammatory 'classical' macrophages, in contrast with M2 or 'alternatively activated' macrophages which have an anti-inflammatory phenotype [11]. In obesity, there is increased macrophage recruitment and retention with a shift toward the more pro-inflammatory M1 phenotype [12]. Macrophage phenotype also plays a role in the stability of atherosclerotic plaques [13]. The alteration in the cellularity of adipose tissue contributes to adipose inflammation and altered production of adipokines. It also promotes insulin resistance through dysregulation of glucose and lipid metabolism and inhibition of insulin signaling, via paracrine and endocrine effects [14]. The action of inflammatory macrophages may represent one of the key links between adiposity and its metabolic complications including those occurring in pregnancy.

Recently soluble fms-like tyrosine kinase-1 (sFlt-1), a soluble form of the vascular endothelial growth factor (VEGF) receptor has been shown to be secreted by adipocytes in the non-pregnant [15]. VEGF is a key cytokine that regulates angiogenesis, is produced by the placenta and other tissues, and is critical for placental development. Plasma sFlt-1 levels are significantly higher in PE compared with controls [16]. Excessive sFlt-1 can bind VEGF thus neutralizing its effects, inhibiting angiogenesis and contributing to the development of PE [17]. Plasma sFlt-1, expressed as a ratio to placental growth factor, is proposed as a prognostic test for PE [18]. sFlt-1 production by adipocytes in PE has not been studied.

We hypothesized that adipocyte release of pro-inflammatory adipokines is exaggerated under both basal and stressed conditions in PE compared controls. We also hypothesized that adipose tissue macrophage infiltration is increased in women with PE, thereby implicating adipocyte function in its pathophysiology. This pathological response may be more apparent in one particular adipose depot i.e. visceral adipose tissue rather than subcutaneous adipose tissue. The aims of the study were to determine the release of IL-6, TNFα, CRP, CCL-2, and sFlt-1 from subcutaneous and visceral adipocytes under basal conditions and when stimulated by lipopolysaccharide (LPS). In addition, macrophage counts of both total ($CD68^+$) and activated ($cfms^+$) macrophages in subcutaneous and visceral adipose tissue were compared. The gene expression of relevant adipokines was also measured in adipose biopsies.

Materials and methods

Recruitment and tissue collection

Healthy singleton non-laboring women at term (n=13) and non-laboring women with PE undergoing caesarean section (n=13) were recruited from the Princess Royal Maternity Hospital, Glasgow. Controls were age- and body mass index (BMI)-matched with women with PE. The study was approved by the Local Research Ethics Committee and all women gave written informed consent. PE 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. Subject characteristics, including age, booking (at first antenatal visit) BMI, were recorded at time of sampling and smoking status recorded as current smoker or non-smoker. Details of maternal blood pressure around time of delivery, gestation of delivery, mode of delivery, fetal sex, birth weight, and placental weight were recorded from the patients' notes. Early onset PE was defined as onset prior to 34 completed weeks of gestation. Severe PE was defined by criteria outlined in the Hypertension in Pregnancy NICE clinical guideline 107. Customized birth weight centiles were calculated using the Gestation Network Centile Calculator 5.4 http://www.gestation.net/birthweight_centiles/centile_online.htm). Fasting venous blood was collected and harvested at 5°C by low speed centrifugation within 30 min of collection and plasma was stored at −80°C until analysis. Adipose tissue biopsies (subcutaneous and visceral) were collected by the surgeon and a small section of each sample was 'flash-frozen' in liquid nitrogen and then placed in pre-cooled metal containers to be stored at −80°C until analysis. The remaining fresh sample was used to prepare adipocytes.

Plasma metabolites

Plasma total cholesterol, triglyceride, and HDL cholesterol, glucose, and high sensitivity CRP assays were performed by the Department of Clinical Biochemistry, Glasgow Royal Infirmary as previously described [19]. Plasma non-esterified fatty acids (NEFA) were quantitated by commercial colorimetric assay (Wako, Alpha Laboratories, Eastleigh, U.K.). Insulin (Mercodia, Sweden) was performed by commercial ELISA according to the manufacturer's

instructions. HOMA was calculated as follows: [fasting insulin (mU/l) × fasting glucose (mmol/l)]/22.5. Plasma leptin, adiponectin, IL-6, TNFα, and VEGF R1/sFlt1 were carried out using commercial ELISA kits (all R&D Systems, Abingdon, U.K.).

Adipose tissue lipolysis assay

Adipocyte preparation was by a modification of the method described by Rodbell [20], with temperature maintained as near to 37°C throughout. The method is described in detail in Huda et al. [5]. Fat cell number was measured indirectly by quantitating the DNA content in a known volume of adipocyte suspension. The number of adipocytes are directly proportional to the amount of adipocyte DNA content and lipolysis rates are expressed per μg of DNA. The DNA was isolated from a known quantity of adipocyte suspension that was previously frozen at −70°C and thawed at room temperature using the Blood Prep DNA Purification protocol on the ABI Prism™ 6100 Nucleic Acid PrepStation (Applied Biosystems). The concentration of DNA was quantitated using Nanodrop® ND 100. The diameters of 100 adipocytes were manually measured using a stage micrometer and a mean calculated for each preparation (Supplementary Figure S1). For information on the adipocyte profile from control and PE biopsies please refer to the Supplementary Results (Supplementary Figure S1) of Huda et al. [5]. The fat cell insulin sensitivity index (FCISI) was calculated as the percentage inhibition of isoproterenol-stimulated lipolysis by insulin.

Adipokine analysis

Paired samples of both basal (no reagent added to adipocyte cell suspension and buffer) and LPS (LPS concentration of 1 μM to adipocyte cell suspension) were incubated. An aliquot of the buffer layer below the adipocyte suspension at time 120 min was frozen at −80°C for later analysis of cytokines. Adipokine quantitation, apart from sFlt-1, was carried out with Bio-Plex (BIO-RAD®) system and suspension array technology. The assay was customized to detect and measure multiple adipokines (PAI-1 LOB1786, CCL-2 LUH279, CRP LOB1707, IL-6 LUH206, Leptin LUB398, TNFα LUH210, Adiponectin LOB1065, IL-110 LUH217 R&D Systems) using the R&D Systems Obesity Base Kit (LOB000). VEGF R1/sFlt1 was quantitated using a commercial ELISA kit (R&D Systems, Abingdon, U.K.).

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, U.K.). cDNA was reverse transcribed from RNA using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington, U.K.) according to manufacturer's instructions. Target gene expression was quantitated relative to a control gene [5,21] (*PPIA* Hs99999904_m1) using commercial primer probe sets (*CD68* Hs00154355-m1, *CSF1R* Hs99999197-m1, *IL6* HS00174131-ms, *TNF* Hs00174128-m1, *CCL2* Hs00234140_m1, *FLT1* Hs01052961_m1) Applied Biosystems, Warrington, U.K.) in a final volume of 25 μl on an 7900HT Sequence Detection System (Applied Biosystems, Warrington, U.K.) according to manufacturer's instructions. Quantitation 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 normalized 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.

Macrophage cell densities

Immunocytochemistry was performed on 7 μM sections of paraffin-embedded adipose tissue biopsies using the avidin: biotinylated enzyme complex method (see Supplementary Methods). The sample size was smaller (n=9) as there was insufficient stored adipose tissue in four of the PE cases. The antibodies used were polyclonal anti-human cfms (CBL776 Chemicon) and monoclonal anti-human macrophage CD68 (Dako-CD68, PG-M1 M876) which identify activated and total macrophages respectively. Macrophages were identified using histological analysis in ten randomly selected high powered fields (×400 objective magnification) and were counted by two independent observers who were blinded to the specimen details. The area for each high-powered field was 0.23 mm^2. Macrophages within the blood vessels were not included in the counts. Tissue macrophage densities were expressed as cell count per adipocyte.

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Table 1 Characteristics of women with pre-eclampsia and age- and body mass index-matched controls

Characteristics	Controls (*n*=13)	PE (*n*=13)	*P*-value PE versus controls
Demographic data			
Age (years)	30.0 (5.9)	31.1 (6.3)	0.66
BMI (kg/m^2)†	29.6 (6.4)	31.1 (8.3)	0.70
Smokers, number (%)§	1 (7.6)	2 (15.4)	0.54
DEPCAT*	4 (4–6)	6 (4–7)	0.12
Gestation at delivery (weeks)	38.9 (1.4)	35.6 (3.2)	0.001
Primigravidae, number (%)§	4 (30.8)	7 (53.8)	0.23
First antenatal visit systolic pressure (mmHg)	116 (13)	127 (14)	0.054
First antenatal visit diastolic pressure (mmHg)	71 (9)	78 (9)	0.060
Systolic Pressure at delivery (mmHg)	118 (14)	152 (29)	0.006
Diastolic Pressure at delivery (mmHg)	71 (8)	98 (7)	<0.0001
Birthweight (g)	3414 (547)	2330 (926)	0.002
Birthweight centile	53 (32)	26 (33)	0.045
Biochemical data			
Total cholesterol (mmol/l)	6.45 (0.90)	6.41 (1.45)	0.93
Triglyceride (mmol/l)†	2.57 (0.65)	3.73 (2.36)	0.044
HDL cholesterol (mmol/l)	1.84 (0.41)	1.71 (0.42)	0.43
NEFA (mmol/l)‡	0.39 (0.20)	0.58 (0.24)	0.037
Glucose (mmol/l)	4.88 (0.52)	5.31 (1.24)	0.26
Insulin (mU/l)†	12 (9)	17 (13)	0.33
HOMA†	2.7(1.9)	4.3 (3.8)	0.29
Leptin (mg/ml)†	50 (23)	85 (42)	0.078
Adiponectin (μg/ml)	9.9 (4.5)	9.0 (4.8)	0.62
IL-6 (pg/ml)†	2.7 (1.0)	3.8 (3.2)	0.91
TNFα (pg/ml)†	0.94 (0.4)	1.54 (0.7)	0.024
CRP (mg/l)†	5.6 (4.3)	23.5 (55.5)	0.90
sFlt-1 (ng/ml)†	3.4 (1.8)	18.6 (14.7)	<0.001
SAT cell size (μm)	110 (10)	110 (11)	0.94
VAT cell size (μm)	86 (11.4)	87.4 (16)	0.80

Continuous values are expressed as mean and standard deviation (*median and interquartile range) and categorical variables as number (percent). Comparisons between women with PE and controls were carried out by two sample *t*-test, on †log or ‡square root transformed data if necessary, or by *Mann–Whitney or §chi-squared test.
(SAT: subcutaneous adipose tissue, VAT: visceral adipose tissue, DEPCAT: Carstairs and Morris index of deprivation 1-most affluent, 7-most deprived)

Statistical analysis

Data were assessed for normal distribution using a Ryan-Joiner test and transformed to achieve a normal distribution where necessary. Means with SD are presented for normalized data. Median and interquartile range are presented for non-parametric data. Comparison within individuals was by paired *t*-test (subcutaneous adipose tissue versus visceral adipose tissue and basal versus LPS stimulated) and between PE and control groups by two sample *t*-test or Mann–Whitney test as appropriate. Mann–Whitney and $\chi 2$ tests were used to test between differences in semi-categorical and categorical variables respectively. For adipocyte sFlt-1 secretion, which included many zero values, the Wilcoxon rank sum test was used to test differences between groups. Pearson's correlation coefficients and regression analysis were calculated to assess associations between variables, and results were expressed as *r* value, R^2 and *P*-value. A *P*-value of <0.05 was considered significant. The data were adjusted for potential cofounders using the General Linear Model. All statistical analysis was carried out in Minitab (version 17).

Results

Maternal antenatal booking characteristics and third trimester plasma metabolic and inflammatory profile

Maternal antenatal booking characteristics (11–14 weeks) for women with PE and controls matched for age, BMI, and smoking are shown in Table 1. Women with PE had a tendency for a higher booking systolic and diastolic blood pres-

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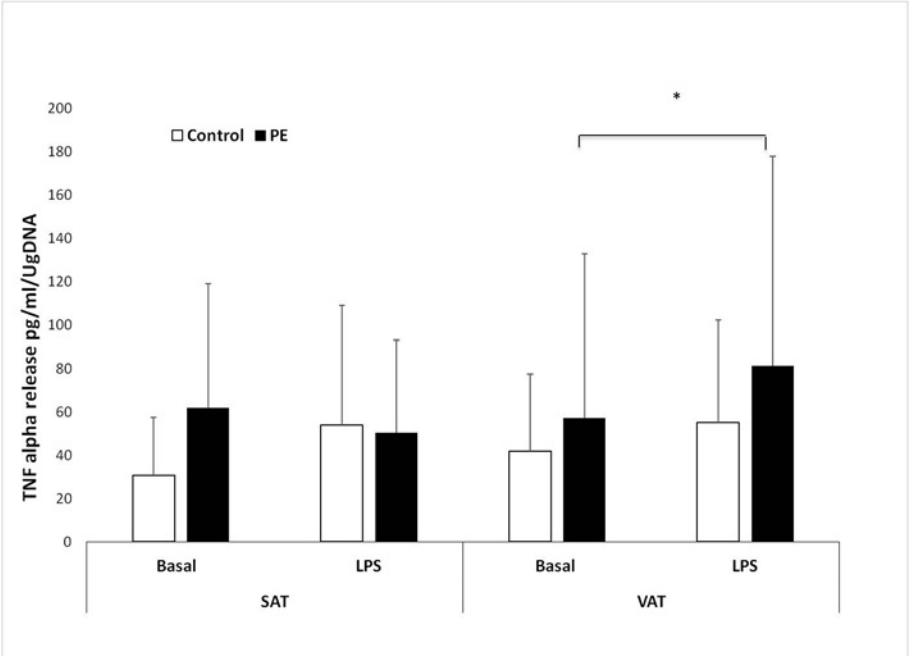

Figure 1. Adipocyte TNFα release

Basal and lipopolysaccahride (LPS) stimulated TNFα (pg/ml/μgDNA) secreted by maternal subcutaneous adipose tissue (SAT) adipocytes and visceral adipose tissue (VAT) adipocytes in control pregnancy (n=13) and in pregnancies complicated by PE (n=13) is shown. Means and standard deviations are presented. *P<0.05 basal versus LPS stimulated TNFα release analyzed by paired t-test on square root transformed data.

sure, were delivered at an earlier gestation and offspring were of lower birth weight centile than controls. Pre-delivery diagnostic blood pressure was higher in PE (Table 1). Twelve out of thirteen had late onset PE (onset after 35 weeks of gestation) and eight out of thirteen had severe PE as defined in NICE guideline 107. Plasma sFlt-1 levels were significantly higher in PE than control women (P<0.001). In addition, mothers with PE had higher plasma triglyceride and NEFA than controls. Maternal serum TNFα was also 64% higher in PE than controls (P=0.024).

Adipocyte TNFα release

There were no differences in either basal or stimulated TNFα release between subcutaneous and visceral adipocytes. Basal subcutaneous adipocyte TNFα release in in PE was higher than in controls (control mean [SD] 31 [27] versus PE 62 [57] pg/ml/μg DNA) but this was not significant (P=0.09) (Figure 1). In controls there was a trend for subcutaneous adipocyte TNFα release to increase after stimulation with LPS (31 [27] versus 54 [55] pg/ml/μgDNA, P=0.051), reaching similar levels to that released by unstimulated PE subcutaneous adipocytes. In PE, subcutaneous adipocyte TNFα release was high under both unstimulated and stimulated conditions (54 [55] versuss 51 [43]) pg/ml/μgDNA, P=0.86). In visceral adipocytes from women with PE, there was a significant increase in TNFα release in response to LPS stimulation (57 [76] versus 81 [97] pg/ml/μg DNA, P=0.030) which did not occur in controls (42 [36] versus 55 [47] pg/ml/μgDNA, P=0.19) (Figure 1).

In controls, both basal and stimulated TNFα release from subcutaneous adipocytes correlated with maternal BMI (r=0.73, P=0.005, and r=0.69, P=0.009 respectively) (Supplementary Figure S2). This was independent of subcutaneous adipocyte size (P=0.002 and P=0.006 respectively). This association with BMI was not seen in subcutaneous adipocytes in PE (Supplementary Figure S1). In PE but not controls, both basal and stimulated TNFα release from visceral adipocytes negatively correlated with visceral adipocyte size ($r = -0.65$, $R^2 = 41.8\%$, P=0.017, and $r = -0.65$, $R^2 = 42.2\%$, P=0.016). Basal release of TNFα negatively correlated with a direct measure of visceral adipocyte insulin sensitivity (FCISI) in this depot ($r = -0.60$, $R^2 = 30.3$, P=0.030). This relationship was independent of BMI (P=0.023), but not of cell size, (P=0.060).

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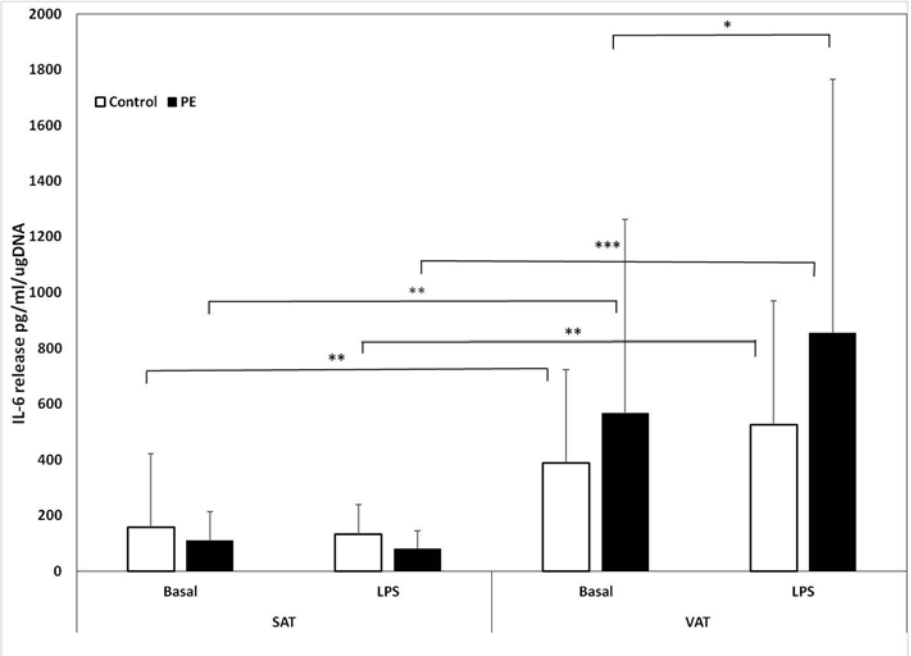

Figure 2. Adipocyte IL-6 release

Basal and LPS stimulated IL-6 (pg/ml/μgDNA) secreted by maternal SAT and visceral VAT adipocytes in control pregnancy (n=13) and in pregnancies complicated by PE (n=13) is shown. Means and standard deviations are presented. *P<0.05, **P<0.010, ***P<0.001. Analysis by two sample (PE versus controls) or paired (SAT versus VAT) t-test on log transformed data.

Adipocyte IL-6 release

Basal and stimulated release of IL-6 was greater in adipocytes from visceral adipose tissue than from subcutaneous adipose tissue in both the control group (basal P=0.001, LPS-stimulated P=0.002) and the PE group (P=0.005 and P<0.001 respectively) (Figure 2). Basal subcutaneous adipocyte IL-6 release was similar in PE and controls and was unaffected by LPS exposure. In visceral adipocytes, IL-6 release was significantly increased after stimulation with LPS in PE (566 [696] versus 852 [914] pg/ml/μgDNA, P=0.019) but not in controls (389 [334] versus 526 [443] pg/ml/μgDNA, P=0.092) (Figure 2). In control subcutaneous adipocytes, both basal and LPS-stimulated release of IL-6 was correlated with maternal BMI (r=0.75, R^2 49.5%, P=0.003, and r=0.88, R^2 55.3%, P<0.001 respectively), an effect not seen in PE (Supplementary Figure S3). This correlation was independent of cell size.

Adipocyte CCL-2 release

Basal and stimulated CCL-2 from visceral adipocytes was higher than that from subcutaneous adipocytes in PE (basal; subcutaneous 67 [61] versus visceral 187 [219] pg/ml/μgDNA, P=0.049; and stimulated (46 [46] versus 224 [271]) pg/ml/μgDNA, P=0.003) (Figure 3). There were no differences between basal and LPS stimulated release of CCL-2 from either subcutaneous adipocytes or visceral adipocytes in either the controls or the PE group. Basal and stimulated release of CCL-2 from subcutaneous adipocytes was correlated with maternal BMI (r=0.79, R^2 = 59.1%, P=0.01 basal, and r=0.82, R^2 64.9%, P=0.001 stimulated) in controls only (Supplementary Figure S4). This relationship was independent of cell size.

Adipocyte CRP release

There was no difference between subcutaneous adipocyte and visceral adipocyte CRP release in either pregnancy group. Nor was there any difference between basal and stimulated release of CRP from either subcutaneous adipocytes or visceral adipocytes in control and PE group (Supplementary Figure S5). Basal and stimulated CRP release from subcutaneous adipocytes were correlated with circulating maternal plasma levels of CRP in controls (basal r=0.69, P=0.010, and stimulated r=0.70, P=0.008). In PE this association appeared to be stronger (basal P=0.91, P<0.001, and stimulated r=0.91, P<0.001). CRP release from subcutaneous adipocytes in PE was correlated with maternal plasma IL-6 concentrations (basal r=0.72, P=0.006, and stimulated r=0.70, P=0.008). Basal and stimulated CRP

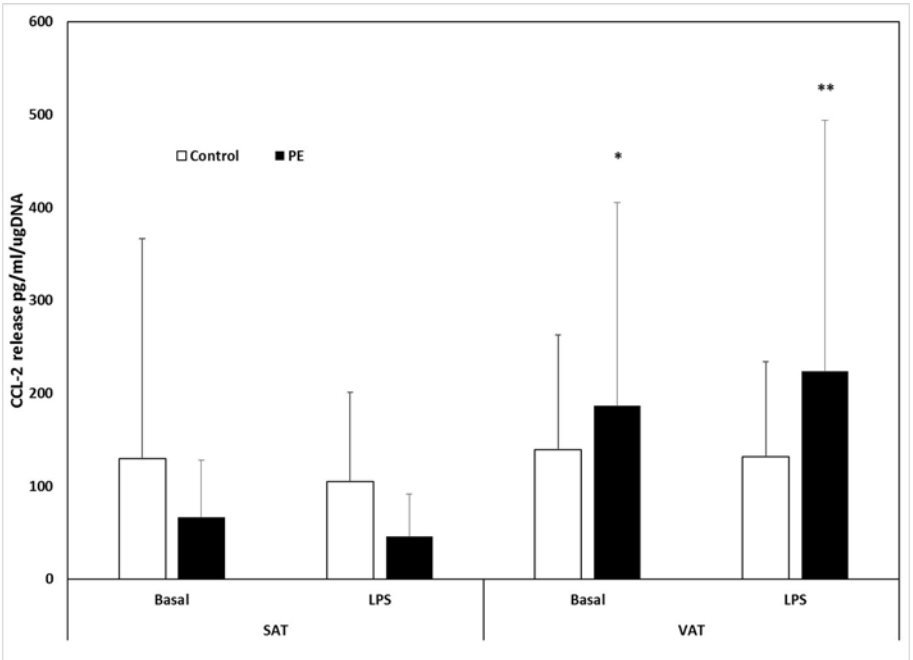

Figure 3. Adipocyte CCL-2 release

Basal and LPS stimulated CCL-2 secreted from maternal SAT and VAT adipocytes in control pregnancy (n=13) and in pregnancies complicated by PE (n=13) is shown. Means and standard deviations are presented. *P<0.05 and **P<0.01 SAT versus VAT using paired t-test on log transformed data.

release from visceral adipocytes correlated with basal net lipolysis in visceral adipocytes in both control (basal r=0.76, P=0.004, and stimulated r=0.81, P=0.001) and PE (basal r=0.76, P=0.004 and stimulated r=0.81, P=0.001) groups.

Adipocyte sFlt-1 release

Data were available for n=7 control and n=11 PE samples. Median sFlt-1 release under all conditions was 0 pg/ml. There was no sFlt-1 release into medium from either subcutaneous of visceral adipocytes under basal conditions in controls. After exposure to LPS there was no sFlt-1 release from subcutaneous adipocyte and only one visceral adipocyte sample had measureable sFlt-1 release (median [interquartile range (IQR)] 0.0 [0.0–0.0] pg/ml). In PE, there was no sflt-1 release from subcutaneous adipocytes under basal or stimulated conditions, however sFlt-1 was released under both basal and stimulated conditions from visceral adipocytes only in three samples (median [IQR] basal 0.0 [0.0–40.4] versus stimulated 0.0 [00 – 36.0] pg/ml, P=0.58).

Adipokine gene expression

There was no difference in subcutaneous adipose tissue *TNF* mRNA expression between PE and controls. Median *TNF* mRNA expression in visceral adipose tissue was higher in PE compared with controls (1.94 [1.13–4.14] versus 0.8 [0.00–1.27] *TNF/PPIA* ratio, P=0.006) (Supplementary Figure S6). Median *FLT1* mRNA expression in subcutaneous adipose tissue was higher in PE compared with controls (6.77 [5.0–13.3] versus 3.5 [1.9–4.8] *FLT1/PPIA* ratio, P=0.023) (Supplementary Figure S7). Median *FLT1* mRNA expression in visceral adipose tissue was higher in PE compared with controls (8.6 [2.7–11.9] versus 2.1 [1.7–3.0] *FLT1/PPIA* ratio, P=0.005) (Supplementary Figure S7). Median *CCL2* mRNA expression was higher in visceral adipose tissue relative to subcutaneous adipose tissue in PE (15.1 [7.3–32.1] versus 45.0 [27.7–118.5] *CCL2/PPIA* ratio, P=0.04). This difference is not seen in controls. Median *IL6* expression was greater in visceral adipose tissue in controls relative to subcutaneous adipose tissue (0.0 [0.0–11.1] versus 9.51[2.2416.8] *IL6/PPIA* ratio, P=0.04) an effect also seen in PE (0.0 [0.0–2.6] versus 11.1 [4.5–26.9] *IL6/PPIA* ratio, P=0.004). No other differences in gene expression were found.

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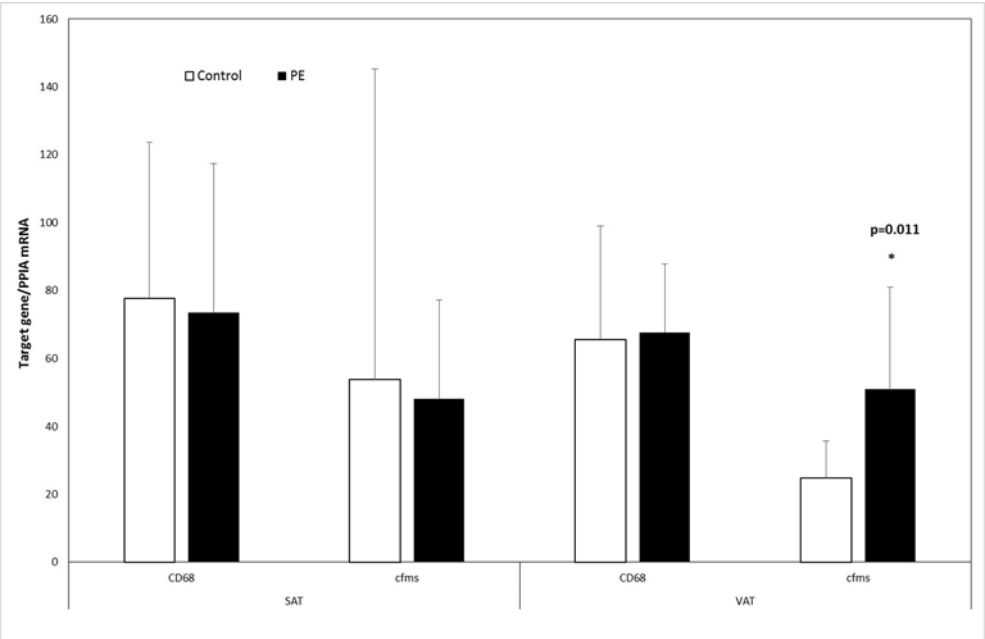

Figure 4. Adipose tissue macrophage marker messenger RNA expression

Messenger RNA expression of *CD68* (n=13) and *CSF1R* (n=13) relative to *PPIA* expression in control and PE SAT and VAT is shown. Means and standard deviations are presented. *P<0.05 *CSF1R* mRNA expression in PE VAT compared with control VAT by two sample t-test on log transformed data.

Adipose tissue macrophage densities and mRNA expression in pre-eclampsia and controls

The mean *CSF1R* mRNA expression in visceral adipose tissue, but not subcutaneous adipose tissue, was higher in PE relative to controls matched for BMI (24.8[11.0] versus 51.0[29.9] *CSF1R/PPIA* ratio, P=0.011) (Figure 4). *CD68* expression was not significantly different in subcutaneous adipose tissue or visceral adipose tissue between PE and controls. Similarly the mean percentage of cfms+ macrophages in visceral adipose tissue, but not subcutaneous adipose tissue, was higher in PE relative to controls (P=0.032) (Figure 5). There were no differences in tissue densities of CD68+ macrophages between PE and controls in either subcutaneous adipose tissue or visceral adipose tissue.

Discussion

The key observations of our study were that, in PE, visceral adipose tissue had a higher activated macrophage content and higher mRNA expression of TNFα than in controls and that adipocytes from visceral adipose tissue in PE were more responsive to LPS stimulation in terms of releasing higher levels of TNFα and IL-6. This suggests that inflammation in visceral adipose tissue may be a hallmark of PE. Human fat compartments can be classified into lower body subcutaneous adipose tissue, upper body subcutaneous adipose tissue and visceral adipose tissue and each depot has different characteristics. Upper body subcutaneous adipose tissue (from which our biopsy of subcutaneous adipose tissue was taken) is relatively resistant to insulin suppression of lipolysis and is estimated to be the source of 60% of circulating NEFA. Visceral adipose tissue can be regarded as an 'ectopic' site of fat accumulation and high levels of visceral adipose tissue are related to metabolic obesity even at a low BMI [22]. We have previously shown that in pregnancy visceral adipose tissue adipocytes are more insulin resistant than subcutaneous adipose tissue adipocytes and that in PE subcutaneous adipose tissue adipocytes become as insulin resistant as their visceral adipose tissue counterparts [5]. Together with the data presented here, this focuses attention on adipocyte dysfunction as a key pathogenic pathway in PE.

TNFα appears to play a central role in the adipocyte inflammatory response in pregnancies complicated by PE. TNFα transcription was higher in PE adipose biopsies compared with controls and adipocytes exposed to LPS were

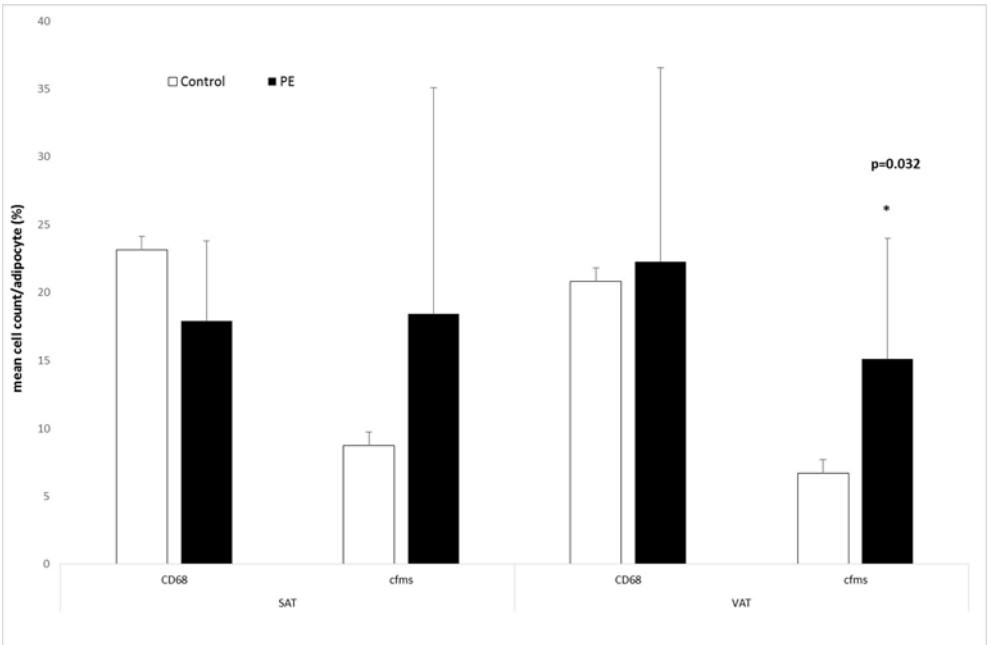

Figure 5. Adipose tissue macrophage content

SAT and VAT macrophage densities expressed as mean cell count per adipocyte in controls (n=9) and PE (n=9) matched for BMI are shown. Means and standard deviations are presented. *P<0.05 between the mean percentage of VAT cfms$^+$/adipocyte in PE compared to controls by two sample t-test on log transformed data.

stimulated to secrete significantly higher levels of TNFα. TNFα is known to be an important determinant of insulin sensitivity in pregnancy and can lead directly to insulin resistance by inhibiting insulin signaling through several mechanisms including inducing serine phosphorylation of the insulin receptor substrate 1 (IRS1) [23]. TNFα is also a potent stimulator of lipolysis through down-regulation of perilipin and suppression of the anti-lipolytic GTP-binding membrane proteins $G\alpha_i$ [24]. In our study, TNFα release from visceral adipose tissue was negatively correlated with adipocyte insulin sensitivity (as assessed by the ability of insulin to suppress lipolysis) of visceral adipose tissue adipocytes in PE thus linking inflammation, insulin resistance and lipolysis in PE. Furthermore, TNFα release was negatively correlated with fat cell size in visceral adipose tissue in PE in this cohort. We have previously shown that in PE visceral adipose tissue has a significantly larger proportion of small adipocytes than controls and our current data are consistent with observations in the non-pregnant that insulin resistance is associated with a greater proportion of small adipose cells. Higher visceral adipose tissue TNFα release in smaller adipocytes may contribute to their insulin resistance in PE.

We showed for the first time that *FLT1* expression was higher in both subcutaneous and visceral adipose tissue in PE compared with control women although there was little evidence for secretion of sFlt-1 from adipocytes in culture. This would suggest the adipocyte does not act as a source additional to the placenta for circulating sFlt-1 in PE. Other stromal cells located in adipose tissue may express *FLT1* and a potential paracrine role for adipose tissue VEGF receptor in angiogenesis related to gestational adipose tissue expansion is possible.

In addition to the higher production of TNFα in visceral adipose tissue, the mRNA expression of other adipokines in visceral adipose tissue biopsies, namely IL-6 and CCL-2, was also higher in PE than controls. Furthermore, secretion of both adipokines from visceral adipocytes was higher than in subcutaneous adipocytes regardless of whether from PE or control women. In PE, IL-6 release from visceral adipose tissue adipocytes was more responsive to stimulation with LPS. IL-6 is a stimulator of whole body lipolysis with anti-insulin effects [25,26] and has also been related to pregnancy-associated insulin resistance. Unlike with TNFα, we did not observe a relationship between IL-6 and direct measures of insulin sensitivity in adipose tissue in our cohort. *In vivo*, the CCL2 signal is important for macrophage infiltration of white adipose tissue. Indeed knockout of the receptor for CCL2 in mice protects against obesity induced inflammation and insulin resistance [27]. The higher expression of *CCL2* in visceral adipose tissue may have important implications for macrophage recruitment.

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We observed higher mRNA expression of *CSF1R* and a higher density of cfms positive macrophages in the visceral adipose tissue of women with PE, while the total number of macrophages both in terms of *CD68* gene expression, and of tissue CD68 positive macrophage content, did not differ. These data suggest that there is no additional recruitment of macrophages to visceral fat in PE, despite the increased *CCL2* expression, but that macrophages become activated to a more pro-inflammatory macrophage phenotype. Women with PE have more pro-inflammatory/activated macrophages in visceral adipose tissue than controls and these cells are another source of cytokines, including TNFα and IL-6, in PE. We observed higher maternal plasma concentrations of TNFα in this small cohort and previously we and others have found plasma levels of both TNFα and IL-6 to be elevated in PE [28,29]. In addition to the potential paracrine and endocrine effects on lipid and glucose metabolism discussed above, TNFα and IL-6 are also implicated in endothelial dysfunction, leukocyte activation, and alterations in coagulation—all features of PE [30-32]. The chronic infusion of TNFα or IL-6 into normal pregnant rodents significantly increases arterial pressure and impairs renal haemodynamics [33]. Previously, the placenta has been suggested as a source of maternal plasma TNFα and IL-6 with its overproduction being secondary to placental hypoxia. However, expression has not consistently been seen to be higher in the placentae of women with PE thereby implicating another tissue source for the elevated concentrations found in peripheral blood [34,35]. Visceral adipose tissue could be an alternative source for these cytokines in PE although it is yet to be determined whether adipocytes or infiltrating macrophages, or both, produce the cytokines.

While the adipose tissue inflammatory changes associated with PE appear to be located predominantly at visceral adipose tissue, there were also some important observations regarding subcutaneous adipose tissue. Basal levels of adipokine release were generally lower in subcutaneous adipose tissue compared to visceral adipose tissue and were refractory to stimulation by LPS, apart from TNFα. Interestingly subcutaneous adipose tissue adipocyte TNFα, IL-6, and CCL-2 release was strongly correlated with maternal BMI possibly reflective of the link between obesity and chronic low-grade inflammation. This association with maternal BMI was lost in PE suggesting that visceral adipose tissue located inflammatory mechanisms override the background chronic inflammation of obesity. This is consistent with the concept of 'benign' and 'metabolic' obesity where the amount of visceral adipose tissue is associated with the metabolic consequences of obesity rather than obesity *per se* [22]. The higher basal release of TNFα from subcutaneous adipose tissue in PE (equivalent to the amounts released from visceral adipose tissue), due to the large size of the subcutaneous adipose tissue depot, could potentially impact significantly on circulating NEFA and may be a mechanism through which early exaggerated rise of NEFA in PE occurs. We observed that in PE, TNFα release from visceral adipose tissue does relate to the insulin sensitivity of lipolysis in this adipose depot. The association between subcutaneous adipose tissue adipocyte CRP release and maternal plasma CRP concentrations suggests that subcutaneous adipose tissue is an important determinant of circulating CRP in the third trimester of pregnancy in both healthy and PE pregnancies. It is interesting to note that in PE this association also extends to maternal plasma IL-6 levels perhaps reflecting the wider metabolic disturbances associated with PE.

The strengths of the present study were the direct *ex vivo* assessment of adipocyte inflammatory function in subcutaneous adipose tissue and visceral adipose tissue derived from the same women. This could be related to the lipolytic function of the same adipose tissue and to plasma concentrations of inflammatory markers. Both total and activated macrophage content was assessed by two independent methods (immunohistochemistry and gene expression) and the data were consistent. We did not establish the M1/M2 phenotype of the infiltrating macrophages and this would be a useful future analysis to carry out. Although the sample size was small, BMI, smoking and age-matching with controls reduced confounding and there was sufficient power to detect consistent statistically significant differences. The gestational age was different between the two groups and this is a limitation of our study that is difficult to avoid due to the characteristics of the condition under study. If we had used preterm matched controls, this also raises issues as to whether these control can be considered as 'healthy'. However, the majority of women were late onset PE and near to term. The present study is a cross-sectional comparison therefore it is difficult to draw conclusions regarding which pathways are causal in PE and which are consequences of the condition and our data should be interpreted accordingly.

Conclusion

Our study demonstrates that in PE, dysregulation of inflammatory pathways is located predominately in visceral adipose tissue and not subcutaneous adipose tissue. In PE, visceral adipose tissue has a higher activated macrophage content, higher mRNA expression of TNFα and is more responsive to LPS stimulation in terms of releasing higher levels of TNFα and IL-6 than in control pregnancy. Inflammation at visceral adipose tissue may be a hallmark of

PE and TNFα may mediate many of the adverse metabolic effects associated with PE. This understanding of pathophysiology of the disease may facilitate better models of prediction of PE and provide the basis for pharmacological intervention in the prevention or attenuation of the disease.

Clinical persepectives

- Pre-eclampsia (PE) is a leading cause of maternal and neonatal morbidity and mortality. There is considerable evidence that maternal obesity, increased insulin resistance, inflammation, and aberrant fatty acid metabolism are involved in the pathogenesis of PE. We hypothesized that adipocyte release of pro-inflammatory adipokines is exaggerated under both basal and stressed conditions and that adipose tissue macrophage infiltration is increased in women with PE, thereby implicating adipocyte function in its pathophysiology.

- We found that in PE compared with controls, visceral adipose tissue has higher activated macrophage content and higher mRNA expression of TNF α, and is more responsive to lipopolysaccharide stimulation in terms of releasing higher levels of TNF α and IL-6.

- Inflammation of visceral adipose tissue may provide one of the key mechanisms in the pathogenesis of PE. This may facilitate better models of prediction of PE and provide the basis for pharmacological intervention in the prevention or attenuation of the disease.

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

Author Contribution
None.S.S.H. recruited patients, collected tissue samples, performed all laboratory work not attributed elsewhere (except immunocytochemistry), analysed data and wrote manuscript; J.B. performed macrophage counts, F.J. carried out real time PCR and bioplex work of adipokines except that undertaken by R.B. and G.L.; R.B. and G.L. real time PCR of *FLT 1* and ELISA of sFlt-1 and summarised that data; D.F. and N.S. obtained original BHF grant which funded much of this work, supervised S.S.H. and revised paper.

Abbreviations
BMI, body mass index; ELISA, enzyme linked immunosorbent assay; HOMA, homeostasis model assessment; NICE, National Institue for Health and Care Excellence; CCL, chemokine ligand; FCISI, fat cell insulin sensitivity index; IQR, interquartile range; IRS1, insulin receptor substrate 1; LPS , lipopolysaccharide; NEFA, non-esterified fatty acids; PE, pre-eclampsia; SAT , subcutaneous adipose tissue; sFlt-1, fms-like tyrosine kinase-1; VAT, visceral adipose tissue; VEGF, vascular endothelial growth factor.

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