Metabolic Pathways in Normal and Pre-eclamptic Pregnancies

MD Thesis

Faculty of Medicine

University of Glasgow

2010

by

Shahzya Shahnaz Huda

MBChB (Hons), MRCP, MRCOG

Division of Developmental Medicine
Reproductive and Maternal Medicine

University of Glasgow

Abstract

Maternal metabolism undergoes dramatic changes in pregnancy in order to sustain and nourish the developing fetus. During healthy pregnancy the mother goes from an anabolic state in early pregnancy to a state of catabolism in late pregnancy with increased lipolysis together with a significant reduction in insulin sensitivity. Pre-eclampsia (PE) characterised by hypertension and proteinuria is a major cause of maternal and perinatal morbidity. There is acute 'atherosis' in PE placenta, and lipid accumulation within glomerular cells and liver. PE women have an early, excessive triglyceride and free fatty acid (FFA) rise and greater cardiovascular disease (CVD) risk in later life. The cause of these lipid abnormalities in PE is unknown but disordered adipocyte function including exaggerated lipolysis and aberrant release of adipokines (such as IL-6 and TNF alpha) is a major candidate pathway. Elevations in FFAs, and pro-inflammatory adipokines could underpin the oxidative stress, endothelial dysfunction, inflammation, and insulin resistance - characteristic features of PE.

The aims of this thesis were to acquire a better understanding of lipid metabolism and function in normal pregnancy, to determine if adipocyte function was altered in PE and, if so, to establish mechanisms. In addition I planned to corroborate epidemiological evidence of increased future CVD risk and to establish which risk factors accounted for this increased risk.

I collected subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) biopsies in non-labouring pregnant healthy (n=31) and PE (n=14) women who underwent caesarean section. Maternal blood was collected prior to delivery and phenotyping of the mother was performed including plasma assay for cholesterol, triglyceride, HDL-cholesterol, IL-6, TNF-α, leptin, adiponectin, high sensitivity CRP, glucose and insulin concentrations. Maternal BMI at booking, standardised blood pressure measurements and birth weight centile were also recorded. I determined ex vivo lipolytic activity (basal, isoprotenerol stimulated and insulin suppression of lipolysis) and adipokine production in response to lipopolysaccharide (LPS) stimulation from these biopsies. The gene expression of relevant target genes and macrophage densities in each adipose depot by immunocytochemistry (ICC) was also performed. In addition I performed carotid ultrasound assessment of women with a previous history of PE (n=31) and

matched controls (n=29). Ethical approval was obtained from Glasgow Royal Infirmary LREC and all patients gave their informed consent.

I found that in normal pregnancy, adipocyte lipolytic function is independent of maternal BMI. Adipocyte lipolytic function of SAT and VAT are also independent of each other. Adipose tissue is very metabolically flexible and the rate of whole body lipolysis is still insulin sensitive in late gestation. VAT is more closely related to markers of maternal insulin resistance (IR) and is more sensitive to catecholamine stimulation and less sensitive to insulin suppression of lipolysis than SAT, the basis of the "portal paradigm". Increasing BMI is associated with an increase in VAT cell size, with increased lipolysis and an increase in pro-inflammatory adipokines, a potential mechanism through which increasing obesity could predispose to metabolic complications of pregnancy. In contrast SAT cell size is not closely related to BMI and this may reflect the adaptation of this depot to increasing fat mass through both hypertrophy and hyperplasia, a metabolically advantageous response. TNF alpha is an important correlate of basal lipolysis in SAT.

In PE there is decreased insulin sensitivity of both SAT and VAT compared to controls as calculated by the fat cell insulin sensitivity index (or responsiveness to insulin once the tissue is stimulated by isoproterenol). This would potentially make a significant impact on total circulating FFA as almost 60% of circulating FFA are from these adipose depots. The rise in FFA in PE occurs early in pregnancy and contributes significantly to IR. Therefore the IR of adipose tissue could lead to a vicious cycle of increased lipolysis, increased FFA and further exacerbation of IR. In contrast to controls, SAT cell size is intimately related to BMI suggesting that adaptation to increasing fat mass is mainly through adipocyte hypertrophy which could lead to increased endoplasmic reticulum stress, increased IR and increased release of inflammatory adipokines. I have shown that SAT cell size does relate to adipokine release in PE, with increased release of leptin, CRP and PAI-1 and paradoxical increase in the anti-inflammatory IL-10. I had hypothesised that in addition to an inherent defect in adipocyte function there was an additional factor present in maternal serum of women with PE released from the placenta which excessively stimulated lipolysis. I failed to demonstrate any effect of maternal serum on adipocyte lipolysis in either controls or PE.

I also found that after stimulation with LPS, there was increased release of TNF alpha and IL-6 in VAT in PE but not in controls, with higher gene expression of these adipokines. TNF alpha release also correlated negatively with the fat cell insulin sensitivity index (FCISI) of VAT implicating a paracrine effect in this tissue. I also demonstrated an increase in gene expression of cfms (activated macrophages) relative to control gene, and increased density of $cfms^{+}$ macrophages/adipocytes in the VAT of PE women implicating activated adipose tissue macrophages as a potential source of the increased release of inflammatory adipokines.

Lastly I attempted to corroborate epidemiological evidence for the increase future risk of CVD women with a history of PE by assessing two surrogate markers for atherosclerosis - carotid IMT and carotid plaque scores. Both were found to be increased, with plaque scores significantly so. Classic risk factors such as age, lipids, BP and smoking did not attenuate this effect and BMI only marginally attenuated it, therefore only partially explaining this increased risk.

In summary the data presented in this thesis provides further evidence that PE is a “metabolic syndrome of pregnancy” with disordered adipocyte function and metabolism, with an increased future risk of CVD in later life. Further studies on adipose accumulation, function and composition in normal and complicated human pregnancy are warranted.

Table of Contents

List of Tables

List of Figures

List of Publications

Full Publications

Huda SS, Brodie LE, Sattar N Setting the Scene: Obesity in Pregnancy. Prevalence and Metabolic Consequences. Semin Fetal Neonatal Med. 2009 Nov 5 [Epub ahead of print]

Huda SS, Sattar N, Freeman DJ Lipoprotein metabolism and Vascular Complications in Pregnancy. Clin.Lipidol 2009 Feb;4(1):91-102

Published abstracts

SS Huda, J Bray, F Jordan, N Sattar, DJ Freeman Aberrant release of adipokines from fat tissue in pre-eclampsia. Reproductive Sciences 2009; 16 (3) (Supplement):173A

J Bray, SS Huda, A Young, F Jordan, SM Nelson, N Sattar, DJ Freeman Placental, but not Visceral or Subcutaneous Adipose Tissue, Macrophage Density is Associated with Maternal Obesity. Reproductive Sciences 2009; 16 (3) (Supplement):168A

DJ Freeman, SS Huda, F Stewart, VA Mackay, MG Kanagalingam, EK Tan, R Lindsay, N Sattar, SM Nelson Maternal Smoking, but not body mass index or social deprivation status, is associated with increased inflammation, adverse lipid profile and markers of endothelial dysfunction in cord blood of the offspring. Reproductive Sciences 2009; 16 (3) (Supplement):224A

SS Huda, K Deans, DJ Freeman, N Sattar Adverse Pregnancy Outcomes and Cardiovascular Disease: A pilot study of carotid ultrasound assessment in women with a history of pre-eclampsia Hypertens Pregnancy. 2008;27(4):512-711

CC Onyiaodike, VA Mackay, SS Huda, EA Brown, DJ Freeman Pregnancy complicated by pre-eclampsia is associated with low cord plasma C-reactive protein and tumour necrosis factor α levels Hypertens Pregnancy. 2008;27(4):512-711

Huda SS, Tan EK, Perry C, Greer IA, Dominiczak AF, Freeman DJ, Sattar N A Comparison of Visceral and Subcutaneous Adipocyte Function in Healthy Pregnant Women. Reproductive Sciences 2007; 14 (1)(Supplement): P204

SS Huda, E K Tan, C Perry, I Greer, N Sattar, D Freeman. Relationship of BMI to Fat Cell Function in Normal Pregnancy Scottish Medical Journal 2007

Acknowledgements

Firstly I wish to thank both my supervisors, Dr Dilys Freeman and Professor Naveed Sattar for their continued guidance, support, encouragement and invaluable advice without which I would not have been able to complete this thesis.

I would like to thank EK Tan who showed me how to perform the lipolysis experiments and who did a lot of the preliminary work in determining the lipolysis methods. Many thanks are also due to Mrs Fiona Jordan, Ann Brown and Anne Young who helped with the tissue processing, and allowed me to immediately start on the processing of the adipose tissue. In particular I would like to thank Ann Brown for help with ELISA, DNA extraction and use of the spectrophotometer. She also performed or organised the processing and phenotyping maternal and cord blood samples, for which I am very grateful.

I would like to acknowledge that Mrs Fiona Jordan carried out all the RT-PCR work and bioplex assays. Mrs Anne Young carried out the immunocytochemisty. Mrs Anne Young, Mr Jack Bray and Miss Clare Tannahill carried out the laborious task of macrophage quantification. I am extremely grateful for all of the work that they carried out.

I would like to thank all the medical and midwifery staff at the Princess Royal Maternity Hospital for their assistance in the recruitment of patients and collection of tissue. I am also grateful to the British Heart Foundation for funding this research project.

Lastly I must acknowledge my wonderful and generous family whose unwavering support has allowed me to complete this thesis. My parents who have always provided me with encouragement and love, my parents-in law who did some much needed babysitting when deadlines were approaching and lastly my beloved husband whose great patience, help and understanding made this possible.

This thesis is dedicated to my son Adam who did his best to throw a spanner in the works by his arrival in the middle of things.

Author's Declaration

The contents of this thesis have not been submitted elsewhere for any other degree, diploma or professional qualification.

This thesis has been composed by me, and I have been responsible for patient recruitment, tissue collection and laboratory studies unless otherwise acknowledged.

Shahzya Huda, February 2010.

Abbreviations

ABCA1	ATP-binding cassette transporter 1
ACBG1	Acyl-CoA synthetase bubblegum 1
AR	adrenoreceptor
ATM	adipose tissue macrophages
ATGL	adipose tissue triglyceride lipase
BMI	body mass index
BMR	basal metabolic rate
cAMP	cyclic adenomonophosphate
CCR2	chemokine receptor 2
cfms	macrophage colony stimulating factor
CEBPα	CCAAT enhancer binding protein alpha
CHD	coronary heart disease
CIMT	carotid intima media thickness
CVD	cardiovascular disease
CRP	c-reactive protein
CS	caesarean section
CSF-1	colony stimulating factor 1
DAG	diacylglycerol

DBP	diastolic blood pressure
DHA	docosahexanoic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FABP4	fatty acid binding protein 4
FCISI	fat cell insulin sensitivity index
GDM	gestational diabetes mellitus
GC	guanylyl cyclise
GLUT4	glucose transporter type 4
GTP	guanosine triphosphate
HOMA	homeostasis model assessment
HDL	high density lipoprotein
HELLP	haemolysis elevated liver enzymes low platelets
HSL	hormone sensitive lipase
HMW	high molecular weight
ICAM-1	intercellular adhesion molecules
ICC	immunocytochemistry
IDL	intermediate density lipoprotein
IFNγ	interferon gamma
IL-6	interleukin 6

INS	insulin
IR	insulin resistance
IRS-1	insulin receptor substrate 1
ISO	isoproterenol
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	intrauterine growth restriction
LCPUFA	long chain polyunsaturated fatty acids
LDL	low density lipoprotein
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MAG	monoacylglycerol
MCP-1	monocyte chemotactic protein 1
mRNA	messenger ribonucleic acide
NEFA	non esterified fatty acids
NFKB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGT	normal glucose tolerance
NP	natriuretic peptides
NPR	natriuetic peptide receptors
PAI-1	plasminogen activator inhibitor 1
PDE	phosphodiesterase

PE	pre-eclampsia
PKA	protein kinase A
PKG	protein kinase G
PPARγ	peroxisome proliferator-activated receptor gamma
SAT	subcutaneous adipose tissue
SBA	basal lipolysis in subcutaneous fat
SREBP1c	sterol regulatory element binding protein-1c
TAG	triacylglycerol
TC	total cholesterol
TG	triglycerides
TGFβ	Transforming growth factor beta
TLR	toll like receptors
TNF alpha	tumour necrosis factor alpha
VAT	visceral adipose tissue
VBA	basal lipolysis in visceral fat
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low density lipoproteins
WAT	white adipose tissue

1 Introduction and Literature Review

1.1 Introduction

Human pregnancy is characterised by striking changes in maternal metabolism and body composition in order to provide sufficient energy and nutrients to the developing fetus and later for lactation. In this review, I will describe the physiology of lipolysis in human adipose tissue as a prelude to the examination lipoprotein metabolism during normal pregnancy and in the presence of additional maternal metabolic stresses such as obesity and other risk factors for diabetes. The evidence regarding the contribution of lipids to the pathogenesis of pre-eclampsia will be assessed, and the relationship of pregnancy with cardiovascular disease in later life explored. Finally the potential areas in this field necessitating further research will be considered.

1.2 Physiology of lipolysis in human adipose tissue

The major area of storage of triglycerides (TG) is white adipose tissue (WAT) and it allows surplus fuel to be stored in times of calorific excess and expended during times of need such as fasting or prolonged exercise. Lipolysis is the catabolic process through which TG are broken down into glycerol and non-esterified fatty acids (NEFA). Three main organs produce and export fatty acids (FA): white adipose tissue (WAT), the intestine and liver. In humans, the main source for adipocyte TG is from chylomicrons and very low density lipoproteins (VLDL). Lipoprotein lipase produced in the adipocyte and transported to the endothelial surface of capillaries. TG in the lipoprotein particles are hydrolyzed by LPL releasing NEFA for uptake and storage by adipocytes. In WAT, LPL activity is cyclical, being highest after meals and lowest after fasting. [1 2]In normal subjects there is fine-tuning of TG synthesis and lipolysis in response to hormonal and neural influences and is innervated by the autonomic system (parasympathetic and sympathetic nervous system). Lipolysis leads to release of NEFA which are not only energy substrates but are also highly toxic to cells and have been implicated in most of the pathological processes involved in obesity and insulin resistance.[3 4]

The mobilization of fat stored in AT is mediated through both hormone sensitive lipase (HSL) and the more recently characterised adipose triglyceride lipase (ATGL).5 HSL was considered the key enzyme catalysing the rate-limiting step of AT lipolysis but this concept has recently been challenged with the finding that

ATGL is the predominant lipase in AT and an important regulator of TG degradation in skeletal muscle. ATGL was independently reported by three groups and is also subject to a recent review.6 It is thought to be important in both basal and catecholamine-stimulated lipolysis. ATGL knockout mice show blunted fat cell lipolysis and as a consequence become obese with deposition of TAGs in multiple sites, particularly the heart.7 The sequential hydrolysis of TAG is regulated by the lipases and results in the liberation of FA at each step with the generation of DAG (diacylglycerol), MAG (monoacylglycerol), and glycerol. The current view is that ATGL and HSL work in a serial manner- ATGL initiates lipolysis by acting on TAG to produce DAG, which is then hydrolysed by HSL to MAG, which are finally converted to FA and glycerol by monacylglycerol lipase (MGL) [8](Figure 1)

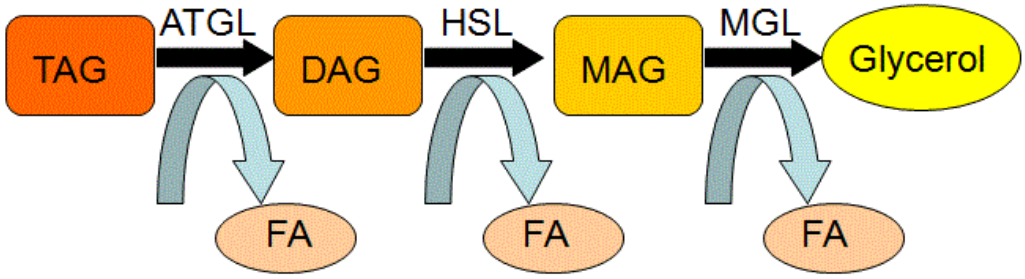

Figure 1 Sequential hydrolysis of triacylglycerides

ATGL initiates lipolysis by acting on TAG to produce DAG, which is then hydrolysed by HSL to MAG, which are finally converted to FA and glycerol by MGL.

Catecholamines, insulin and natriuretic peptides are considered to represent the major regulators of lipolysis in humans (Figure 2). Several novel lipolytic and anti-lypolytic agents have been discovered but their function *in vivo* are still to be clarified. Catecholamines are the most important stimulator of lipolysis and act via the symphathetic nervous system through $ß_1$, $ß_2$ and $ß_3$ adrenoreceptors (AR) in the human fat cell. They initiate activation of lipolysis by stimulation of cAMP production via G_s-proteins and activation of protein kinase A (PKA).[9] The two main targets for PKA phosphorylation are HSL and the perilipins [10], and this results in a dramatic increase in lipolysis. Perilipins are a family of proteins which regulate coordination of lipid storage and utilization in various cell types.[11] Perilipin A is the predominant form which covers the lipid droplets in mature adipocytes. Perilipin phosphorylation results in important physiological

alteration of the droplet surface that facilitates the action of HSL and ATGL.[11] Coexisting on the human fat cell are α_2-adrenoceptors which when stimulated inhibit cAMP production and lipolysis through G_I-inhibitory proteins[12]. Thus in humans, the balance of beta and alpha adrenoreceptors mediates the net effect of catecholamines on lipolysis. The relative importance of ß and α-adrenergic effects in human fat depots varies with age, adipose mass, WAT location and sex.

Insulin is a potent mediator of fat metabolism and promotes glucose uptake by adipocytes and re-esterification of NEFA. Insulin is the main inhibitor of lipolysis and acts by it ability to lower cAMP levels via activation of phosphodiesterase 3B and therefore reducing PKA activity. [13]

Natriuretic Peptides (NP) are known to have a key role in the regulation of salt and water homeostasis and the control of blood pressure. They have also been found to exert a powerful lipolytic response in human AT via activation of a cGMP dependent pathway in contrast to cAMP seen with catecholamines.[14] Expression of atrial natriuretic peptides (ANP) receptor mRNA and binding studies have confirmed the presence of types A and C ANP-receptors (NPR-A and NPR-C).[15] Not surprisingly it appears that insulin (as it acts via PD 3B to lower cAMP) has no effect on ANP stimulated lipolysis in contrast to its potent inhibition of catecholamine induced lipolysis.[16] Real-time PCR has shown that large adipocytes express higher mRNA levels of NPR-A than small adipocytes on their cell membrane.[17] NP appears to contribute to the physiological response of increased lipolysis during exercise [18] and this is particularly relevant in subjects receiving ß AR blockade.[19 20]

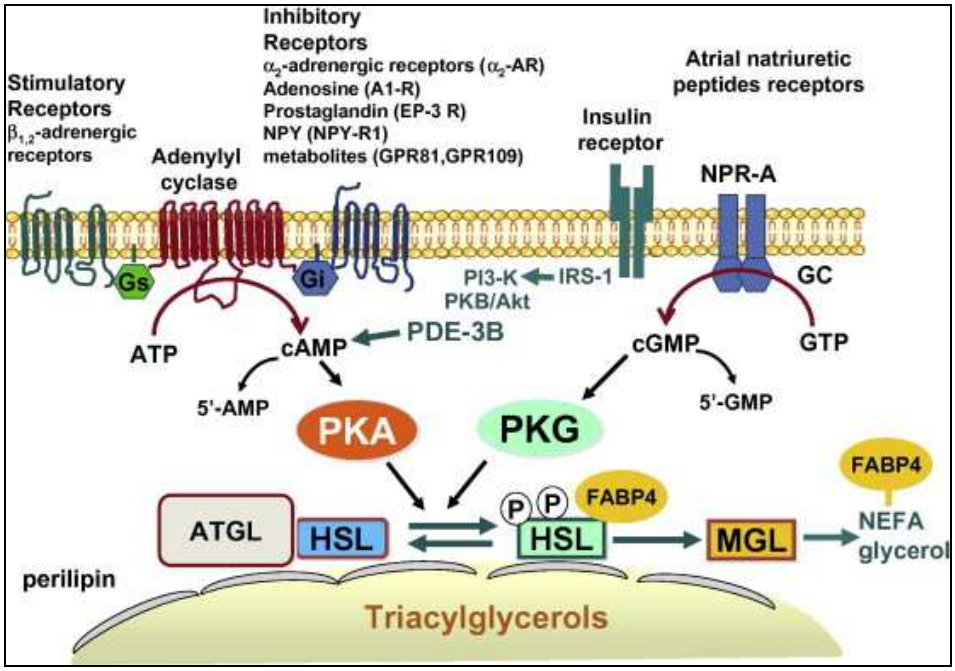

Figure 2 Major pathways involved in the stimulation of human fat cell lipolysis. Adapted from [21]

Signal transduction pathways for catecholamines via adrenergic receptors, metabolite-driven inhibitory receptors and atrial natriuretic peptides via type A receptor (NPR-A). Protein kinases (PKA and PKG (cGK-I)) are involved in target protein phosphorylation. HSL phosphorylation promotes its translocation from the cytosol to the surface of the lipid droplet. Perilipin phosphorylation induces an important physical alteration of the droplet surface that facilitates the action of HSL. ATGL acts to initiate lipolysis by acting on TAG to produce DAG, which is then hydrolysed by HSL to MAG. Docking of adipocyte lipid-binding protein (FABP4) to HSL favours the outflow from the cell of NEFAs released by the hydrolysis of triacylglycerols. Insulin, via stimulation of fat cell insulin receptors and phosphodiesterase-3B stimulation promotes cAMP degradation and antilipolytic effects while it is not active on cGMP-dependent pathways (not shown in the diagram). ATGL, adipose triglyceride lipase; FABP4, adipocyte fatty acid binding protein 4; GC, guanylyl cyclase; Gi, inhibitory GTP-binding protein; Gs, stimulatory GTP-binding protein; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase; NEFA, nonesterified fatty acid; NPR-A, type A natriuretic peptide receptor.

1.3 Lipid Metabolism in Normal Pregnancy

1.3.1 Early Pregnancy

Maternal metabolism during pregnancy adapts to benefit the growth and development of the fetus and can be divided into two phases. During the initial two thirds of gestation, when fetal energy demands are limited, maternal fat stores increase.[22] This is attributable in part to maternal behavioural change including hyperphagia [23] and to increased adipose tissue lipogenesis.[24] In early

pregnancy insulin sensitivity is normal or even slightly improved with normal peripheral sensitivity to insulin and hepatic basal glucose production.[25] This metabolic environment together with pregnancy related endocrine changes including increasing levels of oestrogen, progesterone and cortisol favours lipogenesis and fat accumulation. [26]

1.3.2 Late Pregnancy

During the latter stages of pregnancy this anabolic state switches to a state of catabolism with a marked increase in lipolysis rates and a corresponding rise in maternal free fatty acids (FFA) and glycerol.[27] [28](Figure 3) This change is enhanced by an increase in hormone-sensitive lipase (HSL) activity and mRNA expression and a decrease in lipoprotein lipase (LPL) activity.[29] Exaggerated catecholamine release in response to even modest maternal hypoglycaemia and the insulin resistant state of late pregnancy contribute to this switch.[30] [31] Insulin effects on lipolysis (adipose tissue) and fat oxidation (in liver and muscle) are significantly impaired during the 3rd trimester compared to earlier in pregnancy and also post partum.[31] Reduced expression of PPARγ and its target genes may also contribute to accelerated fat metabolism in late pregnancy.[32] This catabolic state corresponds to the time of maximum fetal growth and by increasing FFA use in the mother, increases availability of glucose and amino acids for the fetus.[33] Increased lipolysis and therefore maternal glycerol production supports gluconeogenesis as glycerol is preferentially used as a substrate for glucose which easily transfers to the fetus.[27] [34] During times of fasting or starvation in late pregnancy there is increased ketogenesis from FFA in the maternal liver.[35] Ketone bodies are readily transferred to the fetus and can be utilized for energy or for fetal lipogenesis.[36] [37] There is an excellent review by Herrera et al exploring lipid metabolism in normal pregnancy in further detail.[38]

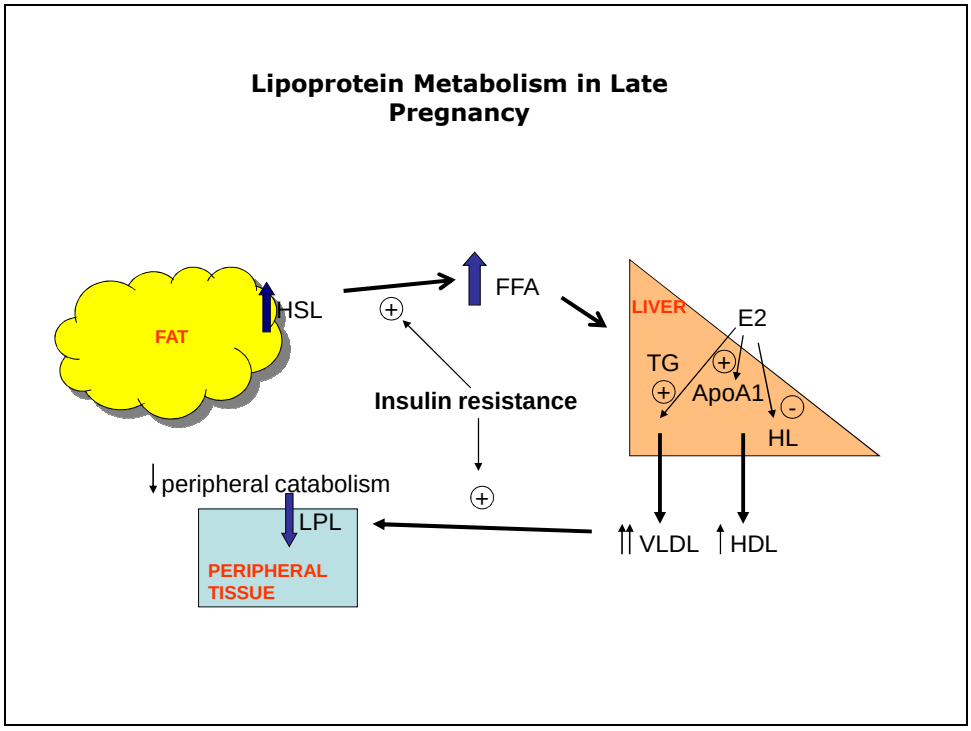

Figure 3 Lipoprotein metabolism in late pregnancy

This diagram summarises the main changes in lipoprotein metabolism which occur in advancing gestation. Due to increasing insulin resistance there is an increase in hormone-sensitive lipase (HPL) activity and a decrease in lipoprotein lipase (LPL) activity. This results in a marked increase in lipolysis rates and corresponding increase in free fatty acids (FFA), delivered to the liver. These are channeled into hepatic triglyceride synthesis and increased secretion of VLDL. Oestrogen (E2) is the primary determinant of increased hepatic VLDL production. E2 also acts to promote Apo A1 production and reduce hepatic lipase (HL) activity with a resultant increase in HDL production. The reduced LPL activity contributes to the increase in plasma VLDL levels by reducing the peripheral catabolism of this lipoprotein.

1.4 Body Composition

All women increase maternal fat stores in early pregnancy irrespective of pre-pregnancy adiposity to meet the feto-placental and maternal demands of late gestation and lactation. Women of normal weight gain around 3.8 kg of fat [39] during pregnancy although there is substantial variation. [40-42] Total fat appears to increase to a peak toward the end of the second trimester before diminishing which corresponds to the period of increased lipolytic activity.[43 44] In women of normal weight the majority of fat is accumulated centrally in the subcutaneous compartment of the trunk and upper thigh.[45 46] In later stages of pregnancy there is an increase in both the thickness of pre-peritoneal fat (visceral) and the ratio

of pre-peritoneal to subcutaneous fat as measured by ultrasound.[47] This pattern may be relevant to increasing insulin resistance and lipid changes that occur as pregnancy progresses. Indeed accumulation of hepatic fat has been shown to be an important mediator of insulin resistance during pregnancy in the rat model.[48] Regionality of fat accumulation may be important due to regional variations in metabolic function as highlighted previously. Obese women, who will have more saturated subcutaneous fat stores, tend to accumulate fat more centrally than lean women, at least as estimated by using the skin-fold thickness technique, an observation which may reflect their more insulin-resistant state.[46] Visceral adiposity appears correlated more strongly to adverse metabolic outcomes in pregnancy including gestational diabetes mellitus, gestational hypertension and pre-eclampsia.[49-51] Furthermore visceral adiposity in early pregnancy appears to correlate better than subcutaneous fat or body mass index (BMI) with metabolic risk factors such as blood pressure, insulin resistance and lipids.[52] Lean subjects have a greater increase in percent of body fat in pregnancy compared with obese subjects but there is no difference in actual total fat mass.[46]

A large population-based Swedish study of 151 025 women highlighted the relevance of inter-pregnancy weight gain.[53] It demonstrated that an increase of BMI of 3kg/m2 between two consecutive pregnancies resulted in an increased risk of pre-eclampsia, gestational diabetes, gestational hypertension, caesarean delivery, still birth and large for gestation age births even if a women has a healthy BMI for both pregnancies. This study re-inforces the evidence for causality between being overweight and obese and adverse pregnancy outcome

1.5 Changes in Lipoprotein Profile during Pregnancy

Pregnancy is characterized by marked increases in plasma lipid concentrations as gestation advances. Plasma cholesterol and triglyceride concentrations rise by 25-50% and 200-400% respectively. The increase in triglyceride is mainly due to VLDL triglyceride which shows a three fold increase from 14 weeks' gestation to late pregnancy.[54] VLDL comprises two fractions, VLDL1 which is secreted by the liver to supply tissues with triglyceride fatty acids in the post-absorptive state and VLDL2 which is the major precursor of the major cholesterol transporting particles IDL and LDL. VLDL1 and VLDL2 increase in parallel by an average of 4-

fold as plasma triglyceride increases with advancing gestation.[55] These changes are due to increased adipose tissue lipolysis resulting in increased delivery of FFA and glycerol to the liver where they are re-esterified for the synthesis of triglycerides and incorporated into VLDL. The insulin resistant condition of pregnancy may contribute to the increased VLDL production but the effect of oestrogen is more likely the primary determinant of increased VLDL production by the liver.[55 56] In addition to increased VLDL production there appears to be a decrease in maternal VLDL catabolism which may be due to a reduction in LPL activity in the third trimester.[56] Oestradiol concentration rises steadily throughout pregnancy which suppresses postheparin hepatic lipase activity which in turn results in reduced triglyceride hydrolysis in IDL and LDL particles. Moreover there is an increase in cholesteryl ester transfer protein activity in mid trimester of pregnancy which would, in the presence of high plasma TG, contribute to enrichment of lipoprotein fractions with triglyceride.[55 56]

Despite a rise in TG in normal pregnancy, HDL-cholesterol levels are elevated by the 14th week and rise by a maximum of around 40% at 28 weeks' gestation mainly due to an increase in the HDL2 subfraction with a proportional fall in HDL3a and HDL3b. [54 56] The mean concentration of HDL-cholesterol is around 2 mmol/L compared to around 1.5 mmol/L in the non-pregnant.[55] This increase in HDL is driven by rising oestrogen concentration which acts on the liver to promote apo AI production and a simultaneous fall in hepatic lipase activity (which is responsible for hydrolysis of HDL2 to smaller HDL3 which is more rapidly removed from the circulation).[55 56]

During normal pregnancy there is a rise in LDL of around 70%. [55]. Although this increase is less marked than TG there are some important qualitative changes in the LDL composition favouring a more "atherogenic" profile with a proportional increase in small dense LDL (LDLIII) in late pregnancy.[57 55] In keeping with the non-pregnant population, this is driven by higher plasma triglycerides where a "threshold" effect may be seen.[55]

1.6 Placental Transfer of Lipids

Fatty acids are required by the developing fetus as they are important constituents of cell membranes.[58] The fetus can produce some of the FFA from

fetal lipogenesis but the majority is acquired via the maternal circulation via the placenta. All of the essential fatty acids must be acquired from the mother from which the long chain polyunsaturated fatty acids (LCPUFA) are derived.[59] These are of particular importance as they form membranes within the brain and nervous system. FFA can be released from circulating maternal TG-rich lipoproteins by placental lipoprotein lipase. Fatty acids cross the placenta by simple diffusion and more importantly through fatty acid binding proteins located in placental membranes and cytoplasm.[60] The maternal LCPUFA status is a key determinant of FA status in the fetus although the placenta is able to selectively transfer important PUFA.[59] Maternal supplementation with LCPUFA such as docosahexanoic acid (DHA) found in oily fish can also influence preferential uptake from mother to fetus.[61]

Cholesterol, essential for fetal growth, is also transported from mother to fetus although it has been shown in animal models that fetal synthesis can provide a significant proportion required for fetal growth.[62] Cholesterol is transported across the placenta from maternal LDL and HDL via LDL receptors and scavenger receptor B1 respectively. Cholesterol is transferred to fetal HDL probably via membrane localized efflux cholesterol transporter proteins ACBG1 and ABCA1.[63] Maternal hypercholesterolaemia can result in the presence of fatty steaks in the fetal aorta and may have implications for future disease in the offspring, although this remains to be definitely proven[64]

1.7 Lipid Metabolism Obese vs Lean Pregnancy

Maternal obesity is a well established and potentially modifiable risk factor for adverse pregnancy outcome including hypertensive disorders of pregnancy, fetal macrosomia and gestational diabetes mellitus (GDM).[65] Rates of obesity are rising within the obstetric population with evidence of a 2-fold increase in women attending for antenatal care in the last decade.[66] Therefore an understanding of metabolic differences between lean and obese women during pregnancy is useful. Lean subjects have a greater increase in percent of body fat in pregnancy compared with obese subjects but there is no difference in actual total fat mass.[46] Obese women tend to accumulate fat more centrally than lean women, at least as estimated by using the skin-fold thickness technique, an observation which may reflect their more insulin-resistant state.[46] In lean women there is an inverse relationship between changes in fat mass and

changes in insulin sensitivity in early pregnancy.[28] In contrast in obese women there was no such relationship in early gestation but one does materialise over the longer gestation period from pre-pregnancy to late gestation.[42] This suggests a slower metabolic responsiveness of obese women during pregnancy. Indeed in obese women there was an initial increase in insulin sensitivity from pre-pregnancy to early pregnancy.[42]

Interestingly obese women do not show any alteration in either basal carbohydrate oxidation or non-oxidisable carbohydrate metabolism from early to late pregnancy in contrast to a 50-80% increase in basal fat oxidation.[42] This corresponds to the period of fat accumulation and reduced insulin sensitivity. In addition women with a high BMI have the largest increase in their basal metabolic rate (BMR) compared to women with normal or low BMI. [67] [68] These differences lend support to the hypothesis that there is an adaptive process in obese individuals who are insulin resistant to prevent additional weight gain.[69]

Maternal obesity results in alteration of the plasma lipid profile with higher serum triglyceride and VLDL cholesterol concentrations than those observed in lean women.[70] [71] This is seen together with lower HDL-cholesterol although LDL-cholesterol and total cholesterol concentrations remain similar.[71]. This pattern of dyslipidaemia is similar to that of the metabolic syndrome in the non-pregnant population.[72]

1.8 Lipid Metabolism GDM vs NGT

Gestational diabetes mellitus (GDM) is defined as a glucose intolerance at the upper end of the population distribution that is first detected during pregnancy.[73] It is present in around 4-9% of all pregnancies depending on the population studied. [74] [75] Its clinical importance reflects an increased perinatal morbidity related to fetal macrosomia, increased maternal risk of type 2 DM in later life and long term risk to offspring including obesity, sustained glucose intolerance and impaired intellectual ability.[76] [77] A detailed discussion of carbohydrate metabolism in pregnancy is beyond the scope of this review. In summary, normal pregnancy is characterized by a progressive increase in insulin secretion by 3-3.5 fold in late pregnancy coupled with increasing insulin resistance to levels around 50-70% less than that of non pregnant women.[25] [78] In

addition there is an increase in basal hepatic glucose production in spite of increased fasting insulin concentration.[78] In GDM there is a similar increase in first-phase insulin response but the increase is attenuated compared to normal controls.[79] However obese women with GDM have an exaggerated second-phase response compared to obese controls.[80] As expected, insulin resistance is also greater in GDM than controls as measured by insulin suppression of glucose production.[79 80] Thus, women who develop GDM begin pregnancy far closer to the metabolic thresholds beyond which they would develop glucose intolerance; pregnancy simply pushes these women beyond this threshold.

In GDM there is dyslipidaemia consistent with insulin resistance. Plasma TG levels are significantly higher than in normal pregnancy and there is enrichment of lipoprotein fractions with TG within VLDL and HDL particles.[81] There is also evidence that GDM increases LDL susceptibility to oxidation.[82] As previously described post-prandial free fatty acids (FFA) are increased in late pregnancy due to insulin resistance and this effect is more pronounced in GDM.[83] Women with GDM were found to have lower levels of insulin receptor substrate 1 (IRS-1) which may contribute to reduced suppression of lipolysis.[32]

Lean women with impaired glucose tolerance prior to pregnancy had a smaller increase in fat mass compared to lean women with NGT.[28] In obese women there was no apparent difference between women with NGT and GDM in the amount of or distribution of accumulated fat.[42 46]

1.9 Lipid Metabolism in Pre-eclampsia

Pre-eclampsia (PE) occurs in 2-4% of pregnancies and is a leading cause of maternal and neonatal morbidity and mortality in the developed world. It is a multi-system disorder resulting in the classic manifestations of hypertension due to vasoconstriction, proteinuria due to glomerular damage and oedema due to increased vascular permeability.[84] Placental damage associated with the condition can result in intrauterine growth restriction. Delivery is the only known cure - thus, PE is a frequent cause of premature delivery and low birth weight. As yet the underlying pathogenesis of the disorder had not been completely understood. The clinical signs of PE are relatively simple manifestations of a complex underlying pathological process with activation of the coagulation

system, platelets and leukocytes and disturbances in metabolism which combine to provoke widespread endothelial damage and dysfunction. This in turn augments further activation of leukocytes and coagulation resulting in a vicious cycle of vascular injury. The disorder is likely to be a result of heterogeneous causes resulting from the interaction of placental and maternal factors.[85] In the presence of a placental trigger the maternal response will depend on the maternal genotype and phenotype resulting in the clinical syndrome of PE.[86]

1.9.1 Excess fat accumulation in several tissues

The classic pathological lesion seen in the placental bed in PE is 'acute atherosis' resulting from the accumulation of lipid-laden macrophages surrounded by areas of fibrinoid necrosis in the spiral arteries – features comparable to atherogenesis in non-pregnant women.[84] Similarly, the characteristic lesion in the glomerulus – endotheliosis - involves the accumulation of lipids within glomerular endothelial cells while the liver also accumulates excess fat.[84] This process is even more marked in HELLP syndrome and acute fatty liver of pregnancy, which are severe complications related to PE. Lipid accumulation at sites of endothelial damage emphasise the potential role of lipid disturbance in the vascular injury of PE. Therefore, whatever the precise nature of the 'triggering' placental factor in PE, it almost certainly provokes a disturbance in lipid metabolism which contributes to vascular damage

1.9.2 Dyslipidaemia of pre-eclampsia

In PE the hyperlipidaemia of normal pregnancy is exaggerated further via greater synthesis and potentially via lower peripheral catabolism (see Figure 2 for summary). Although direct evidence for impairment of peripheral catabolism is lacking there is a suggestion of the over representation of common mutations in the LPL gene in women with PE.[87] These mutations are associated with a reduction in LPL activity and dyslipidaemia in the non-pregnant. It has long been recognised that maternal hypertriglyceridaemia is significantly higher among pre-eclamptic women than normal matched controls.[88-91] Importantly this rise occurs well in advance of manifestations of the disease.[92 93] Furthermore there are some important qualitative changes in lipid composition as a consequence of high plasma TG. There is an almost three fold higher VLDL1 and an almost two

fold higher VLDL2 concentration than in control healthy pregnancy.[89] Total LDL concentrations are similar but the exaggerated rise in TG drives increased production of small dense LDL in PE pregnancy to almost three times that seen in normal pregnancy with a reduction in large, buoyant LDL subfractions.[89 94-96] It has been established in cardiovascular disease that hyperlipidaemia results in endothelial damage via oxidative stress and parallels exist between the atherogenic lipid profile seen in PE. Small, dense LDL exhibit enhanced oxidative potential and once oxidised these particles are believed to be highly atherogenic promoting foam cell formation and endothelial dysfunction.[97] Soluble vascular cell adhesion molecule-1 (VCAM-1), a marker of vascular dysfunction, is elevated in PE and correlates with LDL cholesterol in pregnancy.[98] Plasma triglycerides also correlate with the lipid peroxidation metabolite malondialdehyde.[99] Oxidised LDL but not native LDL inhibits trophoblastic cell invasion in a concentration dependent manner and this may be a further mechanism by which dyslipidaemia leads to impaired placentation and PE.[100] Furthermore there is a reduction in endothelial protective HDL cholesterol.[89 94 101]

1.9.3 Elevated Fatty Acids and their effects

Together with an early rise of plasma triglycerides in women who go on to develop PE, a rise in FFA is also observed independent of maternal adiposity suggesting early exaggerated adipocyte lipolysis.[92] Elevated FFA can be detrimental in several ways. Exposure of the liver to elevated FFA leads to reduced hepatic insulin extraction leading to systemic hyperinsulinaemia and accelerated gluconeogenesis.[102] There is also increased esterification of FFA and reduced hepatic degradation of apolipoprotein B which leads to an increased synthesis and secretion of small VLDL particles and triglycerides. In addition to the effects on the liver, increases in FFA promote peripheral insulin resistance by reduction of insulin mediated glucose uptake (primarily in skeletal muscle).[103] Elevation of FFA has also been shown to impair endothelial function, via several potential mediatory mechanisms, through blunting of nitric oxide dependent tone via increases in the formation of reactive oxygen species in endothelial and vascular smooth muscle cells.[104 105]

These adverse effects have also been confirmed in pregnancy and PE women. Increased FFA are an important mediator of insulin resistance in pregnancy.[106]

The high ratio of FFA to albumin in serum of PE women compared to normal controls resulted in increased uptake of FFA by cultured endothelial cells which are further esterified into TG.[107] In addition serum of PE women induces VCAM-1 expression in endothelial cells an effect mimicked by the addition of FFA to serum of normal pregnancy.[108]

FFA are also implicated in inflammation and have been shown to be an important mediator of inflammation in macrophages and are associated with increased CRP levels in obese women.[109] [110] Thus the combination of insulin resistance, dyslipidaemia, oxidative stress, endothelial dysfunction and inflammation apparent in PE could be in part be attributable to this early elevation in FFA. There is preliminary evidence that the trigger for this increase in FFA and adipocyte lipolysis is present in serum of women with PE.[107]

1.10 Factors influencing adipocyte function

1.10.1 Anatomical Location

There are important differences between visceral or intra-abdominal and subcutaneous WAT depots. At the same BMI, women on average have greater subcutaneous fat mass and higher plasma levels of FFA than men. Visceral adiposity is more closely related to adverse metabolic outcomes including insulin resistance, hyperinsulinaemia, dyslipidaemia, hypertension and the metabolic syndrome.[111] In visceral fat there is a higher turnover of lipids due to its greater sensitivity to catecholamine-induced lipolysis and decreased sensitivity to insulin.[112] Visceral fat is in direct contact with the liver via the portal venous system. The liver is therefore exposed to chronic elevation of NEFA which as outlined above produces alteration in liver metabolism and promotes hepatic insulin resistance - the basis for the 'portal paradigm'. In addition, visceral fat may further influence increased insulin resistance through a variety of inflammatory pathways.[113] The differences are enhanced in obesity and polycystic ovarian syndrome. Alternatively, rather than visceral fat being directly or fully responsible for metabolic dysregulation of obesity, its rising volume may also simply signal saturation of 'good' fat storage capacity. Drolet et al have proposed a model whereby subcutaneous fat acts as the primary fat depot and when the storage capacity of this depot is reached "overspill" into

secondary fat depots including, amongst other tissues, visceral fat, occurs.[114] Excess subcutaneous fat appears to be metabolically favourable.[115] The much higher subcutaneous storage capacity of women compared to men and therefore less propensity to accumulate fat in the visceral compartment likely explains the lower prevalence of metabolic disturbances, and diabetes in middle-age, in women compared to men. This theory may also in part explain why certain racial groups such as South Asians are at increased susceptibility to central obesity and its metabolic consequences. [116] Similarly, in certain chronic illnesses, e.g. HIV, a loss of subcutaneous fat storage capacity may 'push' fat more centrally into key metabolic organs and instigate greater insulin resistance.[117] Interestingly, fat from 'ectopic sites' such as visceral fat is preferentially lost with modest weight loss and may explain how modest weight loss appears to provide significant metabolic and clinical benefits.[118]

1.10.2 Fat cell size and function.

The adipocyte is the only cell type whose size may vary considerably in physiological conditions. Regional growth of AT is determined by the capacity of the mature adipocyte to accumulate and mobilize TG. Sex differences in body fat distribution and adipocyte metabolism suggest that the storage capacity and propensity of fat cells to enlarge (fat cell hypertrophy) or to differentiate (fat cell hyperplasia) may be regulated in a depot specific fashion. In women adipocytes from subcutaneous depot are larger than visceral fat, with marked regional differences in AT metabolic function in these two depots.[114] Pre-adipocytes produce two functionally distinct forms of adipocytes which characteristically predominate at different sites.[119] Differentiation of these pre-adipocytes into lipid-storing, mature adipocytes are dependent on the expression of numerous transcription factors including CEBPα, PPARγ and SREBPIc which are differentially expressed in subcutaneous and visceral AT.[114] As adipocytes increase in size both lipogenesis and lipolysis become increasingly active with increasing fatty acid flux across their cell membranes. Key enzymes utilized in AT metabolism have increased activity and mRNA levels in larger adipocytes compared to smaller adipocytes such as fatty acid synthase, HSL, LPL and GLUT4, with upregulation of genes required for lipid metabolism as determined in microarray analysis.

Adipocyte size in fat is also related to adverse metabolic complications. Individuals with type II diabetes and dyslipidaemia had larger subcutaneous adipocytes, and adipocyte size in femoral fat is related to fasting plasma insulin, TGs and HDL- cholesterol ratios in men and women. Increasing adipocyte size is associated in a shift toward dominance of pro-inflammatory adipokines including TNF alpha and IL-6. This may be a result of dysregulated release of adipokines in hypertrophic larger adipocytes.[120] Adipocyte hypertrophy is also thought to result in endoplasmic reticulum (ER) stress which results in activation of metabolic factors that trigger insulin resistance, with release of inflammatory cytokines and increased macrophage recruitment.[121]

1.11 Adipokines and Pregnancy

The view that adipose tissue is simply a storage organ of excess triglycerides has changed dramatically over recent years. It has been shown to secrete a diverse range of cytokines, proteins and signals which have both paracrine and endocrine actions and a wide-ranging influence on the metabolic and physiological function of other organs.[122] In particular fat cells secrete factors involved in inflammation (TNFα, IL-6), haemostasis (PAI-1), insulin sensitivity (adiponectin) and energy balance and control of appetite (leptin). Adipokines including adiponectin, leptin, TNF alpha and IL-6 are increasingly implicated as important mediators of maternal metabolism particularly in relation to insulin resistance (IR) and lipid metabolism.

1.11.1 Adiponectin

Adiponectin is the most abundant adipokine in circulation and is synthesized exclusively in adipocytes and in contrast to other adipokines it is negatively correlated with adiposity.[123] In humans, low plasma adiponectin concentrations are highly correlated with insulin resistance in obesity and type 2 diabetes.[124] Adiponectin is an insulin-sensitizing agent and mediates its effects through activation of adensosine monophosphate (AMP) protein kinase leading to increased uptake of glucose by myocytes and reduction in hepatic gluconeogenesis[125] and by increasing fatty acid oxidation in skeletal muscle by the sequential activation of AMP activated protein kinase, p38 mitogen-activated protein kinase and PAR alpha phosphorylation. Human adiponectin circulates in 3

isoforms- high molecular weight (HMW), middle MW and low MW. Although the total adiponectin level correlates well with insulin sensitivity, it has been found that the HMW isoform is an even better correlate.[126] Maternal HMW adiponectin is the most prevalent adiponectin isoform regardless of gestational age or BMI status.[127]. The relevance of adiponectin in lipid and glucose metabolism in pregnancy is still to be elucidated. Catalano et al have demonstrated that total adiponectin secretion and adiponectin mRNA levels in WAT decline with advancing gestation in lean women, associated with a 25% increase in fat mass [128]. Adiponectin is negatively correlated with HOMA in late pregnancy in healthy women without GDM.[129] Similar to subjects with obesity or type 2 diabetes, studies have shown that adiponectin is reduced in women who have had GDM[130] and in women with GDM during pregnancy compared to controls matched for BMI. In human and animal studies, maternal adiponectin levels were found to decreased or unchanged during normal pregnancy.[131 132] Reduced adiponectin concentration may be consistent with increasing IR of pregnancy, whereas a lack of association may represent an independent role of adiponectin in pregnancy. As PE is a state of IR it was assumed that adiponectin levels would be lower, however Ramsay et al were the first to show that maternal adiponectin levels were marked elevated by nearly 50% in PE.[133] However since then although several studies supported this finding, others showed the converse and no change in adiponectin levels.[131 132] The lack of correlation between adiponectin levels and markers of IR in PE may suggests an atypical role of adiponectin in this syndrome. Interestingly adiponectin mRNA expression in adipose tissue of PE women was found to be similar to healthy controls.[134]

1.11.2 Leptin

Leptin is mainly synthesized and secreted by adipose tissue and is involved in involved in control of food intake and energy balance. Without functional leptin severe obesity occurs as in the ob/ob mouse.[135] Adipocytes secrete leptin in direct proportion to adipose tissue mass as well as nutritional status. Plasma leptin concentrations positively correlate with subcutaneous fat due to its mass effect and higher secretion rate as compared to visceral fat.[136] Insulin is a potent activator of leptin mRNA expression and protein secretion and is the major mediator of increased postprandial leptin concentrations.[137] Obese individuals have higher leptin mRNA and protein levels than lean individuals.[138]

Serum leptin levels are elevated throughout normal human pregnancy and concentrations increase with advancing gestational age.[139 140] Although the origin of leptin is still to be established, the placenta is thought to be a major contributor of maternal hyperleptinaemia [141]. Leptin has wide ranging effects in reproduction including maternal physiology, implantation, paracrine effects in the placenta and fetal development and growth.[142] Leptin has some important effects on lipid metabolism acting peripherally to prevent TG accumulation in peripheral tissues, but also regulates fuel partitioning by promoting lipid oxidation and protein synthesis and by curtailing lipogenesis, resulting in a selective loss of adiposity while preserving lean body mass.[143 144] It mediates its liporegulatory actions both centrally and directly on the liver and WAT indicated by reduced expression of key enzymes involved in fatty acid synthesis in these tissues [145 146].Therefore one potential role of hyperleptinaemia in pregnancy may be to enhance mobilization of maternal fat stores to provide energy substrates for the developing fetus.

The majority of studies suggest that maternal leptin concentrations and placental leptin synthesis are increased in pregnancies complicated by PE, with levels correlating with the severity of disease even before its clinical onset.[147-149] The role of leptin in the pathogenesis of PE is still to be elucidated but increased leptin could be considered to be a compensatory response aiming to increase nutrient delivery to an underperfused, hypoxic placenta. Leptin does correlated with other inflammatory markers including TNF alpha and IL-6 in normal and PE pregnancy, and may therefore be involved in the inflammatory and metabolic processes of both.[150]

1.11.3 TNF alpha

The inflammatory cytokine TNF alpha is associated with obesity and insulin resistance[151] and is an independent predictor of CHD and CVD events and total mortality among men.[152] Absolute levels and expression of TNF alpha in obese individuals compared to lean are higher. In addition TNF alpha correlates with insulin levels and decreased insulin sensitivity in IR individuals.[153 154] It can lead directly to insulin resistance by inhibiting insulin signaling through several mechanisms including inducing serine phosphorylation of the insulin receptor IRS1.[155] TNF alpha can also induce IR by stimulation of adipocyte lipolysis, and

through modulation of other adipokines including adiponectin and leptin. TNF alpha contributes to endothelial dysfunction via inhibition of endothelium dependent vasodilatation[156] and triggers pro-coagulant activity and fibrin deposition.[157] It is produced widely in immune cells, placenta and adipose tissue. TNF alpha is a potent regulator of lipid metabolism and induces lipolysis through multiple signaling pathways.[158] TNF alpha rises during normal pregnancy primarily thought to be due to placental production, and is an important independent predictor of insulin sensitivity in late pregnancy.[159] Maternal TNF-alpha levels are higher in PE compared to normal pregnancy and could contribute to the pathogenesis of the condition through its effects on IR and endothelial dysfunction. Despite higher levels in PE, placental expression of TNF-alpha does not appear to be higher therefore suggestive of an alternative source for the increased plasma levels.[160]

1.11.4 IL-6

IL-6, another proinflammatory cytokine implicated in IR, is secreted in significant amounts by SAT, in addition to a wide range of other cells and correlates with BMI in non-pregnant.[161] In vivo, administration of IL-6 stimulates whole body lipolysis and exerts anti-insulin effects.[162 163] TNF alpha induces Il-6 gene transcription and protein secretion in differentiated adipocytes.[164] Similar to TNF alpha, IL-6 is produced by the placenta, immume cells and AT and serum levels increases during pregnancy.[165] IL-6 is increased in obese compared to lean women in pregnancy [166]. IL-6 has also been related to pregnancy-associated insulin resistance.[167]

1.11.5 CRP

The best characterized biomarker of inflammation is C-reactive protein (CRP) produced predominantly by the liver. CRP is an acute-phase reactant synthesized mainly in the liver and is regulated by circulating levels of IL-6, although IL-1 and TNF alpha can also induce hepatic CRP mRNA expression.[168] The liver was believed to be the major source of CRP, with its synthesis mainly under transcriptional control by IL-6 and, to a lesser extent, by other cytokines. However, now adipose tissue-derived IL-6 appears to be a major regulator of hepatic CRP production. CRP is also produced by adipose tissue, but it is unclear

to what extent AT contributes to circulating CRP levels. Elevated CRP is a strong independent predictor of the metabolic syndrome, cardiovascular disease and diabetes [169 170]. CRP is positively correlated with plasma FFA in non-pregnant women.[110]CRP levels in normal pregnancy fluctuate widely but median values are consistently elevated throughout pregnancy.[171] In addition, CRP is elevated in metabolic complications of pregnancy including PE and GDM, although this may be in part secondary to increased adiposity [172 173].

1.11.6 Plasminogen Activator Inhibitor-1

PAI-1 is a regulatory serine-protease inhibitor that decreases fibrinolysis and correlates well with visceral adiposity, hyperinsulinaemia and the expression of which is increased in the VAT of obese individuals.[174 175] The link between PAI-1 and the metabolic syndrome is now well established, with the more severe the syndrome the higher the plasma level of PAI-1.[176]. The mechanisms of PAI-1 over expression in obesity are complex and it is likely that a variety of inducers at a variety of sites are involved, including ectopic fat in the liver and visceral fat.[177 178] PAI-1 is also implicated in adipose tissue differentiation and in the control of insulin signalling in adipocytes.[179 180] In normal pregnancy in order to prepare the body for the haemostatic challenge of delivery fibrinolytic capacity is diminished, mainly due to markedly increased levels of PAI-1 from endothelial cells (a marker of endothelial dysfunction) and PAI-2 from the placenta. The ratio of PAI-1/PAI-2 is elevated in women with PE and in particular early onset disease and obese relative to lean pregnant women.[181 182]

1.11.7 Pregnancy, pre-eclampsia and cardiovascular disease

There is increasing epidemiological evidence to suggest that adverse pregnancy outcomes such as PE, preterm delivery and low birth weight are associated with increased risk in later life of cardiovascular disease (CVD) in the mother. [183-186] Jonsdottier et al, in a population based study investigated the association between hypertensive complications in pregnancy and death rates from ischemic heart disease (IHD). They found that the relative risk (RR) of dying from IHD was significantly higher among eclamptic women (RR=2.61; 1.11-6.123) and those with PE (RR=1.90; 1.02-3.52) than those with hypertension alone.[184] In a retrospective cohort study in Scotland using discharge data of almost 130,000

women, PE was associated with a two-fold increased risk of subsequent IHD (RR2.0;1.5-2.5).[185] More alarmingly if a woman had a combination of PE, preterm delivery and a baby of low birth weight she had a risk of IHD admission or death seven times that of controls (95% CI 3.3-14.5). A recent meta-analysis combining eight studies (2 346 997 women) with a mean follow up of 11.7 years demonstrated a relative risk of 2.16 (1.86-2.52) of IHD in women with pre-eclampsia substantiating previous evidence.[187] This doubling of risk remains robust even after adjusting for pre-pregnancy hypertension, diabetes mellitus, obesity, dyslipidaemia, the metabolic syndrome and smoking.[186]

Gestation of onset also appears to influence the risk - if PE occurred prior to 37 weeks' gestation the risk of IHD was almost eight-fold (7.71,4.4-13.5).[187] Indeed parity itself is associated with increased risk of CVD with prospective studies finding a positive association.[188] [189] A study by Lawlor and colleagues found a "J" shaped association between number of children and CHD, with the lowest prevalence among those with two children and a linear increase with subsequent children.[190] Although the association was attenuated by adjustment for obesity and metabolic risk factors it was not completely obliterated. The authors suggest that normal pregnancy is a state of insulin resistance and dyslipidaemia and repeated pregnancies may have adverse long-term effects. These epidemiological links are biologically plausible as PE and CVD share many features including dyslipidaemia, insulin resistance, endothelial dysfunction, inflammation and oxidative stress common risk factors, either genotypic or phenotypic, might underlie both PE and CHD. Greer and Sattar proposed a model whereby pregnancy with its concomitant digression into a metabolic syndrome is a "stress test" of maternal metabolic response (Figure 4).[191] Women who develop adverse pregnancy outcomes such as PE make greater excursions into metabolic disturbances during pregnancy and are predisposed to metabolic and vascular disease in later life.

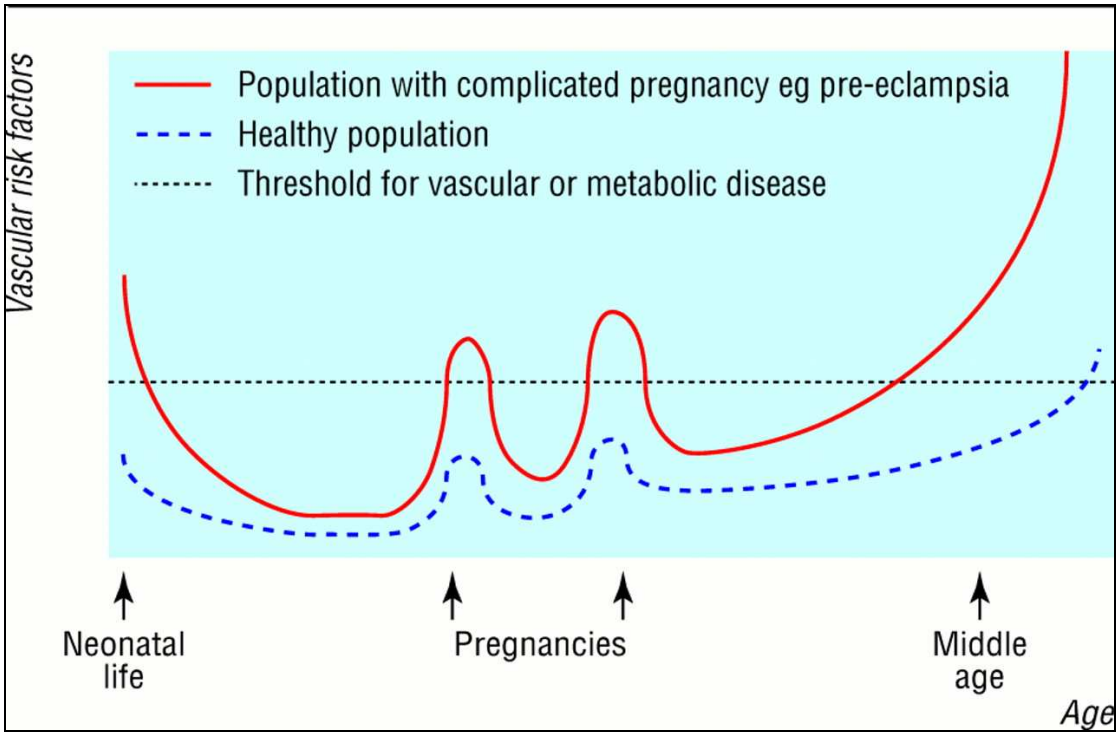

Figure 4 Risk factors for vascular disease are identifiable during excursions into the metabolic syndrome of pregnancy.

Adapted from [191].

1.11.8 Summary of aims and objectives:

There are several directions in which further research would be beneficial and will be explored in this thesis and are summarized as follows:

- To investigate the lipolytic function and properties of adipocytes in normal human pregnancy including its relationship to BMI, differences in function depending on regionality of the adipose tissue, and relationship to maternal markers of inflammation and insulin resistance.
- To compare adipocyte lipolytic function between PE and normal pregnancy and to determine whether the basal or stimulated adipocyte release of FFA is exaggerated in women with PE.
- To determine if plasma from women with PE excessively stimulates adipocytes suggesting the presence of a stimulatory lipolytic factor in PE plasma.
- To determine if adipocyte release of adipokines is exaggerated under either basal or stressed conditions in women with PE compared to controls.
- To determine if macrophage infiltration of adipose tissue, as a marker of tissue inflammation, is increased in women with PE compared to controls

- To corroborate epidemiological evidence that women with PE have an increased cardiovascular risk in later life using carotid ultrasound surrogate markers for atherosclerosis and to determine which risk factors can account for any observed difference.

2 Materials and Methods

2.1 Subjects

Ethical approval was obtained from the Glasgow Royal Infirmary Local Research Ethics Committee 1 (REC reference no 06/S0704/14). All subjects were given an information leaflet (case or control appropriate) and gave written informed consent to participate.

2.1.1 Cases

We recruited non-labouring pregnant women with pre-eclampsia (PE) undergoing caesarean section. PE was defined according to the International Society for the Study of Hypertension in Pregnancy (ISSHP)[192] criteria i.e. DBP greater than 110mm Hg on one reading or > 90mm Hg on repeated readings with proteinuria (≥0.3g of protein per 24 hours or ≥2+ protein on dipstick testing, in the absence of infection or renal disease). All gestations were included however gestation matched controls less than 32 weeks were difficult to find. In total 14 cases were recruited.

2.1.2 Controls

Healthy non-labouring pregnant women undergoing elective CS, either at term (>37 completed weeks of gestation) or pre-term but without hypertension or intra-uterine growth restriction (IUGR) being delivered for other obstetric indications were recruited as controls. IUGR was defined as an estimated fetal weight less than the 5th centile or less than the 10th centile plus either oligohydramnios or abnormal umbilical artery doppler. In the preterm group this was for problems such as placenta praevia or antepartum haemorrhage. Control women were matched to PE cases for smoking habit, age (+/- 5 years) and booking BMI (+/- 2 kg/m^2). In total 36 healthy pregnant women were recruited. A subgroup of matched controls were used for comparison in Chapters 4,5,and 6.

2.2 Buffers

The following physiological buffers using distilled water were prepared using chemicals from Sigma Ltd, Poole Dorset unless otherwise stated.

2.2.1 KRH Buffer

KRH buffer (NaCl 118mM, $NaHCO_3$ 5mM, KCL 4.7 mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, HEPES 25mM, pH adjusted to 7.4) was prepared in advance, autoclaved and stored at 4°C. Wash, collection and digestion buffers were prepared on the day of tissue collection.

2.2.2 Wash Buffer

The wash buffer was derived form the KRH buffer and contained in addition 2.5 mM $CaCl_2$ and 151 mM bovine serum albumin (Sigma Ltd, Poole, Dorset, UK) with pH adjusted to 7.4.

2.2.3 Collection Buffer

Wash buffer with addition of glucose to obtain concentration of 3mM.

2.2.4 Digestion Buffer

For each gram of fat tissue 4ml of collection buffer with 2mg/ml collagenase (Collagenase, Worthington Type 1, Lorne Laboratories, Twyford, Essex, UK) was added.

2.3 Tissue collection

Maternal blood (20ml) was obtained prior to caesarean section into a selection of blood bottles with varying additives including K_2EDTA, lithium heparin, clot activator, buffered sodium citrate and potassium oxalate. They were transferred to the laboratory to be immediately centrifuged at 3000rpm for 15 minutes and the resulting plasma and serum pipetted into aliquots (colour-coded to appropriate additive) and stored at -70°C. Tissue collection buffer was prepared fresh on day of collection and pre-warmed to 37°C. The surgeon was briefed to obtain a sample of subcutaneous adipose tissue (SAT) around the size of a 50 pence coin (around 6-8g) on entry into abdominal cavity and this was placed in the collection buffer. Immediately after delivery of the cord and placenta, a sample of cord blood was obtained and transferred into pre-prepared blood bottles. A sample of visceral adipose tissue (VAT) was obtained from the

omentum after closure of the uterus and haemostasis secured. This was again the size of a 50 pence coin and was also placed in collection buffer. Both samples were transferred to the laboratory where processing of fat was immediately commenced. A research technician was available on return to the laboratory to process the cord blood and other tissue. Cord blood was centrifuged and the resulting serum/plasma was divided into aliquots to be stored at -70°C. Two samples of cord around 2cm in length, four samples of full thickness placenta, paired samples of SAT and VAT when excess tissue was available were "flash-frozen" in liquid nitrogen and then placed in pre-cooled metal containers to be stored at -70°C. A further 2 samples of placenta were fixed in 10% buffered paraffin prior to being embedded in paraffin, cut into sections and mounted on slides for later use.

2.4 Processing the fat sample

The fat sample was placed in a petri dish with warm wash buffer and any large blood vessels, fibrous tissue or skin was removed before weighing the sample. Two sections of both SAT and VAT of around 1g were flash frozen and placed in pre-cooled metal containters and stored at -70°C for later total RNA extraction. Adipocyte cell suspensions were prepared in accordance with the method described by Rodbell.[193] In brief the fat sample was added to the appropriate volume of digestion buffer and cut into smaller pieces with scissors before agitating in a 37°C water bath (Grant, OPS-200) for 30 minutes. At the end of the 30 minutes the digestate was passed through a metal tea strainer (pore size 600uM). The filtrate comprised a layer of adipocytes floating on top of the digestion buffer.

Using a needle and syringe, the digestion buffer was replaced with warm wash buffer 5 times prior to aspirating as much buffer as possible below the adipocyte layer to leave adipocytes suspended at approximately 90% cytocrit.

Adipocyte cell suspension (100ul) was added to 900ul of warm wash buffer in a 15ml Falcon centrifuge tube. All assays were carried out in duplicate. All reagents were added to the relevant tubes and the timing of the assay was commenced. The tubes were placed in a 37°C shaking water bath at 91 cycles per minute and incubated for 120 minutes.

The remaining adipocyte suspension was divided into 150ul aliquots and frozen at -70°C for later quantification of DNA.

2.5 Lipolysis Assay - Conditions and reagents

2.5.1 Basal lipolysis

No reagent was added to adipocyte cell suspension and warm wash buffer

2.5.2 Insulin

Insulin (Human Actrapid® Novo Nordisk)(10ul) was added to 590μl of KRH buffer to produce the insulin STOCK (10μM).This was diluted by a factor of 10 to a concentration of 1μM. 10ul of 1μM solution was added to the adipocyte cell suspension (total volume 1ml) to give a final concentration of 10nM.

2.5.3 Isoproterenol

Isoproterenol is a non-specific beta adrenergic agonist which stimulates lipolysis in the adipocyte. Isoproterenol (49.5mg) was added to 100ml of KRH buffer to obtain a STOCK of 2mM. This was diluted by a factor of 100. 10ul of this solution was added to the adipocyte cell suspension to give a final concentration of 200nM.

2.5.4 Isoproterenol and insulin

Insulin and isoproterenol was prepared as above and 10 ul of each solution was added to give a final concentration of 10nM and 200nM respectively.

2.5.5 Lipopolysaccharide

Sterile balanced salt solution (1 ml of autoclaved KRH buffer) was added to a vial of 1mg LPS powder. Concentration of LPS STOCK: 1mg / ml. This was diluted by a factor of 10 in KRH buffer and 10ul of this solution was added to the final adipocyte cell suspension to give a concentration of 1μM.

2.5.6 Determining concentrations and time lines

A time course of lipolysis and dose response curves for isoproterenol and insulin were determined by Dr EK Tan who had worked on refining the methods for submission as a thesis for a degree of Master of Science.

2.5.6.1Time course

Using adipose tissue of normal healthy pregnant women at term a time course of lipolysis was determined by measuring NEFA release with isoproterenol stimulation. Maximum isoproterenol stimulated release was at 180 minutes and then plateaued. A time point of 120 minutes which was within the linear phase of stimulation was chosen as the sampling time point

2.5.6.2 Isoproterenol and Insulin dose-response

Increasing concentrations of isoproterenol resulted in increasing release of NEFA as calculated as percentage stimulation above basal release to a maximum at 2uM, with a plateau after that. A concentration of 200nM which was within the linear phase of the dose- response curve was used in the lipolysis assay. Similarly the concentration of insulin was chosen based on a dose response curve determining percentage suppression below basal release.

2.5.6.3 Lipopolysaccharide

Lipopolysaccharide (LPS) is an endotoxin and constitutes the lipid portion of the outer leaflet of Gram-negative bacteria. It acts on toll-like receptor (TLR) 4 on adipocytes. It was shown to neither stimulate nor suppress lipolysis (Dr EK Tan). A time-course and dose–response curve was performed to determine the concentration of LPS to use in the final lipolysis assay by measuring the cytokine IL-6 by ELISA (R&D Systems, Abindgdon, UK)

An initial experiment was performed using increasing concentrations of LPS (0,1nM,10nM,100nM,1000nM,10000nM) added to adipocyte suspensions prepared as before. Samples of the supernatant were taken at time points 0, 30 mins, 60 mins, 120 mins and 18 hours for analysis of IL-6 concentrations. This showed that IL-6 did not appear to be released from the adipocytes until at least 2 hours

incubation. Time points 120 mins and 18 hours were chosen for practicality of sampling. At 18 hours all concentrations were greater than 400 pg/ml. Dilutions were not performed. (Figure 5)

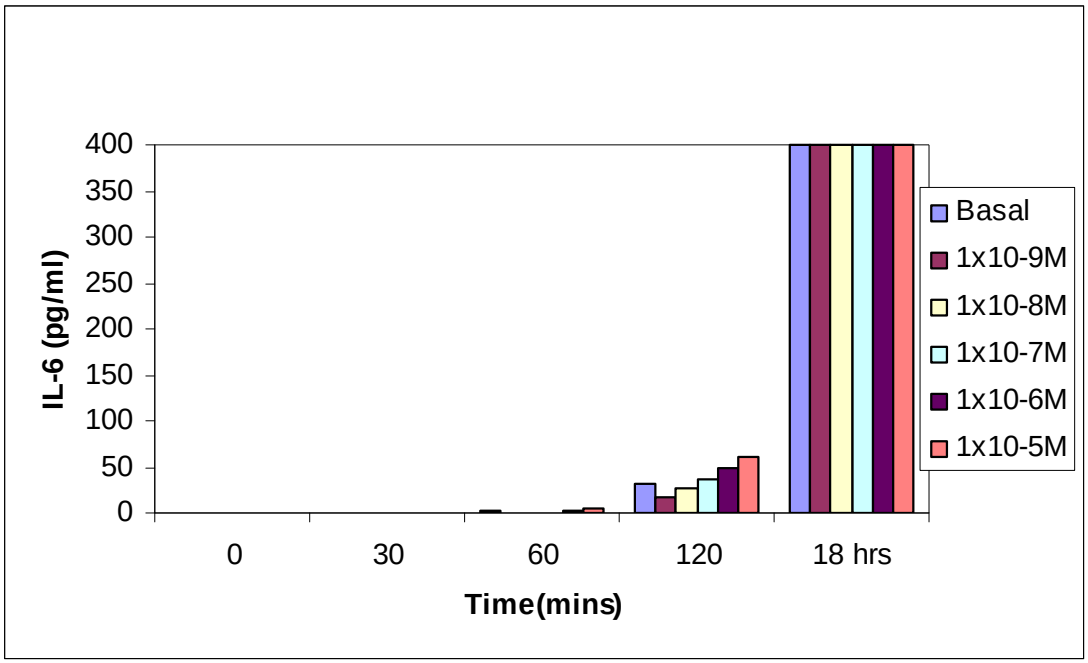

Figure 5 Release of IL-6 from adipocytes

IL-6 release did not appear to occur until at least 120 minutes incubation. The maximum sensitivity of the ELISA was 400 pg/ml. Time points 120 mins and 18 hours were chosen for practicality of sampling. At 18 hours all concentrations were greater than 400 pg/ml. Dilutions were not performed.

A time course was performed (n=3) with sampling taken at 2, 4, 6, and 18 hours (Figure 6)

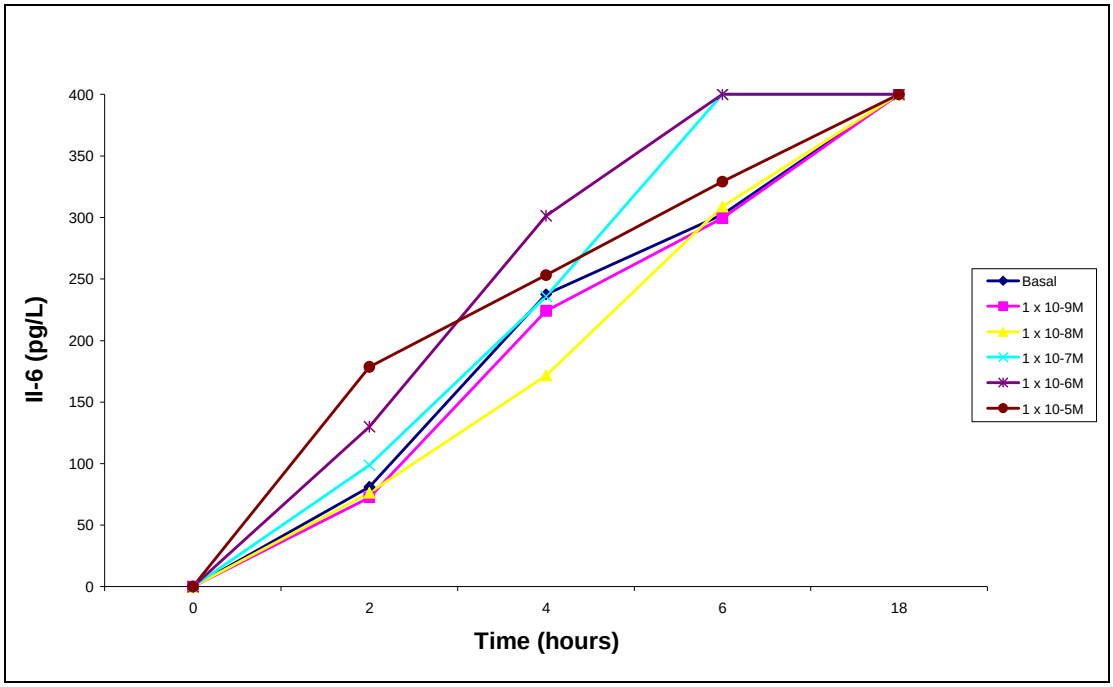

Figure 6 Time course of IL-6 release with increasing concentrations of LPS

From a practical point of view the best sampling time of the supernatant during the lipolysis experiments would either be at 2 hours or 18 hours (overnight). The 2 hours time point was chosen as it was in the linear portion of the graph.

A dose-response curve was also performed (n=4)(Figure 7).

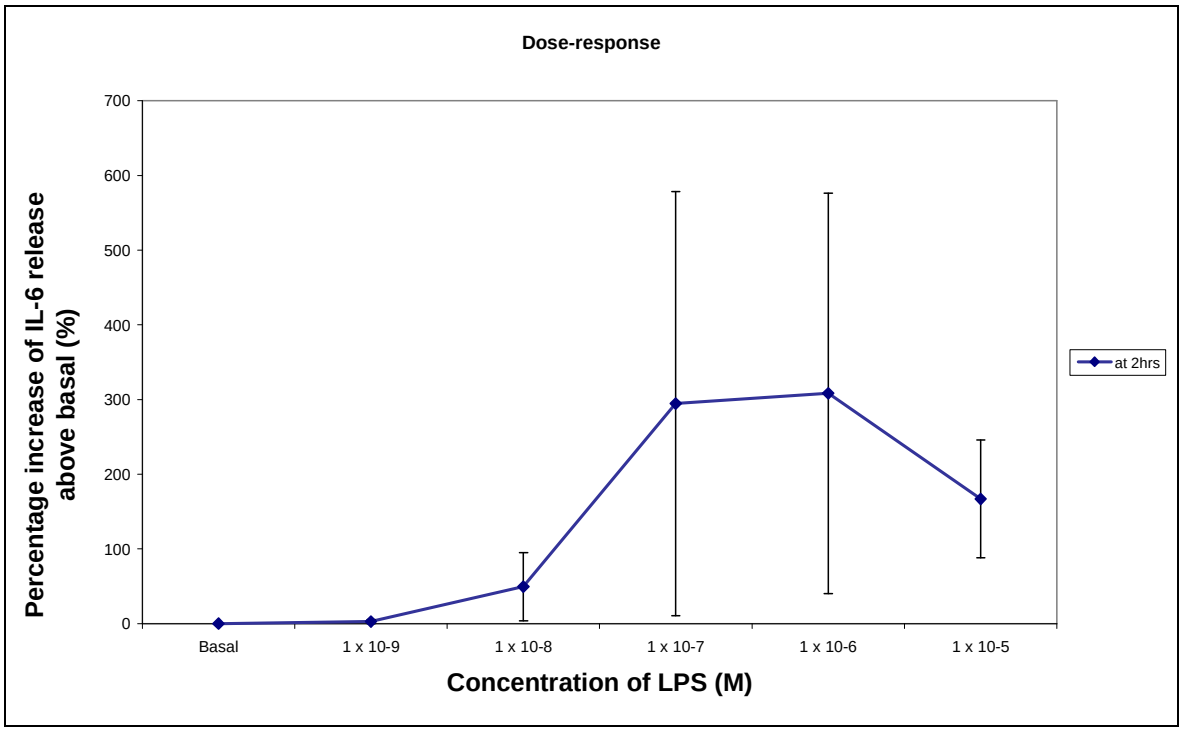

Figure 7 Effect of increasing concentration of LPS on IL-6 release sampled at 2 hours

We therefore determined that a concentration of 10^{-6} M LPS to be used in the final methodology. At levels of 10^{-5} M of LPS concentrations of IL-6 paradoxically fell implying a possible toxic effect of very high concentrations of LPS on the adipocyte.

2.5.7 Measuring Fatty Acid Concentration

At the end of the assay (time = 120mins) aliquots (5ul) obtained from the buffer layer below the cellular suspension were obtained for estimation of non-esterified fatty acid concentration (NEFA) using a Wako NEFA-C Assay kits (Alpha laboratories, Eastleigh, Hampshire, UK). The Wako enzymatic method relies upon the acylation of coenzyme A(CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA produced is oxidized by added acyl-CoA oxidase (ACOD) with the generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(b-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple colour. This was measured by a microplate spectrophotometer (Multiscan EX, Thermo Electron Corporation) at 550nm. The within-run precision of the assay is 2.7%CV, 1.1%CV and 1.1%CV for mean concentrations of 0.33mmol/L, 0.62mmol/L and 0.99mmol/L, respectively (manufacturer). The linear range of the assay is 0-2.0mmol/L.

2.5.8 Measuring Glycerol Concentration

At the end of the assay (time = 120 minutes) aliquots (10ul) obtained from the buffer layer below the cellular suspension were obtained for estimation of glycerol concentration using a colorimetric glycerol assay kit (Randox Laboratories Ltd, Co Antrim, UK). This utilizes a quinoneimine chromagen system in the presence of glycerol kinase, peroxidise and glycerol phosphate oxidase which results in the formation from glycerol of n-(4-antipyryl)-3-chloro-5-sulphonate-p-benzoquinoneimine to produce an orange colour. The optical density was measured at 520nm using a microplate spectrophotometer.

2.6 Fat cell sizing

An unfixed fresh cellular suspension of adipocytes (10ul with 10ul KRH buffer) was prepared on a glass slide. An Olympus BX50 microscope using a x10 lens connected to 3-CCD colour camera (JVC) was used for digital image capture. Computer visualisation of the images were achieved with the image analysis program Image-Pro Plus 4.0 (Figure 8) and later analysed with Adobe Photoshop Version 7.0. An image of a stage micrometer with 100um markings was taken to convert pixels as measured by Adobe Photoshop to microns.(Figure 9)

Diameter of adipocyte in um = y/x*100 when y= cell diameter in pixels and x= number of pixels between markings in stage micrometer ie 100um

At least 100 adipocytes on the digital images were manually measured to derive the mean diameter of each adipocyte preparation.

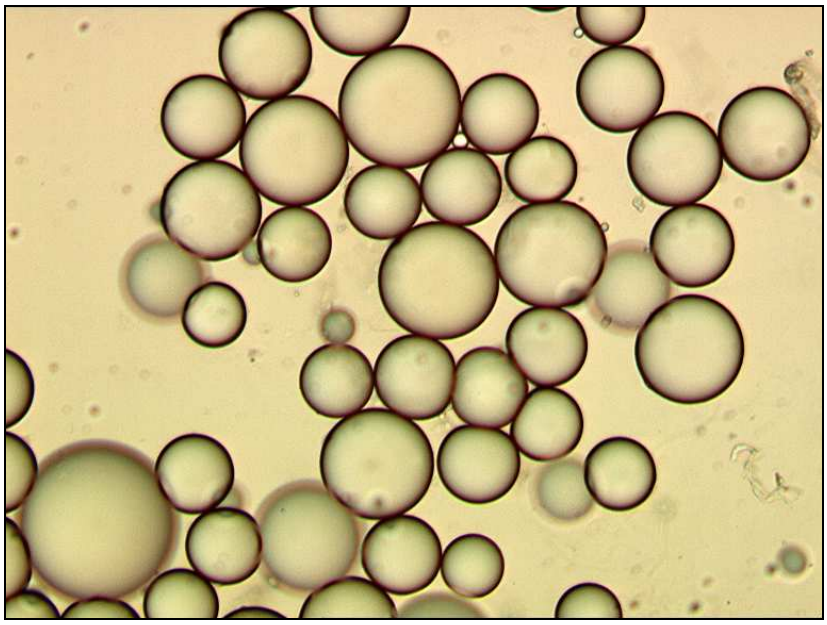

Figure 8 Image of cellular suspension of adipocytes (x100 magnification)

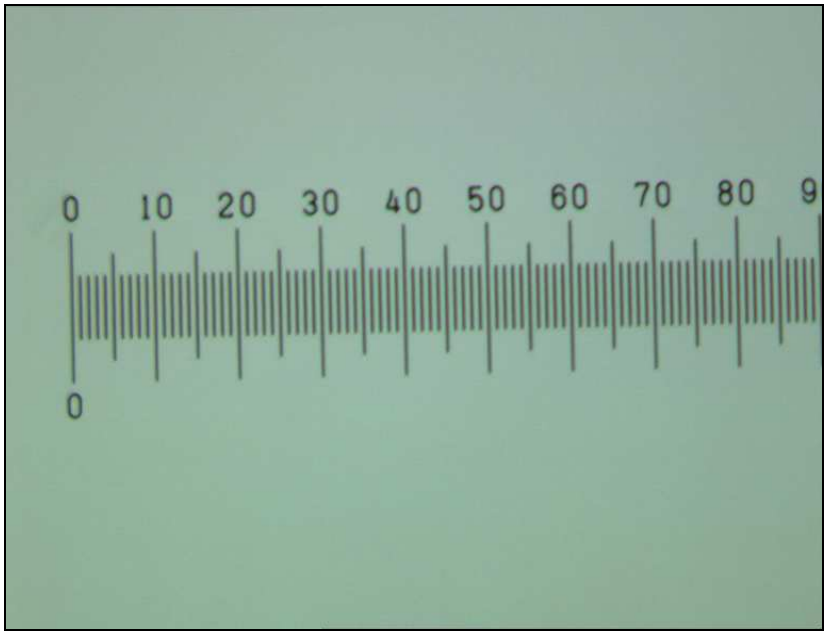

Figure 9 Image of stage micrometer with 100um markings (x100 magnification)

2.7 Determining Fat Cell Numbers

Fat cell number is important to determine as cellular density will influence the rate of lipolysis as measured by fatty acid and glycerol concentration in aliquots of the supernatant. Fat cell number was measured indirectly by quantifying the DNA content in a known volume of adipcotye suspension. The number of adipocytes would be directly proportional to the amount of adipocyte DNA content and lipolysis rates are expressed per ug 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)(Figure 10).

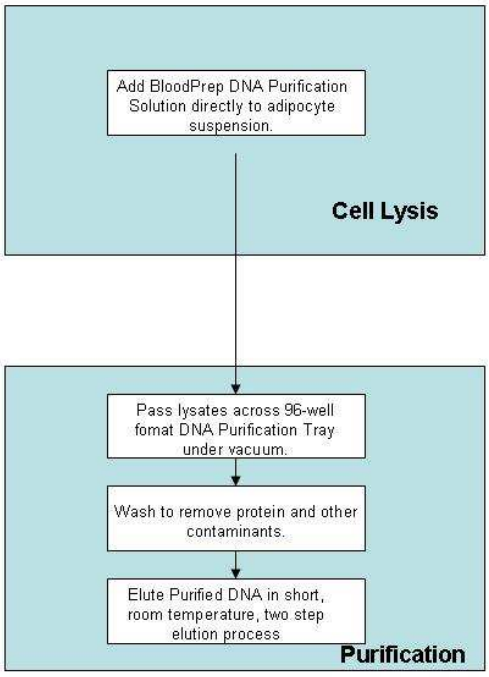

Figure 10 Protocol for DNA isolation using the ABI Prism™ 6100 Nucleic Acid PrepStation

Purification solution was added to adipocyte suspensions (2 x 150 ul of adipocytes for each specimen ie total of 300ul of cells) in eppendorf tubes. This was spun at 13,000 rpm for 2 minutes to separate lipid and water phase. Using fine-tip pastettes the water phase was carefully pipetted into the relevant well leaving the lipid phase behind in order to minimise clogging. The standard BloodPrep™ protocol was adapted below (Table 1).

Step	*Description*	*Volume*	*Position*	*Incubation*	*Vacuum*	*Time(sec)*
1	Load 1st sample	650ul	Waste	0	80	300
Repeat Step 1 [a]	Load 2nd sample	650ul	Waste	0	80	300
2	Add purification solution	650ul	Waste	0	80	400
Repeat Step 2 [a]						
3	Add Wash solution	650ul	Waste	0	80	60
Repeat Step 3 [b]						
4	Add Wash solution	600ul	Waste	0	80	60
5	Add Wash Solution	300ul	Waste	0	80	60
6	Preelution Vacuum	-	Waste	0	100	120
7(ensure collection tray in place)	Touch off (rock gently)		Waste			
8	Elution solution 1	100ul	Collection	180	0	-
9	Collection		Collection	0	60	120
10	Elution solution 2	100ul	Collection	0	60	120

[a] 300ul of adipocyte cell suspension was used to improve DNA yield above the limit of Nanodrop sensitivity (ie 5ng/ul). A max of 150ul of sample can be added at Step 1

[b] An additional wash step was added to minimise contamination with guanidine HCL present in purification solution

Table 1 Adapted BloodPrep™ protocol to isolate DNA from adipocytes.

The concentration of DNA was quantified using Nanodrop® ND 100 which is a cuvette free spectrophotometer which can accurately measure nucleic acid concentrations in small volumes (from 1ul). Absorbance readings are performed at 260nm where DNA absorbs light most strongly, and the number generated allows one to estimate the concentration of the solution. A "blank" measurement with a 50:50 mixture of elution buffer I and II was performed to minimise high absorbance at 230nm due to salts in the elution buffers. Measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280nm is used as a measure of DNA purity. DNA absorbs UV light at 260 and 280 nm, and aromatic proteins absorbs UV light at 280 nm; a pure sample of DNA has the 260/280 ratio at 1.8 and is relatively free from protein contamination. A DNA

preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8.

The results were deemed acceptable if the concentration of DNA was > 5ng/ul and absorbance ratio at 260/280 was between 1.60 -2.00 (Figure 11). If not, the graphs were individually assessed to determine if there was an identifiable peak at absorbance 260nm, and if so the result was accepted. (Figure 12). If not, the process was repeated again using stored 150ul aliquots of adipocyte suspensions.

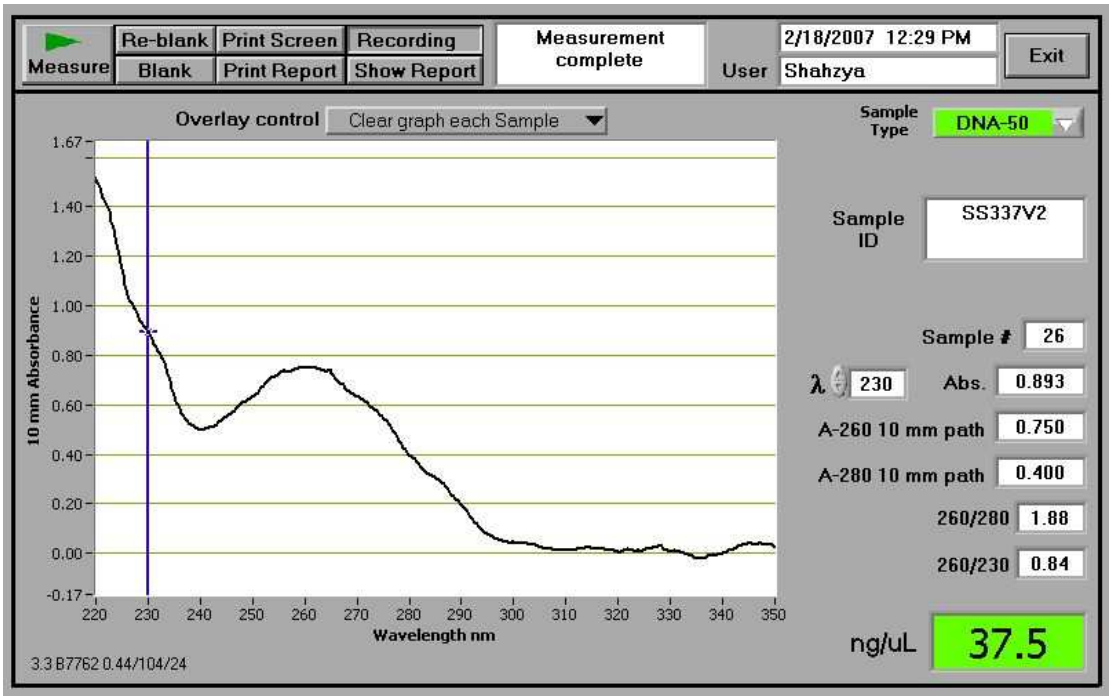

Figure 11 Concentration of DNA of adipocyte preparation as measured on nanodrop

The concentration of DNA was >5ng/ul and the absorbance ration at 260/280 was between 1.60-2.00 (1.88).

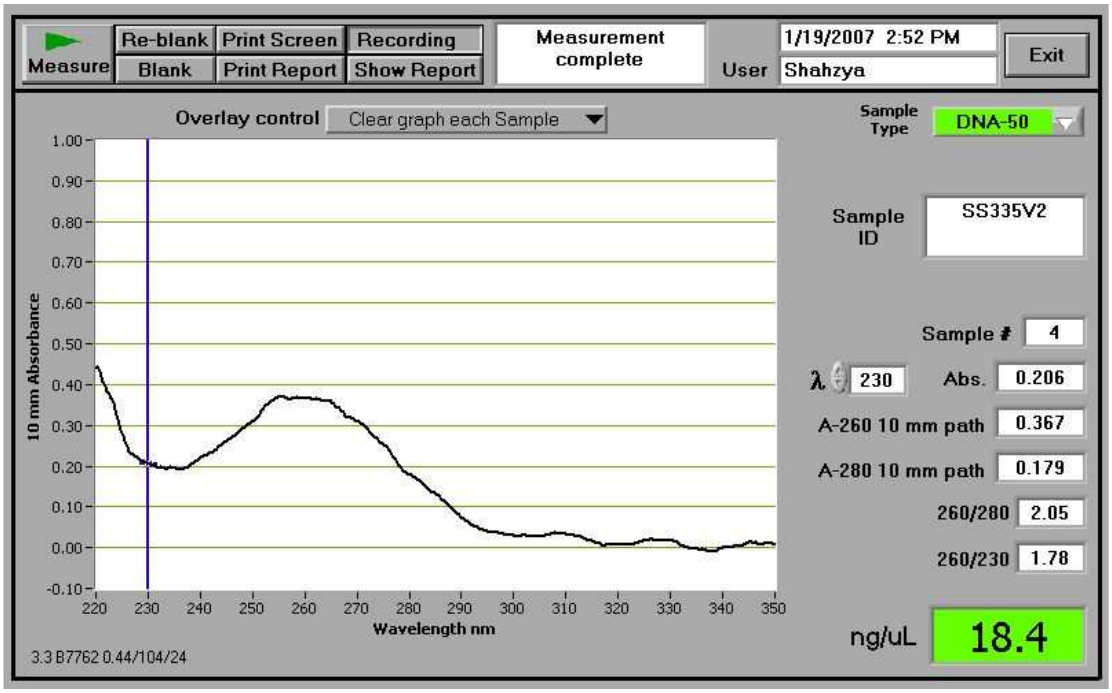

Figure 12 Concentration of DNA of adipocyte preparation as measured on nanodrop

The concentration of DNA was >5ng/ul. The absorbance at 260/280 was greater than 2.00 (2.05) but there was an identifiable peak at absorbance 260nm. Therefore the result was deemed acceptable.

2.8 Bioplex

At time=120min paired 120ul aliquots of the buffer layer below the adipocyte layer was frozen at -80˚C for later analysis of cytokines. Cytokine quantification was carried out with Bio-Plex (BIO-RAD®) system and suspension array technology. This is a multiplexed, particle based, flow cytometric assay which utilises anti-cytokine monocloncal antibodies linked to microspheres incorporating distinct proportion of two fluorescent dyes. Each of the 100 spectrally addressed bead sets can contain a captive antibody specific for a unique target protein. Fluorescent intensity of the bead identifies the reaction. Analyte-specific antibodies are pre-coated onto colour-coded microparticles. Microparticles, standards and samples are pipetted into wells and the immobilised antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the captured biotinylated antibody, is added to each well. A final wash removes unbound Streptavidin-PE and the microparticles are resuspended in buffer and read using the Luminex analyzer. One laser is microparticle-

specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound. Our assay was customised to detect and measure multiple adipokines (Table 2) using the R + D Systems Obesity Base Kit (cat no LOB000).

Adipokine	*Cat No:*
Serpin E1/PAI-1	LOB1786
CCL2/MCP-1	LUH279
IL-6	LUH206
Leptin	LUB398
TNF alpha	LUH210
Adiponectin	LOB1065
IL-10	LUH217

Table 2 Adipokine antibodies and catalogue numbers

The previously frozen samples were thawed at room temperature and spun at 10,000rpm for 1 min then diluted in Calibrator diluent RD5K in a ratio of 1:20. All standards, buffers and reagents were prepared as per the manufacturers protocol. The desired number of wells of 96 well filter plate was prewet with 100ul wash buffer and then removed by vacuum filtration. The bottom of the filter plate was dried thoroughly with a clean paper towel (lint free). The diluted microparticle mixture was resuspended and 50ul pipetted into each well. Standard or sample (50ul) was added into each well. The plate was covered with foil and shaken at 500rpm for 3 hours at room temperature. Buffer was removed by vacuum filtration and washed 3 times with 100ul Bio-Plex wash buffer blotting the bottom of filter plate with clean paper towel after every wash to prevent cross-contamination. Diluted biotin antibody cocktail (50ul) was added to each well and then covered with foil and shaken at 500rpm for 1 hour at room temperature. The buffer was removed by vacuum filtration and a further 3 washes were performed. Diluted streptavidin-PE (50ul) was added to each well, covered with foil and shaken at 500rpm for 30 mins at room temperature. At the end of the incubation the buffer was removed and a further 3 washes performed. The microparticles were then resuspended in each well with 100ul wash buffer and the plate covered with sealing tape and shaken at 500rpm for 2mins. The plate was read on Biorad Bioplex analyser Luminex 100 within 90 minutes. All results were in pg/ml.

2.9 Isolation of Total RNA

RNA was isolated using the Tissue RNA Isolation protocol from Applied Biosystems for ABI PRISM 6100 Nucleic Acid Prepstation. Keeping the tissue frozen using dry ice and liquid nitrogen 50mg tissue was weighed into a pre-cooled universal and the exact weight was noted. Nucleic acid purification lysis (2.5ml) (1:1 PBS) was added. This was homogenised for 30 secs and left on wet ice for 30 mins (placenta) or 1 hour (adipose tissue). The homogenized lysate was stored at -20^{o}C until purification. The lysate underwent prefiltration according to the manufacturer's protocol in order to increase the yield of total RNA and reduce clogging in the purification tray. In this technique the homogenised lysate was passed across the tissue pre-filter tray and collected. Purification of the filtered tissue lysate was achieved by passing the lysate through a purification tray containing an application-specific membrane. Wash solutions (AbsoluteRNA Wash solution) were applied to the membrane and the purified RNA was eluted into a 96-well PCR plate. This process was performed using the manufacturer's semi-automated protocol.

2.10 Quantitative real time PCR

RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington , UK) according to the manufacturer's instructions. A No RT control was also performed (Table 3).

Preparation	*Sample mix with RT(ul)*	*No RT control (ul)*
10 x RT buffer	1	1
25 x dNTPs	0.4	0.4
10 x random primers	1	1
Multiscribe reverse transcriptase	0.5	0
Superasein (1U/ul)	0.5	0.5
Nuclease free water	1.6	2.1
Total volume per reaction	5	5
	+ 5ul RNA	+ 5ul RNA
Total voume	10 ul	10ul

Table 3 Constituents of assay.

cDNA was quantitated using TaqMan® technology on an ABI Prism 7900HT (Applied Biosystems).The target assays were purchased from Applied Biosystems and listed in Table 4. Briefly 1.25ul of 20 x target assay or control assay mix was

added to 12.5ul of 2 x Mastermix (Applied Biosystems), 10.25ul deionised distilled water and 1ul cDNA. The thermal cycler conditions were 50°C 2min, 95°C for 10 min then 40 x 95°C for 15 secs and 60°C for 1min. Data was analysed using the Sequence Detection software, which calculated the threshold cycle (C_T) values. The expression of the target assays were normalized by subtracting the C_T value of the endogenous control (18s and PPIA) from the C_T value of the relevant target assay. The fold increase relative to the control was obtained by using the formula $2^{-\Delta CT}$.

Gene symbol	TaqMan® gene expression assay	Assay ID	Source
CD68	CD68 molecule	Hs00154355-m1	Applied Biosystems
PP1A	peptidylprolyl isomerase A (cyclophilin A)	Hs99999904-m1	Applied Biosystems
18S	Endogenous control	4310893E	Applied Biosystems
IL-6	Interleukin-6	Hs00174131-m1	Applied Biosystems
TNF-α	Tumour necrosis factor- alpha	Hs00174128-m1	
c-fms	colony stimulating factor 1 receptor	Hs99999197-m1	Applied Biosystems

Table 4 Taqman gene expression assays

2.11 Immunocytochemistry

2.11.1 Processing of biopsies

Biopsies of SAT and VAT which had previously been flash frozen in liquid nitrogen and stored at -70°C were defrosted at room temperature. Approximately 1cm^3 section was fixed in 30 ml of zinc formalin (Cellpath Acetic Zinc Formalin) for 24 hours. It was then processed on a Leica ASP overnight and taken through the following solutions: zinc formalin for 1 hour 15 minutes, 70% methylated spirit for 1 hour, 90% methylated spirit for 1 hour, methylated spirit phenol for 1 hour, industrial alcohol for 3 x 1 hour, xylene for 45 minutes, then 2 x 1 hours and 3 wax treatments for 1 hour, 1 hour 30 minutes, and 1 hour 30 minutes. The paraffin blocks were subsequently used in immunocytochemistry (ICC). Sections 7 um thick were cut from the paraffin embedded tissue on a microtome (Leica

RM 2135) and mounted on electrostatic superfrost slides, heated to 60°C overnight and stored in slide boxes until used. Placental tissue as was fixed in 10% buffered formalin prior to being embedded in paraffin, cut into 5um sections and mounted on slides for later use.

2.11.2 ABC method

The antibodies used in ICC were polyclonal anti human cFMS (Chemicon cat no.CBL776) and monoclonal anti-human macrophage CD68 (Dako-CD68, PG-M1 code no. M876) which represent activated and total macrophages respectively.

The sections were heated to 60°C for 35 min, depariffinized in xylene, and rehydrated in a graded alcohol series. Endogenous peroxidise activity was quenched using 0.5% hydrogen peroxide in methanol. Sections were washed in PBS. In sections to be incubated with anti-cfms, antigen was retrieved by microwaving in a pressure cooker (Lakeland Plastics Ltd., Cumbria, UK) at full power for 5 min in citrate buffer (10 mM, pH 6.0). The sections were washed in H_20 then blocked with 20% goat/20% human serum. Section to be incubated with anti CD68, antigen was retrieved by pre-treatment with 0.1% trypsin in TRIS buffer containing 0.1% calcium chloride for 15 minutes and then washed in PBS. They were blocked by adding 20% horse/20% human serum. Sections were then incubated overnight for 16 hours at 4°C with the primary antibody diluted either in 2% goat serum at a dilution of 1:25 (cfms) or in 2% horse serum at a dilution of 1:50(CD68). Slides were washed in PBS and were incubated for 30 minutes with 2°Ab biotinylated anti-rabbit IgG (Vector laboratories) diluted 1:200 in 2% goat serum (cfms) or biotinylated horse anti-mouse (Vector) diluted 1:200 in 2% horse serum both with 5% human serum added. Sections were washed in PBS and then incubated with avidin DH/biotinylated horseradish peroxidase H reagent (Vector) in PBS for 30 min before final washing. The antigen was localized using 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 0.2% hydrogen peroxide in 50 mM Tris HCl, pH 7.6, and appeared as a brown end product. Sections were counterstained with Harris hematoxylin (Sigma-Aldrich). In sections using anti – CD68 negative controls included slides incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 (Dako-

X931), an enzyme that is neither present nor inducible in mammalian tissue. Tonsillar tissue was used as a positive control for all primary antibodies used.

2.12 Quantification of macrophage cell density

Macrophages were identified using histological analysis in ten randomly selected high powered fields (x 400 objective magnification) and were counted by two independent observers who were blinded to the specimen details. The area for each high-powered filed was 0.23mm^2. Macrophages within the blood vessels were not included in the counts. Tissue macrophage densities were expressed as cell count per field (placenta) and cell count per adipocyte.

2.13 Maternal and Cord Blood Phenotyping

Maternal blood (20ml) was obtained prior to caesarean section into a selection of blood bottles with varying additives including K_2EDTA, lithium heparin, clot activator, buffered sodium citrate and potassium oxalate. They were transferred to the laboratory to be immediately centrifuged at 3000rpm for 15 minutes and the resulting plasma and serum pipetted into aliquots (colour-coded to appropriate additive) and stored at -70°C. Cord blood was obtained from the cord with a needle and syringe after delivery of the placenta. The sample was also centrifuged and stored as aliquots at -70°C.

Cholesterol and triglyceride were determined by enzymatic colorimetric assays on a Roche 917 analyser (Roche Diagnostics Ltd., Burgess Hill, United Kingdom). LDL and HDL were measured after ultracentrifugation at 105,000g at 4°C for 16 hours, followed by precipitation of the LDL fraction using a solution of heparin and manganous chloride. Glucose was measured by hexokinase/glucose-6-phosphate dehydrogenase assay on an Abbott c8000 analyser (Abbott Diagnostics, Maidenhead, United Kingdom). High sensitivity C-reactive protein (CRP) was measured by an immunoturbidimetric assay (Roche Diagnostics Ltd., Burgess Hill, United Kingdom). Insulin was measured by a direct sandwich Enzyme-Linked Immunosorbent Assay (ELISA) (Mercodia AB, Uppsala, Sweden). IL-6 (Cat No:HS600B), TNF-alpha (Cat No: HSTA00D), leptin (Cat No: DLPOO) and adiponectin (Cat No:DRP300) were all by the quantitative sandwich ELISA (R&D systems, Abingdon, UK). NEFA in serum was measured using a Wako NEFA-C Assay kits (Alpha Laboratories, Eastleigh, Hampshire, UK) and read on a

microplate spectrophotometer (Multiscan EX, Thermo Electron Corporation) at 550nm.

2.14 Statistics

Data was assessed for normal distribution using a Ryan-Joiner test and transformed to achieve a normal distribution where necessary. Comparisons between groups was made by two sample t-test (control vs PE) or paired t test (SAT vs VAT), and expressed as means with standard error for the mean (SEM). For non-parametric data comparisons were made using Mann-Whitney U test and data expressed as median and inter-quartile range. Pearson's correlation coefficients 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 was adjusted for potential cofounders using the General Linear Model. All statistical analysis was carried out in Minitab (version 15).

3 Adipocyte Lipolytic Function in Normal Pregnancy

3.1 Introduction

Obesity, which is characterized by an increase in adipose tissue, is increasing in the western world to epidemic proportions. It is widely shown that increasing adiposity is linked to a broad range of metabolic abnormalities including insulin resistance hypertension, dyslipidaemia and increase risk of cardiovascular disease. Obesity or adiposity affects almost all aspect of female reproductive life including the metabolic complications of gestational diabetes and pre-eclampsia. Adipose tissue itself has increasingly been found to have far reaching metabolic and endocrine effects and alterations in adipose tissue function with increasing adiposity may provide, at least in part, the link between adiposity and metabolic complications of pregnancy. It is therefore important that we have a better understanding of its function in both normal and abnormal states.

I have investigated the lipolytic properties of adipocytes taken at elective caesarean section (CS) from women with healthy pregnancies of varying BMI. Much of the work on adipocyte function in pregnancy from AT explants has been on animal models and the following investigations represent the first detailed examination of adipocyte characteristics in normal human pregnancy in over 30 years[194]. This will provide the basis for future comparisons with complications of human pregnancy involving adipocyte metabolism and function including pre-eclampsia, IUGR and GDM.

3.2 Specific Research Questions

1. Do adipocytes respond to catecholamines and insulin in a similar manner to those of the non-pregnant ie isoproterenol stimulates lipolysis and insulin suppresses lipolysis?

2. Are direct measures of adipocyte lipolytic function related to maternal BMI?

3. Are direct measures of adipoyte lipolytic function related to plasma measures of insulin resistance?

4. What are the relationships between maternal adipokine and inflammatory markers and direct measures of adipocyte lipolytic function?

5. What is the relationship of adipocyte cell size and measures of adipocyte function?

6. Are there functional differences in AT metabolism in pregnancy depending on their anatomical locations and does this pattern reflect other insulin resistant states.

3.3 Results

3.3.1 Subjects

Thirty six non-labouring women were recruited as described in methods section 2.1.2. The characteristics are described in Table 5.

Characteristics	*Cohort (n=36)*
Age, y	**31.2(5.3)**
BMI, kg/m^2	**28.2(5.4)**
Lean <30kg/ m^2 (%)	**72.2**
Smokers (%)	**8.3**
DEPCAT*	**4(4-6)**
Gestation at delivery, wk	**38.9(1.2)**
Parity (0,1,2)	**7,22,7**
Systolic pressure, mmHg	**114.6(13.4)**
Diastolic pressure, mmHg	**69.9(8.2)**
Birthweight, g	**3466.8(532)**
Birthweight centile	**57.7(28.4)**

Table 5 Characteristics of control women.

All values expressed as mean and standard deviation (*median and interquartile range)

Lipolysis assays were undertaken as set out in methods section 2.5, 2.6 and 2.7. Maternal biochemical markers were determined as detailed in methods section 2.12.

3.3.2 Action of Isoproterenol and Insulin on Lipolysis

In SAT when lipolysis rates are expressed as the release of NEFA isoproterenol stimulates lipolysis (0.36[0.05] vs 0.76[0.07] mmol/L/ugDNA, p<0.001) and insulin suppresses basal lipolysis (0.36[0.05] vs 0.24[0.02] mmol/L/ugDNA, p=0.001). Insulin also attenuates the effect of catecholamine stimulation of lipolysis (0.76[0.07] vs 0.56[0.06] mmol/L/ugDNA, p <0.001)(Figure 13).

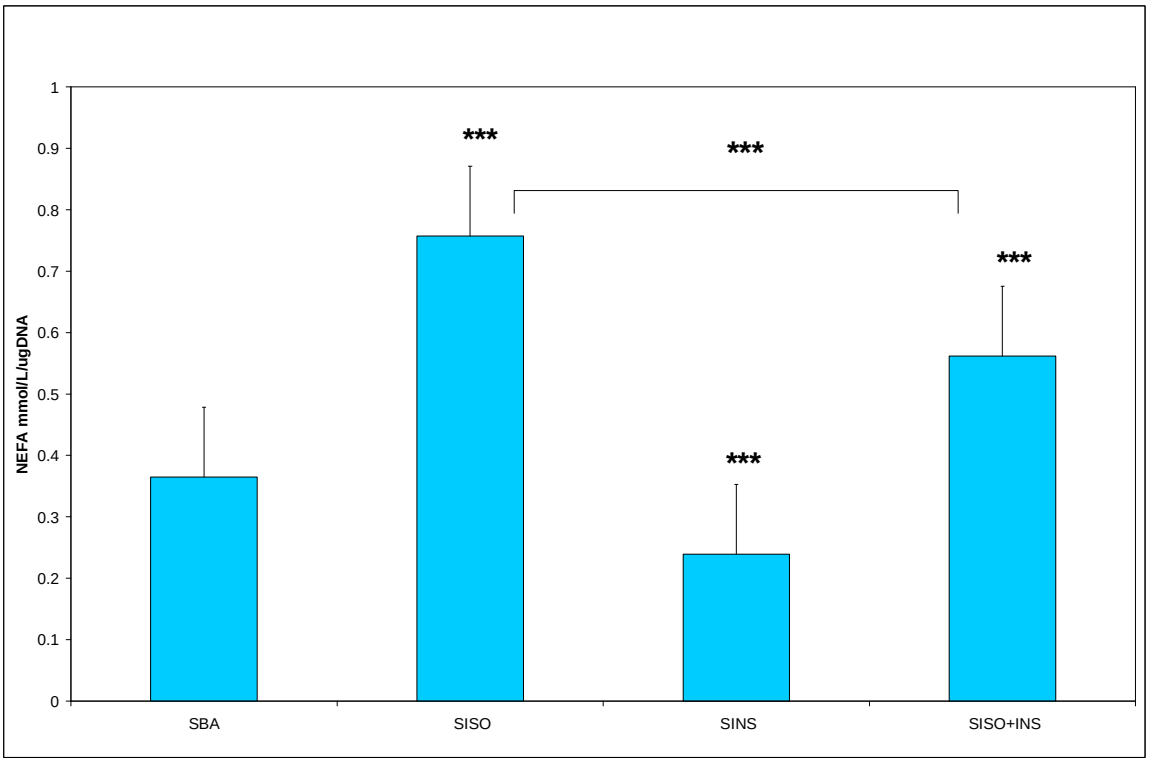

Figure 13 Comparison of NEFA release in subcutaneous fat in healthy pregnancy.

Isoproterenol stimulates release over basal (p<0.001), insulin suppresses release (p=0.001), and insulin attenuates isoproterenol stimulation (p<0.001) (n=36). SBA =basal liploysis in SAT, SISO=lipolysis in presence of isoproterenol in SAT, SINS=lipolysis in the presence of insulin in SAT, SINS=lipolysis in the presence of isoproterenol and insulin in SAT. Values displayed as mean and SEM. Comparisons made using paired t-test to basal release unless otherwise indicated. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

A similar effect in subcutaneous fat of healthy controls is seen if lipolysis rates are calculated from glycerol release. Isoproterenol stimulates lipolysis (114.6[14.6] vs 270.1[19.4] umol/L/ugDNA, p<0.001), no effect is seen with insulin on basal release (114.6[14.6] vs 127[18.2] umol/L/ugDNA, p=0.5) but insulin does attenuate catecholamine stimulation of lipoloysis (270.1[19.4] vs 242.4[25.4] umol/L/ugDNA, p=0.034).(Figure 14)

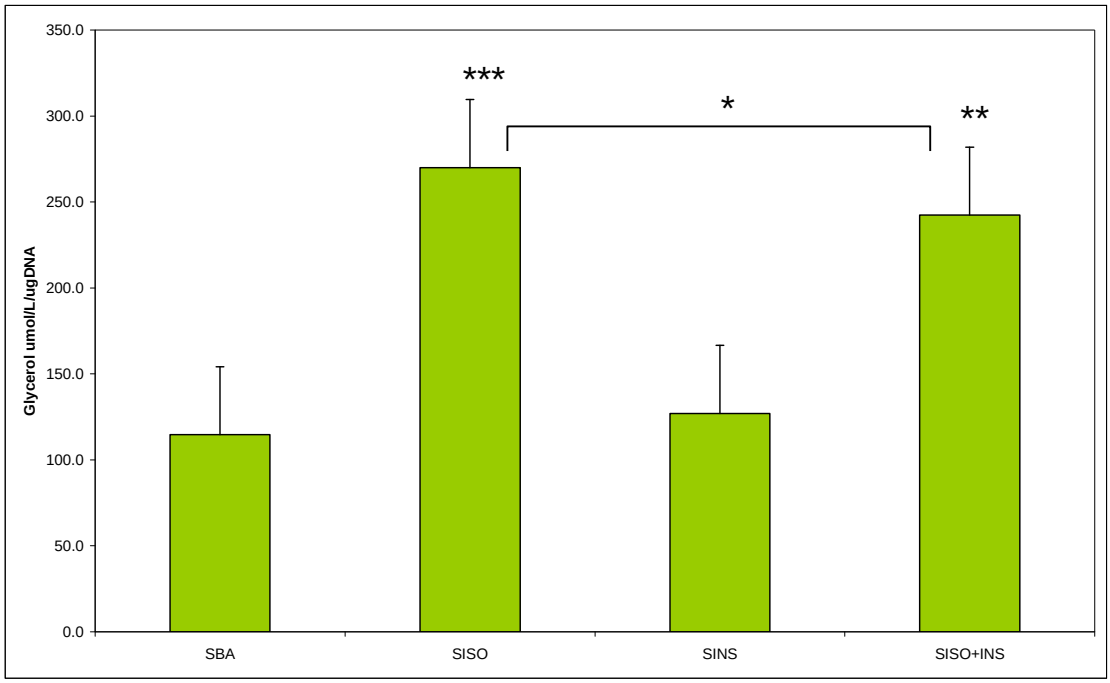

Figure 14 Comparison of glycerol release in subcutaneous fat in healthy pregnancy

Isoproterenol stimulates release (p<0.001) and insulin attenuates isoproterenol stimulation (p=0.034)(n=36). SBA =basal liplolysis in SAT, SISO=lipolysis in presence of isoproterenol in SAT, SINS=lipolysis in the presence of insulin in SAT, SINS=lipolysis in the presence of isoproterenol and insulin in SAT. Values displayed as mean and SEM. Comparisons made using paired t-test to basal release unless otherwise indicated. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

Similarly in visceral fat of control women isoproterenol stimulates lipolysis when calculated from NEFA release (0.25[0.05] vs 0.61[0.11] mmol/L/ugDNA, p<0.001), insulin suppresses basal release (0.25[0.05] vs 0.20[0.04] mmol/L/ugDNA, p=0.034) and insulin attenuates the effect of isoproterenol (0.61[0.11] vs 0.51[0.09] mmol/L/ugDNA, p<0.001) (Figure **15**)

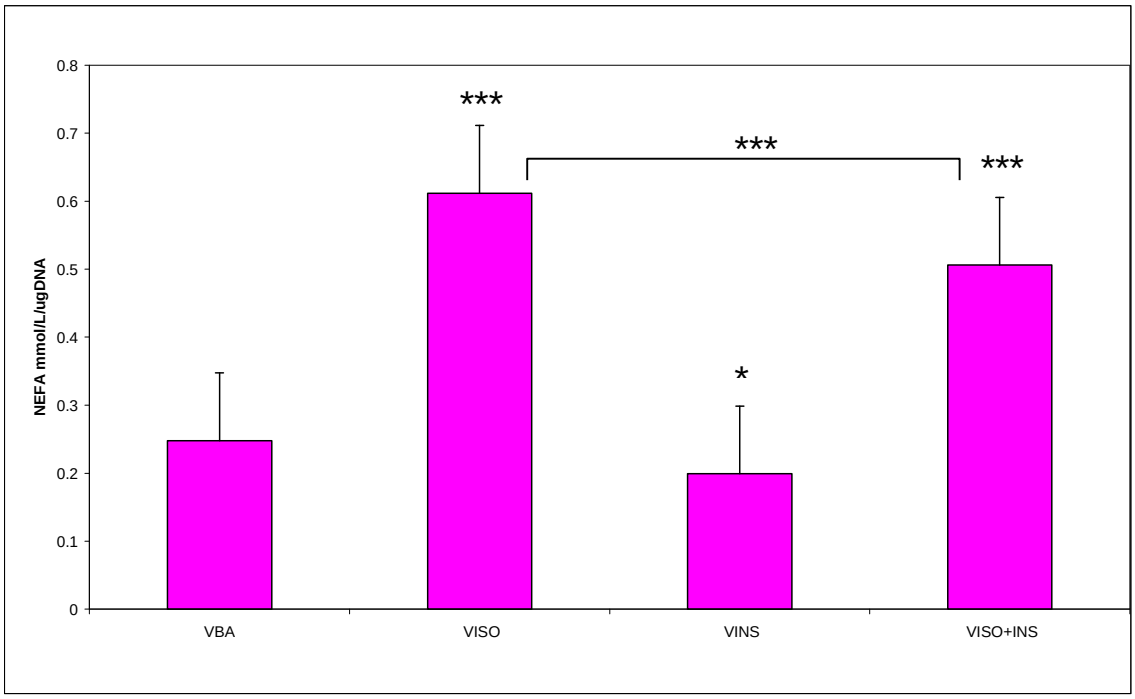

Figure 15 Comparison of NEFA release in visceral fat in healthy pregnancy.

Isoproterenol stimulates release (p<0.001), insulin suppresses release (p=0.034) and insulin attenuates isoproterenol stimulated release (p<0.001)(n=36). VBA =basal liploysis in VAT, VISO=lipolysis in presence of isoproterenol in VAT, VINS=lipolysis in the presence of insulin in VAT, VINS=lipolysis in the presence of isoproterenol and insulin in VAT. Values displayed as mean and SEM. Comparisons made using paired t-test to basal release unless otherwise indicated. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

When lipolysis rates are expressed as glycerol release isoproterenol stimulates release (80[13.3] vs 189.5[17.5] umol/L/ugDNA, p<0.001), insulin has no effect on basal release (80[13.3] vs 80.6[11.9] umol/L/ugDNA p=0.95) and insulin appears to attenuate isoproterenol stimulated release (189.5[17.5] vs 172.3[19] umol/L/ugDNA, p=0.111)(Figure 16).

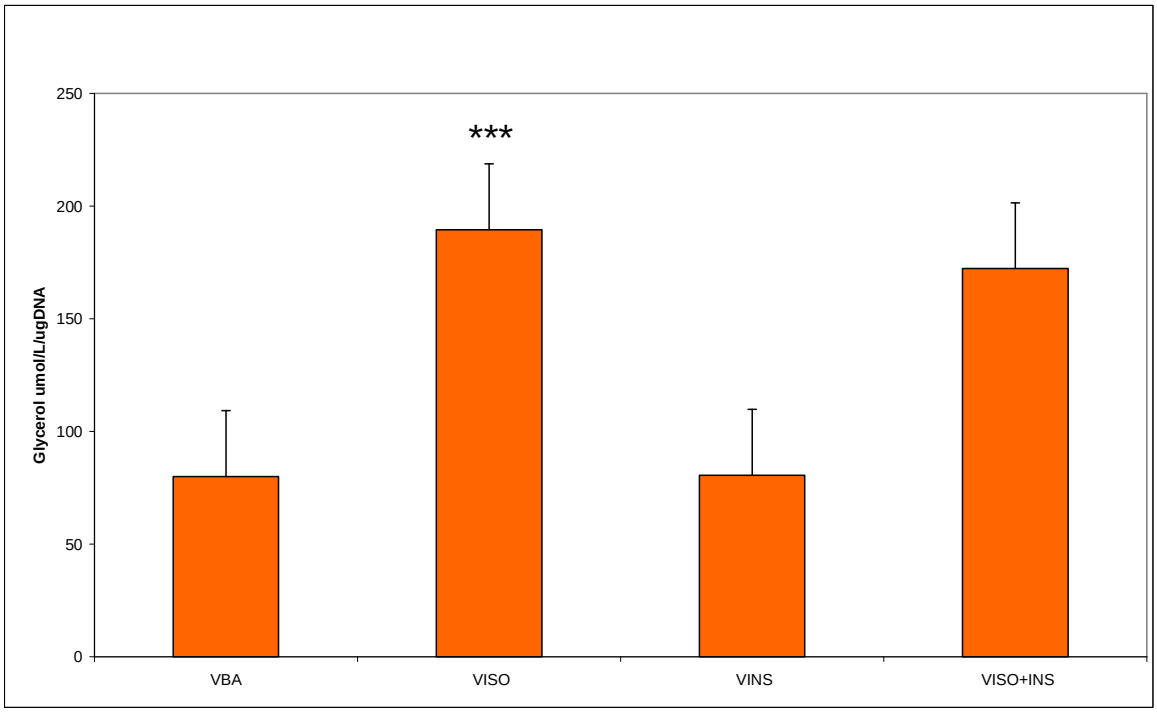

Figure 16 Comparison of glycerol release in visceral fat in healthy pregnancy.

Isoproterenol stimulates release (p<0.001) and there is a trend for insulin to attenuate the effect of isoproterenol (p=0.111)(n=36). VBA =basal liplolysis in VAT, VISO=lipolysis in presence of isoproterenol in VAT, VINS=lipolysis in the presence of insulin in VAT, VINS=lipolysis in the presence of isoproterenol and insulin in VAT. Values displayed as mean and SEM. Comparisons made using paired t-test to basal release unless otherwise indicated. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

The degree of stimulation by isoproterenol was calculated as a percentage of basal release of NEFA ie percentage stimulation = (SISO-SBA)/SBA)*100 and the degree of suppression by insulin was calculated as a proportion of basal release ie :(SBA-SINS/SBA)*100. Visceral fat is more responsive to stimulation by isoproterenol compared to subcutaneous fat (239[32] vs 159 [20]%, p=0.04) (Figure 17) and this trend was also apparent when lipolysis was expressed by glycerol release (337[83] vs 236[34]%, p=0.265).(Figure 18). There was a trend for basal suppression of lipolysis to be lower in VAT although this did not reach significance (8.8[7.6] vs 22.7[4.6]%, p=0.123).

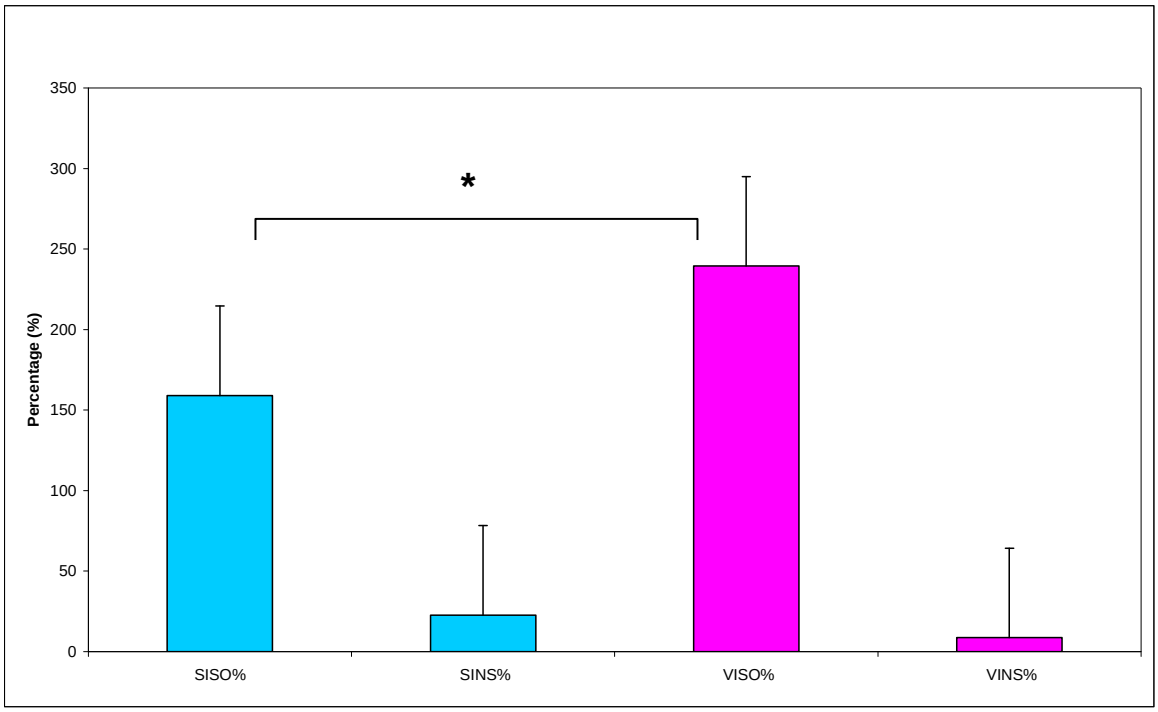

Figure 17 Percentage stimulation and inhibition of NEFA release in SAT and VAT in healthy pregnancy.

Visceral fat is more responsive to stimulation by isoproterenol (p=0.04)(n=36). SISO% - percentage stimulation of lipolysis by isoproterenol in SAT, SINS%-percentage inhibition of lipolysis by insulin in SAT, VISO% -percentage stimulation of lipolysis by isoproterenol in VAT, VINS%- percentage inhibition of lipolysis by insulin in VAT. Values displayed as mean and SEM. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

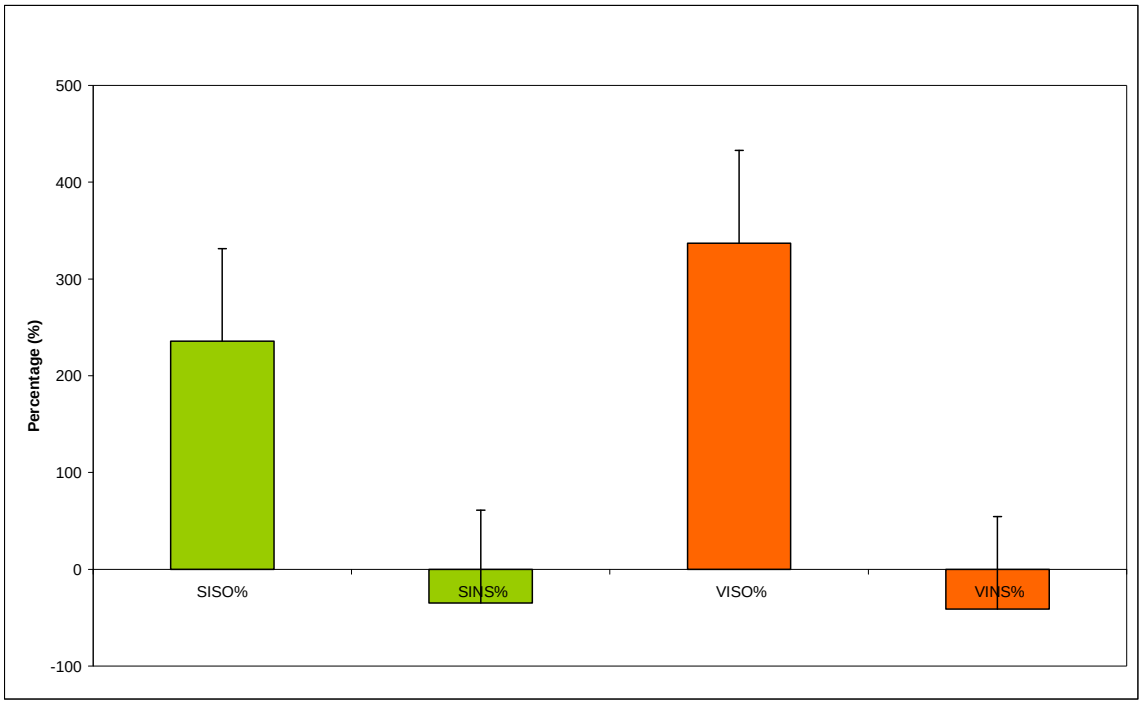

Figure 18 Percentage stimulation and inhibition of glycerol release of SAT and VAT in healthy pregnancy.

There was a non significant trend for VAT to be more sensitive to isoproterenol stimulation than SAT when expressed as glycerol release. SISO% -percentage stimulation of lipolysis by isoproterenol in SAT, SINS%-percentage inhibition of lipolysis by insulin in SAT, VISO% - percentage stimulation of lipolysis by isoproterenol in VAT, VINS%- percentage inhibition of lipolysis by insulin in VAT. Values displayed as mean and SEM.

3.3.3 Relationship of stimulated, inhibited and basal lipolysis

In SAT, the metabolic flexibility (ie its responsiveness to stimulation and inhibition) of the adipocytes is related to the basal lipolysis of the cell. The lower the basal lipolysis of the AT the increased susceptibility it is to stimulation by isoproterenol ($r=-0.056$, R^2 31.8%, $p<0.001$) (Figure 19).

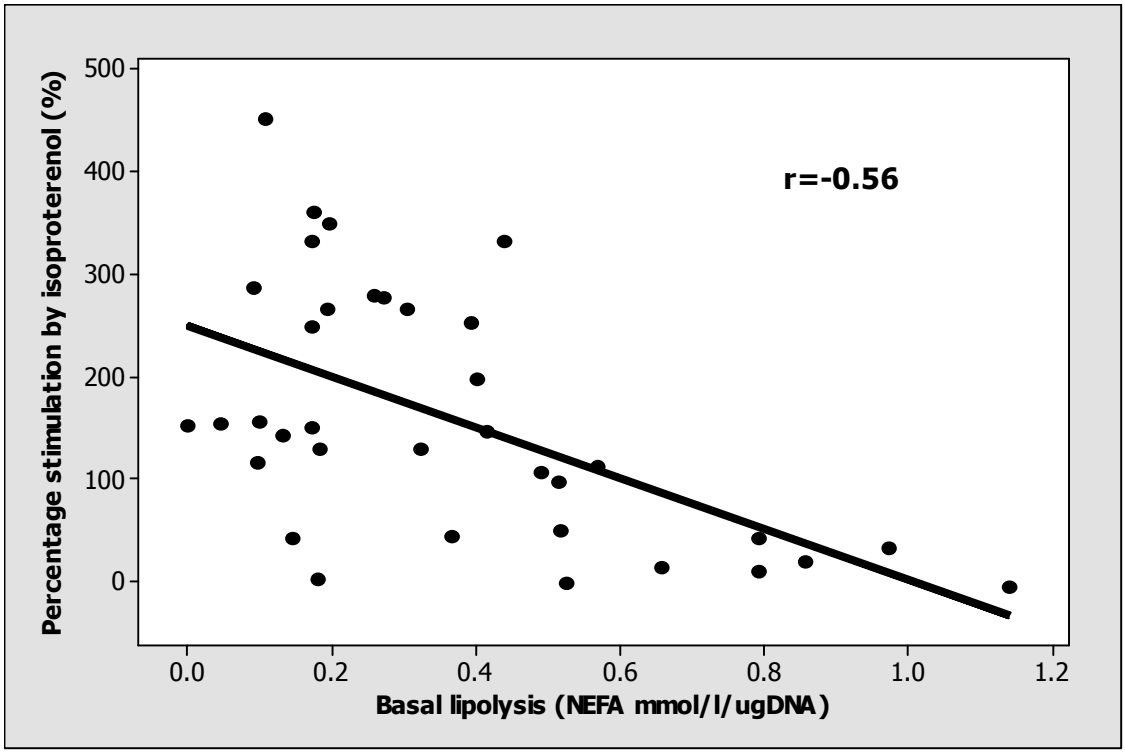

Figure 19 Basal Lipolysis versus percentage stimulation in SAT in healthy pregnancy.

The higher the basal lipolysis the lower the percentage stimulation by isoproterenol ($p<0.001$)($n=36$).

In addition in SAT basal lipolysis is related to sensitivity of the fat to suppression by insulin. As basal lipolysis increases so does the degree of suppression by insulin ($r=0.58$, $R^2 = 33.5\%$, $p<0.001$) (Figure 20).

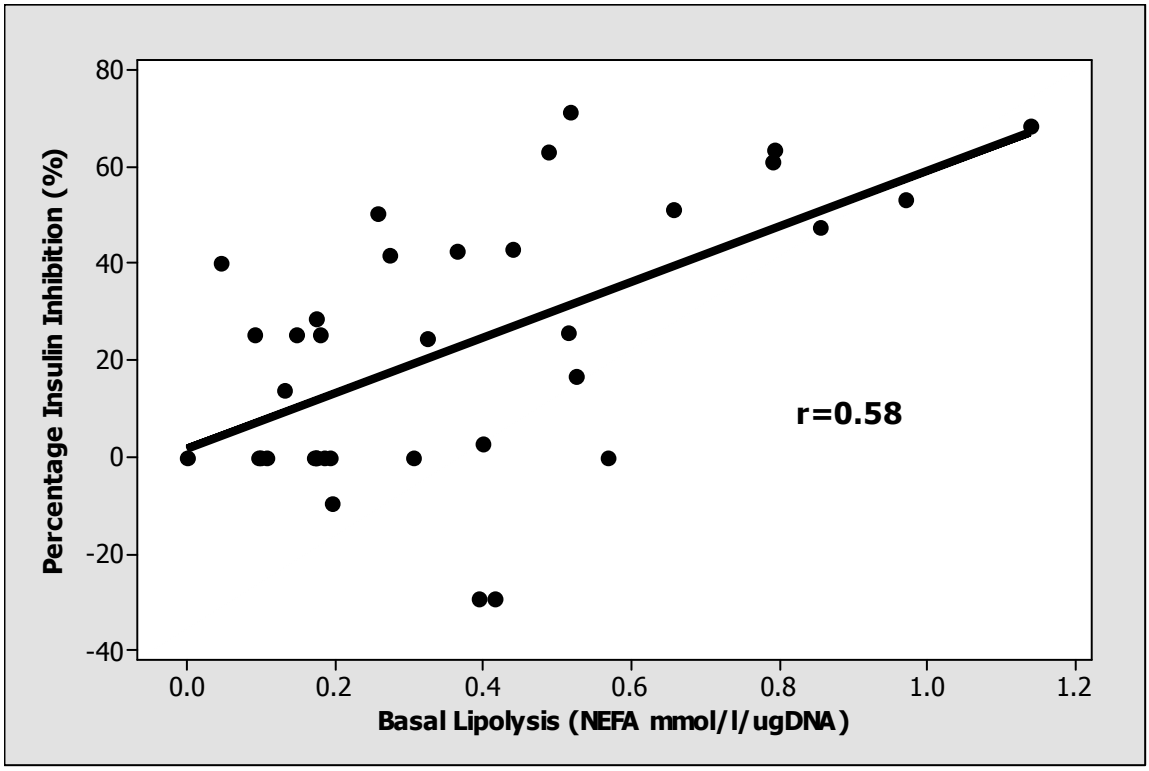

Figure 20 Basal lipolysis versus percentage inhibition in SAT in healthy pregnancy.

The higher the basal lipolysis the higher the percentage inhibition by insulin (p<0.001) (n=36).

A similar effect is apparent in VAT – increasing basal lipolysis is related to reduced susceptibility to stimulation by isoproterenol. (r=-0.45, R^2 = 20.5%, p=0.009)(Figure 21).

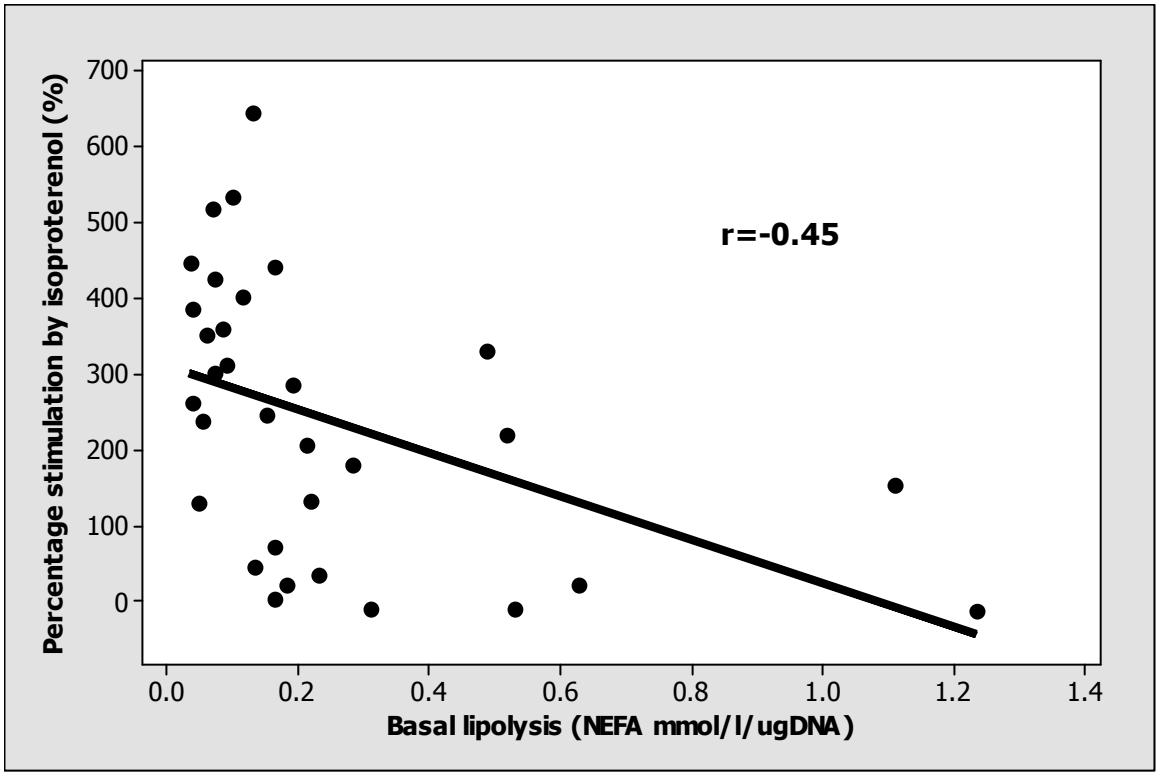

Figure 21 Basal lipolysis versus percentage stimulation in VAT in healthy pregnancy.

As basal lipolysis increases the less responsive VAT is to stimulation by isoproterenol (p=0.009)(n=36).

However there is no apparent relationship between basal lipolysis and sensitivity to suppression with insulin in contrast to that seen in subcutaneous fat (Figure 22).

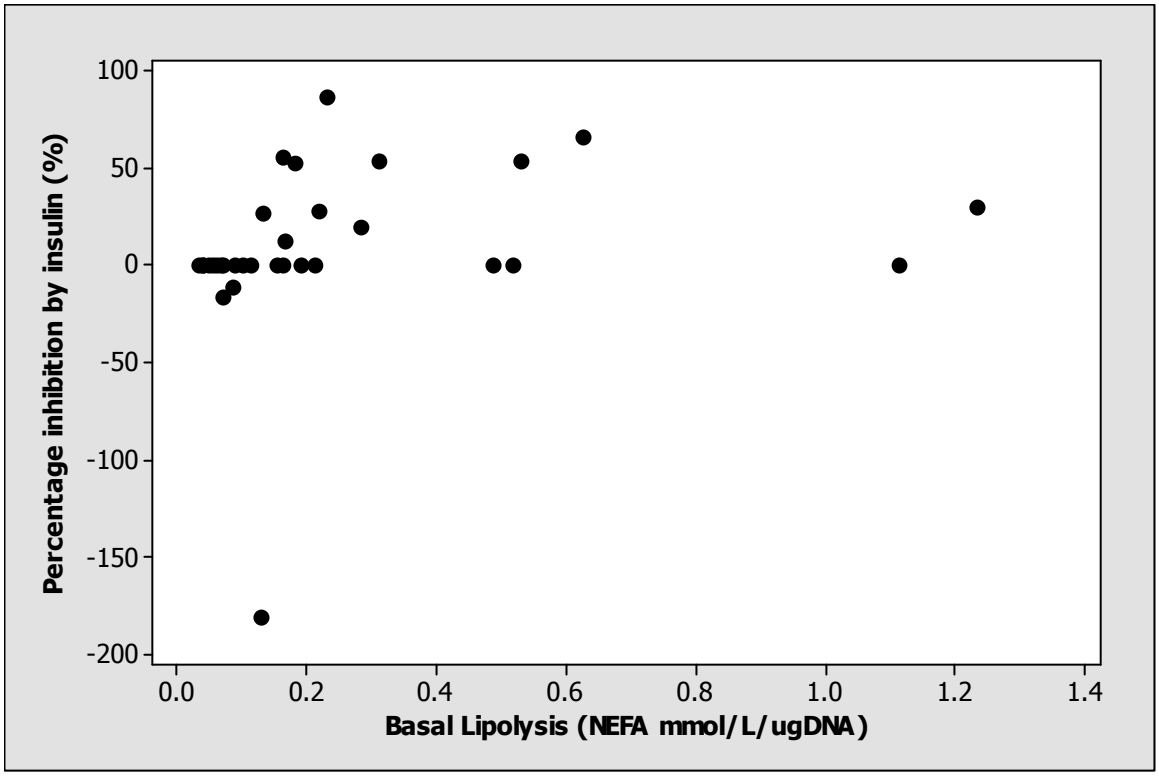

Figure 22 Basal lipolysis versus percentage inhibition in VAT in healthy pregnancy.

There is no apparent relationship (p=0.219)(n=36).

There is also an inverse relationship between the sensitivity of SAT and VAT to stimulation by catecholamines and inhibition by insulin. ($r=-0.46, R^2 = 20.7\%$, $p=0.005$ and $r=-0.74$, $R^2 = 54.5\%$, $p<0.000$ respectively) (Figure 23 & Figure 24).

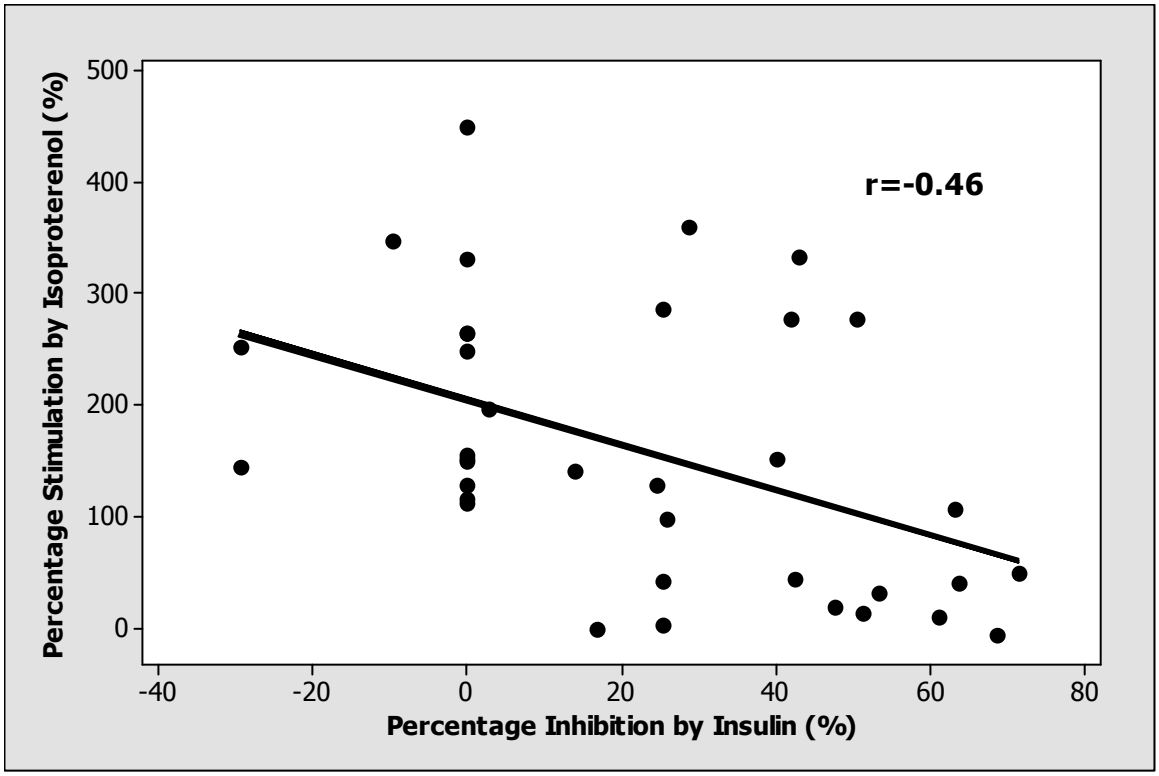

Figure 23 Percentage stimulation of lipolysis by isoproterenol vs percentage inhibition by insulin in SAT.

There is an inverse relationship (p=0.005) (n=36).

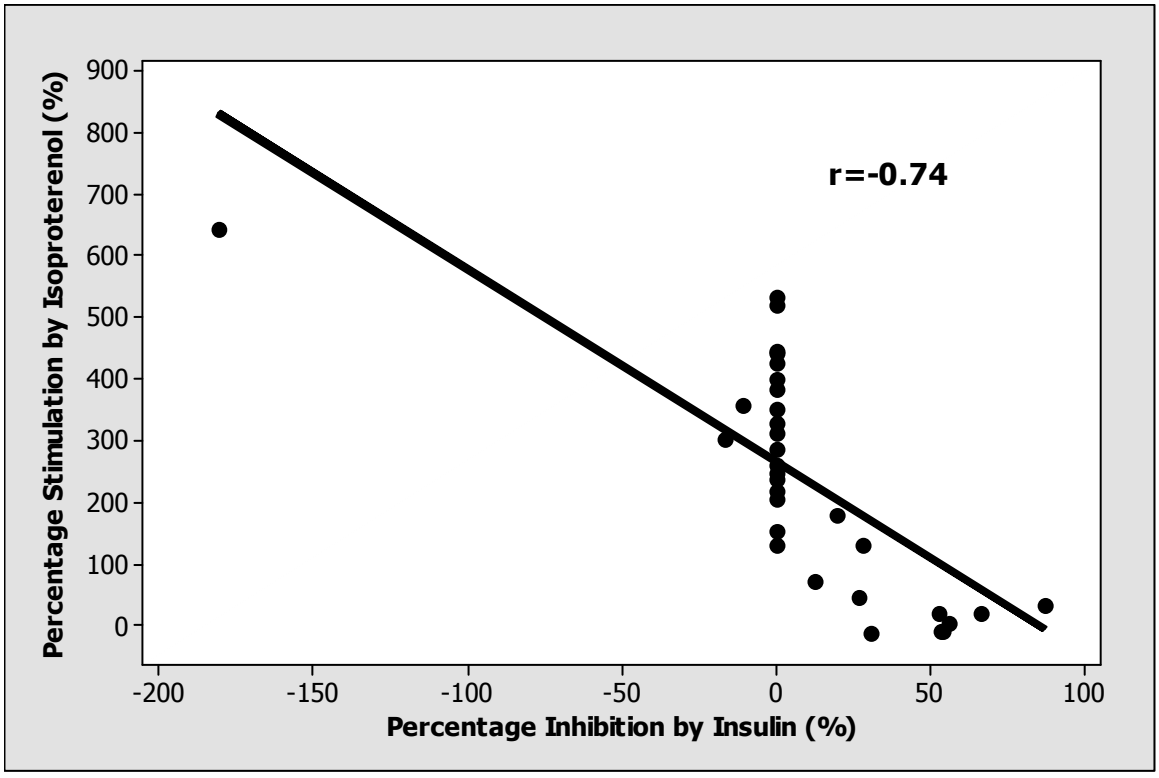

Figure 24 Percentage stimulation of lipolysis by isoproterenol versus percentage inhibition by insulin in VAT.

There is an inverse relationship (p<0.000)(n=36). Relationship remains robust when re-tested without apparent outlier (r=-0.75, p=0.000).

3.3.4 Relationship of lipolytic function between VAT and SAT.

There is no relationship between basal lipolysis in SAT and VAT when expressed as either release of NEFA or glycerol. The lipolytic function of SAT and VAT appear to be independent. The only apparent correlation is between that of isoproternol stimulation of lipolysis when expressed as NEFA release/ugDNA – the sensitivity of catecholamine stimulation of SAT is positively associated with that in VAT (r=0.46, R^2=21.5, p=0.008)(Figure 25).

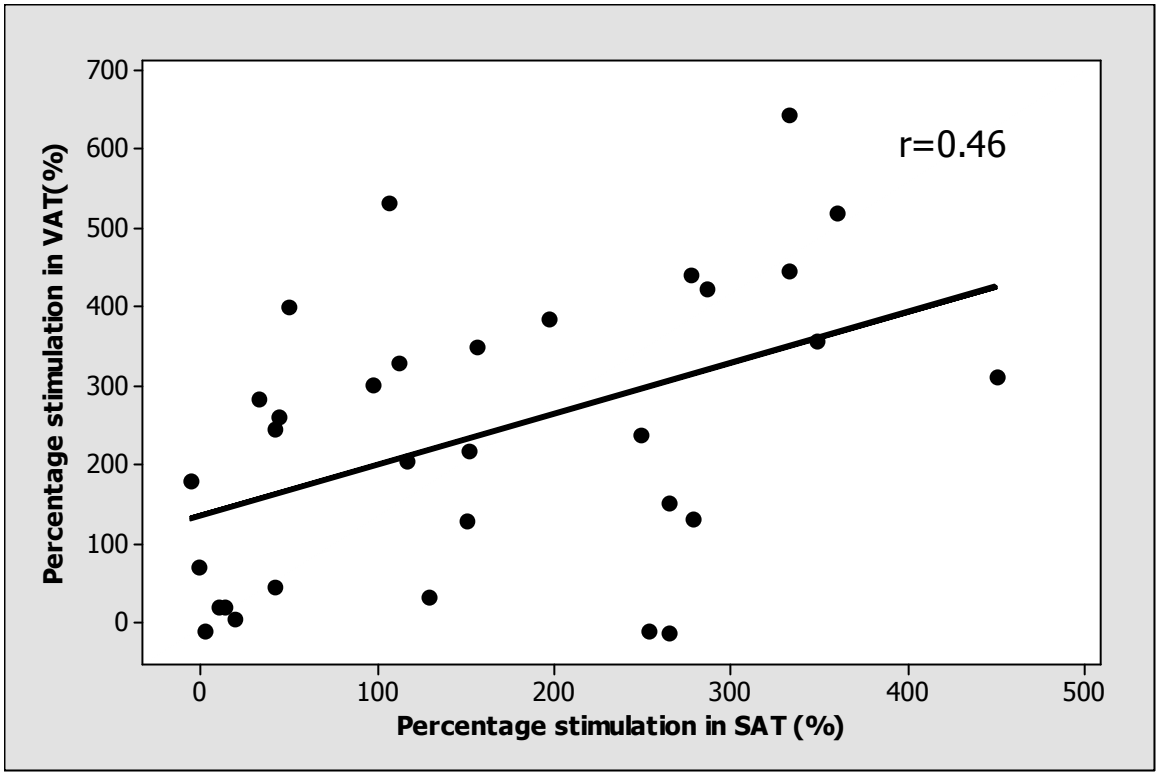

Figure 25 Percentage stimulation of lipolysis versus percentage stimulation in SAT.

There is positive correlation between percentage stimulation of lipolysis in VAT and SAT (p=0.008)(n=36).

3.3.5 Relationship between lipolytic function of AT and maternal and fetal characteristics.

There was no correlation between any measure of lipolytic function (ie absolute levels of basal, insulin suppressed or isoproterenol stimulated lipolysis or percentage stimulation or inhibition of lipolysis) and maternal BMI, age, parity, systolic BP ,diastolic BP, gestational age at delivery, birth weight and birth weight centile.

Maternal plasma levels of NEFA were positively correlated with gestational age at delivery (r=0.37, R^2=14.0%, p=0.025) (Figure 26).

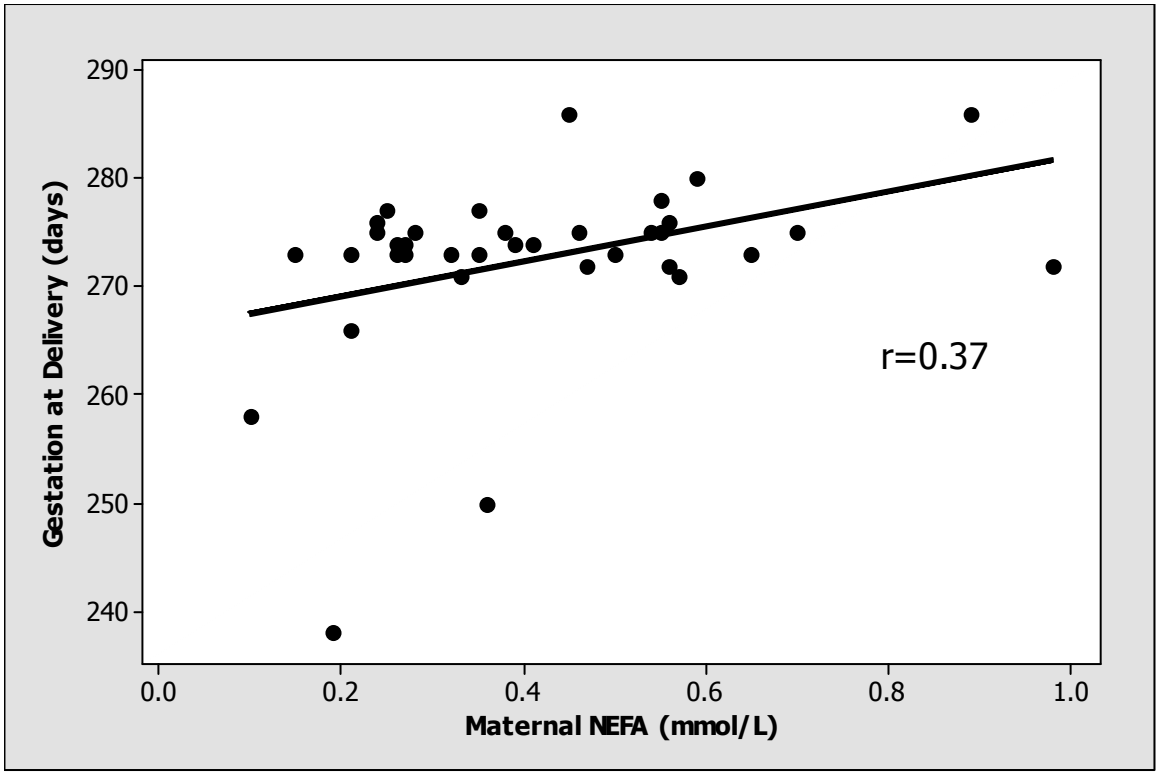

Figure 26 Relationship between gestational age at delivery and maternal NEFA.

There is a positive correlation between gestational age at delivery and maternal NEFA (p=0.025)(n=36).

3.3.6 Fat Cell Insulin Sensitivity

A direct measure of insulin sensitivity of the fat cell was calculated from the percentage inhibition of catecholamine stimulated lipolysis by insulin measured from the release of NEFA. This was derived from the following calculation (Figure 27):

(SISO-SISO+INS)/(SISO-SBA)*100

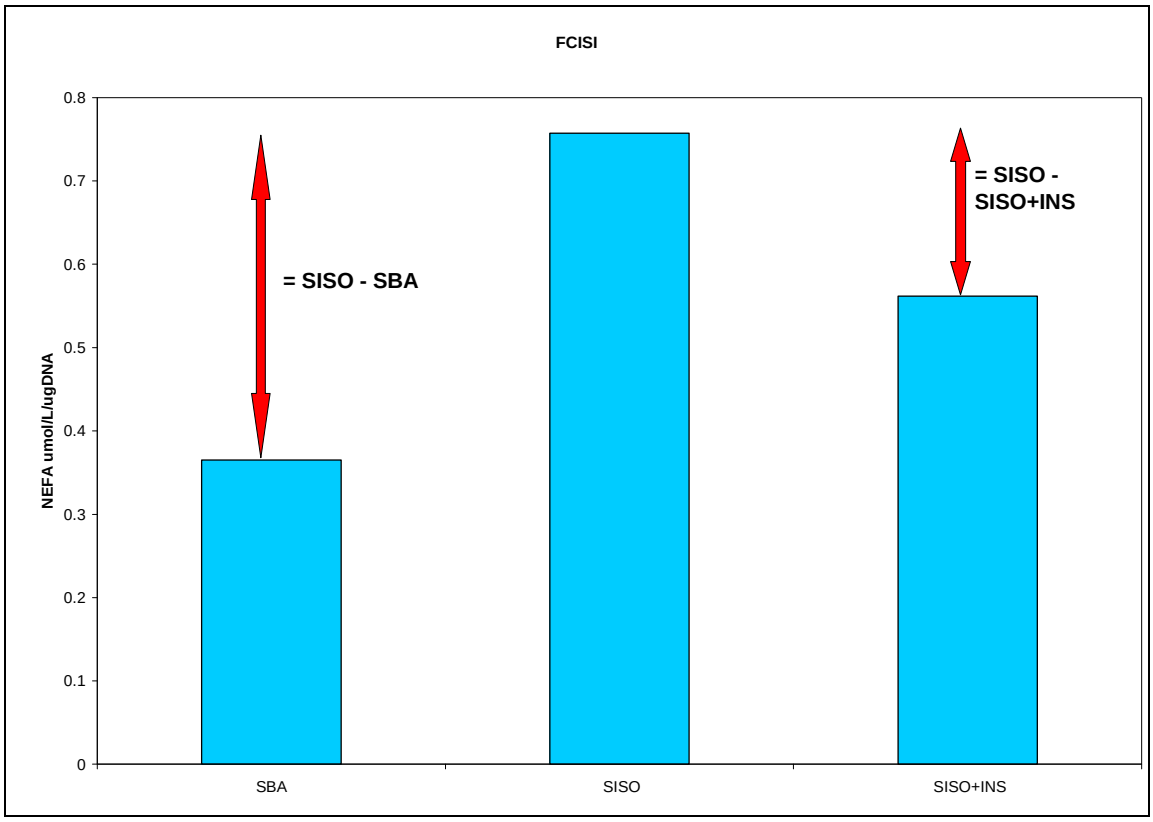

Figure 27 Fat cell insulin sensitivity index.

The fat cell insulin sensitivity index (FCISI) is calculated from the percentage inhibition of catecholamine stimulated lipolysis by insulin ie (SISO-SISO+INS)/(SISO-SBA)*100.

This was called the fat cell insulin sensitivity index (FCISI).

Using this measure, visceral fat was less insulin sensitive than subcutaneous fat (11%[27] vs 82%[21] p=0.04) in the normal pregnant woman.(Figure 28)

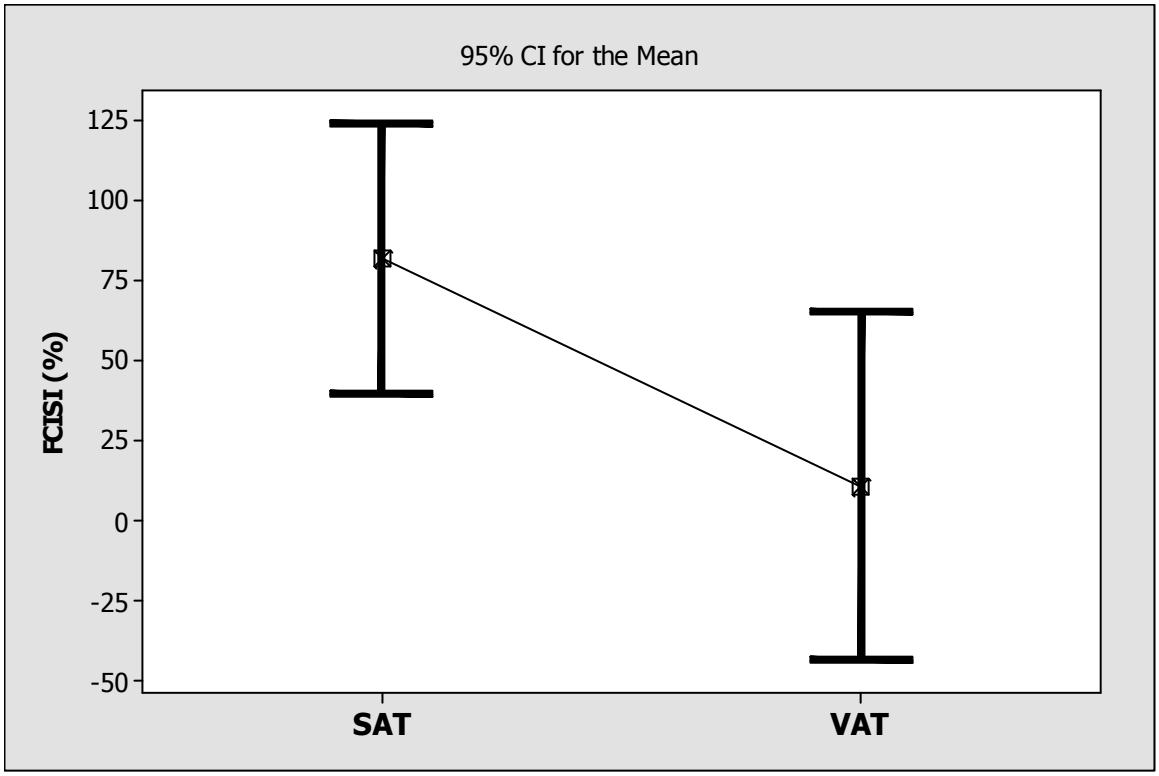

Figure 28 Comparison of FCISI between VAT and SAT.

Visceral fat is less insulin sensitive as measured by the FCISI (11%vs82% p=0.04)(n=36). Values displayed as mean and 95% confidence interval.

There was no correlation between FCISI in SAT and VAT (r=-0.05, p=0.79).

Maternal plasma glucose, insulin and subsequent HOMA (Homeostatis Model Assessment) did not correlate with absolute values for basal lipolysis, stimulated lipolysis or insulin attenuated catecholamine stimulated lipolysis in either SAT or VAT. HOMA is a measure of whole body insulin resistance and is calculated by the product of the fasting concentrations of glucose and insulin divided by a constant (22.5)[195]. FCISI in visceral fat was negatively correlated with maternal glucose (r=-0.46, R^2 = 21.5%, p=0.008) which remained robust after adjustment for age, BMI and parity (p=0.016).(Figure 29) FCISI in visceral fat was also negatively correlated with HOMA (r=-0.32), R^2=10.3%, p=0.073) and this association became more robust after adjusting for age, BMI and parity (p=0.05).(Figure 30) Interestingly the converse was true for subcutaneous fat. There was a positive correlation between FCISI in SAT and maternal glucose (ie the more insulin sensitive the SAT the higher the maternal glucose) (r=0.49, R^2=23.6, p=0.003), which persisted after adjustment for age, BMI and parity (p=0.005).(Figure 31) In addition there was also a positive correlation with SAT

FCISI and HOMA (r=0.34, R^2=11.2%, p=0.046) which again remained robust after adjustment (p=0.047).(Figure 32)

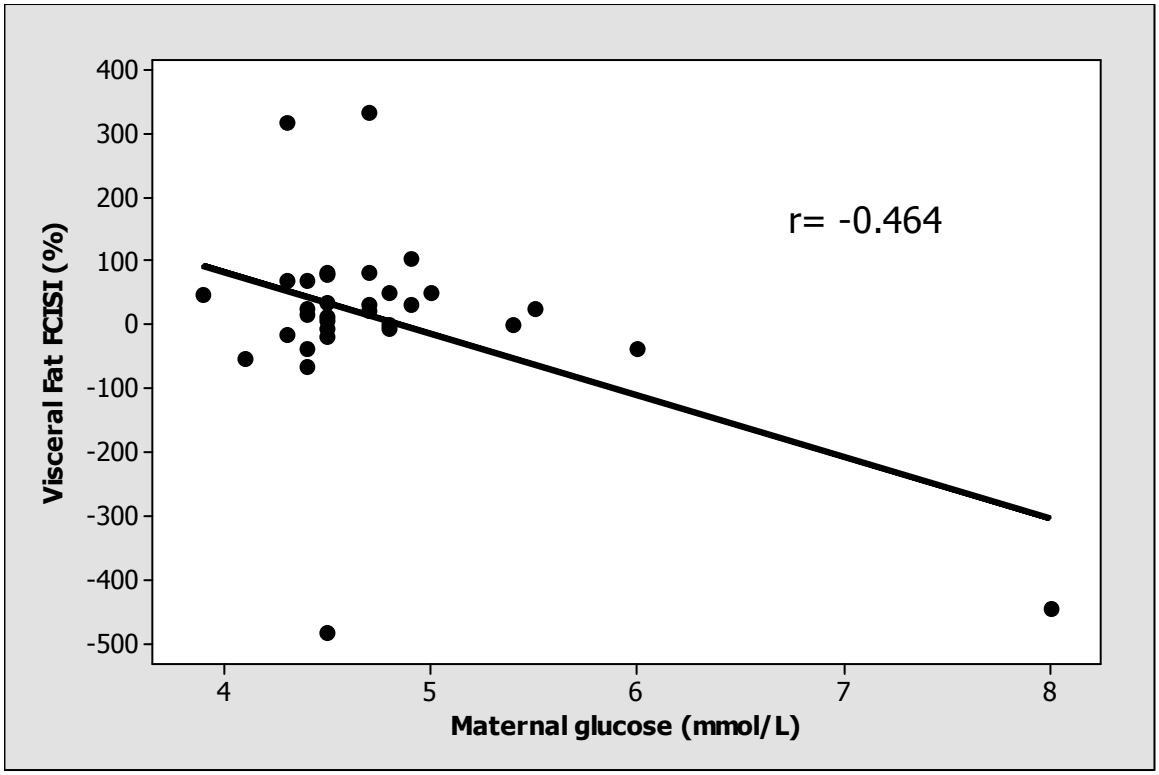

Figure 29 Relationship between FCISI in VAT and maternal glucose.

FCIS in VAT is negatively correlated with maternal glucose r=-0,464, p=0.016 adjusted for age, BMI and parity (n=36).

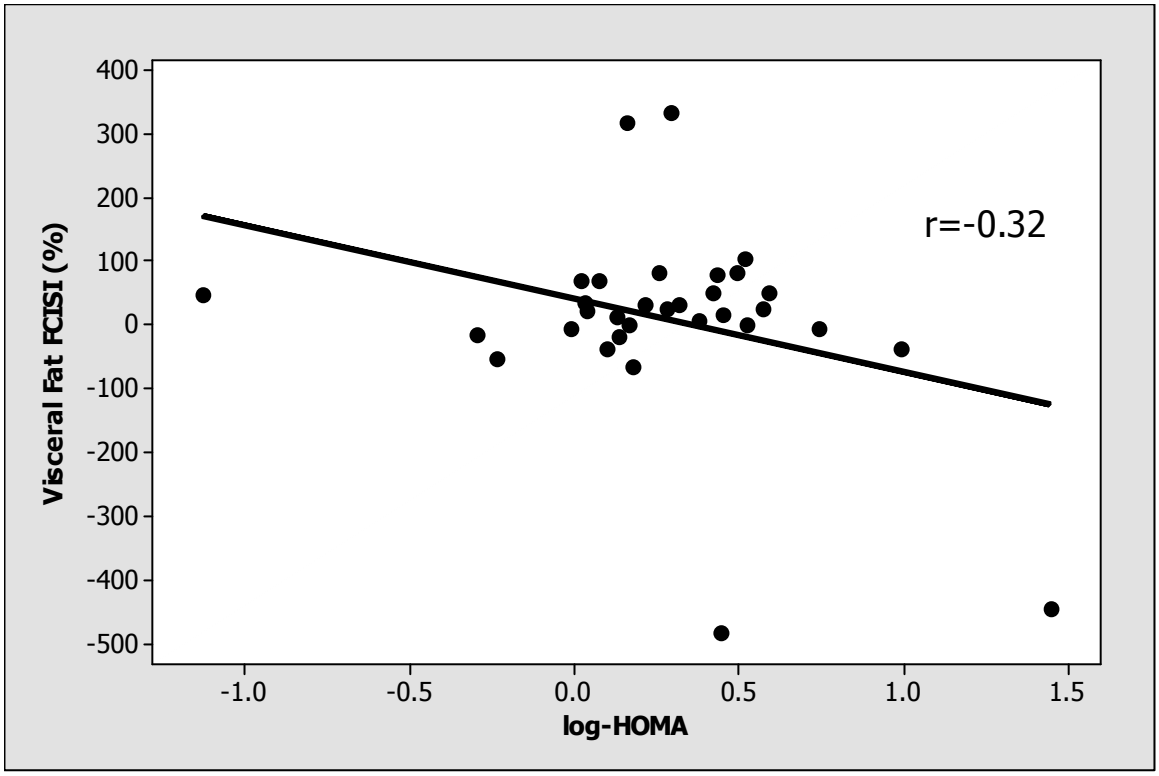

Figure 30 Relationship between FCIS in VAT and maternal HOMA.

FCISI in VAT is negatively correlated with maternal HOMA, r=-0.32, p=0.05 after adjustment for age, BMI and parity (n=36).

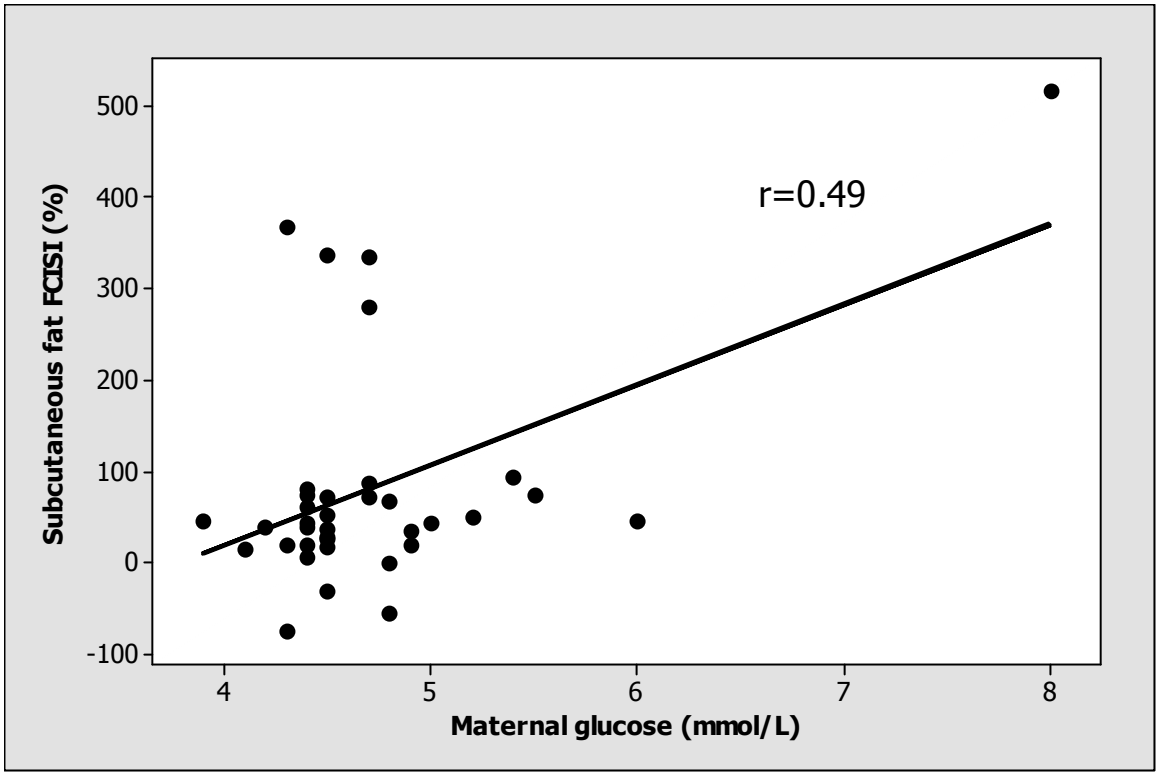

Figure 31 Relationship between FCISI in SAT and maternal glucose.

FCISI in SAT is positively correlated with maternal glucose r=0.49, p=0.005 adjusted for age, BMI and parity (n=36).

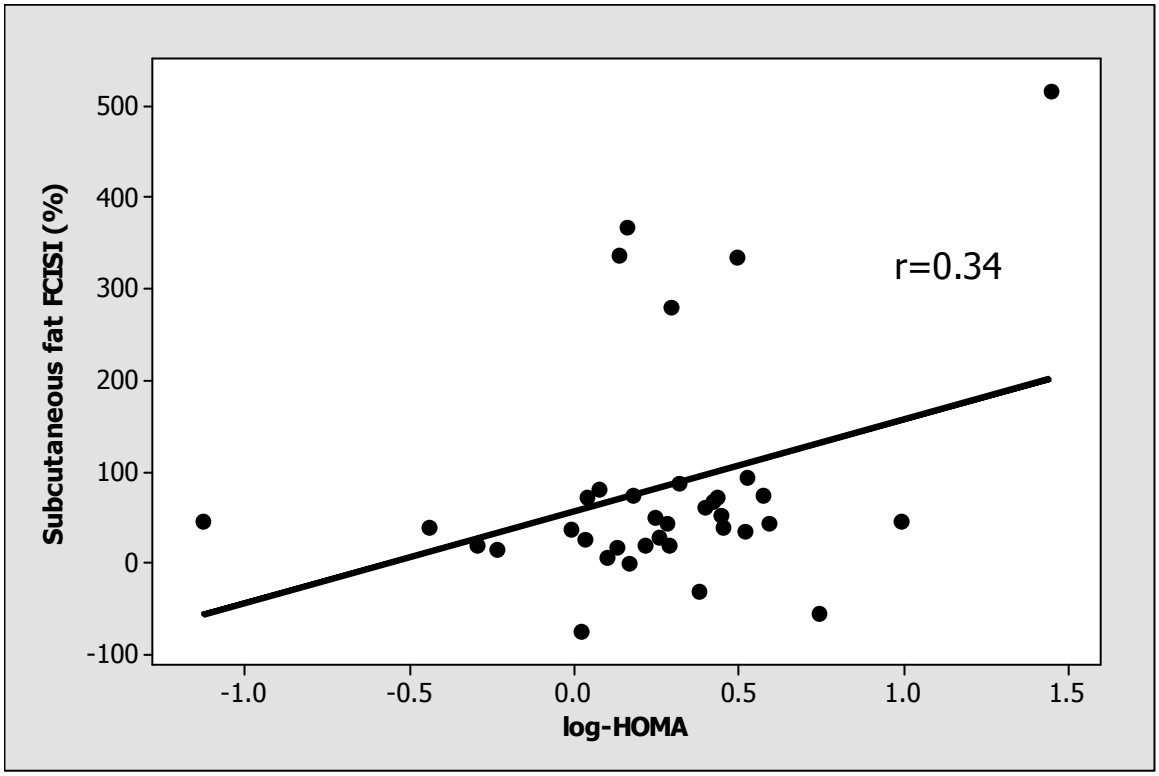

Figure 32 Relationsip between FCISI in SAT and maternal HOMA.

FCISI in SAT is positively correlated with maternal HOMA r=0.34,p=0.047 adjusted for age BMI and parity (n=36).

Absolute values of basal lipolsis, isoproterenol stimulated lipolysis and insulin suppressed lipolysis in ether SAT or VAT did not correlate with plasma markers of maternal glucose, insulin or HOMA.

3.3.7 Relationship of serum lipids and lipolytic activity

Maternal serum TGs increase as visceral fat cell insulin sensitivity decreases (r=-0.5, R^2=24.8%, p=0.004, which is independent of BMI (p<0.001) (Figure 33).

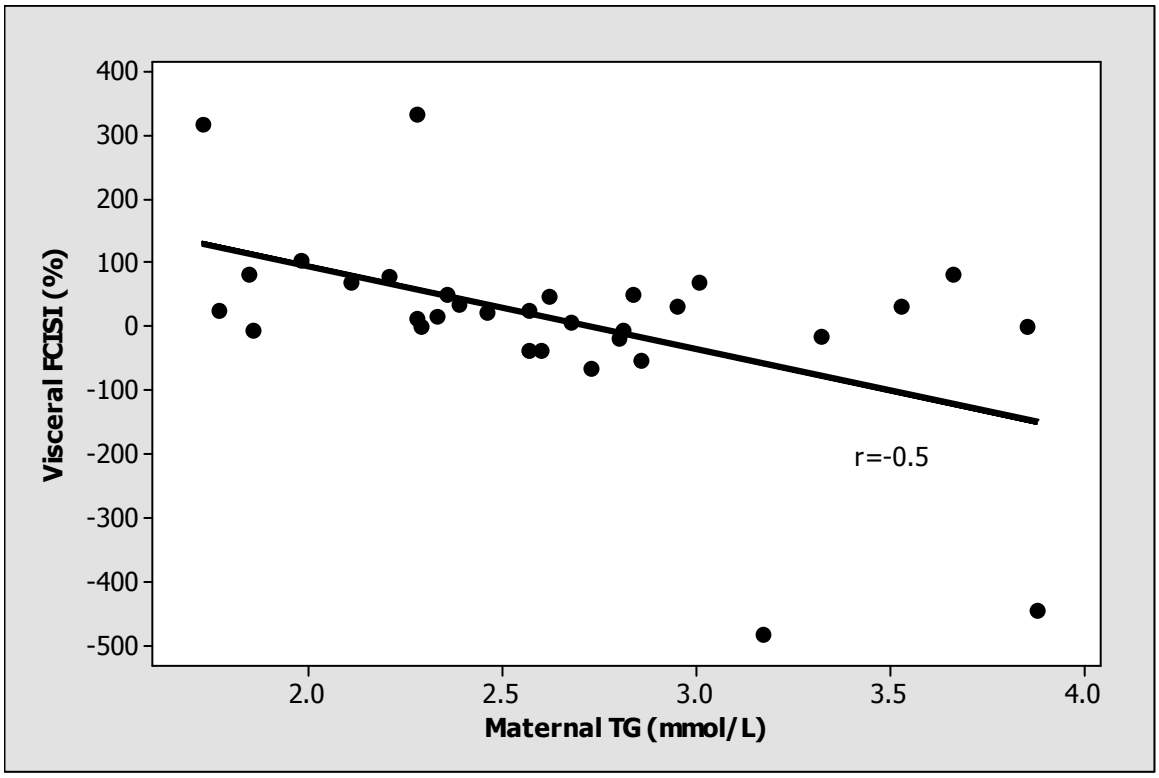

Figure 33 Relationship between maternal TG and FCISI in VAT.

Visceral fat cell insulin sensitivity is negatively correlated with maternal plasmaTG (p=0.004)(n=36).

3.3.8 Relationship of Maternal NEFA and Maternal Glucose, Insulin and HOMA

Maternal fasting levels of NEFA are inversely related to maternal glucose levels (r=-0.47, R^2 = 21.9%, p=0.004)(Figure 34) and maternal insulin levels (r=-0.59,R^2=34.5%, p<0.001)(Figure 35), and therefore not surprisingly with HOMA

(r=-0.59,R^2=35.2%,p<0.001)(Figure 36). All relationships remained robust after adjustment for age, BMI and parity (p=0.002;p<0.001;p<0.001 respectively). In addition, as expected, maternal glucose was positively correlated with maternal insulin levels (r=0.7, R^2=49.1% p<0.001)(Figure 37).

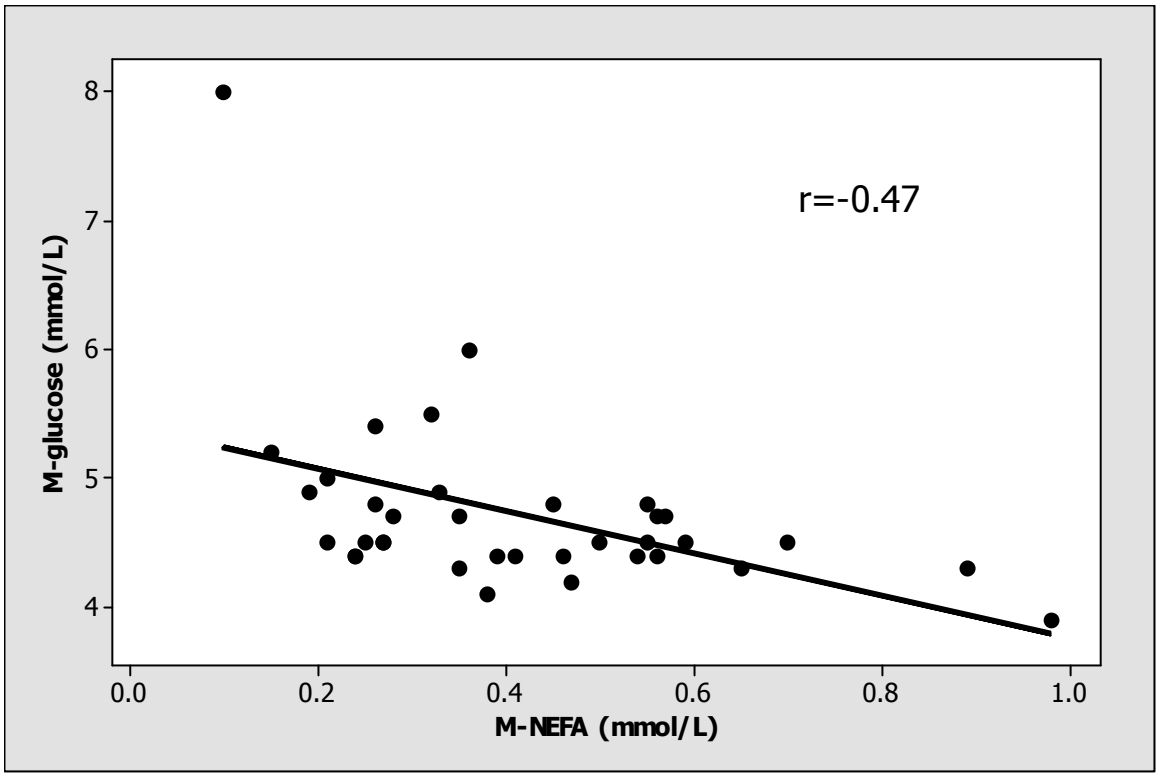

Figure 34 Relationship between maternal NEFA and maternal glucose.

Maternal fasting NEFA is negatively correlated with maternal fasting glucose (p=0.004)(n=36).

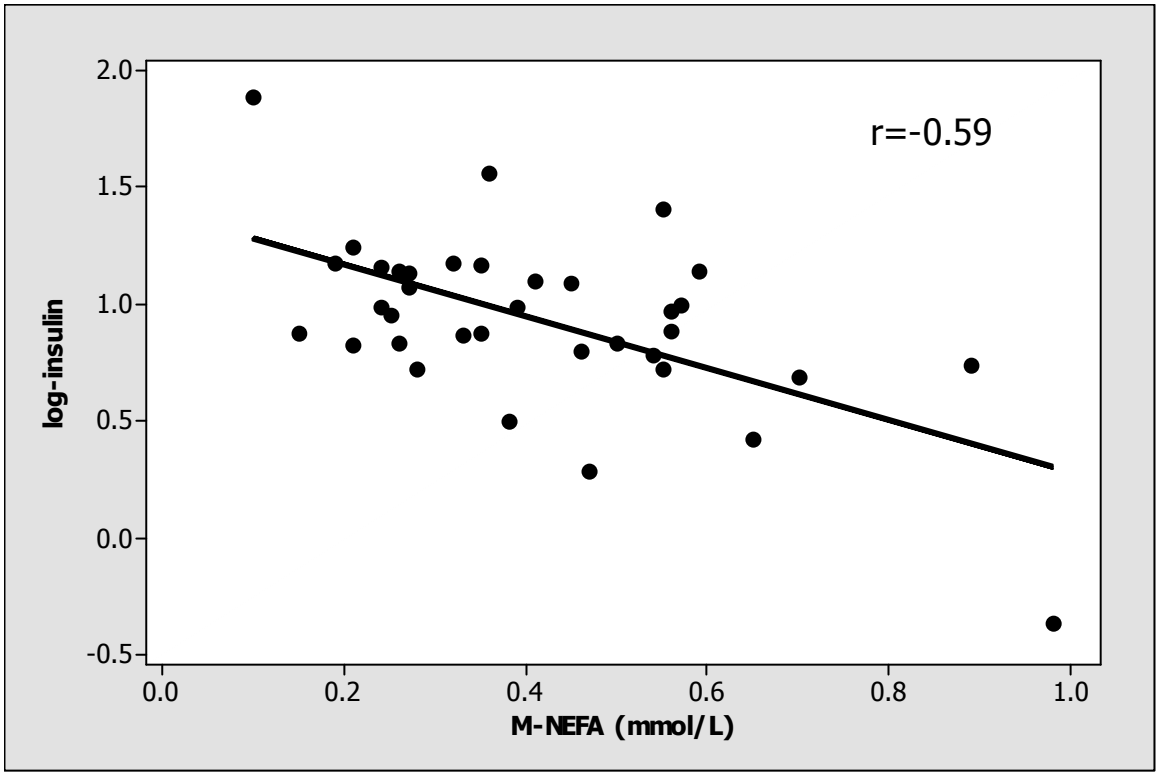

Figure 35 Relationship between maternal NEFA and maternal insulin.

Maternal fasting NEFA are negatively correlated with maternal fasting insulin (p<0.001)(n=36).

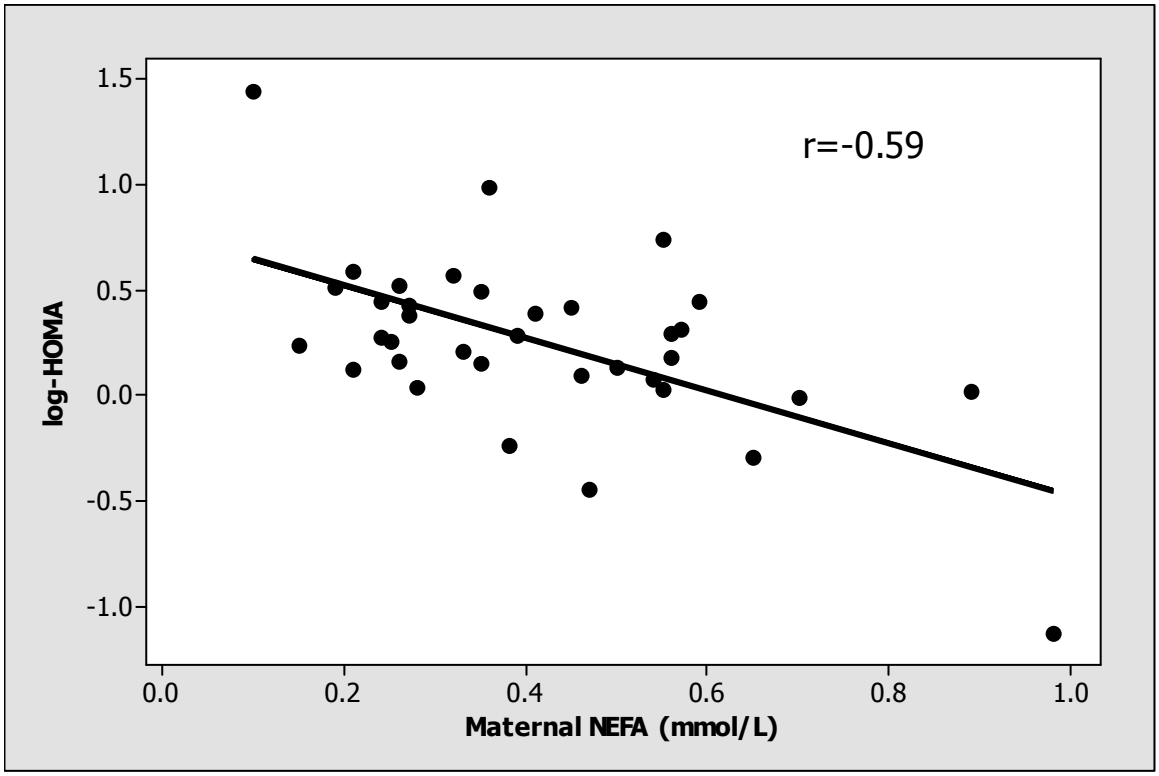

Figure 36 Relationship between maternal NEFA and maternal HOMA.

Maternal fasting NEFA are negatively correlated with maternal HOMA (p<0.001)(n=36).

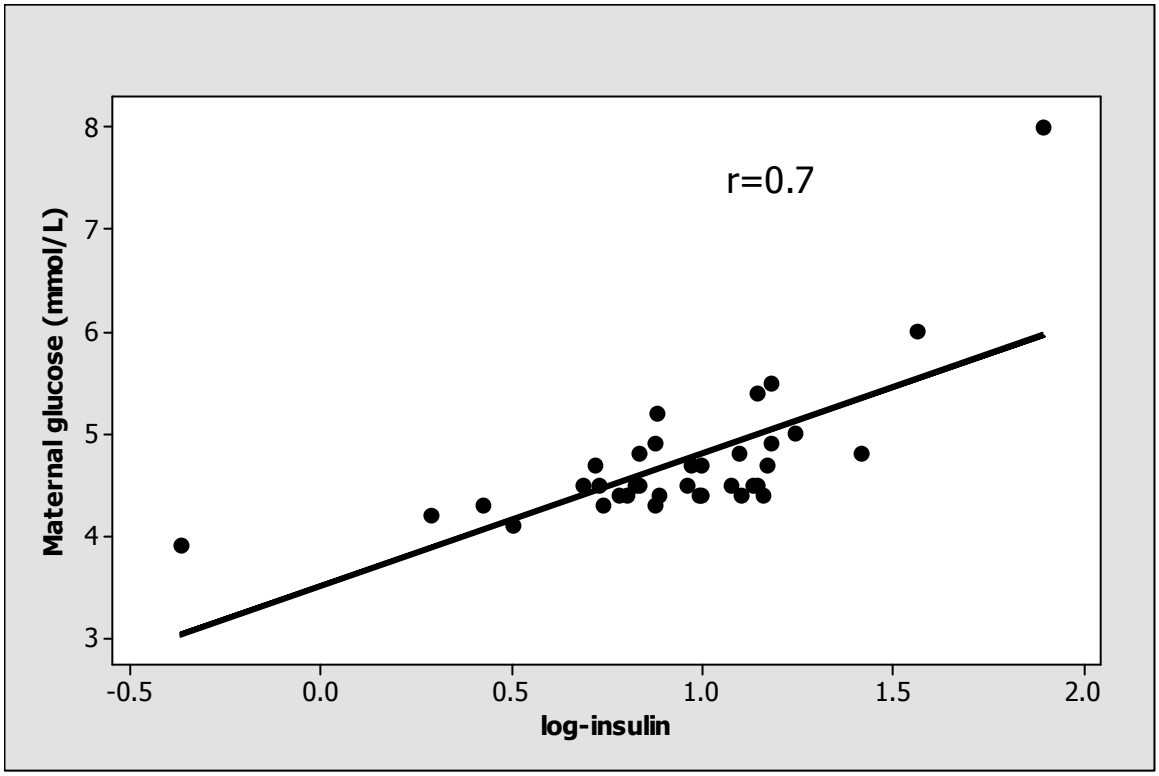

Figure 37 Relationship between maternal insulin and maternal glucose.

Maternal fasting insulin is positively related to maternal fasting glucose (p<0.001) (n=36).

3.3.9 Relationship of adipokines and lipolysis

3.3.9.1Adiponectin

In this cohort, although there was a trend for a negative association with BMI this did not reach significance (p=0.07). There was no correlation with other maternal and fetal characteristics ie age, parity, BP, gestational age at delivery, birth weight and birth weight centile.

There was no correlation between adiponectin and any measure of lipolytic function in either SAT or VAT (ie absolute levels of basal, insulin suppressed or isoproterenol stimulated lipolysis or percentage stimulation or inhibition of lipolysis).

A positive correlation was seen with progesterone (r=0.44, R^2=19.2%, p=0.007) but not with either oestradiol or hPL (human placental lactogen). This relationship remained robust after adjustment for age, parity, BMI, smoking and gestation at delivery (p=0.026)(Figure 38).

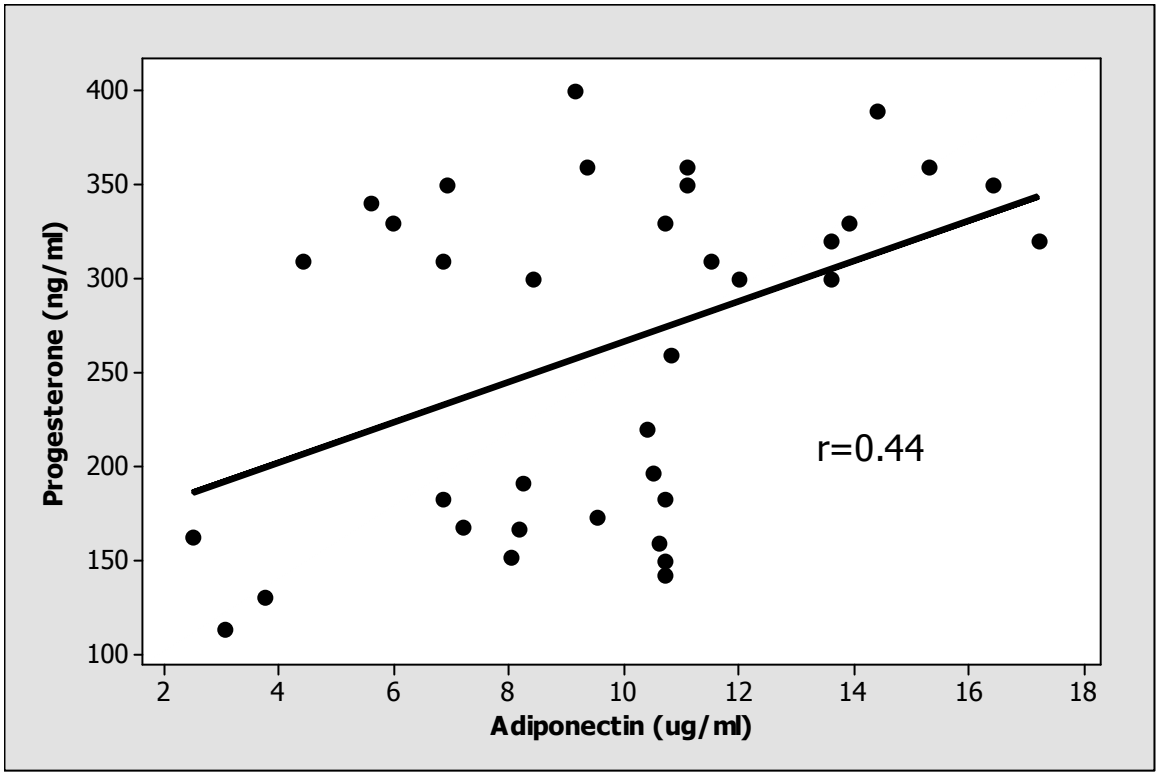

Figure 38 Relationship between maternal plasma adiponectin and maternal progesterone.

Adiponectin is positively correlated with maternal progesterone (p=0.026) after adjustment for age, parity, smoking and gestation at delivery (n=36).

There was no correlation between adiponectin with markers of insulin resistance including maternal glucose, insulin or HOMA in this cohort of normal pregnant women at term.

There was no correlation between adiponectin with maternal NEFA, total cholesterol (TC), triglceride(TG), nor high density lipoprotein cholesterol (HDL).

Adiponectin was negatively correlated with log CRP (r=-0.33, R^2=11% p=0.048) but this relationship was attenuated when adjusted for maternal BMI (p=0.151). No association was found between adiponectin and TNF alpha, IL-6 or leptin.

3.3.9.2Leptin

There is a positive association between leptin and BMI (r=0.5, R^2=24.5%, p=0.002)(Figure 39). There was no association between leptin and maternal age, parity, systolic and diastolic BP, gestation at sampling, birthweight and birthweight centile.

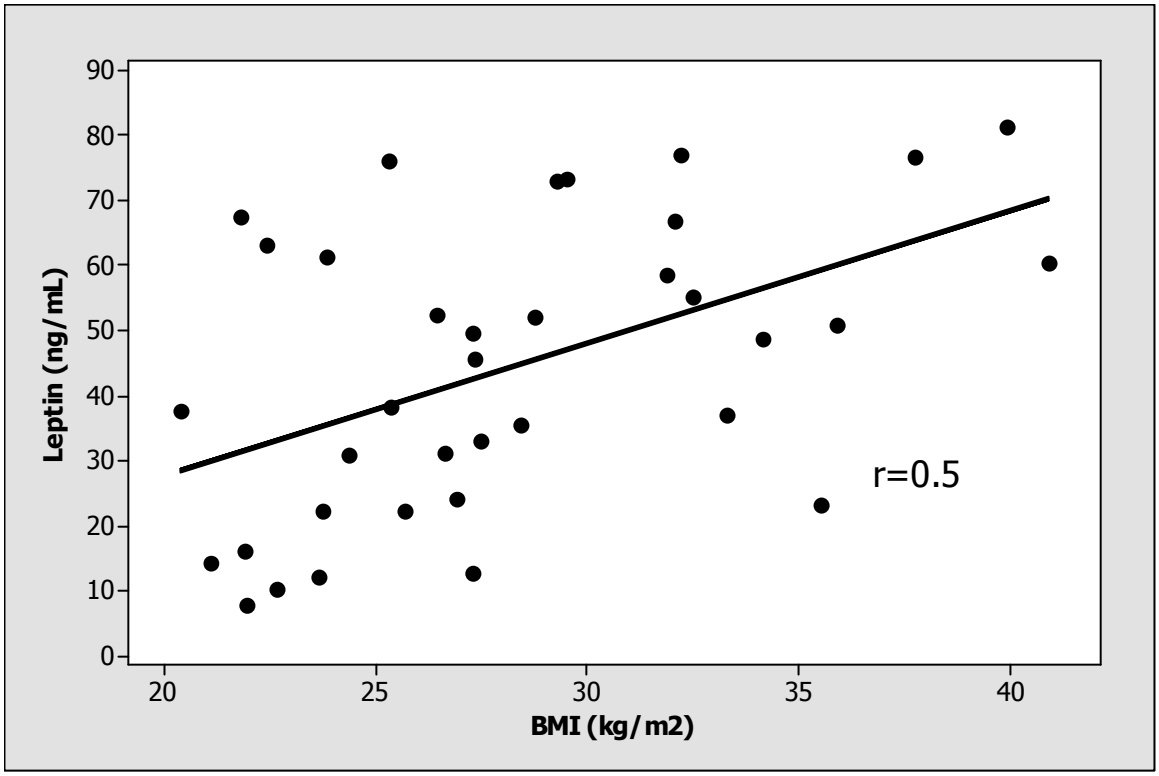

Figure 39 Relationship between plasma leptin and maternal BMI.

Leptin is positively correlated with BMI (r=0.5, p=0.002) in normal pregnant women (n=36).

Leptin correlates with measures of lipolytic function in VAT but not SAT. There is a positive correlation between maternal leptin and absolute values of lipolysis in the presence of isoproterenol expressed as NEFA release (r=0.36, R^2=12.6%,p=0.046)(Figure 40) and the presence of isoproterenol and insulin expressed as NEFA release (r=0.38, R^2 =14.%, p=0.034.(Figure 41) These associations remain after adjustment for age, parity, BMI and smoking (p=0.04 and p= 0.05 respectively).

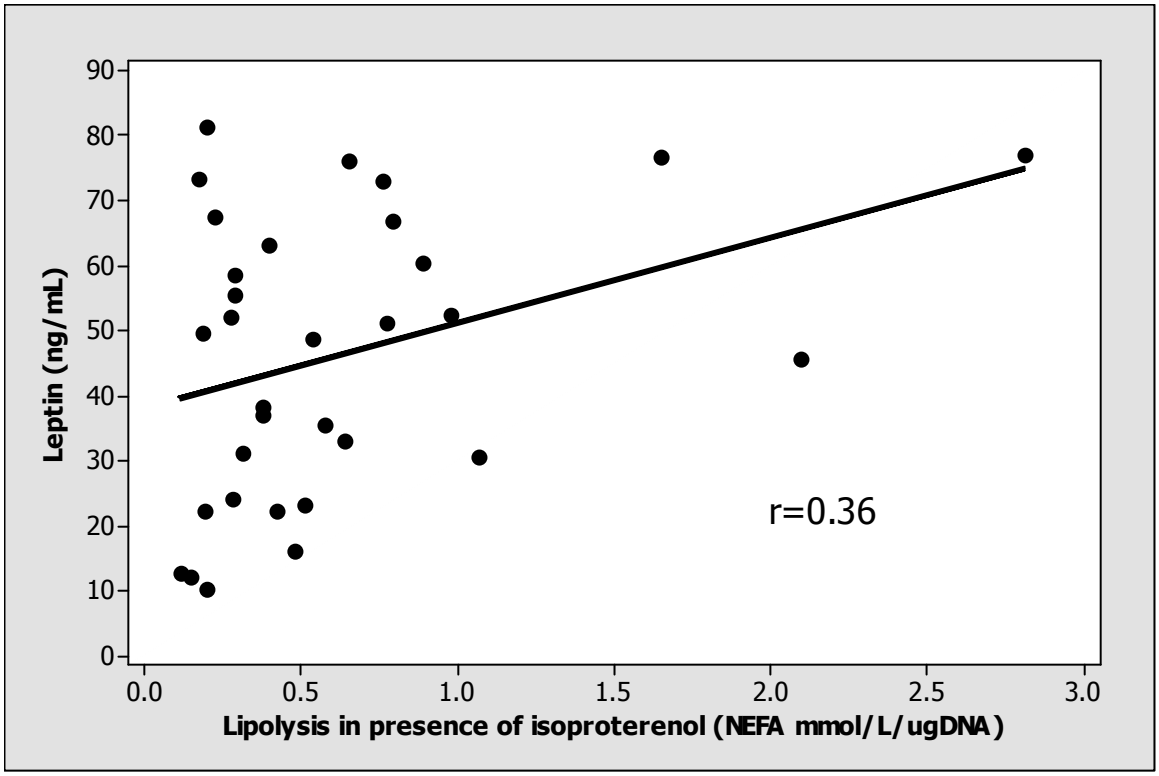

Figure 40 Relationship between maternal leptin and stimulated NEFA release in VAT.

Leptin is positively correlated with NEFA release in the presence of isoproterenol in VAT (r=0.36, p=0.040) adjusted for age, parity, BMI and smoking (n=36).

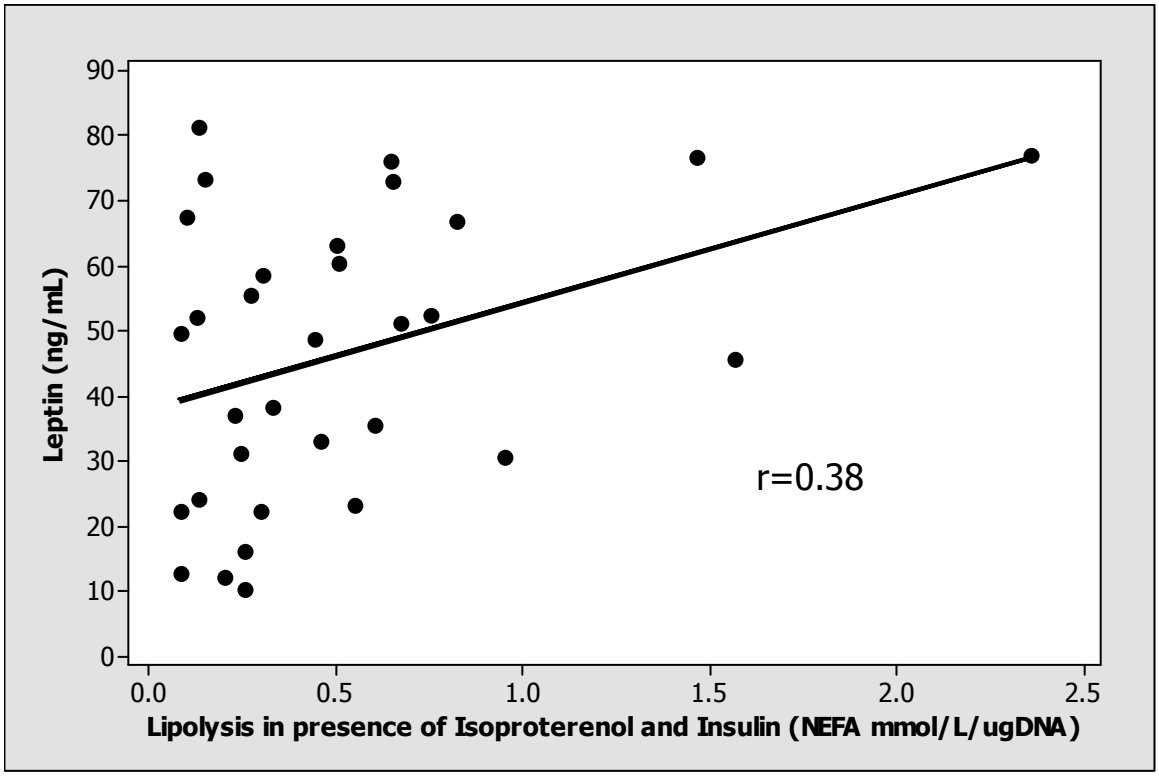

Figure 41 Relationship between maternal leptin and NEFA release in presence of isoproterenol and insulin in VAT.

Leptin is positively correlated with NEFA release in the presence of isoproterenol and insulin in VAT (r=0.38, p=0.05) adjusted for age, parity, BMI and smoking (n=36).

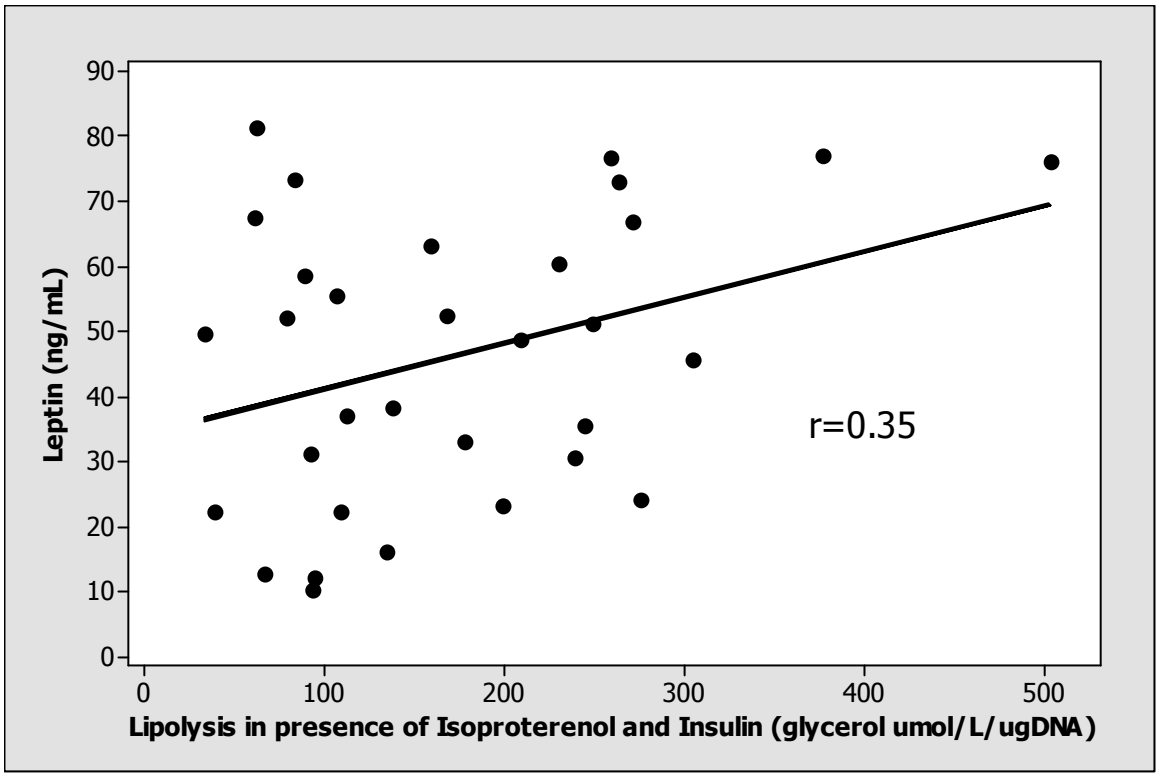

Figure 42 Relationship between maternal leptin and glycerol release in VAT.

Leptin is positively correlated with glycerol release in the presence of isoproterenol and insulin in VAT (r=0.35, p=0.05) (n=36).

There is no correlation between leptin and maternal progesterone, oestradiol and hPL.

Leptin is positively correlated with markers of plasma insulin resistance: maternal insulin (r=0.45, R^2=20.4%, p=0.006) and maternal HOMA (r=0.42, R^2=17.9%, p=0.01) and remains after adjustment for age, BMI, parity and smoking (p=0.01 and p=0.015).

There is a strong positive association of leptin with log CRP (r=0.59, R^2=33.4%, p<0.001) but no association was seen with IL-6 and TNFα (Figure 43). This relationship remains after adjustment for age, parity, BMI and smoking (p=0.006).

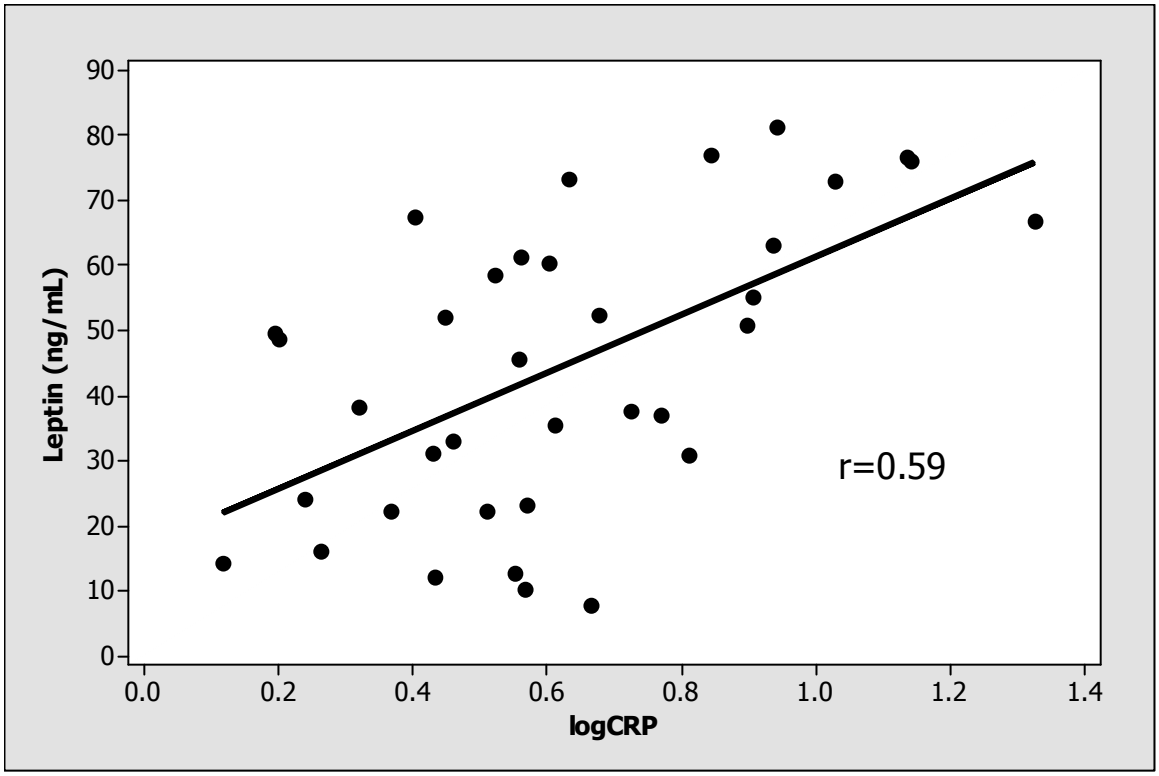

Figure 43 Relationship between plasma leptin and plasma CRP in healthy pregnancy.

Leptin is positively associated with log CRP (r=0.59, p=0.006) after adjustment for age, parity, BMI and smoking (n=36).

There was no relationship between leptin and maternal NEFA and lipids in this cohort.

For a summary of the correlations between maternal plasma adipokines and inflammatory markers with fetal and maternal characteristics, and markers of lipid and glucose metabolism see Table 6 and Table 7.

Plasma Marker	Age	BMI	Systolic BP	Diastolic BP	Gestational Age	Birthweight	Birthweight centile
Adiponectin	NS	r=-0.30 p=0.07	NS	NS	r=0.32 p=0.058	NS	NS
Leptin	NS	r=0.5 p=0.002	NS	NS	NS	NS	NS
Il-6	NS	r=-0.35 p=0.037	NS	NS	NS	r=-0.39 p=0.02	r=-0.38 p=0.026
TNF-α	NS	r=-0.34 p=0.04	NS	NS	NS	NS	NS
CRP	NS	r=0.37 p=0.027	NS	NS	NS	NS	NS

Table 6 Summary of correlations between maternal plasma adipokines and inflammatory markers with maternal and fetal characterisitics (n=36).

Plasma Marker	SAT Lipolytic Measures	VAT Lipolytic Measures	Glucose	Insulin	HOMA	SAT FCISI	VAT FCISI	NEFA	TG	TC	HDL
Adiponectin	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Leptin	NS	ISO:r=0.36 p=0.046 ISO+INS: r=0.38 p=0.034	NS	r=0.45 p=0.006	r=0.42 p=0.01	NS	NS	NS	NS	NS	NS
Il-6	NS	NS	r=0.35 p=0.03	NS	NS	NS	NS	NS	NS	NS	NS
TNFapha	Basal: r=0.44 p=0.008 ISO%: r=-0.35 p=0.036 INS%: r=0.4 p=0.015	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CRP	NS	ISO: r=0.35 p=0.05 ISO+INS: r=0.45 p=0.01	NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 7 Summary of correlations between maternal plasma markers of inflammation and adipokines with measures of lipid and glucose metabolism (n=36).

All lipolytic measures are expressed as NEFA release/ugDNA. ISO-in presence of isoproterenol; ISO+INS- in presence of isoproterenol and insulin; ISO% -percentage stimulation by isoproterenol; INS%-percentage inhibition by insulin.

3.3.10 Regional differences in the relationship between adipose lipolytic function and plasma markers of inflammation

3.3.10.1 C-reactive protein

CRP is positively correlated with BMI (r=0.37, R^2=13.6%, p=0.027) (Figure 44). There is no association between CRP and parity, smoking, DEPCAT and gestational age of sampling in this cohort. As previously described CRP is positively correlated with leptin.

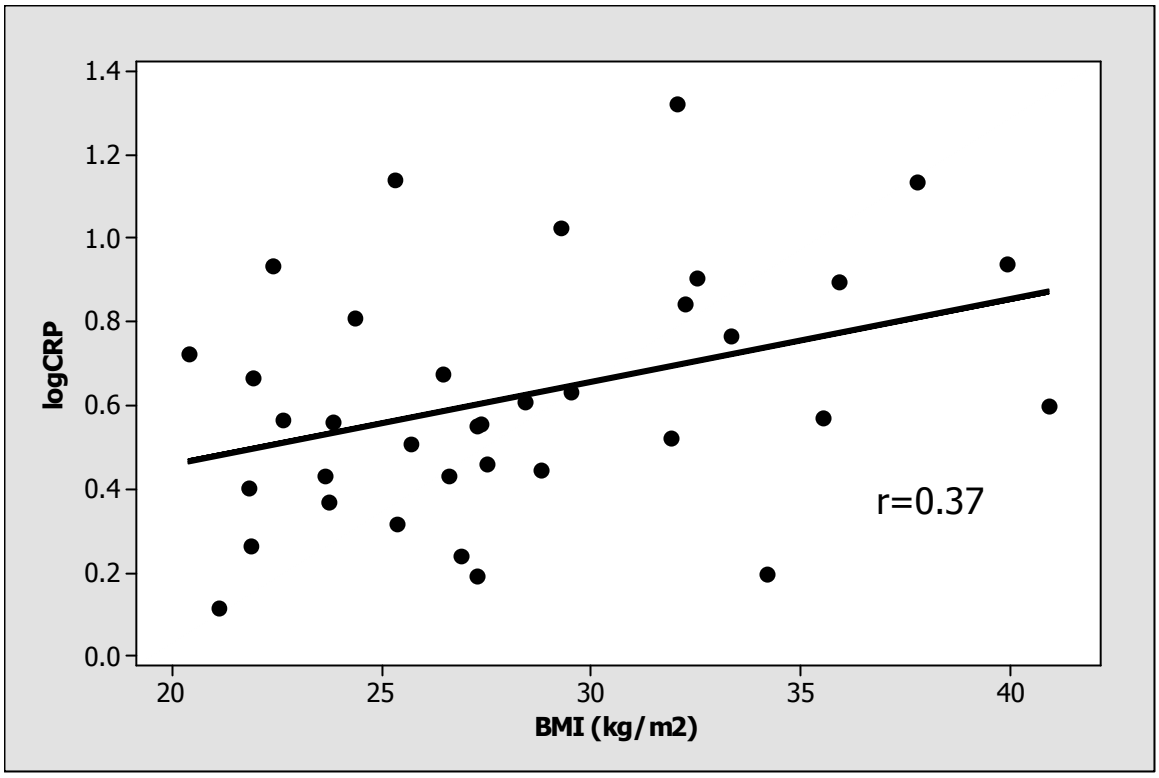

Figure 44 Relationship between BMI and maternal CRP.

BMI is positively correlated with log CRP (p=0.027) (n=36).

In addition, maternal log CRP is related to VAT function but not SAT function. There is a positive correlation between log CRP and NEFA release in the presence of isoproterenol (r=0.35, R^2=12.2%,p=0.05) (Figure 45) and this association becomes more robust when adjusted for age, BMI and parity (p=0.024). In addition it is also positively associated with NEFA release in the presence of isoproterenol and insulin (r=0.45, R^2=19.9%, p=0.010) and this remains robust after adjustment for age, parity and BMI (P=0.008)(Figure 46).

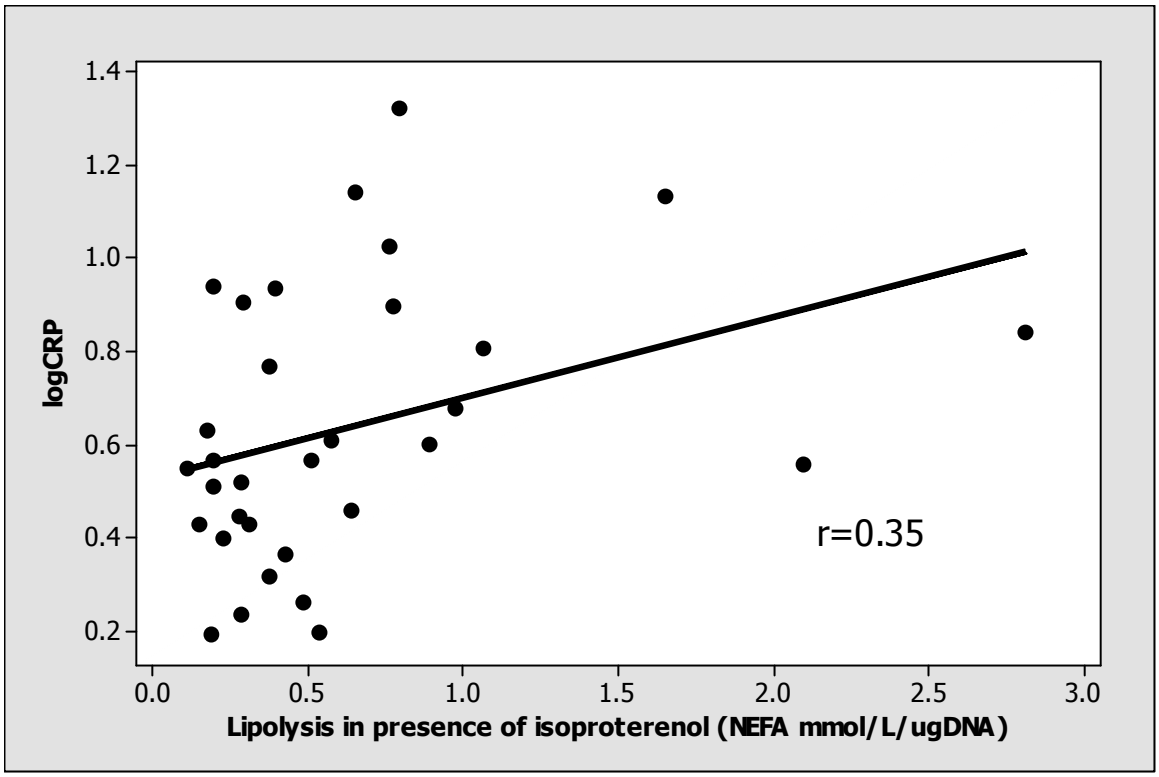

Figure 45 Relationship between maternal CRP and stimulated lipolysis in VAT.

Maternal log CRP is positively associated with NEFA release in the presence of isoproterenol in VAT (p=0.024 adjusted for age, BMI and parity) (n=36).

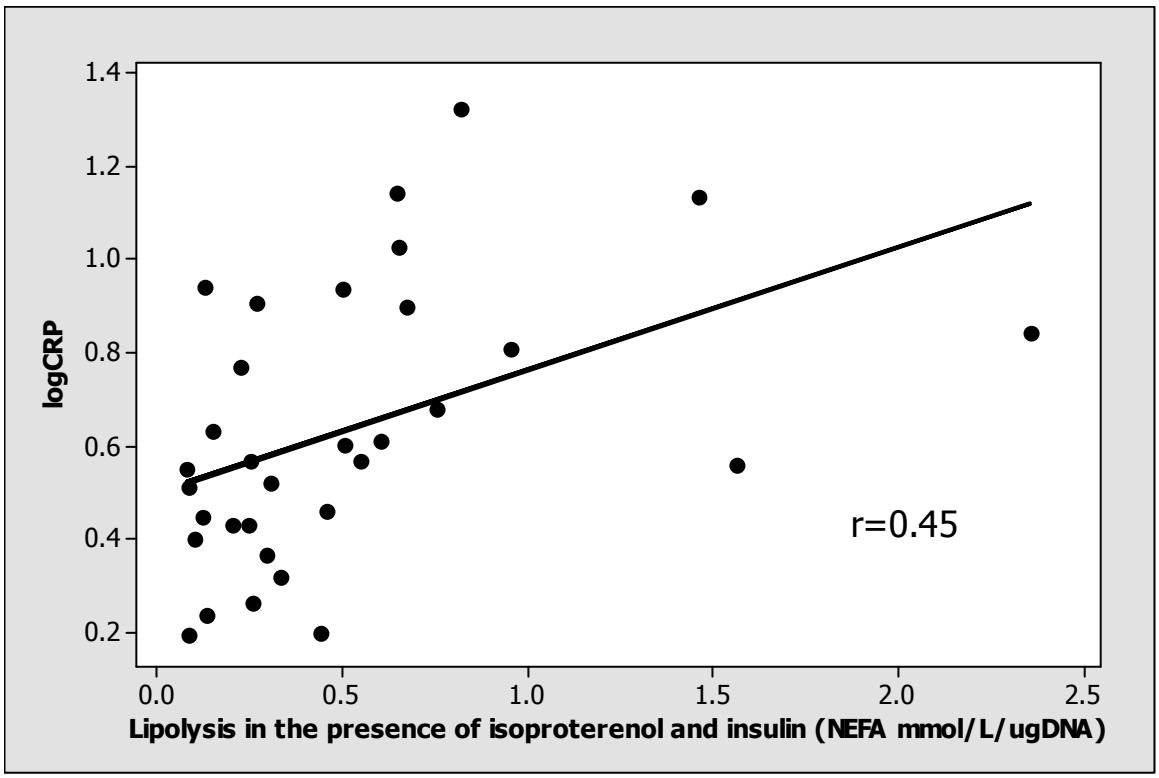

Figure 46 Relationship between maternal CRP and lipolysis in presence of isoproterenol and insulin.

Maternal log CRP is positively associated with NEFA release in the presence of isoproterenol and insulin in VAT (p=0.008 adjusted for age, BMI and parity) (n=36).

These correlations are also apparent between log CRP and glycerol release in presence of isoprtoerenol and isoproterenol and insulin in VAT (p=0.024 and p=0.005 respectively).

3.3.10.2 TNF-alpha

TNF alpha strongly correlates with SAT basal lipolysis when expressed as NEFA release mmol/l/ugDNA.(r=0.44, R^2=18.9%, p=0.008) This is more robust when adjusted for age, BMI and parity (p<0.001).(Figure 47) This relationship is also apparent when basal lipolysis is expressed as glycerol release/ug DNA (p=0.04).

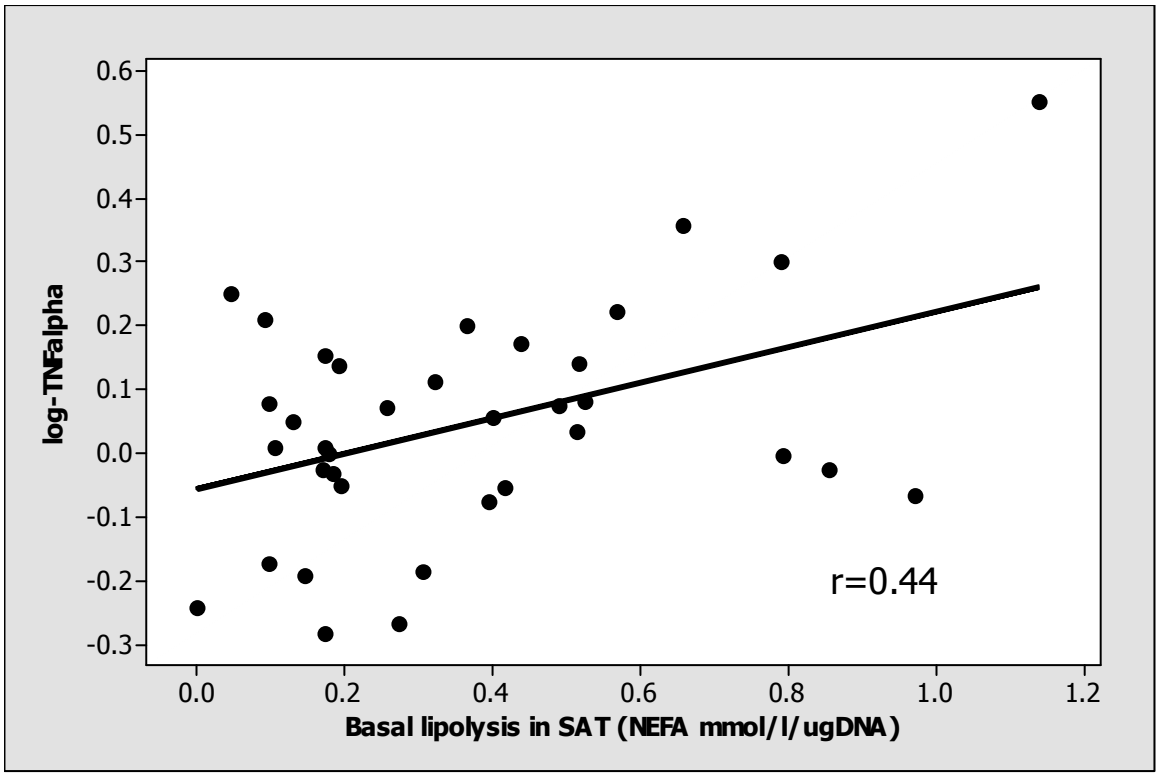

Figure 47 Relationship between maternal TNF alpha and basal lipolysis in SAT.

Maternal log TNF alpha is positively correlated with basal release of NEFA in SAT (r=0.44, p<0.001 adjusted for age, BMI and parity) (n=36).

In addition, TNF alpha is also correlated with other measures of lipolytic function in SAT including percentage stimulation of NEFA release by isoproterenol (r=-0.35, p=0.036) and percentage suppression of NEFA release by insulin (r=0.4, p=0.015). Similarly an association is seen with percentage stimulation of glycerol release by isoproterenol (r=-0.33, p=0.05).

There are no associations between plasma TNF alpha and measures of lipolytic adipocyte function in VAT.

In addition there is no relationships between maternal plasma TNF alpha and plasma markers of insulin resistance in this cohort.

3.3.10.3 IL-6

Maternal plasma IL-6 correlates with the birth weight centile of infants born to women with healthy pregnancies (r=0.37, R^2=13.5%, p=0.03) which remains robust after adjustment for age, BMI and parity (p=0.01).

Maternal IL-6 is positively correlated with maternal TNF alpha (r=0.45, R^2=19.8%, p=0.006).

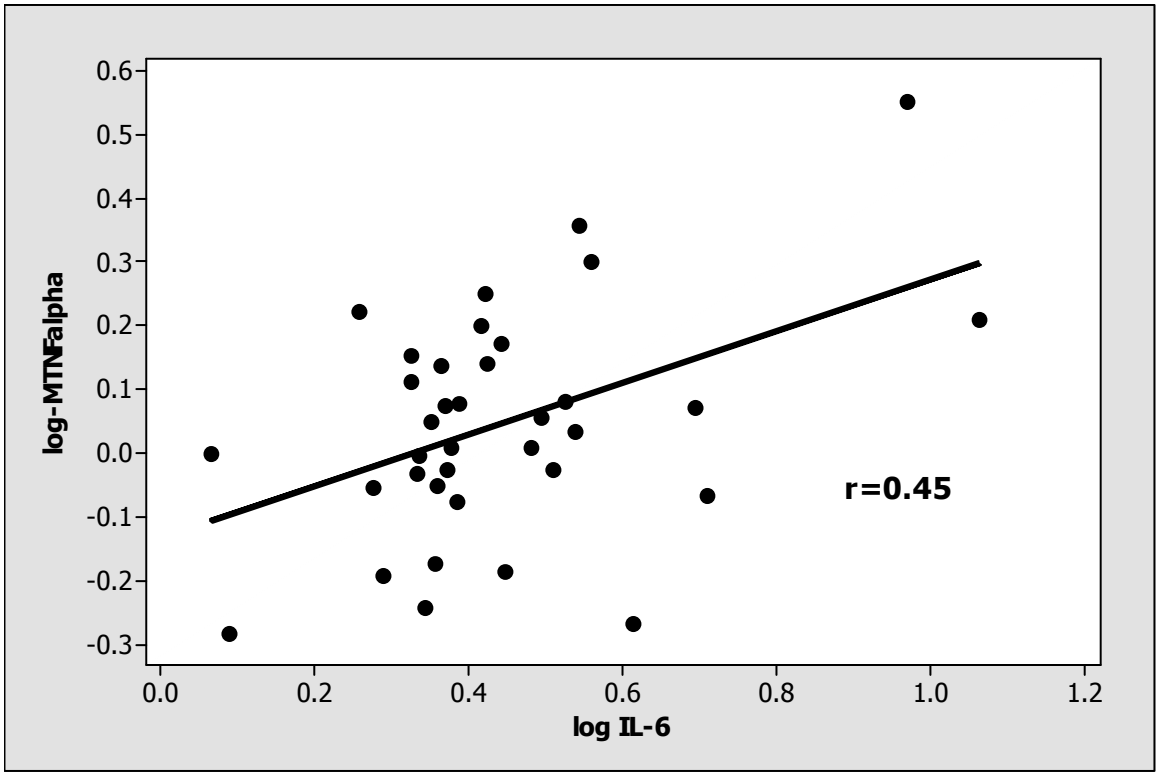

Figure 48 Relationship between maternal plasma IL-6 and TNF alpha.

Maternal IL-6 is positively correlated with maternal log TNF alpha (r=0.45, p=0.006) (n=36).

Maternal IL-6 is not correlated with any measures of lipolytic function.

3.3.11 Relationship of fat cell size with maternal and fetal characterstics

Subcutaneous fat cells are larger than visceral fat cells (109.1[1.8] vs 85.6[2.4] um, p=0.000) (Figure 49)

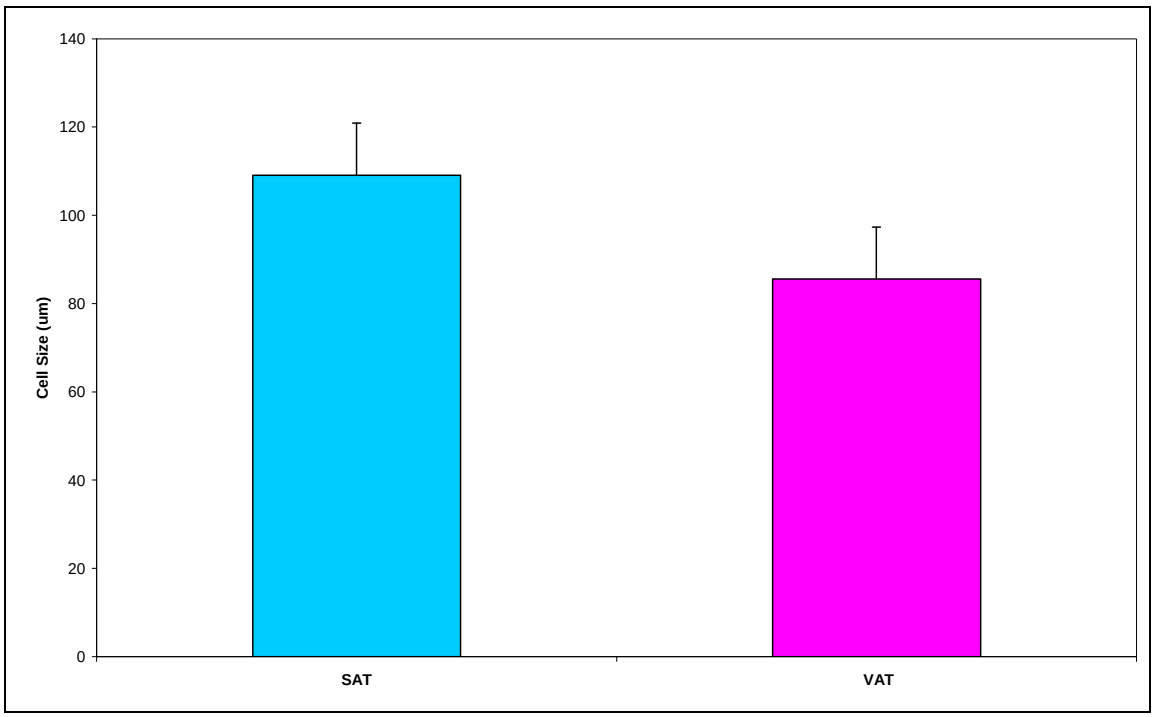

Figure 49 Comparison of size of adipocytes in SAT and VAT.

Subcutaneous fat cells are larger than visceral fat cells (mean 109.1 vs 85.6 um p=0.000) (n=36). Values displayed as mean and SEM.

Visceral fat cell size is strongly correlated with maternal BMI (r=0.50, R^2=24.8%, p=0.003) which remains robust after adjustment for age and parity (p=0.008) (Figure 50). This relationship is lacking in subcutaneous fat (p=0.085). There is no correlation of either VAT or SAT cell size and maternal BP, DEPCAT, gestation at delivery and birth-weight. Subcutaneous and visceral fat size are closely related (r=0.71, R^2=50.6%, p<0.001) independent of BMI (p<0.001) (Figure 51).

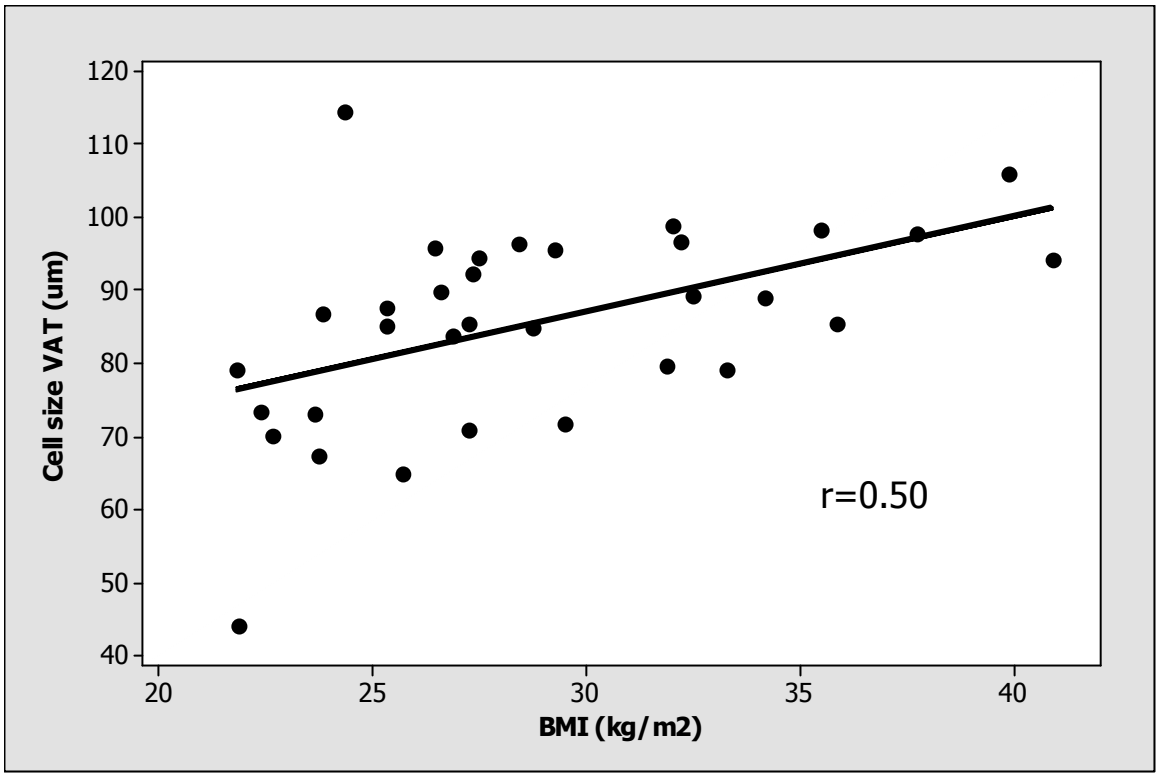

Figure 50 Relationship of BMI with VAT cell size.

Visceral fat cell size is positively correlated with BMI independent of age and parity (r=0.50, p=0.008) (n=36).

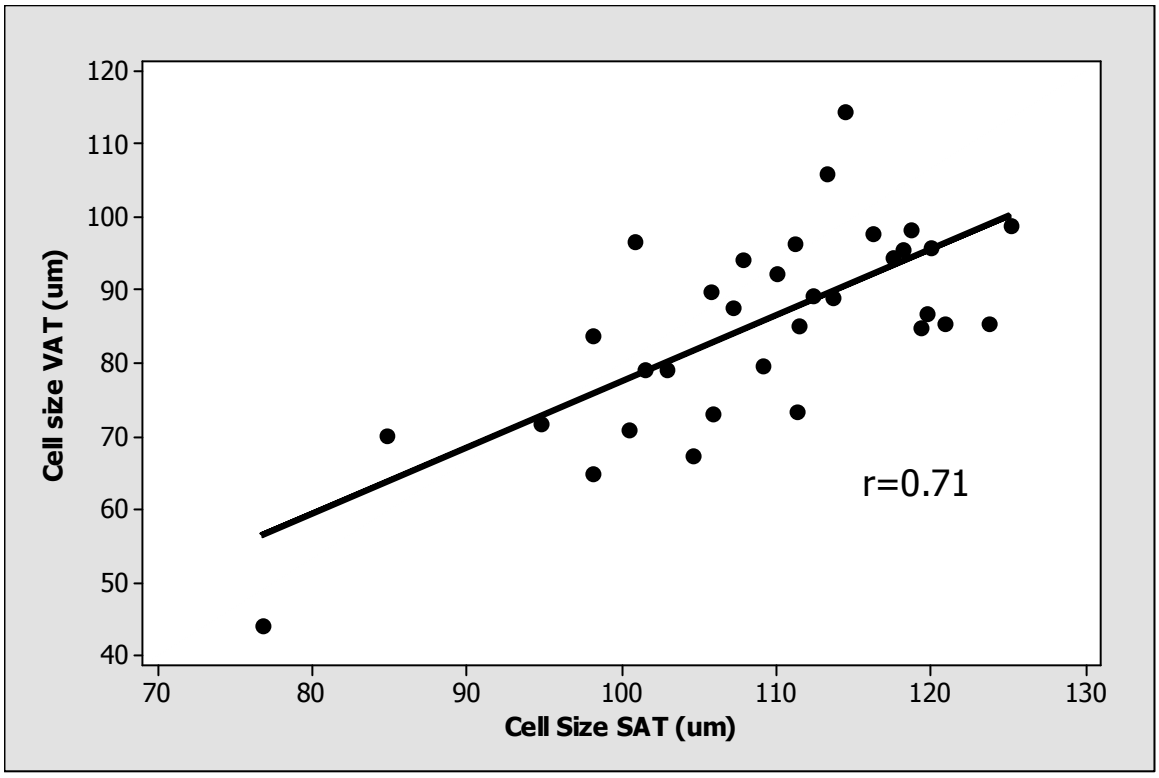

Figure 51 Relationship of VAT and SAT cell size.

Subcutaneous and visceral fat cell size are positively correlated (r=0.71, p<0.001) (n=36).

There is no correlation between fat cell size in SAT and VAT and plasma measures of insulin resistance.

3.3.12 Relationship of fat cell size with plasma inflammatory markers and adipokines

Matenal plasma leptin is positively correlated with SAT (r=0.35, R^2=12.2%, p=0.037) and VAT cell size (r=0.44, R^2=19.6%, p=0.01), but this relationship is not independent of BMI (p=0.15 and p=0.11 respectively when adjusted for BMI). Similarly maternal plasma adiponectin is negatively correlated with VAT cell size r=-0.36, R^2=12.9%, p=0.04), but this relationship is attenuated when adjusted for BMI (p=0.19). Maternal plasma IL-6 (1/1L-6) is also negatively correlated with SAT r=-0.4, R^2=15.7%, p=0.017) and VAT cell size (r=-0.41, R^2=17.0, p=0.017) but this relationship is not independent of BMI (p=0.054 and p=0.097 respectively).

Maternal plasma log CRP is positively correlated with VAT cell size but not SAT (r=0.44, R^2=19.4%, p=0.01). This is again attenuated when adjusted for BMI (p=0.07). There is no correlation between maternal TNF alpha and VAT or SAT cell size.

3.3.13 Relationship of fat cell size with measures of adipocyte lipolytic function

SAT cell size is negatively correlated with the FCISI of SAT (r=-0.41, R^2=16.5%, p=0.014) independent of age, parity and BMI (p=0.031) (Figure 52).

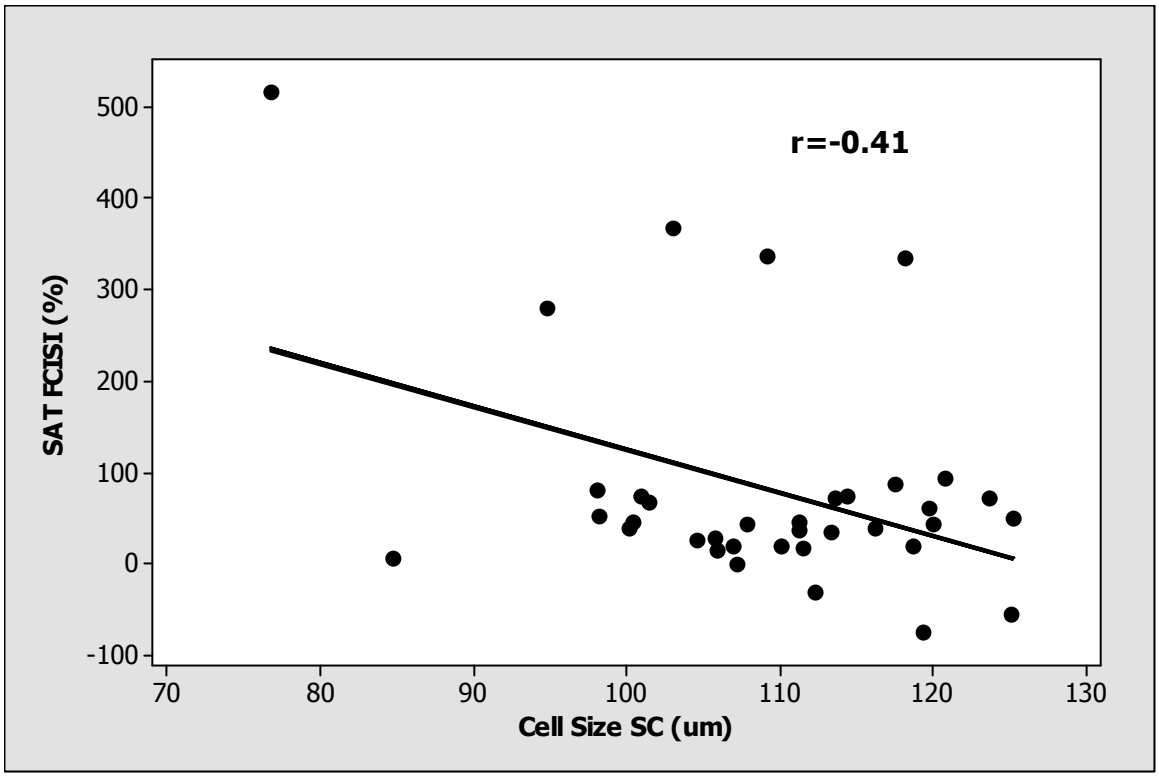

Figure 52 Relationship between fat cell size and fat cell insulin sensitivity in SAT.

Subcutaneous fat cell size is negatively correlated with SAT fat cell insulin sensitivity index (r=-0.41, p=0.031 adjusted for age, BMI and parity) (n=36).

There is no relationship between SAT cell size and absolute levels of basal lipolysis, isoproterenol stimulated lipolysis and insulin suppressed lipolysis. VAT cell size is positively correlated with basal lipolysis in VAT(r=0.39, R^2= 18.8, p=0.013)(Figure 53), lipolysis in the presence of isoproterenol (r=0.43, R^2= 26.7, p=0.002), lipolysis in the presence of insulin (r=0.45, R^2= 24.5, p=0.004) and lipolysis in the presence of isoproterenol and insulin(r=0.47, R^2= 27.1%, p=0.002) (Figure 54). There is no relationship between VAT cell size and VAT FCISI.

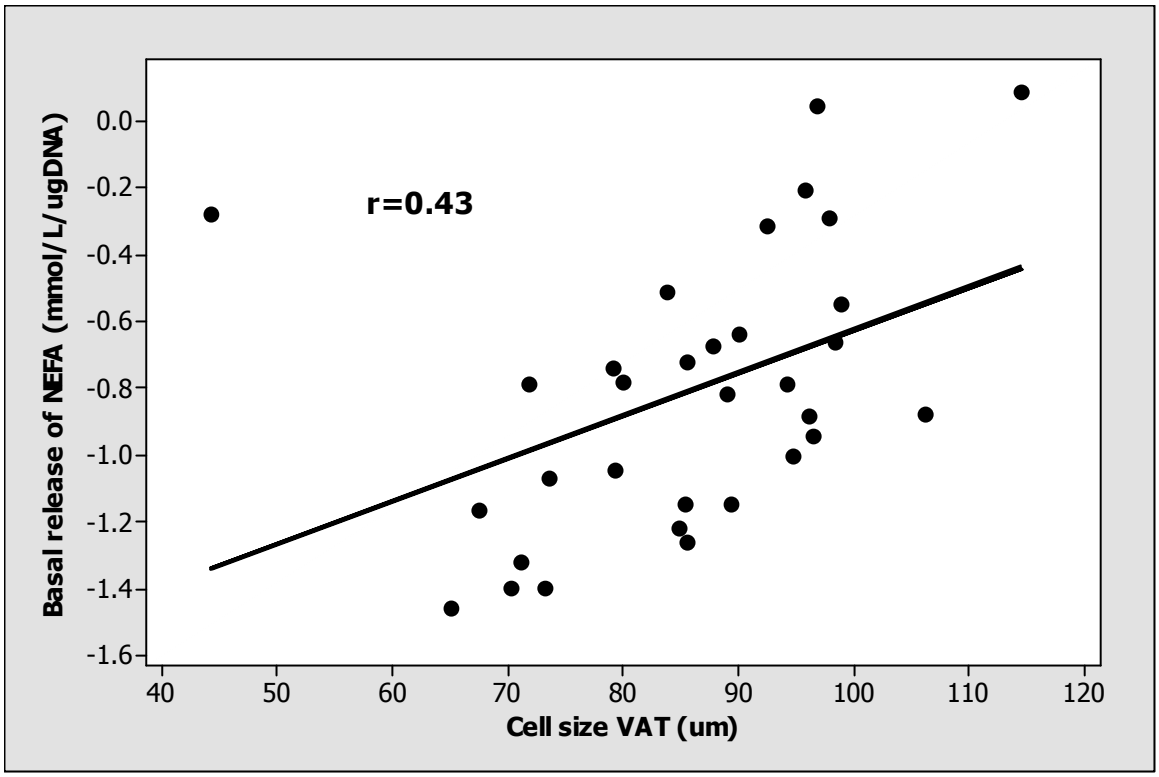

Figure 53 Relationship between VAT cell size and basal lipolysis.

Basal lipolysis when expressed as NEFA release in VAT is positively correlated with VAT cell size (p=0.013) (n=36).

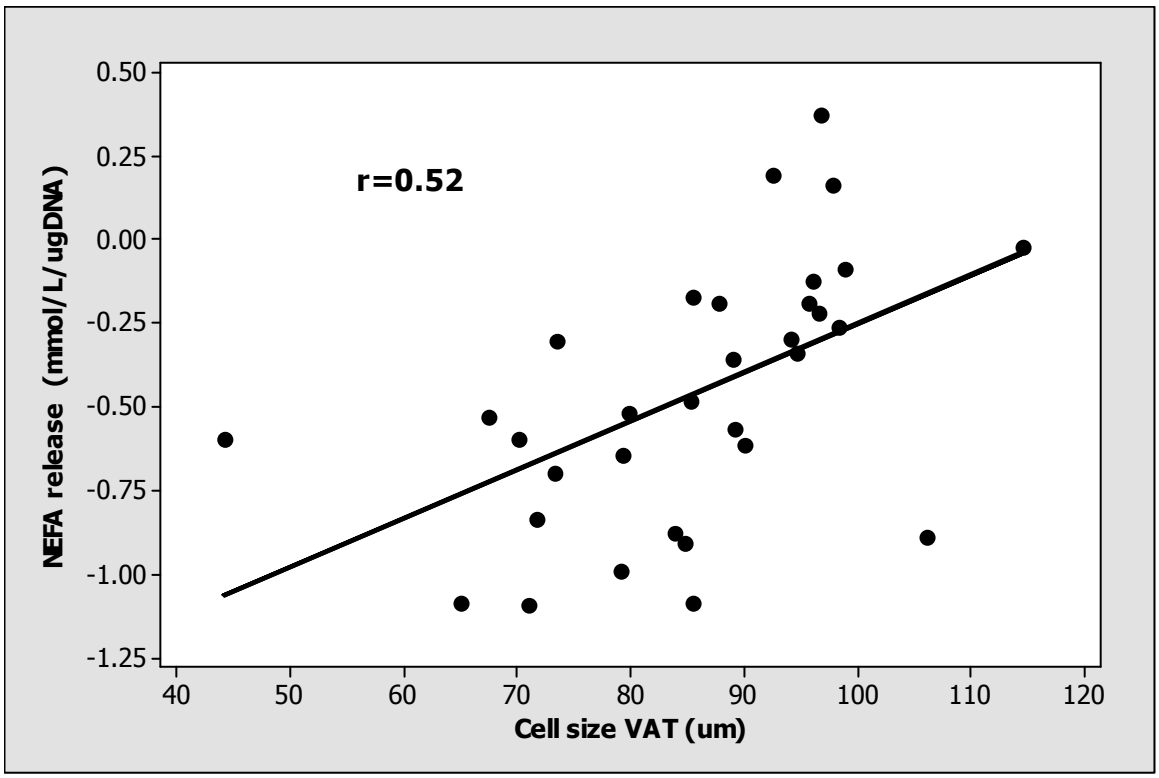

Figure 54 Relationship between VAT cell size and lipolysis in presence of isoproterenol and insulin.

VAT cell size is positively correlated with VAT lipolysis in presence of isoproterenol and insulin (p=0.002) (n=36).

3.4 Discussion

This is the first detailed examination of adipocyte lipolytic function from primary adipocytes in normal human pregnancy for over 30 years.

3.4.1 Metabolic Flexibility

We have confirmed that isoproterenol, a synthetic catecholamine that stimulates both beta1 and beta2 adrenergic receptors (with no alpha receptor capabilities) stimulated lipolysis in both subcutaneous and visceral AT. Insulin not only suppresses basal lipolysis in these two fat depots but also attenuates the effect of catecholamine stimulation. In our cohort the release of NEFA appears to be a more sensitive measure of adipocyte lipolysis than glycerol release, particularly when assessing insulin suppression of basal lipolysis. This is possibly secondary to differential effects of insulin on HSL and ATGL, with HSL being of greater importance in stimulated lipolysis and ATGL having greater emphasis in basal lipolysis[8]. In addition glycerol is the end product of lipolysis after three separate consecutive steps, whereas NEFA is released after each consecutive step (Figure 1). Our data suggests that the lower the basal lipolysis of the adipocyte in SAT the increased susceptibility to stimulation by catecholamines and reduced sensitivity to suppression by insulin. This suggests an inherent metabolic flexibility of the tissue ie those with low basal lipolysis are more readily stimulated by catecholamines and respond more effectively to 'stressful' stimuli and correspondingly as basal rates of lipolysis are already low they are less readily suppressed further by insulin. This would be metabolically advantageous in pregnancy in the third trimester to facilitate energy supply to the fetus. A similar effect is seen in VAT in that the adipocytes are more responsive to catecholamine stimulation if basal lipolysis is low, and there is an inverse relationship between the sensitivity of VAT to stimulation by catecholamines and inhibition by insulin.

3.4.2 Insulin and catecholamine sensitivity

We have found some important regional differences in adipocyte function in pregnancy. It appears that VAT and SAT function independently of each other. In our cohort, VAT is more sensitive to stimulation with isoproterenol than SAT. We have also demonstrated that VAT is less insulin sensitive than SAT. This is similar

to that seen in the non-pregnant and forms the basis of the portal paradigm. Visceral fat is more sensitive to the lipolytic action of catecholamines and less responsive to the anti-lipolytic effects of insulin thereby leading to increased NEFA release. Visceral fat is drained by the portal vein and increased NEFA have direct effects on hepatic function including hyperinsulinaemia, hyperglycaemia and dyslipidaemia. The FCISI in VAT (our measure for insulin sensitivity of the fat depot) is also related to maternal plasma markers of insulin resistance with an inverse relationship with plasma glucose and HOMA. Interestingly the converse was true for SAT. This suggests that visceral fat insulin sensitivity may be a better marker for maternal metabolic parameters than subcutaneous fat. This is supported by a study by Bartha et al which demonstrated that measures of visceral fat thickness by ultrasound correlated better with diastolic BP, glycaemia, insulinaemia, HOMA, triglycerides and HDL cholesterol than BMI as measure in early pregnancy.[52] Similarly we have also demonstrated that visceral fat insulin sensitivity is inversely correlated with maternal plasma triglycerides. NEFA supply to the liver is a major determinant of VLDL TG production[196], therefore if VAT is insulin resistant, this would result in increased delivery of NEFA from this depot thereby contributing to hyper-triglyceridamia. Indeed, splanchnic fat (a combination of VAT and liver fat) contributes to a greater degree to VLDL TG in insulin resistant men and women in the post-prandial state.[197]

3.4.3 Lipid metabolism in late pregnancy

Circulating NEFA arises from lipolysis in adipose tissue and therefore maternal plasma levels of NEFA provides a relatively crude measure of whole body lipolysis. During the latter stages of pregnancy there is a marked increase in lipolysis rates and a corresponding rise in maternal free fatty acids (FFA) and glycerol.[27 28] This change is enhanced by an increase in hormone-sensitive lipase (HSL) activity and mRNA expression and a decrease in lipoprotein lipase (LPL) activity.[29] Reduced expression of PPARγ and its target genes may also contribute to accelerated fat metabolism in late pregnancy.[32] Exaggerated catecholamine release in response to even modest maternal hypoglycaemia and the insulin resistant state of late pregnancy contribute to this switch.[30 31] Insulin effects on lipolysis (adipose tissue) and fat oxidation (in liver and muscle) are significantly impaired during the 3rd trimester compared to earlier in pregnancy and also post

partum.[31] In keeping with this, in our cohort lipolysis rates appear to increase as pregnancy advances which is reflected by the positive correlation seen between gestational age and maternal NEFA. Interestingly we also found a strong negative correlation with fasting levels of maternal NEFA and maternal levels of insulin, HOMA and glucose. This suggests that even in the late 3rd trimester of women with healthy pregnancies, AT is still sensitive to the anti-lipolytic effects of insulin in the fasting state. Similarly Frayn et al have shown that NEFA release from subcutaneous abdominal AT in IR men is still suppressed by high insulin concentrations in the fasting state.[198]

3.4.4 Adipokines, lipid metabolism and normal pregnancy

Adipokines including adiponectin, leptin, TNF alpha and IL-6 are increasingly implicated as important mediators of maternal metabolism particularly in relation to insulin resistance (IR) and lipid metabolism as detailed previously. In our cohort we have found no correlation between adiponectin and any measure of lipolytic function in either SAT or VAT in late pregnancy nor maternal NEFA and lipids. Furthermore our data are suggestive of a negative association of adiponectin with BMI (p=0.07). Similarly Catalano et al also showed no relationship with measures of lipid metabolism including maternal plasma NEFA under conditions of hyperinsulinaemia and the rate of lipid oxidation in basal and insulin-stimulated conditions. Despite in-vitro data of adiponectin influencing lipid oxidation, its action is impaired in obese subjects.[199 200] This disparity could be related to the action of other pro-inflammatory adipokines such as IL-6 or TNF alpha, which are increased in normal pregnancy. We also found no relationship between maternal markers of glucose metabolism and adiponectin. Adiponectin as previously detailed has insulin –sensitizing properties and levels are lower in insulin resistant states including type II DM and obesity. Its role in pregnancy however is still to be defined. Several studies have already suggested lack of correlation between adiponectin levels and markers of insulin resistance, particularly in complications of pregnancy including PE and IUGR.[133 201 202] In our study adiponectin is positively associated with progesterone, a pregnancy specific hormone, suggesting a possible pregnancy specific function for this adipokine. It is hypothesised that adiponectin may act in a compensatory fashion in an effort to counteract the effect of inflammation

and insulin resistance particularly when these conditions are exaggerated such as complicated human pregnancy.

As expected, there was a strong positive correlation between leptin and BMI in this cohort of normal pregnant women, consistent with increasing body fat mass. Interestingly maternal serum levels of leptin correlated with measures of VAT lipolytic function in normal pregnancy and not SAT. This suggests that in pregnancy, leptin may exert its liporegulatory effects more predominantly in VAT compared to SAT which may be due either to increased expression and secretion from this adipose depot or increased sensitivity to its effects. This may also be relevant in complicated human pregnancy - expression of leptin was upregulated in VAT but not in SAT in women with GDM. [203] Similarly despite hyperleptinaemia in pregnancies complicated by PE, there was no increased mRNA expression of leptin in SAT, suggesting either placenta or VAT as the source of excess leptin[134]. As yet no studies have examined expression of leptin in visceral fat of normal or complicated human pregnancy. Leptin appears to be a significant correlate for markers of insulin resistance in pregnancy including maternal insulin and maternal HOMA. Our data are consistent with previous studies that demonstrated correlation between measure of insulin sensitivity in pregnancy and leptin. [129 204]Although this relationship is apparent, it is still unclear whether leptin directly influences insulin resistance in pregnancy.

In our cohort, CRP is associated with increasing BMI. As in the non –pregnant, we have shown that CRP is independently associated with leptin and negatively correlated with adiponectin (although not independent of BMI), which underscores the potential role of interactions between adipokines.[205] Our data suggests that CRP is more closely related to visceral adiposity (and in particular lipolytic function), a feature already demonstrated in both healthy and IR states in the non-pregnant.[206-208] The direct anatomical relationship of VAT and the liver, the major source of CRP, may underlie this apparent association.

Our data again highlights regional differences in AT functionality in normal pregnancy. TNF alpha is strongly correlated with basal lipolysis in SAT and other measures of lipolytic function in this fat depot such as percentage stimulation of lipolysis by catecholamines and percentage suppression by insulin. There are no apparent correlations with measures of VAT lipolytic function. Regional

differences in TNF alpha and its effects on lipolysis have not been well characterized in the non-pregnant, although our data suggest a more dominant role for TNF alpha in SAT lipid metabolism in late pregnancy. Kirwan et al found that TNF –alpha was correlated with insulin sensitivity in late pregnancy and was the most important predictor of insulin sensitivity when compared to the reproductive hormones, leptin and cortisol. In contrast we found no correlation between TNF alpha and plasma markers of insulin resistance including glucose, insulin and HOMA.

We have shown that IL-6 is positively correlated with the birth weight centile of women with normal healthy pregnancies. IL-6 has been implicated in fetal growth although the studies examining this relationship are few and contradictory.[209 210] Recently Catalano et al have shown that IL-6 is higher in both maternal and cord blood of women who are obese compared to lean, with obese women having bigger babies.[211] Increased Il-6 may reflect increased insulin resistance rather than increasing fat mass per se with a corresponding increase in fetal insulin and size.

3.4.5 Adipocyte cell size

Fat cell size and relation to adipocyte function in pregnancy also demonstrates important variations according to AT location. We have demonstrated that booking BMI does not seem to be an important determinant of adipocyte lipolytic function unlike fat cell size. As in the non-pregnant woman, visceral adipocytes are smaller than subcutaneous adipocytes, and more closely related to BMI than subcutaneous fat. This may in part be explained by the finding in non-pregnant: women with higher subcutaneous fat mass exhibit both adipocyte hypertrophy and hyperplasia, whereas increased omental fat was primarily due to hypertrophy.[114] Visceral fat cell size appear to be more closely related to direct measures of lipolytic function including basal lipolysis and absolute levels of stimulated, suppressed and insulin attenuated stimulated lipolysis, than subcutaneous fat cell size. In contrast subcutaneous fat cell size is related to the FCISI of the tissue with larger fat cells being less insulin sensitive than their smaller counterparts. This is consistent to previous studies which have shown that enlarged subcutaneous adipocytes independently predict insulin sensitivity and type 2 DM.[212 213] Our data demonstrate correlations between adipokines and

fat cell size in both visceral and subcutaneous fat although not independent of BMI. Increasing cell size is predominantly correlated with the pro-inflammatory adipokines of leptin, and CRP but negatively correlated with the anti-inflammatory adiponectin. This may be one mechanism through which obesity leads to increased inflammation and IR in pregnancy resulting in adverse metabolic complications.

3.4.6 Summary

My findings are summarised by the following diagrams:

Error! Objects cannot be created from editing field codes.

Figure 55 Visceral fat function in normal pregnancy

As BMI increases in normal pregnancy so does visceral fat cell size, with resultant increase in maternal leptin, CRP, IL-6 and lower adiponectin levels. Leptin and CRP correlate with lipolytic function in visceral fat. The insulin sensitivity of visceral fat lipolysis is related to measures of maternal insulin resistance including HOMA, glucose and triglycerides.

In subcutaneous fat there is no relationship with maternal BMI and fat cell size, although cell size does correlate with the insulin sensitivity of the fat depot. Maternal TNF alpha is closely related to lipolytic function in SAT in normal pregnancy. However lipolysis in SAT does not appear to influence maternal markers of insulin resistance and glucose metabolism.

4 A Comparison of Adipocyte Lipolytic Function between Normal and Pre-eclamptic Pregnancies

4.1 Introduction

Pre-eclampsia (PE) occurs in 2-4% of pregnancies and is a leading cause of maternal and neonatal morbidity and mortality in the developed world. It is a multi-system disorder resulting in the classic manifestations of hypertension due to vasoconstriction, proteinuria due to glomerular damage and oedema due to increased vascular permeability.[84] As yet the underlying pathogenesis of the disorder had not been completely understood. The clinical signs of PE are relatively simple manifestations of a complex underlying pathological process with activation of the coagulation system, platelets and leukocytes and disturbances in metabolism which combine to provoke widespread endothelial damage and dysfunction. This in turn augments further activation of leukocytes and coagulation resulting in a vicious cycle of vascular injury. The disorder is likely to be a result of heterogeneous causes resulting from the interaction of placental and maternal factors.[85] In the presence of a placental trigger the maternal response will depend on the maternal genotype and phenotype resulting in the clinical syndrome of PE.[86] There are several reasons, as discussed in Chapter 1, why disordered lipid and adipocyte metabolism could contribute to the pathogenesis of this condition and include lipid accumulation at sites of endothelial damage, exaggerated dyslipidaemia and an early rise in FFA independent of maternal adiposity suggesting early exaggerated adipocyte lipolysis. There is preliminary evidence that the trigger for this increase in FFA and adipocyte lipolysis is present in serum of women with PE.[107] These hypotheses are summarised in Figure 56.

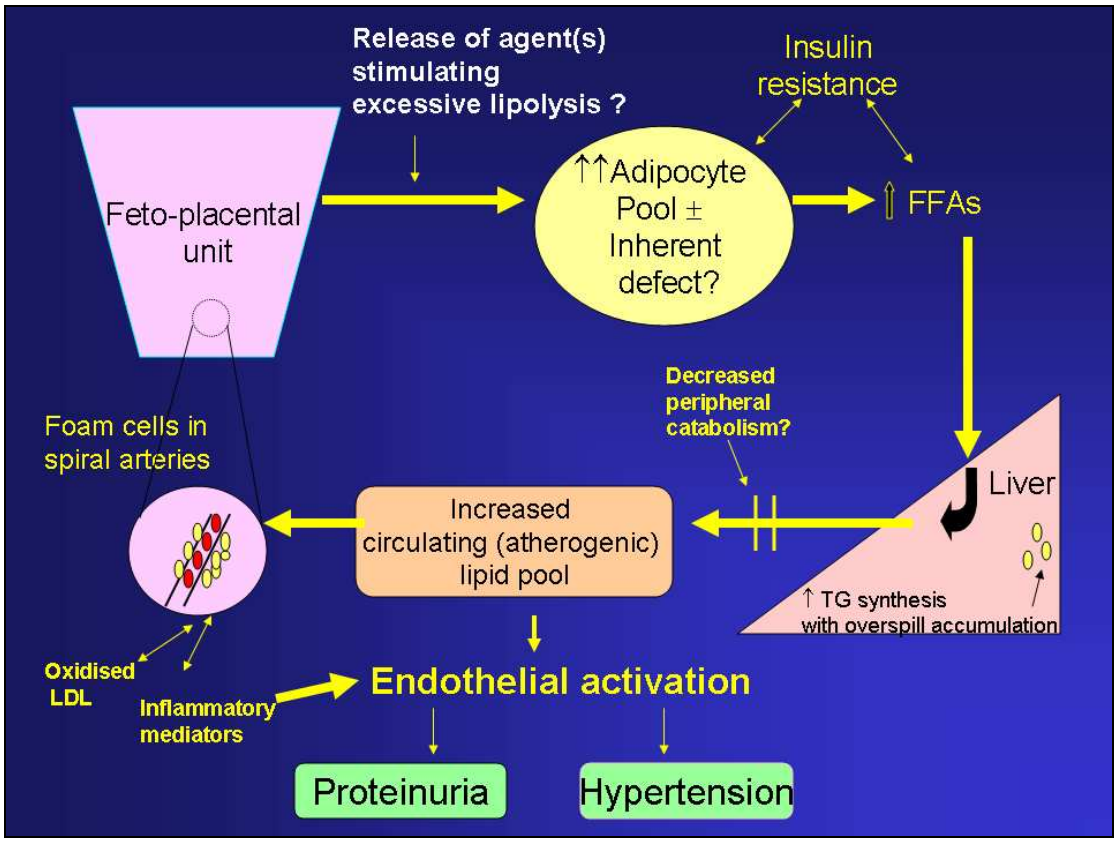

Figure 56 A summary of the potential role of a disturbance in lipid metabolism in the pathogenesis of pre-eclampsia.

A factor(s) released from the placenta enhances peripheral lipolysis which is already stimulated in normal pregnancy by HPL. This results in an increased flux of free fatty acids to the liver. These are channelled predominantly into hepatic triglyceride synthesis so there is increased secretion (over and above normal pregnancy) of triglyceride-rich lipoproteins ($VLDL_1$). Accumulation of triglyceride occurs in the hepatocyte when this pathway is saturated. Increased concentrations of $VLDL_1$ in the circulation drives the production of an atherogenic lipoprotein profile by stimulating excessive synthesis of small, dense LDL (LDL-III) and by lowering HDL-cholesterol. This lipid profile may contribute to endothelial dysfunction and therefore the expression of pre-eclampsia in the mother. Finally, this pathway plays a part in the formation of lipid-laden macrophages (foam cells) in the spiral arteries of the decidua basalis, and as a result, may be involved in the enhanced placental production of pro-inflammatory mediators in PE.

4.2 Aims and Objectives

4.2.1 Hypotheses

1. That adipocyte release of fatty acids is exaggerated under either basal or stimulated conditions in women with PE, thereby implicating adipocyte function in its pathophysiology.

2. That plasma from women with PE excessively stimulates adipocytes from healthy women as compared to plasma taken from healthy controls thereby suggesting the presence of an excessive lipolytic factor in PE plasma.

4.2.2 Specific Research Questions

1. Are there differences in basal or isoproterenol-stimulated lipolysis between adipocytes derived from women with pregnancies complicated by PE compared to healthy pregnant women matched for age, smoking and booking BMI?

2. Is insulin-suppression of isoproterenol-stimulated lipolysis impaired in PE, and does the pattern of defects in PE reflect that in other insulin resistant conditions?

3. Does plasma from women with PE stimulate adipocyte lipolysis excessively in comparison to plasma from healthy pregnant women matched for age, BMI, and smoking?

4. Is the adipocyte defect dependent predominantly in one functional tissue depot - i.e. visceral vs subcutaneous?

4.3 Results

Fourteen cases each with two matched controls for age, BMI and smoking were recruited as described in methods 2.1. Processing of tissue and lipolysis assays were carried as detailed in methods sections 2.2 -2.7. Maternal biochemical markers were determined as detailed in methods section 2.12.

4.3.1 Subjects

The characteristics of the subjects are described in Table 8.

Characteristics	*Controls (n=28)*	*PE (n=14)*	*P value*
Age, y	**30.8(5.1)**	**31.5(6.3)**	**0.72**
BMI, kg/m^2	**29.6(5.25)**	**31.1(8.0)**	**0.53**
Smokers (non,current)**	**26,2**	**11,2**	**0.41**
DEPCAT*	**5(4-6)**	**6(4-7)**	**0.14**
Gestation at delivery, days	**272.5(9.1)**	**249.4(21.5)**	**0.002**
Parity (0,≥1)**	**6,22**	**8,6**	**0.02**
Systolic pressure, mmHg	**115.3(14.4)**	**127.1(13.1)**	**0.013**
Diastolic pressure, mmHg	**69.6(8.6)**	**79.1(9.0)**	**0.003**
Birthweight, g	**3525(533)**	**2330(926)**	**0.001**
Birthweight centile	**60.3(28.1)**	**26.0(31.2)**	**0.003**

Table 8 Characteristics of cases and controls.

Blood pressure refers to booking values. All values expressed as mean and standard deviation (*median and interquartile range). Comparisons made by paired t test except * Mann-whitney, and ** chi-squared test.

Subjects were matched for age, BMI and smoking. Due to the most common indication for elective caesarean section being previous caesarean section it was difficult to obtain sufficient primiparous women in the control group to match for parity. Women with PE had significantly higher systolic and diastolic BP at booking. They were more likely to deliver earlier than the control group and had babies with lower birthweight and birthweight centile (adjusting for gestational age).

4.3.2 Maternal Lipids and Plasma Markers of Insulin Resistance

Maternal triglycerides were significantly elevated in PE compared to controls. There was no difference in total cholesterol or HDL between the two groups. Maternal NEFA were also significantly elevated in PE compared to controls. Although maternal glucose, insulin and HOMA were all higher in PE compared to controls, these differences did not reach significance. These results are summarised in Table 9.

Plasma Markers	*Controls (n=28)*	*PE (n=14)*	*P value*
Total Cholesterol (mmol/L)	6.24 (0.97)	6.53 (1.48)	0.53
Triglycerides (mmol/L)*	2.6 0.58)	3.9 (2.3)	0.02
HDL (mmol/L)	1.86 (0.36)	1.7 (0.41)	0.24
NEFA (mmol/L)*	0.4 (0.18)	0.55 (0.24)	0.02
Glucose (mmol/L)	4.7 (0.41)	5.3 (1.2)	0.09
Insulin (mU/L)*	10.9 (7.2)	16.6 (12.3)	0.20
HOMA*	2.38 (1.8)	4.2 (0.98)	0.14

Table 9 Comparison of maternal lipids and plasma markers of insulin rsistance.

All values expressed as mean and standard deviation. Statistical analysis by paired t-test.

***Data transformed to normal distribution but expressed as non-transformed values.**

4.3.3 Differences in Lipolysis

4.3.3.1Subcutaneous Adipose Tissue

There is no difference in basal SAT lipolysis between control and PE when expressed either as release of NEFA (0.56[0.25] vs 0.68[0.29] sqrt NEFA mmol/L/ugDNA, p=0.23) (Figure 57) or glycerol release (9.88[4.25] vs 12.1[6.1] sqrt glycerol umol/L/ugDNA, p=0.24).

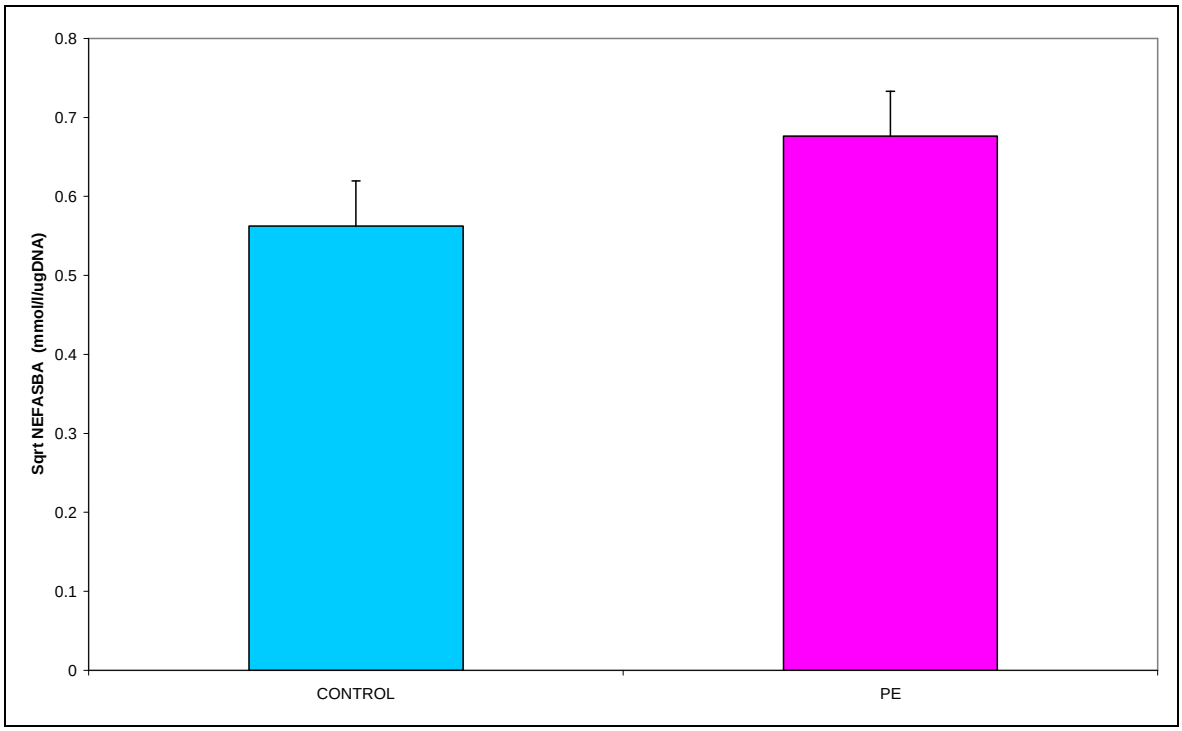

Figure 57 Basal Lipolysis in SAT between controls and PE.

There is no significant difference in basal lipolysis when expressed as NEFA release between controls and PE (p=0.226) (PE:n=14, controls:n=28). NEFA SBA = SAT basal release of NEFA. Comparisons made using student t-test and results displayed as mean and SEM.

There is no difference in rates of lipolysis in the presence of isoproterenol in SAT when expressed as either release of NEFA (0.77[0.42] vs 1.0[0.72] mmol/L/ugDNA, p=0.27) (Figure 58) or release of glycerol(267[108] vs 344[214] umol/L/ugDNA, p=0.22). Similarly there is no difference in rates of lipolysis between controls and PE in the presence of insulin or insulin and isoproterenol when expressed as either release of NEFA or release of glycerol. (Figure 59)

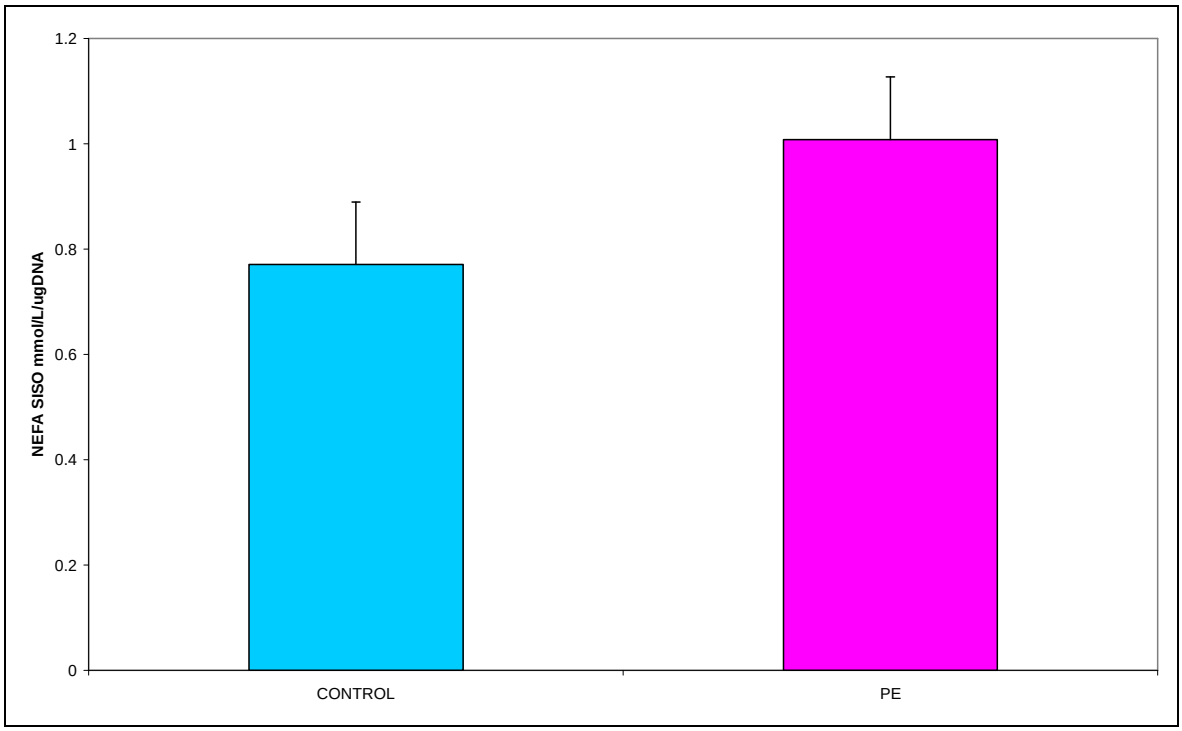

Figure 58 Lipolysis in presence of isoproterenol in controls and PE.

There is no significant difference in NEFA release in the presence of isoproterenol between controls and PE (p=0.27) (PE:n=14, controls:n=28). Comparisons using student t-test and results displayed as mean and SEM. NEFA SISO = SAT in the presence of isoproterenol.

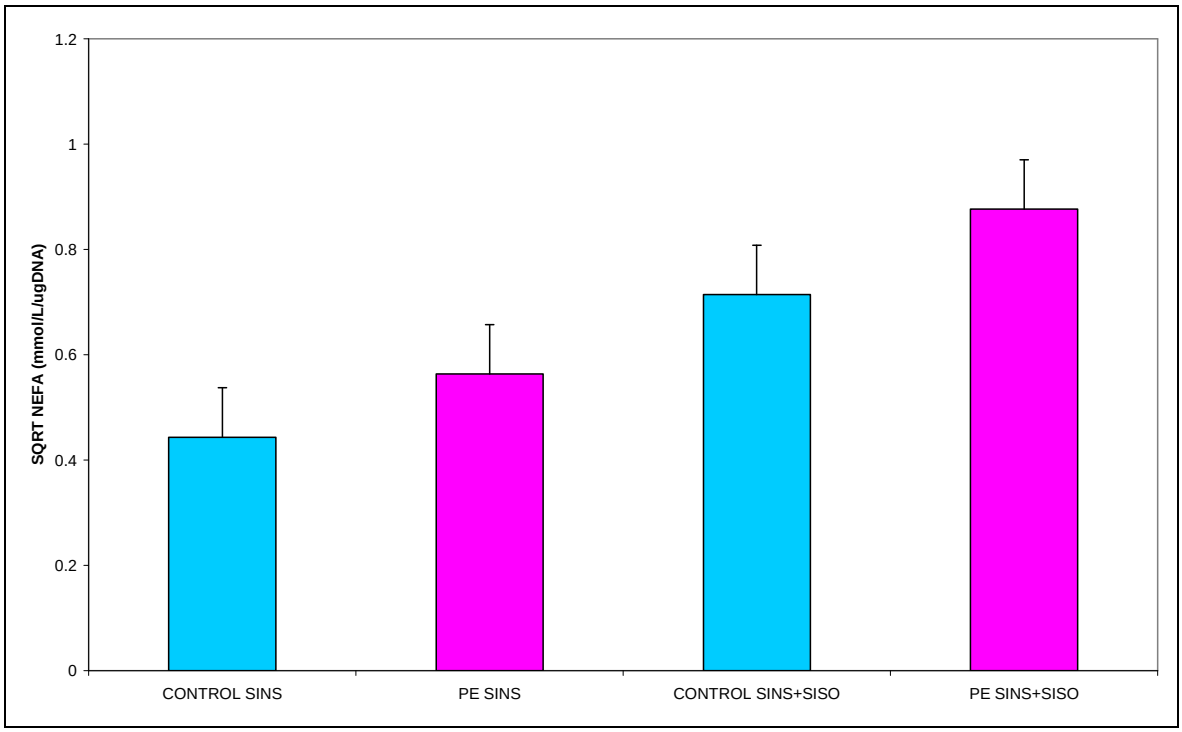

Figure 59 Lipolysis in presence of insulin in controls and PE.

There is no significant difference in NEFA release between controls and PE in the presence of insulin (p=0.133) or in the presence of insulin and isoproterenol (p=0.14) (PE:n=14, controls:n=28). Comparisons using student t-test and results displayed as mean and SEM. SINS= SAT in presence of insulin; SINS+SIO=SAT in the presence of insulin and isoproterenol.

There is no difference in percentage stimulation by isoproterenol in SAT between controls and PE (168[132] vs 130[142] %, p=0.42)(Figure 60) when related to lipolysis expressed as NEFA release. The same holds true for lipolysis expressed as glycereol release (252[217]% vs 235[313]%,p=0.85).

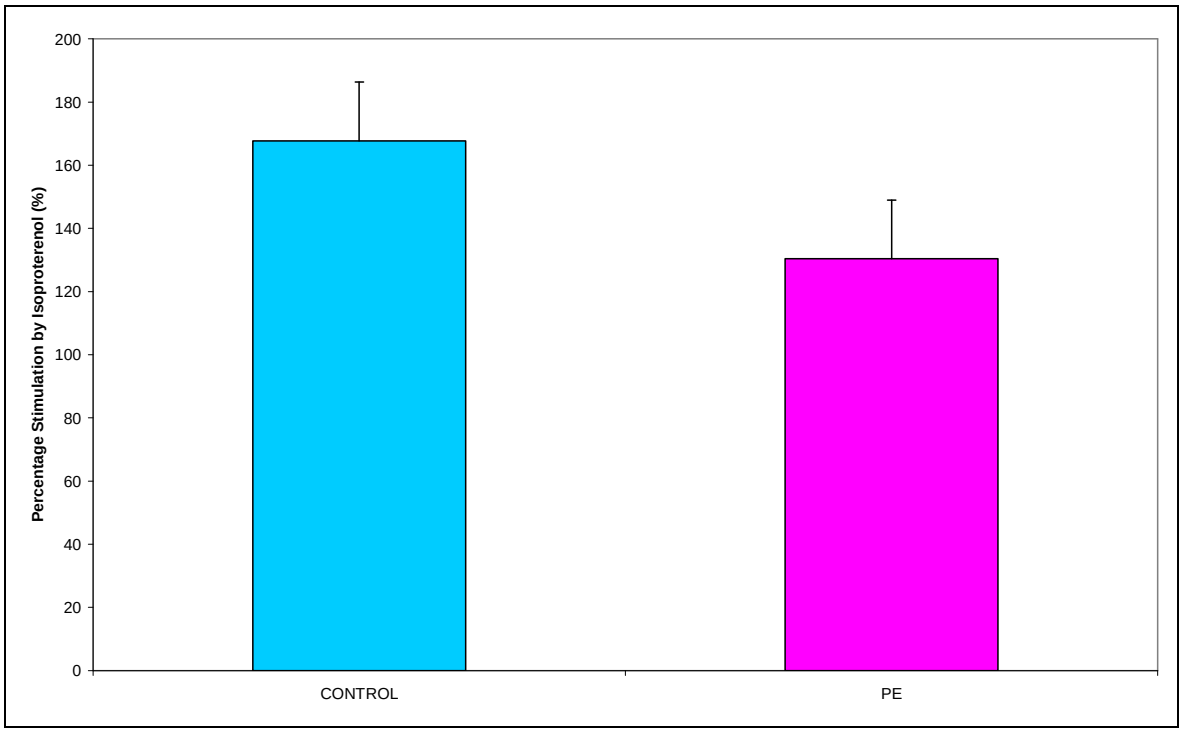

Figure 60 Percentage stimulation by isoproterenol in controls and PE.

There is no difference in percentage stimulation by isoproterenol between controls and PE (p=0.42) (PE:n=14, controls:n=28). Comparisons using student t-test and results displayed as mean and SEM.

There is no difference in percentage suppression by insulin of lipolysis between controls and PE (28[25.9] vs 22.8[29.6]%, p=0.59)(Figure 61) when expressed as NEFA release. The same holds true for glycerol release (-15.5[56.4] vs -36[107]%, p=0.51)

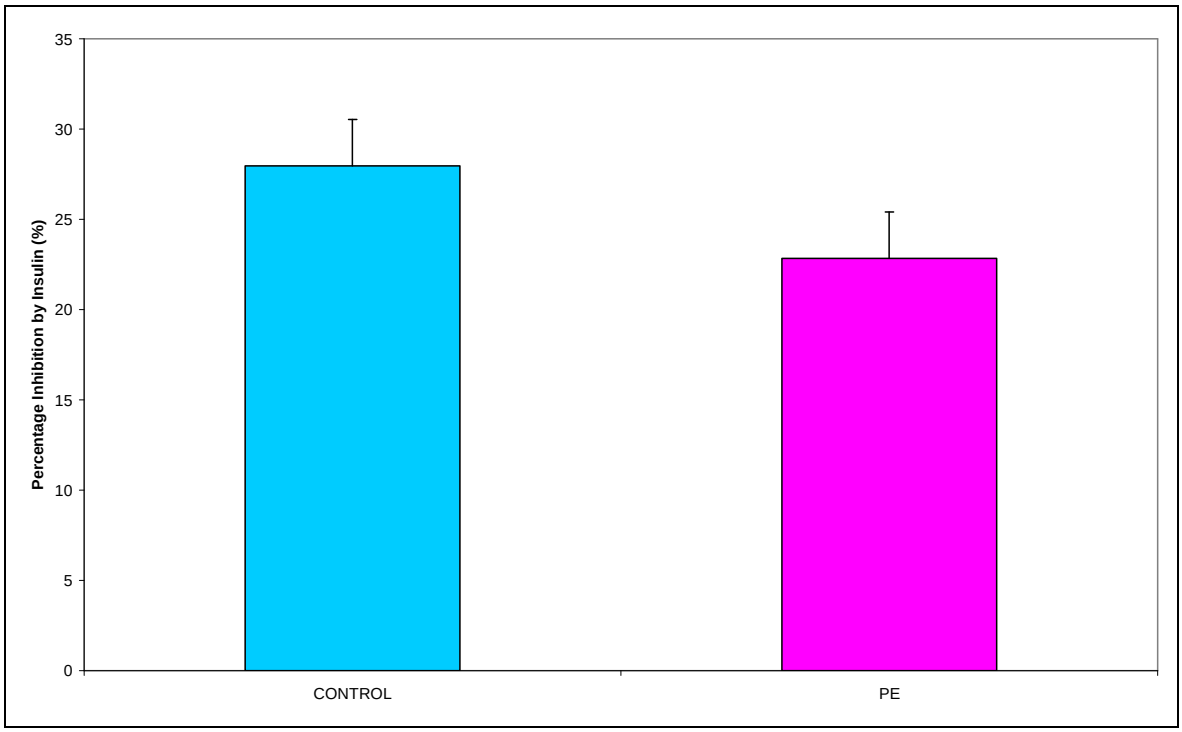

Figure 61 Percentage suppression of lipolysis by insulin in controls and PE.

There is no difference in percentage suppression of lipolysis by insulin between controls and PE when expressed as NEFA release (p=0.59) (PE:n=14, controls:n=28). Comparisons using student t-test and results displayed as mean and SEM.

4.3.3.2 Visceral Fat

In VAT there is no difference in basal lipolysis between controls and PE when expressed as release of NEFA (-0.8[0.41] vs -0.77[0.35] log NEFA mmol/L/ugDNA, p=0.81) (Figure 62) or glycerol (7.9[3.6] vs 9.54[3.6] sqrt glycerol umol/L/ugDNA, p=0.18).

There is no difference in lipolysis in presence of isoproterenol between controls and PE when expressed as release of NEFA (-0.3[0.34] vs -0.47[0.35] log NEFA mmol/L/ugDNA, p=0.16) or glycerol (13.6[3.7] vs 13.2[4.2] sqrt glycerol umol/L/ugDNA, p=0.8). Similarly there is no difference in lipolysis in presence of insulin (-0.87[0.4] vs -0.84[0.33] log NEFA mmol/L/ugDNA, p=0.8) or insulin and isoproterenol (-0.4[0.38] vs -0.48[0.33] log NEFA umol/L/ugDNA, p=0.46) between controls and PE when expressed as NEFA release (Figure 63). This is also the case when expressed as glycerol release (1.74[0.37] vs 1.9[0.34] log glycerol umol/L/ugDNA, p=0.11 and 2.17[0.29] vs 2.3[0.24] log glycerol umol/L/ugDNA, p=0.3 respectively).

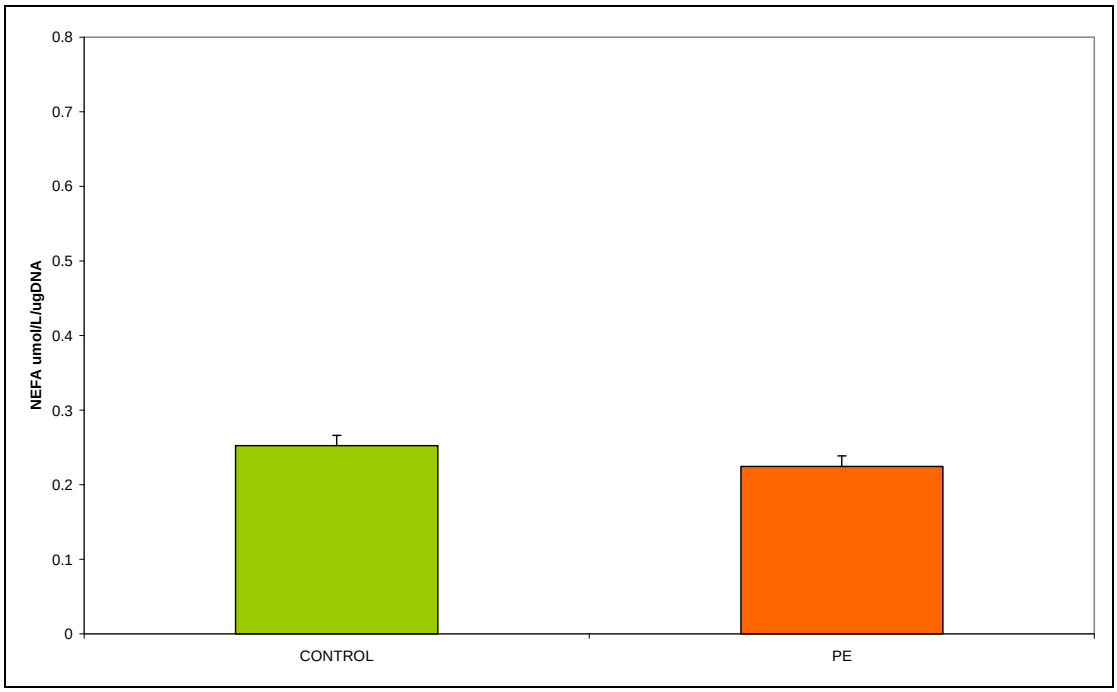

Figure 62 Basal lipolysis in visceral fat in controls and PE

There is no difference in basal lipolysis when expressed as release of NEFA between controls and PE in VAT (p=0.81) (PE:n=14, controls:n=28). Values displayed as mean and SEM of non-transformed data. Comparison made on log transformed values by student t-test.

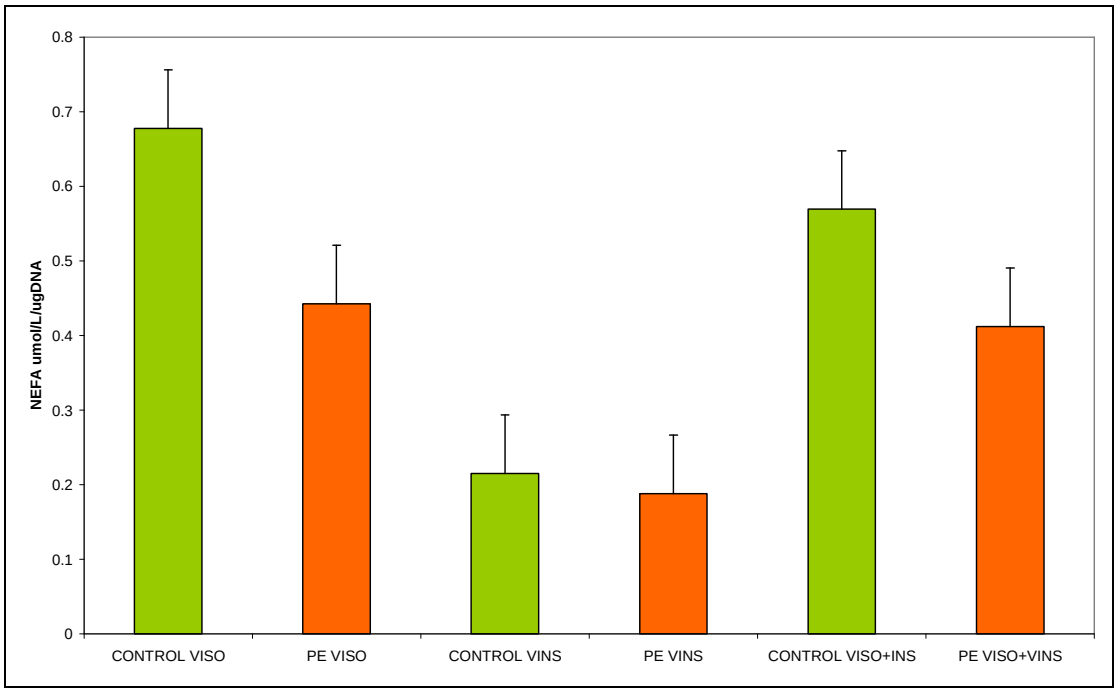

Figure 63 Lipolysis in visceral fat in controls and PE in the presence of isoproterenol, insulin and isoproterenol and insulin.

There is no difference in lipolysis between controls and PE in the presence of isoproterenol (p=0.16), insulin (p=0.8) or isoproterenol and insulin (p=0.46), when expressed as release of NEFA (PE:n=14, controls:n=28). Values displayed as mean and SEM on non-transformed data, but comparisons made by transformed data using student t-test. VISO= VAT in presence of isoproterenol, VINS= VAT in presence of insulin, VISO+INS= VAT in presence of isoproterenol and insulin.

There is no statistical difference in percentage stimulation by isoproterenol between controls and PE when expressed as NEFA release (265[176] vs 152[177]%, p=0.06) or glycerol release (385[498] vs 369[1087]%, p=0.96).

Furthermore there is no statistical difference in percentage inhibition by insulin between controls and PE when expressed as NEFA release (4.5[44.1] vs 11.6[20.5]%, p=0.48) or glycerol release (-44[179] vs -25.9[89.7]%, p=0.67)

4.3.4 Fat Cell Insulin Sensitivity Index

A direct measure of insulin sensitivity of the fat cell was calculated from the percentage inhibition of catecholamine stimulated lipolysis by insulin measured from the release of NEFA. This was derived from the following calculation (Figure 27):

(SISO-SISO+INS)/(SISO-SBA)*100

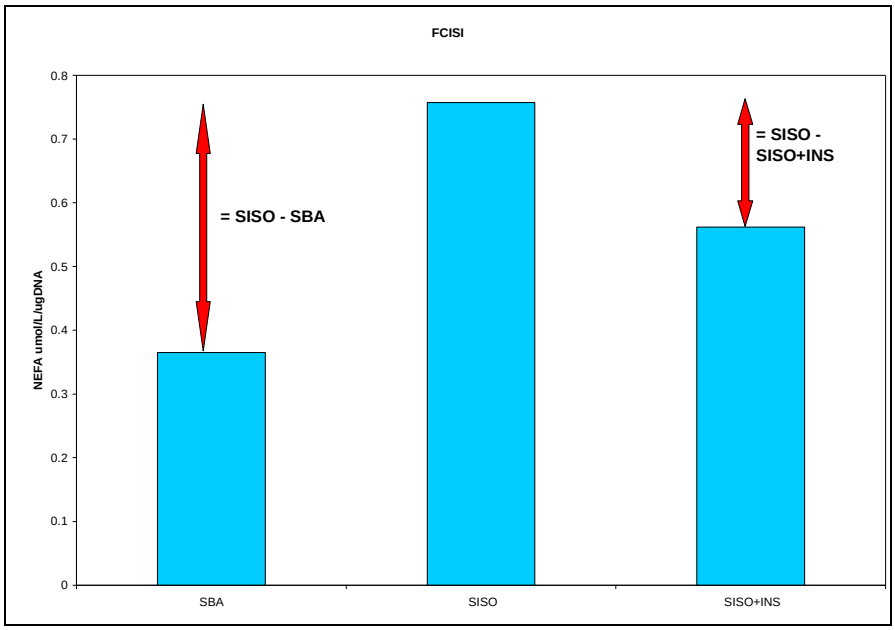

Figure 64 The fat cell insulin sensitivity index

Fat cell insulin sensitivity index is calculated from the percentage inhibition of catecholamine stimulated lipolysis by insulin ie (SISO-SISO+INS)/(SISO-SBA)*100. SBA= SAT basal lipolysis, SISO= SAT in presence of isoproterenol, SISO +INS=SAT in presence of isoproterenol and insulin.

This was called the fat cell insulin sensitivity index (FCISI).

Using this index we found that SAT in controls was more insulin sensitive than in PE (65.7[18] vs 15.8[13], p=0.032) (Figure 65).

Furthermore, VAT also appears to be more insulin sensitive in controls compared to PE although this did not quite reach significance (35.2[14] vs -3.0[14], p=0.06)(Figure 66)

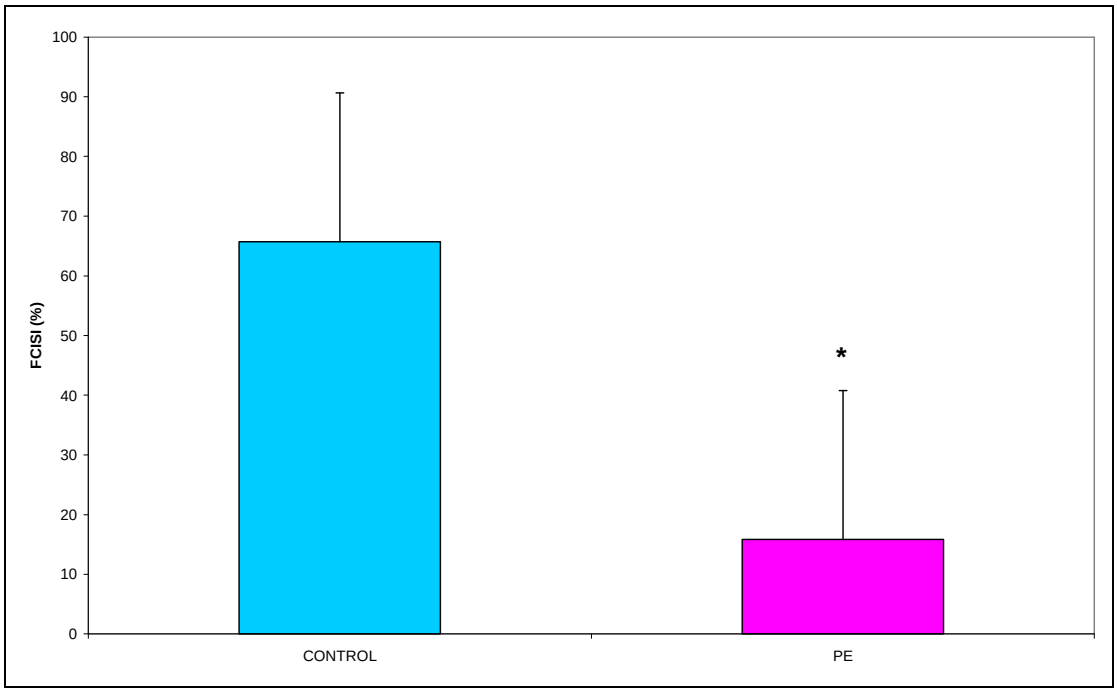

Figure 65 The fat cell insulin sensitivity index of SAT in controls and PE.

The FCISI of SAT is greater in controls than in PE, p=0.032 (PE:n=14, controls:n=28). Comparisons using student t-test and results displayed as mean and SEM.

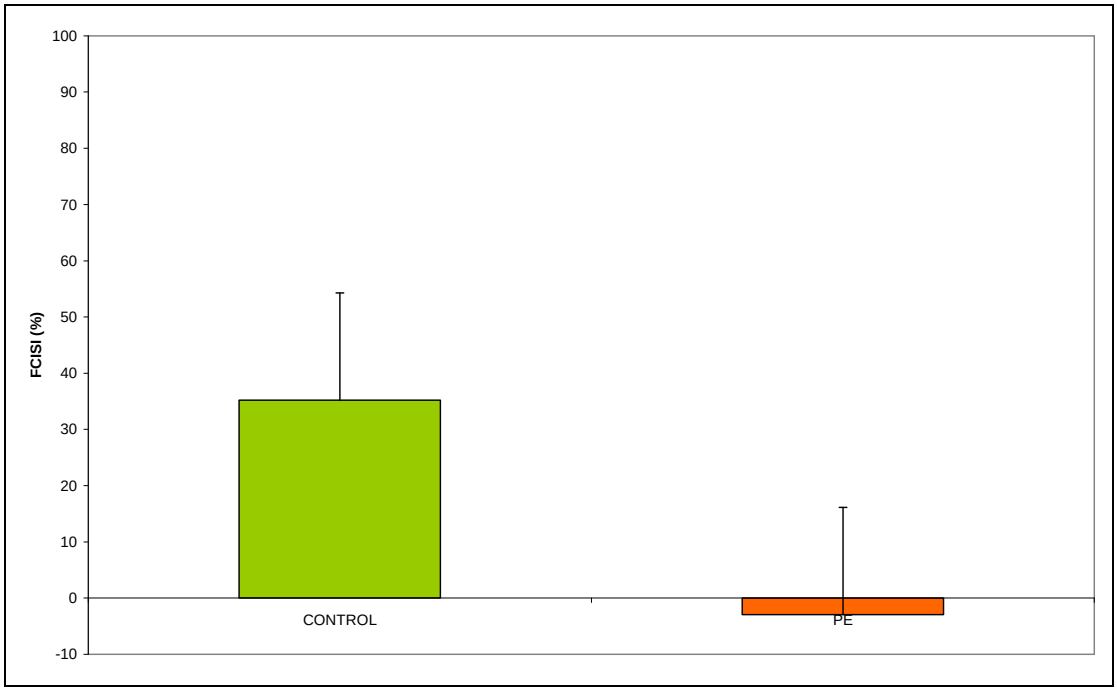

Figure 66 The fat cell insulin sensitivity index of VAT in controls and PE.

The FCISI (fat cell insulin sensitivity index) of VAT appears greater in controls than in PE (p=0.06) (PE:n=14, controls:n=28). Comparisons using student t-test and results displayed as mean and SEM.

4.3.5 Regional differences in adipocyte cell size and adipocyte function between controls and PE.

4.3.5.1 Relationship of adipose fat cell size and BMI

SAT cell size is closely correlated with BMI in PE (r=0.69, R^2=47.1%, p=0.007)(Figure 67) as is VAT cell size (r=0.83, R^2=69.5%, p<0.0001)(Figure 68). This is in contrast to that seen in the normal population as described in Chapter 3 where only VAT cell size correlates with BMI. Indeed when we looked at the 28 matched controls (a sub population of the "normal population") the same lack of association between SAT cell size and BMI is seen (p=0.82)(Figure 69), but is present in VAT (r=0.39, R2=15.2%, p=0.04).

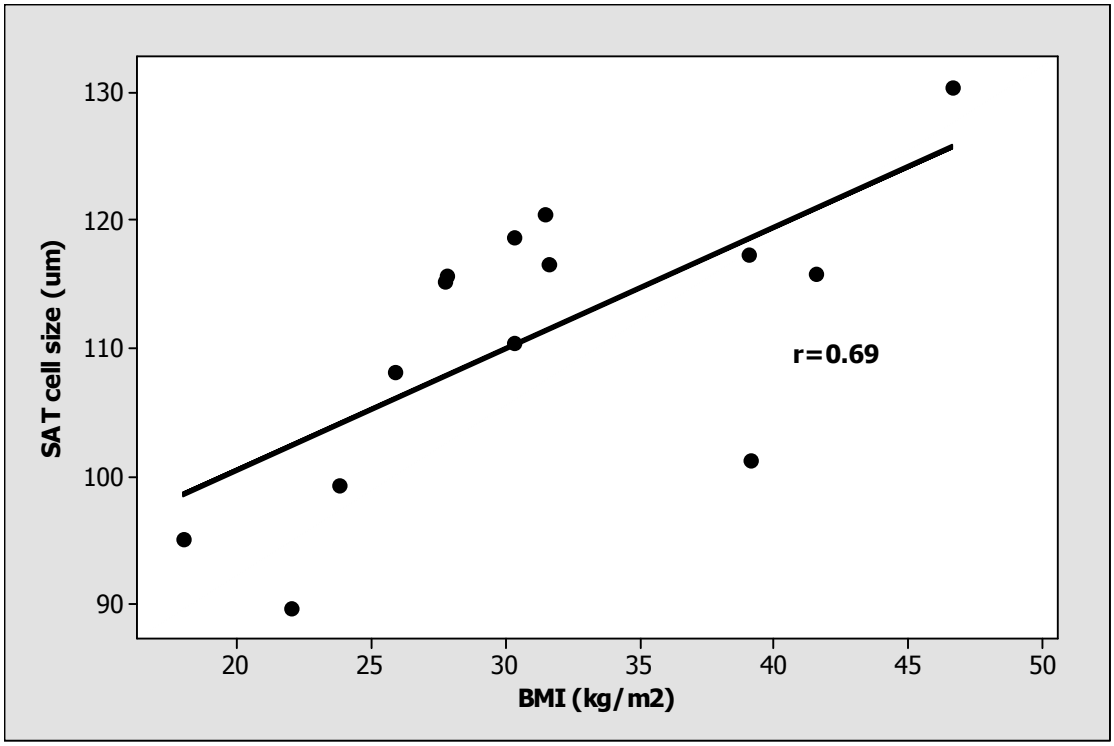

Figure 67 The relationship between maternal BMI and SAT cell size in PE.

There is a positive correlation between maternal BMI and SAT cell size in PE (p=0.007) (n=14).

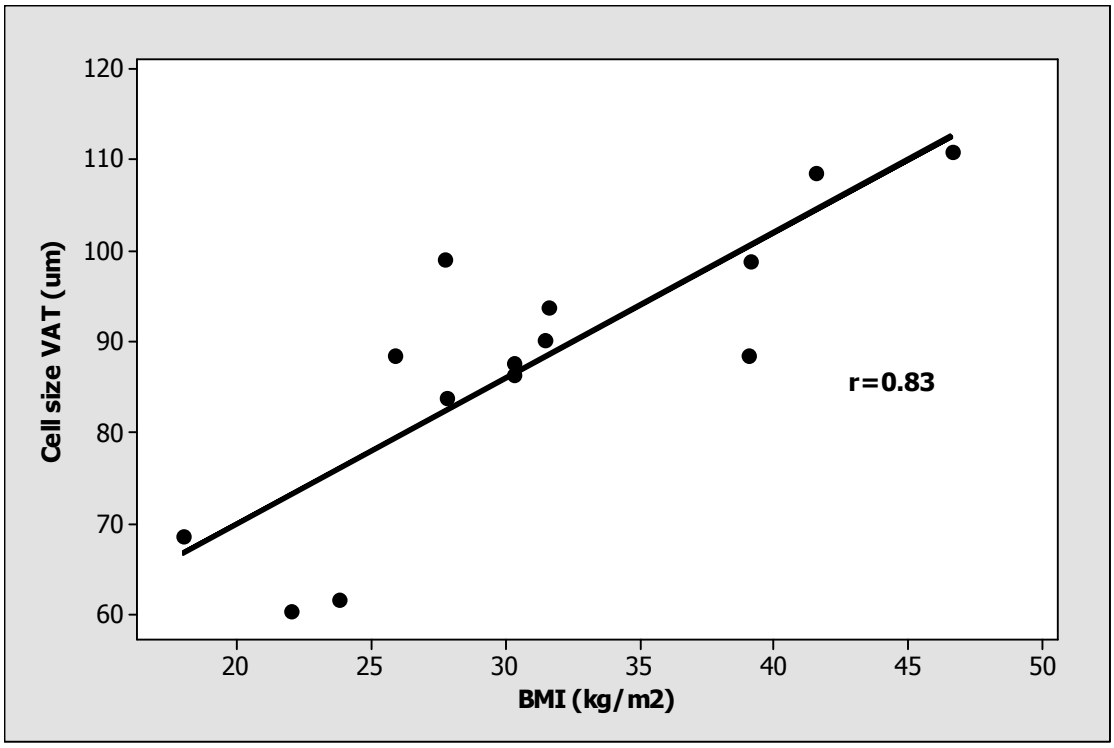

Figure 68 The relationship between maternal BMI and VAT cell size in PE.

There is a positive correlation between maternal BMI and VAT cell size in PE (p<0.0001) (n=14).

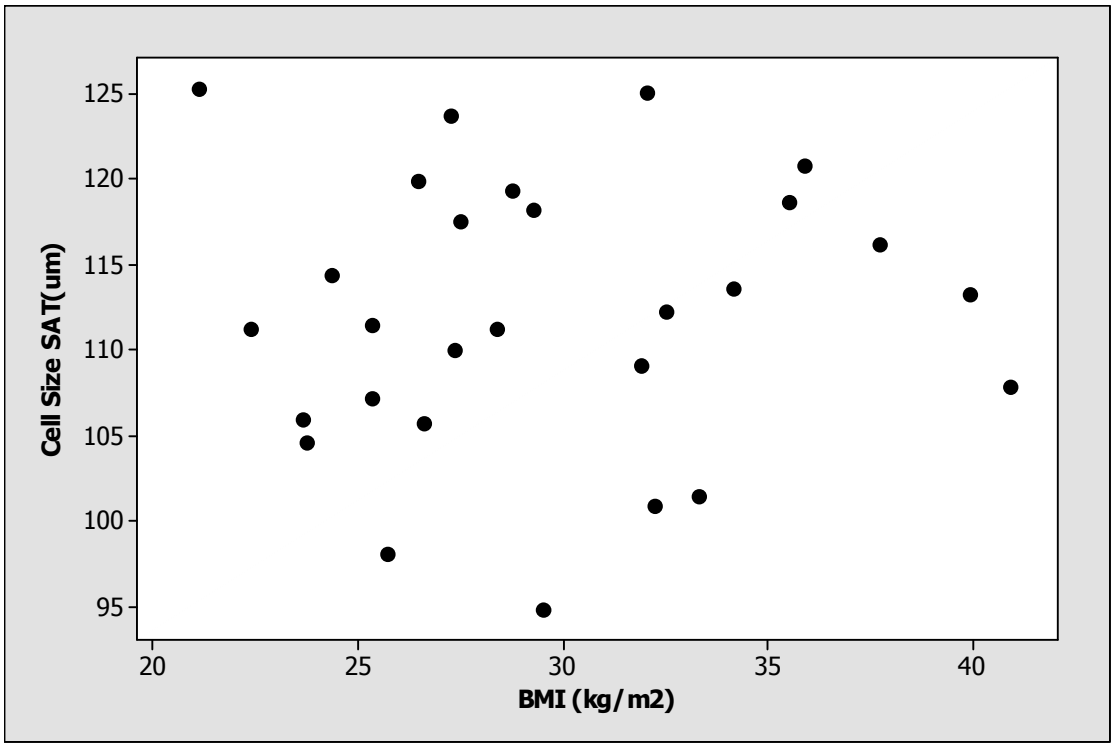

Figure 69 The relationship between BMI and SAT cell size in controls.

There is no correlation between maternal BMI and SAT cell size in controls (p=0.82) (n=28).

4.3.5.2 Relationship of adipocyte cell size and lipolytic function

Also interestingly we have demonstrated that SAT cell size is closely correlated with SAT lipolytic function in PE including basal lipolysis(r=0.67, R^2=45.1%,p=0.009)(Figure 70), lipolysis in presence of isoproterenol (r=0.6, R^2=35.8%,p=0.024) and in presence of isoproterenol and insulin (r=0.66, R^2=43.3%, p=0.01) which becomes more robust after adjustment for BMI and parity(p=0.007, p=0.004 and p=0.004 respectively). Conversely these associations are lacking in normal controls. However VAT cell size in controls strongly correlates with VAT cell function including basal lipolysis (r=0.67,R2=45.5%, p=0.001 adjusted for BMI and parity)(Figure 71), lipolysis in presence of isoproterenol (r=0.56, R2=31.1%,p=0.015 adjusted for BMI and parity), lipolysis in presence of insulin(r=0.64, R2=41.2%,p=0.003 adjusted for BMI and parity), and lipolysis in presence of isoproterenol and insulin (r=0.54, R2=28.6%, p=0.036 adjusted for BMI and parity) suggesting that in normal pregnancy VAT cell size is an important determinant of visceral fat cell function.

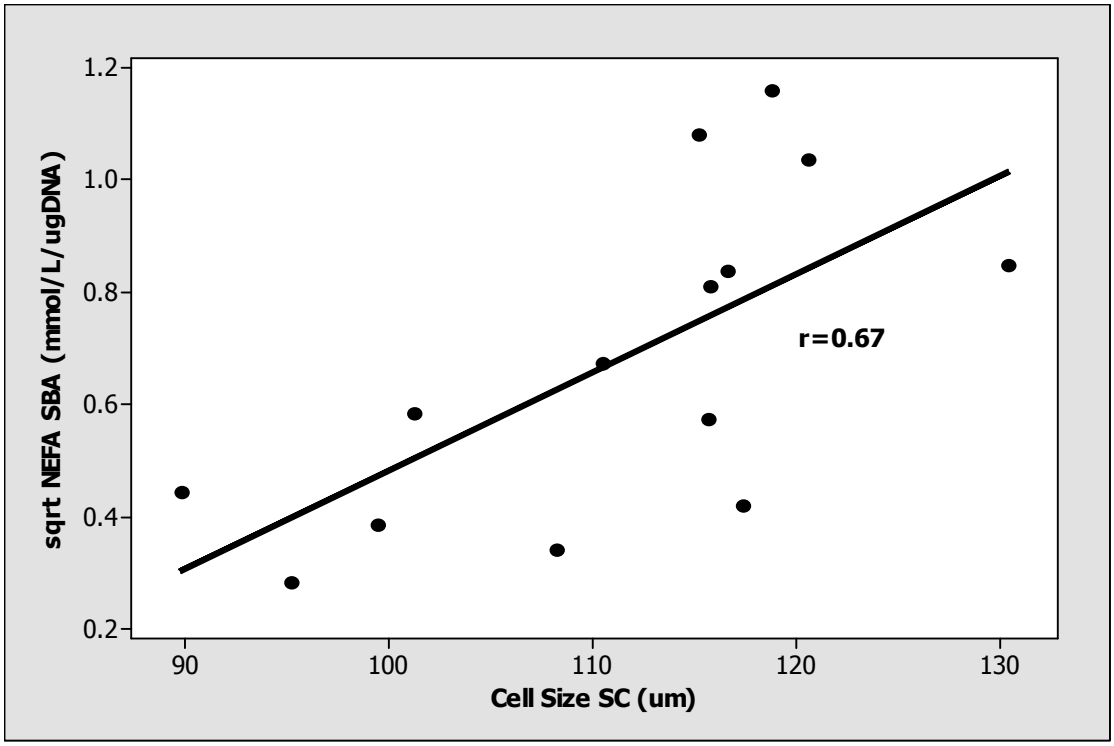

Figure 70 Relationship between SAT cell size and basal lipolysis.

SAT cell size is positively correlated with SAT basal lipolysis in PE (p=0.009) (n=14). NEFA SBA= SAT basal release of NEFA

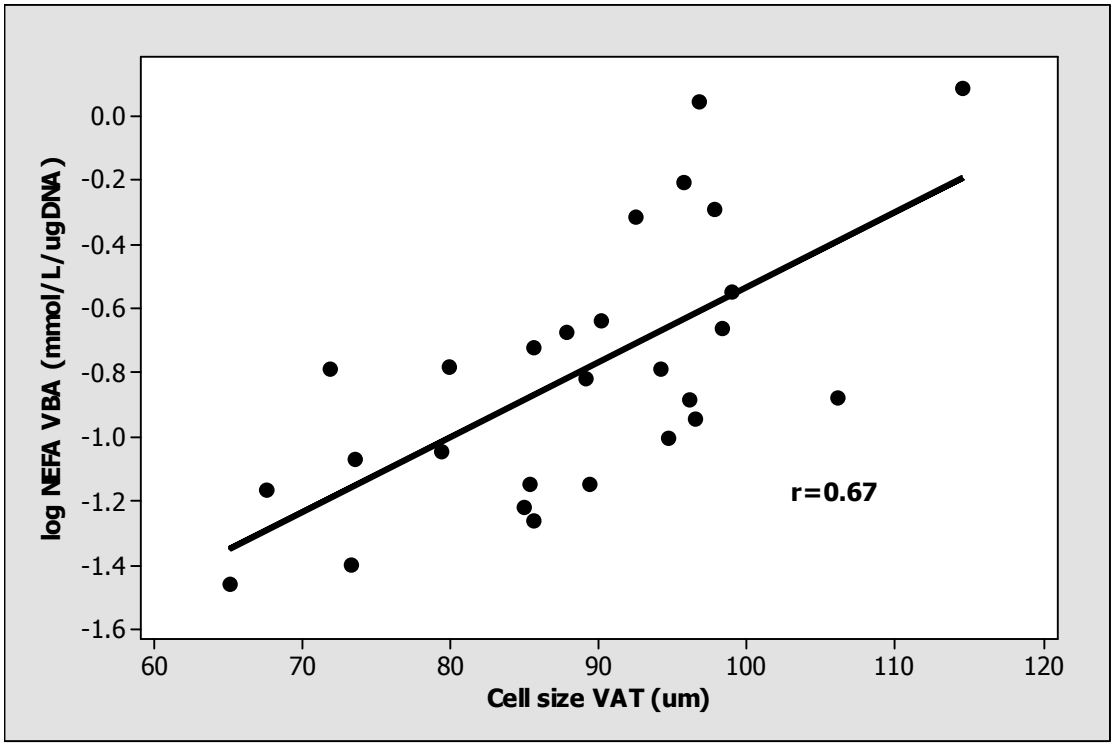

Figure 71 Relationship between VAT cell size and basal lipolysis

VAT cell size is positively correlated with VAT basal lipolysis in controls (p=0.015) (n=28). NEFA VBA = VAT basal release of NEFA.

4.4 The Effect of Maternal Serum on Adipocyte Lipolysis: Pre-eclamptic vs Controls.

I wanted to determine whether serum from women with PE stimulates adipocyte lipolysis excessively in comparison to serum from healthy pregnant women matched for smoking, BMI, age.

4.4.1 Methods

Healthy women from singleton pregnancies undergoing elective caesarean section at term were recruited as outlined in methods section 2.1. Lipolysis experiments were carried out as outlined in methods section 2.3 and 2.4. Only subcutaneous fat biopsies were obtained. As SAT from the same subject was used for all conditions in each experiment correction for fat cell number was not required.

All assays were carried out in duplicate. The reagents were prepared as outlined in methods section 1.5. All reagents were added to the relevant tubes and the timing of the assay was commenced. The tubes were placed in a 37°C shaking water bath at 91 cycles per minute and incubated for 120 minutes. Control and pre-eclamptic serum was obtained from previously stored serum samples frozen at -80°C and defrosted at room temperature. An aliquot from tubes containing either control or PE serum was taken at time=0 to correct for NEFA and glycerol already present in the serum. Comparisons between the groups was perfomed using the paired t-test.

4.4.2 The effect of control serum on lipolysis in adipose tissue explants.

I attempted to determine whether maternal serum per se has any effect on rates of lipolysis in SAT in vitro. The condition of each tube used in this experiment is outlined in Table 10 Conditions of assay The number of subjects in this experiment was n=4.

Condition	*Control* *900ul Buffer* *100ul adipocyte cells*	*Serum* *800ul Buffer* *100ul adipocyte cells* *100ul control serum*
Basal	No reagent	No reagent
Isoproterenol 200nM	Isoproterenol	Isoproterenol
Insulin 10nM	Insulin	Insulin

Table 10 Conditions of assay

4.4.2.1Results

There was no difference in basal lipolysis rates when expressed as NEFA release (1.12[0.02] vs 1.1[0.01] mmol/L, p=0.39) or glycerol release (20.6[10.7] vs 27.3[17.3] μmol/L, p=0.39) in the presence of or absence of control serum. (Figure 72 and Figure 73) Similarly, there was no difference between lipolysis rates in the presence of insulin when expressed as NEFA release (0.1[0.0] vs

0.09[0.02] mmol/L, p=0.61) or glycerol release (23.4[8.1] vs 29.6[19.6] µmol/L, p=0.64). However lipolysis rates in the presence of isoproterenol were increased in the control serum group when expressed as NEFA release (0.32[0.12] vs 0.56[0.14] mmol/L, p=0.004)(Figure 74) or glycerol release (81.8[41] vs 156[51.5] µmol/L, p=0.04). This was also true of percentage stimulation of lipolysis by isoproterenol when calculated from NEFA release (157%[97.9]vs 379%[96.6], p=0.002) and glycerol release (375%[291] vs 809%[269], p=0.08)(Figure 75).

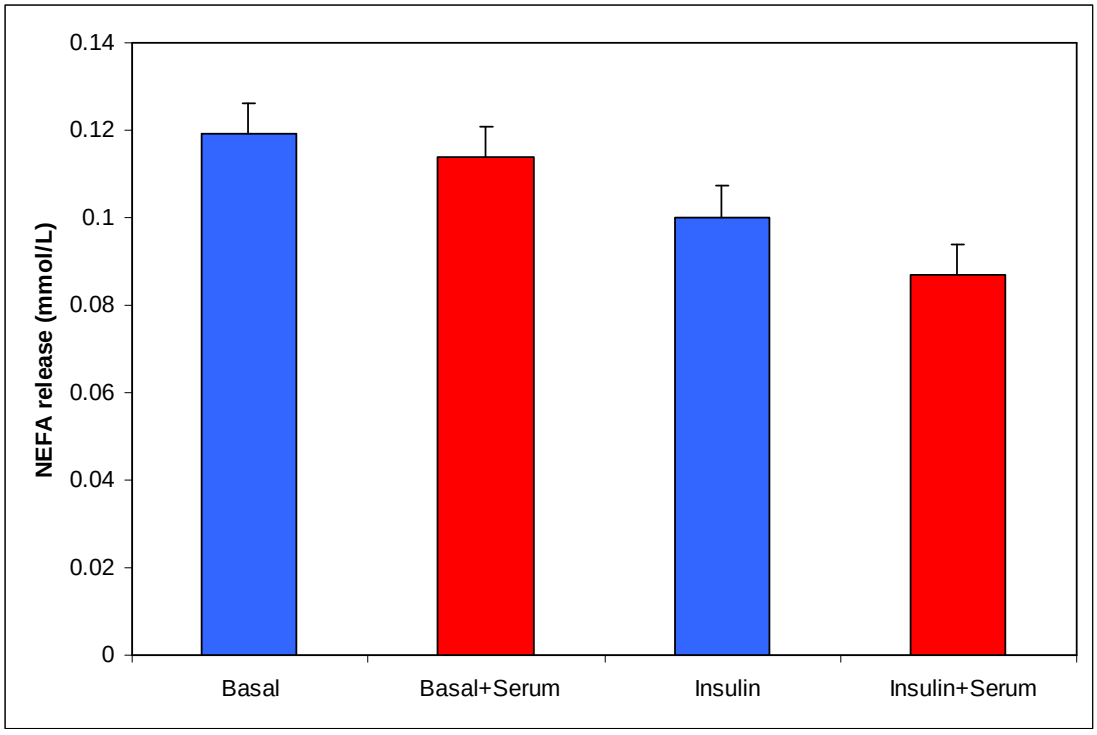

Figure 72 The effect of control serum on basal lipolysis and in the presence of insulin (NEFA).

There was no difference in basal lipolysis (p=0.39) or insulin suppressed lipolysis (p=0.61) with the addition of maternal serum when expressed as NEFA release (n=4).

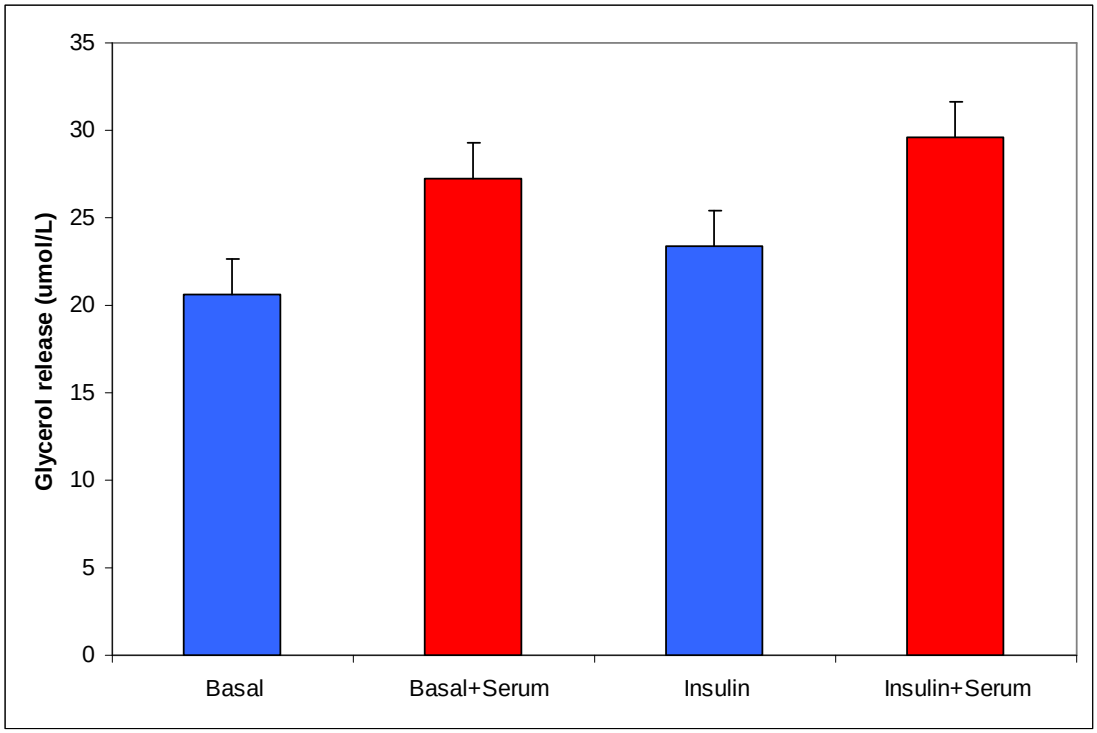

Figure 73 The effect of control serum on basal lipolysis and in the presence of insulin (glycerol).

There was no difference in basal lipolysis (p=0.39) or insulin suppressed lipolysis (p=0.64) with the addition of maternal serum when expressed as glycerol release, n=4.

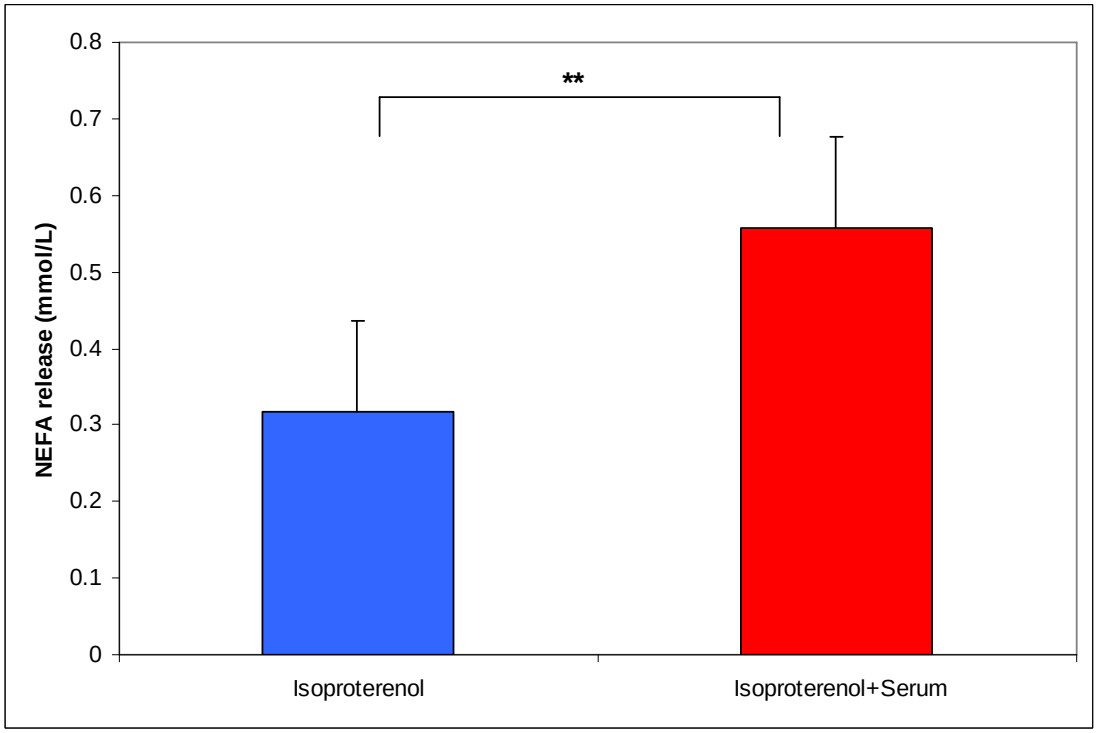

Figure 74 The effect of control serum on lipolysis in the presence of isoproterenol.

Lipoloysis was increased in SAT exposed to isoproterenol and maternal serum compared to isoproterenol alone (p=0.004) (n=4). **p=≤0.01

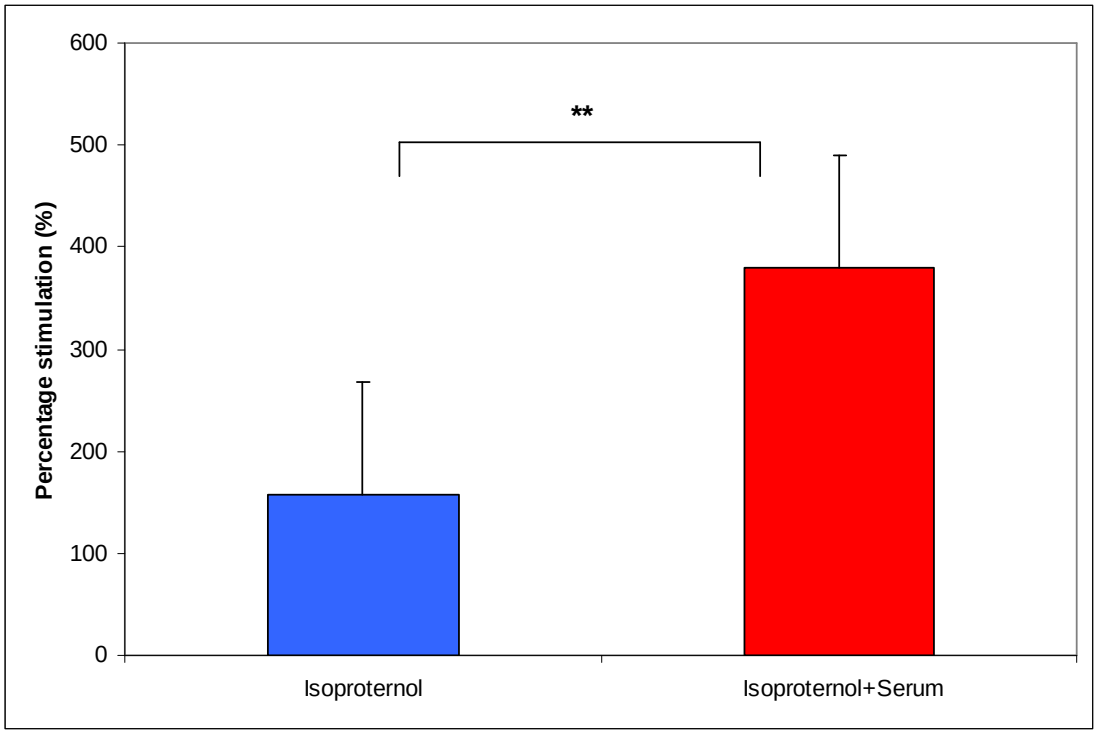

Figure 75 The effect of control serum on the percentage stimulation of lipolysis

The percentage stimulation of lipolysis was increased in SAT exposed to maternal serum in addition to isoproterenol when calculated from NEFA release (p=0.002) (n=4). **p=≤0.01.

4.4.3 Does PE serum excessively stimulate lipolysis in AT compared to serum from healthy controls

A preliminary experiment was performed to determine whether the effect of PE serum on lipolysis rates in SAT explants was different to that of serum from control women. The conditions of each tube are outlined in Table 11. All tubes contained 800ul warm wash buffer and 100ul adipocyte cells, giving a concentration of 10% volume/volume of maternal serum. All conditions were carried out in duplicate. Only NEFA concentration at time=120 minutes was determined. The number of subjects in this experiment was n=6.

Conditions	*Control* *800ul Buffer* *100ul adipocyte cells*	*PE* *800ul Buffer* *100ul adipocyte cells*
Basal	100ul control serum	100ul PE serum
Isoproterenol	100ul control serum + isoproterenol 200nM	100ul PE serum+ Isoproterenol 200nM

Table 11 Conditions of each assay.

4.4.3.1Results

There was no difference between the release of NEFA from adipocytes exposed to control serum compared to PE serum with a concentration odf 10% volume/volume (0.17[0.04] vs 0.14[0.04] mmol/L, p=0.44) with . There was no difference between isoproterenol stimulated NEFA release in adipocytes exposed to control serum compared to PE serum (0.66[0.05] vs 0.6[0.05] mmol/L, p=0.3). Furthermore there was no difference in percentage stimulation of NEFA release by isoproterenol in the two groups (383%[156] vs 390%[94], p=0.97)

4.4.4 A comparison of pooled PE serum and pooled control serum on adipocyte lipolysis.

Aliquots (500ul) of serum from 20 PE and 20 controls matched for BMI were thawed and pooled. This pooled serum was aliquoted (500ul) and refrozen at -80ºC. The experiment was carried out as outlined in the methods above. The conditions of each tube is outlined in Table 12. The number of subjects in this experiment is n=4.

Condition (% volume/volume)	*Serum*	*Wash Buffer*	*Adipocytes*
Basal	Nil	900ul	100ul
10%	100ul control	800ul	100ul
20%	200ul control	700ul	100ul
30%	300ul control	600ul	100ul
10% + isoproterenol (200nM)	100ul control	800ul	100ul
10%	100ul PE	800ul	100ul
20%	200ul PE	700ul	100ul
30%	300ul PE	600ul	100ul
10% + isoproterenol (200nM)	100ul PE	800ul	100ul

Table 12 The condition of each assay.

The total volume in each falcon tube was 1 ml. All conditions were carried out in duplicate

4.4.4.1Results

There was no statistical difference in release of NEFA from adipocytes incubated in increasing concentrations of control and PE serum (10% volume/volume 0.14[0.06] vs 0.06[0.09]mmol/L, p=0.3; 20% volume/volume 0.11[0.1] vs 0.1[0.09]mmol/L, p=0.85; 30% volume/volume dilution 0.2[0.09] vs 0.015[0.05] mmol/L p=0.16 control vs PE), when these concentrations are used.(Figure 76) In addition there was no dose response effect in the ranges of concentrations used for either control serum(10% volume/volume vs 30% volume/volume, p=0.24) or PE serum (10% volume/volume vs 30% volume/volume, p=0.38) (Figure 76). Furthermore no difference was seen in lipolysis rates after stimulation with isoproterenol in the presence of serum (0.5[0.13] vs. 0.39[0.13] mmol/L, p=0.46) nor percentage stimulation (968[302] vs 1268[713]%, p=0.54).

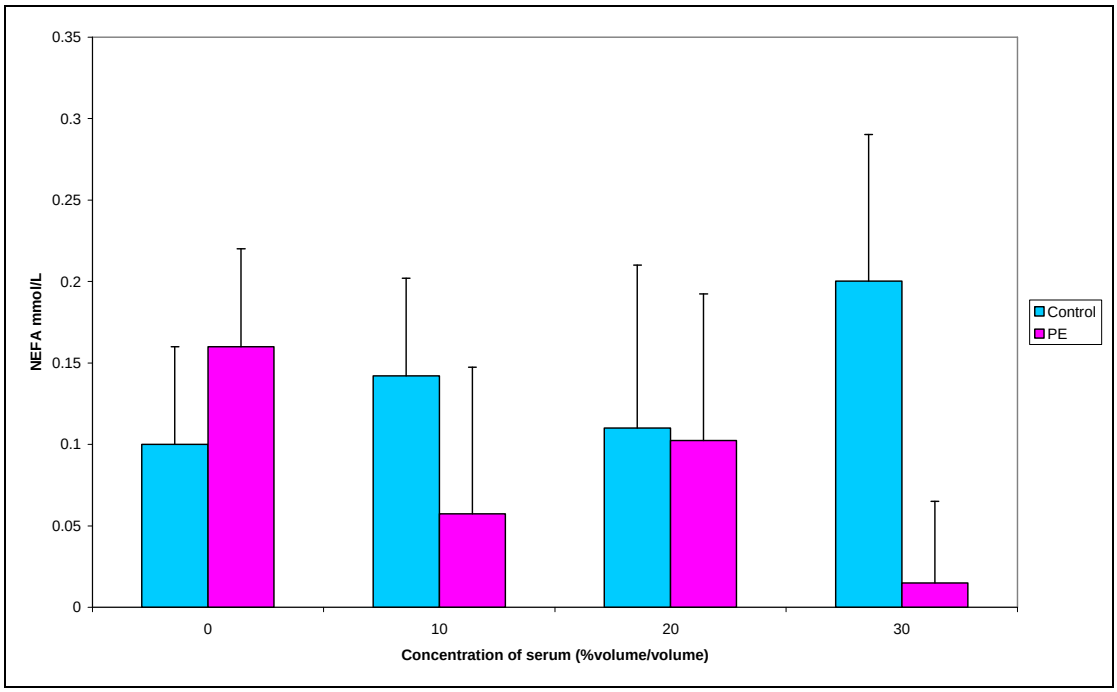

Figure 76 Effect on increasing concentrations of control and PE serum on basal lipolysis.

There is no difference in lipolysis rates of adipocytes exposed to control or PE serum in increasing concentrations (10%, 20%, 30% volume/volume, p=0.3, p=0.85 and p= 0.16 respectively).Comparisons made using paired t-test and results displayed as mean and SEM.

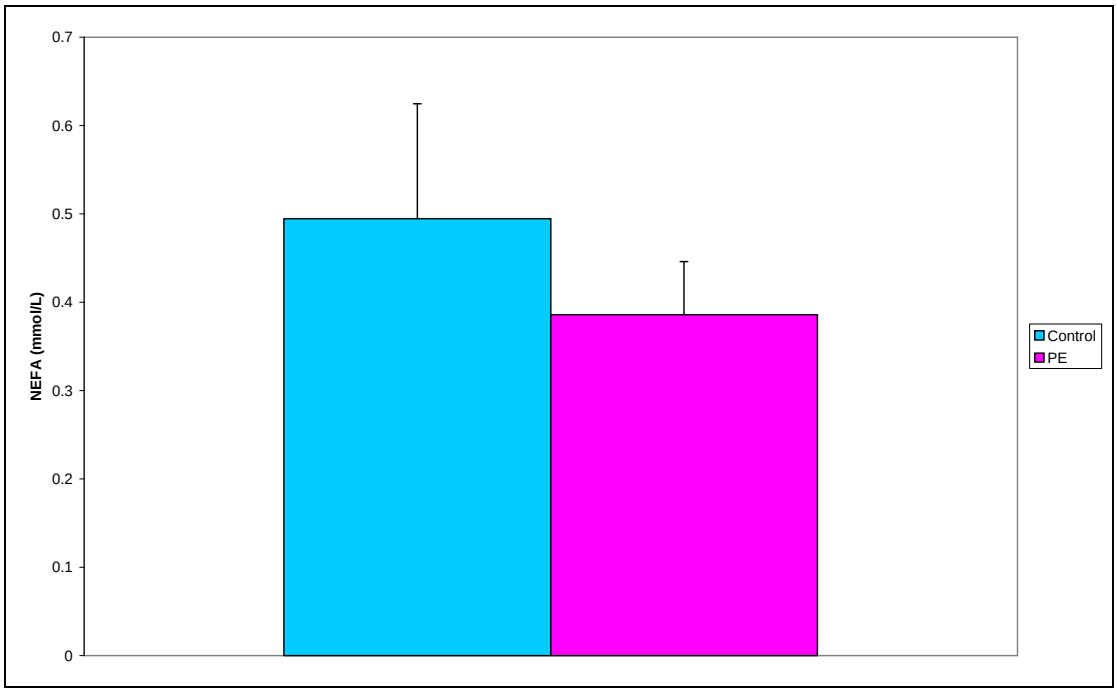

Figure 77 A comparison of the effect of control and PE serum on lipolysis in the presence of isoproterenol.

There is no difference in lipolysis rates when expressed as release of NEFA between adipocytes incubated with isoproterenol (200 nM) together with either control or PE serum in a concentration of 10% of total volume (p=0.46). Comparison made using paired-t-test and results displayed as mean and SEM.

4.5 Discussion

4.5.1 Metabolic Phenotype

The pre-eclamptic women in our cohort conform to the described metabolic phenotype[86]. They have a higher booking blood pressure, with an exaggerated hyperlipidaemia of pregnancy consisting of higher maternal serum TG and FFA. There is also a suggestion of increased insulin resistance with higher maternal glucose, insulin and HOMA compared to controls, despite an earlier gestational age at sampling. As expected our cases tend to deliver earlier and have babies of lower birth-weight centile than the controls.

4.5.2 Lipolytic function of SAT and VAT

Our data suggests that SAT and VAT in *vitro* does not differ in basal lipolysis, nor have an exaggerated response to catecholamine stimulation in women with PE

compared to controls. In addition there was no difference in either AT depot in the absolute response to insulin inhibition. However, we have demonstrated by using our FCISI that SAT in women with PE is less insulin sensitive than that in control women and a similar tendency is seen in VAT. Our FCISI reflects responsiveness to insulin once the tissue is stimulated by the catecholamine isoproterenol – a non-selective ß-adrenergic agonist which acts to promote lipolysis through elevating cellular cAMP production and activation of protein kinase A (PKA) and HSL. Insulin inhibits lipolysis by its ability to lower cAMP levels via activation of phosphodiesterase 3B (PDE 3B) and therefore reduce PKA activity. FCISI may be more relevant *in vivo* than simple stimulation or suppression of adipocyte lipolysis as it represents a functional test of AT lipolysis. There may be a dysregulated response in SAT in women with PE whereby stimulated fat cells are less sensitive to effects of insulin resulting in excessive release of FFA. The mechanisms through which this occurs may be secondary to several factors including autocrine and paracrine effects of other "lipases" including the inflammatory adipokines such as TNF alpha which can attenuate the anti-lipolytic effect of insulin by suppressing PDE 3B activity and expression[214]. Metformin, a drug which is an insulin sensitizing agent and lowers circulating FFA inhibits isoproterenol stimulated lipolysis by reducing cellular cAMP production and PKA activity and attenuates the phosphorylation of perilipiin during isoproterenol-stimulated lipolysis in primary rat adipocytes illustrating potential interactions between insulin sensitivity and catecholamine stimulated lipolysis.[215] In addition the rise of FFA in PE occurs in early pregnancy well in advance of manifestations of the disease. Sivan et al demonstrated that FFA in early pregnancy induces insulin resistance to levels similar to that seen in late pregnancy[106]. This may therefore result in a "vicious" cycle of reduced insulin sensitivity of the tissue resulting in increased release of FFA in early pregnancy with further exacerbation of insulin resistance in PE.

4.5.3 Fat Cell Size

Interestingly we see a discordant relationship between BMI and adipocyte size in controls and PE. As we have previously demonstrated, BMI is not as closely related to adipocyte cell size in SAT compared to VAT in normal controls. This may be explained by this depot adapting to increased fat mass and accumulation of lipid by both hyperplasia and hypertrophy of the cells, whereas increased VAT

is primarily due to hypertrophy, a phenomenon seen in non-pregnant women[114]. However in PE, SAT cell size is intimately related to BMI. This is possibly due to differences in the behaviour of this tissue in these metabolically challenged women to accumulate excess lipid by cell hypertrophy. This process is metabolically detrimental. Individuals with type 2 diabetes and dyslipidaemia have larger adipocytes. Furthermore increasing adipocyte size is associated in a shift toward dominance of proinflammatory adipokines including TNF alpha and IL-6.[120] Adipocyte hypertrophy is also thought to result in endoplasmic reticulum (ER) stress which results in activation of metabolic factors that trigger insulin resistance, with release of inflammatory cytokines and increased macrophage recruitment.[121] Thus the propensity for SAT to accumulate fat mass by hypertrophy as opposed to hypertrophy and hyperplasia would potentially predispose them to metabolic complications of pregnancy such as PE. We also have shown that SAT cell size in PE is closely related to lipolytic function of the tissue independent of BMI, a relationship lacking in normal controls. Conversely VAT cell size is an important determinant of VAT function in normal controls but not in PE. This highlights potential disparate functional metabolic roles of each tissue depot in each group of women.

4.5.4 The Effect of Maternal Serum

Although we have show in *vitro* that AT does not have increased basal lipolysis or responsiveness to catecholamines per se, we hypothesised that there may be a factor originating from the placenta present in the maternal serum of PE which excessively stimulates lipolysis over and above that seen in normal pregnant women. We based this on a study by Endresen et al who showed that incubation of endothelial cells with sera from women with PE resulted in increased uptake of TG, with increased lipolytic activity of PE sera of 3-4 fold as measured by release of FFA [107]. Serum from control women did not appear to have an effect on basal lipolysis in SAT explants nor lipolysis in presence of insulin. Preliminary results indicated an exaggerated response to isoproterenol in the presence of control serum at 10% (volume/volume) but this was not reproduced in subsequent experiments. When using 10% serum (volume/volume) from PE and controls we found no difference in either basal or stimulated lipolysis. In addition there was no effect from increasing concentrations of serum between PE and controls on basal or stimulated lipolysis. There was an apparent

statistical difference in basal lipolysis in the presence PE serum at concentration of 30% but this was probably secondary to correcting for FFA already present in the serum. As FFA concentration is higher in PE serum and due to the margin of error of the NEFA assay its likely that this resulted in an "over-correction". Overall we failed to demonstrate an effect of serum on lipolysis on SAT explants in *vitro* nor any differences between PE and controls, suggesting either an absence of a factor influencing lipolysis in serum of PE women or a lack of effect in *vitro* as compared to in *vivo*. Alternatively the processing of the blood sample and incubation with AT in buffer may have altered or inactivated such a factor.

4.5.5 Limitations

One limitation of our study is the disparity in gestational age of the cases and controls as there are some important metabolic changes in late pregnancy including the switch to a state of catabolism with a marked increase in lipolysis rates and increased insulin resistance. Unfortunately it was very difficult to obtain "pre-term" samples from healthy women being delivered for reasons other than IUGR or PE in a non-urgent manner to allow recruitment to a study. However, the mean gestational age of our cases was 35 weeks which is well into the third trimester by which time the majority of changes in carbohydrate and lipid metabolism have occurred. [27 28 80] Also in normal pregnancy IR increases as pregnancy advances. Therefore the effect of increased IR in adipose tissue PE is likely to be even more apparent if matched for gestational age.

In summary, we have found some important differences in adipocyte cell function between controls and PE which could contribute to the metabolic challenges and pathophysiology of this disease. This is summarised in Figure 78 and Figure 79.

.

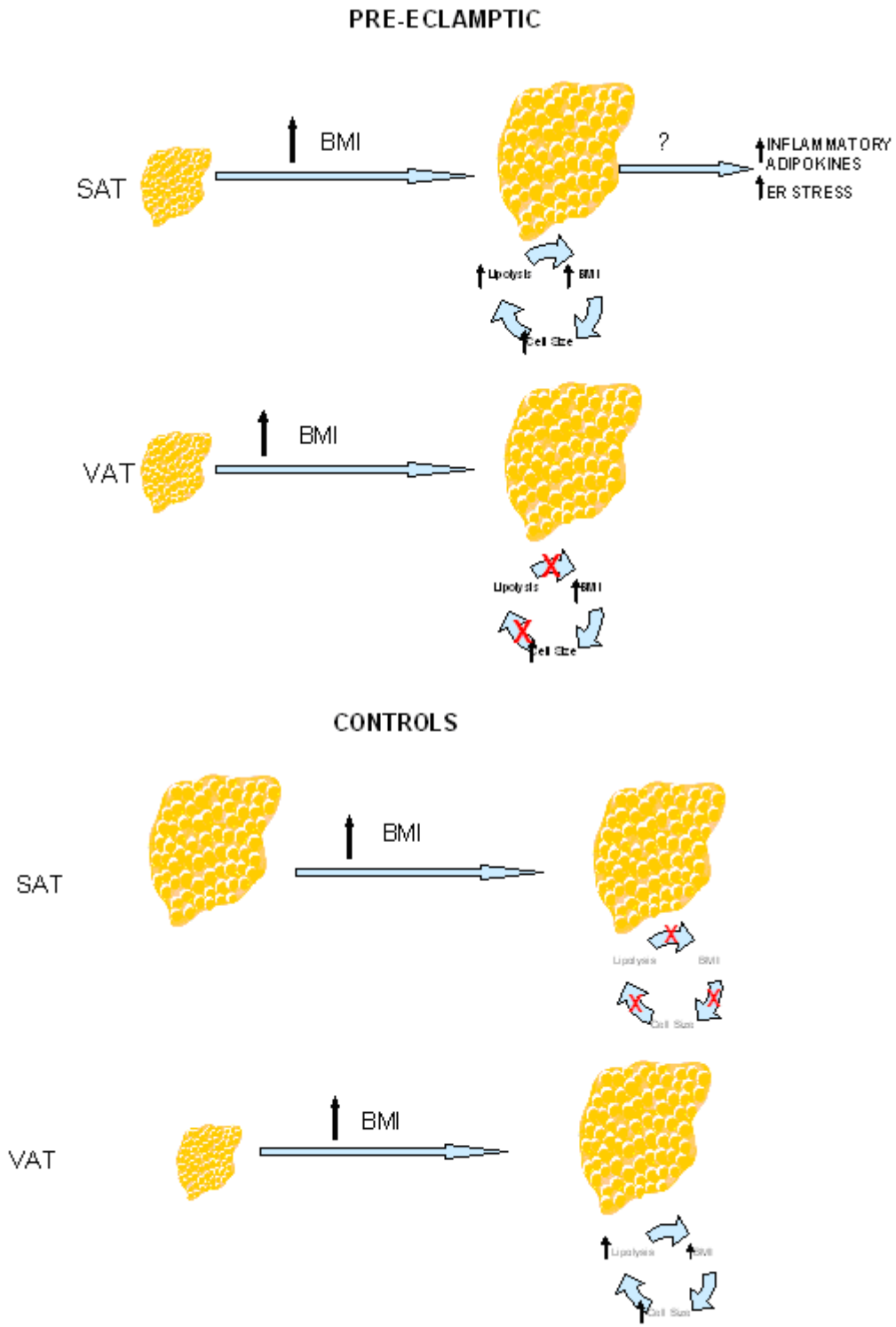

Figure 78 Comparison of SAT and VAT cell size and lipolytic function in controls and PE

In PE, SAT cell size is closely correlated with BMI, an effect not seen in controls. This maybe due to the adaptation of this fat depot to an increase in fat mass by adipocyte hypertrophy which could result in increased ER stress and release of pro-inflammatory adipokines. Although VAT cell size is closely related to BMI in PE, it is not related to lipolytic function in this depot unlike in controls.

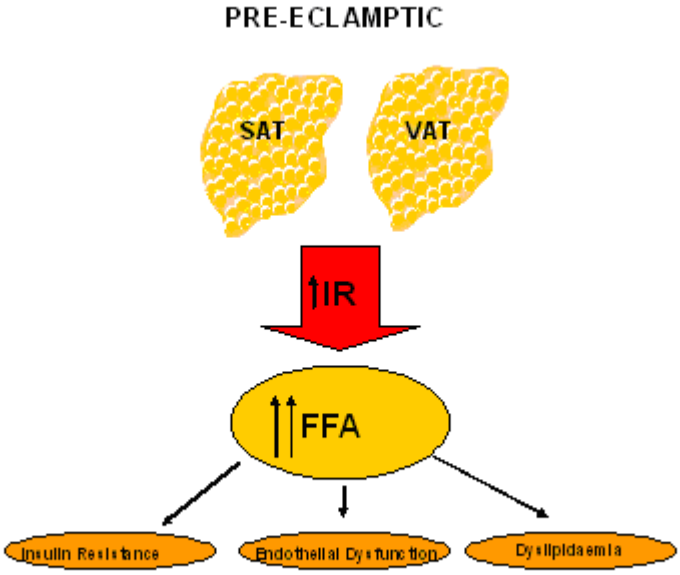

Figure 79 SAT and VAT in PE is less insulin sensitive than controls.

Decreased insulin sensitivity of SAT and VAT could potentially lead to exaggerated lipolysis with increase release of circulating FFA. FFA can further contribute to insulin resistance, endothelial dysfunction and dyslipidaemia, all features of the syndrome of PE.

5 Adipose Tissue, Inflammation and Pre-eclampsia

5.1 Introduction

Endothelial dysfunction as part of an overall more general inflammatory reaction is a hallmark of pre-eclampsia and involves placental and systemic circulations[216] [217]. Healthy pregnancy is a state of systemic inflammation and therefore pre-eclampsia may represent an extreme end of maternal systemic inflammatory responses engendered by the pregnancy itself[216]. Notably all the inflammatory changes of normal pregnancy are exaggerated in pre-eclampsia and features of the disease are derived not only from endothelial dysfunction but a wider stress response including the acute phase response and effects on metabolism.[217] The corollary is that any maternal or fetal factor, which would enhance this inflammatory response, would predispose to endothelial dysfunction and pre-eclampsia. For example maternal diabetes and maternal obesity are associated with an increased risk of endothelial dysfunction with a concomitant increase in the risk of pre-eclampsia[71 218].Many similarities between the metabolic syndrome and pre-eclampsia can be found including insulin resistance, dyslipidaemia, inflammation and endothelial dysfunction. The link between adiposity, inflammation and insulin resistance has been increasingly defined since Hotamisligil first demonstrated this relationship in 1993[219]. White adipose tissue (WAT) secretes a number of pro-inflammatory mediators which contributes significantly to the chronic inflammatory state and metabolic complications of obesity[113]. Therefore it is plausible that similar disturbances in adipocyte function could contribute to the development of the clinical syndrome of PE.

5.1.1 Adipokines

WAT secretes a diverse range of cytokines, proteins and signals which have both paracrine and endocrine actions and a wide-ranging influence on the metabolic and physiological function of other organs[122]. It releases a host of pro-inflammatory cytokines (e.g. TNF alpha, IL-1,IL-6,IL-8, IL-10), chemokines (e.g. MCP1, MIP-1 alpha) and acute phase proteins (e.g. serum amyloid A, C-reactive protein, haptoglobin, PAI-1). In addition there are AT specific adipokines such as leptin which acts not only in the control of appetite and energy balance, but contributes to inflammation through modulation of T-cell and monocyte functions[220], and the anti-inflammatory and potent insulin-sensitising agent adiponectin. The function of important adipokines and their potential roles in normal and complicated pregnancy have been detailed previously.

5.1.2 Adipose Tissue Macrophages

WAT is a heterogeneous tissue composed of several cell types including mature adipocytes, pre-adipocytes, fibroblasts,endothelial cells, histiocytes, and adipose tissue macrophages (ATM).The non-adipocyte cells are termed the 'stroma vascular fraction' (SVF). Many of the pro-inflammatory factors are secreted by the non-adipocyte cells, and this is of particular relevance in the pathological condition of obesity [221]. Similar to the Th1/Th2 concept of T-cell activation, a concept of M1/M2 polarization has been described for macrophages. Macrophages are classically stimulated by IFNγ alone or in combination with LPS and produce inflammatory cytokines, reactive oxygen species such as NO and are capable of inducing Th1-polarized T-cell responses. These pro-inflammatory "classical" macrophages are named M1.[222] In contrast M2 or "alternatively activated" macrophages are induced by IL-4 and IL-13 and have an anti-inflammatory phenotype.[223] They down-regulate inflammatory processes that are initiated by M1 by production of IL-10, TGFβ, and the IL-1 receptor antagonist.[222]. In obesity, there is increased macrophage recruitment and retention with a shift toward the more pro-inflammatory M1 phenotype. The driving force behind this is thought to be secondary to adipocyte hypertrophy, cell death and local hypoxia within expanding WAT.[224-226] The alteration in the cellularity of WAT contributes to adipose inflammation, altered production of adipokines and promotion of insulin resistance through dysregulation of glucose and lipid metabolism[227]. Monocyte chemotactic protein-1, acting through its receptor CCR2 is strongly implicated in ATM recruitment and remodelling[228]. It is over-expressed in obese rodents and obese diabetic humans, and is implicated in insulin resistance [229 230]. In addition macrophage accumulation is also required for angiogenesis at sites of inflammation and ischaemia in AT. The action of these inflammatory cells may represent one of the key links between adiposity and its metabolic complications including those occurring in pregnancy.

5.1.3 Toll-like receptors and innate immunity

In addition to adipokine receptors, adipocytes and the SVF express all toll-like receptors (TLR) except 5 and 7[231]. TLRs are part of the innate immune system that classically alert the immune system to the presence of pathogens. TLR4 is an LPS receptor which is also activated by long-chain fatty acids that transduce

cytokine expression [232]. Activation of TLR4 in adipocytes alters key mediators of insulin signalling and glucose uptake including NFKB target genes and via an MyD88 independent pathway [233]. TLR4 deficient mice were protected from diet induced obesity and IR [234]. Previous groups have reported that experimental human endotoxinemia promotes adipose inflammation and alters adipokine function coincident with systemic IR [235].

5.1.4 Macrophage Markers

Cfms is a type III receptor tyrosine kinase that is selectively expressed on macrophages and their progenitor cells and serves as the exclusive receptor for colony-stimulating factor (CSF-1), a cytokine which controls the production, differentiation and function of macrophages[236].Upon binding to CSF-1, cfms undergoes autophosphorylation and dimerization, and ultimately induces the phosphorylation of downstream signalling proteins, thereby driving the differentiation and activation of these cells[237]. Thus, ligation of cfms by CSF-1 results in activation and proliferation of macrophages and their subsequent release of inflammatory mediators. Therefore the identification of $cfms^{+}$ macrophages would represent activated ATM. All tissue macrophages highly express CD68, a transmembrane glycoprotein of unknown function, and anti-CD68 is commonly used as a macrophage marker in ICC.

5.2 Aims and Objectives

5.2.1 Hypotheses

1. That adipocyte release of adipokines is exaggerated under either basal or stressed conditions in women with PE, thereby implicating adipocyte function in its pathophysiology.
2. That macrophage infiltration of adipose tissue, a marker of tissue inflammation, is increased in women with PE compared to controls.

5.2.2 Specific Research Questions

1. Is the release of the adipokines IL-6, TNF-alpha, IL-10, CRP, leptin, adiponectin and PAI-1 altered in adipocytes from women with PE under basal conditions or when stimulated by LPS?

2. Is the basal release of MCP-1 altered in adipocytes in women with PE or when stimulated by LPS?
3. Is the adipocyte defect dependent predominantly on one functional tissue depot - i.e. visceral vs subcutaneous?
4. If differences in adipokine release are found, is this reflected by differences in the gene expression in adipose tissue?
5. Does either basal or stimulated adipokine release relate to maternal markers of insulin resistance and adipocyte lipolysis?
6. Does either basal or stimulated adipokine release relate to adipocyte cell size?
7. Is there a higher percentage of macrophages ($CD68^+$) and activated macrophages ($cfms^+$) in the adipose tissue of women with PE?

5.3 Methods

Fourteen PE and matched controls for age, BMI, smoking and parity were recruited as outlined in Methods section 2.1. Adipose tissue was processed as outlined in methods section 2.3-2.5. At time=120min paired 120ul aliquots of the buffer layer below the adipocyte layer was frozen at -80°C for later analysis of adipokines by Bio-Plex (BIO-RAD®) system a suspension array technology as outlined in section 2.8. Adipokines were corrected for cell number by dividing by the quantity of DNA in a known volume of adipocytes as outlined in methods section 2.7. The values are therefore expressed as pg/ml/ugDNA. RNA was isolated from adipose tissue using methods described in 2.9. Reverse transcribed RNA to cDNA was synthesised and cDNA was quantitated using TaqMan technology as outlined in section 2.10.

Immunocytochemistry and quantification of macrophage cell density was performed as outlined in methods section 2.11 and 2.12 .

Statistical analysis was performed as per methods section 2.14.

5.4 Results

5.4.1 Subjects

The characteristics of the subjects are described in Table 13. Subjects were matched for age, BMI and smoking.

Characteristics	*Controls (n=14)*	*PE (n=14)*	*P value*
Age, y	**30.0(5.8)**	**31.5(6.3)**	**0.51**
BMI, kg/m^2	**29.7(6.6)**	**31.1(8.0)**	**0.62**
Smokers (non,current)**	**12,2**	**11,2**	**0.82**
DEPCAT*	**4(4-5.75)**	**6(4-7)**	**0.06**
Gestation at delivery, days	**270.3(11.2)**	**249.4(21.5)**	**0.004**
Systolic pressure, mmHg	**114.7(12.3)**	**127.1(13.1)**	**0.013**
Diastolic pressure, mmHg	**69.9(9.0)**	**79.1(9.0)**	**0.009**
Birthweight, g	**3304(633)**	**2330(926)**	**0.004**
Birthweight centile	**50.7(31.7)**	**26(31.2)**	**0.046**

Table 13 Characteristics of cases and controls. Blood pressure refers to booking values. All values expressed as mean and standard deviation (*median and interquartile range). Comparisons made by paired t test except * Mann-whitney, and ** chi-squared test.

5.4.2 Adipokines

5.4.2.1TNF-alpha

There was no difference in subcutaneous adipose tissue (SAT) of basal release of TNF-alpha (p=0.10) or LPS stimulated release (p=0.97) between controls and PE. Similiarly there was no difference in visceral adipose tissue (VAT) of basal release (p=0.64) or LPS stimulated release (p=0.39) between controls and PE. In addition there was no significant difference in basal release of TNF-alpha between SAT and VAT in controls (p=0.23) or PE (0.88).

TNF-alpha secretion was significantly increased in VAT after stimulation by LPS in PE (64.1[20.6] vs 89.1[26.0] pg/ml/ugDNA, p=0.018), but not in controls (45.4[9.2] vs 55.5[11.8] pg/ml.ugDNA, p=0.16)(Figure 80).

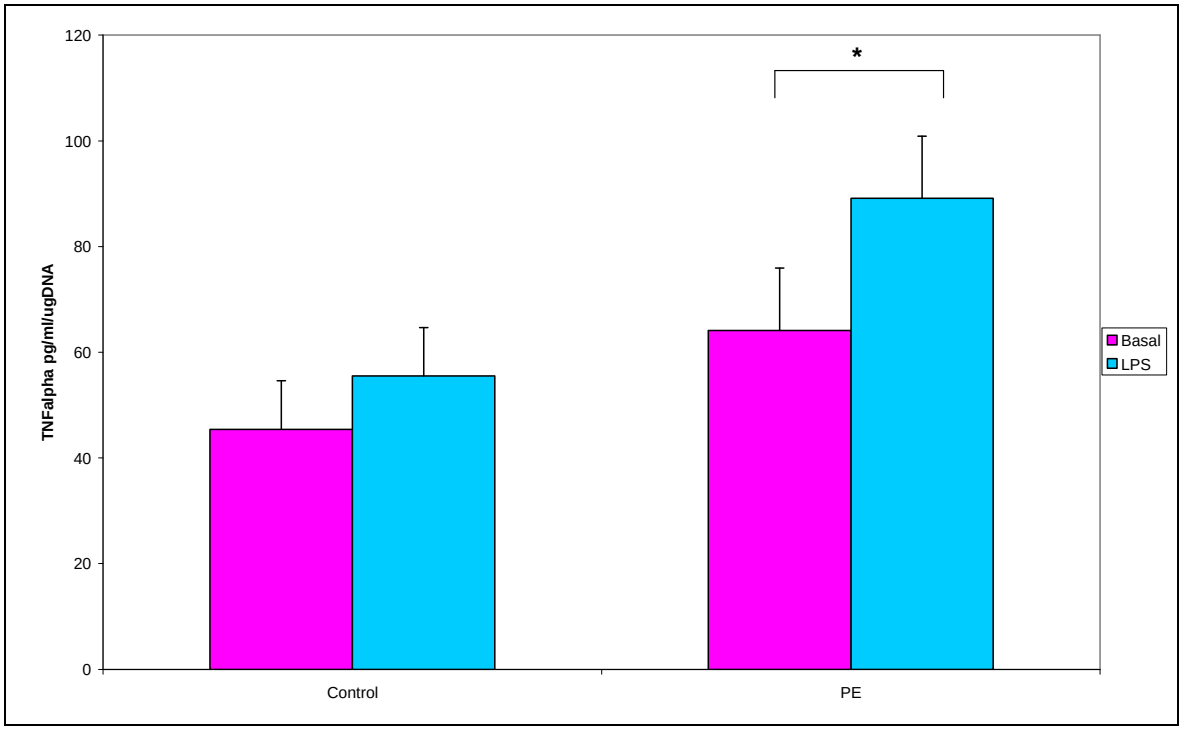

Figure 80 Comparison of basal release and stimulated release of TNF-alpha in visceral adipose tissue.

TNF-alpha secretion was significantly increased in PE (p=0.018)(n=14) but not in controls (n=14). Raw data shown but analysis performed on transformed data by paired t-test, and expressed as mean and SEM. *=p≤0.05.

Conversely, TNF- alpha secretion was significantly increased in SAT after stimulation by LPS in controls (34.0[7.7] vs 57.7[13.6] pg/ml/ugDNA, p=0.02) but not in PE (62.1[14.6] vs 49.0[11.0] pg/ml/ugDNA, p=0.26) (Figure 81). Basal release of TNF-alpha from SAT in PE is almost double that of controls although not significantly so (62.1[14.6] vs 34.0[7.7] pg/ml/ugDNA, p=0.097).

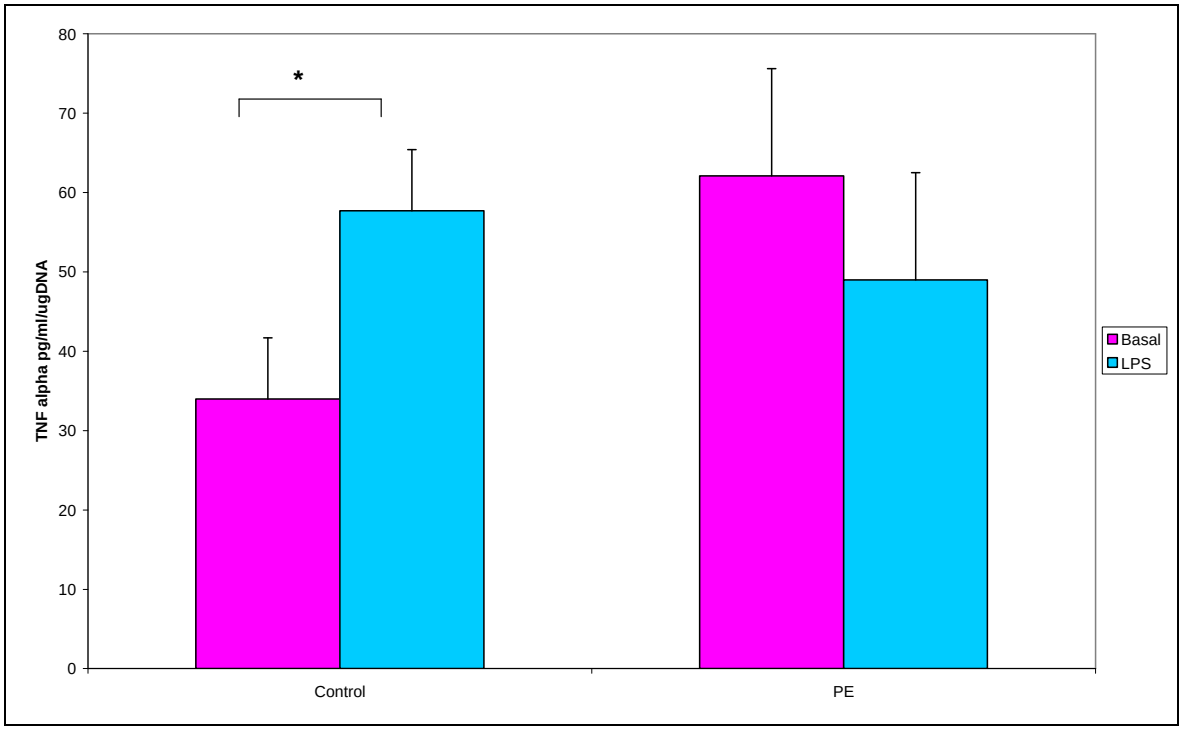

Figure 81 Comparison of basal release and LPS stimulated release of TNF-alpha in subcutaneous adipose tissue.

TNF-alpha secretion was significantly increased in controls (p=0.02) (n=14) but not in PE (n=14). Analysis performed on transformed data by paired t-test, but raw data shown and expressed as mean and SEM. *=p≤0.05.

In controls, TNF-alpha basal secretion from SAT is positively correlated with BMI ($r=0.63$, $R^2=39.4\%$, $p=0.009$) as is TNF-alpha secretion after stimulation by LPS ($r=0.59$, $R^2=34.9$, $p=0.016$)(Figure 82 and Figure 83). This relationship is not affected when adjusted for cell size ($p=0.014$ and $p=0.018$ respectively).There is no correlation with BMI and VAT basal or stimulated TNF-alpha secretion.

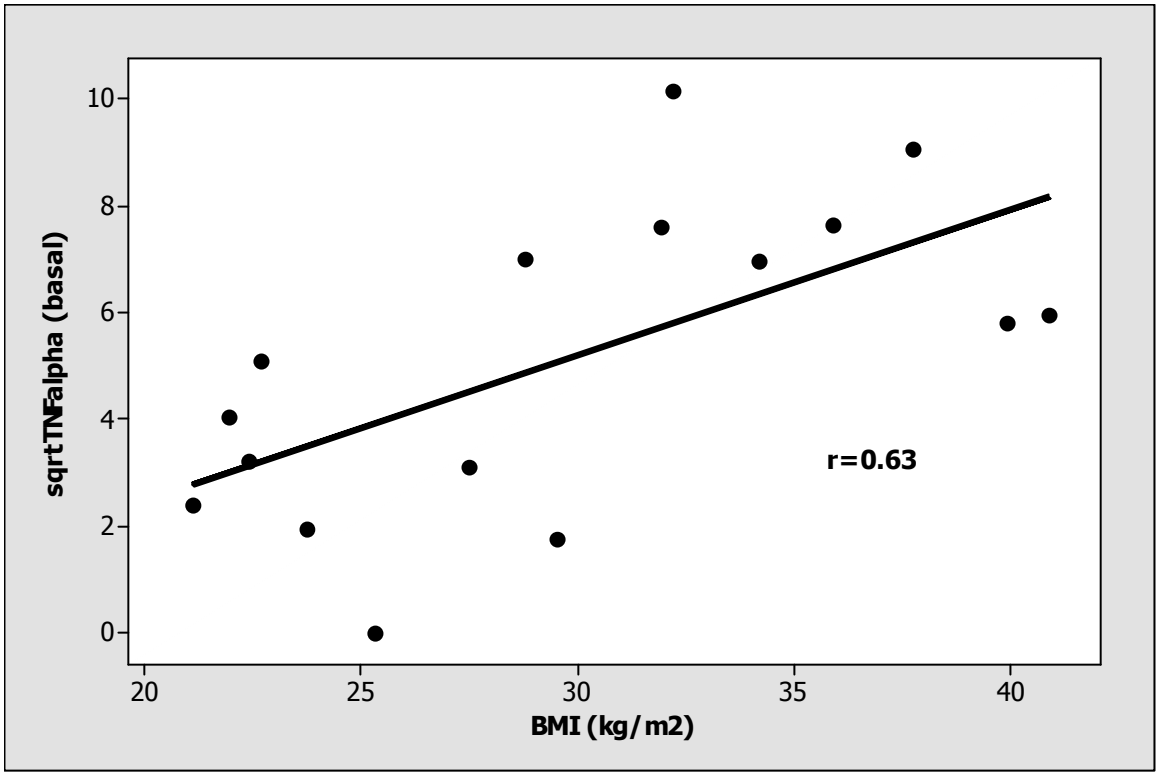

Figure 82 Relationship between maternal BMI and basal release of TNF-alpha in SAT of controls.

There is a positive correlation between BMI and basal release of TNF-alpha from SAT in controls (p=0.009). SQRT TNF-alpha= square root TNF-alpha.

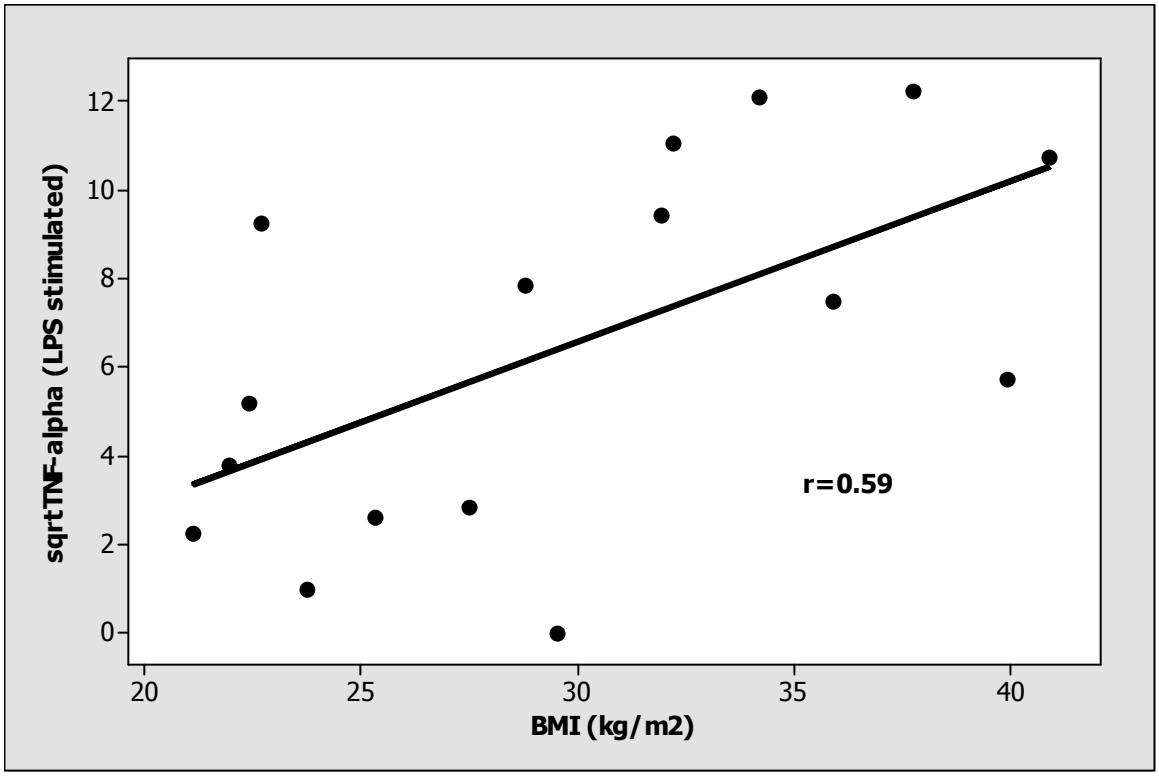

Figure 83 Relationship between maternal BMI and LPS stimulated release of TNF-alpha from SAT of controls.

There is a positive relationship between TNF-alpha and BMI (p=0.016). SQRT TNF-alpha= square root TNF-alpha.

There is no correlation with SAT or VAT TNF-alpha secretion (either basal or stimulated) and maternal triglycerides, maternal NEFA, maternal glucose, insulin or HOMA. SAT or VAT TNF-alpha secretion (basal or stimulated) is not correlated with maternal plasma TNF-alpha, leptin or adiponectin or CRP. In addition there appears to be no apparent relationship between SAT or VAT TNF-alpha secretion (basal or stimulated) and direct measures of adipocyte lipolysis and adipose tissue insulin sensitivity. However VAT TNF-alpha secretion, both basal and stimulated, correlates with maternal IL-6 plasma levels ($r=0.55$, $R^2=30.0\%$, $p=0.042$ and $r=0.54$, $R^2=29.3\%$, $p=0.045$ respectively).

Conversely, unlike in controls, in PE there is no apparent correlation between SAT TNF-alpha release (basal or stimulated) and maternal BMI ($p=0.40$ and $p=0.88$ respectively). In addition, VAT TNF-alpha release does not correlate with maternal BMI in PE.

Similar to data from controls, there is no correlation in PE between TNF-alpha release (basal or stimulated) and maternal TG, maternal NEFA, glucose, insulin or HOMA. Furthermore no relationship was found with maternal serum leptin,

adiponectin, maternal TNF-alpha, IL-6 and maternal CRP. However SAT basal and stimulated release of TNF alpha was positively correlated with NEFA release in the presence of isoproterenol (r=0.63, p=0.017 and r=0.62, p=0.017 respectively) and NEFA release in the presence of isoproterenol and insulin (r=0.58, p=0.03 and r=0.55, p=0.04 respectively).

In PE, VAT basal release of TNF-alpha is negatively correlated with FCISI (fat cell insulin sensitivity) of VAT(r=-0.60, R^2=41.5%, p=0.018 adjusted for BMI), as is stimulated release of TNF-alpha(r=-0.53, R^2=34.3%, p=0.037 adjusted for BMI) (Figure 84 & Figure 85). The FCISI is a direct measure of insulin sensitivity of adipose tissue and is calculated from the percentage inhibition of catecholamine stimulated lipolysis by insulin measured from the release of NEFA (see section 3.3.6). This relationship is not seen in SAT in PE, nor SAT and VAT in controls.

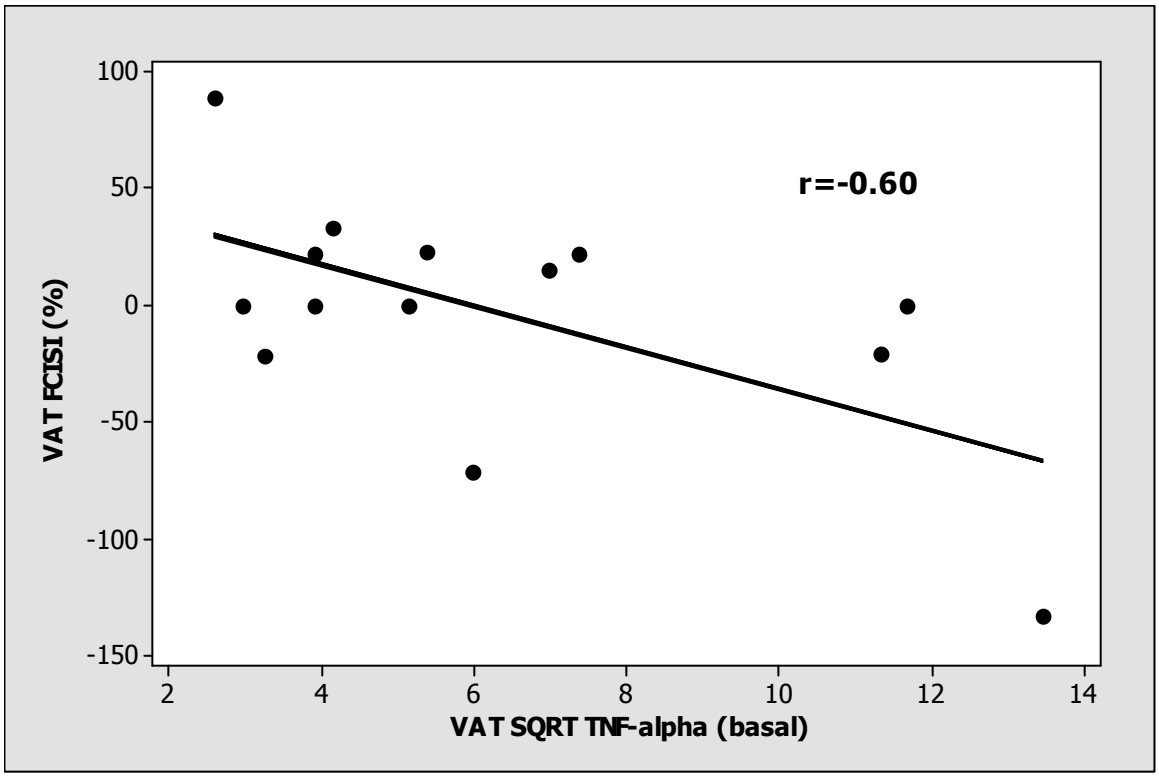

Figure 84 Relationship between basal release of VAT TNF-alpha and FCISI.

Basal release of VAT TNF-alpha is negatively correlated with FCISI of VAT in PE (p=0.018 adjusted for BMI) (n=14). SQRT TNF-alpha = square root TNF-alpha, FCISI=fat cell insulin sensitivity index.

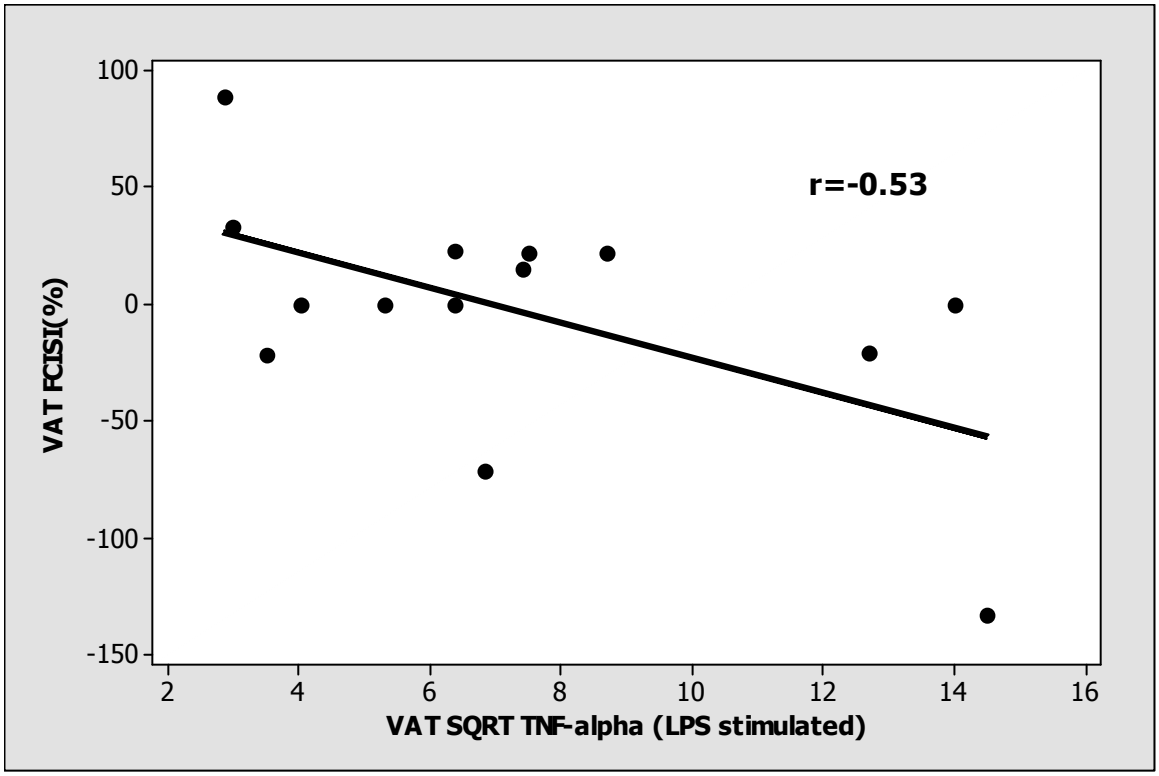

Figure 85 Relationship between stimulated release of VAT TNF-alpha and FCISI.

Stimulated release of VAT TNF-alpha by LPS is negatively correlated with FCISI of VAT in PE (p=0.037 adjusted for BMI) (n=14). SQRT TNF-alpha = square root TNF-alpha, FCISI=fat cell insulin sensitivity index

5.4.2.2 IL-6

Statistical analysis was performed on log transformed data to ensure normal distribution. Values are expressed as mean and SEM of untransformed data unless otherwise stated. There was no significant difference between basal release of IL-6 in SAT between controls and PE (169.1[61.3] vs 107.8[27.0] pg/ml/ugDNA, p=0.74), nor after stimulation with LPS (173.5[40.6] vs 76.8[16.9] pg/ml/ugDNA, p=0.12). In VAT, there was no difference between basal release of IL-6 (454[93.8] vs 584[180] pg/ml/ugDNA, p=0.90) or after stimulation with LPS (581[117] vs 912[242] pg/ml/ugDNA, p=0.65) between controls and PE.

IL-6 secretion was significantly increased in VAT after stimulation by LPS in PE (584[180] vs 912[242] pg/ml/ugDNA, p=0.007), but not in controls (454[9308] vs 581[117] pg/ml/ugDNA, p=0.09) (Figure 86). There was no significant difference in IL-6 secretion in SAT after stimulation by LPS in controls and PE.

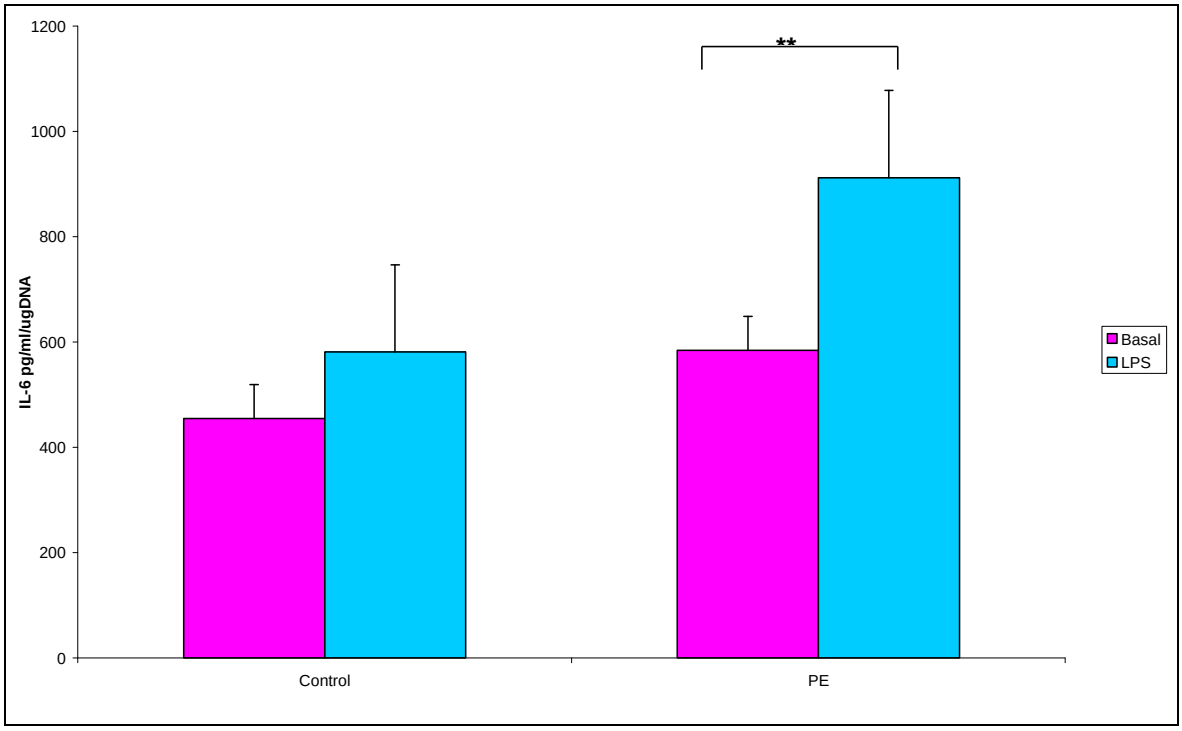

Figure 86 Comparison of the basal release of IL-6 and LPS stimulated release in visceral adipose tissue.

IL-6 release was significantly increased in PE (n=14) but not in controls (n=14) (p=0.007). Data normalized by log transformation prior to analysis by paired t-test but displayed as untransformed data as mean and SEM. **=p≤0.01.

Basal and LPS stimulated release of IL-6 in SAT positively correlated with BMI in controls(r=0.60, R^2=35.9%, p=0.014 and r=0.65, R^2=41.7%, p=0.007 respectively)(Figure 87).

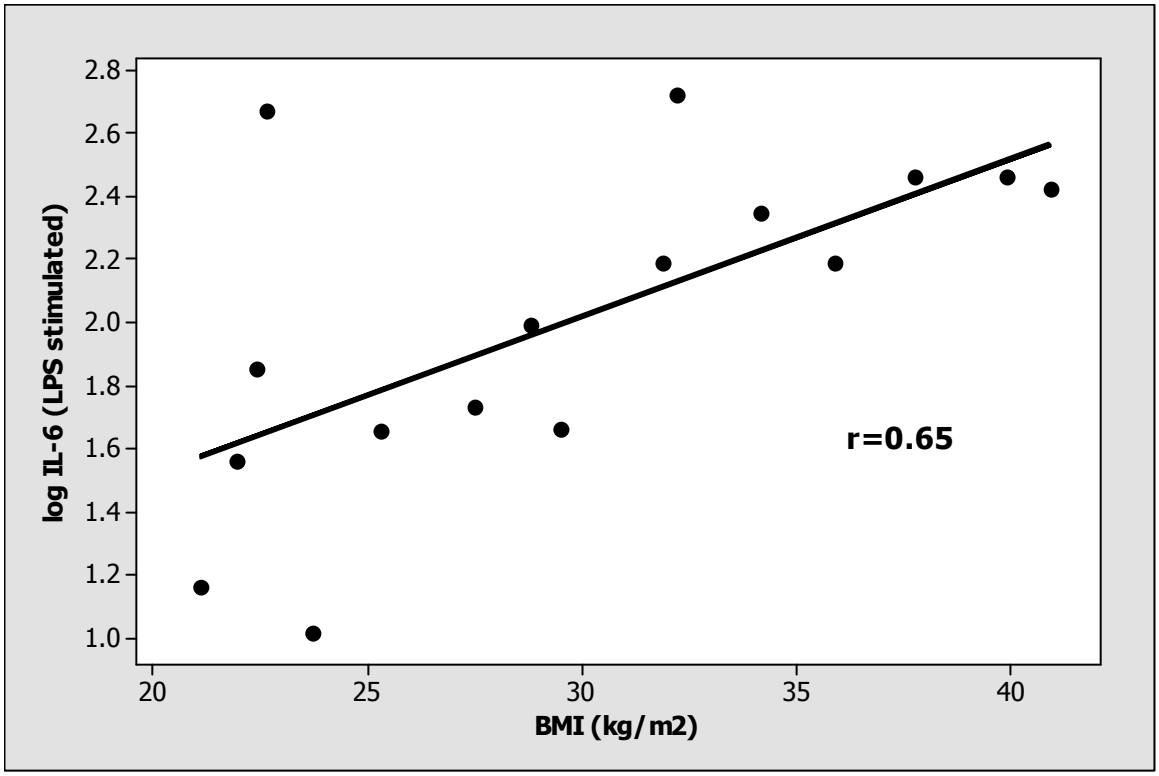

Figure 87 Correlation between BMI and LPS stimulated release of IL-6 in controls.

LPS stimulated release of IL-6 in SAT positively correlated with BMI in controls (p=0.007).

There was no correlation between SAT and VAT basal and stimulated release of IL-6 and maternal total cholesterol and TG, maternal NEFA, and maternal serum markers of insulin resistance including glucose, insulin and HOMA. There was no relationship between SAT and VAT basal and stimulated release of IL-6 and maternal serum IL-6, TNF alpha, CRP, leptin or adiponectin. Furthermore there was no correlation between IL-6 release in SAT and VAT and direct markers of adipocyte lipolysis or FCISI. There was no correlation between SAT and VAT cell size and IL-6 release. Basal and stimulated release of IL-6 from SAT was negatively correlated with maternal HDL (r=-0.59,R^2=34.8%, p=0.016 and r=-0.52, R^2=26.8%, p=0.04 respectively) an effect which is attenuated when adjusted for BMI (p=0.053 and p=0.13 respectively).

Basal release of IL-6 is higher in VAT than SAT in controls (169.1[61.3] vs 454.7[93.8] pg/ml/ugDNA, p=0.009), as is IL-6 release after stimulation by LPS (173.5[40.6] vs 581[117] pg/ml/ugDNA, p=0.003)(Figure 88).

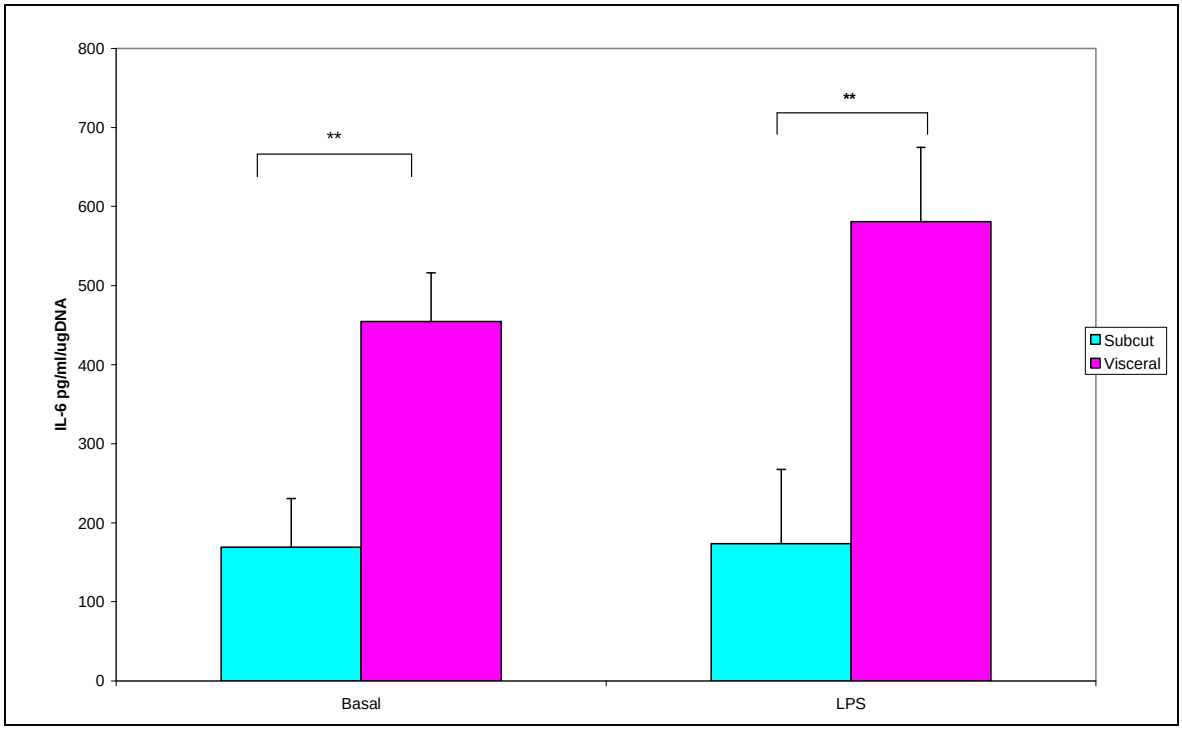

Figure 88 Comparison of basal and LPS stimulated release of IL-6 from SAT and VAT.

Basal and stimulated release of IL-6 is higher in VAT than in SAT (p=0.009 and p=0.003 respectivey) (n=14). Data displayed as mean and SEM. **=p≤0.01.

In PET, there is no correlation between basal and stimulated IL-6 release and BMI in SAT and VAT. There was no correlation between IL-6 release in SAT and VAT and maternal lipids, NEFA or maternal markers of insulin resistance. There was no correlation between SAT and VAT basal and stimulated IL-6 release and maternal plasma IL-6, TNF-alpha, CRP, leptin or adiponectin. There was no association between SAT and VAT basal and stimulated IL-6 release and direct measures of adipocyte lipolysis and insulin sensitivity.

Basal and LPS stimulated release of TNF-alpha and IL-6 are closely related in SAT and VAT in controls (Table 14) and PE (Table 15).

CONTROLS	SAT TNF-alpha Basal	SAT TNF-alpha LPS stimulated	VAT TNF alpha Basal	VAT TNF alpha LPS stimulated
SAT IL-6 Basal	r=0.71 R^2=50.4% p=0.002			
SAT IL-6 LPS stimulated		r=0.87 R^2= 74.9% p<0.001		
VAT IL-6 Basal			r=0.79 R^2=61.6% p=0.001	
VAT IL-6 LPS stimulated				r=0.79 R^2=63.0% p=0.001

Table 14 Correlations between basal and stimulated TNF-alpha and IL-6 release from SAT and VAT in controls (n=14)

PRE-ECLAMPTICS	SAT TNF-alpha Basal	SAT TNF-alpha LPS stimulated	VAT TNF alpha Basal	VAT TNF alpha LPS stimulated
SAT IL-6 Basal	r=0.79 R^2= 63.2% p=0.001			
SAT IL-6 LPS stimulated		r=0.74 R^2= 55.0% p=0.002		
VAT IL-6 Basal			r=0.72 R^2= 52.2% p=0.004	
VAT IL-6 LPS stimulated				r=0.86 R^2= 73.5% p<0.001

Table 15 Correlations between basal and stimulated TNF-alpha and IL-6 release from SAT and VAT in controls (n=14)

5.4.2.3Adiponectin

Basal and LPS stimulated release of adiponectin from VAT in controls is positively correlated with measures of visceral lipolytic function (see Figure 89 and Table 16). No apparent relationship in PE is found.

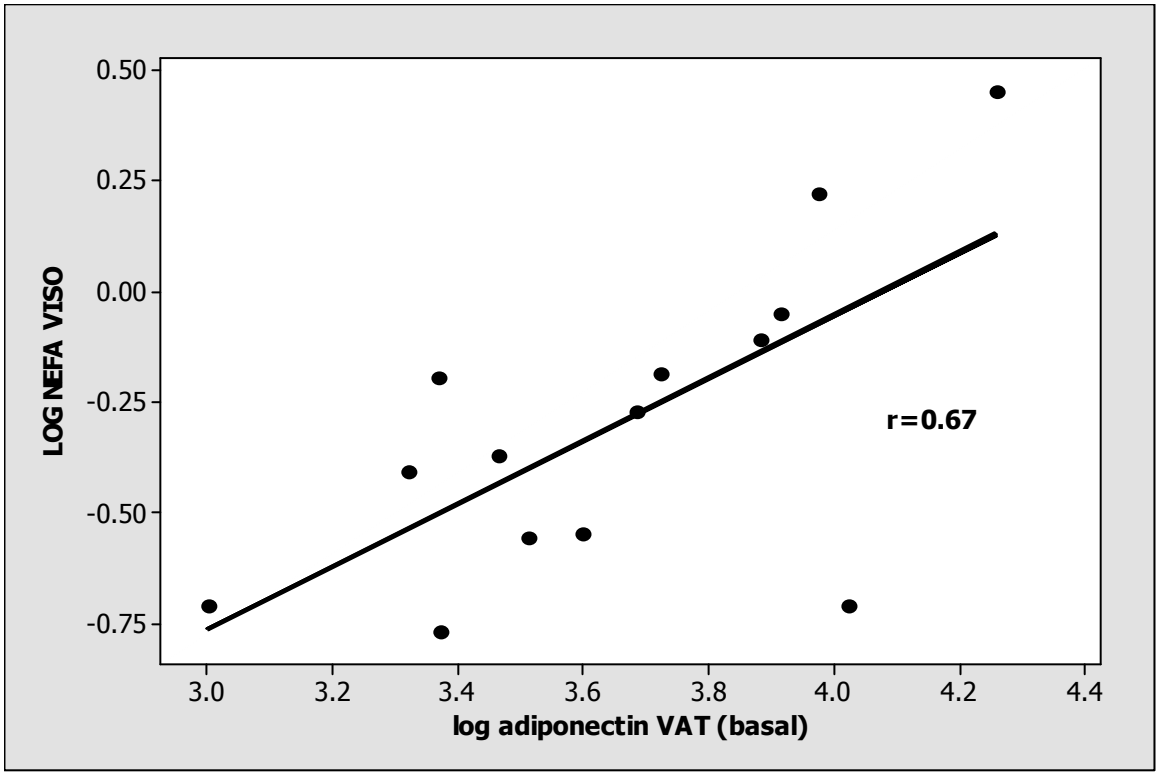

Figure 89 Relationship of basal release of adiponectin and VAT stimulated lipolysis.

Basal release of adiponectin is positively correlated with NEFA release in the presence of isoproternol (p=0.008). Data normalised by log transformation prior to statistical analysis. VISO = VAT in the presence of isoproterenol.

Controls (VAT)	*VISO*	*VINS*	*VISO+ VINS*
Basal release of adiponectin	r=0.67, p=0.008	r=0.59, p=0.027	r=0.71, p=0.004
Stimulated release of adiponectin	r=0.65, p=0.011	r=0.57, p=0.034	r=0.72, p=0.004

Table 16 Correlations between basal and stimulated release of adiponectin and measures of lipolytic function in VAT.

VISO= NEFA release from VAT in presence of isoproternol. VINS = NEFA release from VAT in presence of insulin. VISO + VINS = NEFA release from VAT in presence of isoproterenol and insulin.

No other significant differences between controls or PE were found, nor were there any other significant correlations (data not shown).

5.4.2.4PAI-1

In controls, there was a greater amount of PAI-1 released from VAT than SAT both basally (median 26.1 vs 110.0 pg/ml/ugDNA, p=0.01, Mann-Whitney) and after stimulation with LPS (median 32.2 vs 81.8 pg/ml/ugDNA, p=0.02, Mann-Whitney)(Figure 90). Similarly in PE there is a greater amount of PAI-1 released from VAT than SAT both basally(median 35.2 vs 123.8 pg/ml/ugDNA, p=0.03) and after stimulation(median 34.0 vs 144.6 pg/ml/ugDNA, p=0.01 Mann-Whitney)(Figure 91).

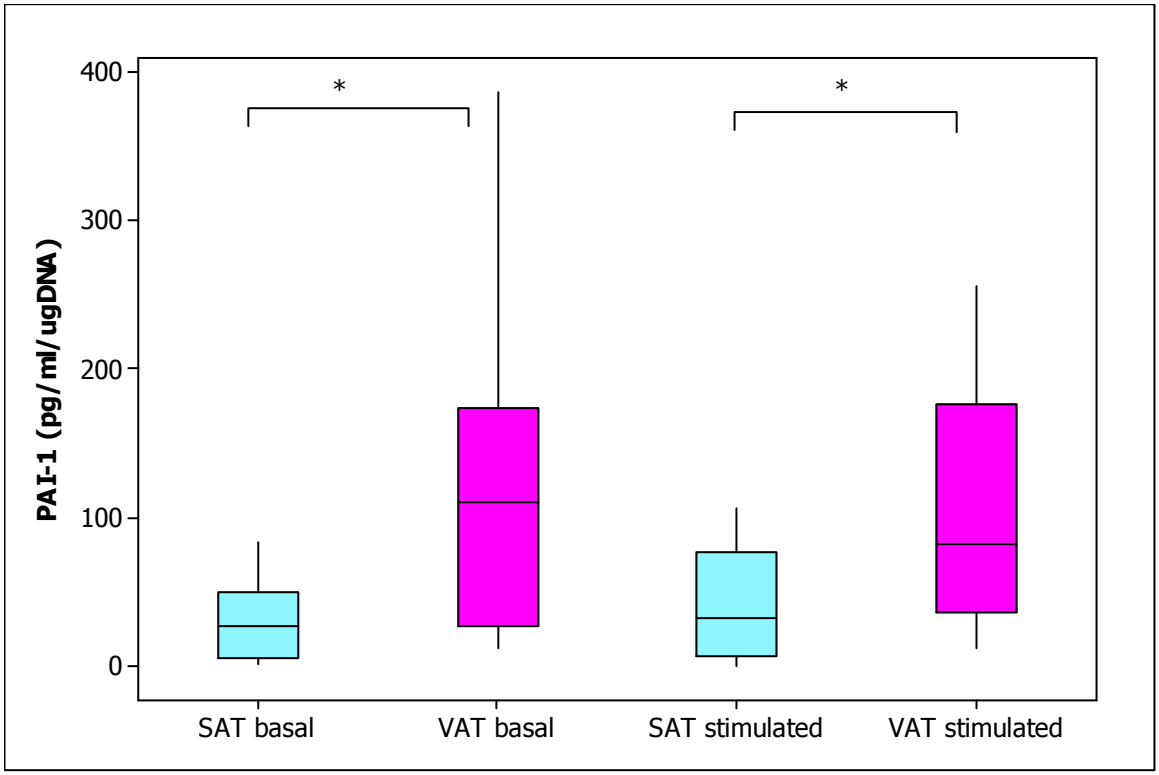

Figure 90 Difference in PAI-1 release between VAT and SAT in controls.

There is a greater amount of PAI-1 released from VAT compared to SAT both basally (p=0.01) and after stimulation with LPS (p=0.02) in controls (n=14). Data displayed as median and interquartile range. Analysis by Mann-Whitney. *=p≤0.05.

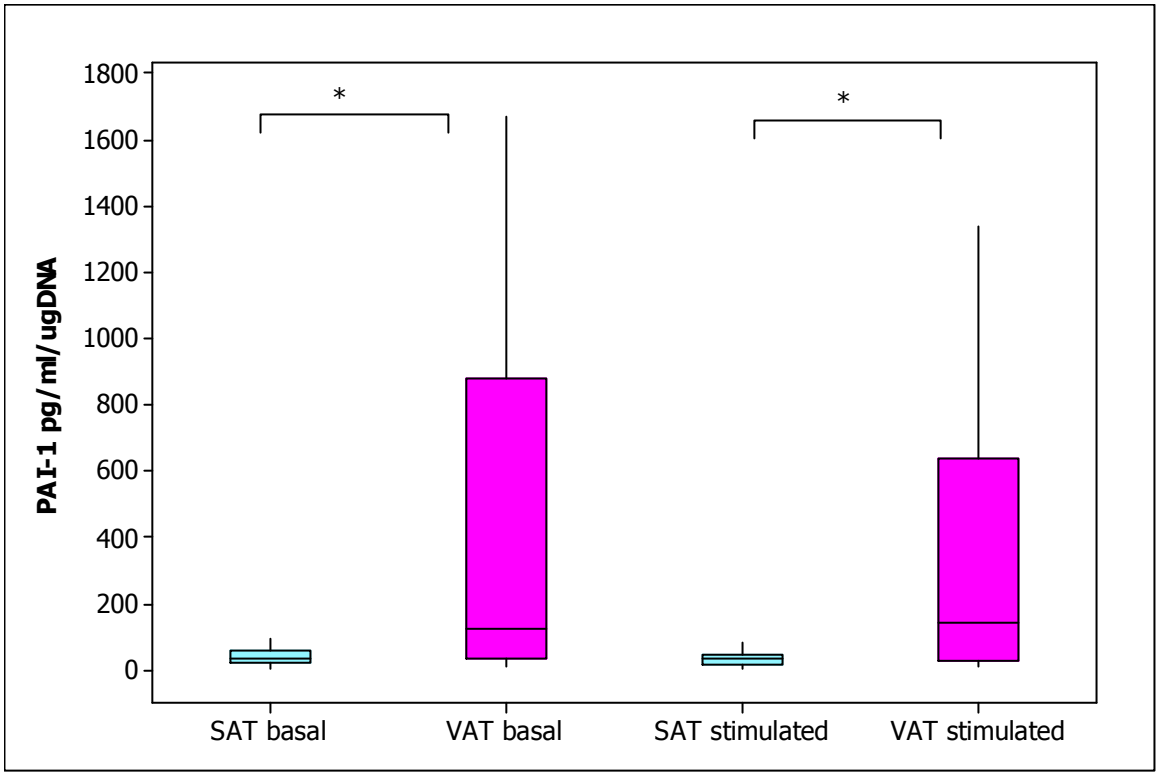

Figure 91 Difference in PAI-1 release between VAT and SAT in PE.

There is a greater amount of PAI-1 released from VAT compared to SAT both basally (p=0.03) and after stimulation with LPS (p=0.01) in PE (n=14). Data displayed as median and interquartile range. Analysis by Mann-Whitney. *=p≤0.05.

Basal PAI-1 release from SAT is positively correlated with BMI in controls (r=0.64, R^2=41.3%, p=0.007) and PE(r=0.63, R^2=40.2%, p=0.015). HDL is negatively correlated with PAI-1 release from SAT in PE only (r=0.60, R^2= 35.6%, p=0.024) which is attenuated but not obliterated when adjusted for BMI (p=0.036).

No other significant differences between controls and PE were found, nor were there any other significant correlations (data not shown).

5.4.2.5Leptin

In controls, basal release of leptin from SAT is higher than VAT (1033[191] vs 268 [72] pg/ml/ugDNA, p=0.002), as is release after LPS stimulation (973[165] vs 261.1[72.1] pg/ml/ugDNA, p=0.005)(Figure 92).

This effect is similar in PE (1334[191] vs 220.4[55.3] pg/ml/ugDNA, p=0.001 and 1269[399] vs 182.2[47.2] pg/ml/ugDNA, p<0.001)(Figure 93).

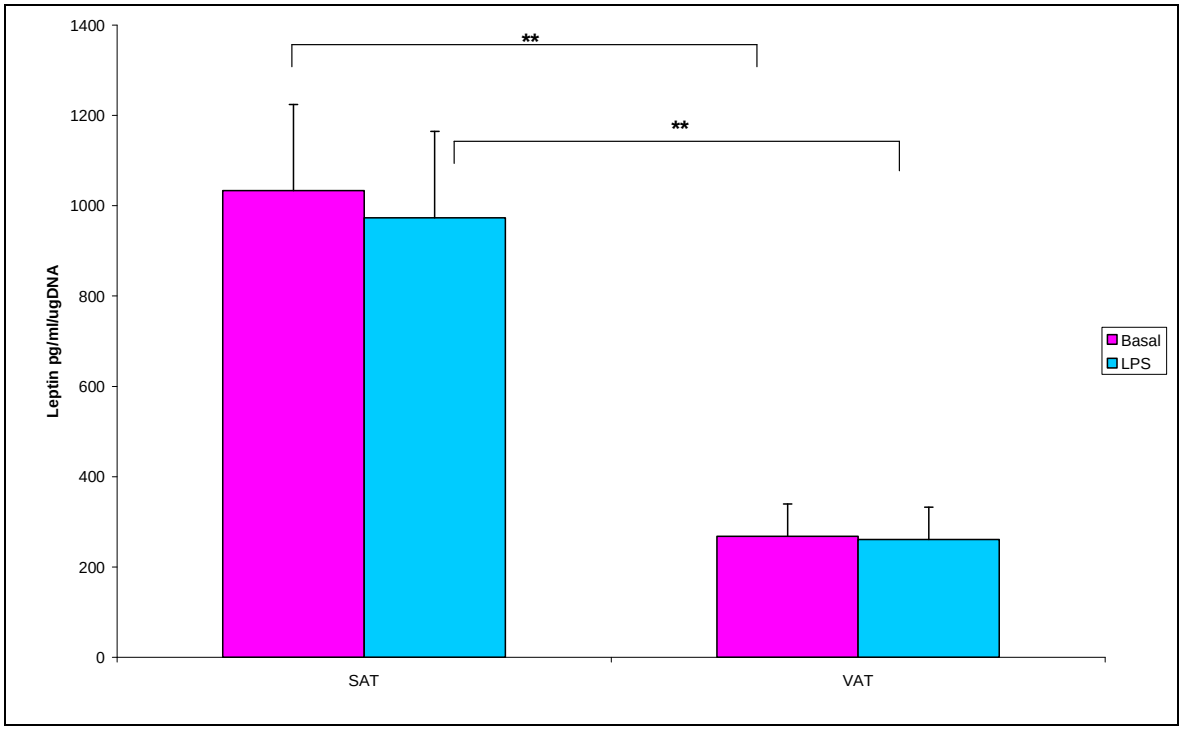

Figure 92 Comparison of release of leptin from SAT and VAT in controls.

Release of leptin from SAT compared to VAT is greater both basally (p=0.002) and after stimulation with LPS (p=0.005)(n=14). Comparison made using unpaired t-test. **=p≤0.01.

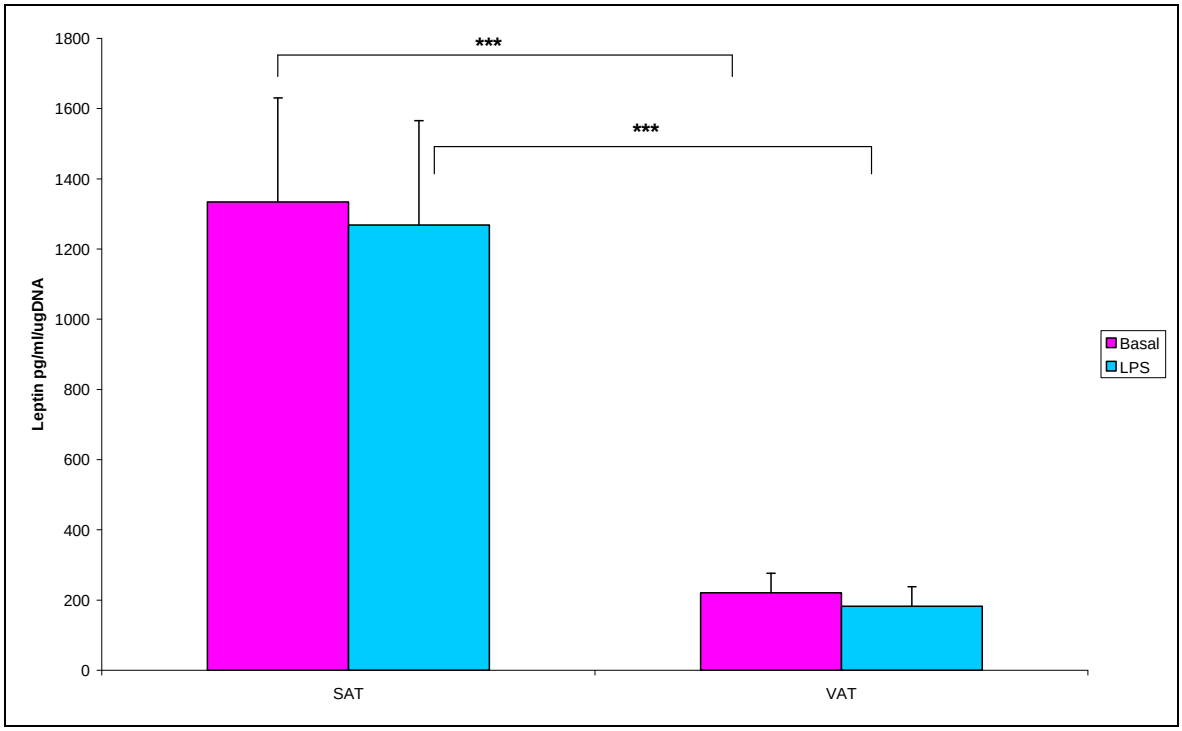

Figure 93 Comparison of release of leptin from SAT and VAT in PE.

Release of leptin from SAT compared to VAT is greater both basally (p=0.001) and after stimulation with LPS (p<0.001) (n=14). Comparisons made using un-paired t-test. ***=p≤0.001.

BMI does not correlate with leptin release from SAT or VAT in PE or controls.

In PE, basal and stimulated release of leptin from SAT is negatively correlated with maternal serum adiponectin (r=-0.78, R^2= 61.2%, p=0.001 and r=-0.76, R^2=57.0%, p=0.001 respectively)(Figure 94). This is independent of BMI (p=0.007 and 0.009). This relationship is not apparent in controls (p=0.82 and p=0.73).

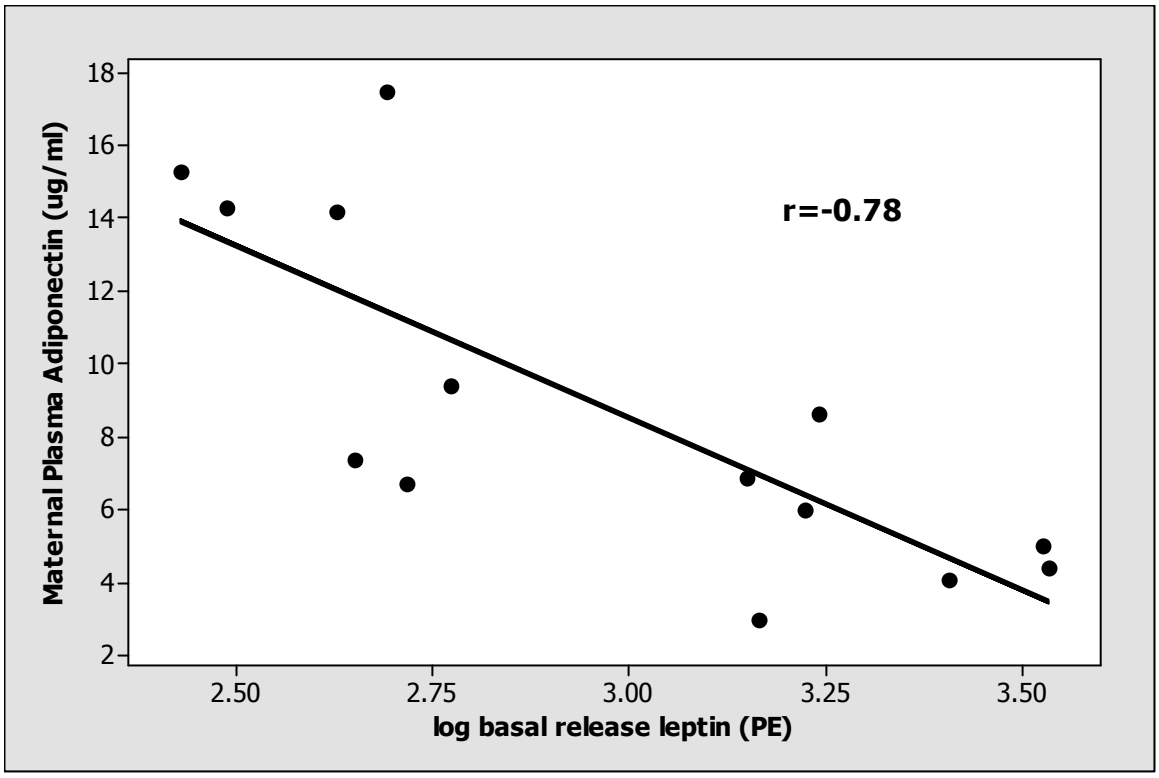

Figure 94 Relationship of basal release of leptin from SAT and maternal plasma levels of adiponectin in PE.

There is a negative correlation between basal release of leptin from SAT and maternal plasma adiponectin independent of BMI (p=0.007) (n=14).

In PE, both basal and stimulated release of leptin from SAT positively correlates with direct measures of adipocyte lipolytic function in this adipose depot (Figure 95 and Table 17).

PE (SAT)	*SBA*	*SISO*	*SINS*	*SISO+INS*
Leptin basal	r=0.85, p<0.001	r=0.60, p=0.024	r=0.73, p=0.003	r=0.74, p=0.002
Leptin stimulated	r=0.79, p=0.001	r=0.55, p=0.042	r=0.71, p=0.004	r=0.69, p=0.006

Table 17 Table of correlations between basal and LPS stimulated release of leptin and measures of lipolytic function (release of NEFA ug/ml/udDNA) in SAT in PE.

SBA= basal lipolysis, SISO=lipolysis in presence of isoproterenol, SINS= lipolysis in presence of insulin, SISO+SINS= lipolysis in presence of isoproterenol and insulin.

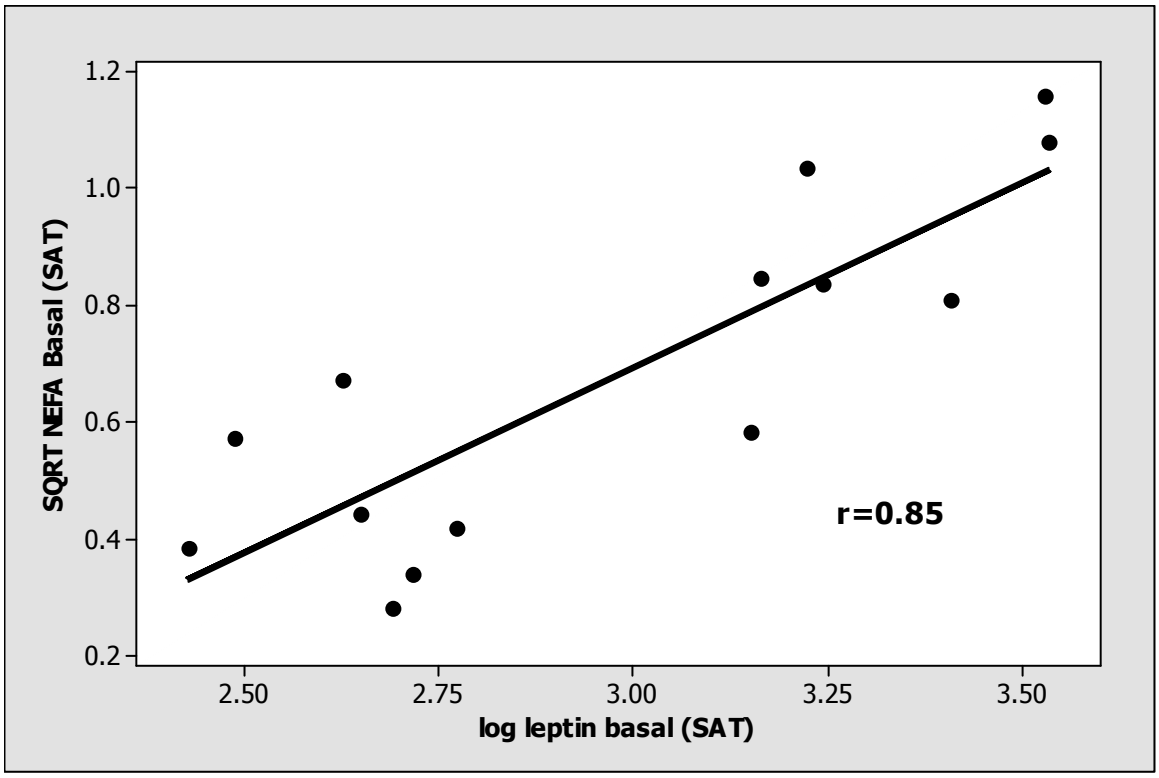

Figure 95 Relationship between basal release of leptin and basal lipolysis in SAT in PE.

There is a positive relationship between the basal release of leptin and basal lipolysis in SAT (p<0.001) (n=14).

Release of leptin from VAT is also correlated with direct measures of adipocyte lipolytic function in this adipose depot (Table 18).

PE (VAT)	*VBA*	*VISO*	*VINS*	*VISO+INS*
Leptin basal	r=0.57, p=0.05	r=0.70, p=0.01	NS	r=0.66, p=0.02
Leptin stimulated	r=0.62, p=0.03	r=0.65, p=0.02	r=0.62, p=0.03	r=0.62, p=0.03

Table 18 Table of correlations between basal and LPS stimulated release of leptin and measures of lipolytic fuction (release of NEFA ug/ml/ugDNA) in VAT in PE.

VBA= basal lipolysis, VISO=lipolysis in presence of isoproterenol, VINS= lipolysis in presence of insulin, VISO+INS= lipolysis in presence of isoproterenol and insulin.

In contrast, in controls, SAT release of leptin is not a significant correlate of lipolytic function (data not shown). However VAT release of leptin does relate to measures of lipolysis (Table 19).

Controls (VAT	*VBA*	*VISO*	*VINS*	*VISO+INS*
Leptin basal	NS	r=0.89, p<0.001	r=0.68, p=0.02	r=0.80, p=0.002
Leptin stimulated	NS	r=0.79, p=0.002	r=0.59, p=0.04	r=0.72, p=0.009

Table 19 Table of correlations between basal and LPS stimulated release of leptin and measures of lipolytic fuction (release of NEFA ug/ml/ugDNA) in VAT in controls.

VBA= basal lipolysis, VISO=lipolysis in presence of isoproterenol, VINS= lipolysis in presence of insulin, VISO+INS= lipolysis in presence of isoproterenol and insulin.

No other significant differences in PE and controls were found, nor were there any other significant correlations.

5.4.2.6IL-10

In PE, basal and stimulated release of IL-10 from SAT is closely correlated with BMI (r=0.81, R^2= 65.6%, p<0.001 and r=0.73, R^2=53.1%, p=0.003), a relationship which is not seen in controls.

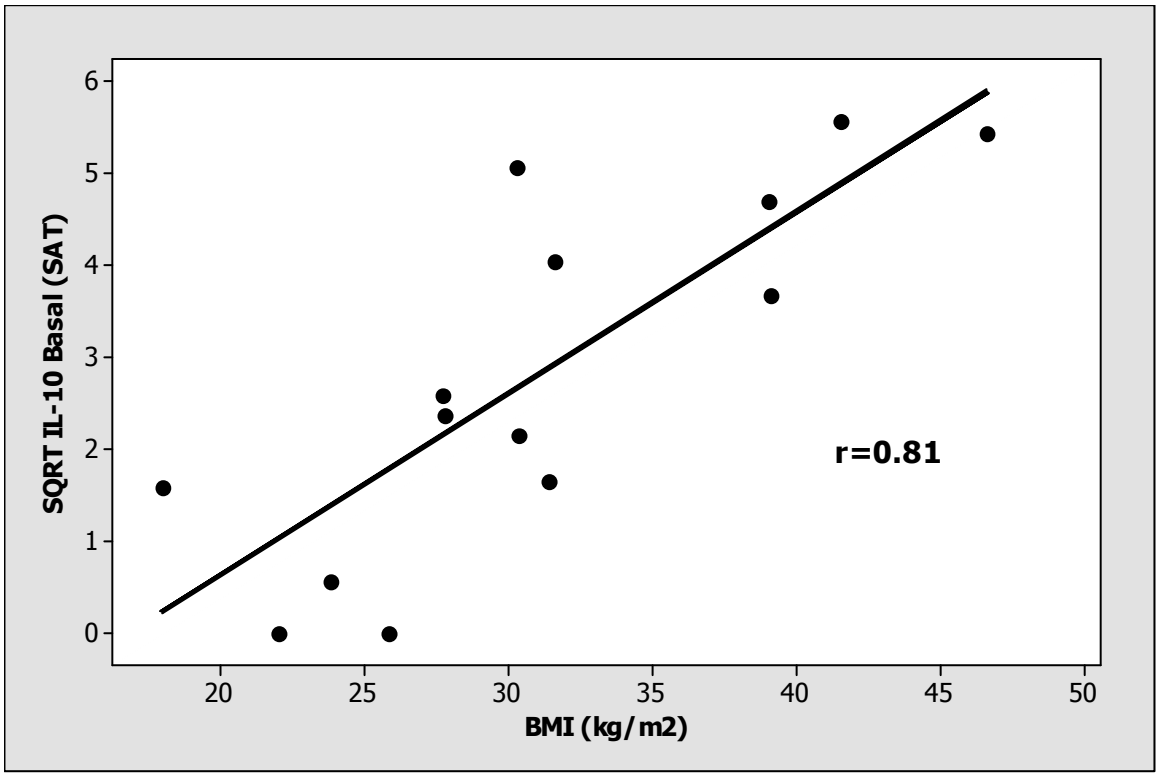

Figure 96 Correlation between BMI and basal release of IL-10 from SAT in PE.

There is a positive correlation between BMI and basal release of IL-10 from SAT in PE (p<0.001) (n=14).

Indeed in PE, IL-10 release from SAT correlates with SAT cell size (p=0.008) but this is not independent of BMI (p=0.33).IL-10 release from SAT negatively

correlates with the insulin sensitivity of the tissue as calculated by FCISI (r=-0.58, p=0.03), but again not independent of BMI (p=0.36).

No other significant differences between controls and PE, nor any other significant correlations were found (data not shown).

5.4.2.7CRP

Release of CRP from SAT is a significant determinant of maternal plasma CRP in both controls (r=0.66, R^2=43.0%, p=0.006) and PE (r=0.89, R2=78.9%, p<0.001) independent of BMI (p=0.015 and p<0.001 respectively)(Figure 97).

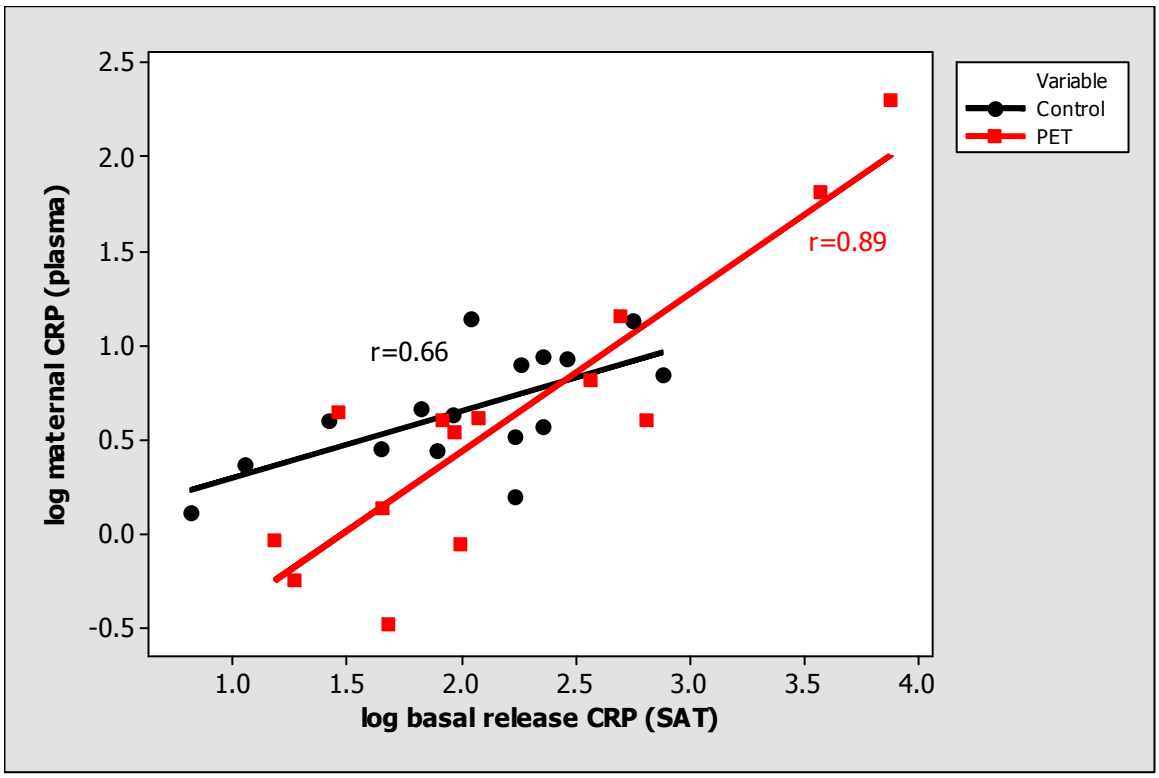

Figure 97 Correlation between basal release of CRP and maternal plasma CRP from SAT in controls and PE.

There is a positive correlation between basal release of CRP from SAT and maternal plasma levels of CRP in controls (p=0.006) (n=14) and PET (p<0.001)(n=14) .

Both basal and stimulated release of CRP from VAT is correlated with measures of VAT lipolysis in controls. This effect is not seen in PE (data not shown).

Controls (VAT)	*VBA*	*VISO*	*VINS*	*VISO+INS*
CRP basal	r=0.60, p=0.02	r=0.66, p=0.01	r=0.65, p=0.01	r=0.75, p=0.002
CRP stimulated	r=0.61, p=0.02	r=0.63, p=0.02	r=0.63, p=0.02	r=0.76, p=0.001

Table 20 Table of correlations between basal and LPS stimulated release of CRP and measures of lipolytic function (release of NEFA ug/ml/ugDNA) in VAT in controls.

VBA= basal lipolysis, VISO=lipolysis in presence of isoproterenol, VINS= lipolysis in presence of insulin, VISO+INS= lipolysis in presence of isoproterenol and insulin.

No other significant differences between controls and PE, nor any further significant correlations were found (data not shown).

5.4.2.8MCP-1

Stimulated release of MCP-1 was higher in VAT than SAT in PE (44.3[11.8] vs 248.5[73.8] pg/ml/ugDNA, p=0.004). There was also a trend for basal release of MCP to be greater in VAT than SAT but did not reach significance (68.6[15.9] VS 195.2[57.0] pg/ml/ugDNA, p=0.07)(Figure 98). There was no significant difference in basal or stimulated MCP-1 release between SAT and VAT in controls (Figure 99).

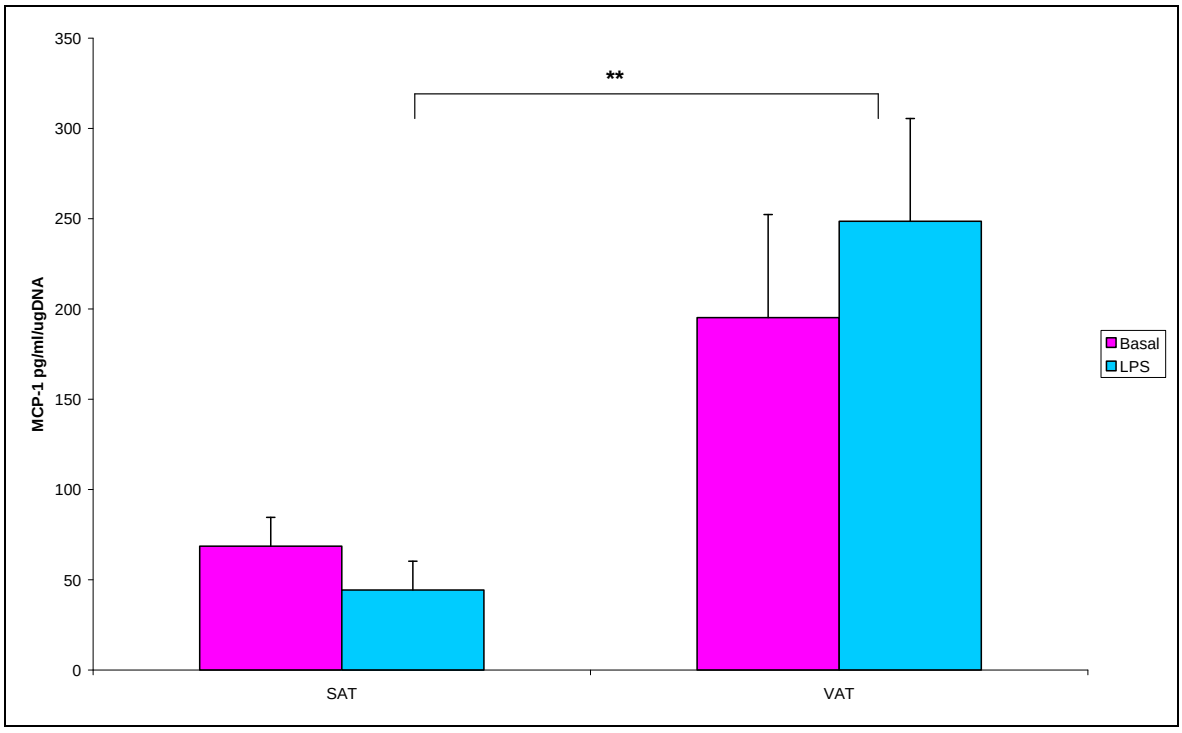

Figure 98 Comparison of SAT and VAT basal and stimulated release of MCP-1 in PE.

Stimulated release of MCP-1 is greater from VAT than SAT (p=0.004) (n=14). Comparisons made using student t-test and data displayed as mean and SEM. **=p≤0.01.

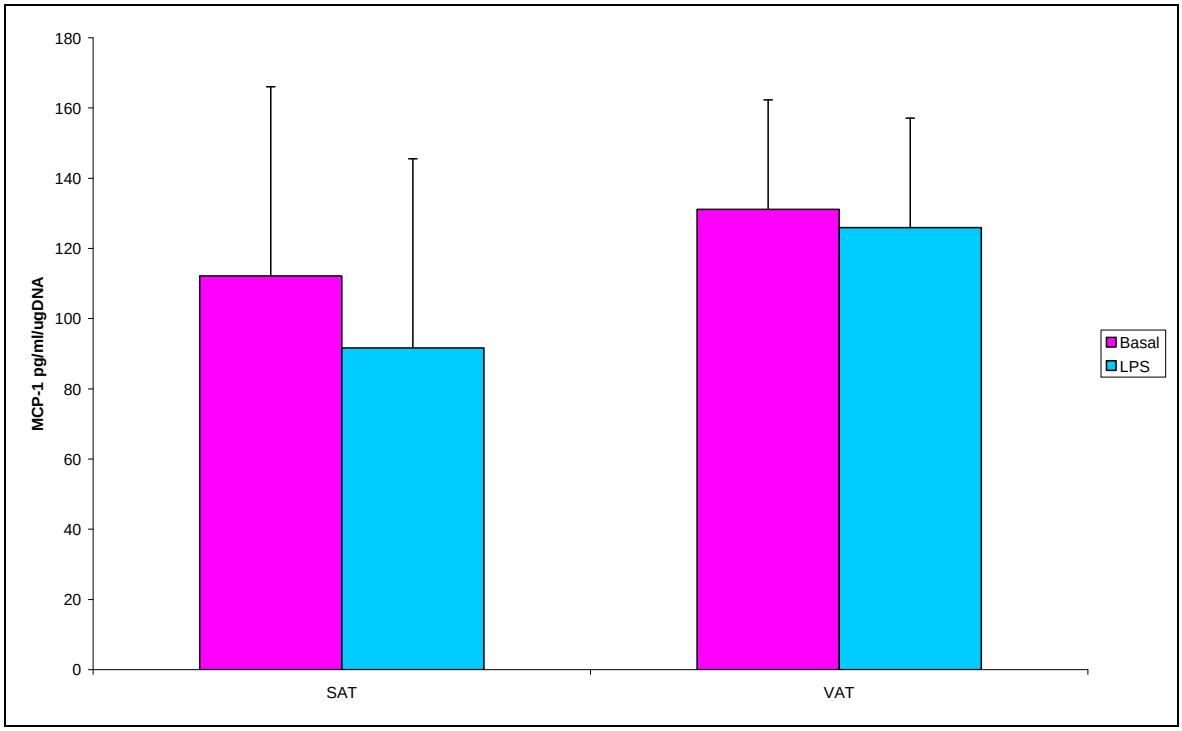

Figure 99 Comparison of SAT and VAT basal and stimulated release of MCP-1 in controls.

There is no significant difference between SAT and VAT (p=0.18 and p=0.15) (n=14). Comparisons made using student t-test and data displayed as mean and SEM.

In controls, there was a positive correlation between release of MCP-1 in SAT and BMI (r=0.68, R^2=46.2%, p=0.004). No such association was apparent in PE.

No other significant differences between PE or controls, nor any other significant correlations were found (data not shown).

5.4.3 Relationship of adipocyte cell size and release of adipokines

5.4.3.1Subcutaneous Adipose Tissue

In controls, no correlation with SAT cell size and release of adipokines was found (data not shown). However in PE several associations were apparent. CRP basal release was positively correlated with SAT cell size (r=0.60, R^2=36.4%, p=0.02) although not independent of BMI (p=0.10). Leptin basal release was also positively correlated with SAT cell size (r=0.53, R^2=28.1%, p=0.05) again not independent of BMI (p=0.28). PAI-1 basal release was also positively correlated with SAT cell size (r=0.53, R^2=27.7%, p=0.05) not independent of BMI (p=0.59).

Furthermore the release of IL-10 was also positively correlated with cell size in this depot in PE only (r=0.68, R^2=46.2%, p=0.008) not independent of BMI (p=0.34). No other associations were found.

5.4.3.2 Visceral adipose tissue

No significant correlations with VAT cell size and release of adipokines were apparent (data not shown). VAT cell size negatively correlated with the release of adiponectin in PE (r=-0.56, R^2=30.9%, p=0.04) independent of BMI (p=0.045).

5.4.4 Messenger RNA expression

5.4.4.1 TNF-alpha

All comparisons were made using Mann-Whitney test for non-parametric data. Median TNF-alpha mRNA expression in VAT was higher in PE relative to controls (2.2 vs 0.9 TNF-alpha to PPIA ratio, p=0.039)(Figure 100). There was no difference in TNF-alpha mRNA expression in SAT between PE and controls (p=0.94).

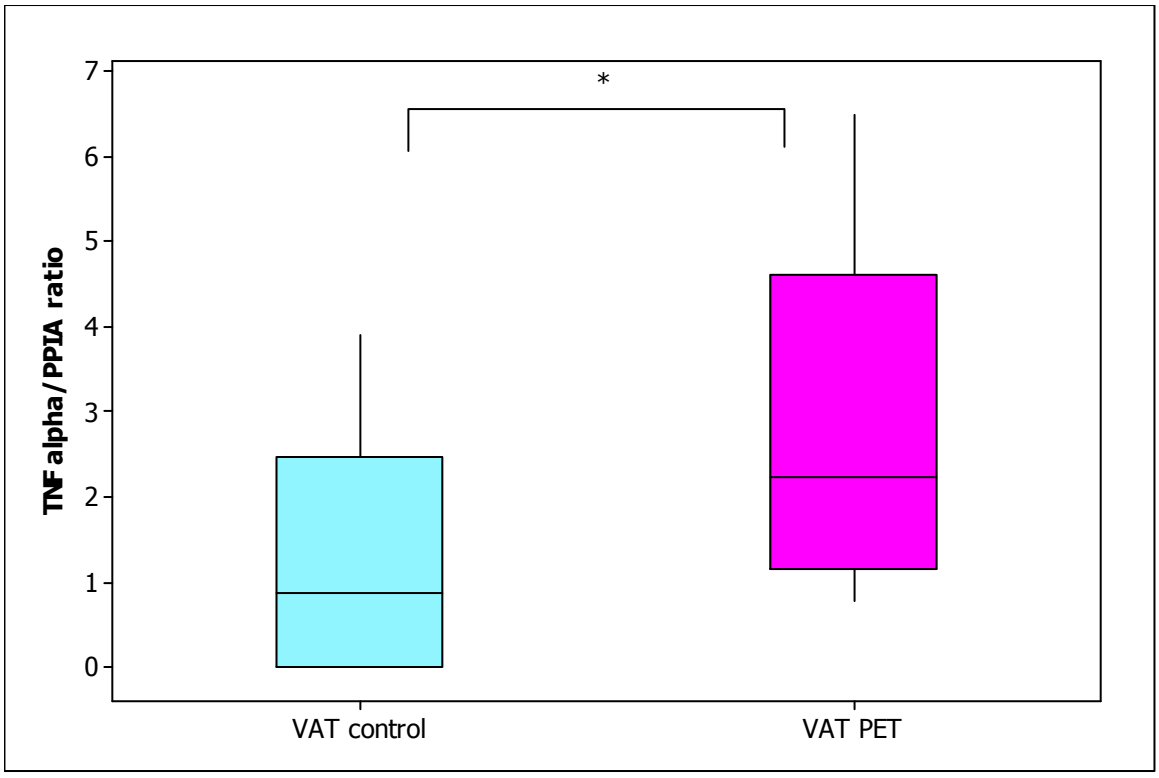

Figure 100 TNF-alpha gene expression in VAT in controls and PE.

Median TNF- alpha gene to PPIA ratio is greater in PE relative to controls (p=0.039) (n=14). Data expressed as median and interquartile range and analysis by Mann-Whitney.*=p≤0.05

5.4.4.2 IL-6

Median IL-6 mRNA expression in VAT appeared higher in PE relative to controls but did not reach significance (11.8 vs 4.0 target gene to PPIA ratio, p=0.11)(Figure 101). There was no difference in IL-6 mRNA expression in SAT between PE and controls (p=0.72).

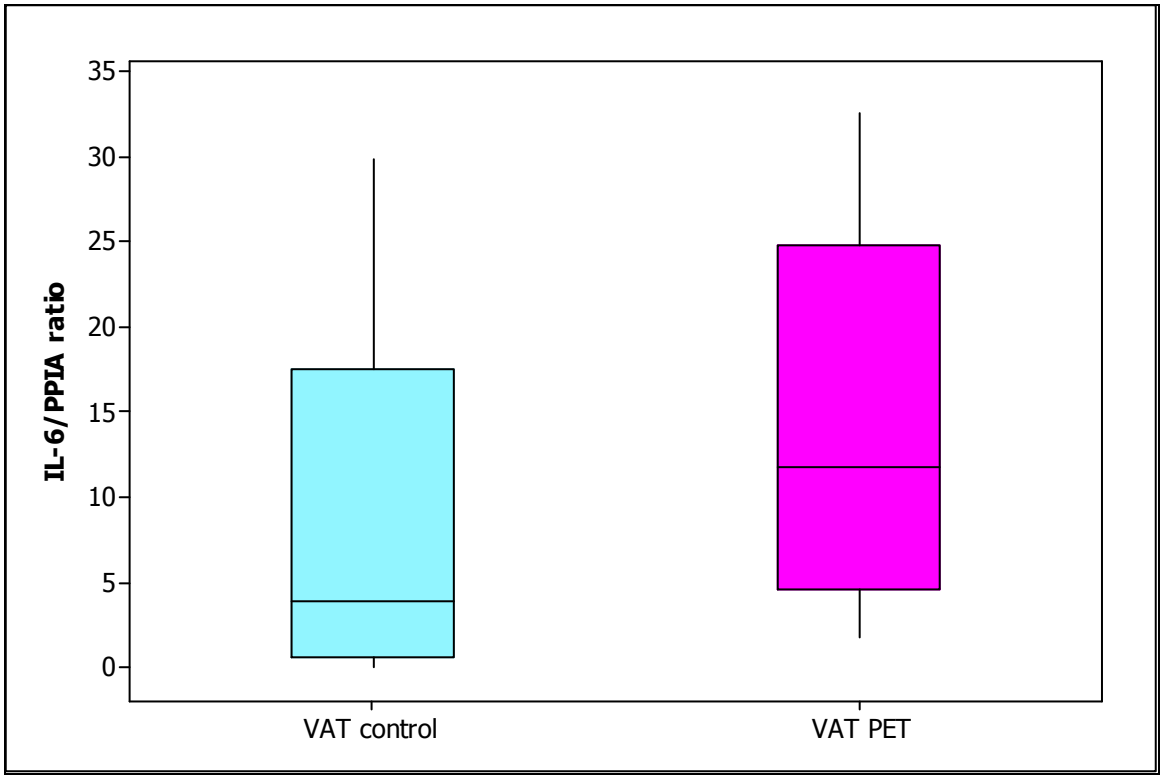

Figure 101 IL-6 gene expression in VAT in controls and PE.

Median Il-6 to PPIA ratio appears greater in PE (n=14) relative to controls (p=0.11) (n=14). Data expressed as median and interquartile range and analysis by Mann-Whitney.

5.4.4.3MCP-1

Comparisons made on log transformed data by unpaired t-test (control vs PE) and paired t-test (SAT vs VAT). Data expressed as absolute values. There was no statistical difference between MCP-1 mRNA expression relative to PPIA in VAT (40.5 vs 67.4 MCP1to PPIA ratio, p=0.15) or SAT (55.8 vs 52.2 MCP1 to PPIA ratio, p=0.42) between controls and PE. However MCP1 mRNA expression appeared higher in VAT compared to SAT in PE (67.4 vs 52.2 MCP1 to PPIA ratio, p=0.049) but not in controls (40.5 vs 55.8 MCP1 to PPIA ratio, p=0.55), although caution

needs to used when comparing gene expression between the different adipose depots.

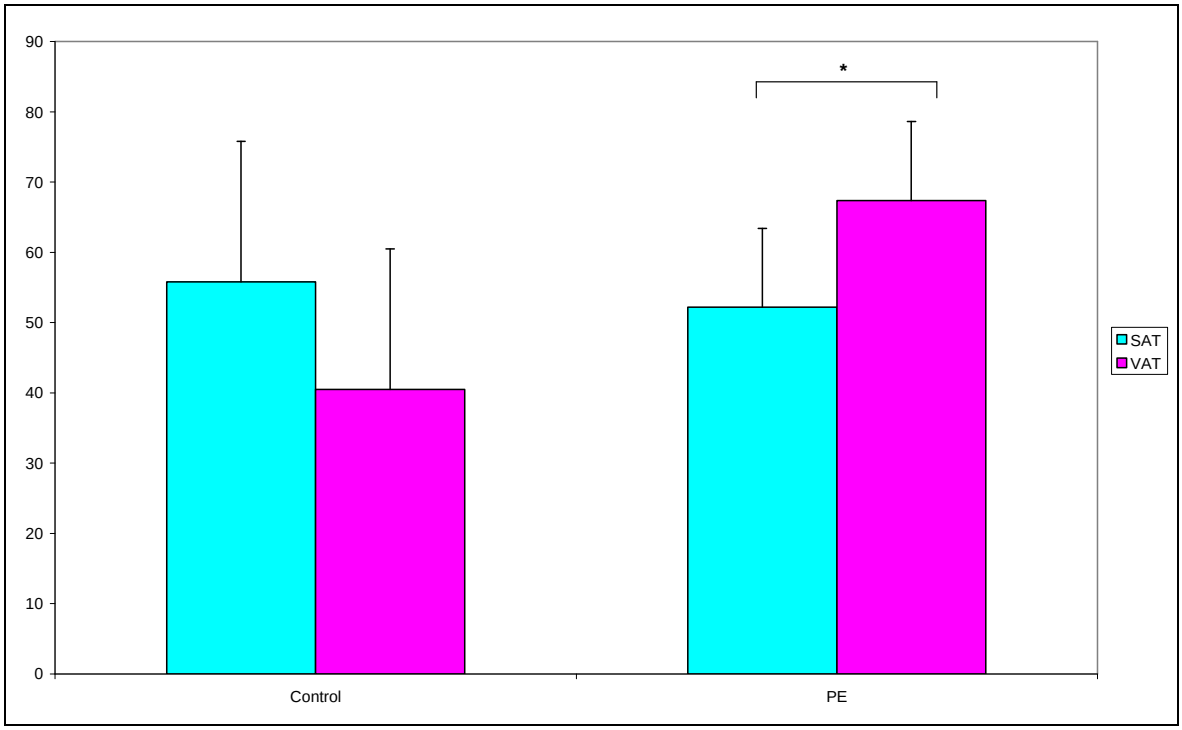

Figure 102 Expression of MCP1 in SAT and VAT.

There is a greater expression of MCP1 from VAT relative to SAT in PE (p=0.049) (n=14) but not in controls (p=0.55) (n=14). Comparisons made using paired t-test and data displayed as mean and SEM. *=p≤0.05.

5.4.5 Adipose Tissue Macrophage Infiltration

5.4.5.1Messenger RNA expression

The mean cfms mRNA expression in VAT was higher in PE relative to controls matched for BMI (28.1[3.9] vs 57.2[10.1], p=0.033)(Figure 103). No other differences between CD68 expression in VAT or SAT or cfms expression in SAT was seen (Table 21). All data is expressed and displayed as absolute values but analysis was performed using unpaired t-test on log transformed data.

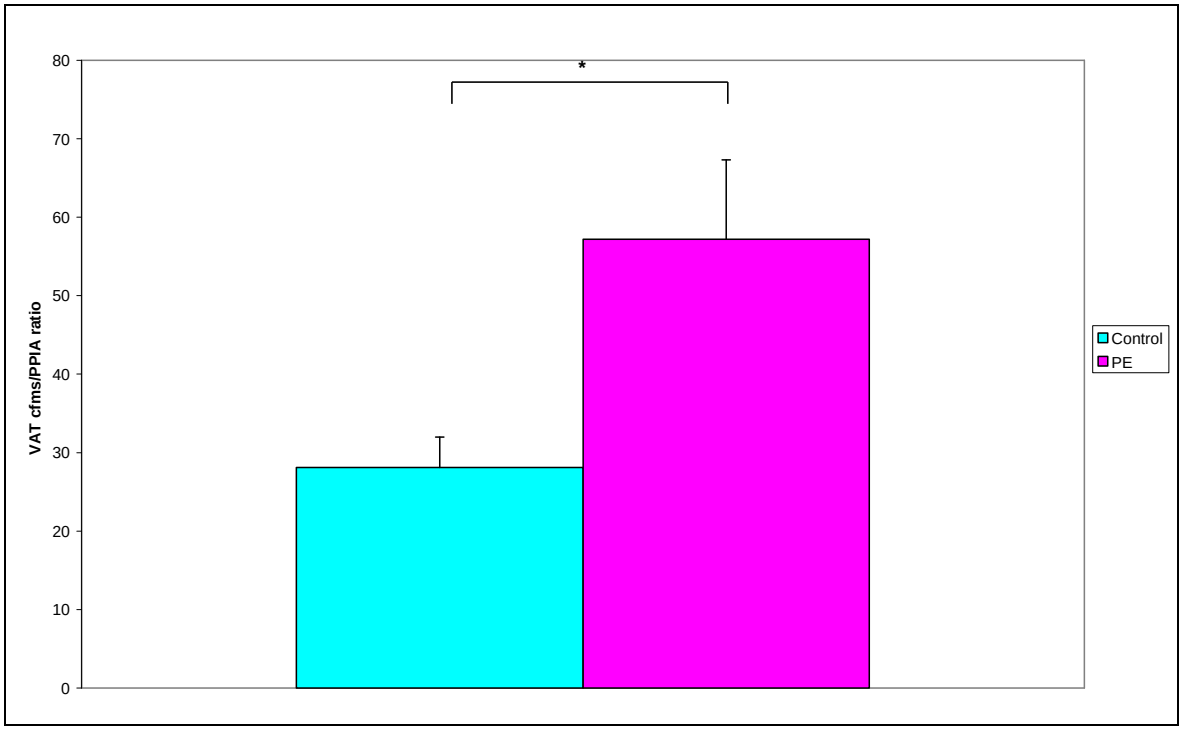

Figure 103 VAT cfms gene expression in controls and PE.

The cfms to PPIA ratio was greater in PE (n=14) than in controls (p=0.033)(n=14)in VAT. Comparisons made using student t test and expressed as mean and SEM. *=p≤0.05.

	Control (n=14)	***PE (n=14)***	***P***
VAT CD68/PPIA mRNA ratio	57.6(9.2)	67.9(5.2)	0.342
SAT CD68/PPIA mRNA ratio	73.4(11.2)	76.8(11.8)	0.494
VAT cfms/PPIA mRNA ratio	28.1(3.9)	57.2(10.1)	**0.033**
SAT cfms/PPIA mRNA ratio	70.0(25.2)	57.4(12.1)	0.855
	n=9	**n=9**	
VAT $CD68^+$/adipocyte(%)	19.6(1.7)	23.1(4.5)	0.491
SAT $CD68^+$/adipocyte(%)	22.3(3.2)	18.8(6.1)	0.379
VAT $cfms^+$/adipocyte(%)	8.2(1.5)	16.4(3.0)	**0.032**
SAT $cfms^+$/adipocyte (%)	8.2(1.3)	18.2(5.5)	0.118

Table 21 Table summarising differences between VAT and SAT mRNA expression and cell density of cfms and CD68 between PE and controls matched for BMI.

5.4.5.2Adipose tissue macrophage density

Adipose tissue macrophage densities were quantified as outlined in methods section 2.11, and expressed as mean cell count per adipocyte. PE (n=9) were matched with controls for BMI (p=0.66).

The mean percentage of $cfms^+$/adipocyte in VAT was higher in PE relative to controls (8.2[1.5] vs 16.4[3.0] %, p=0.032). No other differences between cfms counts in SAT or CD68 in SAT or VAT were found.

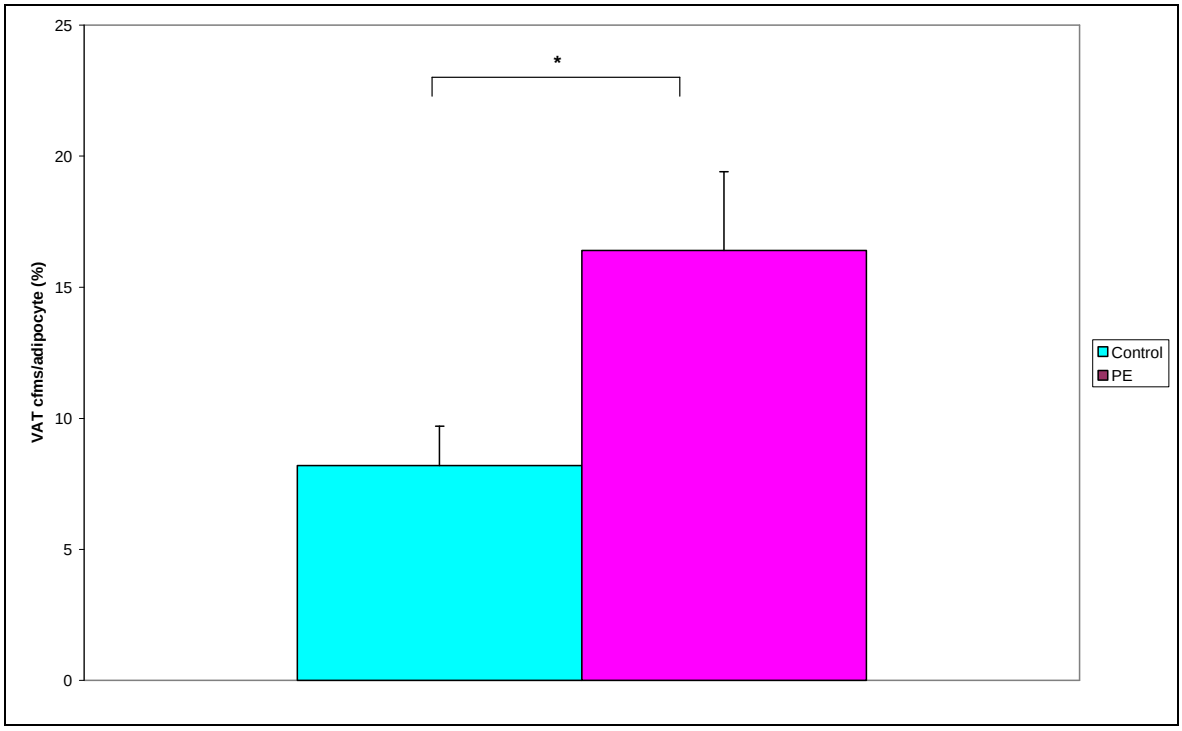

Figure 104 Mean cfms^{+}/adipocyte counts in VAT in PE and controls.

There is a higher percentage of cfms^{+}/adipocyte in PE (n=14) than in controls (p=0.032)(n=14). Comparisons made using student t test and expressed as mean and SEM. *=p≤0.05.

There was no correlation between basal or stimulated MCP1 release and macrophage counts in VAT or SAT in controls or PE.

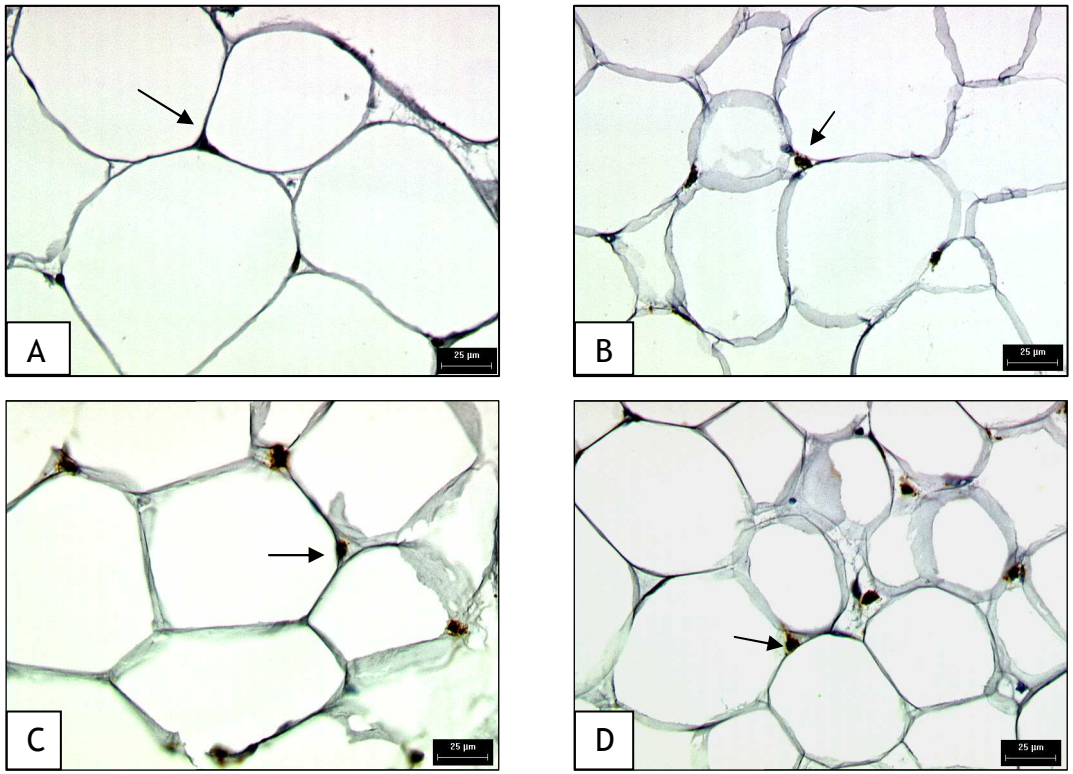

Figure 105 CD68 staining of adipose tissue (at x 400).

A-SAT of control, B- SAT of PE, C- VAT of control, D- VAT of PE. Arrow highlights CD68 positive staining of macrophage. Tissue macrophage densities were expressed as cell count per adipocyte.

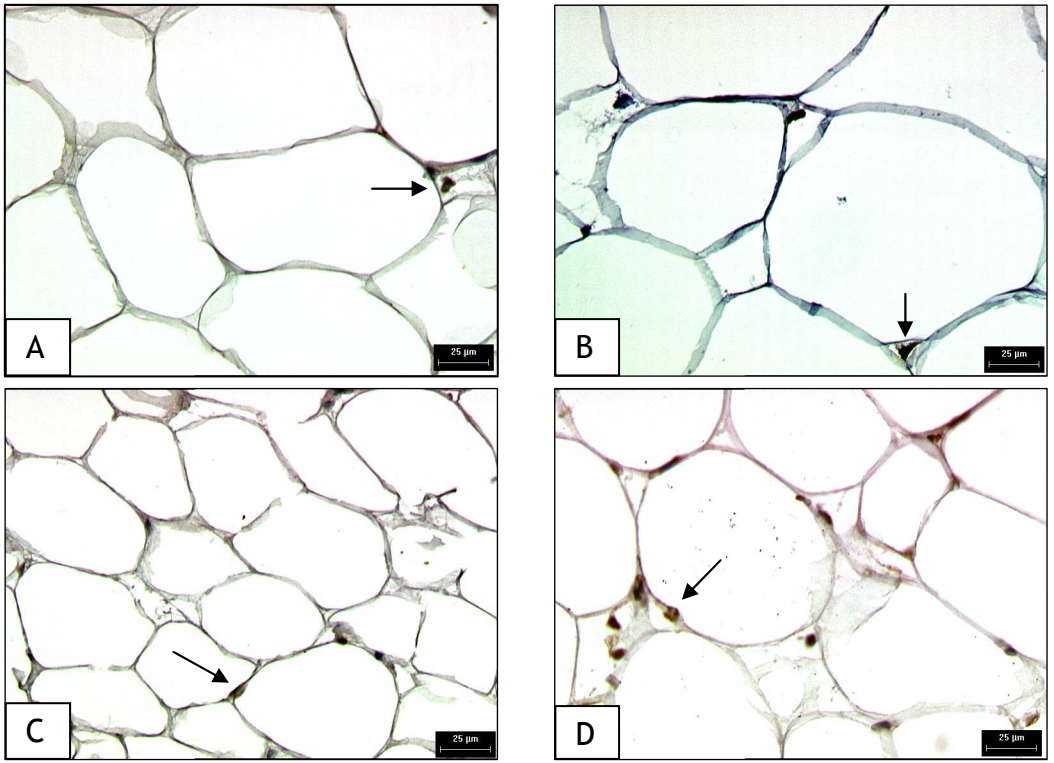

Figure 106 Cfms staining of adipose tissue (at x400).

A-SAT of control, B- SAT of PE, C- VAT of control, D- VAT of PE. Arrow highlights cfms positive staining of cell (activated macrophage). Tissue macrophage densities were expressed as cell count per adipocyte.

5.5 Discussion

We have demonstrated clear differences in adipose tissue function between PE and controls including important regional differences.

There is an increased tendency to secrete the inflammatory adipokines of TNF alpha and IL-6 from visceral fat in PE, with increased gene expression in this fat depot. TNF alpha is 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 IRS1.[155] Correspondingly, we have also shown that TNF-alpha release from VAT is negatively correlated with the fat-cell insulin sensitivity of this depot in PE providing evidence of a potentially more pathogenic role of VAT in PE. IL-6, a stimulator of whole body lipolysis with anti-insulin effects,[162 163] has been related to pregnancy-associated insulin resistance, although we did not determine a relationship between IL-6 and direct measures of insulin sensitivity in adipose tissue in our cohort. [167] Important regional difference in the release of these inflammatory adipokines are also apparent between controls and PE. In healthy pregnancy, BMI is an important determinant of both TNF alpha and IL-6 release from SAT, a relationship not seen in PE pregnancies, implicating disordered release from this depot. In PE, TNF alpha release from SAT, although not determined by BMI, does relate to lipolytic function of this adipose depot. TNF alpha itself is a potent stimulator of lipolysis through down regulation of perilipin and suppression of the anti-lipolytic GTP-binding membrane proteins GA_i.[238] It may therefore be a more important determinant of lipolysis in SAT in PE compared to controls. TNF alpha release and IL-6 release are closely correlated in both PE and controls in both SAT and VAT. TNF alpha has been found to induce other pro-inflammatory adipokines including IL-6, and both are produced by activated macrophages present in adipose tissue which may explain their close association.[239]

The source of excess release of TNF-alpha and IL-6 form VAT is still to be determined as both adipocytes and macrophages produce these cytokines. We have demonstrated both an increase in the mRNA expression of cfms relative to control gene and an increased density of cfms+ macrophages/adipocyte in the

visceral fat of PE women implicating adipose tissue macrophages as the potential source of increased release of pro-inflammatory adipokines. Moreover, MCP-1 release from VAT is higher relative to SAT in PE only with a similar pattern of gene expression of MCP-1 in VAT. Our data suggests that PE women have more pro-inflammatory/activated macrophages in VAT than controls potentially implicating this group of cells as the source of excess TNF alpha and IL-6 production from this depot. Higher maternal concentrations of TNF-alpha and IL-6 have been demonstrated in PE [240 241]. In addition to potential paracrine and autocrine effects on lipid and glucose metabolism, TNF-alpha in particular is implicated in endothelial dysfunction, leukocyte activation, and alterations in coagulation – all characteristic of PE. [156 157 242] Furthermore, the chronic infusion of TNF-alpha or Il-6 into normal pregnant rodents significantly increases arterial pressure and impairs renal haemodynamics.[243] Although TNF alpha and IL-6 may be overproduced by the placenta secondary to hypoxia, their expression has not consistently been seen to by higher in the placentae of women with PE thereby implicating another source for the elevated concentrations found in peripheral blood.[160 244]

PAI-1 is a regulatory serine-protease inhibitor that decreases fibrinolysis and correlates well with visceral adiposity, hyperinsulinaemia and the expression of which is increased in the SAT of obese individuals.[174 175] In keeping with this we found a positive correlation with basal PAI-release from SAT and BMI in both PE and controls, with increased release from VAT than SAT. Interestingly PAI-1 is known to be an independent risk factor for the metabolic syndrome and correlates closely with features such as waist circumferences, plasma fasting glucose, TG and negatively with HDL.[245] In women with PE we determined further parallels with the metabolic syndrome, namely increasing PAI-1 release from SAT is negatively correlated with HDL. Low levels of HDL are an independent risk factor for atherosclerosis. HDL particles are believed to be anti-atherogenic secondary to their capacity to drive reverse cholesterol transport and antagonize pathways of inflammation, thrombosis, and oxidation [246]. HDL increases in normal pregnancy which may impart a protective role on the endothelium and this rise is attenuated in pre-eclampsia. We have shown a negative correlation with IL-6 release from SAT and maternal plasma HDL in controls, which could represent an appropriate anti-inflammatory effect of HDL on SAT.

Leptin correlates with VAT lipolytic function not only in healthy pregnant women as shown in Chapter 3 but also is an important determinant of VAT lipolysis in women with PE. We found no influence of leptin of SAT lipolytic function in healthy controls in spite of its release being higher from this depot than VAT. However leptin release does correlate closely with lipolytic function in SAT in PE, particularly basal lipolysis. This is perhaps surprising as previous studies have shown that despite hyperleptinaemia in pregnancies complicated by PE, there was no increased expression of leptin in SAT.[134] However the function or expression of leptin receptors in adipose tissue has not been studied in normal or complicated human pregnancy. Human adipocytes express the long form of the leptin receptor (OB-R) and two of the short forms (OB-R 219.1 and 219.3).[247] Leptin is thought to have autocrine/paracrine actions on adipose tissue. In rodents leptin has been demonstrated to have lipolytic actions[248] but this has not been reproduced in human primary adipocyte culture.[249] Our data is suggestive of an autocrine/paracrine action of leptin on basal lipolysis in SAT in PE but further studies are required to determine this and potential underlying mechanisms.

We hypothesised in Chapter 4 that a tendency for SAT in PE to adapt to increasing fat mass by adipocyte hypertrophy rather than hyperplasia and hypertrophy may in part contribute to disordered metabolism of this adipose depot. Our data does show some relationships between fat cell size and adipokine release which is more apparent in PE. We have demonstrated that leptin release in SAT in PE is correlated with cell size but not in controls. Basal release of leptin from VAT also appears to be positively related to fat cell size, although not independent of BMI. In addition other pro-inflammatory adipokines including PAI-1 and CRP release from SAT are closely related to cell size in PE and not controls, again not independent of BMI. There appears to be no correlation between TNF alpha and IL-6 release and fat cell size in SAT in either controls of PE. The anti-inflammatory IL-10 release is related to increasing fat cell-size in SAT in PE, not independent of BMI, and this paradoxical increase may represent a compensatory mechanism in this tissue.

In summary, we have identified a more pathogenic role of VAT in PE with increased infiltration of activated macrophages and corresponding increased release of inflammatory adipokines which could contribute to the disturbances in

lipid and glucose metabolism and vascular dysfunction apparent in this maternal syndrome.

6 Adverse Pregnancy Outcomes and Maternal Cardiovascular Risk: A pilot study of carotid ultrasound assessment in women with a history of pre-eclampsia.

6.1 Introduction

6.1.1 Coronary heart disease in women: the extent of the problem

Coronary heart disease (CHD) is the commonest single cause of death among women in Britain, with 41,796 British women dying of CHD in 2006.[250] There is growing evidence of some unique sex-specific features of CHD.[251] Women with CHD more often present atypically with a greater frequency of non-exertional chest pain, and this preponderance of atypical presentation may have scontributed to findings of poorer uptake of primary and secondary prevention initiatives among women compared to men.[252-254] Furthermore women with established CVD are twice as likely to have associated metabolic syndrome than men, and when present the risk of death is over 10-fold compared to two to threefold in men.[255] Thus more proactive approaches to identifying those at high risk at an early stage in their life course are particularly important for women. The US Institute of Medicine report Exploring the Biological contributions to Human Health: Does Sex Matter? called for increased research into the aetiology, diagnosis and management of CHD in women.[256]

6.1.2 Pregnancy, Preeclampsia and cardiovascular disease

There is increasing epidemiological evidence to suggest that adverse pregnancy outcomes such as pre-eclampsia (PE), preterm delivery and low birth weight are associated with increased risk in later life of cardiovascular disease (CVD) in the mother. [183-186] Jonsdottier et al, in a population based study investigated the association between hypertensive complications in pregnancy and death rates from ischemic heart disease (IHD). They found that the relative risk (RR) of dying from IHD was significantly higher among eclamptic women (RR=2.61; 1.11-6.123) and those with pre-eclampsia (RR=1.90; 1.02-3.52) than those with hypertension alone.[184] In a retrospective cohort study in Scotland using discharge data of almost 130,000 women, PE was associated with a two-fold increased risk of subsequent IHD (RR2.0;1.5-2.5).[185] More alarmingly if a woman had a combination of PE, preterm delivery and a baby of low birth weight she had a risk of IHD admission or death seven times that of controls (95% CI 3.3-14.5). A recent meta-analysis combining eight studies (2 346 997 women) with a mean follow up of 11.7 years demonstrated a relative risk of 2.16 (1.86-2.52) of IHD in women with PE substantiating previous evidence.[187] This doubling of risk remains

robust even after adjusting for pre-pregnancy hypertension, diabetes mellitus, obesity, dyslipidaemia, the metabolic syndrome and smoking.[186] Gestation of onset also appears to influence the risk - if PE occurred prior to 37 weeks' gestation the risk of IHD was almost eight-fold (7.71,4.4-13.5).[187] Indeed parity itself is associated with increased risk of CVD with prospective studies finding a positive association.[188 189] A study by Lawlor and colleagues found a "J" shaped association between number of children and CHD, with the lowest prevalence among those with two children and a linear increase with subsequent children.[190] Although the association was attenuated by adjustment for obesity and metabolic risk factors it was not completely obliterated. The authors suggest that normal pregnancy is a state of insulin resistance and dyslipidaemia and repeated pregnancies may have adverse long-term effects. Hypertensive disorders of pregnancy have also been shown to predispose to diabetes in later life.[257] A genetic predisposition to hypertensive disorders of pregnancy is also suggested by family-linkage studies with Inheritance followed both through sons and daughters, potentially through a single gene.[258-260]Greer and Sattar proposed a model whereby pregnancy with its concomitant digression into a metabolic syndrome is a "stress test" of maternal metabolic response.[191] Women who develop adverse pregnancy outcomes such as PE make greater excursions into metabolic disturbances during pregnancy and are predisposed to metabolic and vascular disease in later life.

Furthermore there is evidence of impaired vascular function in women with a history of PE potentially predisposing to an increased risk of CVD. Laser Doppler imaging in vivo has confirmed impaired microvascular function in women with PE[90] and these differences are maintained 15-25 years after pregnancies complicated by PE[261]. In addition, there is evidence of impaired vascular dilatation in women several years after a pre-eclamptic pregnancy.[262]

6.1.3 Carotid ultrasound: a predictor of cardiovascular disease

Epidemiological evidence for the increase risk of CVD in women with pregnancies complicated by PE is not able to adjust for all potential confounders and there is very little direct evidence for this increased risk. Furthermore there is minimal data on the underlying mechanisms for this apparent increase in risk of CVD. Carotid intima media thickness (IMT) is a validated non-invasive surrogate

marker for the presence and progression of atherosclerosis. Carotid IMT correlates well with traditional cardiovascular risk factors and is relatively easy to perform. A recent meta-analysis showed that the age and sex adjusted relative risk for myocardial infarction increases by 1.15% (95% CI 1.16-1.21) for every 0.10 mm increase in carotid IMT.[263] The adjusted relative risk for stroke increases by 1.18 (95% CI 1.16 to 1.21). The detection of carotid plaques by ultrasound is also extremely informative.[264] Plaque score has been show to be associated with risk of myocardial infarction [265 266] and stroke[267]. In the Rotterdam study, the hazard ratio for myocardial infarction for a plaque score of three or more compared with one of zero was 1.83 (95% CI 1.27-2.62), and relative risk for stroke was 1.61 (95% CI 1.16 to 2.23) when comparing the highest tertile of plaque score to the lowest tertile.[265 267]

6.2 Objectives

6.2.1 Primary Objective

To determine whether carotid IMT and plaque counts, as accepted surrogate markers for atherosclerosis, are increased in women with a history of PE compared to age-matched women with history of normal pregnancies. As such our work would help corroborate data from epidemiological and observations studies, which although consistent, can on occasions be misleading.

6.2.2 Secondary Objectives

To determine which risk factors best correlate with carotid IMT and plaque counts in cases and controls and in particular whether any established or novel risk factors can account for any observed difference in carotid IMT or plaque counts.

6.3 Materials and Methods

6.3.1 Study Population

We initially recruited primigravid women who delivered between 1975 and 1985 with PE and matched controls for time of index pregnancy, age and smoking. The diagnosis of PE was in line with International Society for the Study of

Hypertension in Pregnancy (ISSHP) criteria. These women had previously been identified and recruited for a study by our group. The relevant data was originally taken from the maternity records which were recorded at the time of the index pregnancy available from 1975. Thus all women with PE were primigravid, had a diastolic blood pressure ≥90 mmHg on 2 occasions more than 4 hours apart (but normal blood pressure at booking) and had ≥ 2+ proteinuria on dipstick in the absence of renal disease or infection. Due to lack of initial response to recruit these women we also extended recruitment to include women from the GOAL [268] database which prospectively examined the impact of the Factor V Leiden mutation on the vascular complication of pregnancy. 4250 unselected subjects who consecutively attended for routine antenatal care at the Glasgow Royal Maternity Hospital between May 1997 and May1999 were recruited to the study. Case records were examined 6 weeks after delivery to determine outcomes of the index pregnancy and 70 subjects with a diagnosis of PE were identified using the criteria outlined above. Controls were matched as above for time of index pregnancy, age and smoking.

6.3.2 Study Protocol

The women were approached initially by letter requesting if they would participate in the study. One visit was required which lasted between 30 -45 minutes. Standardised measures of height, weight, waist circumference and blood pressure were taken. Subjects then completed a brief questionnaire regarding history of cardiovascular disease, lifestyle and family history.

Venous blood was taken for analysis of lipids and lipoproteins, glucose, and CRP. These samples were non-fasting as patients were required to attend in the afternoon due to limitations of use of the equipment. This was justifiable as non-fasting measurements do not alter cholesterol, HDL-C, CRP or glycosylated haemoglobin and both non-fasting triglyceride[269] and non-fasting insulin[270] predict CHD in large studies in the non-pregnant populations, with magnitudes of effect that are similar to those found for associations with fasting levels.

The Carstairs Indicator is a measure of deprivation, produced decennially using data collected by the UK Censuses on the basis of four components: car ownership, low social class, male unemployment and overcrowding. The 1991

and 2001 Carstairs Indicators, calculated from the 1991 and 2001 Censuses were both included in this analysis, in categorical form, ranging from most affluent (DepCat 1) to most deprived (DepCat 7). [271]

All ultrasound scans were performed on a Siemens Acuson Sequoia 512 scanner with an L7 5-12MHz linear array broadband transducer (Siemens Medical Solutions, Erlangen, Germany). The scans were performed by myself (SSH) in the unit following a protocol developed by colleagues in Amsterdam. Before the study commenced scanning reproducibility was assessed by repeat scanning of staff volunteers. The sonographer (SSH) mean absolute difference per subject for mean common carotid artery IMT was 0.036mm based on the quality control scans which is less than the minimum standard of <0.15mm required.

The scanning protocol involved initially measuring the Doppler velocity in the internal carotid artery in order to exclude significant internal carotid artery stenosis. Thereafter, still B-mode images and video clips were recorded of the distal 1cm of the common carotid artery, the carotid bulb and the proximal internal carotid artery. The same series of images and clips was recorded on both right and left sides. All images were saved in Digital Imaging and Communications in Medicine (DICOM) format for later off-line analysis.

Scans were read using the eTrack software provided by the Department of Physiology, Academic Medical Centre, Amsterdam. All scans were read by the same independent reader (KD), who was blinded to the identities of the participants.

6.3.3 Outcome measures

The pre-specified primary outcome was mean common carotid intima-media thickness and plaque counts. Intima-media thickness was measured on the far wall of each arterial segment, averaged along a 1cm length of the segment, or as much of this as was able to be read[265]. Number of plaques per subject was counted, with plaque being defined as a focal structure encroaching into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value, or demonstrating a thickness >1.5 mm as measured from the media-adventitia interface to the intima-lumen interface[264]. In order to adjust for images which could not be read, the total plaque count for each subject was divided by the

number of readable images present and multiplied by 6 (the maximum possible number of images per subject) giving a plaque score[265].

6.3.4 Biochemical analysis

All blood samples were centrifuged, separated and frozen at -80°C within 1 hour of venepuncture. Cholesterol and triglyceride were determined by enzymatic colorimetric assays on a Roche 917 analyser (Roche Diagnostics Ltd., Burgess Hill, United Kingdom). LDL and HDL were measured after ultracentrifugation at 105,000g at 4°C for 16 hours, followed by precipitation of the LDL fraction using a solution of heparin and manganous chloride. Glucose was measured by hexokinase/glucose-6-phosphate dehydrogenase assay on an Abbott c8000 analyser (Abbott Diagnostics, Maidenhead, United Kingdom). High sensitivity C-reactive protein (CRP) was measured by an immunoturbidimetric assay (Roche Diagnostics Ltd., Burgess Hill, United Kingdom).

6.3.5 Statistical Power

At the time of commencement of the study there were no prior published data on IMT and plaque counts in women with a history of PE. Hence, we extrapolated from carotid IMT data on women of similar age with PCOS[272 273] (a group with perhaps slightly lower CHD risk [HR ~ 1.5 based on the best epidemiological findings[274]]), and we anticipated a minimum difference in carotid IMT of 0.05mm between PE and normal women. Therefore, we would require 25 subjects in each group to provide greater than 90% power to detect this difference with α=0.05. Despite using data from pathology with lower CHD risk, given the potential for overestimation of any difference in IMT between cases and controls, we selected to recruit 40 women from each group in order to be confident that the study was of sufficient size to detect meaningful difference in carotid IMT.

The data in two groups was compared by unpaired t-test using where necessary normalised data, Mann-Whitney or Chi-squared test where appropriate. Adjustment for potential explanatory factors or confounders was made using general linear model. Values were expressed as mean and SEM unless otherwise stated.

6.4 Results

6.4.1 Subjects

A total of 60 subjects who delivered between 1975 and 1998 with PE (n=31) and matched controls (n=29). The characteristics of the two groups are shown in Table 22. There was no difference in risk factors for CVD including age, BMI, smoking, deprivation scores, systolic or diastolic blood pressure. The gestational age at index pregnancy was significantly different with PE delivering around 4 weeks earlier.

Characteristics	*PE (n=31)*	*Control (n=29)*	*p*
Index Pregnancy data			
Age, y	25.4(1.10)	27.3(0.89)	0.17
BMI, kg/m^2	23.4(0.81)	22.3(0.55)	0.18
Smokers (non,current)*	22,9	22,7	0.67
DEPCAT**	4(3-6)	4(2-5)	0.30
Gestation at delivery, wk	35.1(0.74)	39.4(0.35)	P<0.0001
Time since index pregnancy	23.4(1.50)	22.6(1.60)	0.70
Data at recall			
Age, y	48.6(1.12)	49.6(1.24)	0.44
BMI, kg/m^2	28.8(1.24)	27.7(1.01)	0.81
Waist circumference, cm	91.5(2.61)	90.1(2.55)	0.69
Parity (1,2, ≥2)*	7,13,10	9,11,6	0.57
Systolic pressure, mmHg	127.0(2.81)	125.1(2.23)	0.55
Diastolic pressure, mmHg	79.5(2.05)	75.7(1.88)	0.17
Smokers (non,current)	6,25	5,24	0.83
Antihypertensive Rx (no,yes)*	19,12	23,6	0.13
Lipid lowering Rx (no,yes)*	24,7	26,3	0.20
Hormone replacement therapy	0	0	NS

Table 22 Characteristics of PE and controls.

All values expressed as mean and SEM except DEPCAT expressed as median and interquartile range. Comparisons made using unpaired t test except * Chi-squared test and ** Mann-Whitney.

6.4.2 Biochemical Analysis

There was no significant difference in traditional biochemical risk factors for cardiovascular disease including cholesterol, triglycerides, HDL and glucose and CRP (Table 23).

Plasma markers (non-fasting)	*PE (n=31)*	*Control (n=29)*	*p*
Cholesterol	5.00 (0.18)	5.32 (0.21)	0.25
Triglycerides	1.57 (0.15)	1.31(0.11)	0.71
HDL	1.40(0.06)	1.55(0.09)	0.20
Glucose*	5.50(5.10-5.80)	5.70(5.20-6.25)	0.30
CRP	3.89(0.80)	2.78(0.60)	0.46

Table 23 Biochemical plasma markers in PE and controls.

All values expressed as mean and SEM and statistical analysis using paired t-test except *glucose which is expressed as median and interquartile range and analysis by Mann-Whitney.

6.4.3 Carotid Ultrasound

Although the carotid IMT was greater in PE than controls (0.66[0.02] vs 0.63[0.02]), there was no statistical difference (p=0.17).

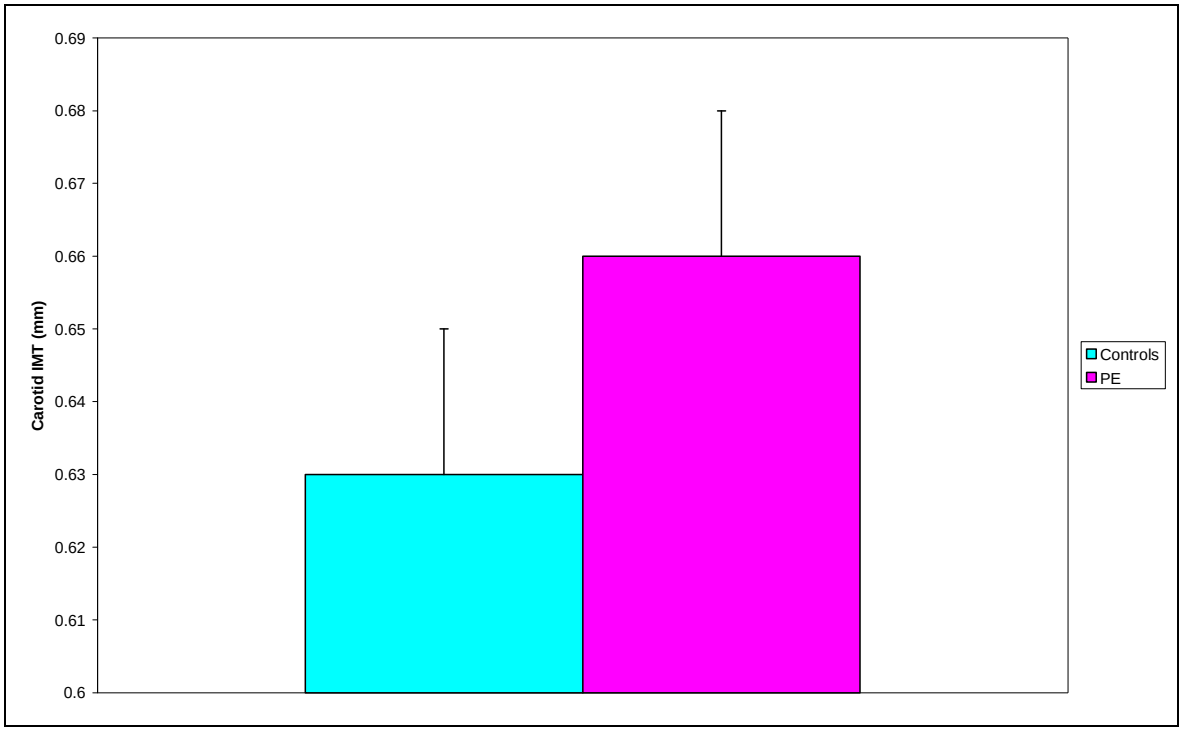

Figure 107 Difference in carotid IMT between controls and PE.

Carotid IMT is not significantly greater in PE compared to controls (p=0.17).

Of those women in whom plaque counts could be assessed, 50% of women with a history of PE (14 out of 28) had one or more plaques compared to only 25% of normal controls (6 out of 24) (p=0.065, chi squared test).(Figure 108)

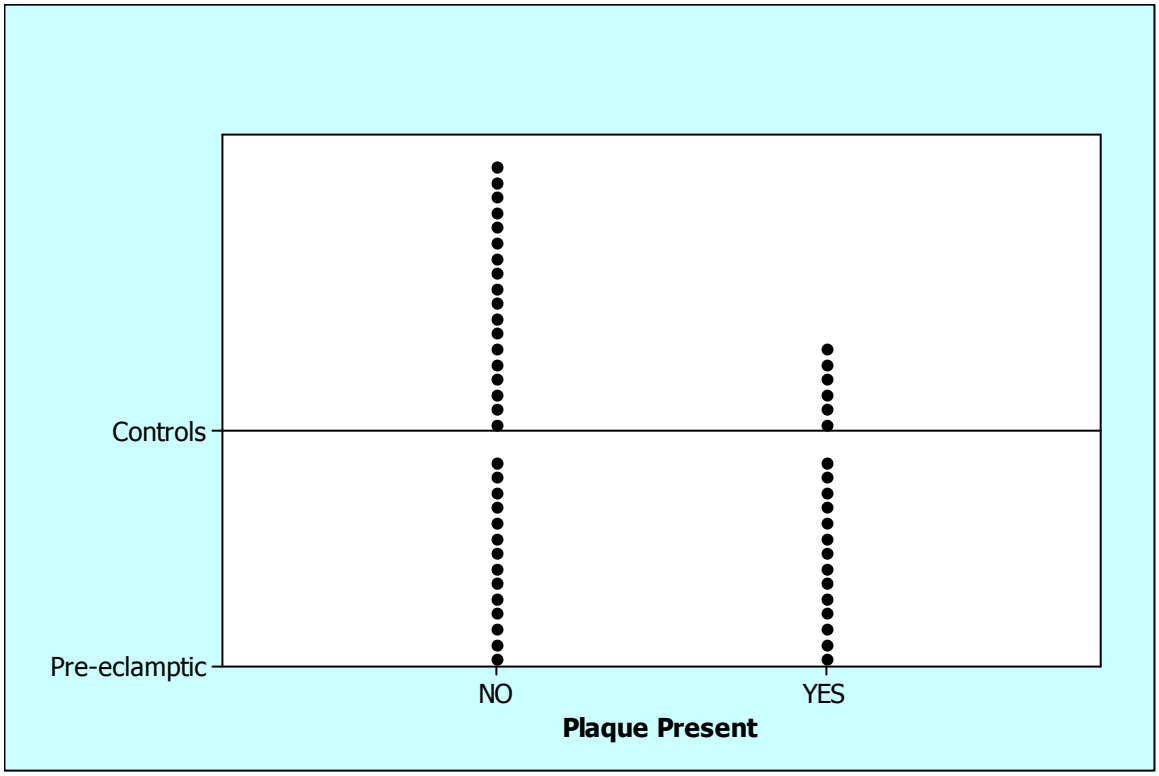

Figure 108 Dotplot of the presence of plaques in controls and PE.

Dotplot of the presence of plaques in controls (6 out of 24) and PE (14 out of 28), p=0.065, chi-squared test.

The mean number of plaques per subject (plaque score) was greater in PE than in controls (0.81[0.19] vs 0.34[0.13], p=0.043 unpaired t-test).

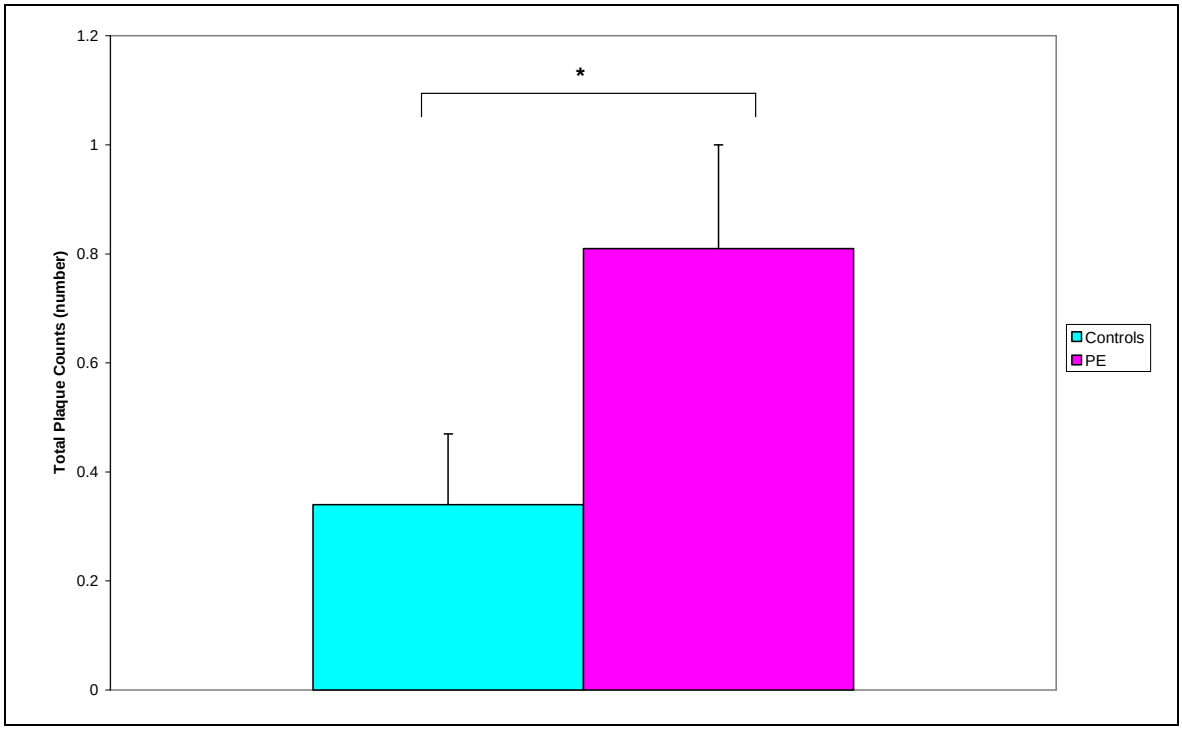

Figure 109 A comparison of plaque scores between controls and PE.

The plaque score (mean number of plaques per subject) was higher in PE than in controls (p=0.043).

When adjusted for classical risk factors including age, triglycerides, cholesterol, HDL cholesterol, systolic blood pressure, diastolic blood pressure, smoking and history of hypertension there was no attenuation of this effect (p=0.042)(Table 24). However the addition of BMI to classical risk factors (p=0.076) did partially attenuate the difference between PE and controls.

Model	*P value*
Unadjusted	0.043
Model 1 (classic)	0.042
Model 2 (classic + BMI)	0.076

Table 24 Multivariate analysis by general linear model for difference in plaque score between PE and controls.

Model 1 (classic): adjusted for age, triglycerides, cholesterol, HDL,systolic BP, diastolic BP, smoking and history of hypertension.: Model 2 = Model 1 +BMI.

In the group as a whole age (r=0.37, R^2=13.4%, p=0.004), systolic BP (r=0.43, R^2=18.9%, p=0.001), cholesterol(r=0.25,R^2=6.38%, p=0.051), triglycerides (r=0.38,R^2=14.7%, p=0.003), CRP (r=0.34,R^2=13.5%, p=0.012) and family history of CVD (r=0.40, R^2=15.93%, p=0.002) were predictive of carotid IMT whereas smoking (r=0.33,R^2=11.1%, p=0.016), HDL (r=-0.31,R^2=9.7%, p=0.025), triglycerides (r=0.30, R^2=8.4%, p=0.037) and glucose (r=0.29, R^2=8.1%, p=0.040) were predictive of total plaque numbers.

6.5 Discussion

CHD is an important cause of morbidity and mortality among women. Due to the atypical presentation of CHD in women and the perception that CHD is predominantly a male disease rates of primary and secondary prevention in women is lower compared to men. Identifying additional risk factors such as PE which is an independent risk factor for coronary artery disease may help target and improve primary prevention strategies.[275] It is important to corroborate epidemiological evidence of this increased associated risk.

Our study has demonstrated direct evidence that atherosclerosis is increased in women with a history of PE compared to women with healthy pregnancies. We have shown that the mean plaque score is higher in women with a history of PE compared to controls. Although carotid IMT was higher in PE, this was not statistically significant.

Plaque counts appear to be a better discriminator and predictor of CVD than carotid IMT in women with a history of PE. This may be due to several factors. The median age of the women undergoing carotid artery ultrasound in our study was around 50. Carotid IMT is age related, with an estimated increase in thickness of around 6.5–10.1 μm/year.[276] In healthy individuals the average IMT is 0.4mm at birth and 0.8mm by age 80 if no risk factors are present. Women have thinner IMT compared to men (and atherosclerosis tends to develop about 10 years later in women) and it may not be until later in life that we would see a significant divergence of IMT in these two groups.

The pathological processes resulting in increased intima media thickening in the common carotid and plaque formation may differ significantly and reflect distinct aspects of atherogenesis and therefore clinical manifestations of

disease. Carotid plaque presence is more strongly predictive of future cardiovascular events and in particular acute myocardial infarction[277] and is more biologically and mechanistically similar to the development of atherosclerosis.[278] Similar to previous data we have shown that different risk factors are predictive of either carotid IMT or plaque counts.

Previous studies have suggested that plaque scores may be a better discriminator of future CVD than carotid IMT in women compared to men, and at an earlier age. A recent study by Deans et al which compared the prevalence of carotid atherosclerosis in participants at extremes of the socioeconomic gradient in Glasgow (the incidence of CVD is higher in areas of socioeconomic deprivation) demonstrated that differences in plaque scores appeared at an earlier age than a difference in carotid IMT.[279] In this study, the population was subdivided into three age groups – 35-44years, 45-54 years, and 55-64years. Furthermore differences in IMT between the study groups did not reach statistical significance in women at any age tertile, and only in the highest age tertile in men. In contrast differences in plaque scores were highly statistically significant in the two highest age tertiles in men and the highest age tertile in women. Another population based study of 6226 men and women aged 25 to 84 demonstrated that carotid plaque was a stronger predictor of first ever MI than was carotid IMT, and this was more striking in women than in men, with a relative risk for MI of 2.92(95% CI 2.04-4.17) in men and 7.8(95% CI 4.46-13.34) in women when comparing the top tertile of plaque area to those without plaques.[266]

In a similar cohort, our group have previously shown a long term differences in inflammatory markers including significantly higher IL-6/IL-10 ratio, an index of proinflammatory cytokine (IL-6) status to anti-inflammatory cytokine (IL-10) status, in PE women compared with matched controls (PE 3.96 [6.07] versus control 2.12 [1.89]; P=0.034), independent of smoking, current BMI, and menopause status (P=0.03)[240], with higher concentrations of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1), by 14% (P=0.038) and 44% (P=0.002), respectively. The differences in ICAM-1 concentration persisted (P=0.010) after adjustment for potential confounders, including hormonal use/menopausal status, antihypertensive or lipid-lowering therapy, and social class.[280] Interestingly there were no significant differences in

fasting lipoprotein concentrations (P>0.20). The cases also demonstrated a tendency toward higher fasting insulin (P=0.08) concentrations and had higher glycosylated hemoglobin levels (P=0.004). Leptin concentrations were not significantly elevated. These data are suggestive that classic risk factors alone cannot fully explain the elevated CHD risk in women with a history of PE, with long –term changes in inflammation, endothelial dysfunction and subtle features of insulin resistance perhaps underpinning this apparent relationship. Our data are in-keeping with this, as adjustment for classical risk factors did not obliterate the difference in plaque scores between PE and controls. Disturbance in inflammation are important contributors to the pathogenesis of both PE and atherosclerosis and may provide one of the key links between these two conditions[216 281]. Although CRP, an inflammatory biomarker is an important predictor of CVD, the contribution of other emerging novel inflammatory factors including TNF-alpha and IL-6 have not been explored in this study. In addition, further investigation of markers of insulin resistance (which potentially links PE, obesity and CVD) and vascular dysfunction and their relationship to plaque counts would be valuable. Although increasing obesity is risk factor for both PE and CVD, adjusting for BMI only partially attenuated the effect on plaque numbers and therefore only partly explains the discrepancy in risk.

A limitation of our study was that despite endeavouring to recruit 80 individuals we only recruited 60. It is therefore possible that the study is underpowered to show a difference in carotid IMT between the groups, one of our primary outcomes measures. A post hoc power calculation estimated that a sample size of 64 in each group would have been required to detect a difference in IMT of 0.05 with a power of 80%. However as we have previously described this lack of significant difference may also be due to the mean age of the cohort, and therefore studying older women may also have been of benefit. In addition due to limitations on the use of the equipment, plasma samples were non-fasting which may explain the lack of difference seen in plasma lipids and glucose.

There are several particular strengths of the study. The methodology of CIMT and plaque assessment is robust with close following of established protocols with excellent reproducibility. In addition, both the sonographer and the independent reader of scans were blinded to the category of the subjects. In addition the identification of subjects in the control and cases groups was

rigorous as data was either collected prospectively or medical records retrospectively checked to confirm the diagnosis of PE.

In summary we have shown direct evidence that a prior history of PE is associated with increased atherosclerosis, which cannot be fully explained by classic cardiovascular risk factors. Further analysis is required to help determine the mechanisms for this difference. Future studies on women with adverse pregnancy complications with even higher risk of CVD (PE, preterm delivery and small for gestational age) is warranted. Carotid ultrasound may provide a valuable screening tool in this high risk group of women in addition to more traditional risk assessment.

7 Final Discussion and Future Research

7.1 Discussion

Maternal metabolism undergoes dramatic changes in pregnancy in order to sustain and nourish the developing fetus. During healthy pregnancy the mother goes from an anabolic state in early pregnancy to a state of catabolism in late pregnancy with increased lipolysis together with a significant reduction in insulin sensitivity. The mother accumulates fat in the first and second trimester of pregnancy in preparation for the increased energy demands of the fetus in the third trimester and for lactation. Increased FFA use in the mother increases the availability of glucose and other substrates for the fetus. Pregnancy is characterised by hyperlipidaemia with a pronounced rise in TG and lesser rise in cholesterol and LDL-cholesterol. However this is combined with an increase in HDL-cholesterol which may have a protective role on the endothelium.

Adipose tissue has increasingly been recognised as an important endocrine organ. It releases a wide array of cytokines and proteins with corresponding autocrine, paracrine and endocrine functions. Metabolism in pregnancy has been extensively studied but much of the work has focused on carbohydrate metabolism with lesser knowledge regarding lipid metabolism and in particular adipose tissue function in both normal and complicated human pregnancy. I have therefore endeavoured in this thesis to acquire a better understanding of adipocyte lipid metabolism and function in normal pregnancy as a basis for further understanding metabolic complications of pregnancy and in particular PE.

In this thesis I have focussed on the hypothesis that PE is “metabolic syndrome” that develops in pregnancy. PE shares many risk factors with CVD and similarities between the metabolic syndrome – namely dyslipidaemia, insulin resistance, inflammation and endothelial dysfunction – are striking. Important predisposing factors for PE include obesity and dyslipidaemia. The pathogenesis of PE is still not fully understood and it is likely the disorder is multifactorial with several underlying contributing mechanisms manifesting as this maternal syndrome. I have concentrated on the “lipid-centric” view that the development of PE is in part related to alterations in lipid and adipocyte metabolism. These perturbances in lipid and glucose metabolism not only may predispose these women to PE but also to CVD in later life.

In chapter 1 I examined lipolytic function of adipocytes in normal pregnancy. The most surprising finding is the lack of association between maternal booking BMI and lipolytic function of adipocytes. This may be accounted for by several factors. Firstly booking BMI, although a reflection of pre-gravid status, may not accurately reflect BMI in late pregnancy. Sattar et al has shown that waist circumference may be a better indicator of risk of development of hypertensive disorders of pregnancy than BMI and indeed it may be the distribution of fat rather than total fat mass which is relevant.[51] Visceral adiposity is linked to adverse metabolic outcomes both in the non-pregnant and pregnant including gestational diabetes mellitus, gestational hypertension and pre-eclampsia.[49-51] Visceral adiposity in early pregnancy appears to correlate better than subcutaneous fat or body mass index (BMI) with metabolic risk factors such as blood pressure, insulin resistance and lipids.[52]Furthermore it may be the total amount of fat mass gained during pregnancy that critically determines the lipolytic function in pregnancy which would not be reflected by a booking BMI.

I have shown that adipose tissue in pregnancy is very metabolically flexible, particularly that stored in the subcutaneous compartment. This is of particular benefit to the fetus in that those mothers with lower basal lipolysis respond quickly to times of 'stress' facilitating increased utilisation of FFA in the mother, and thereby increasing the availability of glucose and amino acids for the fetus. The subcutaneous compartment is the largest maternal store of adipose tissue. In addition, the rate of whole body lipolysis is still insulin sensitive in fasting conditions in normal pregnancy. This might be a reflection of the distribution of fat mass accumulation in women with normal pregnancy, with a greater propensity to store fat in the lower body, which is much more insulin sensitive.[282] Lower body fat is independently associated with a lower risk of lipid and carbohydrate metabolic dysregulation[283] and larger depots of lower body adipose tissue are associated with a more efficient storage of dietary fat. All women accrue fat mass during pregnancy of similar amounts, whether lean or obese, and location of fat storage may be one mechanism through which "metabolically healthy" women can adapt efficiently to pregnancy.

Furthermore I have demonstrated some important regional differences in lipolytic function in normal pregnancy with potential relevance to metabolic complications of pregnancy. Similar to previous literature I have shown that

visceral adiposity is more closely related to measures of maternal insulin resistance. In visceral fat there is a higher turnover of lipids due to its greater sensitivity to catecholamine-induced lipolysis and decreased sensitivity to insulin, both demonstrated by the data presented here. Visceral fat is in direct contact with the liver via the portal venous system. The liver is therefore exposed to chronic elevation of NEFA (non-esterified fatty acids) which can produce alteration in liver metabolism and promotes hepatic IR – the basis for the 'portal paradigm'. This is illustrated by the inverse correlation seen between visceral fat insulin sensitivity and maternal TG in this cohort. In later stages of pregnancy there is an increase in both the thickness of pre-peritoneal fat (visceral) and the ratio of pre-peritoneal to subcutaneous fat as measured by ultrasound.[47] This may be relevant to increasing insulin resistance and lipid changes that occur as pregnancy progresses. Indeed accumulation of hepatic fat has been shown to be an important mediator of insulin resistance during pregnancy in the rat model.[48] I have also shown that increasing BMI is associated with an increase in visceral fat cell size, with increased lipolysis and an increase in plasma levels of the pro-inflammatory adipokines leptin, IL-6 and CRP and a reduction in the anti-inflammatory adiponectin. This may be one potential mechanism through which increasing obesity predisposes to metabolic complications of pregnancy through increased inflammation and insulin resistance.

In contrast, the subcutaneous depot appears to work independently to visceral fat. Fat cell size is not a closely related to BMI and this may reflect the propensity of this fat depot to increase by both adipose cell hyperplasia and hypertrophy, a metabolically advantageous response. Fat cell size in SAT does however correlate with the insulin sensitivity of the cell. It appears that maternal TNF-alpha is a significant correlate of adipocyte lipolytic function in this depot and in particular basal lipolysis. TNF alpha has been shown to stimulate lipolysis through several mechanisms including inhibition of insulin receptor signalling, interaction with adenosine, and direct stimulation of basal lipolysis through phosphorylation and decreased expression of perilipin. Although we failed to demonstrate any relationship between TNF-alpha and maternal markers of insulin resistance in our cohort, Kirwan et al demonstrated that TNF-alpha was the most significant independent predictor of insulin sensitivity in

human pregnancy.[159] TNF-alpha not only exerts its effects on the adipocyte but also influences insulin signalling in skeletal muscle and the liver.

Pre-eclampsia is a multi-system disorder with complex underlying mechanisms. We know that both the rise in maternal TG and FFA is exaggerated in PE and occurs well in advance manifestation of the disease suggesting disordered adipocyte metabolism. In chapter 4 I attempted to explore this hypothesis and to determine if there were any significant underlying differences in adipocyte lipolytic function between PE and control women. Interestingly we found that there was no inherent difference in the effect of catecholamines or insulin in either adipose depot *in vitro*. However the insulin sensitivity as calculated by the FCISI (or responsiveness to insulin once the tissue is stimulated by isoproterenol) of SAT and probably VAT was lower in women with PE than controls. This is likely to be more relevant *in vivo* as this is a functional test of adipose tissue function. Almost 60% of circulating NEFA is from upper body fat ie abdominal subcutaneous and visceral fat.[282] I have shown that both these depots are insulin resistant and this potentially would make a significant impact on total circulating FFA. The rise of FFA in PE occurs early in pregnancy and contributes significantly to IR in pregnancy.[106] Therefore the IR of adipose tissue could lead to a vicious cycle of increased lipolysis and release of FFA with further exacerbation of IR.

In addition to increasing IR of adipose tissue we have shown a difference in SAT response to increasing BMI. I have previously highlighted that SAT cell size does not closely correlate with BMI in controls and theorised that this is potentially a healthy adaptive response by increasing fat mass by both hypertrophy and hyperplasia. However in PE, SAT cell size is intimately related to BMI suggesting that they increase fat mass predominantly by adipocyte hypertrophy. This is a maladaptive response and can result in increased ER stress, increased IR, increased release of inflammatory cytokines and increased macrophage recruitment. SAT cell size is closely related to lipolytic function of the tissue in PE but not in controls.

I also hypothesised that in addition to an inherent defect in adipocyte function, there was an additional factor present in maternal serum of women with PE

released from the placenta which excessively stimulates lipolysis. I failed to demonstrate any effect of serum on adipocyte lipolysis in either controls or PE.

An alternative mechanism through which adipose tissue could affect maternal metabolism resulting in PE would be through dysregulated release of adipokines and increased tissue inflammation similar to process of chronic inflammation in obesity. In Chapter 5 I examined differences in adipokine release from SAT and VAT between controls and PE. I found that there was an increased tendency for the pro-inflammatory adipokines TNF-alpha and IL-6 to be released after stimulation of VAT, with increased gene expression in this fat depot. TNF-alpha release also correlated negatively with the fat cell insulin sensitivity of VAT implicating a paracrine effect in this tissue potentially due to its known effect on insulin signalling. TNF-alpha also appears to influence SAT lipolytic function in PE. Both TNF alpha and IL-6 are released from adipocytes and macrophages and the actual source of excess release is still to be identified. However I have demonstrated increased release of MCP-1 from VAT relative to SAT in PE only. Furthermore there is both an increase in gene expression of cfms and increased density of $cfms^{+}$ macrophages/adipocytes in the VAT of PE women implicating activated adipose tissue macrophages as a potential source of increased release of these pro-inflammatory adipokines. TNF-alpha and IL-6 have been shown to be elevated in women with PE and their effects of metabolism, endothelial dysfunction and inflammation may provide one of the mechanisms through which the maternal syndrome develops.

Following on from data in Chapter 4 which showed that SAT cell size was closely related to BMI in PE but not in controls, I have shown that SAT cell size does relate to adipokine release in PE. The basal release of leptin, PAI-1 and CRP are closely related to SAT cell size in PE but not in controls. In addition the anti-inflammatory IL-10 release paradoxically increases with increasing SAT cell size in PE only suggesting a possible compensatory mechanism.

Leptin also appears to have disparate roles on lipid metabolism in PE and healthy pregnancy. Leptin, secreted from adipose tissue, decreases lipid accumulation in the liver by promoting FA oxidation and in adipose tissue via a direct autocrine effect [284 285]. I previously identified that leptin is an important correlate of VAT and not SAT lipolytic function in normal pregnancy. However in PE women it is

closely related to basal lipolysis in SAT. Although previous studies have shown no increased expression of leptin in SAT complicated by PE, the expression or function of leptin receptors in adipose tissue in normal or complicated pregnancy has yet to be determined. My data is suggestive of an autocrine/paracrine effect of leptin not only in VAT in PE but also in SAT, but further studies are required define the role of leptin in pregnancy adipocyte function.

There is increasing epidemiological evidence that PE is associated with an increase risk of CVD in later life. Greer and Sattar proposed a model whereby pregnancy with its concomitant digression into a metabolic syndrome is a "stress test" of maternal metabolic response.[191] Women who develop adverse pregnancy outcomes such as PE make greater excursions into metabolic disturbances during pregnancy and are predisposed to metabolic and vascular disease in later life. There are some unique sex-specific characteristics of CVD. Women with established CVD are twice as likely to have associated metabolic syndrome than men, and when this cluster of features is present the risk of death is over 10-fold compared to two to threefold in men.[255] The purpose of Chapter 6 was to corroborate the increasing epidemiological evidence and to determine which risk factors could account for any observed differences. Two surrogate markers of atherosclerosis in the carotid artery were assessed- namely carotid IMT and plaque scores. Both were found to be increased in women with a history of PE, with plaque scores significantly so, corroborating evidence of increased atherosclerotic burden in these women. Classic risk factors such as age, lipids, BP and smoking did not attenuate this effect. Interestingly BMI only marginally attenuated this relationship, therefore only partially explaining this increased risk. Women who develop early onset pre-eclampsia with associated IUGR and preterm delivery are epidemiologically at much higher risk of CVD than those with late onset disease suggestive of differing underlying pathological processes. It is possible that long-term changes in inflammation, endothelial and vascular dysfunction and features of insulin resistance which have previously been identified in women with a history of PE underlie the increased risk of CVD in later life.

7.2 Future Research

This thesis has determined some important aspects of adipocyte and lipid metabolism in normal pregnancy and PE, and established direct evidence for an increased risk of CVD in women with a history of PE. However the data from this thesis has clearly stimulated further avenues for future research.

Maternal obesity has far reaching consequences for both mother and offspring and predisposes to metabolic complications of pregnancy including GDM and PE.[286] Independent of maternal pregnancy BMI, gestational weight gain (GWG) has also been associated with adverse outcomes. However the evidence for the association of GWG with adverse pregnancy outcome is somewhat weaker than the evidence for pre-pregnancy BMI.[287] In addition, although obese women have and exaggerated metabolic response in pregnancy, lean and obese women put on similar fat mass, although obese women tend to accumulate fat more centrally than lean women, which may reflect their more insulin-resistant state. [70 71 288] Clearly there is a gap in our knowledge to explain these apparently contradictory facts.

Human fat can be subdivided into lower body subcutaneous fat, upper body subcutaneous fat and intra-abdominal/visceral fat as previously detailed. In general visceral fat is thought to be more pathogenic and associated with an abnormal metabolic and adipokine profile with is supported by data in this thesis. However upper body subcutaneous fat, as studied here, and visceral fat stores are often not differentiated and both are relatively resistant to insulin suppression of lipolysis[282]. Conversely lower body fat subcutaneous fat (eg gluteal-femoral fat) is much more insulin sensitive and is independently associated with a reduced risk of lipid and carbohydrate metabolic dysregulation.[283 289] Thus the compartment in which fat is stored during pregnancy may impact on the metabolic response to pregnancy.

Important avenues for further research therefore would include determining the relationships between the pattern and mass of adipose tissue as it accumulates in pregnancy in both lean and obese women and determine the relationships between maternal energy metabolism and markers of “lipotoxicity” or disorderd lipid metabolism. Similar studies on comparing adipocyte function of subcutaneous abdominal AT and visceral AT with biopsies of femoral fat would

be of particular value. Other areas of interest would be to further examine the relationships between fat cell size and maternal metabolism. In particular further examination of the differences in adipose tissue adaptation to the normal increase in fat mass in pregnancy in lean and obese women would be of value. Furthermore, it is not known whether the nature of AT in obese pregnant women, which was mostly pre-existing before pregnancy, is different to AT in lean pregnant women which has been accumulated in the gestational period. Adipose tissue from lean women not only may be deposited in different subcutaneous compartments than obese women, but the composition of the AT may also differ significantly. One component of the insulin resistant state apparent in obesity is disordered fatty acid (FA) metabolism whereby fewer FA are diverted down the elongation and desaturation pathways to form LC-PUFA (long chain polyunsaturated fatty acids). Desaturases are key enzymes in the remodelling of FA by introducing a double-bond in the FA chain. Therefore it would be of important to determine the fatty acid composition and desaturase activity of AT in lean and obese women, and women with metabolic complications of pregnancy and relate them to measures of adipocyte lipolysis and insulin sensitivity.

I have also identified that leptin appears to play an important regulatory role in VAT lipolysis in normal pregnancy and both SAT and VAT lipolysis in PE. The mechanism through which this occurs is still to be determined. There is evidence in humans that adipose tissue leptin and leptin receptor mRNA expression is related to changes in insulin sensitivity in a physiologically dynamic situation – exercise. Barwell et al have shown that daughters of women with type 2 diabetes have a significantly greater improvement in insulin sensitivity in response to an exercise training programme than controls and that this is accompanied by a significant decrease in plasma leptin, whereas leptin was unchanged in controls.[290] Adipose tissue leptin expression was not different between controls and offspring of type 2 diabetics but the offspring had increased levels of both long and short forms of the leptin receptor[291]. In a multivariate analysis leptin and leptin receptor mRNA expression contributed significantly to insulin sensitivity index at baseline and change in insulin sensitivity index in response to exercise. Parallels with the dynamic situation of pregnancy may occur with adipocyte first trimester accumulation of fat and third trimester depletion of fat being regulated by leptin in an

autocrine/paracrine manner. It would therefore be valuable to measure leptin and leptin receptor mRNA levels in subcutaneous and visceral fat in normal (lean and obese) pregnancy and PE and relate this to adipocyte lipolysis and maternal insulin resistance.

Furthermore although I have determined that SAT and probably VAT is less insulin sensitive in PE than controls perhaps contributing to the early exaggerated rise in FFA, further investigation to determine the mechanism for this would be of great importance. This would help establish whether this "defect" would be amenable to interventions such as pharmacological or lifestyle.

Lastly, using carotid ultrasound assessment, and in particular plaque counts in other groups of women with a history of adverse pregnancy outcome would be of interest. It would also be useful to distinguish between early (with its particularly high risk of CVD) and late onset PE to determine which risk factors (traditional or novel) best account for any observed difference to help corroborate increasing support that these two conditions are distinct pathological processes.

In conclusion the data in this thesis provides further evidence that PE is "metabolic syndrome of pregnancy" with disordered adipocyte function and metabolism. Further studies on adipose accumulation, function, and composition in normal and complicated human pregnancy are warranted.

Bibliography

1. Bergo M, Wu G, Ruge T, Olivecrona T. Down-regulation of adipose tissue lipoprotein lipase during fasting requires that a gene, separate from the lipase gene, is switched on. *J Biol Chem* 2002;277(14):11927-32.
2. Bergo M, Olivecrona G, Olivecrona T. Forms of lipoprotein lipase in rat tissues: in adipose tissue the proportion of inactive lipase increases on fasting. *Biochem J* 1996;313 (Pt 3):893-8.
3. Boden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* 1997;46(1):3-10.
4. Frayn KN. Adipose tissue and the insulin resistance syndrome. *Proc Nutr Soc* 2001;60(3):375-80.
5. Langin D, Dicker A, Tavernier G, Hoffstedt J, Mairal A, Ryden M, et al. Adipocyte lipases and defect of lipolysis in human obesity. *Diabetes* 2005;54(11):3190-7.
6. Zechner R, Kienesberger PC, Haemmerle G, Zimmermann R, Lass A. Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J Lipid Res* 2009;50(1):3-21.
7. Haemmerle G, Lass A, Zimmermann R, Gorkiewicz G, Meyer C, Rozman J, et al. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* 2006;312(5774):734-7.
8. Watt MJ, Steinberg GR. Regulation and function of triacylglycerol lipases in cellular metabolism. *Biochem J* 2008;414(3):313-25.
9. Holm C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem Soc Trans* 2003;31(Pt 6):1120-4.
10. Londos C, Brasaemle DL, Schultz CJ, Segrest JP, Kimmel AR. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin Cell Dev Biol* 1999;10(1):51-8.
11. Brasaemle DL. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *J Lipid Res* 2007;48(12):2547-59.
12. Lafontan M, Berlan M. Fat cell alpha 2-adrenoceptors: the regulation of fat cell function and lipolysis. *Endocr Rev* 1995;16(6):716-38.
13. Shakur Y, Holst LS, Landstrom TR, Movsesian M, Degerman E, Manganiello V. Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. *Prog Nucleic Acid Res Mol Biol* 2001;66:241-77.
14. Sengenes C, Bouloumie A, Hauner H, Berlan M, Busse R, Lafontan M, et al. Involvement of a cGMP-dependent pathway in the natriuretic peptide-mediated hormone-sensitive lipase phosphorylation in human adipocytes. *J Biol Chem* 2003;278(49):48617-26.
15. Sengenes C, Berlan M, De Glisezinski I, Lafontan M, Galitzky J. Natriuretic peptides: a new lipolytic pathway in human adipocytes. *FASEB J* 2000;14(10):1345-51.
16. Moro C, Polak J, Richterova B, Sengenes C, Pelikanova T, Galitzky J, et al. Differential regulation of atrial natriuretic peptide- and adrenergic receptor-dependent lipolytic pathways in human adipose tissue. *Metabolism* 2005;54(1):122-31.
17. Yu J, Yu HC, Kim KA, Kwon KB, Park JW, Kim SZ, et al. Differences in the amount of lipolysis induced by atrial natriuretic peptide in small and large adipocytes. *J Pept Sci* 2008;14(8):972-7.

18. Moro C, Crampes F, Sengenes C, De Glisezinski I, Galitzky J, Thalamas C, et al. Atrial natriuretic peptide contributes to physiological control of lipid mobilization in humans. *Faseb J* 2004;18(7):908-10.
19. Berlin I, Lechat P, Deray G, Landault C, Maistre G, Chermat V, et al. Beta-adrenoceptor blockade potentiates acute exercise-induced release of atrial natriuretic peptide by increasing atrial diameter in normotensive healthy subjects. *Eur J Clin Pharmacol* 1993;44(2):127-33.
20. Luchner A, Burnett JC, Jr., Jougasaki M, Hense HW, Riegger GA, Schunkert H. Augmentation of the cardiac natriuretic peptides by beta-receptor antagonism: evidence from a population-based study. *J Am Coll Cardiol* 1998;32(7):1839-44.
21. Lafontan M, Langin D. Lipolysis and lipid mobilization in human adipose tissue. *Prog Lipid Res* 2009;48(5):275-97.
22. Villar J, Cogswell M, Kestler E, Castillo P, Menendez R, Repke JT. Effect of fat and fat-free mass deposition during pregnancy on birth weight. *Am J Obstet Gynecol* 1992;167(5):1344-52.
23. Douglas AJ, Johnstone LE, Leng G. Neuroendocrine mechanisms of change in food intake during pregnancy: a potential role for brain oxytocin. *Physiol Behav* 2007;91(4):352-65.
24. Ramos MP, Crespo-Solans MD, del Campo S, Cacho J, Herrera E. Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness. *Am J Physiol Endocrinol Metab* 2003;285(2):E318-28.
25. Catalano PM, Tyzbir ED, Roman NM, Amini SB, Sims EA. Longitudinal changes in insulin release and insulin resistance in nonobese pregnant women. *Am J Obstet Gynecol* 1991;165(6 Pt 1):1667-72.
26. Ryan EA, Enns L. Role of gestational hormones in the induction of insulin resistance. *J Clin Endocrinol Metab* 1988;67(2):341-7.
27. Diderholm B, Stridsberg M, Ewald U, Lindeberg-Norden S, Gustafsson J. Increased lipolysis in non-obese pregnant women studied in the third trimester. *Bjog* 2005;112(6):713-8.
28. Catalano PM, Roman-Drago NM, Amini SB, Sims EA. Longitudinal changes in body composition and energy balance in lean women with normal and abnormal glucose tolerance during pregnancy. *Am J Obstet Gynecol* 1998;179(1):156-65.
29. Martin-Hidalgo A, Holm C, Belfrage P, Schotz MC, Herrera E. Lipoprotein lipase and hormone-sensitive lipase activity and mRNA in rat adipose tissue during pregnancy. *Am J Physiol* 1994;266(6 Pt 1):E930-5.
30. Herrera EM, Knopp RH, Freinkel N. Urinary excretion of epinephrine and norepinephrine during fasting in late pregnancy in the rat. *Endocrinology* 1969;84(2):447-50.
31. Sivan E, Homko CJ, Chen X, Reece EA, Boden G. Effect of insulin on fat metabolism during and after normal pregnancy. *Diabetes* 1999;48(4):834-8.
32. Catalano PM, Nizielski SE, Shao J, Preston L, Qiao L, Friedman JE. Downregulated IRS-1 and PPARgamma in obese women with gestational diabetes: relationship to FFA during pregnancy. *Am J Physiol Endocrinol Metab* 2002;282(3):E522-33.
33. Freinkel N. Banting Lecture 1980. Of pregnancy and progeny. *Diabetes* 1980;29(12):1023-35.
34. Zorzano A, Herrera E. Comparative utilization of glycerol and alanine as liver gluconeogenic substrates in the fed late pregnant rat. *Int J Biochem* 1986;18(7):583-7.

35. Herrera E, Knopp RH, Freinkel N. Carbohydrate metabolism in pregnancy. VI. Plasma fuels, insulin, liver composition, gluconeogenesis, and nitrogen metabolism during late gestation in the fed and fasted rat. *J Clin Invest* 1969;48(12):2260-72.
36. Shambaugh GE, Mrozak SC, Freinkel N. Fetal fuels. I. Utilization of ketones by isolated tissues at various stages of maturation and maternal nutrition during late gestation. *Metabolism* 1977;26(6):623-35.
37. Seccombe DW, Harding PG, Possmayer F. Fetal utilization of maternally derived ketone bodies for lipogenesis in the rat. *Biochim Biophys Acta* 1977;488(3):402-16.
38. Herrera E, Amusquivar E, Lopez-Soldado I, Ortega H. Maternal lipid metabolism and placental lipid transfer. *Horm Res* 2006;65 Suppl 3:59-64.
39. Lederman SA, Paxton A, Heymsfield SB, Wang J, Thornton J, Pierson RN, Jr. Body fat and water changes during pregnancy in women with different body weight and weight gain. *Obstet Gynecol* 1997;90(4 Pt 1):483-8.
40. Lawrence M, Coward WA, Lawrence F, Cole TJ, Whitehead RG. Fat gain during pregnancy in rural African women: the effect of season and dietary status. *Am J Clin Nutr* 1987;45(6):1442-50.
41. Goldberg GR, Prentice AM, Coward WA, Davies HL, Murgatroyd PR, Wensing C, et al. Longitudinal assessment of energy expenditure in pregnancy by the doubly labeled water method. *Am J Clin Nutr* 1993;57(4):494-505.
42. Okereke NC, Huston-Presley L, Amini SB, Kalhan S, Catalano PM. Longitudinal changes in energy expenditure and body composition in obese women with normal and impaired glucose tolerance. *Am J Physiol Endocrinol Metab* 2004;287(3):E472-9.
43. Kopp-Hoolihan LE, van Loan MD, Wong WW, King JC. Fat mass deposition during pregnancy using a four-component model. *J Appl Physiol* 1999;87(1):196-202.
44. Pipe NG, Smith T, Halliday D, Edmonds CJ, Williams C, Coltart TM. Changes in fat, fat-free mass and body water in human normal pregnancy. *Br J Obstet Gynaecol* 1979;86(12):929-40.
45. Sohlstrom A, Wahlund LO, Forsum E. Total body fat and its distribution during human reproduction as assessed by magnetic resonance imaging. *Basic Life Sci* 1993;60:181-4.
46. Ehrenberg HM, Huston-Presley L, Catalano PM. The influence of obesity and gestational diabetes mellitus on accretion and the distribution of adipose tissue in pregnancy. *Am J Obstet Gynecol* 2003;189(4):944-8.
47. Kinoshita T, Itoh M. Longitudinal variance of fat mass deposition during pregnancy evaluated by ultrasonography: the ratio of visceral fat to subcutaneous fat in the abdomen. *Gynecol Obstet Invest* 2006;61(2):115-8.
48. Einstein FH, Fishman S, Muzumdar RH, Yang XM, Atzmon G, Barzilai N. Accretion of visceral fat and hepatic insulin resistance in pregnant rats. *Am J Physiol Endocrinol Metab* 2008;294(2):E451-5.
49. Zhang S, Folsom AR, Flack JM, Liu K. Body fat distribution before pregnancy and gestational diabetes: findings from coronary artery risk development in young adults (CARDIA) study. *BMJ* 1995;311(7013):1139-40.
50. Ijuin H, Douchi T, Nakamura S, Oki T, Yamamoto S, Nagata Y. Possible association of body-fat distribution with preeclampsia. *J Obstet Gynaecol Res* 1997;23(1):45-9.
51. Sattar N, Clark P, Holmes A, Lean ME, Walker I, Greer IA. Antenatal waist circumference and hypertension risk. *Obstet Gynecol* 2001;97(2):268-71.
52. Bartha JL, Marin-Segura P, Gonzalez-Gonzalez NL, Wagner F, Aguilar-Diosdado M, Hervias-Vivancos B. Ultrasound evaluation of visceral fat and

metabolic risk factors during early pregnancy. *Obesity (Silver Spring)* 2007;15(9):2233-9.
53. Villamor E, Cnattingius S. Interpregnancy weight change and risk of adverse pregnancy outcomes: a population-based study. *Lancet* 2006;368(9542):1164-70.
54. Fahraeus L, Larsson-Cohn U, Wallentin L. Plasma lipoproteins including high density lipoprotein subfractions during normal pregnancy. *Obstet Gynecol* 1985;66(4):468-72.
55. Sattar N, Greer IA, Louden J, Lindsay G, McConnell M, Shepherd J, et al. Lipoprotein subfraction changes in normal pregnancy: threshold effect of plasma triglyceride on appearance of small, dense low density lipoprotein. *J Clin Endocrinol Metab* 1997;82(8):2483-91.
56. Alvarez JJ, Montelongo A, Iglesias A, Lasuncion MA, Herrera E. Longitudinal study on lipoprotein profile, high density lipoprotein subclass, and postheparin lipases during gestation in women. *J Lipid Res* 1996;37(2):299-308.
57. Silliman K, Shore V, Forte TM. Hypertriglyceridemia during late pregnancy is associated with the formation of small dense low-density lipoproteins and the presence of large buoyant high-density lipoproteins. *Metabolism* 1994;43(8):1035-41.
58. Stewart F, Rodie VA, Ramsay JE, Greer IA, Freeman DJ, Meyer BJ. Longitudinal assessment of erythrocyte fatty acid composition throughout pregnancy and post partum. *Lipids* 2007;42(4):335-44.
59. Sattar N, Berry C, Greer IA. Essential fatty acids in relation to pregnancy complications and fetal development. *Br J Obstet Gynaecol* 1998;105(12):1248-55.
60. Haggarty P. Placental regulation of fatty acid delivery and its effect on fetal growth--a review. *Placenta* 2002;23 Suppl A:S28-38.
61. Montgomery C, Speake BK, Cameron A, Sattar N, Weaver LT. Maternal docosahexaenoic acid supplementation and fetal accretion. *Br J Nutr* 2003;90(1):135-45.
62. Woollett LA. Origin of cholesterol in the fetal golden Syrian hamster: contribution of de novo sterol synthesis and maternal-derived lipoprotein cholesterol. *J Lipid Res* 1996;37(6):1246-57.
63. Plosch T, van Straten EM, Kuipers F. Cholesterol transport by the placenta: placental liver X receptor activity as a modulator of fetal cholesterol metabolism? *Placenta* 2007;28(7):604-10.
64. Napoli C, D'Armiento FP, Mancini FP, Postiglione A, Witztum JL, Palumbo G, et al. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. *J Clin Invest* 1997;100(11):2680-90.
65. Ramsay JE, Greer I, Sattar N. ABC of obesity. Obesity and reproduction. *Bmj* 2006;333(7579):1159-62.
66. Kanagalingam MG, Forouhi NG, Greer IA, Sattar N. Changes in booking body mass index over a decade: retrospective analysis from a Glasgow Maternity Hospital. *Bjog* 2005;112(10):1431-3.
67. Butte NF, Wong WW, Treuth MS, Ellis KJ, O'Brian Smith E. Energy requirements during pregnancy based on total energy expenditure and energy deposition. *Am J Clin Nutr* 2004;79(6):1078-87.
68. Prentice AM, Goldberg GR, Davies HL, Murgatroyd PR, Scott W. Energy-sparing adaptations in human pregnancy assessed by whole-body calorimetry. *Br J Nutr* 1989;62(1):5-22.

69. Eckel RH. Insulin resistance: an adaptation for weight maintenance. *Lancet* 1992;340(8833):1452-3.
70. Merzouk H, Meghelli-Bouchenak M, Loukidi B, Prost J, Belleville J. Impaired serum lipids and lipoproteins in fetal macrosomia related to maternal obesity. *Biol Neonate* 2000;77(1):17-24.
71. Ramsay JE, Ferrell WR, Crawford L, Wallace AM, Greer IA, Sattar N. Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways. *J Clin Endocrinol Metab* 2002;87(9):4231-7.
72. Sattar N, Tan CE, Han TS, Forster L, Lean ME, Shepherd J, et al. Associations of indices of adiposity with atherogenic lipoprotein subfractions. *Int J Obes Relat Metab Disord* 1998;22(5):432-9.
73. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998;15(7):539-53.
74. Sermer M, Naylor CD, Farine D, Kenshole AB, Ritchie JW, Gare DJ, et al. The Toronto Tri-Hospital Gestational Diabetes Project. A preliminary review. *Diabetes Care* 1998;21 Suppl 2:B33-42.
75. Hunt KJ, Schuller KL. The increasing prevalence of diabetes in pregnancy. *Obstet Gynecol Clin North Am* 2007;34(2):173-99, vii.
76. Crowther CA, Hiller JE, Moss JR, McPhee AJ, Jeffries WS, Robinson JS. Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. *N Engl J Med* 2005;352(24):2477-86.
77. Metzger BE. Long-term outcomes in mothers diagnosed with gestational diabetes mellitus and their offspring. *Clin Obstet Gynecol* 2007;50(4):972-9.
78. Catalano PM, Tyzbir ED, Wolfe RR, Roman NM, Amini SB, Sims EA. Longitudinal changes in basal hepatic glucose production and suppression during insulin infusion in normal pregnant women. *Am J Obstet Gynecol* 1992;167(4 Pt 1):913-9.
79. Catalano PM, Tyzbir ED, Wolfe RR, Calles J, Roman NM, Amini SB, et al. Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes. *Am J Physiol* 1993;264(1 Pt 1):E60-7.
80. Catalano PM, Huston L, Amini SB, Kalhan SC. Longitudinal changes in glucose metabolism during pregnancy in obese women with normal glucose tolerance and gestational diabetes mellitus. *Am J Obstet Gynecol* 1999;180(4):903-16.
81. Couch SC, Philipson EH, Bendel RB, Pujda LM, Milvae RA, Lammi-Keefe CJ. Elevated lipoprotein lipids and gestational hormones in women with diet-treated gestational diabetes mellitus compared to healthy pregnant controls. *J Diabetes Complications* 1998;12(1):1-9.
82. Sanchez-Vera I, Bonet B, Viana M, Quintanar A, Martin MD, Blanco P, et al. Changes in plasma lipids and increased low-density lipoprotein susceptibility to oxidation in pregnancies complicated by gestational diabetes: consequences of obesity. *Metabolism* 2007;56(11):1527-33.
83. Xiang AH, Peters RK, Trigo E, Kjos SL, Lee WP, Buchanan TA. Multiple metabolic defects during late pregnancy in women at high risk for type 2 diabetes. *Diabetes* 1999;48(4):848-54.
84. Sattar N, Greer I. Lipids and the pathogenesis of pre-eclampsia. *Current Obstetrics and Gynaecology* 1999;9:190-95.
85. Ness RB, Roberts JM. Heterogeneous causes constituting the single syndrome of preeclampsia: a hypothesis and its implications. *Am J Obstet Gynecol* 1996;175(5):1365-70.

86. Rodie VA, Freeman DJ, Sattar N, Greer IA. Pre-eclampsia and cardiovascular disease: metabolic syndrome of pregnancy? *Atherosclerosis* 2004;175(2):189-202.
87. Zhang C, Austin MA, Edwards KL, Farin FM, Li N, Hsu L, et al. Functional variants of the lipoprotein lipase gene and the risk of preeclampsia among non-Hispanic Caucasian women. *Clin Genet* 2006;69(1):33-9.
88. Potter JM, Nestel PJ. The hyperlipidemia of pregnancy in normal and complicated pregnancies. *Am J Obstet Gynecol* 1979;133(2):165-70.
89. Sattar N, Bendomir A, Berry C, Shepherd J, Greer IA, Packard CJ. Lipoprotein subfraction concentrations in preeclampsia: pathogenic parallels to atherosclerosis. *Obstet Gynecol* 1997;89(3):403-8.
90. Ramsay JE, Ferrell WR, Crawford L, Wallace AM, Greer IA, Sattar N. Divergent metabolic and vascular phenotypes in pre-eclampsia and intrauterine growth restriction: relevance of adiposity. *J Hypertens* 2004;22(11):2177-83.
91. Vadachkoria S, Woelk GB, Mahomed K, Qiu C, Muy-Rivera M, Malinow MR, et al. Elevated soluble vascular cell adhesion molecule-1, elevated Homocyst(e)inemia, and hypertriglyceridemia in relation to preeclampsia risk. *Am J Hypertens* 2006;19(3):235-42.
92. Lorentzen B, Enderson M, Clausen T, Henriksen T. Fasting serum free fatty acids and triglycerides are increased before 20 weeks of gestation in women who later develop pre-eclampsia. *Hypertension in Pregnancy* 1994;13:103-19.
93. Enquobahrie DA, Williams MA, Butler CL, Frederick IO, Miller RS, Luthy DA. Maternal plasma lipid concentrations in early pregnancy and risk of preeclampsia. *Am J Hypertens* 2004;17(7):574-81.
94. Belo L, Caslake M, Gaffney D, Santos-Silva A, Pereira-Leite L, Quintanilha A, et al. Changes in LDL size and HDL concentration in normal and preeclamptic pregnancies. *Atherosclerosis* 2002;162(2):425-32.
95. Llurba E, Casals E, Dominguez C, Delgado J, Mercade I, Crispi F, et al. Atherogenic lipoprotein subfraction profile in preeclamptic women with and without high triglycerides: different pathophysiologic subsets in preeclampsia. *Metabolism* 2005;54(11):1504-9.
96. Hubel CA, Shakir Y, Gallaher MJ, McLaughlin MK, Roberts JM. Low-density lipoprotein particle size decreases during normal pregnancy in association with triglyceride increases. *J Soc Gynecol Investig* 1998;5(5):244-50.
97. Griffin BA, Freeman DJ, Tait GW, Thomson J, Caslake MJ, Packard CJ, et al. Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. *Atherosclerosis* 1994;106(2):241-53.
98. Hubel CA, Lyall F, Weissfeld L, Gandley RE, Roberts JM. Small low-density lipoproteins and vascular cell adhesion molecule-1 are increased in association with hyperlipidemia in preeclampsia. *Metabolism* 1998;47(10):1281-8.
99. Hubel CA, McLaughlin MK, Evans RW, Hauth BA, Sims CJ, Roberts JM. Fasting serum triglycerides, free fatty acids, and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours post partum. *Am J Obstet Gynecol* 1996;174(3):975-82.
100. Pavan L, Tsatsaris V, Hermouet A, Therond P, Evain-Brion D, Fournier T. Oxidized low-density lipoproteins inhibit trophoblastic cell invasion. *J Clin Endocrinol Metab* 2004;89(4):1969-72.
101. Ware-Jauregui S, Sanchez SE, Zhang C, Laraburre G, King IB, Williams MA. Plasma lipid concentrations in pre-eclamptic and normotensive Peruvian women. *Int J Gynaecol Obstet* 1999;67(3):147-55.

102. Homko CJ, Cheung P, Boden G. Effects of free fatty acids on glucose uptake and utilization in healthy women. *Diabetes* 2003;52(2):487-91.
103. Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R, et al. Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* 2005;54(6):1640-8.
104. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 2000;49(11):1939-45.
105. Pleiner J, Schaller G, Mittermayer F, Bayerle-Eder M, Roden M, Wolzt M. FFA-induced endothelial dysfunction can be corrected by vitamin C. *J Clin Endocrinol Metab* 2002;87(6):2913-7.
106. Sivan E, Homko CJ, Whittaker PG, Reece EA, Chen X, Boden G. Free fatty acids and insulin resistance during pregnancy. *J Clin Endocrinol Metab* 1998;83(7):2338-42.
107. Endresen MJ, Lorentzen B, Henriksen T. Increased lipolytic activity and high ratio of free fatty acids to albumin in sera from women with preeclampsia leads to triglyceride accumulation in cultured endothelial cells. *Am J Obstet Gynecol* 1992;167(2):440-7.
108. Endresen MJ, Morris JM, Nobrega AC, Buckley D, Linton EA, Redman CW. Serum from preeclamptic women induces vascular cell adhesion molecule-1 expression on human endothelial cells in vitro: a possible role of increased circulating levels of free fatty acids. *Am J Obstet Gynecol* 1998;179(3 Pt 1):665-70.
109. Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol* 2005;25(10):2062-8.
110. Florez H, Castillo-Florez S, Mendez A, Casanova-Romero P, Larreal-Urdaneta C, Lee D, et al. C-reactive protein is elevated in obese patients with the metabolic syndrome. *Diabetes Res Clin Pract* 2006;71(1):92-100.
111. Bays HE. "Sick fat," metabolic disease, and atherosclerosis. *Am J Med* 2009;122(1 Suppl):S26-37.
112. Arner P. Human fat cell lipolysis: biochemistry, regulation and clinical role. *Best Pract Res Clin Endocrinol Metab* 2005;19(4):471-82.
113. Shah A, Mehta N, Reilly MP. Adipose inflammation, insulin resistance, and cardiovascular disease. *JPEN J Parenter Enteral Nutr* 2008;32(6):638-44.
114. Drolet R, Richard C, Sniderman AD, Mailloux J, Fortier M, Huot C, et al. Hypertrophy and hyperplasia of abdominal adipose tissues in women. *Int J Obes (Lond)* 2008;32(2):283-91.
115. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, et al. Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation* 2007;116(1):39-48.
116. Sniderman AD, Bhopal R, Prabhakaran D, Sarrafzadegan N, Tchernof A. Why might South Asians be so susceptible to central obesity and its atherogenic consequences? The adipose tissue overflow hypothesis. *Int J Epidemiol* 2007;36(1):220-5.
117. Brown TT, Xu X, John M, Singh J, Kingsley LA, Palella FJ, et al. Fat distribution and longitudinal anthropometric changes in HIV-infected men with and without clinical evidence of lipodystrophy and HIV-uninfected controls: a substudy of the Multicenter AIDS Cohort Study. *AIDS Res Ther* 2009;6:8.

118. Chaston TB, Dixon JB. Factors associated with percent change in visceral versus subcutaneous abdominal fat during weight loss: findings from a systematic review. *Int J Obes (Lond)* 2008;32(4):619-28.
119. Tchkonia T, Tchoukalova YD, Giorgadze N, Pirtskhalava T, Karagiannides I, Forse RA, et al. Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots. *Am J Physiol Endocrinol Metab* 2005;288(1):E267-77.
120. Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 2007;92(3):1023-33.
121. Lionetti L, Mollica MP, Lombardi A, Cavaliere G, Gifuni G, Barletta A. From chronic overnutrition to insulin resistance: the role of fat-storing capacity and inflammation. *Nutr Metab Cardiovasc Dis* 2009;19(2):146-52.
122. Halberg N, Wernstedt-Asterholm I, Scherer PE. The adipocyte as an endocrine cell. *Endocrinol Metab Clin North Am* 2008;37(3):753-68, x-xi.
123. Okamoto Y, Kihara S, Funahashi T, Matsuzawa Y, Libby P. Adiponectin: a key adipocytokine in metabolic syndrome. *Clin Sci (Lond)* 2006;110(3):267-78.
124. Altinova AE, Toruner F, Bozkurt N, Bukan N, Karakoc A, Yetkin I, et al. Circulating concentrations of adiponectin and tumor necrosis factor-alpha in gestational diabetes mellitus. *Gynecol Endocrinol* 2007;23(3):161-5.
125. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002;8(11):1288-95.
126. Fisher FF, Trujillo ME, Hanif W, Barnett AH, McTernan PG, Scherer PE, et al. Serum high molecular weight complex of adiponectin correlates better with glucose tolerance than total serum adiponectin in Indo-Asian males. *Diabetologia* 2005;48(6):1084-7.
127. Mazaki-Tovi S, Romero R, Kusanovic JP, Erez O, Vaisbuch E, Gotsch F, et al. Adiponectin multimers in maternal plasma. *J Matern Fetal Neonatal Med* 2008;21(11):796-815.
128. Catalano PM, Hoegh M, Minium J, Huston-Presley L, Bernard S, Kalhan S, et al. Adiponectin in human pregnancy: implications for regulation of glucose and lipid metabolism. *Diabetologia* 2006;49(7):1677-85.
129. Eriksson B, Lof M, Olausson H, Forsum E. Body fat, insulin resistance, energy expenditure and serum concentrations of leptin, adiponectin and resistin before, during and after pregnancy in healthy Swedish women. *Br J Nutr* 2009:1-8.
130. Winzer C, Wagner O, Festa A, Schneider B, Roden M, Bancher-Todesca D, et al. Plasma adiponectin, insulin sensitivity, and subclinical inflammation in women with prior gestational diabetes mellitus. *Diabetes Care* 2004;27(7):1721-7.
131. O'Sullivan AJ, Kriketos AD, Martin A, Brown MA. Serum adiponectin levels in normal and hypertensive pregnancy. *Hypertens Pregnancy* 2006;25(3):193-203.
132. Cortelazzi D, Corbetta S, Ronzoni S, Pelle F, Marconi A, Cozzi V, et al. Maternal and foetal resistin and adiponectin concentrations in normal and complicated pregnancies. *Clin Endocrinol (Oxf)* 2007;66(3):447-53.
133. Ramsay JE, Jamieson N, Greer IA, Sattar N. Paradoxical elevation in adiponectin concentrations in women with preeclampsia. *Hypertension* 2003;42(5):891-4.
134. Haugen F, Ranheim T, Harsem NK, Lips E, Staff AC, Drevon CA. Increased plasma levels of adipokines in preeclampsia: relationship to placenta and

adipose tissue gene expression. *Am J Physiol Endocrinol Metab* 2006;290(2):E326-33.
135. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372(6505):425-32.
136. Van Harmelen V, Reynisdottir S, Eriksson P, Thorne A, Hoffstedt J, Lonnqvist F, et al. Leptin secretion from subcutaneous and visceral adipose tissue in women. *Diabetes* 1998;47(6):913-7.
137. Rentsch J, Chiesi M. Regulation of ob gene mRNA levels in cultured adipocytes. *FEBS Lett* 1996;379(1):55-9.
138. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995;1(11):1155-61.
139. Sagawa N, Yura S, Itoh H, Mise H, Kakui K, Korita D, et al. Role of leptin in pregnancy--a review. *Placenta* 2002;23 Suppl A:S80-6.
140. Domali E, Messinis IE. Leptin in pregnancy. *J Matern Fetal Neonatal Med* 2002;12(4):222-30.
141. Highman TJ, Friedman JE, Huston LP, Wong WW, Catalano PM. Longitudinal changes in maternal serum leptin concentrations, body composition, and resting metabolic rate in pregnancy. *Am J Obstet Gynecol* 1998;178(5):1010-5.
142. Henson MC, Castracane VD. Leptin in pregnancy: an update. *Biol Reprod* 2006;74(2):218-29.
143. Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, et al. Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci U S A* 1997;94(9):4637-41.
144. Wang MY, Lee Y, Unger RH. Novel form of lipolysis induced by leptin. *J Biol Chem* 1999;274(25):17541-4.
145. Jiang L, Wang Q, Yu Y, Zhao F, Huang P, Zeng R, et al. Leptin contributes to the adaptive responses of mice to high-fat diet intake through suppressing the lipogenic pathway. *PLoS One* 2009;4(9):e6884.
146. Buettner C, Muse ED, Cheng A, Chen L, Scherer T, Pocai A, et al. Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. *Nat Med* 2008;14(6):667-75.
147. Li RH, Poon SC, Yu MY, Wong YF. Expression of placental leptin and leptin receptors in preeclampsia. *Int J Gynecol Pathol* 2004;23(4):378-85.
148. Laivuori H, Gallaher MJ, Collura L, Crombleholme WR, Markovic N, Rajakumar A, et al. Relationships between maternal plasma leptin, placental leptin mRNA and protein in normal pregnancy, pre-eclampsia and intrauterine growth restriction without pre-eclampsia. *Mol Hum Reprod* 2006;12(9):551-6.
149. Poston L. Leptin and preeclampsia. *Semin Reprod Med* 2002;20(2):131-8.
150. Bartha JL, Romero-Carmona R, Escobar-Llompart M, Comino-Delgado R. The relationships between leptin and inflammatory cytokines in women with pre-eclampsia. *Bjog* 2001;108(12):1272-6.
151. Hotamisligil GS. The role of TNFalpha and TNF receptors in obesity and insulin resistance. *J Intern Med* 1999;245(6):621-5.
152. Tuomisto K, Jousilahti P, Sundvall J, Pajunen P, Salomaa V. C-reactive protein, interleukin-6 and tumor necrosis factor alpha as predictors of incident coronary and cardiovascular events and total mortality. A population-based, prospective study. *Thromb Haemost* 2006;95(3):511-8.
153. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 1995;95(5):2409-15.

154. Arner P. Insulin resistance in type 2 diabetes -- role of the adipokines. *Curr Mol Med* 2005;5(3):333-9.
155. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996;271(5249):665-8.
156. Bhagat K, Vallance P. Inflammatory cytokines impair endothelium-dependent dilatation in human veins in vivo. *Circulation* 1997;96(9):3042-7.
157. Kirchhofer D, Tschopp TB, Hadvary P, Baumgartner HR. Endothelial cells stimulated with tumor necrosis factor-alpha express varying amounts of tissue factor resulting in inhomogenous fibrin deposition in a native blood flow system. Effects of thrombin inhibitors. *J Clin Invest* 1994;93(5):2073-83.
158. Chen X, Xun K, Chen L, Wang Y. TNF-alpha, a potent lipid metabolism regulator. *Cell Biochem Funct* 2009;27(7):407-16.
159. Kirwan JP, Hauguel-De Mouzon S, Lepercq J, Challier JC, Huston-Presley L, Friedman JE, et al. TNF-alpha is a predictor of insulin resistance in human pregnancy. *Diabetes* 2002;51(7):2207-13.
160. Hayashi M, Ueda Y, Yamaguchi T, Sohma R, Shibazaki M, Ohkura T, et al. Tumor necrosis factor-alpha in the placenta is not elevated in pre-eclamptic patients despite its elevation in peripheral blood. *Am J Reprod Immunol* 2005;53(3):113-9.
161. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, et al. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997;82(12):4196-200.
162. Senn JJ, Klover PJ, Nowak IA, Mooney RA. Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes* 2002;51(12):3391-9.
163. Petersen EW, Carey AL, Sacchetti M, Steinberg GR, Macaulay SL, Febbraio MA, et al. Acute IL-6 treatment increases fatty acid turnover in elderly humans in vivo and in tissue culture in vitro. *Am J Physiol Endocrinol Metab* 2005;288(1):E155-62.
164. Fasshauer M, Klein J, Lossner U, Paschke R. Interleukin (IL)-6 mRNA expression is stimulated by insulin, isoproterenol, tumour necrosis factor alpha, growth hormone, and IL-6 in 3T3-L1 adipocytes. *Horm Metab Res* 2003;35(3):147-52.
165. Montagnana M, Lippi G, Albiero A, Salvagno GL, Franchi M, Guidi GC. Serum pro-inflammatory cytokines in physiological and pre-eclamptic pregnancies. *Gynecol Endocrinol* 2008;24(3):113-6.
166. Challier JC, Basu S, Bintein T, Minium J, Hotmire K, Catalano PM, et al. Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. *Placenta* 2008;29(3):274-81.
167. Kuzmicki M, Telejko B, Zonenberg A, Szamatowicz J, Kretowski A, Nikolajuk A, et al. Circulating pro- and anti-inflammatory cytokines in Polish women with gestational diabetes. *Horm Metab Res* 2008;40(8):556-60.
168. Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 1999;19(4):972-8.
169. Freeman DJ, Norrie J, Caslake MJ, Gaw A, Ford I, Lowe GD, et al. C-reactive protein is an independent predictor of risk for the development of diabetes in the West of Scotland Coronary Prevention Study. *Diabetes* 2002;51(5):1596-600.

170. Ridker PM, Morrow DA. C-reactive protein, inflammation, and coronary risk. *Cardiol Clin* 2003;21(3):315-25.
171. Belo L, Santos-Silva A, Rocha S, Caslake M, Cooney J, Pereira-Leite L, et al. Fluctuations in C-reactive protein concentration and neutrophil activation during normal human pregnancy. *Eur J Obstet Gynecol Reprod Biol* 2005;123(1):46-51.
172. Leipold H, Worda C, Gruber CJ, Prikoszovich T, Wagner O, Kautzky-Willer A. Gestational diabetes mellitus is associated with increased C-reactive protein concentrations in the third but not second trimester. *Eur J Clin Invest* 2005;35(12):752-7.
173. Cebesoy FB, Balat O, Dikensoy E, Kalayci H, Ibar Y. CA-125 and CRP are elevated in preeclampsia. *Hypertens Pregnancy* 2009;28(2):201-11.
174. De Taeye B, Smith LH, Vaughan DE. Plasminogen activator inhibitor-1: a common denominator in obesity, diabetes and cardiovascular disease. *Curr Opin Pharmacol* 2005;5(2):149-54.
175. Koistinen HA, Dusserre E, Ebeling P, Vallier P, Koivisto VA, Vidal H. Subcutaneous adipose tissue expression of plasminogen activator inhibitor-1 (PAI-1) in nondiabetic and Type 2 diabetic subjects. *Diabetes Metab Res Rev* 2000;16(5):364-9.
176. Juhan-Vague I, Alessi MC, Mavri A, Morange PE. Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. *J Thromb Haemost* 2003;1(7):1575-9.
177. Shimomura I, Funahashi T, Takahashi M, Maeda K, Kotani K, Nakamura T, et al. Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* 1996;2(7):800-3.
178. Alessi MC, Bastelica D, Mavri A, Morange P, Berthet B, Grino M, et al. Plasma PAI-1 levels are more strongly related to liver steatosis than to adipose tissue accumulation. *Arterioscler Thromb Vasc Biol* 2003;23(7):1262-8.
179. Lopez-Alemany R, Redondo JM, Nagamine Y, Munoz-Canoves P. Plasminogen activator inhibitor type-1 inhibits insulin signaling by competing with alphavbeta3 integrin for vitronectin binding. *Eur J Biochem* 2003;270(5):814-21.
180. Liang X, Kanjanabuch T, Mao SL, Hao CM, Tang YW, Declerck PJ, et al. Plasminogen activator inhibitor-1 modulates adipocyte differentiation. *Am J Physiol Endocrinol Metab* 2006;290(1):E103-E13.
181. Wikstrom AK, Nash P, Eriksson UJ, Olovsson MH. Evidence of increased oxidative stress and a change in the plasminogen activator inhibitor (PAI)-1 to PAI-2 ratio in early-onset but not late-onset preeclampsia. *Am J Obstet Gynecol* 2009;201(6):597 e1-8.
182. Stewart FM, Freeman DJ, Ramsay JE, Greer IA, Caslake M, Ferrell WR. Longitudinal assessment of maternal endothelial function and markers of inflammation and placental function throughout pregnancy in lean and obese mothers. *J Clin Endocrinol Metab* 2007;92(3):969-75.
183. Hannaford P, Ferry S, Hirsch S. Cardiovascular sequelae of toxaemia of pregnancy. *Heart* 1997;77(2):154-8.
184. Jonsdottir LS, Arngrimsson R, Geirsson RT, Sigvaldason H, Sigfusson N. Death rates from ischemic heart disease in women with a history of hypertension in pregnancy. *Acta Obstet Gynecol Scand* 1995;74(10):772-6.
185. Smith GC, Pell JP, Walsh D. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. *Lancet* 2001;357(9273):2002-6.

186. Ray JG, Vermeulen MJ, Schull MJ, Redelmeier DA. Cardiovascular health after maternal placental syndromes (CHAMPS): population-based retrospective cohort study. *Lancet* 2005;366(9499):1797-803.
187. Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ* 2007;335(7627):974.
188. Green A, Beral V, Moser K. Mortality in women in relation to their childbearing history. *BMJ* 1988;297(6645):391-5.
189. Ness RB, Harris T, Cobb J, Flegal KM, Kelsey JL, Balanger A, et al. Number of pregnancies and the subsequent risk of cardiovascular disease. *N Engl J Med* 1993;328(21):1528-33.
190. Lawlor DA, Emberson JR, Ebrahim S, Whincup PH, Wannamethee SG, Walker M, et al. Is the association between parity and coronary heart disease due to biological effects of pregnancy or adverse lifestyle risk factors associated with child-rearing? Findings from the British Women's Heart and Health Study and the British Regional Heart Study. *Circulation* 2003;107(9):1260-4.
191. Sattar N, Greer IA. Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? *Bmj* 2002;325(7356):157-60.
192. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* 2001;20(1):IX-XIV.
193. Rodbell M. Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis. *J Biol Chem* 1964;239:375-80.
194. Elliott JA. The effect of pregnancy on the control of lipolysis in fat cells isolated from human adipose tissue. *Eur J Clin Invest* 1975;5(2):159-63.
195. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28(7):412-9.
196. Lewis GF, Uffelman KD, Szeto LW, Weller B, Steiner G. Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. *J Clin Invest* 1995;95(1):158-66.
197. Hodson L, Bickerton AS, McQuaid SE, Roberts R, Karpe F, Frayn KN, et al. The contribution of splanchnic fat to VLDL triglyceride is greater in insulin-resistant than insulin-sensitive men and women: studies in the postprandial state. *Diabetes* 2007;56(10):2433-41.
198. Bickerton AS, Roberts R, Fielding BA, Tornqvist H, Blaak EE, Wagenmakers AJ, et al. Adipose tissue fatty acid metabolism in insulin-resistant men. *Diabetologia* 2008;51(8):1466-74.
199. Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang Cc C, Itani SI, et al. Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc Natl Acad Sci U S A* 2002;99(25):16309-13.
200. Stefan N, Vozarova B, Funahashi T, Matsuzawa Y, Ravussin E, Weyer C, et al. Plasma adiponectin levels are not associated with fat oxidation in humans. *Obes Res* 2002;10(10):1016-20.
201. Kajantie E, Kaaja R, Ylikorkala O, Andersson S, Laivuori H. Adiponectin concentrations in maternal serum: elevated in preeclampsia but unrelated to insulin sensitivity. *J Soc Gynecol Investig* 2005;12(6):433-9.
202. Fasshauer M, Bluher M, Stumvoll M, Tonessen P, Faber R, Stepan H. Differential regulation of visfatin and adiponectin in pregnancies with

normal and abnormal placental function. *Clin Endocrinol (Oxf)* 2007;66(3):434-9.
203. Kleiblova P, Dostalova I, Bartlova M, Lacinova Z, Ticha I, Krejci V, et al. Expression of adipokines and estrogen receptors in adipose tissue and placenta of patients with gestational diabetes mellitus. *Mol Cell Endocrinol* 2009.
204. McLachlan KA, O'Neal D, Jenkins A, Alford FP. Do adiponectin, TNFalpha, leptin and CRP relate to insulin resistance in pregnancy? Studies in women with and without gestational diabetes, during and after pregnancy. *Diabetes Metab Res Rev* 2006;22(2):131-8.
205. Sugiura K, Tamakoshi K, Yatsuya H, Otsuka R, Wada K, Matsushita K, et al. Contribution of adipocytokines to low-grade inflammatory state as expressed by circulating C-reactive protein in Japanese men: comparison of leptin and adiponectin. *Int J Cardiol* 2008;130(2):159-64.
206. Iwasaki T, Nakajima A, Yoneda M, Terauchi Y. Relationship between the serum concentrations of C-reactive protein and parameters of adiposity and insulin resistance in patients with type 2 diabetes mellitus. *Endocr J* 2006;53(3):345-56.
207. Saijo Y, Kiyota N, Kawasaki Y, Miyazaki Y, Kashimura J, Fukuda M, et al. Relationship between C-reactive protein and visceral adipose tissue in healthy Japanese subjects. *Diabetes Obes Metab* 2004;6(4):249-58.
208. Park JS, Cho MH, Nam JS, Ahn CW, Cha BS, Lee EJ, et al. Visceral adiposity and leptin are independently associated with C-reactive protein in Korean type 2 diabetic patients. *Acta Diabetol* 2009.
209. Street ME, Seghini P, Fieni S, Ziveri MA, Volta C, Martorana D, et al. Changes in interleukin-6 and IGF system and their relationships in placenta and cord blood in newborns with fetal growth restriction compared with controls. *Eur J Endocrinol* 2006;155(4):567-74.
210. Odegard RA, Vatten LJ, Nilsen ST, Salvesen KA, Vefring H, Austgulen R. Umbilical cord plasma interleukin-6 and fetal growth restriction in preeclampsia: a prospective study in Norway. *Obstet Gynecol* 2001;98(2):289-94.
211. Catalano PM, Presley L, Minium J, Hauguel-de Mouzon S. Fetuses of obese mothers develop insulin resistance in utero. *Diabetes Care* 2009;32(6):1076-80.
212. Weyer C, Wolford JK, Hanson RL, Foley JE, Tataranni PA, Bogardus C, et al. Subcutaneous abdominal adipocyte size, a predictor of type 2 diabetes, is linked to chromosome 1q21--q23 and is associated with a common polymorphism in LMNA in Pima Indians. *Mol Genet Metab* 2001;72(3):231-8.
213. Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia* 2000;43(12):1498-506.
214. Mei J, Holst LS, Landstrom TR, Holm C, Brindley D, Manganiello V, et al. C(2)-ceramide influences the expression and insulin-mediated regulation of cyclic nucleotide phosphodiesterase 3B and lipolysis in 3T3-L1 adipocytes. *Diabetes* 2002;51(3):631-7.
215. Zhang T, He J, Xu C, Zu L, Jiang H, Pu S, et al. Mechanisms of metformin inhibiting lipolytic response to isoproterenol in primary rat adipocytes. *J Mol Endocrinol* 2009;42(1):57-66.
216. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science* 2005;308(5728):1592-4.

217. Redman CW, Sargent IL. Placental stress and pre-eclampsia: a revised view. *Placenta* 2009;30 Suppl A:S38-42.
218. Ramsay JE, Simms RJ, Ferrell WR, Crawford L, Greer IA, Lumsden MA, et al. Enhancement of endothelial function by pregnancy: inadequate response in women with type 1 diabetes. *Diabetes Care* 2003;26(2):475-9.
219. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993;259(5091):87-91.
220. Matarese G, Moschos S, Mantzoros CS. Leptin in immunology. *J Immunol* 2005;174(6):3137-42.
221. Harman-Boehm I, Bluher M, Redel H, Sion-Vardy N, Ovadia S, Avinoach E, et al. Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab* 2007;92(6):2240-7.
222. Zeyda M, Stulnig TM. Adipose tissue macrophages. *Immunol Lett* 2007;112(2):61-7.
223. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;3(1):23-35.
224. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112(12):1796-808.
225. Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, 2nd, DeFuria J, Jick Z, et al. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* 2007;56(12):2910-8.
226. Rausch ME, Weisberg S, Vardhana P, Tortoriello DV. Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. *Int J Obes (Lond)* 2008;32(3):451-63.
227. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005;184(4):285-93.
228. Dahlman I, Kaaman M, Olsson T, Tan GD, Bickerton AS, Wahlen K, et al. A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. *J Clin Endocrinol Metab* 2005;90(10):5834-40.
229. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, et al. Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* 2006;281(36):26602-14.
230. Chacon MR, Fernandez-Real JM, Richart C, Megia A, Gomez JM, Miranda M, et al. Monocyte chemoattractant protein-1 in obesity and type 2 diabetes. Insulin sensitivity study. *Obesity (Silver Spring)* 2007;15(3):664-72.
231. Kopp A, Buechler C, Neumeier M, Weigert J, Aslanidis C, Scholmerich J, et al. Innate immunity and adipocyte function: ligand-specific activation of multiple Toll-like receptors modulates cytokine, adipokine, and chemokine secretion in adipocytes. *Obesity (Silver Spring)* 2009;17(4):648-56.
232. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007;56(7):1761-72.
233. Song MJ, Kim KH, Yoon JM, Kim JB. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. *Biochem Biophys Res Commun* 2006;346(3):739-45.
234. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, et al. Loss-of-function mutation in Toll-like receptor 4

prevents diet-induced obesity and insulin resistance. *Diabetes* 2007;56(8):1986-98.
235. Anderson PD, Mehta NN, Wolfe ML, Hinkle CC, Pruscino L, Comiskey LL, et al. Innate immunity modulates adipokines in humans. *J Clin Endocrinol Metab* 2007;92(6):2272-9.
236. Sherr CJ. The fms oncogene. *Biochim Biophys Acta* 1988;948(2):225-43.
237. Pixley FJ, Stanley ER. CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol* 2004;14(11):628-38.
238. Ryden M, Arner P. Tumour necrosis factor-alpha in human adipose tissue -- from signalling mechanisms to clinical implications. *J Intern Med* 2007;262(4):431-8.
239. Cawthorn WP, Sethi JK. TNF-alpha and adipocyte biology. *FEBS Lett* 2008;582(1):117-31.
240. Freeman DJ, McManus F, Brown EA, Cherry L, Norrie J, Ramsay JE, et al. Short- and long-term changes in plasma inflammatory markers associated with preeclampsia. *Hypertension* 2004;44(5):708-14.
241. Greer IA, Lyall F, Perera T, Boswell F, Macara LM. Increased concentrations of cytokines interleukin-6 and interleukin-1 receptor antagonist in plasma of women with preeclampsia: a mechanism for endothelial dysfunction? *Obstet Gynecol* 1994;84(6):937-40.
242. Zhang H, Park Y, Wu J, Chen X, Lee S, Yang J, et al. Role of TNF-alpha in vascular dysfunction. *Clin Sci (Lond)* 2009;116(3):219-30.
243. LaMarca BD, Ryan MJ, Gilbert JS, Murphy SR, Granger JP. Inflammatory cytokines in the pathophysiology of hypertension during preeclampsia. *Curr Hypertens Rep* 2007;9(6):480-5.
244. Benyo DF, Smarason A, Redman CW, Sims C, Conrad KP. Expression of inflammatory cytokines in placentas from women with preeclampsia. *J Clin Endocrinol Metab* 2001;86(6):2505-12.
245. Chou YY, Sheu WH, Tang YJ, Chen YM, Liao SC, Chuang YW, et al. Plasminogen activator inhibitor type 1 (PAI-1) is a valuable biomarker for predicting the metabolic syndrome (MS) in institutionalized elderly residents in Taiwan. *Arch Gerontol Geriatr* 2009;49 Suppl 2:S41-5.
246. Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ Res* 2005;96(12):1221-32.
247. Aprath-Husmann I, Rohrig K, Gottschling-Zeller H, Skurk T, Scriba D, Birgel M, et al. Effects of leptin on the differentiation and metabolism of human adipocytes. *Int J Obes Relat Metab Disord* 2001;25(10):1465-70.
248. Fruhbeck G, Aguado M, Martinez JA. In vitro lipolytic effect of leptin on mouse adipocytes: evidence for a possible autocrine/paracrine role of leptin. *Biochem Biophys Res Commun* 1997;240(3):590-4.
249. Elimam A, Kamel A, Marcus C. In vitro effects of leptin on human adipocyte metabolism. *Horm Res* 2002;58(2):88-93.
250. Allender S PV, Scarborough P, Kaur A, Rayner M. Coronary Heart Disease Statistics. London: British Heart Foundation, 2008.
251. Levin RI. The puzzle of aspirin and sex. *N Engl J Med* 2005;352(13):1366-8.
252. Diamond GA, Forrester JS. Analysis of probability as an aid in the clinical diagnosis of coronary-artery disease. *N Engl J Med* 1979;300(24):1350-8.
253. Legato MJ. Dyslipidemia, gender, and the role of high-density lipoprotein cholesterol: implications for therapy. *Am J Cardiol* 2000;86(12A):15L-18L.
254. Hippisley-Cox J, Pringle M, Crown N, Meal A, Wynn A. Sex inequalities in ischaemic heart disease in general practice: cross sectional survey. *BMJ* 2001;322(7290):832.
255. Sprecher DL, Pearce GL. How deadly is the "deadly quartet"? A post-CABG evaluation. *J Am Coll Cardiol* 2000;36(4):1159-65.

256. Exploring Biological Contributions to Human Health. Does sex matter? Committee on Understanding the Biology of Sex and Gender Differences. Washington DC: Institute of Medicine, 2001.
257. Callaway LK, Lawlor DA, O'Callaghan M, Williams GM, Najman JM, McIntyre HD. Diabetes mellitus in the 21 years after a pregnancy that was complicated by hypertension: findings from a prospective cohort study. *Am J Obstet Gynecol* 2007;197(5):492 e1-7.
258. Arngrimsson R, Bjornsson S, Geirsson RT, Bjornsson H, Walker JJ, Snaedal G. Genetic and familial predisposition to eclampsia and pre-eclampsia in a defined population. *Br J Obstet Gynaecol* 1990;97(9):762-9.
259. Arngrimsson R, Hayward C, Nadaud S, Baldursdottir A, Walker JJ, Liston WA, et al. Evidence for a familial pregnancy-induced hypertension locus in the eNOS-gene region. *Am J Hum Genet* 1997;61(2):354-62.
260. Chesley LC, Cooper DW. Genetics of hypertension in pregnancy: possible single gene control of pre-eclampsia and eclampsia in the descendants of eclamptic women. *Br J Obstet Gynaecol* 1986;93(9):898-908.
261. Ramsay JE, Stewart F, Greer IA, Sattar N. Microvascular dysfunction: a link between pre-eclampsia and maternal coronary heart disease. *Bjog* 2003;110(11):1029-31.
262. Lampinen KH, Ronnback M, Kaaja RJ, Groop PH. Impaired vascular dilatation in women with a history of pre-eclampsia. *J Hypertens* 2006;24(4):751-6.
263. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. *Circulation* 2007;115(4):459-67.
264. Touboul PJ, Hennerici MG, Meairs S, Adams H, Amarenco P, Bornstein N, et al. Mannheim carotid intima-media thickness consensus (2004-2006). An update on behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium, 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. *Cerebrovasc Dis* 2007;23(1):75-80.
265. van der Meer IM, Bots ML, Hofman A, del Sol AI, van der Kuip DA, Witteman JC. Predictive value of noninvasive measures of atherosclerosis for incident myocardial infarction: the Rotterdam Study. *Circulation* 2004;109(9):1089-94.
266. Johnsen SH, Mathiesen EB, Joakimsen O, Stensland E, Wilsgaard T, Lochen ML, et al. Carotid atherosclerosis is a stronger predictor of myocardial infarction in women than in men: a 6-year follow-up study of 6226 persons: the Tromso Study. *Stroke* 2007;38(11):2873-80.
267. Hollander M, Hak AE, Koudstaal PJ, Bots ML, Grobbee DE, Hofman A, et al. Comparison between measures of atherosclerosis and risk of stroke: the Rotterdam Study. *Stroke* 2003;34(10):2367-72.
268. Clark P, Sattar N, Walker ID, Greer IA. The Glasgow Outcome, APCR and Lipid (GOAL) Pregnancy Study: significance of pregnancy associated activated protein C resistance. *Thromb Haemost* 2001;85(1):30-5.
269. Stensvold I, Tverdal A, Urdal P, Graff-Iversen S. Non-fasting serum triglyceride concentration and mortality from coronary heart disease and any cause in middle aged Norwegian women. *Bmj* 1993;307(6915):1318-22.
270. Ruige JB, Assendelft WJ, Dekker JM, Kostense PJ, Heine RJ, Bouter LM. Insulin and risk of cardiovascular disease: a meta-analysis. *Circulation* 1998;97(10):996-1001.
271. Carstairs VM, R. Deprivation and health in Scotland. Aberdeen, Aberdeen University Press 1991.

272. Vryonidou A, Papatheodorou A, Tavridou A, Terzi T, Loi V, Vatalas IA, et al. Association of hyperandrogenemic and metabolic phenotype with carotid intima-media thickness in young women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2005;90(5):2740-6.
273. Talbott EO, Zborowski JV, Boudreaux MY, McHugh-Pemu KP, Sutton-Tyrrell K, Guzick DS. The relationship between C-reactive protein and carotid intima-media wall thickness in middle-aged women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2004;89(12):6061-7.
274. Solomon CG, Hu FB, Dunaif A, Rich-Edwards JE, Stampfer MJ, Willett WC, et al. Menstrual cycle irregularity and risk for future cardiovascular disease. *J Clin Endocrinol Metab* 2002;87(5):2013-7.
275. Haukkamaa L, Salminen M, Laivuori H, Leinonen H, Hiilesmaa V, Kaaja R. Risk for subsequent coronary artery disease after preeclampsia. *Am J Cardiol* 2004;93(6):805-8.
276. Chambless LE, Folsom AR, Davis V, Sharrett R, Heiss G, Sorlie P, et al. Risk factors for progression of common carotid atherosclerosis: the Atherosclerosis Risk in Communities Study, 1987-1998. *Am J Epidemiol* 2002;155(1):38-47.
277. Belcaro G, Nicolaides AN, Ramaswami G, Cesarone MR, De Sanctis M, Incandela L, et al. Carotid and femoral ultrasound morphology screening and cardiovascular events in low risk subjects: a 10-year follow-up study (the CAFES-CAVE study(1)). *Atherosclerosis* 2001;156(2):379-87.
278. Spence JD. Measurement of intima-media thickness vs. carotid plaque: uses in patient care, genetic research and evaluation of new therapies. *Int J Stroke* 2006;1(4):216-21.
279. Deans KA, Bezlyak V, Ford I, Batty GD, Burns H, Cavanagh J, et al. Differences in atherosclerosis according to area level socioeconomic deprivation: cross sectional, population based study. *Bmj* 2009;339:b4170.
280. Sattar N, Ramsay J, Crawford L, Cheyne H, Greer IA. Classic and novel risk factor parameters in women with a history of preeclampsia. *Hypertension* 2003;42(1):39-42.
281. Libby P. Inflammation in atherosclerosis. *Nature* 2002;420(6917):868-74.
282. Jensen MD. Role of body fat distribution and the metabolic complications of obesity. *J Clin Endocrinol Metab* 2008;93(11 Suppl 1):S57-63.
283. Snijder MB, Visser M, Dekker JM, Goodpaster BH, Harris TB, Kritchevsky SB, et al. Low subcutaneous thigh fat is a risk factor for unfavourable glucose and lipid levels, independently of high abdominal fat. The Health ABC Study. *Diabetologia* 2005;48(2):301-8.
284. Huang W, Dedousis N, Bandi A, Lopaschuk GD, O'Doherty RM. Liver triglyceride secretion and lipid oxidative metabolism are rapidly altered by leptin in vivo. *Endocrinology* 2006;147(3):1480-7.
285. Park BH, Wang MY, Lee Y, Yu X, Ravazzola M, Orci L, et al. Combined leptin actions on adipose tissue and hypothalamus are required to deplete adipocyte fat in lean rats: implications for obesity treatment. *J Biol Chem* 2006;281(52):40283-91.
286. Huda SS, Brodie LE, Sattar N. Obesity in pregnancy: prevalence and metabolic consequences. *Semin Fetal Neonatal Med* 2009.
287. Nelson SM, Matthews P, Poston L. Maternal metabolism and obesity: modifiable determinants of pregnancy outcome. *Hum Reprod Update* 2009.
288. Soltani H, Fraser RB. A longitudinal study of maternal anthropometric changes in normal weight, overweight and obese women during pregnancy and postpartum. *Br J Nutr* 2000;84(1):95-101.

289. Snijder MB, Zimmet PZ, Visser M, Dekker JM, Seidell JC, Shaw JE. Independent and opposite associations of waist and hip circumferences with diabetes, hypertension and dyslipidemia: the AusDiab Study. *Int J Obes Relat Metab Disord* 2004;28(3):402-9.
290. Barwell ND, Malkova D, Moran CN, Cleland SJ, Packard CJ, Zammit VA, et al. Exercise training has greater effects on insulin sensitivity in daughters of patients with type 2 diabetes than in women with no family history of diabetes. *Diabetologia* 2008;51(10):1912-9.
291. Moran CN, Barwell, N.D., Malkova, D.,Cleland, S.J., McPhee, I., Packard, C.J.,Gill, J.M. Effects of diabetes family history and exercise training on the expression of adiponectin and leptin and their receptors. *Metabolism* 2010:In Press

Appendices

Published Papers