Metabolic Complications of Pregnancy and Cardiovascular Disease Risk

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Abstract

Pre-eclampsia (PET) is a multi-system disorder particular to pregnancy. It is characterised by widespread endothelial dysfunction, resulting in hypertension due to vasoconstriction, proteinuria attributable to glomerular damage and oedema secondary to increased vascular permeability. PET has a complex aetiology involving a spectrum of exaggerated disturbances in maternal metabolism, potentially resulting from a trigger from the placenta. PET shares many risk factors with cardiovascular disease (CVD) and may be associated with increased risk of future maternal CVD. The similarities between the metabolic syndrome and PET, has lead to the proposal that PET is metabolic syndrome of pregnancy. It is likely that a combination of environmental, genetic and metabolic parameters have a role in the aetiology of PET, rather than one specific factor. Although there are abundant data on the metabolic pathways and vascular function in the non-pregnant individual, there are little such data with respect to pregnancy and PET. The purpose of this thesis was to concentrate on PET as a metabolic disorder and to focus on the key mediators involved in the metabolic syndrome including PPAR receptors, lipoprotein metabolism, insulin resistance and inflammation.

PPARs are a family of nuclear receptors controlling pathways involved in the metabolic syndrome including adipocyte differentiation, glucose and lipid homeostasis and fatty acid metabolism. Animal and human models have highlighted a role for the PPARs in pregnancy. In this thesis, the placental localisation and expression of each of the PPARs (α, δ and γ) were determined, in uncomplicated and PET and IUGR pregnancy. The PPARs were localised in trophoblasts in each trimester of pregnancy and in PET and IUGR, with sparse PPARα staining in the 3rd trimester. Specifically, PPARδ mRNA and protein expression were higher in the 3rd compared to the 1st trimester. There were no consistent differences in PPAR expression in PET or IUGR placentae compared to controls.

Lipid metabolism is a key element of the metabolic syndrome and maternal and fetal lipid and lipoproteins were studied in a BMI-matched study of uncomplicated 3rd trimester pregnancies, and pregnancies complicated by PET or IUGR. Paraoxonase (PON-1) activity was also determined as a marker of oxidative stress. There was a significantly elevated maternal total cholesterol (TC) in PET compared with controls, and maternal PON-1 activity was significantly lower in PET. In PET and IUGR, there were alterations in fetal lipid profile. In PET, there was a significantly elevated concentration of fetal TC,

triglyceride (TG) and an increased fetal cholesterol/high density lipoprotein (HDL) ratio compared with uncomplicated pregnancies. In IUGR, elevated levels of fetal TG were reported. Fetal PON-1 activity was unaltered between controls, PET or IUGR pregnancy.

The link between insulin resistance and inflammation, components of the metabolic syndrome, was studied in PET. Maternal and fetal inflammatory and insulin resistance markers in PET, IUGR and uncomplicated control pregnancies were compared. Maternal erythrocyte membrane fatty acid composition was also determined as a marker of insulin resistance, and results were related to the plasma lipid, inflammatory and insulin resistance markers. Finally tissue expression of key genes involved in insulin resistance and inflammation was assessed.

Fetal plasma C-reactive protein (CRP) levels were elevated in PET and fetal plasma TNFα levels were increased in IUGR pregnancies compared to uncomplicated control pregnancies. In a longitudinal study of erythrocyte membrane fatty acid status, there was a reduction of 18:0 and elongase activity and a significant increase in maternal docosahexaenoic acid (DHA) with advancing gestation. An increase in total n3 fatty acids and delta 9 desaturase activity from the 1st to the 2nd trimester and a reduction in delta 5 desaturase activity from the 1st to the 2nd trimester were observed. In PET pregnancies, there was a significant reduction in average chain length and elongase activity in PET compared with healthy controls. There were no differences in any of the fatty acids tested between IUGR and controls.

Increased placental leptin mRNA expression in PET was confirmed, and a similar increase in leptin expression was observed in IUGR. These data suggest that the tissue source of fetal leptin production may be adipose tissue rather than placenta, as there was a non-significant reduction in fetal plasma leptin levels in PET and IUGR despite the observed increase in placental mRNA levels. The association of fetal leptin with birth weight centile in uncomplicated pregnancies strengthens this proposal. Placental leptin production correlated with plasma HDL in uncomplicated pregnancy, suggesting a link between placental leptin and fetal lipid stores. The tissue source of maternal plasma interleukin-10 (IL-10) was suggested to be primarily from the maternal adipose tissue.

To study the genetic contribution to PET risk, IL-10, leptin and PPARγ genotypes were assessed in PET and control pregnancies. There was a significant association between the PPARγ Pro12Ala and the leptin 3'tet polymorphisms and risk of PET. Maternal PPARγ

Pro12Ala and leptin 3'tet polymorphisms appeared to be associated with some changes in inflammatory markers.

In conclusion, these data provide evidence that multiple metabolic alterations occur in PET and IUGR, and demonstrate that these changes are also evident in the fetus. These perturbations in fetal lipids and inflammatory markers may be relevant to fetal programming of adult vascular disease. Although PPAR expression is unaltered in PET, these receptors may still have an influence on the aetiology of the condition through genetic effects on metabolism, as the PPARγ P12A polymorphism appears to be related to increased PET risk. A potential research marker for insulin resistance in PET, elongase activity, has been described. Adipose tissue has been highlighted as a potential source of inflammatory mediators. It is suggested that multiple interacting metabolic processes are involved in the aetiology of PET, and that these processes are directly related to the development of CVD later in life. An understanding of the genetic and metabolic mechanisms involved in PET may inform strategies for identification and intervention in individuals at risk. The work produced by this thesis demonstrates the importance of viewing PET as a metabolic disorder rather than searching for a single candidate gene or molecule to account for its aetiology.

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V.A. Rodie, M Caslake, N Sattar, I.A. Greer, DJ Freeman. Smoking during pregnancy: effects on maternal and fetal plasma lipids (2003) *Atherosclerosis:* (169); 361-362.

Presentations of work undertaken in this thesis to learned societies

Erythrocyte membrane composition in healthy and complicated pregnancy. Invited speaker –03/04, McDonald Meeting (BFMS meeting), Glasgow.

The relationship between maternal insulin resistance/dyslipidaemia and maternal and fetal membrane fatty acid composition. Invited speaker – 03-04/07/03, HEART UK, 17th Annual Medical and Scientific Meeting, Manchester.

Smoking during pregnancy: effects on maternal and fetal plasma lipids. Oral presentation – 03-04/07/03. HEART UK, 17th Annual Medical and Scientific Meeting, Manchester.

Localisation and expression of PPARγ in placentae from healthy pregnancies and pregnancies complicated by pre-eclampsia. Poster Presentation – 04.04.01, First International Symposium on PPARs: from basic science to clinical applications, Florence, Italy.

Localisation and Expression of PPARs throughout placental development in Uncomplicated Pregnancies. Oral Presentation - 16/17.06.01, Annual Trainee's Meeting, Pitlochry

Placentae from healthy pregnancies and from pregnancies complicated by pre-eclampsia express PPARγ. Oral presentation – 05.07.01, British Hyperlipidaemia Association Meeting, Edinburgh

Localisation and Expression of PPARs pregnancies complicated by PET or IUGR. Poster Presentation - 14/15.12.01, Blair-Bell Research Competition Meeting, RCOG, London

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A Comparison of PPAR and NFκB Localisation and Expression between Placentae from Healthy Pregnancies and those Complicated by Pre-Eclampsia or IUGR, Poster presentation - 02-05/06/02, 13th World Congress, International Society for the Study of Hypertension in Pregnancy (ISSHP), Toronto, Canada.

Localisation and Expression of PPARs throughout placental development in Uncomplicated Pregnancies , Poster Presentation - 14.05.02, Combined meeting of the Cardiovascular School and Early Origins of Disease Groups, Western Infirmary, Glasgow

Peroxisome Proliferator Activated Receptor Gamma (PPARγ) Polymorphisms and Inflammatory Markers in Pre-eclampsia, Poster Presentation – 05/10.11.02, Clinical Complications of Pregnancy Meeting (IFPA 8), Melbourne, Australia.

Localisation and Expression of PPARα, δ, γ and RXRα in early and late gestation placentae, Poster presentation – 18.03.03, Second International Symposium on PPARs, Florence, Italy.

Fetal cord plasma lipoprotein levels are altered in human pregnancies complicated by intrauterine growth restriction and pre-eclampsia, Poster presentation – 7-10/06/03, Second World Congress on Fetal Origins of Adult Disease, Brighton.

Maternal and fetal erythrocyte membrane fatty acid composition is altered during normal pregnancy and in pregnancies complicated by pre-eclampsia and intrauterine growth restriction, Poster presentation – 7-10/06/03, Second World Congress on Fetal Origins of Adult Disease, Brighton.

Paraoxonase-1 levels in early and late pregnancy and in fetal cord blood at birth, Poster presentation – 10-13/07/03, International Symposium on Triglycerides, Metabolic Disorders and Cardiovascular Disease, New York, USA.

Delta-5 desaturase activity index and insulin resistance in pre-eclampsia, Poster presentation – 11/04, 14th World Congress, International Society for the Study of Hypertension in Pregnancy (ISSHP), Vienna, Austria.

Erythrocyte membrane fatty acid composition in healthy pregnancies and pregnancies complicated by PET or IUGR – 11/04, 14th World Congress, International Society for the Study of Hypertension in Pregnancy (ISSHP), Vienna, Austria.

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Vanessa Rodie

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List of Abbreviations

AA	arachidonic acid
ADMA	asymmetric dimethylarginine
APC	activated protein C
ARDS	adult respiratory distress syndrome
ARMS	amplification refractory mutations system
BMI	body mass index
BSA	bovine serum albumin
CHD	coronary heart disease
CL	chain length
CRP	C-reactive protein
CV	coefficient of variation
CVA	cerebrovascular accident
CVD	cardiovascular disease
CY-7	cytokeratin-7
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
EDHF	endothelial derived hyperpolarizing factor
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMFA	erythrocyte membrane fatty acids
EPA	eicosapentaenoic acid
ET	endothelin
FA	fatty acids
FFA	free fatty acids
GAPDH	glyceraldehyde phosphate dehydrogenase
GC	gas chromatography
GDM	gestational diabetes mellitus
HDL	high density lipoprotein
HOMA	homeostasis assessment model
HPL	human placental lactogen
ICAM	intracellular adhesion molecule
ICC	immunocytochemistry
IHD	ischaemic heart disease
IL-6, -10	interleukin –6, -10
IUGR	intra-uterine growth restriction
IVGTT	intravenous glucose tolerance test
KDa	kilo Daltons
LCPUFA	long chain polyunsaturated fatty acid
LDL	low density lipoprotein
LUSCS	lower uterine Caesarean section
MI	myocardial infarction
NIDDM	non-insulin dependent diabetes mellitus
NO	nitric oxide
OD	optical density
PAF	platelet activating factor
PAI	plasminogen activator inhibitors
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PECAM	platelet endothelial cell adhesion molecule
PET	pre-eclampsia

PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PIH	pregnancy induced hypertension
PON	paraoxonase
PPAR	peroxisome proliferator activated receptor
PUFA	polyunsaturated fatty acid
RBC	red blood cell
RNA	ribonucleic acid
RR	relative risk
RXR	retinoid X receptor
SC	subcutaneous
SD	standard deviation
SGA	small for gestational age
SHBG	sex hormone binding globulin
TC	total cholesterol
TG	triglyceride
TNFα	tumour necrosis factor-α
UI	unsaturation index
VCAM	vascular cell adhesion molecule
VEGF	vascular cell endothelial growth factor
VLDL	very low density lipoprotein
VTE	venous thromboembolism
vWF	von Willebrand syndrome

Author's declaration

I declare that this thesis has been composed by myself and that, other than the TaqMan studies and plasma lipid/PON analyses, it is a record of work performed by myself. It has not been submitted previously for a higher degree.

The work described in this thesis was carried out under the supervision of Dr Dilys Freeman, Division of Developmental Medicine, University of Glasgow, and Professor Greer, Division of Developmental Medicine, University of Glasgow.

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1 Introduction

Complications of pregnancy, particularly pre-eclampsia (PET) and intrauterine growth restriction (IUGR) have been associated with future maternal cardiovascular disease (CVD). Pre-eclampsia, characterised by insulin resistance, widespread endothelial damage and dysfunction, coagulation defects and increased systemic inflammatory response, shares many risk factors with CVD. In this introduction, I will consider the evidence that the metabolic changes seen in normal pregnancy and the exaggerated atherogenic-like response seen in PET persist post partum and are associated with CVD. In addition, I will review the possible underlying mechanisms common to CVD and PET and the potential for interventions based on early assessment of cardiovascular risk. Finally, I will discuss the potential role in pregnancy of a family of nuclear receptors, the peroxisome proliferator activated receptors (PPARs), which are reported to be involved in the metabolic changes associated with atherosclerosis.

1.1 Evidence for an association between the metabolic complications of pregnancy and cardiovascular disease (CVD) risk

1.1.1 Pregnancy and CVD risk

It is long established that female gender-specific risk factors for CVD include menopause, hysterectomy and use of exogenous hormones (Hannaford *et al* 1997). However, evidence is now accumulating to show that pregnancy is associated with an increase in future cardiovascular risk in women. Women, parous at index pregnancy, have a twofold higher risk of dying from ischaemic heart disease relative to primigravid women (relative risk [RR] 2.05; 95%CI 1.19-3.55; p=0.01) (Jonsdottir *et al* 1995). Women who experience an uncomplicated pregnancy have a lower risk of CVD in later life, compared with those who had complicated pregnancies (Fisher *et al* 1981).

1.1.2 Pre-eclampsia and CVD risk

Women with hypertensive problems in pregnancy have an increased risk of CVD later in life (Croft and Hannaford 1989, Mann *et al* 1976, Thorogood *et al* 1992) (Table 1-1). Specifically, an history of PET has been shown to increase a woman's risk of experiencing

a venous thromboembolic event (van Walraven *et al* 2003) or haemorrhagic stroke (WHO Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception 1996). A prospective cohort study of parous women with an history of PET (Hannaford *et al* 1997), reported a significantly increased risk of hypertensive disease, acute MI, chronic ischaemic heart disease, angina pectoris, all ischaemic heart disease and venous thromboembolism, compared to parous women with no history of PET (table 1-1). A more recent retrospective cohort study of women who had PET during their first singleton pregnancy (Wilson *et al* 2003) demonstrated significant positive associations between PET/eclampsia and later hypertension (table 1-1).

Author	Disease	RR (95% CI)
Hannaford et al 1997	Hypertensive disease	2.35 (2.08 – 2.65)
	Acute MI	2.24 (1.42 – 3.53)
	Chronic IHD	1.74 (1.06 – 2.86)
	Angina pectoris	1.53 (1.09 – 2.15)
	All IHD	1.65 (1.26 – 2.16)
	VTE	1.62 (1.09 – 2.41)
		Proportional hazard ratio (95% CI)
Van Walraven et al 2003	Thromboembolism	2.2 (1.3 – 3.7)
		Adj. Odds ratio (95% CI)
Wilson et al 2003	Hypertension	3.98 (2.82 – 5.61)

Table 1-1. Maternal history of PET/eclampsia and risk of cardiovascular disease.

Jonsdottir and colleagues (Jonsdottir *et al* 1995) investigated the potential association between hypertension in pregnancy, PET and eclampsia, with increased death rates from ischaemic heart disease among eclamptic women and those with PET versus those with hypertension alone, in a population-based study with a 16-year follow-up (table 1-2). This study suggests that it is the metabolic disturbances associated with PET and not hypertension *per se* that is associated with cardiovascular risk.

In a retrospective discharge data analysis of all singleton first births in Scotland between 1981 and 1985, with 15 – 19 years follow up, maternal risk of ischaemic heart disease admission or death was associated with PET (adjusted hazard ratio 2.0; CI 1.5 – 2.5) (Smith *et al* 2001). Irgens and colleagues (Irgens *et al* 2001) performed a population based cohort study to assess whether mothers and fathers have a higher long-term risk of death, particularly from CVD, after the mother has had PET (table 1-2). They observed a 1.65-fold higher long-term risk of death from CVD in women with PET in their first pregnancy, who delivered at term. However, mothers with PET who were delivered pre-term demonstrated an 8-fold increase in risk of death from the same cause later in life. There

was no long-term increased risk of death in the fathers from the PET pregnancies compared with the fathers from uncomplicated pregnancies. The authors concluded that there *is* a relationship between PET and maternal CVD later in life, especially relevant to the subset of women who were delivered pre-term. These latter two studies highlight the difficulty in interpreting population studies in the absence of detailed information on the time of presentation and severity of PET. There is also an association with preterm delivery and vascular disease. Davey Smith and colleagues (Davey Smith *et al* 2000b) reported a strong association between preterm delivery and CVD mortality (Table 1-2). However, this association did not consider confounding caused by reason for the preterm delivery, for example, PET, which may have resulted in an even stronger association with CVD.

Author	Category	Maternal mortality due to IHD
Jonsdottir et al 1995	Eclampsia	2.61 (1.11 – 6.12)[a]
	Pre-eclampsia	1.90(1.02 – 3.52)[a]
		Maternal admission or mortality due to IHD (adjusted hazard ratio)
Smith et al 2001	Lowest birth quintile	1.9 (1.5 – 2.4)
	Preterm delivery	1.8 (1.3 – 2.5)
	Lowest birth quintile, preterm delivery & PET	7.0 (3.3 – 14.5)
		Maternal mortality due to IHD
Irgens et al 2001	Pre-eclampsia	1.65 (1.01 – 2.70)[b]
	Pre-eclampsia & preterm delivery	8.12 (4.31 – 15.33)[b]
Davey Smith et al 2000b	Birthweight (offspring)	0.77 (0.65 – 0.50)[b]
	Birth length	0.85 (0.73 – 0.99)[b]
	Ponderal index	0.77 (0.65 – 0.92)[b]
	Preterm delivery	2.06 (1.22 – 3.47)[b]
Davey Smith et al 1997	Birthweight (offspring)	2.00 (1.18 – 3.33)[c]
Davey Smith et al 2000a	Birthweight < 2500g (offspring)	7.05 (2.64 – 18.77)[b]

Table 1-2. Maternal admission or mortality from IHD.
a=relative risk, b=hazard ratio, c=adjusted relative rate.

The classification of hypertension in pregnancy is confusing. A variety of conditions make up the hypertensive disorders of pregnancy and there is not only a lack of knowledge of their aetiology, but also a lack of agreement on their nomenclature and classification. To compound this confusion, the true diagnosis and therefore the classification, may only be evident retrospectively several months after the pregnancy is completed. The clinician, faced with the problem during pregnancy must therefore make a provisional diagnosis.

Here, some form of classification is essential to make diagnoses consistent, to help quantify the risk to mother and fetus, and to guide decisions on the patient's management. The lack of an agreed classification and nomenclature has also hampered research in this area by preventing comparisons between centres and between countries.

1.1.3 Low birthweight offspring and CVD risk

Previous studies have reported increased rates, ranging from 14 – 28.2%, of small for gestational age infants from PET pregnancies, in women who were previously normotensive (Lydakis *et al* 2001, Pietrantoni and O'Brien 1994, Sibai *et al* 1984). In the study by Smith *et al* (Smith *et al* 2001), maternal risk of ischaemic heart disease or death was associated with delivering a baby in the lowest birth weight quintile for gestational age and preterm delivery (table 1-2).

The risks were additive; women with both these characteristics and PET, had a risk of hospital admission for ischaemic heart disease or death that was 7 times greater than the reference category. Davey Smith and colleagues also demonstrated (Davey Smith *et al* 2000b), in a cohort study of 3706 women who gave birth to liveborn singletons, that maternal CVD mortality was inversely related to birthweight, birth length and ponderal index of the offspring, when adjusted for blood pressure in pregnancy (table 1-2). In another study from the same group (Davey Smith *et al* 1997), investigating the association between birth weight of offspring and mortality among mothers and fathers in the West of Scotland, mortality from CVD was inversely related to offspring birthweight for mothers (table 1-2) and fathers (RR 1.52; 95% CI 1.03 – 2.17). These findings were unchanged by correction for social, environmental, behavioural and physiological risk factors. In a prospective observational study, Davey Smith *et al* (Davey Smith *et al* 2000a) demonstrated a hazard ratio of 7.05 (2.64 – 18.77) for death from CVD among women who delivered a baby less than 2500g compared to those delivering babies weighing greater than or equal to 3500g, thus confirming the strong relationship between infants' birthweight and mothers' mortality from CVD (table 1-2). They also described the independent risk from preterm delivery as mentioned previously. To what extent these latter studies might be confounded by PET in the population is unknown.

1.2 Pathology of pre-eclampsia

1.2.1 General overview

In order to understand how PET might be linked to future cardiovascular risk it is important to consider its pathology. Pre-eclampsia, occurring in 2 - 4 % of pregnancies remains one of the leading causes of maternal and neonatal morbidity and mortality in the developed world. The only definitive treatment is to deliver the baby and placenta, often prematurely in the interests of the baby or the mother. Pre-eclampsia is a multi-system disorder. The classical diagnostic criteria of hypertension secondary to vasoconstriction, proteinuria due to glomerular endotheliosis and oedema secondary to increased vascular permeability represent only the 'tip of the iceberg' of widespread pathology arising from endothelial damage and dysfunction. Eclampsia occurs in about 0.1% of pregnancies, and cerebrovascular accident (CVA) is the most common cause of death. Pathological findings in the brain include cerebral oedema, petechial or gross haemorrhage, small hypoxic, ischaemic and perivascular infarcts and arteriolar damage, identified by thrombosis and fibrinoid necrosis. The other complications that can arise such as hepatic dysfunction, necrosis and haemorrhage; pulmonary oedema and adult respiratory distress syndrome (ARDS); and renal failure demonstrate the extent of the pathology. The fetal effects of PET, intrauterine growth restriction (IUGR) and iatrogenic prematurity, arise due to placental infarction and insufficiency.

Pre-eclampsia is often considered to be a disorder with 2 components. The first component is an as yet unidentified signal that arises from the placenta and is associated with either defective implantation or large placental mass such as in twin pregnancy or hydatidiform mole. The second component is the maternal response to this placental signal (Ness and Roberts 1996). The manifestation of the maternal syndrome will depend on maternal genotype and phenotype, which will influence the maternal response to the placental signal. This response involving inflammation, coagulation, and metabolic systems results in endothelial cell activation and dysfunction, which characterises PET.

1.2.2 The placental pathology

In normal pregnancy, the spiral arteries of the placental bed undergo a series of physiological changes (figure 1-1). They are invaded by the cytotrophoblast (Khong and Robertson 1987), which breaks down the endothelium, internal elastic lamina, and

muscular coat of the vessel, which are largely replaced by fibrinoid material. Virtually every spiral artery in the decidua basalis will have undergone these physiological changes by the end of the first trimester (Brosens and Dixon 1966). Early in the second trimester, a second wave of cytotrophoblast invasion occurs and transforms the myometrial segments of the spiral arteries. These physiological changes convert the vessels supplying the placenta from muscular end arteries to wide-mouthed sinusoids. The vascular supply is thus transformed from a high pressure-low flow system to a low pressure-high flow system to meet the needs of the fetus and placenta. Loss of the endothelial and muscular layers render these vessels unable to respond to vasomotor stimuli.

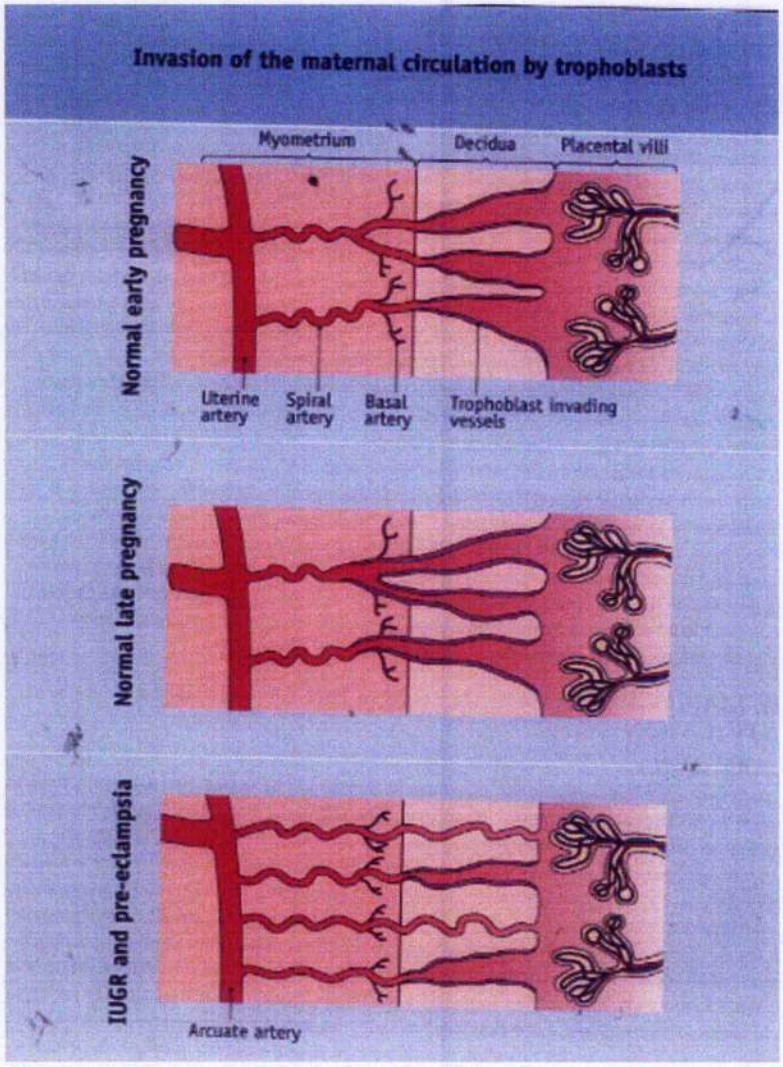


Figure 1-1. Invasion by trophoblasts.

Upper panel shows normal early pregnancy with invasion to level of the decidua. Middle panel shows invasion to level of myometrium following second wave of trophoblast invasion. Lower panel shows inadequate implantation of IUGR and pre-eclampsia. Broken lines indicate trophoblast invading blood vessels.

In pre-eclampsia, only about one-half to two-thirds of the decidual spiral arteries undergo these physiological changes (Khong *et al* 1986), and the conversion of myometrial components of the spiral arteries fails to occur (Sheppard and Bonnar 1981). Thus, the primary invasion of trophoblast is partially impaired and the second wave fails to occur or is limited. This results in restricted placental blood flow, which becomes more critical with advancing gestation as the demands of the conceptus increases. In addition, the vessels maintain their muscular coats and so remain sensitive to vasomotor stimuli (figure 1-1). These changes are not specific to PET and also occur in IUGR without PET.

The typical, though non-specific, vascular lesion found in the placental bed in PET has been termed `acute atherosis' because of the presence of foam cells in the damaged vessel wall (Labarrere 1988). Acute atherosis is a necrotising arteriopathy characterized by fibrinoid necrosis, accumulation of lipid-laden macrophages and damaged cells, fibroblast proliferation and a mononuclear cell perivascular infiltrate. Again, it is not specific to PET being present in IUGR also. These features are similar to those seen in the atherosclerotic plaque (Sattar and Greer 2002). The acute atherosis is associated with endothelial damage which can be seen ultrastructurally in the decidua at sites outside the placental bed throughout the maternal fetal boundary (Shanklin and Sibai 1989) and this correlates with the degree of maternal hypertension. Plasma urate, a marker of disease severity correlates with these vascular changes in the placental bed (McFadyen *et al* 1986). Ischaemia of the fetal placenta will occur because of the restricted blood flow and lead to infarcts, patchy necrosis and an obliterative endarteritis of the fetal stem arteries (Fox 1988, p. 16-37). More recently, it has been suggested that there is incomplete development of the fetal placental microvasculature in PET associated with IUGR, which could account for reduced perfusion of the fetal placenta seen in this condition (Macara *et al* 1995).

1.2.3 The maternal metabolic response to pregnancy and pre-eclampsia

In normal pregnancy, a degree of insulin resistance, hyperlipidaemia and an increase in coagulation factors develops, along with up-regulation of inflammatory markers (Greer 1999, Martin *et al* 1999, Sacks *et al* 1998). This serves to meet the metabolic demands of the growing fetus. However, in PET this normal adaptive metabolic response is greatly exaggerated and can lead to gross metabolic disturbance. I will review these vascular and metabolic changes seen in normal pregnancy and in PET to highlight the similarities to metabolic syndrome.

1.2.3.1 Vasomotor function

In healthy pregnancy, blood pressure falls reaching a nadir at 20 weeks' gestation then rises again reaching levels consistent with non-pregnant values by term. This reflects the marked reduction in total peripheral vascular resistance, which decreases by 25% before increasing again in line with the blood pressure (Clark *et al* 1989, Kirshon and Cotton 1987) more than counteracting the increase in cardiac output. Plasma volume also expands in normal pregnancy by approximately 40% peaking at 24 weeks.

In contrast to the normal pregnant situation, a contracted plasma volume occurs in PET. This is associated with an increase in systemic vascular resistance, a normal or reduced cardiac output and reduced cardiac preload (Clark and Cotton 1988, Groenendijk *et al* 1984). This reflects increased peripheral resistance due to vasoconstriction. Despite increases in plasma renin concentration, renin substrate and angiotensin II (AII) in normal pregnancy, blood pressure falls. This is due to acquired vascular insensitivity to AII, which is maximal in the second trimester, after which it slowly returns towards the non-pregnant situation (Gant *et al* 1974) and is associated with down-regulation of AII receptors (Baker *et al* 1991).

In PET, there is a loss of the acquired insensitivity to AII, which antedates clinical disease, and an increase in AII receptors (Baker *et al* 1991, Gant *et al* 1974). *Ex-vivo* studies suggest that endothelium dependent vascular relaxation is reduced, so implicating the endothelium in the increased vasomotor activity of PET (Ashworth *et al* 1997). The mechanism may be due to disturbance of or damage to key processes relating to vasomotor control. The precise changes that occur in the renin–angiotensin system in pregnancy-induced hypertension are difficult to determine, owing to methodological problems and the study of groups varying in disease severity. However, the changes appear to fall into two distinct patterns. In late-onset, non-proteinuric disease there is an increase in plasma renin activity (Fievet *et al* 1985, Gallery *et al* 1980), while AII may be unchanged or increased (Symonds and Pipkin 1978, Symonds *et al* 1975). In early-onset, proteinuric pregnancy-induced hypertension, plasma renin activity, AII and aldosterone are reduced (Fievet *et al* 1985, Karlberg *et al* 1984, Weir *et al* 1973). In contrast to the suppression of the renin–angiotensin system seen in severe pregnancy-induced hypertension, atrial natiuretic peptide is increased (Fievet *et al* 1988). This may be a compensatory mechanism for hypertension, a supposition supported by the direct correlation that exists between blood pressure and plasma atrial natriuretic peptide in normal and hypertensive pregnancies (Fievet *et al* 1988). Recent data reveal a potential mechanism for the loss of the aquired insensitivity to the pressor agent

AII via increased levels of heterodimers between the vasopressor receptor angiotensin II type 1 receptor (AT1) and the vasodepressor bradykinin receptor (B2) (AbdAlla *et al*, 2001). The receptor heterodimers display increased sensitivity toward AII (AbdAlla *et al*, 2000) and are found in platelets and in omental vessels of pre-eclamptic women. Furthermore, the AT1/B2 receptor heterodimers are resistant to inactivation by reactive oxygen species, which are elevated in PET (AbdAlla *et al*, 2004). This mechanism may provide a plausible explanation for hypertension in pre-eclampsia.

Reduced production of endothelial derived vasodilator prostaglandins, reduced nitric oxide production and increased endothelin in PET have been proposed, although the evidence is conflicting (Choi *et al* 2002, Granger *et al* 2002, Khedun *et al* 2002, Rowe *et al* 2003, Vural 2002). Accurate measurement of these substances or their metabolites is difficult and the conflicting evidence may represent methodological difficulties. However, these results, which assess a variety of vasomotor agonists, are consistent with endothelial damage and/or dysfunction in PET, which is responsible for vasoconstriction and the increased peripheral vascular resistance.

1.2.3.2 Coagulation

In normal pregnancy there are increases in the levels of the coagulation factors V, VII, VIII, von Willebrand Factor (vWF), X and XII (Clark *et al* 1998b, Stirling *et al* 1984), an increase in plasma fibrinogen and suppression of fibrinolysis (Wiman *et al* 1984). There is a decrease in protein S and activated protein C (APC) resistance may occur in the absence of factor V Leiden mutation (Clark *et al* 1998b, Mathonnet *et al* 1996). It is likely that this state of hypercoaguability may serve to limit life-threatening bleeding at delivery but it does mean there is an increased risk of thromboembolism associated with pregnancy.

In PET microvascular thrombi are found in numerous organs including the kidney, liver and brain (Arias and Mancilla-Jimenez 1976, McKay 1972). This increased fibrin deposition in the maternal vasculature is consistent with excessive activation of the coagulation system (Kobayashi *et al* 1999). Activation of the coagulation cascade occurs early in PET and often antedates clinical symptoms. In PET there is increased factor VIII activity (Howie *et al* 1976, Howie *et al* 1971) and increased vWF levels (Redman *et al* 1977). Antithrombin, an endogenous inhibitor of coagulation, is reduced in PET and this correlates with disease severity (Howie *et al* 1971, Weiner and Brandt 1982). There is also increased resistance to the anticoagulant property of activated protein C (APC) and increased levels of prothrombin fragment 1 and 2 and thrombin-antithrombin complex

(TAT) indicating activation of the coagulation cascade (Aznar *et al* 1986, VanWijk *et al* 2002). Tissue plasminogen activator (tPA) is increased in plasma possibly due to stimulation of, or damage to, the endothelium (Estelles *et al* 1998) and there is a simultaneous increase in plasminogen activator inhibitors (PAI) 1 and 2 (Estelles *et al* 1998). Platelet count is reduced in PET, secondary to a reduced lifespan (Redman *et al* 1978) and the reduction in platelet count correlates with disease severity (Rakoczi *et al* 1979). There is also evidence of enhanced platelet activation and increased levels of platelet endothelial cell adhesion molecule-1 (PECAM-1) (Roberts *et al* 1991, Roberts *et al* 1989). In an unselected prospective longitudinal study of pregnant subjects, Clark and colleagues demonstrated that women who subsequently developed PET had lower APC sensitivity ratios, in the absence of Factor V Leiden, at 7-16 weeks gestation, and that this was associated with a 2.95-fold increased risk of PET (Clark *et al* 2001).

1.2.3.3 Lipids

In healthy pregnancy, there is an alteration in lipid profile; a gestational increase of around 300% in triglyceride (TG) levels, a 25-50% increase in total cholesterol (TC), and increases in very low density lipoprotein 1 (VLDL1), VLDL2, high density lipoprotein (HDL) and small dense low density lipoprotein (LDL) (Montelongo *et al* 1992, Sattar *et al* 1997b). These alterations in lipid profile are considered to be under hormonal control (Julius *et al* 1994, Walsh *et al* 1991). Gestational hyperlipidaemia fulfils the physiological role of supplying both cholesterol and triglyceride to the rapidly developing fetus (Dugdale 1986).

In PET, TG levels are further raised especially in the third trimester where median TG concentrations are near double those seen in normal pregnancy (Hubel *et al* 1996, Kaaja *et al* 1995, Sattar *et al* 1997a). This is reflected in a three-fold higher VLDL-1 and a two fold higher VLDL-2 concentration relative to normal pregnancy. A raised low density lipoprotein III (LDL-III) concentration (atherogenic, small dense LDL) and lower LDL peak particle diameter result from the exaggerated TG rise (Belo *et al* 2002a, Lorentzen and Henriksen 1998, Ogura *et al* 2002, Sattar *et al* 1997a). HDL cholesterol levels are reduced, probably as a consequence of the increased TG levels. There have been reports of an elevated TC level and increased levels of lipoprotein (a) (Wang *et al* 1998), which correlates to disease severity, but this is not observed in all cases (Sattar *et al* 2000). Hepatic lipase activity has been shown to be elevated in PET and could contribute to increased LDLIII concentration (Sattar *et al* 1997a), via increased TG exchange into LDL, followed by hepatic lipase induced lipolysis of the particle (Tan *et al* 1995).

The abnormal lipoprotein metabolism in PET may be a compensatory response to placental insufficiency and an increased requirement to deliver fuel to the placenta (Sattar *et al* 1999a). However the marked dyslipidaemia may contribute to endothelial activation and dysfunction and to promotion of oxidative stress (Flavahan 1992, Goode *et al* 1995, Myatt and Miodovnik 1999). Raised TG levels may promote endothelial dysfunction directly or via an increased proportion of small, dense LDL that is easily oxidised. Alternatively the increased Factor VIIa and PAI-1 associated with hypertriglyceridaemia (Sattar *et al* 1999b) may influence endothelial function. Oxidised LDL and VLDL-1 promote leukocyte adhesion by stimulating endothelial expression of adhesion molecules (e.g. VCAM-1) and PAI-1. The marked dyslipidaemia could also contribute to accumulation of lipids within the kidney and spiral arteries of the placenta.

PET is also associated with significantly increased free fatty acid (FFA) levels (Sattar *et al* 1996), even prior to the onset of clinical manifestations of the disease. FFAs are known to be implicated in the development of insulin resistance in muscle and liver, the major regulators of systemic insulin sensitivity. Elevated IL-1 and tissue necrosis factor alpha (TNFα) in PET can induce adipocyte lipolysis (Chajek-Shaul *et al* 1989, Feingold *et al* 1991), and promote *de novo* hepatic fatty acid synthesis. These cytokines can further impair mitochondrial β-oxidation and ketogenesis (Memon *et al* 1992) in subjects with PET relative to normal pregnancy. Thus cytokines may be responsible for the lipid and lipoprotein disturbance in PET.

1.2.3.4 Inflammation

Normal pregnancy is associated with a generalised maternal inflammatory response (Sacks *et al* 1998). Maternal total white cell blood count increases with gestation, largely attributed to neutrophilia (Naccasha *et al* 2001, von Dadelszen *et al* 1999) and there is activation of neutrophils (von Dadelszen *et al* 1999) and circulating leukocytes (Sacks *et al* 1998). Cytokine levels, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are also elevated in healthy pregnancy.

This inflammatory response of pregnancy is exaggerated in PET. Neutrophil activation has been implicated in the pathophysiology of PET, for which several potential mechanisms have been identified. These include up-regulation of cellular adhesion molecules on the endothelial surface, increased generation of TNF-α and endothelial activation from hyperlipidaemia (Clark *et al* 1998a). Greer *et al* demonstrated that concentrations of neutrophil elastase, a specific marker for neutrophil activation *in vivo*, are elevated in the

peripheral circulation of women with PET (Greer *et al* 1989) and is confined to the maternal circulation (Greer *et al* 1991a). This activation has also been demonstrated in IUGR (Johnston *et al* 1991, Sabatier *et al* 2000). Furthermore, there are increased numbers of elastase-positive neutrophils in the placental bed in PET (Butterworth *et al* 1991), the site where acute atherosis is seen. Recently neutrophil and monocyte activation during uteroplacental passage was demonstrated in uterine venous blood from women with PET (Mellembakken *et al* 2002).

Levels of TNFα, IL-6, VCAM-1, ICAM-1, E-selectin, PECAM-1, selectins P and L (Acar *et al* 2001, Bretelle *et al* 2001, Carr *et al* 2001, Greer *et al* 1994, Lyall *et al* 1994, Vince *et al* 1995, Zeisler *et al* 2001) are elevated in peripheral blood in PET. Elevated markers of inflammation such as TNFα, IL-2, and CRP (though not independently of BMI) have been demonstrated in the 1st and early 2nd trimesters of pregnant women who later develop PET (Eneroth *et al* 1998, Hamai *et al* 1997, Williams *et al* 1999, Wolf *et al* 2001). The up-regulation of cytokine expression may contribute to the endothelial damage that occurs in PET and may explain the mechanism underlying leukocyte activation and endothelial adhesion in this disorder (Greer *et al* 1994).

1.3 Common hypotheses underlying metabolic syndrome and PET

There are generally considered to be three main hypotheses regarding the metabolic alterations involved in the aetiology of PET, namely endothelial dysfunction and activation, insulin resistance and oxidative stress. It is unlikely that these mechanisms are independent factors in the aetiology of the disease; rather that they are all related to a certain degree, with the degree of each parameter demonstrating inter-patient variation. Each hypothesis will be considered separately to demonstrate the metabolic changes in PET and to underline their similarities to those changes seen in the metabolic syndrome.

1.3.1 Endothelial dysfunction and activation

In the non-pregnant state endothelial cells release vasodilators, such as nitric oxide (NO) and prostacyclin (PGI_2), and vasoconstrictors, such as endothelin (ET) and platelet activating factor (PAF), for the purpose of blood flow and pressure regulation (Cines *et al* 1998). NO promotes vasodilatation and inhibits inflammation, thrombosis and vascular

smooth muscle cell proliferation (Behrendt and Ganz 2002). Normal pregnancy is associated with reduced vascular reactivity and tone (Granger 2002).

Ramsay and colleagues have described an *in vivo* assessment of endothelial-dependent and independent microvascular function using laser Doppler imaging (Ramsay *et al* 2002). They reported that obesity in pregnancy, a risk factor for PET, is associated with impaired endothelial function, higher blood pressure and inflammatory up-regulation. Similar effects in pre-eclamptic women have been demonstrated using pulsed Doppler ultrasonography of uterine and brachial arteries of pre-eclamptic women (Takata *et al* 2002). Knock and colleagues (Cockell and Poston 1997, Knock and Poston 1996) described enhanced bradykinin-mediated relaxation in *ex-vivo* subcutaneous resistance arteries in women with healthy pregnancy compared with non-pregnant subjects. This enhanced relaxation was not present in women with PET. Kenny *et al* (Kenny *et al* 2002) demonstrated that small myometrial arteries from healthy pregnant women were more responsive to endothelium-derived hyperpolarizing factor (EDHF) than similar arteries derived from pre-eclamptic pregnancies. Recently, Savvidou and colleagues (Savvidou *et al* 2003) demonstrated that the endothelial dysfunction of PET develops before the clinical manifestation of the disease. They reported that women with high resistance placental circulation at risk of PET, IUGR or both, have raised concentrations of asymmetric dimethylarginine (ADMA), the endogenous inhibitor of endothelial nitric oxide synthase. They concluded that this raised ADMA concentration is a potential contributory factor for PET.

Many markers of endothelial dysfunction have been observed in PET. Coagulation activation is often manifest weeks to months before onset of the clinical condition (Leiberman *et al* 1988, Weiner 1991, Greer 1999, p. 163-81). The damaged endothelium of PET is reflected by elevated levels of PAI-1 (Gilabert *et al* 1995, Halligan *et al* 1994) and von Willebrand factor (Greer *et al* 1991b, Redman *et al* 1977). Endothelial dysfunction in PET is indicated by elevated plasma levels of soluble adhesion molecules (Chaiworapongsa *et al* 2002, Lyall *et al* 1994), which may be elevated before PET appears clinically (Krauss *et al* 1997). However, Johnson and colleagues (Johnson *et al* 2002) recently demonstrated that markers of endothelial cell activation (VCAM, ICAM and E-selectin) were elevated in maternal plasma from both pregnancies complicated by PET *and* pregnancies complicated by IUGR, compared to healthy pregnancies. They noted that levels of cytokines (TNFα, IL-6 and IL-8) were only elevated in the PET group, concluding that endothelial activation is a consequence of abnormal trophoblast invasion, but that there are other factors involved in the manifestation of PET, possibly related to cytokine release. The diversity of factors contributing to the endothelial dysfunction of

PET demonstrates the enigmatic nature of PET pathophysiology and emphasizes the difficulty in identifying potential mechanistic links.

Endothelial activation in PET results in an exaggerated release of endothelin, thromboxane and superoxide, increased vascular sensitivity to the pressor effects of angiotensin II, and decreased formation of vasodilators such as nitric oxide and prostacyclin (Granger *et al* 2002) by the damaged endothelium. This may lead to an increase in total peripheral resistance, despite the increasing plasma volume of pregnancy, and thus, vasospasm and hypertension. However recent evidence from a case control study of non-pregnant, normotensive pregnant and PET pregnant women, showed that NO production was actually increased in the PET group compared to the other groups (Vural 2002). The increased formation of the vasodilator NO was considered to be a compensatory response to the vasoconstriction and hypertension.

It has also been proposed that poor placental perfusion causing ischaemia, leads to a release of factors, such as pro-inflammatory cytokines, from the placenta that provoke endothelial activation and dysfunction (Roberts *et al* 1991, Roberts *et al* 1989). Syncytiotrophoblast microvillous membranes (STBM) (Knight *et al* 1998, Smarason *et al* 1993), or microparticles (VanWijk *et al* 2002), released into the maternal circulation in increased amounts in PET have been implicated in the aetiology of PET but it is difficult to differentiate whether these cell fragments may be a cause or an effect of endothelial dysfunction. *In vitro* studies have shown that perfusion of small arteries from pregnant women with STBMs impairs maternal endothelial function (Cockell *et al* 1997).

1.3.2 Insulin resistance

Normal pregnancy is a state of insulin resistance, with a doubling in fasting insulin concentrations. This is likely due to increased production of placental hormones including human placental lactogen (HPL), and possibly progesterone and oestrogen (Kirwan *et al* 2002, Ryan and Enns 1988). The increased insulin resistance reaches a maximum in the 3rd trimester, and improves following delivery (Buchanan *et al* 1990, Catalano *et al* 1993, Cousins *et al* 1980, Kuhl 1991, Yen 1973).

The features of PET - hypertension, endothelial cell dysfunction and lipid alterations are all features of the insulin resistance syndrome (Reaven *et al* 1996), thus insulin resistance may play a pivotal role in the development of PET. It has been demonstrated that plasma glucose levels after a glucose load are elevated in pregnant women who subsequently

develop PET (Solomon *et al* 1994), and that fasting insulin levels are elevated after an oral glucose tolerance test in women with established PET (Kaaja *et al* 1995, Lorentzen *et al* 1998). Wolf and colleagues demonstrated lower first trimester sex hormone binding globulin (SHBG), a negative correlate of insulin resistance, in women who subsequently developed PET, compared with those who had an uncomplicated pregnancy ($P<0.01$) (Wolf *et al* 2002). It is notable that not all studies describe a positive relationship between insulin resistance and PET. Roberts and colleagues reported an increase in insulin sensitivity in women with PET compared to controls and they concluded that there was no association between PET and insulin resistance (Roberts *et al*, 1988). These discrepancies may reflect the application of techniques for assessing insulin resistance that may not have been validated in pregnancy.

Kirwan *et al* (Kirwan *et al* 2002) recently reported that TNFα is a significant predictor of insulin resistance during pregnancy, in a prospective study of women with normal glucose tolerance and with gestational diabetes mellitus, emphasising the close relationships between insulin resistance and inflammation. It has been shown that both PAI-1 (Meigs *et al* 2000) and leptin (Segal *et al* 1996) correlate with insulin resistance in pregnancy, further demonstrating a role for insulin resistance in PET. Laivuori reported that leptin levels were higher in PET women but that insulin sensitivity showed no direct relationship to leptin during the pregnancy. However, leptin and insulin sensitivity correlated directly in PET puerperal women compared to puerperal controls (Laivuori *et al* 2000).

1.3.3 Oxidative stress

Oxidative stress plays a role in the aetiology of atherosclerosis (Heinecke 1998). Similarly it is proposed that oxidative stress is a component of PET (Hubel 1999). The oxidative stress theory of PET involves the hypothesis that the abnormal placentation and dyslipidaemia results in a release of free radicals, particularly superoxide anions, and lipid hydroperoxides, which damage the vascular endothelium (Hubel 1999). Oxidative stress may link the decreased placental perfusion in PET to the maternal response (Ness and Roberts 1996, Roberts and Hubel 1999), via direct vascular damage and endothelial dysfunction. Sources of reactive oxygen species could be derived from the circulation or from the placenta itself. Oxidative stress in the systemic circulation may be explained by free radical generation by activated neutrophils or by formation of products of lipid peroxidation (e.g. malondialdehyde). Placental derived sources may be oxidised fragments of syncytiotrophoblast entering the systemic circulation due to increased placental

apoptosis (Redman and Sargent 2001) or the production of cytokines, for example TNFα, by an hypoxic placenta (Benyo *et al* 1997).

Markers of oxidative stress and placental function are altered in PET and women delivering small for gestational age (SGA) infants. Chappell and colleagues (Chappell *et al* 2002) have demonstrated that plasma ascorbic acid concentrations are decreased in both the SGA pregnancies and pre-eclamptic pregnancies compared to low risk women. Uric acid concentrations were increased only in the PET pregnancies. There was also a trend towards higher values of 8-epi-prostaglandin F 2α (a lipid peroxidation marker) in the pre-eclamptic group. Akyol and colleagues (Akyol *et al* 2000) demonstrated a reduction in serum vitamin E levels in pre-eclamptic women.

Of great interest is a pilot intervention study where pregnant women at high risk of PET (283 cases) were randomised to placebo or treatment with a combination of vitamin C (1000mg/day) and vitamin E (400IU/day) (Chappell *et al* 1999). Placental function was improved (Chappell *et al* 1999) as demonstrated by a decrease in the PAI-1/PAI-2 ratio (Cerneca *et al* 1997, Estelles *et al* 1998, Shaarawy and Didy 1996). An unexpectedly large decrease in the frequency of PET (17% in the placebo group vs 8% in the treatment group (adjusted odds ratio 0.39; 0.17 – 0.90, p=0.02) was reported. In a follow-up study (Chappell *et al* 2002), indices of placental dysfunction and oxidative stress were measured in the same cohort of patients at high risk of PET taking vitamin supplements. These women were compared to the cohort at high risk of PET and to the cohort of women at low risk for PET, both of whom were not taking supplementation. The placebo group demonstrated decreased levels of ascorbic acid, PAI-2 and placental growth factor, and increased levels of 8-epi-prostaglandin F (2alpha), leptin and PAI-1 to PAI-2 ratio compared to the low risk group. In the high-risk group receiving vitamin supplementation, the levels of the above markers were similar to the values seen in the low risk group. Thus, it was concluded that antioxidant supplementation was associated with improvement in biochemical indices of the disease. Antioxidants such as ascorbic acid have also been demonstrated to improve endothelial dysfunction in women with previous PET (Chambers *et al* 2001). Evidently, these findings need further investigation via large randomised controlled trials, but raise the exciting possibility that antioxidants, either dietary or pharmaceutical, may have a role in prevention of PET in high-risk patients. It has been hypothesised that regular exercise enhances antioxidant enzymes in pregnant women, reducing oxidative stress and the incidence of PET, and at the same time promoting a healthy lifestyle (Yeo and Davidge 2001).

1.4 The link between PET and CVD

1.4.1 Pre-pregnancy risk factors for PET and CVD

There is evidence to suggest that the *pre*-pregnancy state may be the contributing factor for the development of future CVD, rather than pregnancy or PET. Non-pregnant women may have characteristics of the metabolic syndrome such as hypertension, increased insulin resistance, obesity and lipid abnormalities which, combined with the metabolic stress of pregnancy, manifest as PET. It is possible that these pre-existing factors may present again later in life, this time as CVD, as it is well established that these abnormalities also predispose to atherosclerotic disease.

Five to ten percent of pregnancies are complicated by chronic hypertension (Vinatier *et al* 1993) and are at increased risk for the development of superimposed PET. Chronic hypertension is also a recognised risk factor for the development of CVD.

In the non-pregnant state, activated protein C (APC) resistance secondary to Factor V Leiden is associated with the development of PET in pregnancy. The degree of resistance to APC also relates to venous thrombosis risk, another risk factor for CVD (Clark *et al* 2001). In line with the suggested involvement of a hypercoaguable state in the actiology of PET, congenital thrombophilias (Factor V Leiden, prothrombin 20210A and antithrombin, protein C and protein S deficiencies, homozygous MTHFR C677T mutation) and acquired thrombophilias (anticardiolipin antibodies and lupus inhibitor) have been associated with PET (Alfirevic *et al* 2002, Arkel and Ku 2001). However an excellent and more recent large population-based study with appropriate inclusion criteria, published in conjunction with a meta analysis, did not find an association between PET and Factor V Leiden, prothrombin G20210A, MTHFR C677T, or platelet collagen receptor $\alpha 2\beta 1$C807T (Morrison *et al* 2002). However, when analysis was restricted to severe PET, there was a significant association with Factor V Leiden and with MTHFR C677T homozygotes (Morrison *et al* 2002).

Similarly, it has been suggested that higher serum cholesterol levels before pregnancy predict the development of PET (Thadhani *et al* 1999). Again, alterations in the lipid profile have been associated with CVD development. In a retrospective analysis, a relative elevation of blood pressure, BMI and lipids in the non-pregnant state were demonstrated as

features of the metabolic syndrome and were proposed to be important sensitising factors contributing to the pathogenesis of PET (Barden *et al* 1999).

Conditions associated with insulin resistance are also associated with an increased risk of PET, and these include polycystic ovarian syndrome (PCOS), increased weight gain and obesity (Solomon and Seely 2001). An increased body mass index (BMI) in early pregnancy or pre-pregnancy is a recognised risk factor for PET (Sattar *et al* 2001, Sibai *et al* 1995). Greater waist circumference and higher BMI was noted in subjects who developed PET (Sattar *et al* 2001). Waist circumference predicts CVD risk, and changes in waist circumference predict changes in this risk (Han *et al* 1997, Han *et al* 1995, Sattar *et al* 2001, Sattar *et al* 1998b). It is known that abdominal obesity is associated with insulin resistance, raised plasma triglycerides and increased sensitivity to lipolysis. This leads to an increased supply of free fatty acids to the liver and increased triglyceride and VLDL synthesis and could be related to the exaggerated dyslipidaemia of PET, as well as that of CVD. It is suggested that the maternal risk of PET increases with the degree of obesity, persists after accounting for other confounding demographic factors and is likely to be related to the altered metabolic state associated with morbid obesity rather than the PET *per se* (Sebire *et al* 2001). Insulin resistance is also associated with changes in a number of metabolic mediators. Elevated levels of PAI-1 (Abbasi *et al* 1999), leptin (Segal *et al* 1996) and TNFα (Fernandez-Real *et al* 1998, Solomon and Seely 2001) are features of insulin resistance. These markers are also increased in PET (Solomon and Seely 2001), which may support the theory that pre-pregnancy insulin resistance predisposes not only to later CVD, but also to PET in the pregnant state.

1.4.2 Do the metabolic and coagulation changes persist post-partum?

The metabolic disturbances of PET and atherosclerosis have many common features. However, it is important to ascertain whether the changes occurring in PET persist post-partum, increasing the risk of future CVD. In a case control study, formerly pre-eclamptic patients had higher systolic and diastolic blood pressures and increased plasma levels of vWF, fibrinogen, cholesterol, triglycerides and VLDL than control pregnant women (He *et al* 1999). The elevations in lipids, vWF and fibrinogen all correlated with the degree of blood pressure elevation in the index pregnancy. Another case control study of women, with similar BMI, approximately seventeen years after index pregnancy found elevated fasting insulin and glucose levels in women with a history of PET (Laivuori *et al* 1996).

The area under the insulin response curve to a glucose tolerance test was larger in women with prior PET. Serum levels of total cholesterol, HDL cholesterol, triglyceride and uric acid did not differ significantly between the study groups, but the area under the insulin response curve was positively related to triglyceride and systolic blood pressure. Van Pampus and colleagues (van Pampus *et al* 1999) have described significantly higher lipoprotein (a) levels in PET women (33%) at least 10 years post partum, compared to a control group (10%). More recently, Sattar and colleagues (Sattar *et al* 2003) have demonstrated significantly elevated levels of VCAM and ICAM, and higher HbA1C levels compared to control subjects, in women with a history of PET up to 15 – 25 years following the pregnancy.

Chambers *et al* (Chambers *et al* 2001) demonstrated impaired vascular reactivity in women with a history of PET. Pre-eclamptic women had a lower mean flow-mediated dilatation than controls, which was improved by ascorbic acid administration in the cases, but not in the controls. The pre-eclamptic group also had higher systolic and diastolic blood pressure, BMI, waist-hip girth ratio, and total cholesterol to HDL-C ratio, and a higher prevalence of hypertension and family history of hypertension. In multivariate regression analysis, the relationship between previous PET and impaired flow-mediated dilatation was independent of these confounders.

1.4.3 How do we interpret these studies?

The list of predisposing factors for PET include hypertension, diabetes, increased insulin resistance, increased testosterone, obesity (Lake *et al* 1997, Sattar *et al* 2001), lipid abnormalities (Sattar *et al* 1997a), black race and increased plasma homocysteine concentration. Interestingly, these are also risk factors for other endothelial diseases, particularly atherosclerosis. There are also notable common features linking established PET and atherosclerosis, namely vasomotor dysfunction and hypertension, platelet and coagulation activation, endothelial damage, inflammation and metabolic disturbance. This serves to highlight the similar pathologies of the two disorders. The 'atherosclerotic' lesion of PET accumulates in the spiral arteries over a short period of time whereas the atherosclerotic lesion in vascular disease develops over decades. However, the features of PET are similar to atherosclerotic changes in the vascular bed in the non-pregnant population, and may highlight common mediators for underlying mechanisms in both diseases.

The link between PET and CVD may be explained by the presence of pre-pregnancy metabolic abnormalities, as discussed in section 1.4.1. Extrapolating from this, it may be considered that those women who are destined to develop PET have a pre-existing susceptibility to 'metabolic stress', either due to pregnancy or simply the ageing process. It is this susceptibility to metabolic stress that underlies the link between PET and CVD. Alternatively, it may be considered that it is the metabolic abnormalities and endothelial damage generated by the onset of PET that results in a susceptibility to CVD later in life. It is likely that the underlying aetiology is a combination of both of these possibilities.

Sattar and Greer have proposed a model where CVD risk changes throughout life (Figure 1-2) (Sattar and Greer 2002). Immediately after birth and weaning cardiovascular risk is low. The risk 'peaks' during pregnancy due to metabolic and vascular changes. This risk decreases once more after delivery, but never quite returns to pre-pregnancy levels. Each subsequent pregnancy will step up the baseline level of risk so that risk increases with parity.

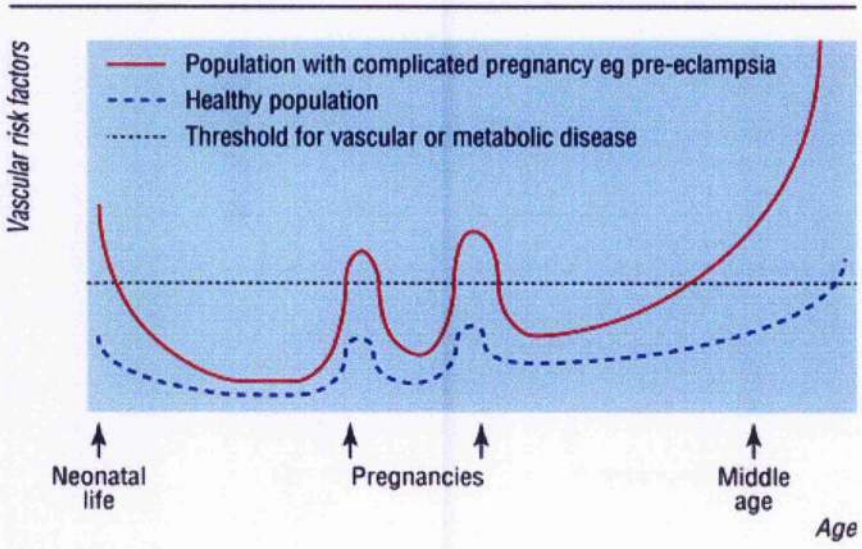


Figure 1-2. Risk factors for vascular disease are identifiable during excursions into the metabolic syndrome of pregnancy.

A positive association between parity and coronary heart disease (CHD) risk in women has been described in previous studies (Dekker and Schouten 1993, Green *et al* 1988, Ness *et al* 1993). In a retrospective cross sectional study, Lawlor and colleagues (Lawlor *et al* 2003) recently reported a 'J' shaped association between number of children and coronary heart disease, with the prevalence lowest among those with two children and increasing linearly with each additional child beyond two. An additional child increased the age-

adjusted odds of coronary heart disease by 30% (odds ratio 1.30; 95% CI 1.17 – 1.44) for women with at least two children. Because adjustment for obesity and metabolic risk factors attenuated the association, this group concluded that lifestyle risk factors associated with child-rearing lead to obesity and thus increased coronary heart disease risk. They acknowledged that the biological response of pregnancy, including insulin resistance, might have an additional adverse effect (Lawlor *et al* 2003). As a woman becomes older, her cardiovascular risk increases with age and accelerates in the post-menopausal years. Potentially some individuals are more susceptible to metabolic stresses than others. Thus a woman who suffers from PET may make greater excursions into metabolic disturbance in each pregnancy and returns to an increased level of risk compared to normal pregnancies thereafter. In later life her phenotypic susceptibility to vascular disease results in a faster acquisition of vascular risk associated with increasing age, resulting in the premature clinical presentation of vascular disease. The model proposed by Sattar and Greer is supported by epidemiological studies, demonstrating the link between the metabolic disturbances of PET and increased cardiovascular risk (Hannaford *et al* 1997, Irgens *et al* 2001, Jonsdottir *et al* 1995, Smith *et al* 2001).

Over the years, there have been many treatments attempted for the control of PET, and yet we still have no cure. We can be certain that there are common mechanisms underlying both CVD and pregnancy complications, clinically manifest as disturbance in metabolism, coagulation and inflammation. The role of insulin resistance in PET provides a possible opportunity for intervention via diet and exercise, in the prevention of both PET and future CVD. Prevention and clinical management of PET may be much informed by atherosclerosis research particularly with respect to lifestyle and diet management. Pre-pregnancy interventions in women at risk of PET may have beneficial effects both on obstetric outcome and on CVD risk later in life.

1.5 Peroxisome proliferator-activated receptors

Peroxisome proliferator activated receptors (PPARs) are a family of intracellular ligand activated nuclear receptors regulating gene transcription (Schoonjans *et al* 1996). On activation, PPAR receptors heterodimerise with their common nuclear receptor binding partner, the retinoid X receptor (RXR) (Torra *et al* 2001). RXRα and PPARγ act synergistically to regulate gene expression and insulin action (Codner *et al* 2001). Three types of PPAR receptor have been identified; α, γ and δ, each differing in their tissue distribution. PPARα is expressed in liver, heart, kidney, muscle, retina and vascular

endothelium (Chinetti *et al* 2001). PPARδ (also designated PPARβ) is distributed in all tissues tested with high expression in placenta and large intestine (Auboeuf *et al* 1997). PPARγ has three isoforms; γ1,γ2 and γ3 and is expressed in adipose tissue (Spiegelman 1998), in large intestine and skeletal muscle (Fajas *et al* 1997, Park *et al* 1997). PPARα is involved in fatty acid metabolism and PPARα activation regulates gene transcription of products involved in pathways such as fatty acid oxidation (Schoonjans *et al* 1996). PPAR γ is involved in adipocyte differentiation (Schoonjans *et al* 1996, Spiegelman 1998) and insulin action (Kubota *et al* 1999, Spiegelman 1998) and agonists are used as insulin sensitizers. Both PPARα and, to a greater extent, PPARγ are implicated in the regulation of inflammatory responses (Chawla *et al* 2001, Clark *et al* 2000, Tontonoz and Nagy 1999) particularly of the macrophage. PPARδ is also known to be involved in lipid metabolism and inflammation (Chawla *et al* 2003, Wang *et al* 2003), as well as keratinocyte differentiation and wound healing (Di-Poi *et al* 2002, Michalik *et al* 2001). The natural ligands for PPARs are not established although 15-deoxy-Δ(12,14)-prostaglandin J_2 and some lipids (e.g. polyunsaturated fatty acids) act as PPARγ agonists (Kliewer *et al* 1997). The fibrate and thiazolidinedione classes of drugs act as PPARα and PPARγ agonists respectively (Schoonjans *et al* 1996).

Evidence for a role for the PPARs in pregnancy has emerged from the PPARγ and PPARδ knockout mice. PPARγ deficiency interfered with terminal differentiation of the trophoblast and placental vascularisation, leading to severe myocardial thinning and death (Barak *et al* 1999, Kubota *et al* 1999). PPARδ was demonstrated to be essential for placentation, with the knockout mice embryos dying in parallel to the appearance of an abnormal gap in the placento-decidual interface (Barak *et al* 2002). High PPARδ expression has been demonstrated at implantation sites and in decidual cells in the rat uterus (Lim and Dey 2000), and thus PPARδ may play an important role during implantation and decidualisation (Ding *et al* 2003).

Human studies have also supported a role for PPARδ and γ in pregnancy. PPARγ is expressed in human cytotrophoblasts, in a choriocarcinoma cell line (JEG-3) and in fetal endothelial cells (Schaiff *et al* 2000, Waite *et al* 2000). Using a luciferase reporter gene construct in the JEG-3 cell line, it was found that PPARγ activation and protein expression increased using sera from pregnant women, possibly by a prostanoid or fatty acid component (Waite *et al* 2000). Non-quantitative assessment of PPARα, δ and γ and RXRα expression has been carried out in cyto- and syncytiotrophoblast cells (Fournier *et al* 2002,

Tarrade *et al* 2001a, Tarrade *et al* 2001b, Wang *et al* 2002) and in trophoblastic disease and hydatidiform molar pregnancies (Capparuccia *et al* 2002). It is interesting to note the report of an index female case from kindred with a dominant negative mutation in human PPARγ (which is associated with severe insulin resistance and hypertension) with a complicated obstetric history (Barroso *et al* 1999).

Because of the role of PPARs in inflammation, insulin and lipid metabolism and placental function, it is possible that they may have a function in the metabolic complications of pregnancy. Recent data suggest that PPARα and γ activation decreases atherosclerotic progression, by correcting metabolic disorders and also through direct effects on the vasculature (Duval *et al* 2002). PPAR agonists are used as insulin sensitising and lipid-lowering agents in the treatment of atherosclerotic diseases. As discussed previously, PET, with widespread endothelial damage and dysfunction and increased systemic inflammatory response, shares many risk factors with cardiovascular disease. There are potential common underlying mechanisms for cardiovascular disease and PET, and there is a link between cardiovascular disease risk and other metabolic complications of pregnancy. There is potential for intervention based on early assessment of cardiovascular risk, among them possible use of the PPAR agonist drugs. PPARs are thus excellent candidate molecules that may be implicated in the development of cardiovascular disease pathology.

1.6 Hypothesis and aims

Based on the evidence demonstrating a role for PPARs in normal pregnancy, in the metabolic complications of pregnancy and in cardiovascular disease, and based on the data linking PET and IUGR with future cardiovascular risk, it was hypothesised that:

- PPAR mRNA and protein expression might increase during gestation and be compromised in complications of pregnancy with placental pathology such as PET and IUGR. Elevated plasma inflammatory and lipid markers in mother and fetus would determine disease severity in the latter groups.

- Analogous to the relationship between inflammation and the development of insulin resistance in type 2 diabetes and CVD, it is proposed that the inflammation seen in PET may be related to the development of insulin resistance and the metabolic syndrome. Inflammatory and metabolic mediators may be produced by the placenta, maternal adipose tissue or both.

- Higher fasting insulin and triglyceride levels in the maternal plasma in PET pregnancies (compared to uncomplicated pregnancies) will result in less unsaturated fatty acids in both maternal erythrocyte membranes, and that the mother and fetus may be at risk for future development of the diseases of insulin resistance, including CVD and PET itself.

- Variation at genes encoding for molecules involved in the metabolic pathways of inflammation and insulin resistance may confer susceptibility to development of PET.

Thus, the aims of this thesis were four fold;

- To determine the localisation and expression of each of the PPARs in uncomplicated pregnancy and in pregnancies complicated by PET and IUGR.

- To determine maternal and fetal lipid and lipoprotein concentrations in a cross-sectional BMI matched case control study of uncomplicated 3rd trimester pregnancies, and pregnancies complicated by PET or IUGR, to assess disease severity and fetal lipid status in these complicated pregnancies. PON-1 activity was also determined, as a marker of oxidative stress in these pregnancies.

- To determine whether there is an aetiological link between the insulin resistance and inflammation seen in PET pregnancies, by comparing maternal and fetal inflammatory markers and markers of insulin resistance in the PET group, with those derived from uncomplicated pregnancies. I also aimed to determine maternal erythrocyte membrane phospholipid fatty acid composition in these pregnancies, as a marker of insulin resistance, relating the findings to the plasma lipid and insulin resistance markers.

- To assess subcutaneous adipose tissue and placental expression of markers of inflammation and insulin resistance (inflammatory cytokines, leptin and PPARγ) in uncomplicated and PET pregnancies, to determine a potential tissue origin.

- To look at the contribution of inflammatory, leptin and PPARγ genotypes on PET risk.

2 Materials and Methods

2.1 Subjects

2.1.1 Cross-sectional study

Twenty-three subjects with PET and 17 subjects with IUGR and their corresponding matched controls (n=40) from third trimester (28-40 weeks gestation) uncomplicated pregnancies were recruited from the Princess Royal Maternity Hospital, Glasgow. Data was normalised using a cohort of 81 maternal and 41 fetal blood samples from uncomplicated 3rd trimester pregnancies, collected in the same manner. Patient characteristics, including booking body mass index (BMI) and mode of delivery, were recorded at time of sampling and smoking status recorded as current smoker or non-smoker. Patients with suspected fetal anomalies likely to contribute to reduced fetal growth were excluded from the study, as were women taking drugs known to affect metabolism. PET was defined according to the International Society for the Study of Hypertension in Pregnancy criteria, that is, a diastolic blood pressure greater than 110 mmHg on one occasion, or exceeding 90 mmHg on repeated readings, with proteinuria of ≥ 0.3g/24 h, or 2+ proteinuria on dipstick testing, in the absence of renal disease or infection. IUGR was defined as having an estimated fetal weight less than the 5th percentile for gestation with associated oligohydramnios (amniotic fluid index <5) and/or abnormal umbilical artery blood flow on Doppler ultrasound. Local birthweight centiles were used. Cases were matched with control subjects for age, parity and BMI. Maternal and fetal blood, placental tissue and subcutaneous adipose tissue were studied (table 2-1). The study was approved by the Ethics Committee of Glasgow Royal Infirmary, and all women gave written informed consent.

2.1.2 Longitudinal study subjects and tissue collection

Subjects with uncomplicated pregnancies were recruited from the Princess Royal Maternity Hospital, Glasgow, and were studied during routine antenatal visits in the 1st, 2nd and 3rd trimesters of pregnancy. Patient characteristics were recorded at the first visit. Maternal and fetal bloods were collected at time of delivery, as in the cross-sectional study (section 2.1.1) (Table 2-1). Samples for the longitudinal study were collected by Dr Frances Stewart, for which I am grateful.

	PET	PET control	IUGR	IUGR control	Longitudinal study (number of samples in each trimester)	Uncomplicated 3rd trimester cohort for data normalisation
Maternal lipids	23	23	17	17	47	81
Fetal lipids	14	14	11	11	n/a	41
Maternal markers of inflammation	23	23	17	17	47 (IL-6 and sCRP)	81
Fetal markers of inflammation	14	14	11	11	n/a	41
Maternal PON-1	23	23	17	17	20	53
Fetal PON-1	14	14	11	11	n/a	n/a
Maternal SHBG	23	23	17	17	47	81
Fetal SHBG	14	14	11	11	n/a	41
Maternal fructosamine	23	23	17	17	n/a	81
Maternal glucose/ insulin	23	23	17	17	47	63
Fetal glucose/ insulin	14	14	11	11	n/a	29
Maternal RBC FA analysis	23	23	17	17	47	47
Placenta – PPAR study	10	10	10	10	n/a	n/a
Placenta – inflammatory study	6	6	6	6	n/a	n/a
Adipose tissue	6	6	6	6	n/a	n/a

Table 2-1. Number of samples collected for analysis in each study group.

2.1.3 Archival placental collection

First, second and third trimester placental samples were obtained from an available archival collection. The first trimester placentae (n=10) (7-12 weeks gestation) had been collected from women undergoing suction termination of pregnancy without pre-treatment for social indications. Second trimester placentae (n=10) (14 - 28 weeks gestation) were similarly collected after medical termination of pregnancy using mifepristone 600mg orally 48 hours prior to receiving a 1mg gemeprost (the synthetic prostaglandin analogue, 16,16-dimethyl-*trans*-Δ^2-PGE_1 methyl ester) vaginal pessary, for social indications. Pregnancies known to have an abnormal fetal karyotype were excluded from the study. Third trimester placental samples (n=10) had been collected at time of delivery in the same manner as for the cross sectional study (section 2.1.1).

2.1.4 Genetic study population

All PET and control samples were collected from the same West of Scotland population attending the Princess Royal Maternity Hospital (formally Glasgow Royal Maternity Hospital) as described in sections 2.1.1 and 2.1.2, and included samples from an archival collection, described in detail in Hypertension (Freeman *et al* 2004). PET was defined as in 2.1.1. Cases were matched with controls for age and parity (PET n=130 and controls n=260).

2.2 Tissue handling and storage

2.2.1 Placental tissue

First and second trimester placentae were washed in phosphate buffered saline and an approximately five-gram sample was fixed in 10% neutral buffered formalin (BDH, Poole, UK). Full thickness biopsy sections of third trimester placentae were obtained (approximately five grams) at time of delivery from four separate pre-determined areas on each placenta, distinct from the umbilical cord insertion, and then samples were randomised. These sections were similarly fixed as before. All samples were paraffin-embedded for immunocytochemistry (ICC). Samples of 1st and 3rd trimester placentae were immediately snap frozen in liquid nitrogen and stored at -70°C for later RNA preparation and protein extraction. Frozen samples for RNA and protein preparation were not available from 2nd trimester samples.

2.2.2 Adipose tissue

Subcutaneous (SC) adipose tissue (approximately $1cm^3$) was collected from the incision site at time of Caesarean section, and washed in phosphate buffered saline. An approximately one-gram sample was fixed in 10% neutral buffered formalin (BDH, Poole, UK). All samples were paraffin-embedded for immunocytochemistry (ICC). Samples were immediately snap frozen in liquid nitrogen and stored at -70°C for later RNA preparation and protein extraction.

2.2.3 Blood

Fifty millilitres of blood was collected at time of recruitment, from the antecubital fossa by venepuncture into K_2EDTA (final concentration 1mg/ml), sodium citrate (3.2%), lithium heparin, fluoride oxalate and plain tubes. None of the samples obtained were taken during labour. Blood samples were not drawn under fasting conditions in the PET and IUGR patients from the cross-sectional study. Patients presenting with PET or IUGR are often admitted on an emergency basis, thus standardised blood sampling after a 12-hour overnight fast is not feasible in these groups. Non-fasting will affect maternal plasma triglyceride (TG), glucose and insulin levels in this thesis. PON-1 activity is unaffected by fasting status. However, it was considered reasonable to observe the non-fasting TG values, as we spend most of our time in the non-fasting state. Blood samples were taken

after a 12-hour overnight fast in the longitudinal study subjects, where samples were drawn under fasting conditions in each trimester of pregnancy. Fetal cord blood was obtained at time of vaginal delivery or lower uterine Caesarean section, from the umbilical vein. Fetal blood was collected into K_2EDTA (final concentration 1mg/ml), fluoride oxalate and lithium heparin tubes. Plasma, serum and blood cells were harvested at 5 °C by low speed centrifugation, and aliquots of plasma, serum and the buffy coat layer were either used immediately or frozen at -70°C until required for use. None of the samples were haemolysed.

2.3 Immunocytochemistry

Immunocytochemistry (ICC) was performed on paraffin embedded placental tissue (10 x 5µm sections from each placenta; 1st trimester, 3rd trimester, PET& IUGR (n=10 placentae in each group, controls n=20). Antibodies used, pre-treatment, blocking solutions, and primary and secondary antibody dilutions are detailed in Table 2-2. Sections were mounted on silane-coated slides, heated to 60°C for 35 minutes, deparaffinised in xylene, and rehydrated in a graded alcohol series. Endogenous peroxidase activity was quenched using 0.5% hydrogen peroxide in methanol. Sections were then washed in phosphate buffered saline (PBS) and antigen was retrieved by pre-treatment as in Table 2-2. Following this, sections were washed in PBS and blocked as in Table 2-2, for 30 minutes at room temperature. They were then incubated for 16 hours at 4°C with the primary antibody diluted as in Table 2-2. Sections were once again washed in PBS then incubated for 30 minutes with the appropriate biotinylated secondary antibody (all Vector Laboratories), as in Table 2-2; all with 5% human serum added. Sections were washed in PBS then incubated with avidin DH/ biotinylated horseradish peroxidase H reagent (Vector Laboratories) in PBS as described by the manufacturer, for 30 minutes before final washing. The antigen was visualised using 1mg/ml diaminobenzidine tetrachloride (Sigma), 0.02% H_2O_2 in 50mM Tris-HCl, pH 7.6, and the antigen appeared as a brown end product. Sections were counterstained with Harris haematoxylin (Sigma). Small bowel tissue sections were used as positive control for each of the PPARs, tonsillar tissue for CD68 and placenta for CY-7, CD31 and RXRα. Negative controls included slides incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 Aspergillus niger glucose oxidase (Dako Ltd., Buckinghamshire, UK), an enzyme that is neither present nor inducible in mammalian tissues. PPAR antibody specificity was confirmed using competing peptides (x1 (0.6µg/ml) – x50 (30µg/ml)) against which the primary antibody was raised (Santa Cruz). The specificity of

the other antibodies used, had previously been verified by the manufacturer using enzyme linked immunosorbent assay (see data sheets).

Antibody	Section type (pre-treatment)	Section blocking solution	1° antibody dilution	1° antibody source	Biotinylated 2° antibody dilution
PPARα	Paraffin [microwave, citrate buffer (pH 6.0)]	20% rabbit/ 20% human serum	1:350 in 2% rabbit	Santa Cruz SC-1985	1:200 rabbit anti-goat in 2% rabbit serum in PBS
PPARδ	Paraffin [microwave, citrate buffer (pH 6.0)]	20% rabbit/ 20% human serum	1:350 in 2% rabbit	Santa Cruz SC-1983	1:200 rabbit anti-goat in 2% rabbit serum in PBS
PPARγ	Paraffin [microwave, citrate buffer (pH 6.0)]	20% rabbit/ 20% human serum	1:350 in 2% rabbit	Santa Cruz SC-1984	1:200 rabbit anti-goat in 2% rabbit serum in PBS
CY-7	Paraffin [microwave, citrate buffer (pH 6.0)]	20% horse/ 20% human serum	1:250 in 2% horse	DAKO M7018	1:200 horse anti-mouse in 2% horse serum in PBS
CD68	Paraffin (Trypsin)	20% horse/ 20% human serum	1:50 in 2% horse	Dako DAKO-X931	1:200 horse anti-mouse in 2% horse serum in PBS
CD31	Paraffin [microwave, citrate buffer (pH 6.0)]	20% horse/ 20% human serum	1:500 in 2% horse	DAKO M0823	1:200 horse anti-mouse in 2% horse serum in PBS
RXRα (D-20)	Paraffin [microwave, citrate buffer (pH 6.0)]	20% goat/ 20% human serum	1:500 in 2% goat	Santa Cruz SC-553	1:200 goat anti-rabbit in 2% goat serum in PBS

Table 2-2. Antibodies used in ICC

2.4 Northern analysis

2.4.1 RNA preparation and electrophoresis

Total RNA was extracted from placenta and adipose tissue using the Trizol ™ method according to the manufacturer's instructions (Life Technologies, Paisley, UK). The isolated RNA was redissolved in diethylpyrocarbonate (DEPC)- treated distilled water and quantified by UV spectrophotometry. The integrity of the RNA was confirmed as being free of protein and DNA contamination by having an optical density at 260nm/280nm (ratio of >1.8) and by the presence of intact 18s and 28s bands on agarose gels. RNA sample loading buffer (Sigma, UK) was added to 10μg of total RNA and electrophoresis carried out in 1.2% agarose gels containing 6% formaldehyde and 20mmol/L MOPS (10x MOPS is 0.2 mol/L 3-[N-Morpholino] propane-sulphonic acid; 0.05 mol/L Na acetate pH

7.0; 0.01 mol/L Na_2EDTA) at 60 volts for 2.5 hours. RNA was transferred overnight onto Hybond-N nylon membranes (Amersham Biosciences, Buckinghamshire, UK) in 20 x SSC (3 mol/L NaCl; 0.3 mol/L $Na_3C_6H_5O_{72}H_2O$, pH 7.0) and fixed to the membrane by ultraviolet irradiation for 40 seconds at 1.2 x 10^5 microjoules. Membranes were pre-hybridised for 1-2 hours, at 42° C, in 14ml of Ultrahyb™ (Ambion (Europe) Ltd., Huntingdon) and then hybridised overnight with the appropriate ^{32}P-labelled probe (Oligolabelling kit, Amersham Biosciences, Buckinghamshire, UK) added to the prehybridisation buffer. Fifty nanograms of cDNA or oligonucleotide were labelled by random priming.

2.4.2 Probes

The PPARγ (1.2Kb) and PPARδ (1.0 Kb) cDNA probes were purchased from Alexis Corporation (Nottingham, UK). Complementary cDNA probes were prepared as follows; a 389 base pair region of the RXRα gene, a 492 base pair region of the PPARα gene, a 359 base pair region of the 18s ribosomal RNA gene and a 469 base pair region of the PAI-2 gene were amplified by polymerase chain reaction (PCR). The forward and reverse primers used are detailed in Table 2-3. PCR products were purified using Wizard PCR Preparations DNA purification system (Promega, UK). Nylon filters were washed in 1 x SSC, 0.1% SDS at 65°C for 20 minutes, then in 0.5 x SSC, 0.1% SDS and if necessary a further wash was carried out in 0.1 x SSC, 0.1% SDS at 65°C. Autoradiography was carried out with Fuji X-ray film at -70°C for between one and four days.

Gene	Forward primer	Reverse primer
PPARα	5'-CCA GTA TTT AGG ACG CTG TCC-3'	5'-AAG TTC TTC AAG TAG GCC TCG-3'
RXRα	5'-AGG AAA CAT GGC TTC CTT CAC CAA G-3'	5'-GTT TGC CTC CAC GTA GGT CTC GGT C-3'
18s	5'-CAA GTC TGG TGC CAG CAG CCG GGG T-3'	5'-TCA CCT CTA GCG GCG CAA TAC GAA T-3'
PAI-2	5'-AAT ATA TTC GAC TCT GTC AGA AAT A-3'	5'-GCT GGT CCA CTT GTT GAG TTT GTC A-3'

Table 2-3. Northern analysis primers for cDNA probes

2.5 Protein extraction and western blot analysis

2.5.1 Protein extraction and SDS-PAGE

Protein was extracted from placenta and adipose tissue using the Trizol ™ method according to the manufacturer's instructions (Life Technologies, Paisley, UK). Protein (50

µg) was mixed with 4 times the volume of 100% acetone, stored for 1 hour at -20°C and then ultracentrifuged at 4°C at 13,000rpm for 10 minutes. The supernatant was removed and the pellet air-dried. The pellet was mixed with loading buffer (0.5M Tris base, 5.2% SDS, 17% v/v glycerol, 8.7% mercaptoethanol, 0.02% Bromophenol Blue, pH 6.8), boiled for 3 minutes and loaded onto a 10% SDS-PAGE gel with a 5% stacking gel. A standard preparation of Jurkat cell nuclear extract (Santa Cruz, SC-2132) (50µg) was used as a positive control. After electrophoresis, proteins were transferred to nitrocellulose using Hoefer 660 transfer apparatus (Amersham Pharmacia Biotech Inc., UK) according to the manufacturer's instructions. The transfer time was 3 hours, and 0.025% SDS was added to the standard buffer.

2.5.2 Western blotting

1° antibody	1° antibody dilution/ duration	2° antibody dilution
PPARα (Santa Cruz Biotechnology Inc. (SC)-1985)	1:100/ 2 hours	Anti-goat (SC-2352) 1:5000
PPARδ (SC-7197)	1:500/ overnight	Anti- rabbit (SC-2054) 1:40 000
PPARγ (SC-7196)	1:500/ overnight	Anti- rabbit (SC-2054) 1:40 000
RXRα (SC-553)	1:200/ overnight	Anti- rabbit (SC-2054) 1:40 000
IL-6 (R&D MAB206)	1:200/overnight	Anti-mouse (SC-2055) 1:2500
IL-10 (R&D MAB217)	1:200/overnight	Anti-mouse (SC-2055) 1:2500
Leptin (OB) (R&D AF389)	1:200/overnight	Anti-goat (SC-2352) 1:2500
TNF-α (R&D MAB610)	1:200/overnight	Anti-mouse (SC-2055) 1:2500

Table 2-4. Antibodies used in Western analysis.

Western blot antibody optimal concentrations were determined using dot-blot apparatus and an antibody dilution series. The PPARγ and δ antibodies used initially (SC-1984 and SC-1983 respectively) demonstrated significant non-specificity for the PPARs and antibody specificity was subsequently obtained using other antibodies from the same company (SC-7196 and SC-7197 respectively). Positive controls for PPARs included Jurkat nuclear extract and a third trimester placenta from an uncomplicated pregnancy, which was known to express high amounts of protein. Recombinant human proteins for IL-10 (50ng, R&D 217-IL-025), leptin (100ng, R&D 398-LP), IL-6 (100ng, R&D 206-IL-05) and TNFα (100ng, R&D 210-TA-050) were used as the appropriate antibody positive control. Blots were developed using standard procedures, with three 15-minute wash steps

in 0.1% TTBS (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0) between each step. Blocking time was 1 hour at room temperature; in 10% skim milk-TTBS. Membranes were subsequently incubated with primary antibody (table 2-4) in 5% skim milk-TTBS at room temperature. After incubation with secondary antibody (table 2-4), for 1 hour at room temperature, blots were developed using ECL reagents (Amersham Pharmacia Biotech, UK) and were exposed to film for 5 minutes.

2.6 Blood analyses

Plasma was harvested at 5 °C by low speed centrifugation, and aliquots of plasma for lipid and lipoprotein measurements were analysed immediately. Plasma total cholesterol, TG, VLDL-C, LDL-C and HDL-C measurements were performed by modification of the standard Lipid Research Clinics Protocol (Lipid Research Clinics Program. Manual of Laboratory Operations. Lipid and lipoprotein analysis, 1975. p. 5) using enzymatic reagents for lipid determinations. IL-6, IL-10, ICAM, VCAM, TNFα, leptin (all R&D Systems Inc., USA), SHBG (IBL Immuno-Biological Labs, Hamburg) and insulin (Mercodia, Sweden) were performed by commercial ELISA according to the manufacturer's instructions. Highly sensitive CRP (sCRP) was performed using a double-antibody sandwich ELISA with rabbit anti-human CRP (Dako Corp. A/S, Glostrup, Denmark, cat. No. A0073) and peroxidase-conjugated rabbit anti-human CRP (DAKO cat. No. P0277). The substrate for the colour was 1,2 phenylaminediamine (DAKO cat. No. 2000) and the standard was human CRP calibrator (DAKO cat. No. X0923). The assay participates in the UK NEQAS quality control scheme (Packard *et al* 2000). Glucose analyses were kindly performed by routine biochemistry (Glasgow Royal Infirmary). Fetal insulin was kindly analysed by Mr Ian Halsall of the Department of Clinical Biochemistry, Addenbrooks, NHS Trust, Cambridge. HOMA analysis was calculated as follows: [fasting insulin (mU/L) x fasting glucose (mmol/L)]/22.5 (Conwell *et al* 2004). Fructosamine analysis was performed using a colorimetric assay (Roche Diagnostics Ltd., East Sussex, UK), on an IL600 autoanalyser. PON1 activity and concentration were kindly analysed by Dr Mike Mackness of the Clinical Research Division II Medicine, Manchester Royal Infirmary. In brief, PON1 activity was measured by adding serum to Tris buffer containing 2 mmol/L $CaCl_2$ and 5.5 mmol/L paraoxon (*O,O*-diethyl-*O*-*p*-nitrophenylphosphate, Sigma Chemical Co). The rate of generation of *p*-nitrophenol was determined at 405 nm, 25°C, with the use of a continuously recording spectrophotometer (Beckman DU-68) (Mackness *et al* 2001). All samples were run in a single assay.

2.7 Red blood cell membrane fatty acid extraction and derivatisation

2.7.1 Red blood cell fatty acid extraction

Preparation of a total fatty acid extract from red blood cell (RBC) membranes was performed with a modified Folch extraction (Folch *et al* 1957, Hoving *et al* 1988). Four hundred microlitres (400µL) of red blood cells were re-suspended in 10mM TRIS buffer (Tris[hydroxymethyl]aminomethane, $C_4H_{11}NO_3$), pH 7, by vortexing in 6ml Beckman Ultraclear Ultracentrifuge tubes (0.5-2.5 inches, part number 344088, Beckman instruments, Inc., Palo Alto, CA 94304, USA). Tubes were filled to the neck, and left at room temperature for 30 minutes to lyse the RBCs. The tubes were centrifuged in a Beckman L8-60M Ultracentrifuge, Type 50.4 rotor, at 49,000 rotations per minute (rpm), at 4 °C for 30 minutes. The supernatant was removed to leave the RBC pellet, which was then re-suspended by vortexing in 200µL of distilled H_2O and 150µL was transferred to a clean glass screw top tube. Two millilitres (ml) of methanol:toluene (4:1) containing heneicosanoic acid ($C_{21}H_{42}O_2$) internal standard (0.2mg $C_{21}H_{42}O_2$/ml toluene), was added, to allow calculation of the absolute fatty acid amounts. While vortexing, 200µL of 100% acetyl chloride was added to each tube, and the tubes capped (screw caps) and sealed with teflon tape. The tubes were heated at 100 °C for one hour in a heating block. The tubes were allowed to cool in cold H_2O in a metal rack. Three millilitres (ml) of 10% K_2CO_3 was slowly added to each tube. One hundred microlitres (µL) of toluene was added. Tubes were centrifuged at 3000rpm for 8 minutes at 5 °C. The upper toluene phase was transferred to GC vials, and stored at minus 20 °C until ready for injection on the gas chromatograph.

2.7.2 Gas chromatography

All analyses were performed in collaboration with Dr Barbara Meyer, University of Wollongong, New South Wales, Australia, and gas chromatography was carried out there, by myself. The fatty acid constituents of the phospholipids and triglycerides were transmethylated by heating as described in section 2.7.1, and the methyl fatty acids (FAs) were separated, identified and quantitated on a Shimadzu GC 17A gas chromatograph with flame ionisation detection and Class VP software. A 30m x 0.25mm-mm DB-23 fused silica capillary column (J&W Scientific, Folson, CA) with a film thickness of 0.25 µm was

used in conjunction with a Hewlett-Packard 7673B on-column auto-injector. Ultra-high purity hydrogen (Commonwealth Industrial Gases, Ermington, NSW, Australia) and air were used as a carrier gas at a flow rate of 2mL/min. A temperature gradient programme was used with an initial temperature of 150°C, increasing at 20°C/min until 190°C, then at 5°C/min until 210°C, then at 2°C/min until 230°C and then at 4°C/min until 240°C (final time 18.5 min) and with an equilibration time of 1 minute. The total programme time was 22 minutes. Identification of fatty acid methyl esters was made by comparison with the retention times of authentic standard mixtures (Fatty acid methyl ester mixture #189-19, product no. L9405, Sigma, Sweden).

2.7.3 Fatty acid data analysis

The results were expressed as a percentage of the total fatty acids (12:0, 14:0, 14:1n7, 16:0, 16:1n7, 17:0, 17:1n7, 18:0, 18:1n6, 18:1n9, 18:1n7, 18:2n6, 18:3n6, 18:3n3, 20:0, 20:1n9, 20:2n6, 20:3n9, 20:3n6, 20:4n6, 20:3n3, 20:5n3, 22:0, 22:1n9, 22:2n6, 22:4n6, 22:3n3, 22:5n6, 22:5n3, 24:0, 22:6n3, 24:1n9). Identified minor peaks (<0.5% of the total) were excluded from the calculation. Three fatty acid indices were derived from the primary data: the average degree of fatty acid unsaturation (the unsaturation index; UI), which was calculated as the average number of double bonds per fatty acid residue multiplied by 100, the average chain length and the total percentage of long chain polyunsaturated fatty acids (PUFA) with ≥20 carbon units (C20-22 PUFA). The activity of certain enzymes of fatty acid biosynthesis was estimated according to the product precursor ratios of the percentage of individual fatty acids. The estimated enzyme activities include; elongase, calculated from the ratio of the percentage of 18:0 (stearic acid) to 16:0 (palmitic acid); and the Δ5 desaturase, calculated from the ratio of 20:4n-6 (arachidonic acid) to 20:3 n-6; the Δ6 desaturase, calculated from the ratio of 20:3n6 to 18:2n6; and Δ9 desaturase, calculated from the ratio of 18:1 n-9 (oleic acid) to 18:0 (Pan *et al* 1995).

2.8 DNA polymorphisms

2.8.1 Preparation of human DNA from blood

Maternal and fetal blood samples were collected in EDTA (1mg/ml). On ice, 1 - 2 millilitres (ml) of packed red cells, or the buffy coat layers, were added to 15 ml of lysis buffer pH 7.5 [sucrose 0.32M 109.5g/L, 1.58gL Tris-HCL or 1.21g/L Tris base 10mM pH 7.5, 1.02 g/L $MgCl_2$ 5mM, 10ml/L Triton X-100 1%], and mixed by inversion. The

samples were centrifuged at 10,000 rpm, for 10 minutes at 4°C, in a JA-17 rotor, in the Beckman J2-21 centrifuge. The supernatant was removed completely, and the pellet samples returned to ice. The pellets were re-suspended in 2.25 ml re-suspension buffer pH 8.0 [4.38g/L NaCl 0.075M, 8.93g/L EDTA 0.024M] using a sterile pastette. SDS (10%, 125µl) and proteinase K 2mg/ml (125µl) were added, and the samples incubated at 37°C overnight in a water bath. The incubated solution was transferred to a clean glass Z10 tube and 2.5ml of water-saturated phenol was added to each, mixing by inversion. The samples were centrifuged at 3000rpm for 10 minutes in a Mistral 3000 centrifuge. The top layer was transferred into a clean Z10 tube, using a long bent glass pasteur pipette, and 2.5ml chloroform: isoamylalcohol (24:1) was added and mixed by inversion. The layers were separated by centrifugation at 3000rpm for 10 minutes, as before, and the top layer transferred to another clean Z10 tube. This step was repeated one further time, and the final top aqueous layer was transferred to a clean Z10 tube. Sodium acetate 0.25ml [3M, pH 7.0 - 8.0] and ethanol 5.5ml were added, and the tubes inverted. The resulting DNA pellet was transferred to an autoclaved 0.5mL plastic tube, using a sealed glass pipette. The tubes were spun in a microfuge using the pulse technique, and any remaining ethanol removed. Autoclaved TE buffer pH 7.6, 0.25ml, [1.58g/L Tris-HCL or 1.21g/L Tris base 10mM, 0.37g/L 1mM EDTA] was added and mixed by pipetting, and the samples left overnight at 4°C. The optical density of a 1/5 dilution of DNA (50µl DNA in 200µl distilled H_2O) was measured at 260nm and 280nm using a 0.5ml quartz cuvette. The DNA concentration was determined using the formula; DNA concentration = OD@260nm x dilution x 50µg/ml DNA. The OD ratio 260/280 was between 1.7 - 2.0. The samples were then stored at -20°C.

2.8.2 Genotype analysis

DNA amplification and digestion was performed using the polymerase chain reaction (PCR) with the appropriate restriction enzyme (see below).

2.8.2.1 PCR and restriction fragment length polymorphism (RFLP)

The polymerase chain reaction (PCR) was used to detect the C161T at exon 6 of the PPARγ gene, the P12A in the PPARγ-2 isoform-specific exon B of the PPARγ gene, the G2548A polymorphism of the LEP gene, a tetranucleotide repeat polymorphism in the 3'-flanking region of the human leptin gene (Shintani *et al* 2002), the IL-10 C592A of the IL-10 gene, the Q192R in the paraoxonase 1 (PON1) gene and the L55M in the PON1 gene.

Primer sequences and the PCR amplification programme were as detailed in table 2-5. The amplifications were each performed in an 11μL volume, using 1μL of DNA per sample, and the reaction mixtures are described in table 2-6. For each polymorphism, the PCR products were digested with the appropriate restriction enzyme (table 2-5), with manufacturer's incubation buffer, 0.4μL acetylated BSA in the case of Lep G2548A and PON L55M, and filtered sterile water in a total volume of 30μL at 37°C for 3 hours. The LEPTET3 polymorphism did not require digestion.

Polymorphism	Primer	PCR programme	Restriction enzyme (recognition site)
PPARγ C161-T	Sense 5'-CAA GAC AAC CTG CTA CAA GC-3' Anti-sense 5'-TCC TTG TAG ATC TCC TGC AG-3'	94°C for 1 min 94°C for 30 sec ⎫ 56°C for 30 sec ⎬ x34 72°C for 1 min ⎭ 72°C for 5 min	4U Eco 72I (MBI Fermentas) (5'....CAC↓GTG....3')
PPARγ P12-A	Sense 5'-GCC AAT TCA AGC CCA GTC-3' Anti-sense 5'-GAT ATG TTT GCA GAC AGT GTA TCA GTG AAG GAA TCG CTT TCC G-3'	94 for 2 min 94°C for 15 sec 64°C for 15 sec 72°C for 30 sec 94°C for 15 sec 62°C for 15 sec 72°C for 30 sec 94°C for 15 sec 60°C for 15 sec 72°C for 30 sec 94°C for 15 sec 58°C for 15 sec 72°C for 30 sec 94°C for 15 sec ⎫ 56°C for 15 sec ⎬ x25 72°C for 30 sec ⎭ 72°C for 5 sec	4U Bst UI (New England Biolabs, Hertfordshire) (5'....CG↓CG....3')
Leptin G-2548A	Sense 5'-TTT CCT GTA ATT TTC CCG TGA G-3' Anti-sense 5'-AAA GCA AAG ACA GGC ATA AAA A-3'	95 for 5 min 94 for 60 sec ⎫ 50 for 60 sec ⎬ x30 72 for 60 sec ⎭ 72 for 5 min	6U HhaI (New England Bio Labs) (5'....GCG↓C....3')
LEPTET3	Sense 5'-AGT TCA AAT AGA GGT CCA AAT CA-3' Anti-sense 5'-TTC TGA GGT TGT GTC ACT GGC A-3'	for 3 min 94 for 30 sec ⎫ 64 for 30 sec ⎬ x35 72 for 1 min ⎭ 72 for 10 min	Nil
IL-10 C592A	Sense 5'-GTT CCT CCC AGT TAC AGT CT-3' Anti-sense 5'-CTG TCT TGT GGT TTG GTT TT-3'	94 for 5 min 94 for 15 sec ⎫ 61 for 15 sec ⎬ x34 72 for 30 sec ⎭ 72 for 5 min	4U RsaI (Abgene, Epsom, Surrey) (5'-....GT↓AC....3')
PON-1 L55M	Sense 5'-GAA GAG TGA TGT ATA GCC CCA G 3' Anti-sense 5'-TTT AAT CCA GAG CTA ATG AAA GCC-3'	95°C for 15 sec ⎫ 55°C for 15 sec ⎬ x34 72°C for 30 sec ⎭ 72°C for 5 min	15U Hsp92 II (Promega, Madison, WI, USA) (5'-CA↓TG-3')
PON-1 Q192R	Sense 5'-TAT TGT TGC TGT GGG ACC TGA G-3' Anti-sense 5'-CAC GCT AAA CCC AAA TAC ATC TC-3'	95°C for 15 sec ⎫ 58°C for 15 sec ⎬ x34 72 °C for 30 sec ⎭ 72°C for 5 min	2U Alw I (New England, Biolabs.) (5'-GGATC (N)$_4$↓-3')

Table 2-5. Polymorphisms, primers, PCR programme and restriction enzyme used in RFLP-PCR.

Reaction constituents (per sample)	PPARγ C161-T	PPARγ P12-A	Lep - G2548A	LEPTET-3	IL-10 C592A	PON1-55	PON1-192
Filtered water	5.5µL	5.3µL	6.6µL	5.5µL	6.6µL	6.5µL	6.5U/L
10x Taq polymerase buffer (Bioline 16mM ammonium sulphate)	1µL	1µL	1µL	1µL	1µL	1 µL	1µL
dNTPs	1nmol/L	1nmol/L	1nmol/L	1nmol/L	1nmol/L	1 nmol/L	1 nmol/L
Primers (forward and reverse)	20pmol/L	20pmol/L	10pmol/L	20pmol/L	10pmol/L	10 pmol/L	10 pmol/L
$MgCl_2$	2 mmol/L	3 mmol/L	1.5 mmol/L	2mmol/L	1.5mmol/L	2mmol/L	2mmol/L
Taq polymerase	0.5U	0.5U	0.5U	0.5U	0.5U	0.5U	0.5U

Table 2-6. PCR reaction constituents per polymorphism.

PCR products were run in a 2.5% agarose gel containing ethidium bromide (0.1µg/ml), along with pBR322 DNA-Msp1 digest (New England Biolabs, Inc., Beverly, MA) as a molecular weight marker for Lep G2548A, and a 100bp ladder (Gibco BRL, Paisley, UK) as a molecular weight marker for the other polymorphisms.

2.8.2.2 Amplification refractory mutation system-PCR

For determination of allelic polymorphisms in the IL-10 promoter gene at positions –1082 and –819, the amplification refractory mutations system (ARMS)-PCR method was employed (Perrey *et al* 1999). The primer sequences and conditions are demonstrated in table 2-7.

	IL-10 819	IL-10 1082
Generic primer (anti-sense)	5'-AGG ATG TGT TCC AGG CTC CT-3'	5'-CAG TGC CAA CTG AGA ATT TGG-3'
Primer C/G sense	5'-CCC TTG TAC AGG TGA TGT AAC-3'	5'-CTA CTA AGG CTT CTT TGG AGA-3'
Primer A/T sense	5'-ACC CTT GTA CAG GTG ATG TAA T-3'	5'-ACT ACT AAG GCT TCT TTG GGA A-3'
Internal control primer 1	5'-GCC TTC CCA ACC ATT CCC TTA-3'	
Internal control primer 2	5'-TCA CGG ATT TCT GTT GTG TTT C-3'	
PCR product size	429bp	233bp

Table 2-7. ARMS-PCR primers.

The DNA was amplified in an 11µl reaction. Final concentrations of reagents were 8.5% (w/v) sucrose (VWR, BDH, Leicestershire), 200µM each dNTPs (Roche), 1.5mM $MgCl_2$ (GeneAmp) (Applied Biosystems), 1µL 10X GeneAmp Gold PCR buffer (Applied Biosystems), 5 µM specific primer mix (TAGN), 4µM internal control primer mix (TAGN) and 1 µM AmpliTaq Gold (Applied Biosystems). The specific primer mix consisted of 10µM generic primer and 10µM of one of the two allele-specific primers. The internal control primers were used to check for successful PCR amplification. These

primers amplify a human growth hormone sequence (Aldener-Cannava and Olerup 1994, Perrey *et al* 1999). The PCR reactions were performed for 30 cycles of 15 seconds at 95°C, 30 cycles of 50 seconds at 59°C and 30 cycles of 45 seconds at 72°C, with an initial denaturation of 1 min at 95°C. Two PCRs were required for each sample (sense and anti-sense). PCR products were run in a 2.5% agarose gel containing ethidium bromide (0.1μg/ml), along with a 100bp ladder (Gibco BRL, Paisley, UK) as a molecular weight marker.

2.9 Quantitative reverse transcription PCR (TaqMan)

RNA was extracted using the Trizol™ method according to the manufacturers instructions (Invitrogen, Paisley UK). RNA was treated with DNAse-1 (2U) using DNA-free kit (Ambion, Huntingdon, UK, cat. no. 1906). cDNA was synthesised from approximately 1μg of RNA using the Superscript First Strand Synthesis RT-PCR kit (Invitrogen, Paisley, UK, 11904-018). PPARα, PPARγ, IL-6, IL-10, TNF-α and leptin cDNA were quantitated using TaqMan technology on an Applied Biosystems 7900. For normalisation of quantitative results, the reference gene 18S was always amplified. The primer probe sets for PPARα, IL-6, IL-10, TNFα and leptin, and the 18s mRNA control probe were purchased from Applied Biosystems (Warrington, UK). The PPARγ primer probe set was designed in-house (NM_005037), using a 26 base pair region of the PPARγ gene as the forward primer [GAA ACT TCA AGA GTA CCA AAG TGC AA (exon 1)] and a 27 base pair region as the reverse primer [AGG CTT ATT GTA GAG CTG AGT CTT CTC (exon 2)]. A 30 base pair region was used as the T probe [CAA AGT GGA GCC CTG CAT CTC CAC CTT ATT].

For all Applied Biosystems probes, 1.25μL of 20 x target assay or control assay mix was added to12.5μL of 2 x TaqMan Mastermix (Applied Biosystems), 10.25μL deionised distilled water and 1μL cDNA. A 1:100,000 dilution of cDNA was required for the 18S control, and data is adjusted accordingly. For the PPARγ probe, 1μL 5μM Probe T, 0.75μL 10μM Primer F and 0.75μL 10μM Primer R was added to 12.5μL of 2x TaqMan Mastermix, 9μL deionised distilled water and 1μL cDNA. Serial dilutions of cDNA were performed in duplicate for each probe (including 18S) on placental and adipose tissue, to determine the optimal dilution to obtain a Ct value of around 28-30. The placental tissue did not require dilution of the 1μL cDNA stock, except in the case of leptin TaqMan analysis, where a 1/10 dilution was used. A 2μL stock solution of cDNA was required for

the adipose tissue in all cases, except for the leptin analysis, where once more, a 1/10 dilution of cDNA was used. Data is adjusted accordingly. The thermal cycler conditions were 50°C 2min, 95°C for 10 min, followed by 40 x 95°C for 15 secs and 60°C for 1min. Each amplification was carried out in triplicate, and each plate had a deionised distilled water control and a no reverse transcriptase control. The cycle threshold (Ct) value between samples measured in triplicate was studied, and any outlier (approximately greater than or less than 1) eliminated, and the mean value calculated from the remaining 2 values.

The TaqMan assay is based on the 5' endonuclease activity of the *Taq* polymerase. Briefly, within the amplicon defined by a gene-specific oligonucleotide primer pair, an oligonucleotide probe labelled with 2 fluorescent dyes is designed. As long as the probe is intact, the emission of a reporter dye (i.e. 6-carbon-flourescein, FAM) at the 5'-end is quenched by the second fluorescence dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3' end. During the extension phase of the PCR, the *Taq* polymerase cleaves the probe, releasing the reported dye. An automated photometric detector combined with special software (ABI Prism 7900 Sequence Detection System, Perkin-Elmer Corp., Foster City, CA) monitors the increasing reporter dye emission. The algorithm normalises the signal to an internal reference (ΔRn) and calculates the threshold cycle number (C_T), when the ΔRn reaches 10 times the standard deviation (SD) of the baseline. The C_T values of target relative to the C_T of the control gene (18s) can be used to measure relative 'fold' difference in gene expression between tissues.

2.10 Autoradiograph scanning and statistical analysis

The intensity of the bands on the autoradiographs, for each of the PPARs and for RXRα, was compared with 18s in the observational study, and both 18s and PAI-2 in the case control study. Ratios determined using the Bio-Rad Multi-Analyst ™/PC version 1.1. Western blots were scanned on the same system. Differences in band intensity were tested using the Mann Whitney-U test, and data are presented as medians and interquartile range. $P<0.05$ was considered to be significant. Correlations were performed using Pearson's coefficient of correlation. Differences in TaqMan analyses were also tested using Mann Whitney-U test as above. A power calculation demonstrated that there was at least 80% power to detect a 50% difference in expression with a standard deviation (SD) of 30%, with a sample size of 6 per group (adipose tissue data) and at least 90% power to detect a 75% difference in expression with a SD of 50%, with a sample size of 10 per group (placental data). The Mann Whitney-U test was employed in the case of the tissue data, as

there was no large comparative dataset from uncomplicated pregnancies, and so data was not normalised. Associations of adipose and placental tissue with plasma inflammatory markers were determined using the raw unadjusted and uncorrected plasma data to correspond with the tissue data. For this reason, differences between these data were also calculated using non-parametric tests.

2.11 Statistical analysis

2.11.1 Bloods

Blood assay data for lipids (TC, TG, VLDL, LDL, HDL), markers of inflammation (IL-6, IL-10, TNFα and leptin) and markers of insulin resistance (SHBG) was tested for normality using the Ryan-Joiner test for normality in MINITAB 13 (MINITAB, State College, PA), using a cohort of 81 maternal blood samples and 41 fetal blood samples from third trimester uncomplicated pregnancies (see table 2-1). For fasting parameters (HOMA, fasting glucose and insulin), a cohort of 63 fasted samples from the original 81 maternal samples was used for normalisation, with 29 fetal samples available from these. Maternal PON-1 data was tested as above, on 53 available results from the 81 maternal samples, but there were only 9 fetal PON-1 values in uncomplicated pregnancy, and so these were logged as for the maternal data. Maternal TG, maternal HDL, maternal PON-1, maternal VCAM, maternal TNFα, maternal IL-6, maternal CRP, maternal SHBG, maternal fasting insulin and glucose, fetal TC, fetal TG, fetal PON-1, fetal ICAM, fetal IL-6, fetal IL-6/IL-10 and fetal CRP were log transformed to achieve normality. Erythrocyte membrane fatty acid data was also tested for normality using the same method, from a cohort of 47 third trimester maternal blood samples obtained from the uncomplicated longitudinal study. Maternal 14:0, 16:0, 18:0, 18:1n9, 20:0, 20:1n9, 20:2n6, 20:3n3, 20:5n3, 22:3n3, 22:5n6, 24:0, % monounsaturated fatty acids, total n9, n6/n3, Δ6 desaturase and Δ9 desaturase were log transformed to achieve normality. The square root of the total n7 fatty acids was used for normality. The data are presented as mean and standard deviation, and additionally for log transformed data, geometric mean and standard deviation. Statistical support was provided for the repeated measures tests in the fatty acid analyses, by Dr Barbara Meyer, University of Wollongong, NSW, Australia. Differences were tested for statistical significance using ANOVA or a 2-sample *t* test for continuous variables and Chi-squared test for categorical variables. Correlations were performed using Pearson's coefficient of correlation. Simple regression analysis or ANOVA was used to examine the effect of maternal age, BMI, parity, gestational age at sampling and smoking

status on all blood analyses. Fetal lipid data was adjusted for the effect of mode of delivery, and maternal PON-1 was adjusted for the effect of gestational age at sampling using the General Linear Model within MINITAB.

2.11.2 DNA polymorphism analyses

For each polymorphism, numbers of individuals of each genotype were counted and allele frequencies calculated. Each polymorphism was tested for Hardy-Weinberg equilibrium using the Chi-squared test (Falconer and Mackay 1996, p5). Individuals who were common homozygotes were termed the referent class for each polymorphism and odds ratio (OR) and confidence intervals (CI) for the development of PET were calculated for the heterozygotes and rare homozygotes respectively. P values were calculated by Chi-squared test or by Fisher's exact test when cells had less than 5 individuals.

For comparison of plasma inflammatory markers between genotypes, the heterozygotes and rare homozygotes (i.e. rare allele carriers) for PPARγ P12A were combined for calculations, due to small numbers available. Baseline (first trimester) characteristics and plasma levels of inflammatory markers by PPARγ P12A were tabulated and compared between the PET and control groups using 2-sample *t*-tests for continuous data or chi-squared test for categorical data. For the Leptin 3'tet polymorphism, statistical analysis was performed using ANOVA, with post-hoc 2-sample *t*-tests. The majority of the inflammatory dataset used for this analysis has been described previously (Freeman *et al* 2004). For the purposes of analysis in the present study, transformed data was used if the previous data (Freeman *et al* 2004) demonstrated a skewed distribution. Within a group (PET or controls), changes at third trimester over first trimester (baseline) by polymorphism were compared using 2-sample *t*-tests on raw data. Due to small sample sizes, data were not adjusted for potential covariates and no adjustments were made for multiple comparisons. Mean with SD and associated *P* values are reported.

3 Immunolocalisation and the mRNA and protein expression of PPARs in placenta

3.1 Introduction

In the Introduction (chapter 1), the role of PPARα and γ agonists in the treatment of atherosclerosis has been discussed. The lipid lowering and insulin sensitising properties of these agonists indicate that their mode of action is through alterations in the metabolic disturbances associated with CVD. A role for PPARs in pregnancy has also been described. As discussed previously, PET, with widespread endothelial damage and dysfunction and increased systemic inflammatory response, shares many metabolic risk factors with cardiovascular disease. Thus potential interventions in this disorder might reasonably be directed at these metabolic disturbances. PPARs are therefore excellent candidates as mechanistic agents underlying the metabolic complications of pregnancy and are amenable to drug intervention.

Based on the evidence demonstrating a role for PPARs in normal pregnancy, in the metabolic complications of pregnancy and in cardiovascular disease, and based on the data linking PET and IUGR with future cardiovascular risk, it was hypothesised that PPAR mRNA and protein expression might increase during gestation and be compromised in complications of pregnancy with placental pathology such as PET and IUGR.

The aim of this chapter was thus to determine the placental localisation and expression of each of the PPARs in uncomplicated pregnancy in the first instance, and then to compare this localisation and expression with that in pregnancies complicated by PET and IUGR in a case control study. The immunocytochemistry and Northern blotting techniques were employed in this study, as they are established techniques within our laboratory, and would either confirm or refute findings from previous studies using these techniques (Wang *et al* 2002). However, quantitative protein expression of the PPARs using western analysis was a technique developed specifically for this study. The quantitative real time (RT)-PCR mRNA expression of PPARα using TaqMan was also developed for this study, as the probe used in the Northern blotting did not provide reproducible results. We also have confirmation of our PPARγ mRNA results using TaqMan in chapter 5. Samples were collected from women in the 3rd trimester of uncomplicated pregnancy and from pregnancies complicated by PET or IUGR at the Princess Royal Maternity Hospital,

Glasgow. These samples were supplemented with samples from an archival collection of 1st, 2nd and 3rd trimester placentae from uncomplicated pregnancies (I.A. Greer, personal collection). In the latter group, only data detailing mode of delivery and gestational age were available. The 18S control gene was used in this study, as no variation of expression under experimental conditions has been reported (de Leeuw *et al* 1989, Zhong and Simons 1999), and consistent levels have been described throughout gestation (Patel *et al* 2002). This control gene attempts to control for cell number, and was considered superior to GAPDH, which has previously been used in similar studies as a constitutively expressed housekeeping gene. It is likely that GAPDH levels may differ between metabolically active cells and quiescent cells. Variation of expression of GAPDH under hypoxic conditions has been reported (Zhong and Simons 1999). This is relevant in placental tissue, which is relatively hypoxic, and may be exacerbated in pathological conditions of pregnancy, which affect the placenta (Heid *et al* 1996, Patel *et al* 2002). The PAI-2 gene was also used as a control gene in the PET/IUGR study, with the aim of controlling for trophoblast cell number. PAI-2 is synthesized by the trophoblast and in uncomplicated pregnancy, concentrations increase progressively as pregnancy develops. However, levels decrease with reduced placental function (Halligan *et al* 1994). This is relevant to the study of metabolic complications of pregnancy, where areas of placenta may be infarcted or structurally damaged. Hence expression of genes relative to the expression of PAI-2 may correct for increased necrosis in pathological placentae.

3.2 Results

3.2.1 Baseline characteristics

Baseline characteristics for the PET, IUGR and controls are demonstrated in table 3-1. There were no demographic data available for the archival collection. PET and IUGR cases had significantly earlier gestational ages at delivery and lower placental weights, fetal weights and birth weight centiles compared with control subjects.

Characteristics	PET			IUGR		
	Case (n=10)	Control (n=10)	p	Case (n=10)	Control (n=10)	p
Age (years)	29.0 24.8-31.3	28.5 23.8-32.0	0.85	31.0 27.8-33.0	31.5 29.5-33.0	0.65
BMI (kg/m^2)	25.5 (22.8-34.0)	26.0 (23.8-29.3)	0.91	25.0 (20.5-30.5)	25.0 20.0-32.5	0.97
Primigravidae n (%)	8 (80%)	8 (80%)	1.00	5 (50%)	5 (50%)	1.00
Smokers n (%)	3 (30%)	3 (30%)	1.00	6 (60%)	4 (40%)	0.37
Gestation at delivery (weeks)	35.5 34.3-39.3	40.5 39.0-41.0	0.008	36.0 34.0-38.0	39.0 38.8-40.3	0.005
Vaginal delivery n (%)	6 (60%)	7 (70%)	0.64	1 (10%)	1 (10%)	1.00
Placental weight (g)	490 308-645	680 588-736	0.008	272 246-417	635 533-762	<0.001
Fetal weight (kg)	2.61 1.87-2.93	3.54 3.33-3.91	<0.001	1.94 1.54-2.07	3.07 2.76-3.91	<0.001
Birth weight centile	10 (8 – 34)	50 (30-75)	0.009	3 (1 – 6)	15 (9-65)	0.003
Fetal sex (%)	30% male 70% female	80% male 20% female	0.03	30% male 70% female	30% male 70% female	1.00

Table 3-1. Baseline characteristics for the PET, IUGR and control subjects (n=10 per group). All values are median and interquartile (IQ) range. Statistical analysis was performed using Mann-Whitney U test for continuous variables, and chi-square for categorical variables.

3.2.2 Immunolocalisation of PPARs

3.2.2.1 PPAR localisation in a gestational series of placental sections

Placental tissue demonstrated maturation from undifferentiated invading trophoblast columns and large stem villi in the 1st trimester, through to formation of outer syncytio- and inner cytotrophoblast layers with intermediate and some terminal villi in the 2nd trimester, to formation of the syncytium with occasional cytotrophoblast cells and many terminal villi in the 3rd trimester. Positive and negative control slides are shown in Plates 1 and 2. In 1st trimester placentae, PPARα, δ, γ and RXRα were localised to the cyto- and syncytiotrophoblast and invading columns (Plates 3 & 4). Occasional isolated stromal cells were stained. In some sections PPARγ staining in the cytotrophoblast was greater than that in the adjacent syncytiotrophoblast (Plate 4A1 & A2). Back to back staining with cytokeratin-7 (CY-7) confirmed that the cells in which PPARs were localised, were of trophoblastic origin (Plate 5). Second trimester localisation of PPARα, δ, γ and RXRα was similar to that in the 1st trimester (Plate 6). Localisation was predominantly cytoplasmic for all of the PPAR antibodies but nuclear staining was also evident. PPARα staining was sparse in the 3rd trimester (Plate 7) with the majority of staining either stromal or within

maternal and fetal blood cells. In some sections faint syncytial staining was observed. Third trimester placentae from uncomplicated pregnancies demonstrated PPARδ, γ and RXRα staining within the syncytium, and localisation within isolated cells in the stroma (Plate 7). This distribution was unaltered by mode of delivery (spontaneous or induced vaginal delivery, elective or emergency Caesarean section – data not shown). The isolated stromal cells to which PPARα, γ and δ were localised in the 3rd trimester were potentially of macrophage origin. In order to clarify this, we co-localised CD68, a macrophage marker, with 'back to back' sections localising each of the PPAR isoforms. There was no obvious relationship demonstrated between the macrophage marker and the stromal cells staining for each of the PPARs (Plate 8). Co-localisation of CD31, an endothelial cell marker, with back-to-back sections of the PPARs confirmed endothelial expression of each of the PPARs, but could not account for all cells staining within the stroma (Plate 8). It is likely that these PPAR-staining stromal cells are of mixed origin.

3.2.2.2 PPAR localisation in PET and IUGR placentae

PPARα staining was sparse in third trimester placentae from pregnancies complicated by PET and IUGR, as for uncomplicated pregnancies (Plate 9). PPARδ and γ and RXRα were localised to the syncytium and cells within the stroma in 3rd trimester placentae from pregnancies complicated by PET or IUGR, similar to findings from uncomplicated pregnancies (Plates 10 - 12). However, staining appeared less abundant and highlighted numerous very thin areas within the syncytium in the PET and IUGR placentae in contrast to sections from uncomplicated pregnancies. Syncytial knots and bridges were also more abundant within the PET sections.

It should be noted that the printed Plates shown below are not truly representative of the quality of the ICC sections at time of image analysis, as transfer of the image on to the printed page is a common problem encountered.

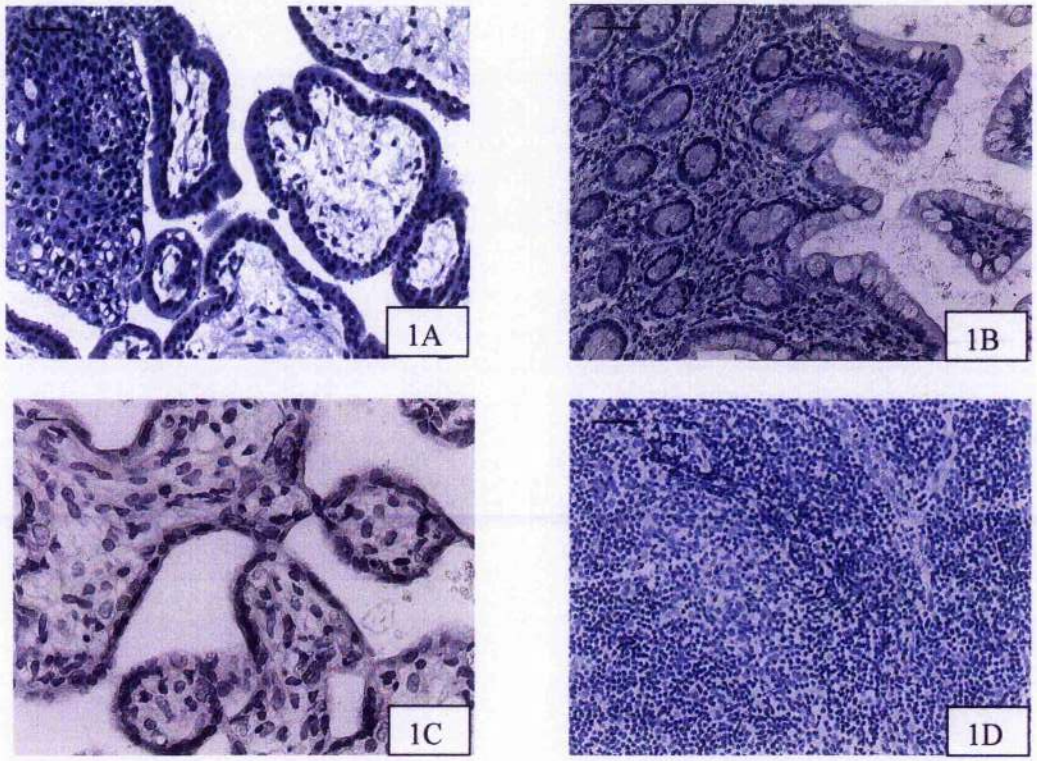


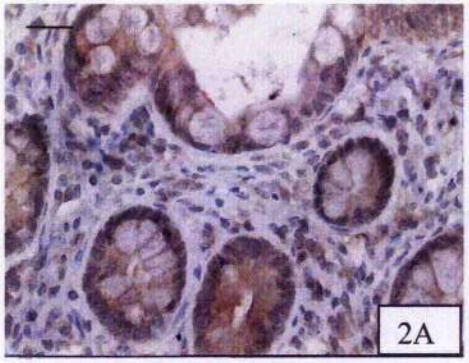
Plate 1. Negative control slides.

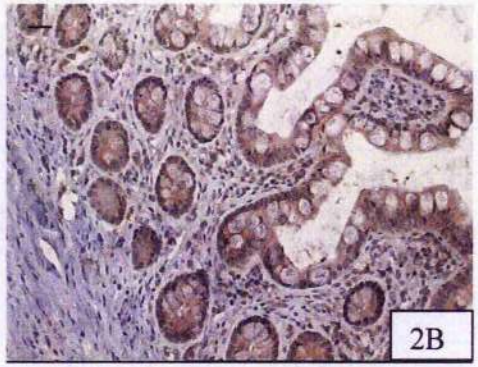
Figure 1A. First trimester (7 weeks gestation) placenta no antibody/2% rabbit serum negative control for PPARα, δ and γ (x20 magnification). Scale bar = 5 μm.

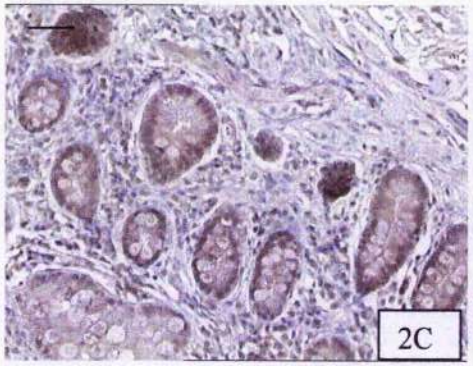
Figure 1B. Small bowel no antibody/2% rabbit serum negative control for PPARα, δ and γ (x20 magnification). Scale bar = 5μm.

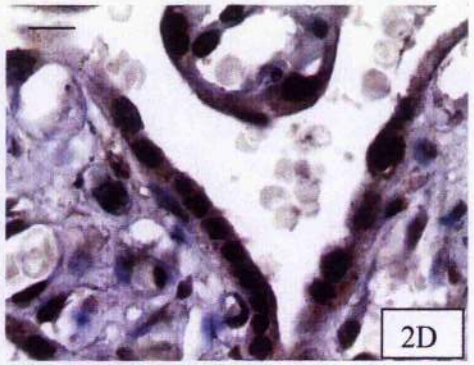
Figure 1C. Third trimester placenta negative control mouse monoclonal antibody against IgG1 Aspergillus Niger glucose oxidase, for monoclonal antibodies i.e. CD31 and CY7 (x40 magnification). Scale bar = 1μm.

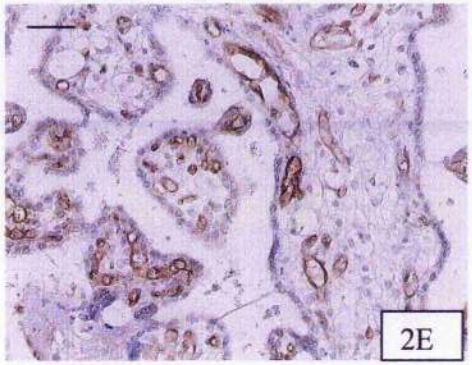
Figure 1D. Tonsil negative control mouse monoclonal antibody against IgG1 Aspergillus Niger glucose oxidase, for CD68 (x20 magnification). Scale bar = 5 μm.

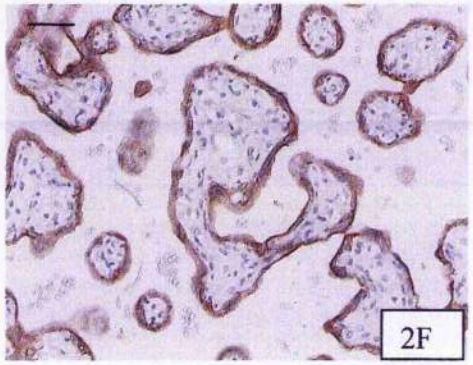












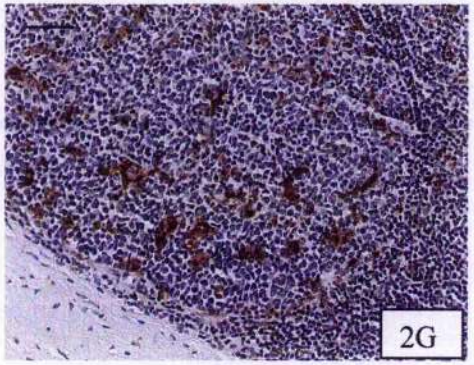


Plate 2. Positive control slides.

Figure 2A. Small bowel PPARα positive control (x100 magnification). Scale bar = 1μm.
Figure 2B. Small bowel PPARδ positive control (x40 magnification). Scale bar = 1μm.
Figure 2C. Small bowel PPARγ positive control (x20 magnification). Scale bar = 5μm.
Figure 2D. Third trimester placental tissue RXRα positive control (x100 magnification). Scale bar = 1μm.
Figure 2E. Third trimester placental tissue CD31 positive control (x20 magnification). Scale bar = 5μm.
Figure 2F. Third trimester placental tissue CY7 positive control (x20 magnification). Scale bar = 5μm.
Figure 2G. Tonsil CD68 positive control (x20 magnification). Scale bar = 5μm.

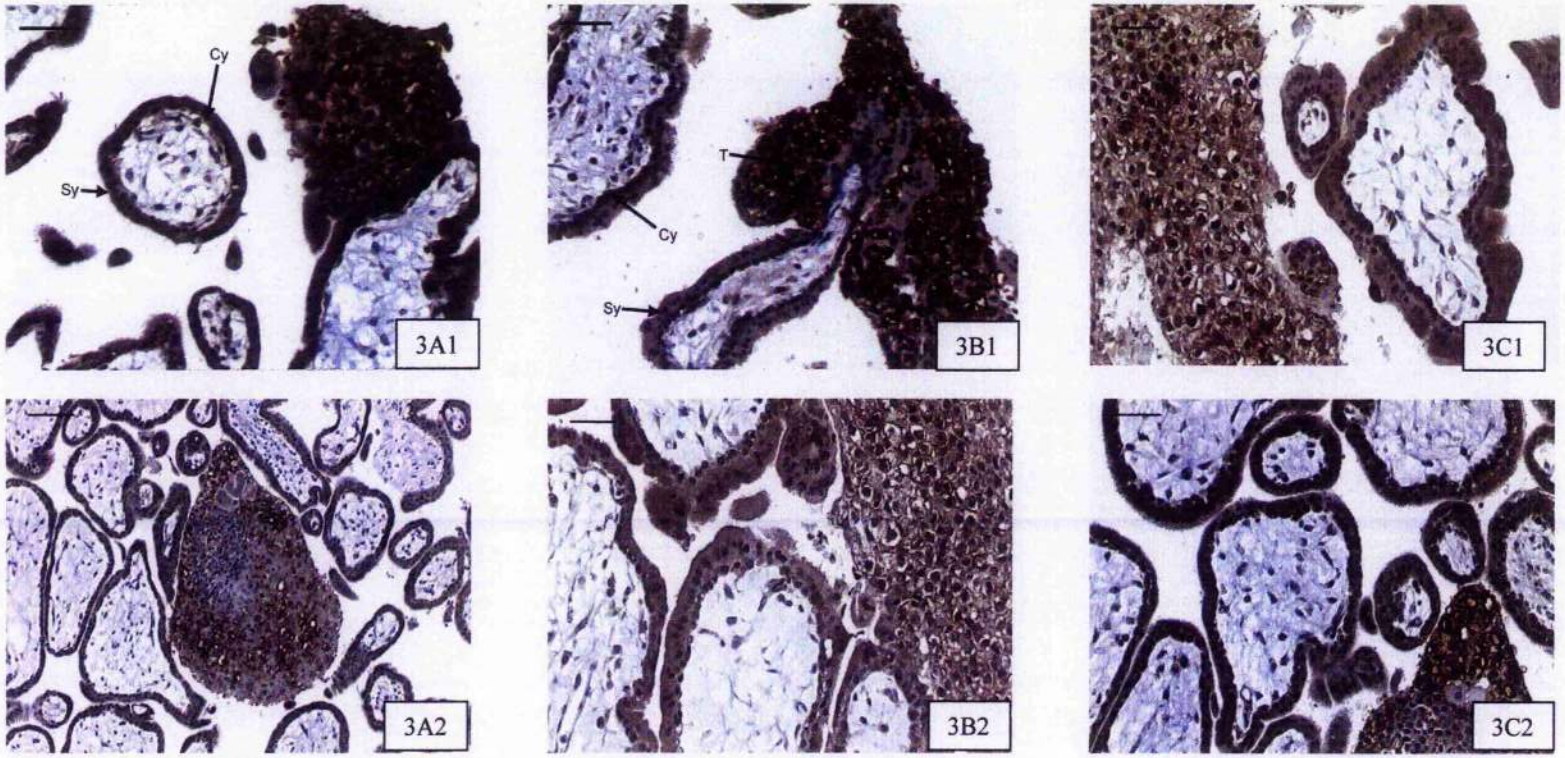


Plate 3.

Figure 3A1 & 3A2. First trimester (7 weeks' gestation) PPARα ICC localisation from 2 different placentae (x20 magnification). Scale bar = 5μm.

Figure 3B1 & 3B2. First trimester (7 weeks' gestation) PPARδ ICC localisation from 2 different placentae (x20 magnification). Scale bar = 5μm.

Figure 3C1 & 3C2. First trimester (7 weeks' gestation) PPARγ ICC localisation from 2 different placentae (x20 magnification). Scale bar = 5μm

Sy = syncytiotrophoblast, Cy = cytotrophoblast, T = trophoblast column.

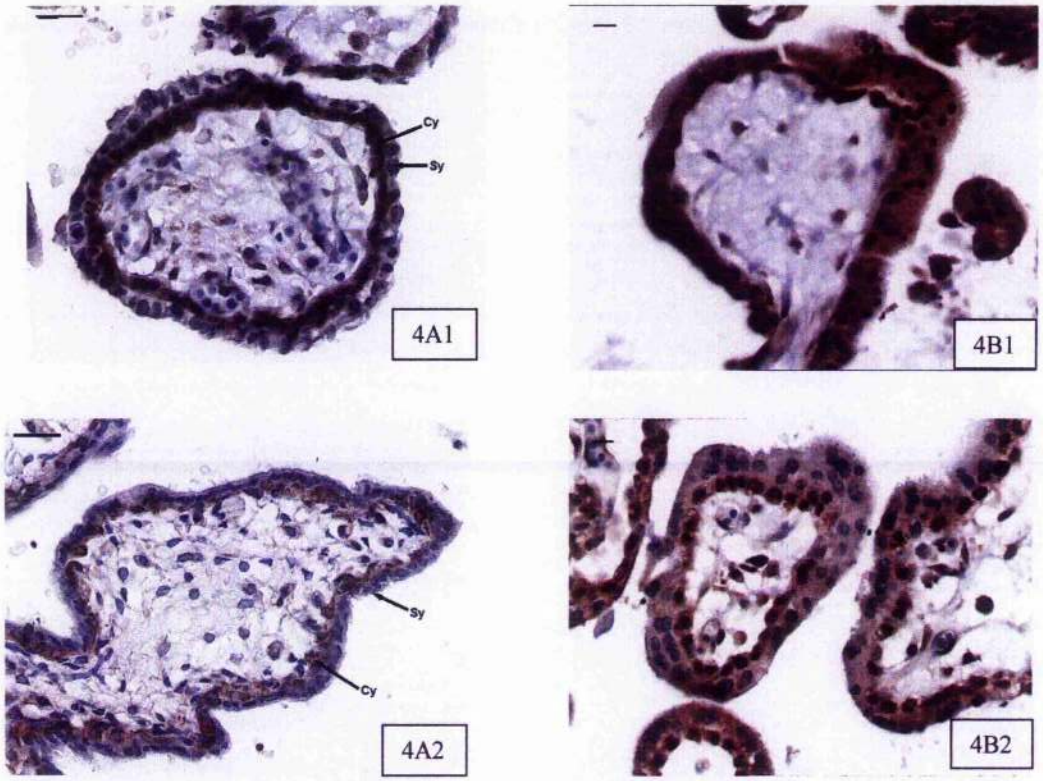


Plate 4.

Figure 4A1 & 4A2. First trimester (7 weeks' gestation) PPARγ ICC localisation from 2 different placentae (x20 magnification). Scale bar = 5μm. Staining is mostly cytotrophoblastic in this section. Sy = syncytiotrophoblast, Cy = cytotrophoblast.

Figure 4B1 & 4B2. First trimester (7 weeks' gestation) RXRα ICC localisation from 2 different placentae (x40 magnification). Scale bar = 1μm.

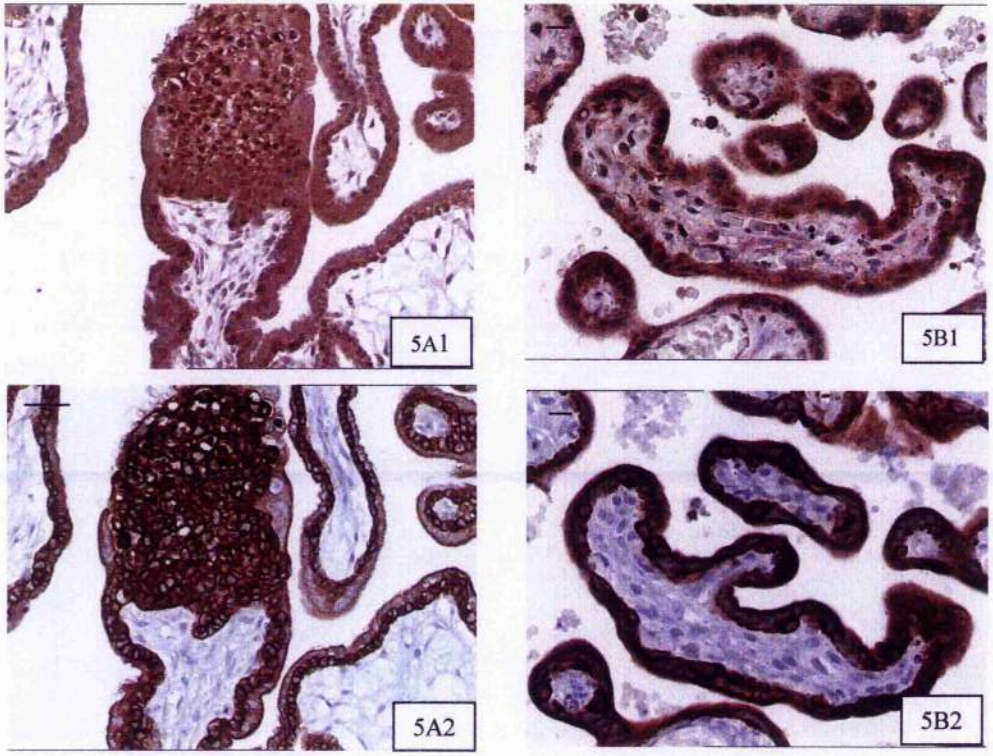


Plate 5.

Figure 5A1 = PPARγ, 5A2 = CY-7 (7 weeks' gestation). An example of PPAR and CY-7 back to back ICC staining (similar to PPARδ) (x 20 magnification). Scale bar = 5 μm.

Figure 5B1 = PPARγ, 5B2 = CY-7 (40 weeks' gestation). An example of PPAR and CY-7 back to back ICC staining (similar to PPARδ) (x 40 magnification). Scale bar = 1 μm.

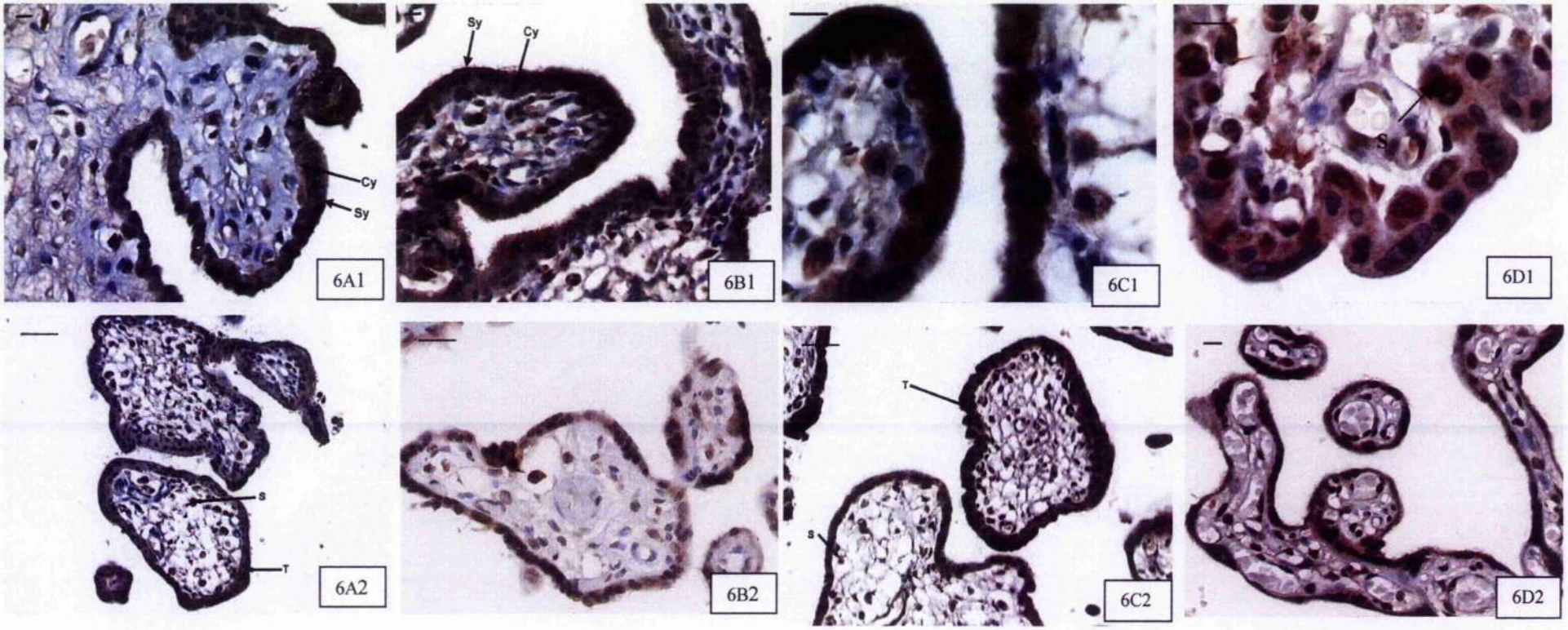


Plate 6.

Figure 6A1 & 6A2. Second trimester (15 weeks' gestation) PPARα ICC localisation from 2 different placentae. A1 = x40 magnification, scale bar = 1μm. A2 = x20 magnification, scale bar = 5 μm.

Figure 6B1 & 6B2. Second trimester (15 weeks' gestation) PPARδ ICC localisation from 2 different placentae. A1 = x40 magnification, scale bar = 1μm. A2 = x20 magnification, scale bar = 5 μm.

Figure 6C1 & 6C2. Second trimester (15 weeks' gestation) PPARγ ICC localisation from 2 different placentae. A1 = x100 magnification, scale bar = 1μm. A2 = x20 magnification, scale bar = 5 μm.

Figure 6D1 & 6D2. Second trimester (15 weeks' gestation) RXRα ICC localisation from 2 different placentae. A1 = x100 magnification, scale bar = 1μm. A2 = x40 magnification, scale bar = 1 μm.

Sy = syncytiotrophoblast, Cy = cytotrophoblast, T = trophoblast column, S = stroma.

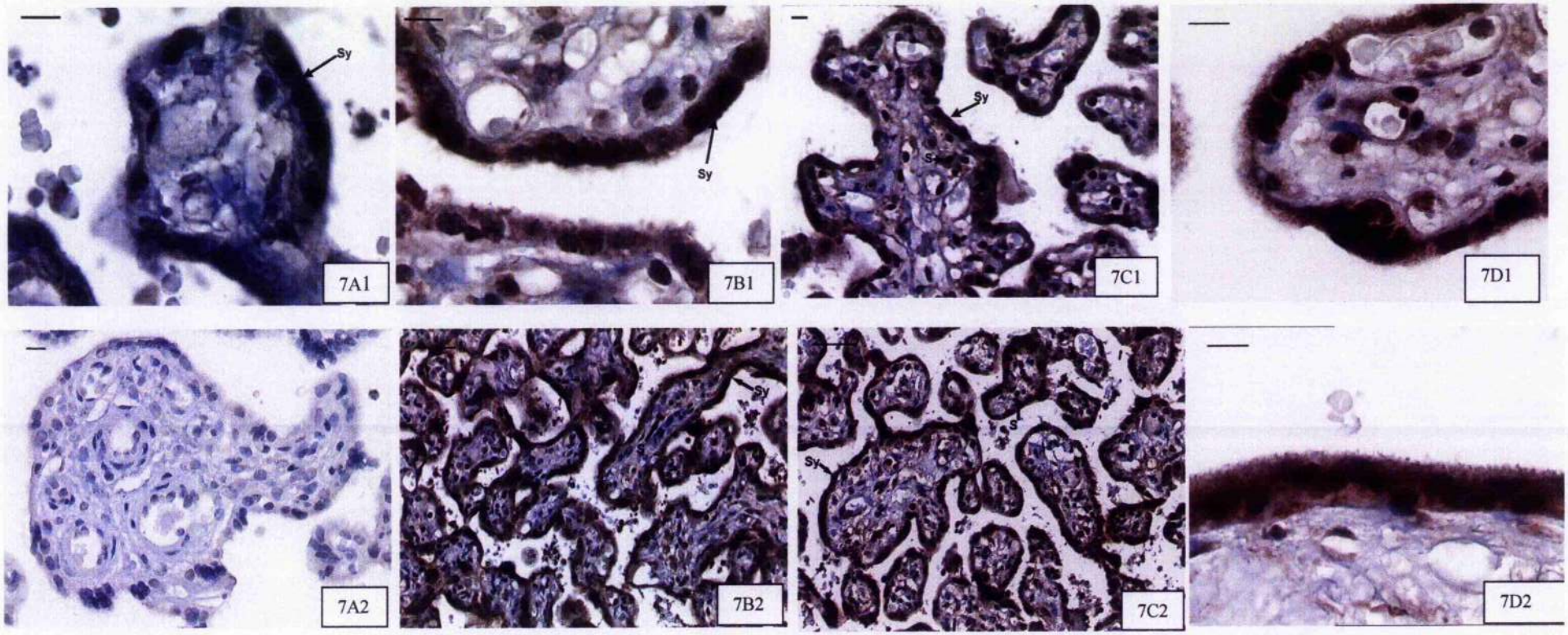


Plate 7.

Figure 7A1 & 7A2. Third trimester (40 weeks' gestation) PPARα ICC localisation from 2 different placentae. A1 = x100 magnification, scale bar = 1μm. A2 = x40 magnification, scale bar = 1 μm.

Figure 7B1 & 7B2. Third trimester (40 weeks' gestation) PPARδ ICC localisation from 2 different placentae. B1 = x100 magnification, scale bar = 1μm. B2 = x20 magnification, scale bar = 5 μm.

Figure 7C1 & 7C2. Third trimester (40 weeks' gestation) PPARγ ICC localisation from 2 different placentae. C1 = x40 magnification, scale bar = 1μm. C2 = x20 magnification, scale bar = 5 μm.

Figure 7D1 & 7D2. Third trimester (40 weeks' gestation) RXRα ICC localisation from 2 different placentae. D1 = x100 magnification, scale bar = 1μm. D2 = x100 magnification, scale bar = 1 μm.

Sy = syncytiotrophoblast.

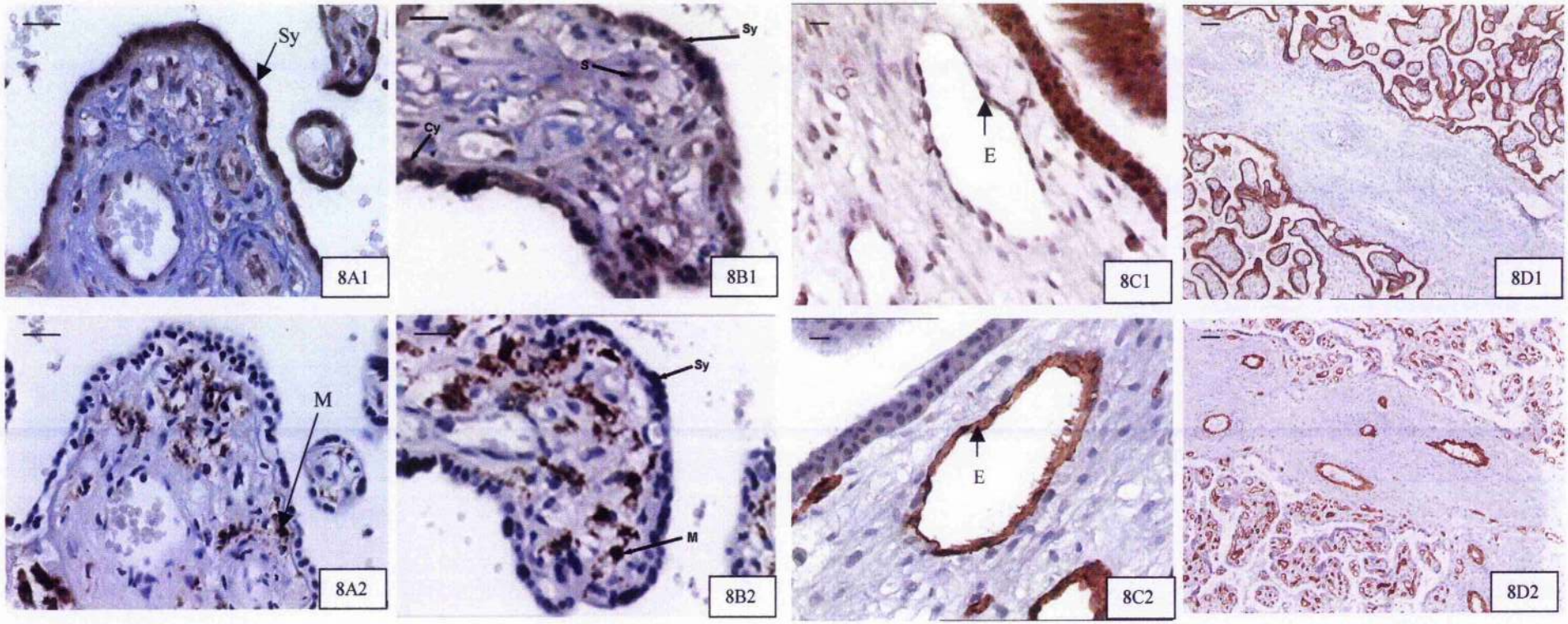


Plate 8.

Figure 8. A1= PPARγ, A2=CD68 (40 weeks' gestation). An example of PPAR and CD68 back to back ICC staining (similar with PPARδ) (x20 magnification). Scale bar = 5μm.

Figure 8. B1= PPARγ, B2=CD68 (40 weeks' gestation). An example of PPAR and CD68 back to back ICC staining (similar with PPARδ) (x20 magnification). Scale bar = 5μm.

Figure 8. C1= PPARγ, C2= CD31 (40 weeks gestation). An example of PPAR and CD31 back to back ICC staining (similar with PPARδ) (x40 magnification). Scale bar = 1μm.

Figure 8. C1= PPARγ, C2= CD31 (40 weeks gestation). An example of PPAR and CD31 back to back ICC staining (similar with PPARδ) (x10 magnification). Scale bar = 5μm.

M = Macrophage, Sy = syncytiotrophoblast, Cy = cytotrophoblast, S = stroma, E = endothelium.

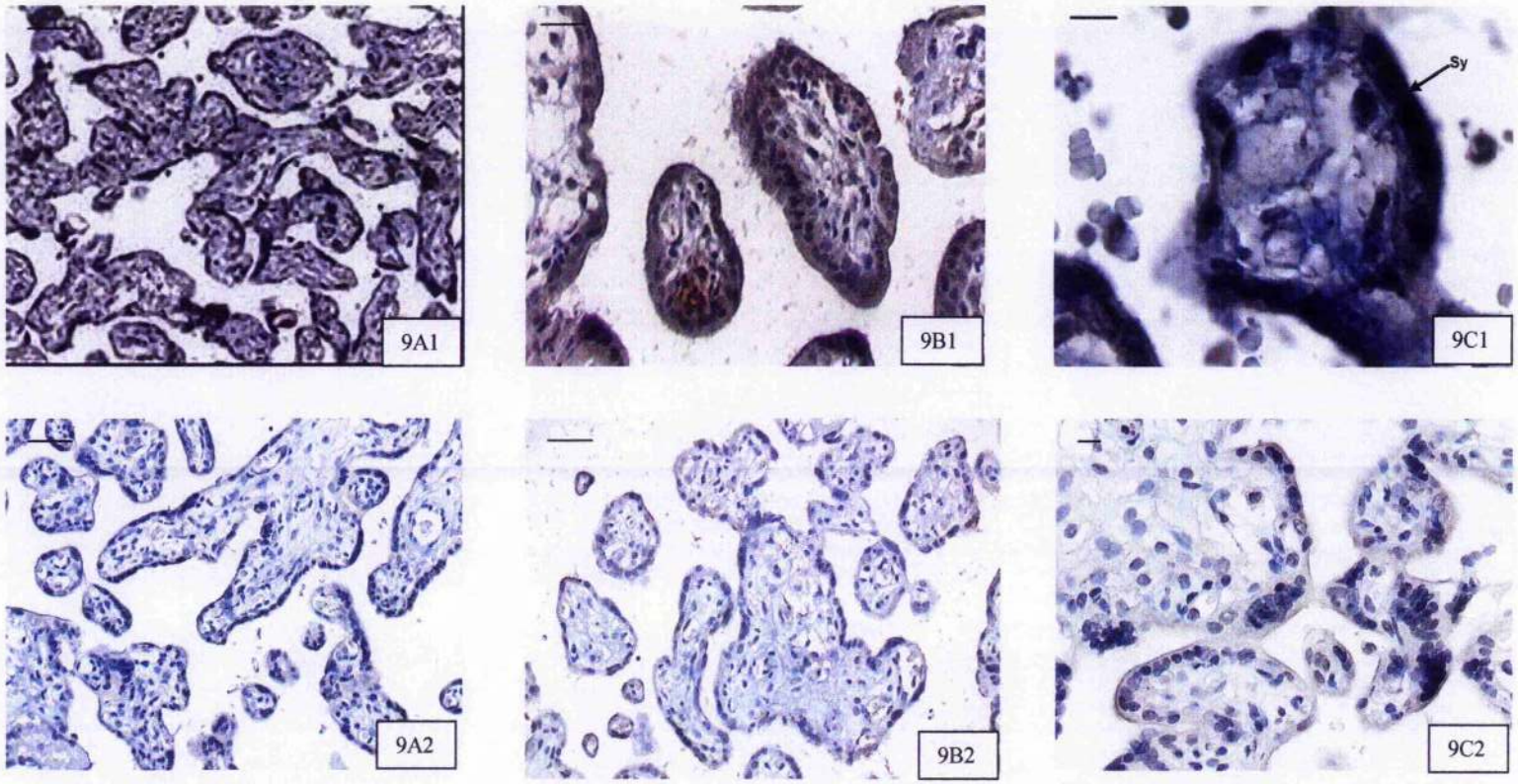


Plate 9.

Figure 9A1 & 9A2. PPARα ICC in 2 different PET placentae (third trimester). A1 & A2 = x20 magnification, scale bar = 5μm.
Figure 9B1 & 9B2. PPARα ICC in 2 different IUGR placentae (third trimester). B1 & B2 = x20 magnification, scale bar = 5μm.
Figure 9C1 & 9C2. PPARα ICC in 2 different uncomplicated third trimester placentae. C1 = x100 magnification, scale bar = 1μm. C2 = x40 magnification, scale bar = 1μm.
Sy = syncytiotrophoblast.

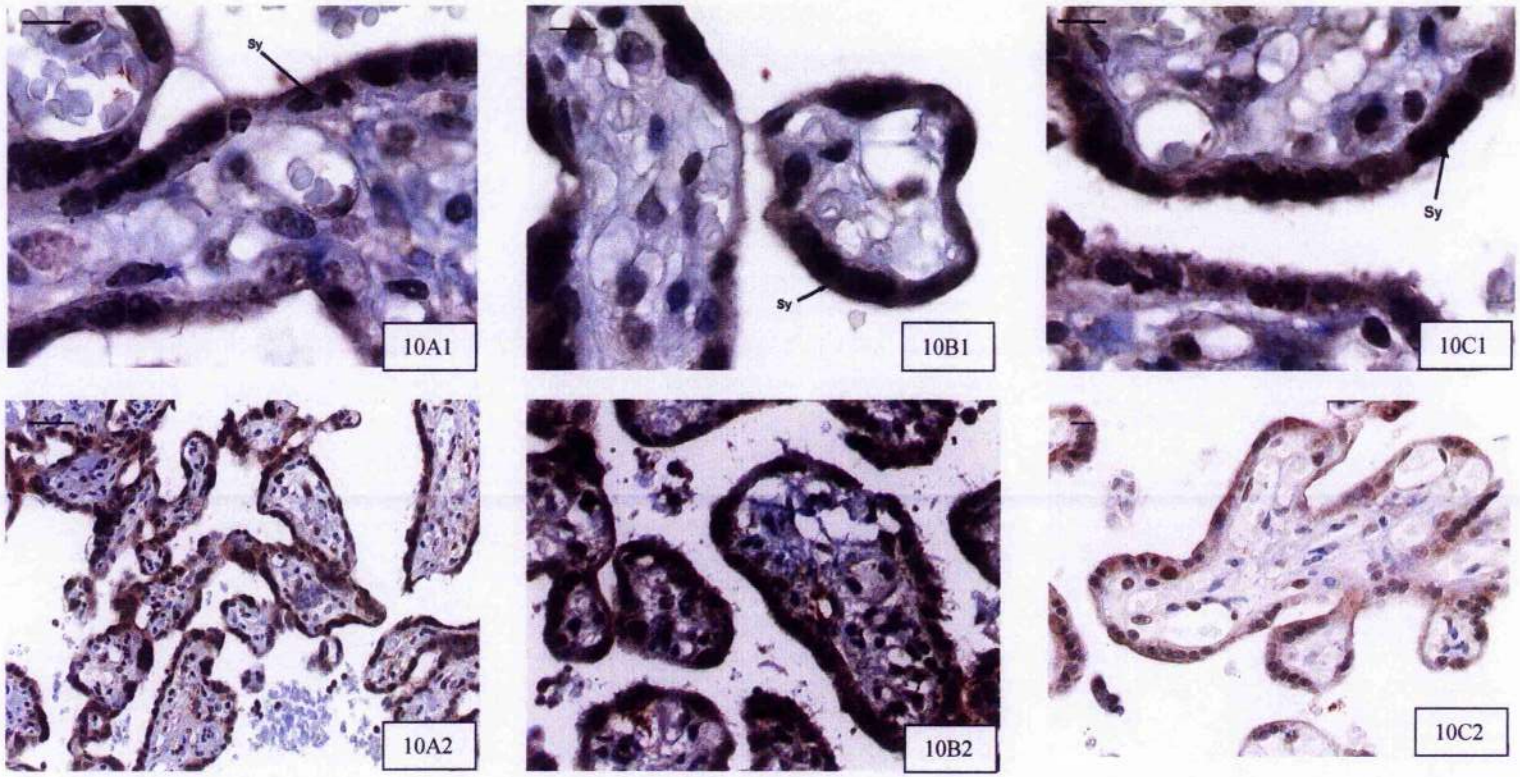


Plate 10.

Figure 10A1 & 10A2. PPARδ ICC in 2 different PET placentae (third trimester). A1 = x100 magnification, scale bar = 1μm. A2 = x20 magnification, scale bar = 5μm.

Figure 10B1 & 10B2. PPARδ ICC in 2 different IUGR placentae (third trimester). B1 = x100 magnification, scale bar = 1μm. B2 = x40 magnification, scale bar = 1μm.

Figure 10C1 & 10C2. PPARδ ICC in 2 different uncomplicated third trimester placentae. C1 = x100 magnification, scale bar = 1μm. C2 = x40 magnification, scale bar = 1μm.

Sy = syncytiotrophoblast.

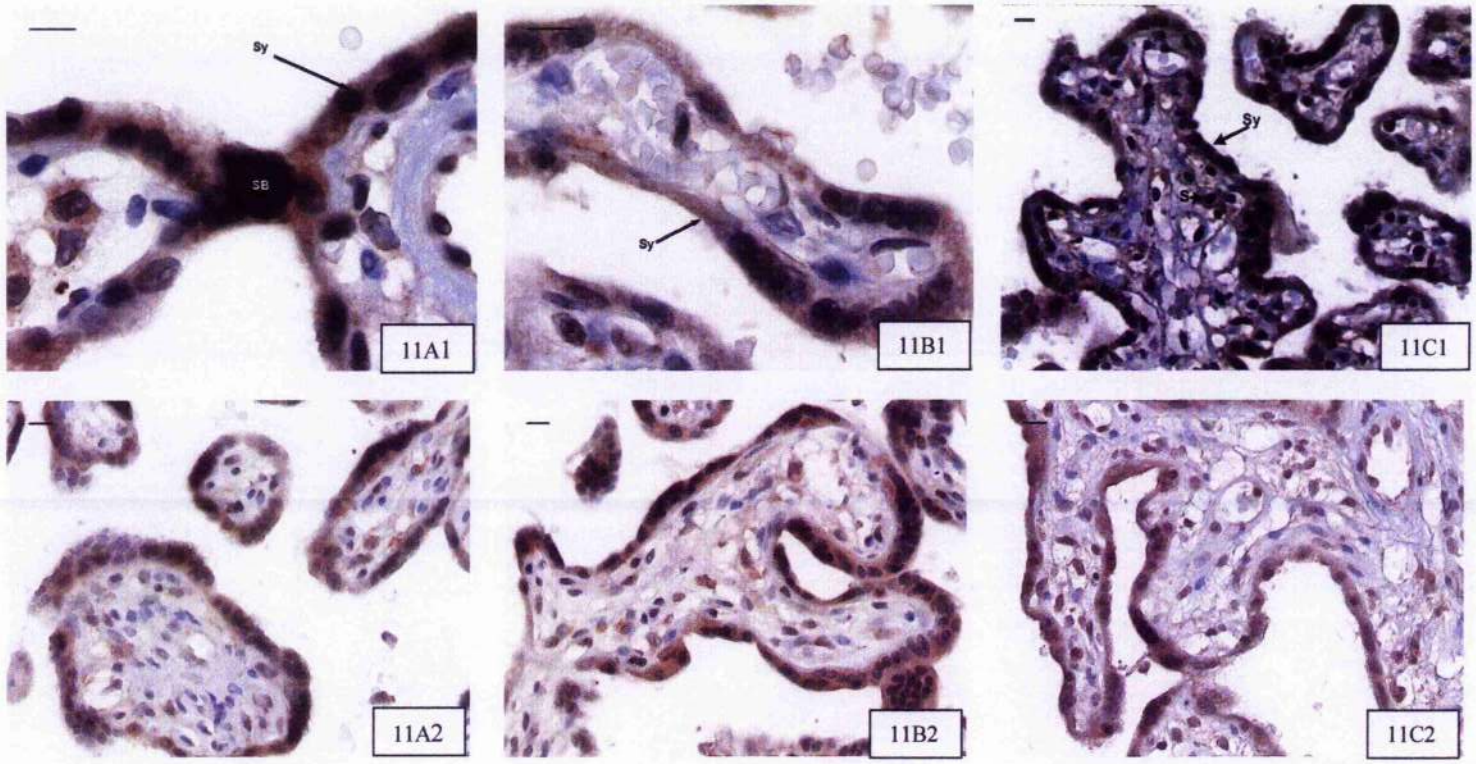


Plate 11.

Figure11A1 & 11A2. PPARγ ICC in 2 different PET placentae (third trimester). A1 = x100 magnification, scale bar = 1μm. A2 = x40 magnification, scale bar = 1 μm.

Figure 11B1 & 11B2. PPARγ ICC in 2 different IUGR placentae (third trimester). B1 = x100 magnification, scale bar = 1μm. B2 = x40 magnification, scale bar = 1 μm.

Figure 11C1 & 11C2. PPARγ ICC in 2 different uncomplicated third trimester placentae. C1& C2 = x40 magnification, scale bar = 1μm.

Sy = syncytiotrophoblast, SB = syncytial bridge.

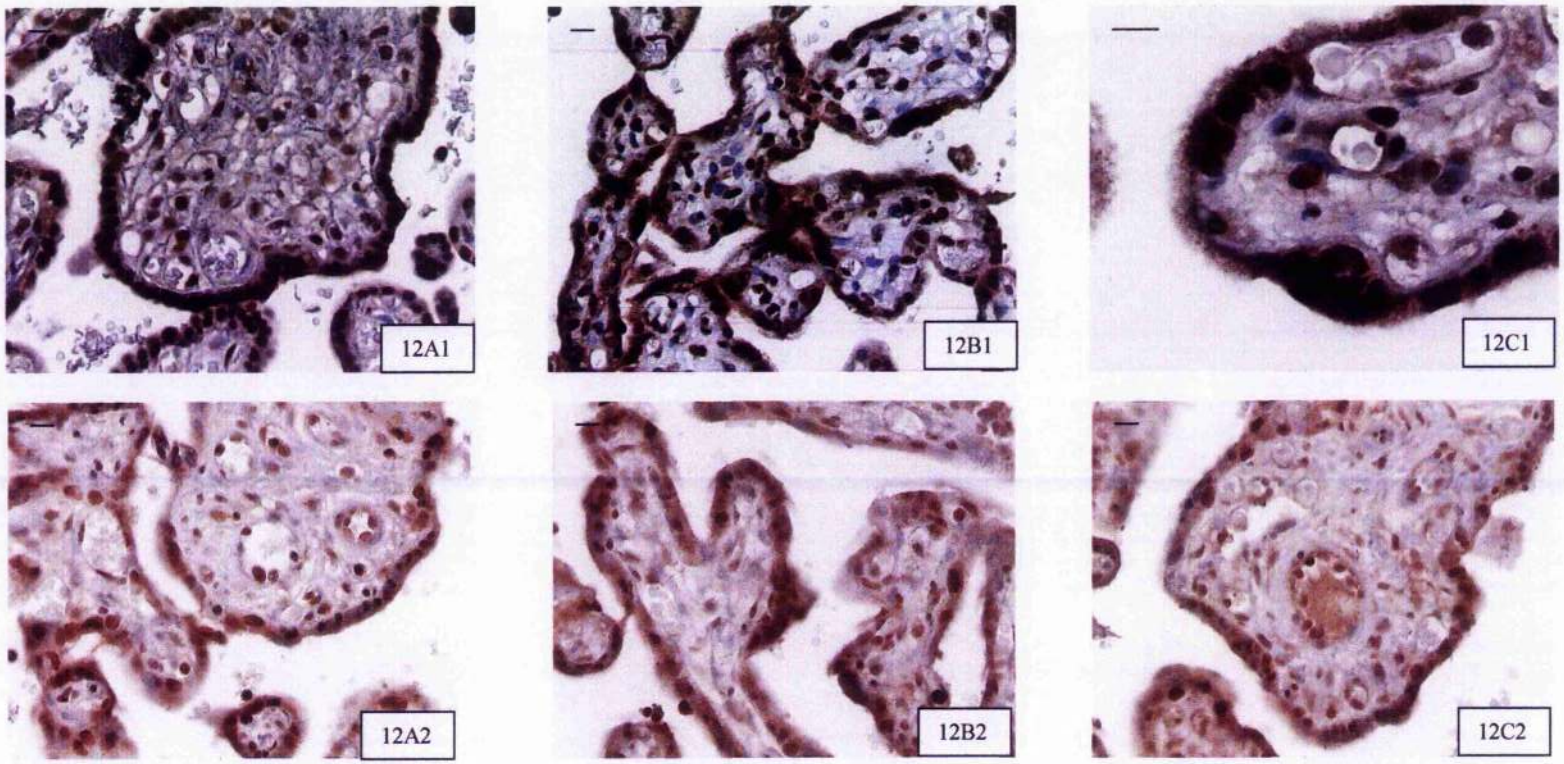


Plate 12.

Figure 12A1 & 12A2. RXRα ICC in 2 different PET placentae (third trimester). A1 & A2 = x40 magnification, scale bar = 1μm.
Figure 12B1 & 12B2. RXR α ICC in 2 different IUGR placentae (third trimester). B1 & B2 = x40 magnification, scale bar = 1μm.
Figure 12C1 & 12C2. RXR α ICC in 2 different uncomplicated third trimester placentae. C1 = x100 magnification, scale bar = 1μm. C2 = x40 magnification, scale bar = 1μm.

3.2.3 PPAR mRNA and protein expression in 1st and 3rd trimester placentae from uncomplicated pregnancies

3.2.3.1 PPAR mRNA expression in 1st and 3rd trimester placentae

Relative to the 18s control gene, PPARδ mRNA demonstrated a 115% higher expression in the 3rd trimester (PPARδ: 18s ratio, median 0.43, IQ range 0.26 – 0.52) compared with the 1st trimester placentae [0.20 (0.00 – 0.26), p=0.03] (Figure 3-1 & 3-2). There was a trend towards a lower placental RXRα mRNA expression in the 3rd trimester [0.72 (0.46-1.19)] compared to the 1st trimester [1.24 (1.15-1.46), p = 0.05] (figure 3-2). PPARγ mRNA expression was not different between the 1st trimester [1.38 (0.75 – 1.50)] and the 3rd trimester placentae [1.00 (0.66 – 1.15), p=0.17] (figure 3-2). As previously reported in the literature (Schultz *et al* 1999), two transcripts of PPARα were demonstrated. One transcript was of very low abundance in our placental samples. Relative to the 18S control gene, the most abundant PPARα mRNA transcript in placenta showed similar expression in the 1st trimester [0.19 (0.16 – 0.23)] and the 3rd trimester [0.14 (0.11 – 0.25), p = 0.30]. It should be noted that results from this PPARα probe were not reproducible after the above finding and it is thus possible that these results are not robust. Therefore no conclusions are drawn from this result.

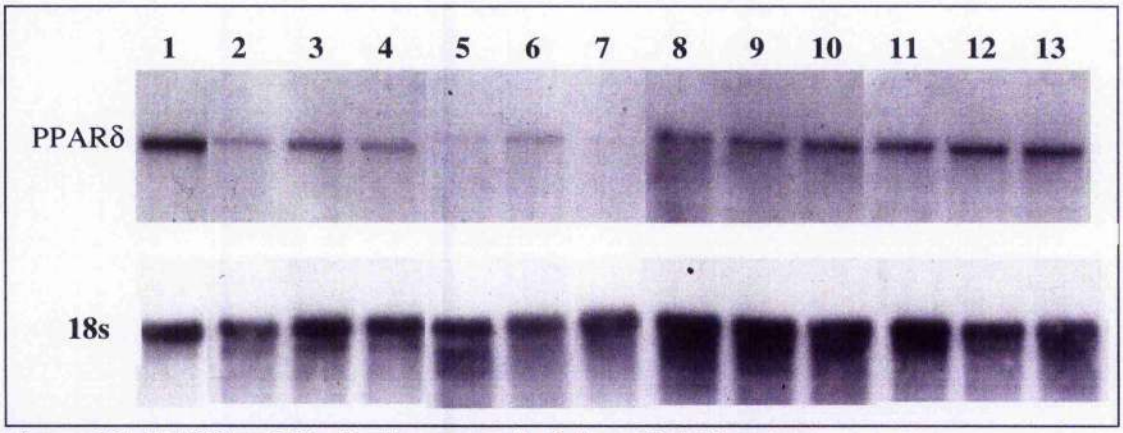


Figure 3-1. PPARδ and 18s Northern scan in 1st and 3rd trimesters.

Lane 1 – positive control (BeWo). Lanes 2-7 - 1st trimester placentae. Lanes 8-13- 3rd trimester placentae.

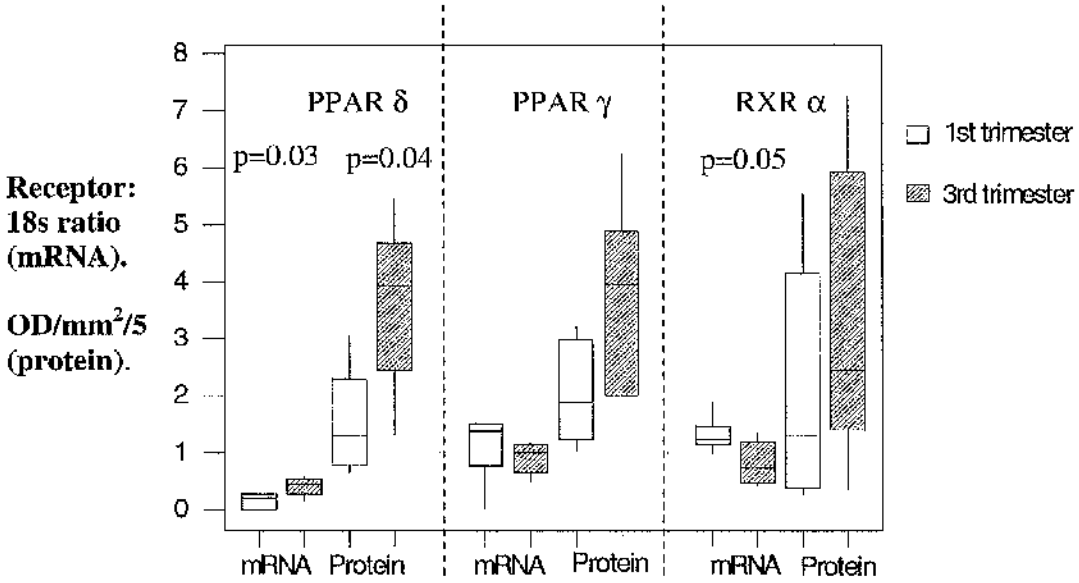


Figure 3-2. mRNA and protein expression in 1st and 3rd trimesters.

Data are presented as median and interquartile (IQ) range. Statistical analysis was performed using Mann-Whitney U test. Protein data values are divided by 5 to allow single graphic representation.

3.2.3.2 PPAR protein expression in 1st and 3rd trimester placentae

PPARδ protein expression was 205% higher in the 3rd trimester [3.94 (2.45-4.68) OD mm^2] compared to the 1st trimester [1.29 (0.78-2.29) OD mm^2, p = 0.04] (Figure 3-2 &3-3). PPARγ protein expression was higher in the 3rd trimester [3.95 OD mm^2 (1.97-4.86 OD mm^2)] than the 1st trimester [1.89 (1.22-2.97) OD mm^2, p=0.06] but did not reach statistical significance. RXRα protein expression was also not significantly different between the 1st [1.28 (0.38-4.14) OD mm^2] and 3rd trimesters [2.44 (1.40-5.92) OD mm^2 p=0.41] (figure 3-2). It was not possible to determine PPARα protein expression, as the bands produced on Western analysis were not consistent with the recognised molecular weight within the literature.

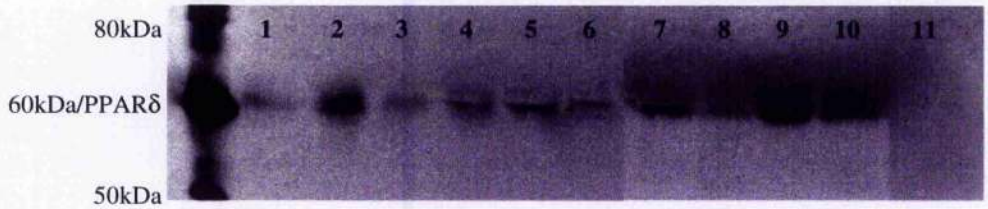


Figure 3-3. PPARδ Western scan in 1st and 3rd trimesters.

Lane 1 – Jurkat nuclear cell extract control, Lane 2 – Standard 3rd trimester placentae sample, Lanes 3-6 – 1st trimester placentae, Lanes 7-10 – 3rd trimester placentae, Lane 11 – blank.

3.2.4 PPAR mRNA and protein expression in placentae from pregnancies complicated by PET or IUGR

3.2.4.1 PPAR mRNA expression in PET and uncomplicated third trimester pregnancies

PPARδ mRNA expression was not different in PET placentae compared to placentae from uncomplicated pregnancies, relative to both 18S [median 0.53 (IQ range 0.25-0.74) vs median 0.71 (IQ range 0.52-0.82), p = 0.09] and PAI-2 [0.81 (0.68-1.20) vs 1.07 (0.91-1.21), p = 0.26]. PPARγ mRNA expression was not different between PET and uncomplicated pregnancies, relative to 18S [1.00 (0.60-1.04) vs 0.69 (0.57-0.80) p = 0.06] and PAI-2 [1.00 (0.65-1.37) vs 1.00 (1.00-1.31) p = 0.29]. Similarly, RXRα mRNA expression was unchanged in PET compared to uncomplicated pregnancies, relative to 18S [2.00 (1.46-2.88) vs 1.50 (1.38-2.25) p = 0.31] but was significantly elevated in PET placentae relative to PAI-2 [1.29 (0.90-2.13) vs 0.68 (0.42-1.00) p = 0.02] (figure 3-4 and 3-5). We were unable to determine PPARα mRNA expression by Northern analysis, as indicated in section 3.3.1 (also see discussion).

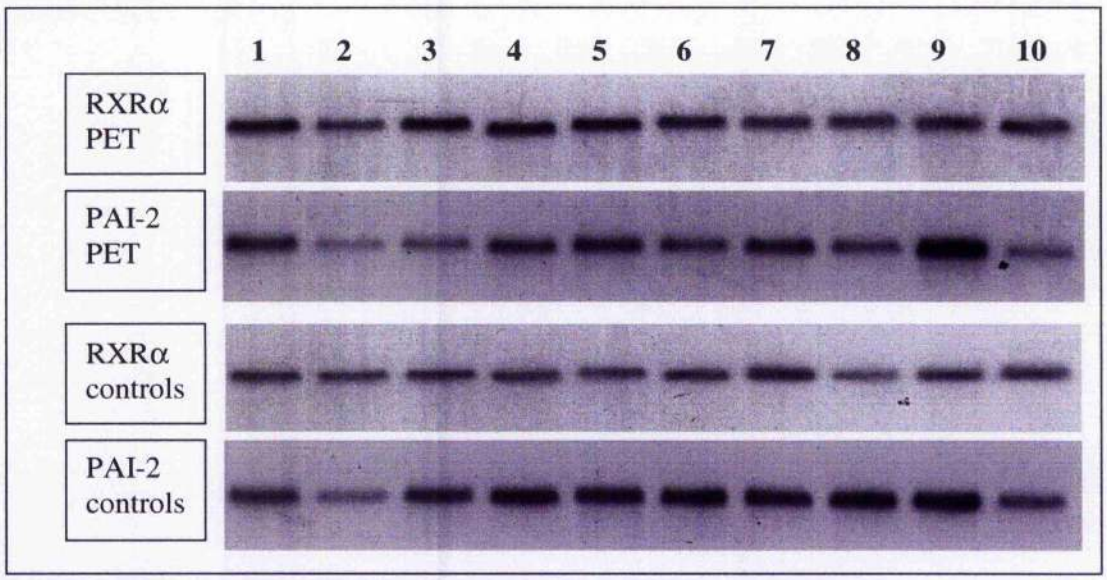


Figure 3-4. RXRα and PAI-2 Northern blot in PET and control placentae.

Lanes 1-10 - n=10 per group.

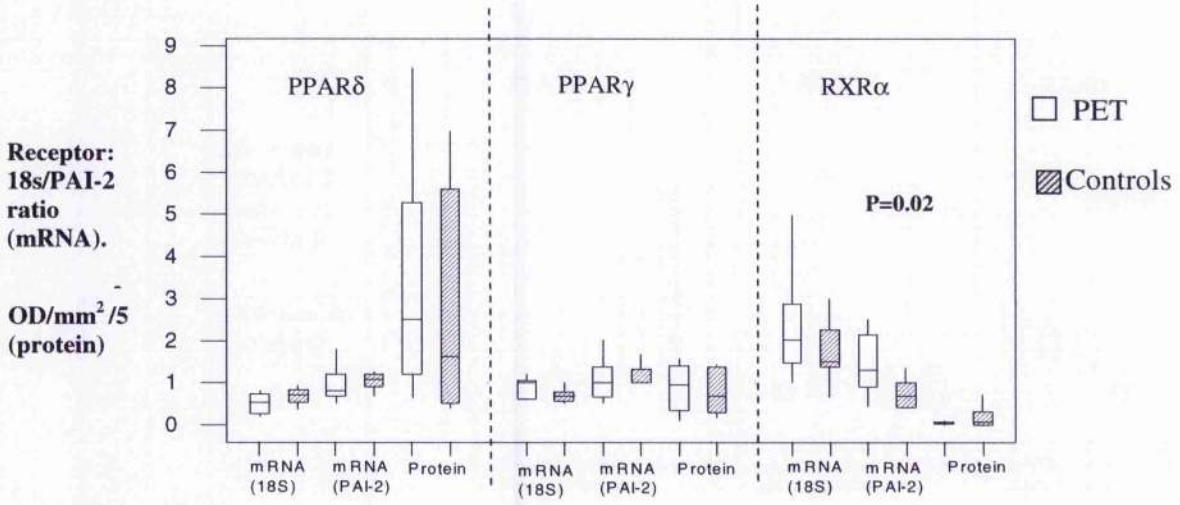


Figure 3-5. mRNA (relative to 18s and PAI-2) and protein expression in PET and control placentae.

Data are presented as median and (interquartile) IQ range. Statistical analysis was performed using Mann-Whitney U test. Protein data values are divided by 5 to allow single graphic representation.

3.2.4.2 PPAR protein expression in PET and uncomplicated third trimester pregnancies

PPARδ protein expression was not different in PET pregnancies [12.53 OD/mm^2 (6.00-26.36 OD/mm^2)] compared to controls [8.06 OD/mm^2 (2.49-28.02 OD/mm^2) p = 0.68]. PPARγ protein expression was not significantly different in PET pregnancies [4.82 (1.71-6.93)] compared to controls [3.43 (1.58-6.96) p=0.91], and similarly, RXRα protein expression was also not different in PET pregnancies [0.16 (0.09-0.35)] compared to controls [0.29 (0.01-0.57) p=0.62] (figure 3-5). PPARα protein expression was not determined, as discussed in section 3.3.2 (see discussion).

3.2.4.3 PPAR mRNA expression in IUGR and uncomplicated third trimester pregnancies

PPARδ mRNA expression was not different in IUGR placentae compared to placentae from uncomplicated pregnancies, relative to both 18S [median 0.16 (IQ range 0.11-0.23) vs median 0.16 (IQ range 0.12-0.21), p = 1.00] and PAI-2 [0.42 (0.25-0.67) vs 0.31 (0.24-0.43), p = 0.41]. PPARγ mRNA expression was significantly higher in IUGR pregnancies relative to 18S [0.42 (0.33-0.70) vs 0.26 (0.19-0.34) p = 0.03], but was not different between IUGR and uncomplicated pregnancies relative to PAI-2 [0.68 (0.50-1.03) vs 0.66 (0.58-0.74) p = 0.97]. RXRα mRNA expression was not different in IUGR compared to uncomplicated pregnancies, relative to 18S [0.24 (0.17-0.46) vs 0.23 (0.20-0.31) p = 0.65] and PAI-2 [0.50 (0.32-1.00) vs 0.46 (0.28-0.70) p = 0.55] (figure 3-6 and 3-7). We were unable to determine PPARα mRNA expression by Northern analysis, as discussed previously (see discussion).

3.2.4.4 PPAR protein expression in IUGR and uncomplicated third trimester pregnancies

PPARδ protein expression was not different in IUGR pregnancies [14.70 OD/mm^2 (6.19-31.75 OD/mm^2)] compared to controls [13.80 OD/mm^2 (4.77-24.14 OD/mm^2) p=1.00]. PPARγ protein expression was also not different in IUGR pregnancies [6.04 (0.89-9.55)] compared to controls [7.12 (3.59-15.36) p=0.34]. Similarly, RXRα protein expression was

not different in IUGR pregnancies [0.52 (0.47-1.45)] compared to controls [0.71 (0.44-2.09) p=0.52] (figure 3-7). PPARα protein expression was not determined, as before (see discussion).

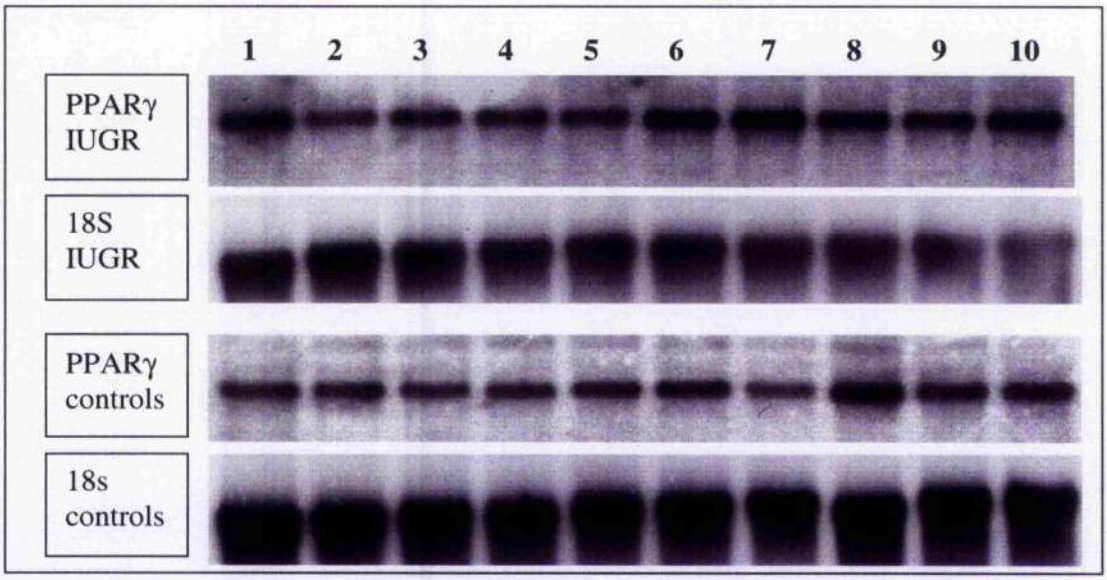


Figure 3-6. PPARγ and 18s Northern blot in IUGR and control placentae.

Lanes 1-10 - n=10 per group.

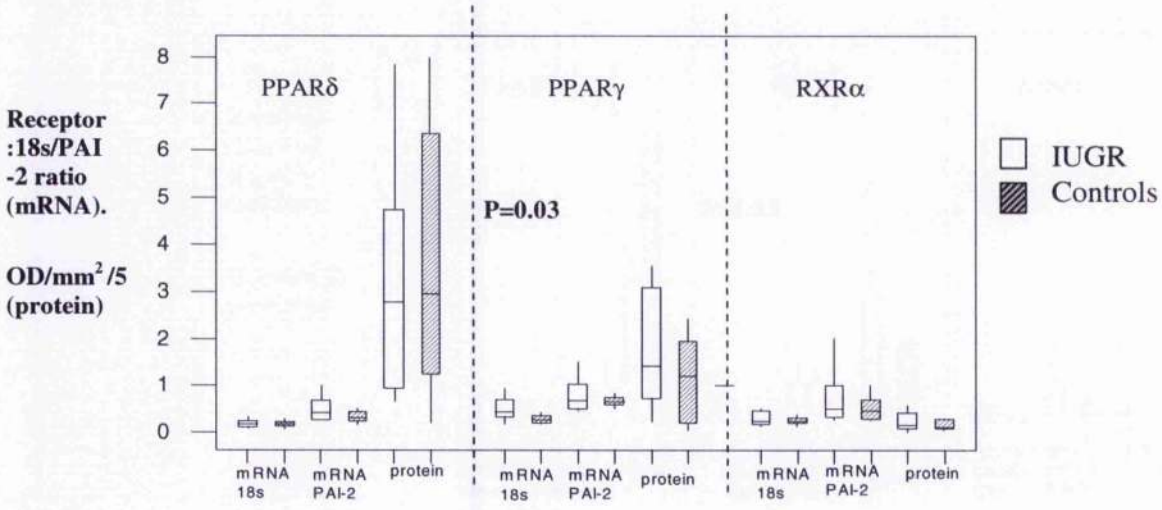


Figure 3-7. mRNA (relative to 18s and PAI-2) and protein expression in IUGR and control placentae.

Data are presented as median and IQ range. Statistical analysis was performed using Mann-Whitney U test. Protein data values are divided by 5 to allow single graphic representation.

3.2.5 RT-PCR (TaqMan) analysis of PPARα in placentae from pregnancies complicated by PET or IUGR

Using the TaqMan method, PPARα mRNA expression was unchanged in PET placentae [PPARα:18s ratio 2.13 (1.75-2.37)] compared to placentae from uncomplicated pregnancies [2.09 (1.63-2.39), p=1.00], and was also not altered between IUGR placentae [2.27 (2.10-1.60)] and control placentae [2.10 (1.60-2.71), p=0.21].

3.3 Discussion

This chapter quantitatively measures higher expression of PPARδ mRNA and protein in 3rd compared to 1st trimester placentae, and demonstrates PPARα, δ, γ and RXRα localisation and expression in human placentae complicated by PET and IUGR. In uncomplicated pregnancies, a 100% higher PPARδ mRNA expression was reported, as was a 200% higher PPARδ protein expression in the 3rd trimester compared to the 1st trimester placentae. Previously PPARδ expression has been described in abundance at implantation sites and in decidual cells in the rat uterus, using *in situ* hybridisation and immunohistochemical techniques (Ding *et al* 2003). Studies of the PPARδ knockout mouse have demonstrated that PPARδ deficiency results in lethality during mid-gestation in over 90% of mouse embryos. PPARδ was demonstrated to be essential for placentation, with the appearance of an abnormal gap in the placento-decidual interface in knockout mice (Barak *et al* 2002). Together with our own findings, these data indicate that PPARδ transcription and translation play an important role in placental development.

Although it was not possible to determine PPARα protein expression between the trimesters, PPARα mRNA levels did not differ between trimesters. The presence of PPARα has been previously confirmed in human placenta (Wang *et al* 2002) but this was not quantitated. PPARα has not been widely studied in pregnancy, but it has been demonstrated that PPARα agonist treatment is associated with alleviation of maternal insulin resistance in the pregnant rat (Sugden *e al* 2003). It is interesting to note that our immunocytochemical data is consistent with that of Waite et *al* (Waite *et al* 2000). However, although trophoblast staining appears less in the 3rd trimester in our study, it is apparent that other cell types are stained positively for PPARα in the 3rd trimester, which may account for lack of differences in mRNA expression between the 1st and 3rd trimesters. In particular it should be noted that both maternal and fetal blood cells were positive for

PPARα and although care was taken to thoroughly wash the placental tissue before freezing, the possibility that changes in maternal and/or fetal blood cell expression of PPARα may be being detected cannot be excluded. It is recognised that lymphocytes express PPARα (Jones *et al* 2002) and PPARα is thought to play a role in macrophage differentiation (Chinetti *et al* 1998). It is possible that PPARα may have a role in placental differentiation but the reduced PPARα localisation in trophoblasts in the 3rd trimester might argue against this. PPARα is also important in inflammation and lipid metabolism and potentially may have roles in immuno-suppression or fuel metabolism in 3rd trimester placentae.

Using the PET and IUGR samples, it was not possible to obtain bands on Northern analysis with either a commercial or in-house PPARα probe. The PPARα probe worked only once, for the 1st to 3rd trimester samples, despite purchasing different probes. The PCR was attempted on four occasions. It was possible to isolate the cDNA from the bands cut from the agarose gel, but this technique was also unsuccessful. A re-probing technique was involved, and it may be that the mRNA had degraded by the time the α probe was utilised. It is also possible that the initial Northern blotting PPARα result is contaminated and therefore cannot be relied upon. It may also be possible that the PPARα probe is extremely sensitive to environmental and experimental conditions, which were not reproduced. For this reason, TaqMan real-time PCR was employed for the measurement of PPARα gene expression in placental tissue in these groups. The TaqMan real-time PCR assay centres on the detection of a fluorescent signal generated from the cleavage of a target sequence specific probe by the Taq polymerase during each cycle of the PCR reaction (Heid *et al* 1996, Li *et al* 2003). As this signal is directly proportional to the PCR product being amplified, it permits very precise quantitation of the amount of initial input template.

Using western blotting, bands for PPARα were obtained at around 90 kDa molecular weight and a Jurkat positive control at the same weight. However, these bands were faint despite optimal antibody concentrations, and previous reports on animal models have demonstrated PPARα protein expression at around 58 kDa (Ibabe *et al* 2002). It is likely that different species and/or tissues may express PPARs with different molecular weights, and it is possible that PPARα has a higher molecular weight in human placenta. However, there are no previous reports to confirm this theory, and results were therefore considered unreliable, and are thus not reported. It should also be considered that the PPARα antibody lacked good specificity. Due to time constraints of this project, it was not possible to attempt to determine further whether this was indeed PPARα or another protein (e.g.

immunoglobulin). However, it may be possible to confirm this by western blotting under the same conditions as in this study, using human placenta as before and the Jurkat positive control, along with a tissue [e.g. mouse liver homogenates or zebrafish (Ibabe *et al* 2002)] of previously confirmed molecular weight which was not 90kDa. If the rodent liver had a band at this predetermined weight and PPARα placental protein was still around 90 kDa, it may be argued with more conviction that this was correct.

The mRNA and protein expression of PPARγ and RXRα at different gestations was also quantitatively assessed. RXRα mRNA expression was decreased from the 1st to the 3rd trimesters; however, this was not confirmed by protein expression. PPARγ mRNA was not altered between the 1st and 3rd trimesters, and the 109% higher 3rd trimester protein was not significant. As differences in specific PPARγ isoform expression were not studied, the possibility of differential expression of PPARγ isoforms cannot be excluded. Other studies of PPARγ and RXRα expression in pregnancy have not been quantitative (Fournier *et al* 2002, Tarrade *et al* 2001a, Tarrade *et al* 2001b, Wang *et al* 2002). Data from knockout mouse models and human pathological placenta indicates that PPARγ plays a role in placental development (Barak *et al* 1999, Capparuccia *et al* 2002). Capparuccia and colleagues (Capparuccia *et al* 2002) demonstrated that PPARγ protein expression is unchanged from the 1st to the 3rd trimester. PPARγ protein expression and activation are dramatically increased by sera from pregnant women (Waite *et al* 2000). Furthermore, exposure of trophoblasts in culture to PPARγ agonists is known to stimulate the production of placental hormones (Tarrade *et al* 2001a, Tarrade *et al* 2001b). In the present study PPAR expression but not activation, has been studied. It is possible that as yet unidentified factors in pregnant serum, such as oxidised lipids, may act as PPARγ agonists. In fact, during the writing of this thesis Waite and colleagues (Waite *et al* 2005) produced data suggesting that previously described PPARγ activators in serum from pregnant women (Waite *et al* 2000) are significantly reduced in those destined to develop PET, up to 15 weeks before the onset of symptoms. This group speculate that the loss of PPAR activation in PET could account for increases seen in endothelial cell activation and inflammatory cytokines.

Placentae from PET and IUGR pregnancies did not demonstrate any clear differences in mRNA or protein expression of PPARδ, γ or RXRα compared to control pregnancies. PPAR and RXRα mRNA expression in PET, IUGR and 3rd trimester uncomplicated control human placentae was analysed, using 18S and PAI-2 as control genes. The 18S control gene was used to control for total cell number, while the PAI-2 gene was used with

the aim of controlling for functional trophoblast. Since in PET and IUGR, placental necrosis is evident, we sought to assess mRNA expression relative to placental function. PAI-2 is synthesized in high amounts by the 3rd trimester trophoblast but levels decrease with reduced placental function (Halligan *et al* 1994). There were no gross differences between the results obtained using either control gene. The lack of distinct down-regulation of placental PPARδ and γ expression in PET and IUGR indicates that expression of these genes does not play a specific role in the aetiology of PET or IUGR. Again it does not rule out the possibility that activation of these nuclear receptors via changes in circulating activator ligands might be changed. We assessed PPAR expression in random placental samples from 10 individuals avoiding areas of overt necrosis and our measurements of mRNA and protein expression in each group has a coefficient of variance (CV) of around 50%. It is possible that gene expression is location-specific within the placenta and there are some data demonstrating variability of PPARγ expression in multiple biopsies from the same individual (Pidoux *et al* 2004) with a CV of the order of 30%. A *post hoc* power calculation using the actual CVs we obtained indicates that we had 90% power to detect a 75% difference between control and PET groups. Thus we would have been unable to detect any smaller differences in expression between groups.

Using immunocytochemical staining with CY-7, cells expressing PPARs were identified to be of trophoblast origin. In this study, PPAR receptors were located both in the cytoplasm and the nuclei of trophoblasts. Co-existing cytoplasmic and nuclear staining has been observed for another member of the PPAR family (PPARα), in macrophages (Chinetti *et al* 1998), but not with other immunocytochemical investigations of placenta (Capparuccia *et al* 2002, Wang *et al* 2002). Stromal cells, which expressed PPARs at all gestations, were less easily identified. It was hypothesised that these cells were Hoffbauer cells of macrophage origin. It was not possible to confirm this by co-localisation with CD68, a macrophage marker. However, due to the predominantly cytoplasmic localisation of CD68 and the predominantly nuclear localisation of the PPARs, visual confirmation of co-localisation on back to back sections was difficult and may be inappropriate given the different intracellular localisation of these molecules. Endothelial cells were found to express PPARs, although these cells could not account for all of the positively stained stromal cells. It is likely that the cells expressing PPARs in the stroma are of mixed origin.

PPAR and RXRα localisation and expression in human placentae complicated by PET and IUGR has not been described prior to this study. These placentae did not demonstrate any clear differences in the immunocytochemical localisation of PPARδ and γ, or RXRα,

compared with placentae from uncomplicated pregnancies. Capparuccia and colleagues (Capparuccia *et al* 2002) have observed reduced immunocytochemical staining for PPARγ in two other pathological conditions of placenta, namely trophoblastic disease and hydatidiform molar pregnancies.

Clearly PPARs have widespread roles in inflammation, differentiation and metabolism, and therefore it is not surprising that many cell types express PPARs. What is striking about the placenta is the distinct pattern of PPAR expression that we have identified. Evidence is provided for the first time that PPARδ expression is independently regulated between the 1st and 3rd trimester. This suggests that PPARs may have differential functional roles in the placenta. PPARδ may play a role in mid to late gestation placenta, possibly in syncytium formation, fuel metabolism or in the inflammatory response. PPARα may have an anti-inflammatory function in the 3rd trimester, potentially at the maternal/fetal interface, although one would expect expression at the syncytium in this situation. Because it was observed that PPARα is expressed in maternal and fetal blood cells, it is possible that the anti-inflammatory functions of PPARα could be systemic. PPARα may also be involved in lipid metabolism and fatty acid oxidation in the 3rd trimester of pregnancy, when there is an increased demand for the supply of energy to the fetus and increased placental transport. Novel evidence is provided, that PPARδ, γ and RXRα protein expression is unaltered in the metabolic complications of pregnancy, in particular, PET and IUGR. This suggests that changes in PPAR expression *per se* may not be involved in the pathophysiology of these conditions, although they may have a role via activation at the ligand binding level.

4 Maternal and fetal plasma lipid metabolism and paraoxonase-1 (PON-1) activity in PET and IUGR pregnancies

4.1 Introduction

4.1.1 Maternal and fetal lipids in PET and IUGR

In the Introduction chapter, the maternal lipid alterations seen in normal pregnancy and in those complicated by PET or IUGR, and the potential consequences of this dyslipidaemia have been discussed. Placental transport of cholesterol to the fetus is likely via uptake of LDL by the LDL-receptor on the maternal side (Schmid *et al* 2003). Maternal TGs are hydrolysed on maternal lipoproteins by lipoprotein lipase and are transported across the placenta via fatty acid binding proteins (Haggarty 2002). There are few data regarding fetal lipid and lipoproteins throughout uncomplicated pregnancy. In general, fetal cord and neonatal plasma lipid levels are reported to be very much lower than those in adults (Averna *et al* 1991, Kilby *et al* 1998, Neary *et al* 1995), with a relatively larger proportion of cholesterol carried in HDL (Kilby *et al* 1998, Neary *et al* 1995) resulting in a lower total cholesterol/HDL ratio (Averna *et al* 1991). HDL cholesterol is not inversely correlated with TG as in the adult but is positively correlated with apolipoprotein (apo) C, apo C-II and apo CIII (Averna *et al* 1991). There are no reports of fetal lipid concentrations in PET pregnancies. It has been shown that in small for gestational age neonates, there is a high triglyceride and low HDL profile, which may result from increased cholesteryl ester transferase activity in the neonate (Kaser *et al* 2001).

Since the placenta can transport both maternal cholesterol and fatty acids to the fetus, it was hypothesised that the maternal dyslipidaemia of PET and IUGR would be reflected in the offspring. It was proposed that disease severity would be associated with abnormal lipid fractions in the mother and her fetus. The aim of this chapter therefore, was to perform a cross-sectional case control study of maternal and fetal lipid and lipoprotein concentrations in third trimester uncomplicated pregnancies and in pregnancies complicated by PET or IUGR. This study was performed in order to confirm previous maternal and fetal lipid values reported in normal pregnancies, and to determine for the first time, fetal lipid values in pregnancies complicated by PET. Samples were obtained

from women recruited from the Princess Royal Maternity Hospital, Glasgow, with uncomplicated 3rd trimester pregnancies and pregnancies complicated by PET or IUGR.

4.1.2 Paraoxonase-1 (PON-1)

4.1.2.1 PON-1 activity and polymorphisms

As an adjunct to the lipid analysis, the activity of paraoxonase-1 (PON-1) in uncomplicated pregnancy and in pregnancies complicated by PET or IUGR was studied. Paraoxonase-1 (PON-1) is a glycoprotein, which in serum is exclusively located on HDL (Durrington *et al* 2001). Paraoxonase-1 hydrolyses organophosphate substrates such as paraoxon and most likely explains the ability of HDL to metabolise lipid peroxides and to protect against their accumulation on LDL (Mackness *et al* 2002) under oxidising conditions. The paraoxonase gene family has three known members, PON1, PON2 and PON3, located on the long arm of chromosome 7 between q21.3 and q22.1 in humans (Primo-Parmo *et al* 1996). There is considerable variation in serum PON-1 activity within and between human populations (Diepgen and Geldmacher-von Mallinckrodt 1986, Roy *et al* 1991), and activity is under genetic and environmental regulation. The PON-1 gene has common polymorphisms in the coding region (L55M, Q192R), and PON-1 enzyme activity for paraoxon as a substrate, includes low paraoxon- and high paraoxon-activity alleles (for example, Q192 and R192 respectively, and M55 and L55 respectively) (Humbert *et al* 1993, Mackness *et al* 1996). However, there is also a 40-fold interindividual variation in PON-1 activity, which is independent of the genotype (Mackness *et al* 2002), and this variation persists even within genotype groups (Richter and Furlong 1999).

4.1.2.2 Antioxidant PON-1 activity and metabolic disorders

Low plasma PON-1 activity has been demonstrated in oxidative stress-associated processes such as dyslipidaemia, diabetes mellitus (Mackness *et al* 1991), advancing age and smoking (Senti *et al* 2003). These findings are independent of PON-1 genotype. High serum cholesterol (Mackness *et al* 1991) and insulin resistance (Kordonouri *et al* 2001) are associated with decreased PON-1 activity. The metabolic abnormalities associated with low PON-1 activity are all components of the metabolic syndrome, which in turn is associated with increased mortality from CVD.

4.1.2.3 Antioxidant PON-1 activity and atherosclerosis

Although PON-1 does not participate in lipid metabolism, it is involved in the development of atherosclerotic lesions (Mackness *et al* 1993), and PON-1 immunoreactivity increases in the arterial wall as atheroma advances (Mackness *et al* 1997). Because serum PON-1 hydrolyses oxidised lipids in LDL, PON-1 may inhibit the development of atherosclerosis (Aviram *et al* 2000). The Q alloenzyme of the Q192R PON-1 polymorphism is more efficient at protecting LDL from oxidation (Mackness *et al* 2002), and the R polymorphism has been more closely associated with CHD than the Q polymorphism. Individuals with the M allele of the L55M PON-1 polymorphism show lower PON-1 activity compared with those who are L-carriers (Leviev and James 2000), and data has shown that MM homozygosity is linked to a 3-fold increased risk of first myocardial infarction in men compared to those without the M allele (Salonen *et al* 1999). However, data in the literature is conflicting as to whether there is an association between the PON-1 55L allele and atherosclerosis (Garin *et al* 1997, Salonen *et al* 1999) or not (Arca *et al* 2002, Sanghera *et al* 1998). A recent meta-analysis of 43 studies of Q192R and L55M was unable to show a strong association between any of the polymorphisms and CHD (Wheeler *et al* 2004). Data suggest that actual levels of PON-1 activity and concentration may be more important in determining the presence of coronary heart disease than PON genetic polymorphisms (Mackness *et al* 2001). When PON-1 activity is measured directly in patients with coronary heart disease, it is about half that of disease free controls (Mackness *et al* 1997). It is possible that the lack of conclusive epidemiological evidence for the association between PON-1 and CHD is based on the fact that most published studies do not take into account individual serum PON-1 levels (Deakin and James 2004).

4.1.2.4 PON-1 activity in pregnancy

There is little information in the literature regarding PON-1 activity in pregnancy. It has been demonstrated that maternal serum PON-1 levels are significantly higher during pregnancy, compared with the non-pregnant state (Roy *et al* 1994). This group also demonstrated a correlation between PON-1 and triglycerides ($r=0.45$-0.60, $p<0.001$) in uncomplicated pregnancy, and maternal PON-1 levels obtained at 28 weeks of gestation were negatively correlated with neonatal birth weight ($r=-0.3$, $p<0.05$). However, previous data using the mouse model suggest that there is a significant reduction in serum PON-1 activity during pregnancy (Weitman *et al* 1983). Neonatal PON-1 activity has been shown to be lower than that in adults (Chen *et al* 2003), as humans have only one fourth to one

third of their adult PON-1 levels at birth (Ecobichon & Stephens 1973). It has been suggested that PON-1 levels are low at birth, and plateau between 6 and 15 months of age, with high inter-individual variability (Cole *et al* 2003).

As discussed in the Introduction (1.3.3), oxidative stress has been proposed as a component of PET (Hubel 1999). The oxidative stress theory of PET involves the hypothesis that the abnormal placentation and dyslipidaemia results in a release of free radicals, particularly superoxide anions and lipid hydroperoxides, which damage the vascular endothelium (Hubel 1999, Hubel *et al* 1989). Increased blood levels of lipid peroxidation products in PET have been reported by several authors (Maseki *et al* 1981, Wang *et al* 1991).

Because low plasma PON-1 activity is associated with less anti-oxidant potential and hence more oxidative stress and production of lipid peroxides, which are features of PET, it was proposed that pregnancies complicated by PET or IUGR would demonstrate lower PON-1 levels than those seen in uncomplicated pregnancies. The link between PON-1 and atherosclerosis might contribute to the further increased risk of cardiovascular disease seen in PET. It was considered that these results might be influenced by genotype, as proposed for CVD. The preliminary data in pregnancy (Roy *et al* 1994) was only assessed in 3rd trimester levels, so it was hypothesised that PON-1 activity would decrease from 1st trimester levels as gestation advanced, in keeping with the increased atherogenic risk of pregnancy (Sattar and Greer 2002). Thus the aim of this study was to determine PON-1 activity, both independent of and related to genotype (PON-1 L55M & PON-1 Q192R) in each trimester of uncomplicated pregnancy in order to clarify the modest data in the literature, and to determine PON-1 activity, independent of and related to genotype in a case control study of PET and IUGR subjects, and corresponding BMI matched controls from 3rd trimester uncomplicated pregnancies.

The PON-1 study was performed in collaboration with Dr Mike Mackness (Clinical Research Division II Medicine, Manchester Royal Infirmary), who kindly performed the assays. Due to small study numbers of subjects with the rare PON-1 alleles, conclusions regarding PON-1 activity related to genotype are limited in this study, and results shown are therefore purely observational.

4.2 Results

4.2.1 Uncomplicated pregnancies

4.2.1.1 Maternal and fetal lipids in uncomplicated pregnancies

Baseline characteristics for eighty-one subjects from uncomplicated pregnancies are shown in Table 4-1.

Characteristics	**n=81**
Age (years)	28.9 (5.3)
BMI (kg/m^2)	27.8 (5.8)
Primigravidae n (%)	44 (54%)
Smokers n (%)	28 (35%)
Gestation at sampling (weeks)	37.3 (2.6)
Vaginal delivery n (%)	53 (65%)
Placental weight (g)	743 (157)
Fetal weight (kg)	3.65 (0.57)
Birth weight centile	56 (31)
Fetal sex n (%)	46 (57%) male
	35 (43%) female
TC (mmol/L)	6.02 (0.92)
Log TG (log mmol/L)	0.43 (0.13)
	[2.69 (1.35)]
VLDL (mmol/L)	0.74 (0.29)
LDL (mmol/L)	3.81 (0.96)
Log HDL (log mmol/L)	0.16 (0.09)
	[1.45 (1.23)]

Table 4-1. Maternal characteristics and lipid concentrations from uncomplicated pregnancies.

All values are mean and standard deviation (S.D.). Log transformed data was used for maternal triglyceride (TG) and high-density lipoprotein (HDL), and is presented as mean (S.D.) and [geometric mean (S.D.)].

Maternal lipid profiles were not affected by BMI (<25 kg/m^2 or >25 kg/m^2), smoking status or parity, using univariate regression analysis or ANOVA. Maternal lipid profiles were also unaffected by gestational age at sampling (range 32 – 42 weeks). Fetal lipids (table 4-2) were available from forty-one of these uncomplicated pregnancies and were unaffected by maternal BMI, age, smoking status, gestational age at delivery or fetal gender.

Fetal lipid	n=41
Fetal log TC (log mmol/L)	0.20 (0.12) [1.58 (1.32)]
Fetal log TG (log mmol/L)	-0.34 (0.26) [0.46 (1.82)]
Fetal HDL (mmol/L)	0.72 (0.19)
Fetal chol/HDL	2.33 (0.62)

Table 4-2. Fetal lipid concentrations from uncomplicated pregnancies.

All values are mean and SD. Log transformed data was used for fetal total cholesterol (TC) and triglycride (TG) and is presented as mean (SD) and [geometric mean (SD)].

However, fetal log TC, log TG and HDL levels all differed significantly (ANOVA, $p<0.001$) between types of delivery; emergency lower uterine Caesarean section (LUSCS), elective LUSCS, vaginal delivery or assisted delivery (figure 4-1). Emergency LUSCS deliveries had the highest fetal log TC, log TG and HDL levels. Placental weight was positively correlated with fetal log TC ($r=0.37$, $p=0.02$), log TG ($r=0.34$, $p=0.04$) (Figure 4-2A) and TC/HDL ratio ($r=0.31$, $p=0.05$) in this group. Maternal TC ($r=0.35$, $p=0.03$) (Figure 4-2B) and LDL ($r=0.36$, $p=0.02$) levels were associated with fetal HDL levels.

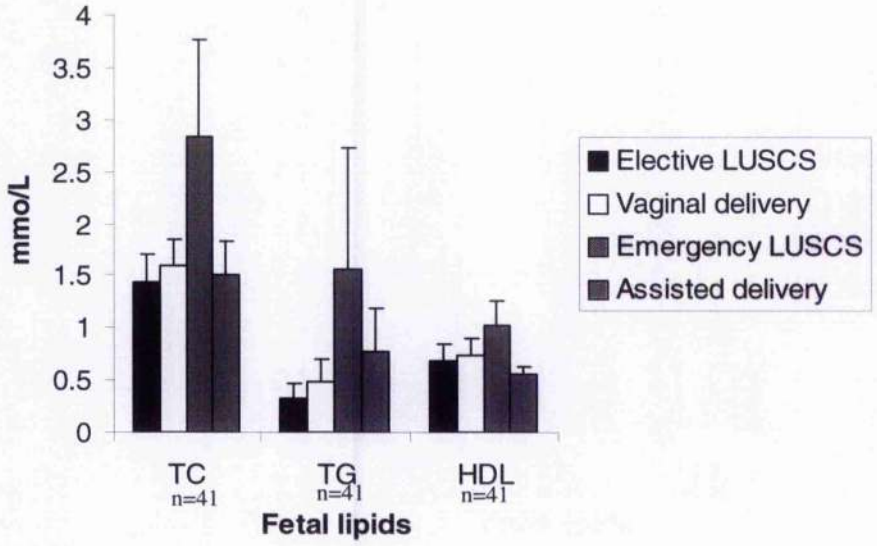


Figure 4-1. Fetal lipids by mode of delivery.

Raw data is shown. Statistical analysis was performed using a one-way ANOVA, using log-transformed data for fetal total cholesterol (TC) and triglyceride (TG). LUSCS-lower uterine Caesarean section. Assisted delivery- forceps or Ventouse.

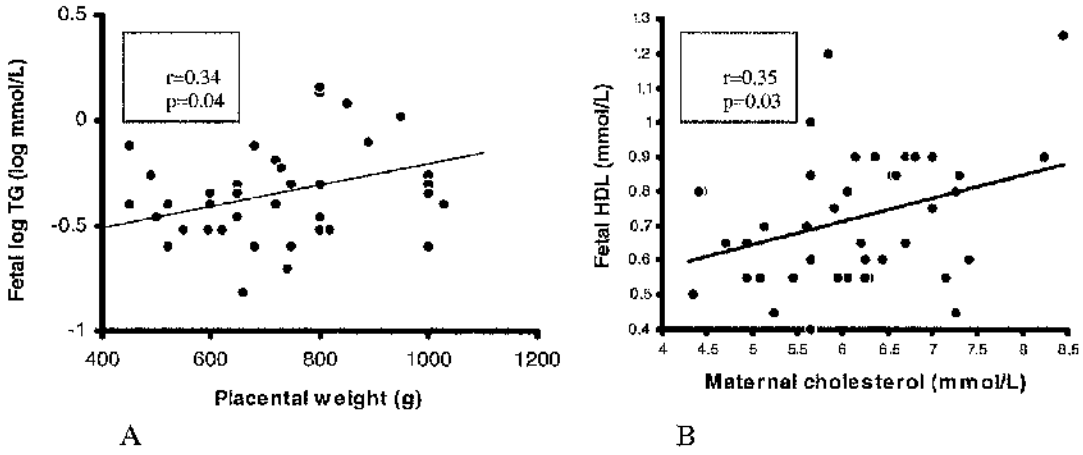


Figure 4-2. A - Association of fetal log triglyceride (TG, n=41) with placental weight. B – Association of fetal high-density lipoprotein (HDL, n=41) with maternal cholesterol (n=81). Statistical analysis was performed using Pearson's coefficient of correlation.

4.2.1.2 Maternal and fetal PON-1 activity in uncomplicated pregnancies

Baseline characteristics for fifty-three subjects in the third trimester of uncomplicated pregnancies, from which samples were available for PON-1 analysis, are shown in Table 4-3.

Maternal log PON-1 values were not affected by BMI (<25 kg/m^2 or >25 kg/m^2), smoking status or parity, using univariate regression analysis or ANOVA. Maternal log PON-1 levels were significantly affected by gestational age at sampling within the third trimester, with lower log PON-1 levels as gestation advanced (univariate regression analysis, p=0.01, R^2 (adj) = 9.9%), and data was adjusted accordingly in the PET/IUGR case control study using a General Linear Model. There were only 9 fetal log PON-1 values available from uncomplicated pregnancies, therefore fetal log PON-1 values remain unadjusted for potential confounders and are logged as maternal values. However, because the fetal lipid values were clearly affected by mode of delivery, this was checked within the available PON-1 samples from these uncomplicated pregnancies. Fetal log PON-1 values were unaffected by mode of delivery (n=9, p=0.51, ANOVA). The mean and S.D. for the 9 fetal log PON-1 values from uncomplicated pregnancies was 1.35 log nmol/min/ml (0.89 log nmol/min/ml), geometric mean 22.38 nmol/min/ml (7.76 nmol/min/ml).

Characteristics	n=53
Age (years)	29.2 (5.48)
BMI (kg/m^2)	27.9 (5.3)
Primigravidae n (%)	28 (53%)
Smokers n (%)	19 (36%)
Gestation at sampling (weeks)	37.5 (2.63)
Vaginal delivery n (%)	31 (58%)
Placental weight (g)	748.4 (0.50)
Fetal weight (kg)	3.69 (0.53)
Birth weight centile	56.6 (30.4)
Fetal sex n (%)	30 (59%) male 21 (41%) female
Log maternal PON (log nmol/min/ml)	2.06 (0.33) [144.82 (2.14)]
Log fetal PON-1 (log nmol/min/ml) *	1.35 (0.89) [22.38 (7.76)]
PON-1 L55M allele frequency	Common (L) 0.61 Rare (M) 0.39
PON-1 Q192R allele frequency	Common (Q) 0.81 Rare (R) 0.19

Table 4-3. Maternal characteristics, paraoxonase-1 (PON-1) activity and allele frequency from uncomplicated pregnancies.

All values are mean and standard deviation (S.D.). Log transformed data was used for maternal PON-1, and is presented as mean (S.D.) and [geometric mean (S.D.)]. *n=9 fetal samples available.

Mean maternal 3rd trimester log PON-1 levels [2.06 (0.33) log nmol/min/ml] demonstrated a trend towards higher values compared to mean fetal log PON-1 levels [1.35 (0.89) log nmol/min/ml, p=0.05] in the uncomplicated group. No difference in maternal PON-1 was detected between either the rare homozygote PON-1 55 MM or the L-carriers, or between the rare homozygote PON-1 192 RR or the Q-carriers. There were no correlations between maternal and fetal log PON-1 activities, or between maternal and fetal log PON-1 activity and any of the lipid parameters. Specifically, maternal and fetal log PON-1 levels were not found to be associated with maternal or fetal log TG or HDL. There were no associations between maternal or fetal log PON-1 and placental weight, fetal weight or birth weight centiles.

4.2.2 Maternal PON-1 activity in a longitudinal study of uncomplicated pregnancies

Baseline maternal characteristics are shown in table 4-4 for 20 subjects studied in each trimester of uncomplicated pregnancy.

Characteristics	n=20
Age (years)	29.1 (5.16)
BMI (kg/m^2)	27.3 (6.96)
Primigravidae n (%)	11 (55%)
Smokers n (%)	6 (30%)
Gestation at sampling (weeks)	36.2 (1.94)
Vaginal delivery n (%)	17 (85%)
Placental weight (g)	798 (204)
Fetal weight (kg)	3.68 (0.67)
Birth weight centile	58 (39)
Fetal sex n (%)	12 (60%) male
	8 (40%) female
PON-1 L55M allele frequency	Common (L) 0.78
	Rare (R) 0.23
PON-1 Q192R allele frequency	Common (L) 0.70
	Rare (R) 0.30

Table 4-4. Maternal baseline characteristics and allele frequency for longitudinal study of paraoxonase-1 (PON-1) activity, taken in the 1st trimester.

All values are mean and S.D.

The mean gestations at sampling in the longitudinal study were; T1 12.1 weeks (SD 1.55 weeks), T2 26.2 weeks (SD 1.07 weeks), T3 35.3 weeks (SD 1.45 weeks). Maternal log PON-1 activity was not altered as gestation advanced [T1 2.12 (0.32) log nmol/min/ml, T2 2.14 (0.31) log nmol/min/ml, T3 2.23 (0.28) log nmol/min/ml, p=0.50] (figure 4-3). Maternal PON-1 activity based on genotype throughout gestation is demonstrated in table 4-5. There were no significant differences in PON-1 activity throughout the trimesters based on genotype.

Interestingly, in each trimester, lower PON-1 activity was observed in the rare homozygotes (MM) for the PON-1 L55M polymorphism, although no statistics were performed due to small sample size (Figure 4-4). For the PON-1 Q192R polymorphism, PON-1 activity was observed to be higher in the rare homozygotes (RR), although as before, statistics were not performed (figure 4-5).

	T1	T2	T3	p
PON-1 55 MM (n=1)	1.86 (*)	1.59 (*)	1.63 (*)	*
PON-1 55 L-carrier (n=19)	2.14 (0.32)	2.17 (0.29)	2.26 (0.25)	0.40
PON-1 192 RR (n=2)	2.64 (0.04)	2.61 (0.05)	2.59 (0.01)	0.53
PON-1 192 Q-carrier (n=18)	2.06 (0.28)	2.09 (0.28)	2.19 (0.27)	0.37

Table 4-5. Maternal log paraoxonase-1 (PON-1) activity (log nmol/min/ml) by genotype in each trimester of uncomplicated pregnancy.

Statistical analysis was performed using ANOVA. Maternal log PON-1 activity was NOT adjusted for gestational age at sampling. *n=1.

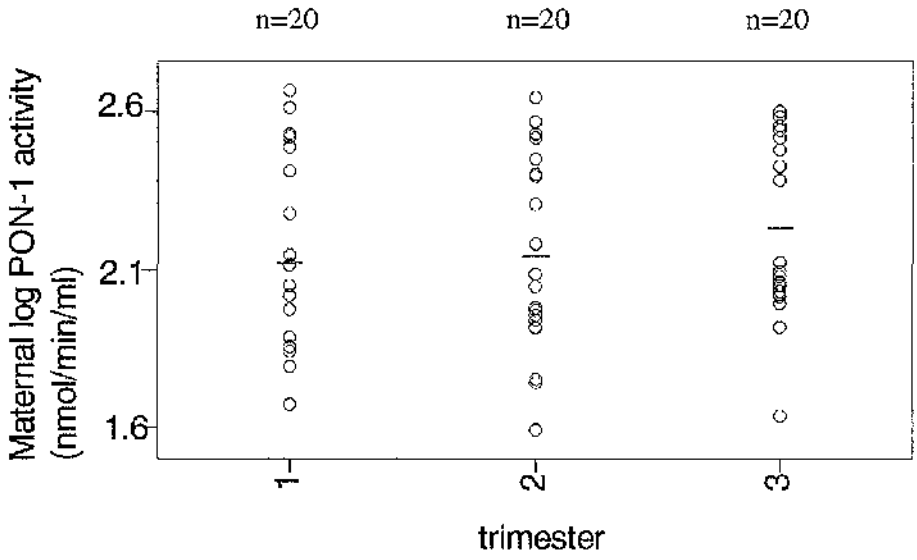


Figure 4-3. Maternal log paraoxonase-1 (PON-1) activity in each trimester of uncomplicated pregnancy. Statistical analysis was performed using ANOVA. Maternal log PON-1 activity was NOT adjusted for gestational age at sampling.

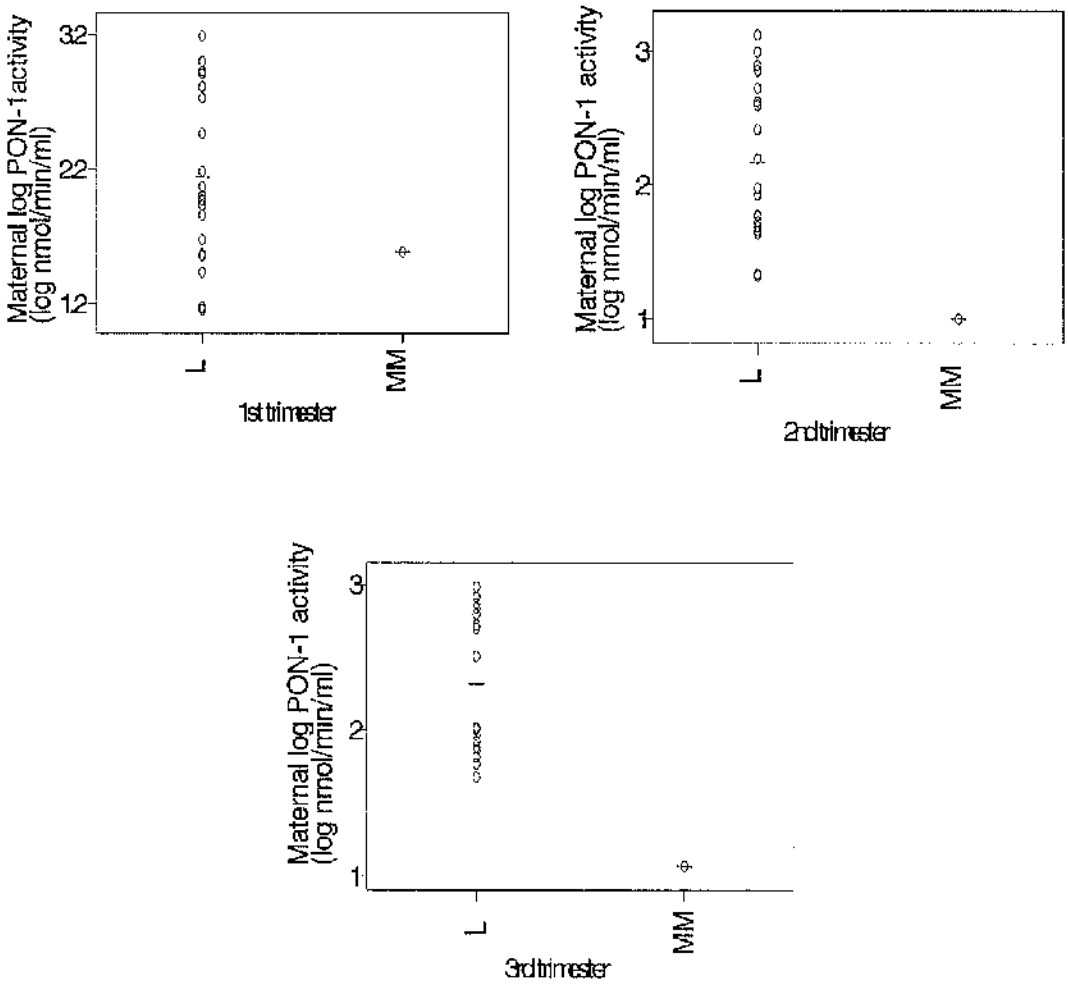


Figure 4-4. Dotplot of differences between paraoxonase-1 (PON-1) activity in PON-1 L55M MM homozygotes or L-carriers, in each trimester.

Mean values are demonstrated by lines.

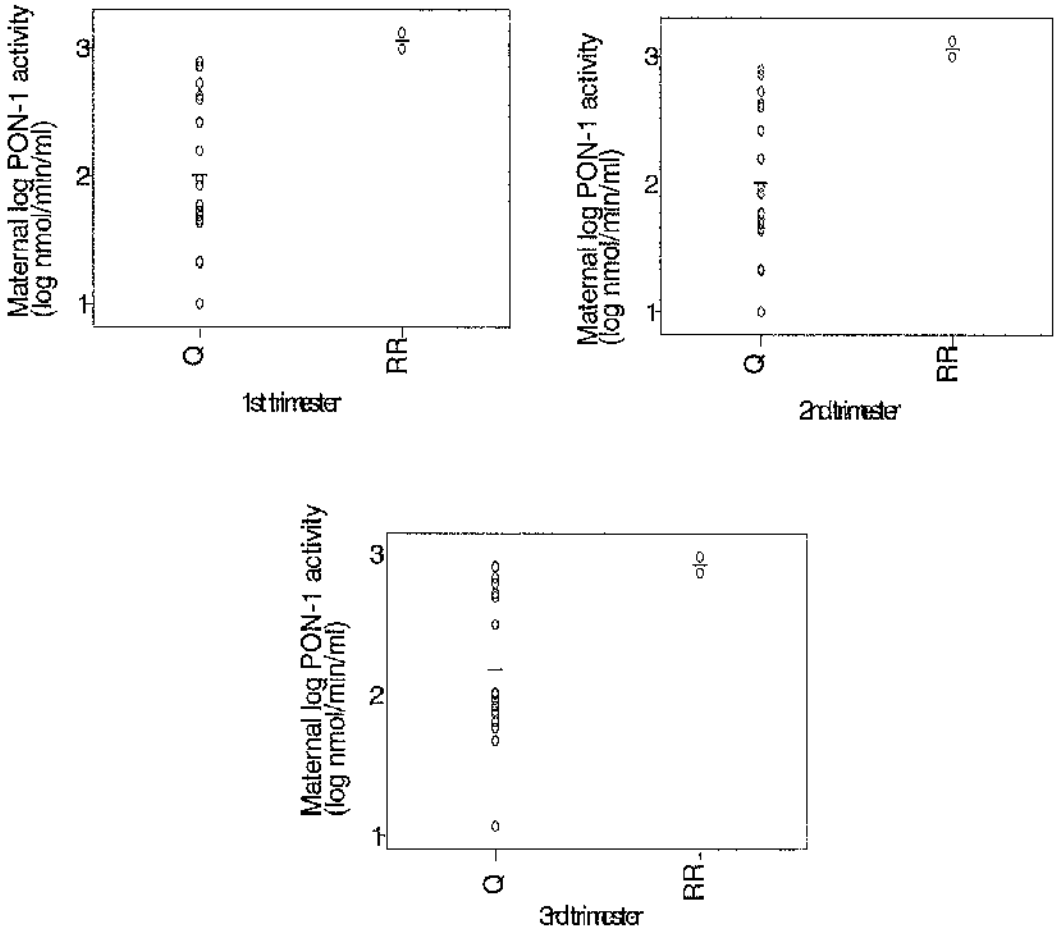


Figure 4-5. Dotplot of differences between paraoxonase-1 (PON-1) activity in PON-1 Q192R RR homozygotes or Q-carriers, in each trimester.

Mean values are demonstrated by lines.

4.2.3 PET and IUGR pregnancies

4.2.3.1 Maternal and fetal characteristics in pregnancies complicated by PET and IUGR

For the analysis of lipids and PON-1 activity in complicated pregnancy, cases were matched for age, parity and BMI with controls. Characteristics of the matched groups are shown in Table 4-6.

Characteristic	PET			IUGR		
	Case n = 23	Control n = 23	p	Case n = 17	Control n = 17	p
Age (years)	29.4 (6.5)	29.7 (6.1)	0.85	29.4 (4.9)	28.3 (5.2)	0.53
BMI (kg/m^2)	27.5 (4.8)	27.8 (4.9)	0.86	24.7 (5.4)	24.8 (5.4)	0.95
Systolic BP (mmHg, max)	161 (14)	129 (17)	<0.001	n/a	n/a	n/a
Diastolic BP (mmHg, max)	108 (8.0)	72 (12)	<0.001	n/a	n/a	n/a
Primigravidae n (%)	17 (74%)	13 (57%)	0.22	10 (59%)	11 (65%)	0.72
Smokers n (%)	5 (22%)	6 (26%)	0.73	11 (65%)	8 (47%)	0.30
Gestation at sampling/ diagnosis (weeks)	36.2 (3.2)	39.6 (2.1)	<0.001	35.8 (3.2)	39.2 (2.4)	0.001
Gestation at delivery (weeks)	36.5 (3.2)	40.0 (1.3)	<0.001	36.7 (2.8)	39.9 (1.4)	<0.001
Vaginal delivery n (%)	12 (52%)	9 (39%)	0.38	5 (29%)	9 (53%)	0.16
Placental weight (g)	516 (190)	702 (84)	0.002	330 (100)	686 (91)	<0.001
Fetal weight (kg)	2.51 (0.77)	3.77 (0.52)	<0.001	2.05 (0.50)	3.63 (0.57)	<0.001
Birth weight centile	22 (22)	57 (27)	<0.001	3 (3)	50 (32)	<0.001
Fetal sex (%)	27% male 73% female	62% male 38% female	0.02	40% male 60% female	81% male 19% female	0.02
TC (mmol/L)	6.57 (1.14)	5.94 (0.89)	0.04	6.32 (1.07)	5.79 (0.77)	0.11
Log TG (log mmol/L)	0.50 (0.14) [3.16 (1.39)]	0.44 (0.10) [2.73 (1.25)]	0.09	0.42 (0.17) [2.62 (1.47)]	0.40 (0.14) [2.48 (1.39)]	0.67
VLDL (mmol/L)	0.96 (0.41)	0.78 (0.27)	0.09	0.77 (0.46)	0.71 (0.21)	0.62
LDL (mmol/L)	3.95 (0.81)	3.64 (0.95)	0.23	3.9 (1.05)	3.54 (0.82)	0.28
Log HDL (mmol/L)	0.19 (0.12) [1.55 (1.31)]	0.17 (0.09) [1.50 (1.23)]	0.53	0.21 (0.09) [1.61 (1.24)]	0.18 (0.08) [1.51 (1.21)]	0.37

Table 4-6. Subject characteristics and maternal lipid profile in pregnancies complicated by PET or IUGR.

All values are mean and standard deviation (S.D.). Log transformed data was used for maternal triglyceride (TG) and high-density lipoprotein (HDL), and is presented as mean (S.D.) and [geometric mean (S.D.)]. Statistical analysis was performed using 2-sample *t*-test for continuous variables and chi-square for categorical variables.

None of the PET subjects had received any anti-hypertensive medication at time of sampling. Systolic and diastolic blood pressure (BP) was significantly higher in PET compared with the control subjects. Women with PET pregnancies (n=23) and women with IUGR pregnancies (n=17) had significantly earlier gestational age of delivery than those of controls, by a mean of 3.5 and 3.2 weeks respectively ($p<0.001$ in both groups). These subjects also had a significantly earlier gestation of blood sampling, at time of recruitment to the study when their condition was diagnosed ($p \leq 0.001$ in both groups). Offspring from women with PET and women with IUGR had significantly lower birth weights ($p<0.001$ for both groups) and significantly lower birth weight centiles ($p<0.001$ for both groups). These groups also had lower placental weights (PET $p = 0.002$, IUGR $p<0.001$), and significantly different gender distribution (PET $p = 0.02$, IUGR $p = 0.02$) with more female offspring in the case groups.

4.2.3.2 Maternal lipid profile in PET, IUGR and uncomplicated control pregnancies

In PET subjects, there was a significantly elevated maternal TC [mean 6.57 (SD 1.14) mmol/L] compared to controls [5.94 (0.89) mmol/L, $p = 0.04$] (Table 4-6 & figure 4-6). There were no significant differences in any of the other lipid fractions in the PET group, although a trend towards increased TG levels was observed. There were no significant differences in the mean concentrations of TC, TG, VLDL-C, LDL-C or HDL-C between the IUGR group and their controls (Table 4-6 & Figure 4-6).

4.2.3.3 Maternal PON-1 activity in PET, IUGR and uncomplicated pregnancy

For analysis, all maternal log PON-1 values were adjusted for gestational age at sampling. In PET subjects, maternal log PON-1 activity was significantly lower [0.37 (3.16) log nmol/min/ml] compared to controls [3.36 (2.04) log nmol/min/ml, $p=0.001$] (Figure 4-7). However, in IUGR subjects, there was no difference between cases [1.97 (0.51) log nmol/min/ml] and controls [2.12 (0.62) log nmol/min/ml, $p=0.49$] (figure 4-7). In PET, the reduction in PON-1 activity was preserved for the L-carriers [PET 0.56 (3.27) vs controls 3.72 (1.29) log nmol/min/ml, $p=0.001$] and the MM homozygotes were observed to have lower PON-1 activity compared to controls [PET –1.44 (0.21) vs controls 2.39 (3.31) log nmol/min/ml] based on the L55M polymorphism (Figure 4-8). However, based on the Q192R polymorphism, PON-1 activity was not different between Q-carriers [PET 1.88

(0.42) vs controls 1.90 (0.34) log nmol/min/ml, p=0.90], and there was only one RR homozygote in each group [PET 2.05 vs control 2.01 log nmol/min/ml] (figure 4-8). In the IUGR group, there were no differences between PON-1 activity based on L-carrier status [IUGR 2.07 (0.42) vs controls 2.21 (0.60) log nmol/min/ml, p=0.51]. Similarly, there were no differences between PON-1 activity based on Q-carrier status [IUGR 1.94 (0.19) vs controls 2.07 (0.31) log nmol/min/ml, p=0.17] (figure 4-9). In the IUGR control group, for the Q192R polymorphism, all subjects were Q-carriers.

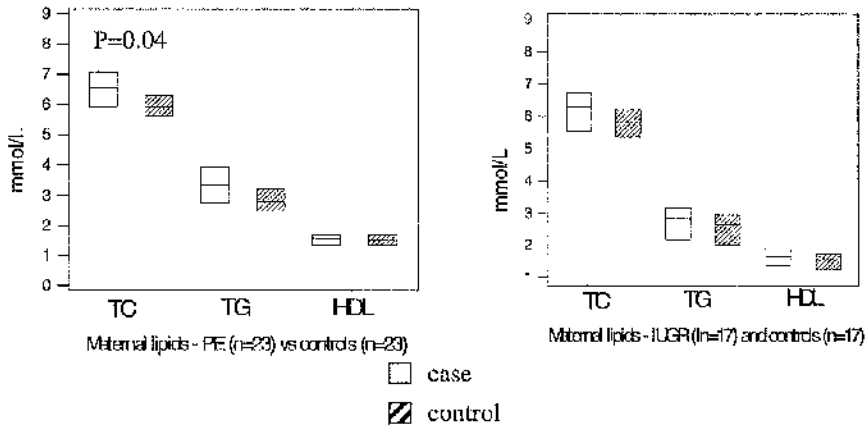


Figure 4-6. Maternal lipids in PET, IUGR and control pregnancies.

Data shown are raw data. All values are mean and 95% confidence intervals. Statistical analysis was performed using a 2-sample *t*-test. Log transformed data was used for statistical analysis of maternal triglyceride (TG) and high-density lipoprotein (HDL).

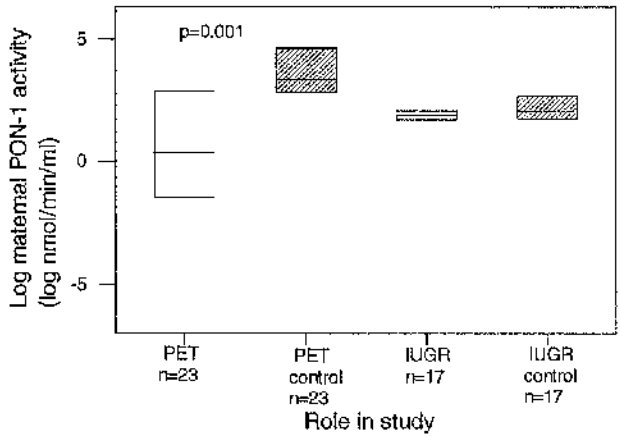


Figure 4-7. Maternal log paraoxonase-1 (PON-1) activity by role in study.

Maternal PON-1 was adjusted for gestational age at sampling, and data was log transformed to achieve normality. Log transformed data is shown to allow graphical representation. All values are mean and 95% confidence intervals. Statistical analysis was performed using a 2-sample *t*-test.

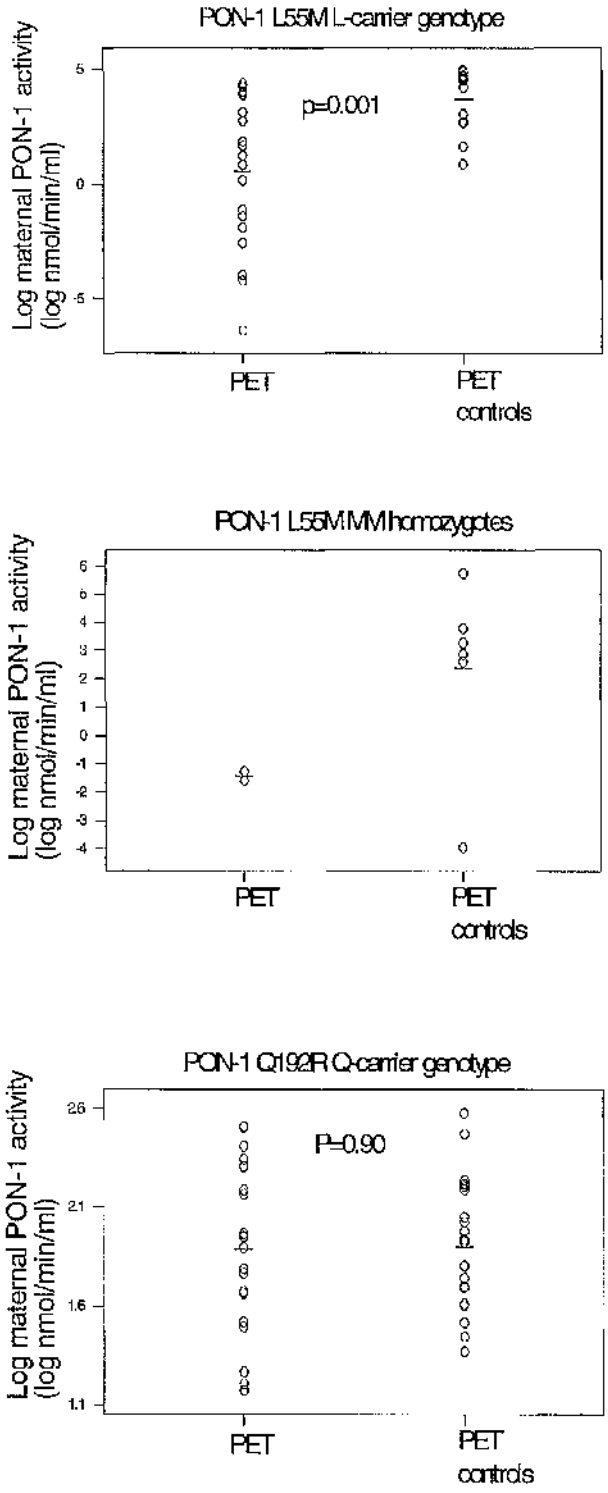


Figure 4-8. Maternal log paraoxonase-1 (PON-1) activity based on genotype (L55M and Q192R) in PET and PET controls.

Statistical analysis was performed using ANOVA. Maternal PON-1 was adjusted for gestational age at sampling, and data was log transformed to achieve normality. Log transformed data is shown to allow graphical representation.

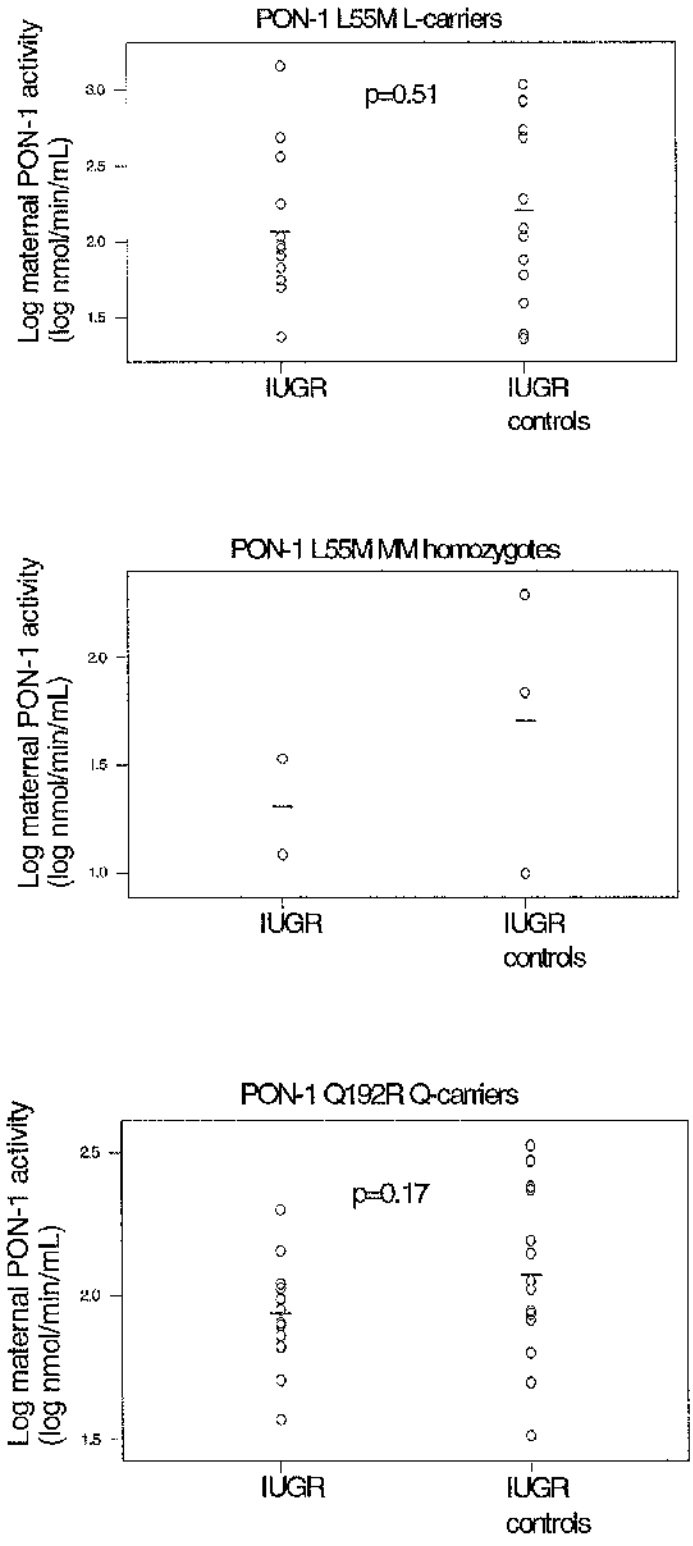


Figure 4-9. Maternal log paraoxonase-1 (PON-1) activity based on genotype (L55M and Q192R) in IUGR and IUGR controls.

Statistical analysis was performed using ANOVA. Maternal PON-1 was adjusted for gestational age at sampling, and data was log transformed to achieve normality. Log transformed data is shown to allow graphical representation.

4.2.3.4 Fetal lipid profile in PET and IUGR and uncomplicated pregnancies

There were 14 PET and 11 IUGR fetal samples available from the maternal cohort. The fetal venous cord blood concentrations of TC, TG and HDL-C were all significantly lower in the fetal group than in their mothers ($p<0.001$ in all groups). For analysis, all fetal lipids were adjusted for the effect of mode of delivery. In the venous cord plasma of pregnancies complicated by PET, there was a significantly increased concentration of log TC [mean 0.36 (SD 0.23) log mmol/L] compared with uncomplicated pregnancies [0.11 (0.15) log mmol/L, $p=0.003$] (Figure 4-10), a significantly increased log TG concentration [-0.21 (0.32) log mmol/L] compared to controls [-0.49 (0.26) log mmol/L, $p = 0.02$] (Figure 4-10) and a significantly increased cholesterol/HDL-C ratio [3.64 (1.62)] compared with controls [1.80 (0.86), $p=0.001$]. However, there was no difference in HDL-C levels. In IUGR pregnancies, a significantly elevated concentration of log TG [-0.17 (0.35) log mmol/L] was noted, compared to the control group [-0.57 (0.10) log mmol/L, $p = 0.01$] (Figure 4-10). There was no difference in TC, HDL-C or the TC/HDL ratio between the groups.

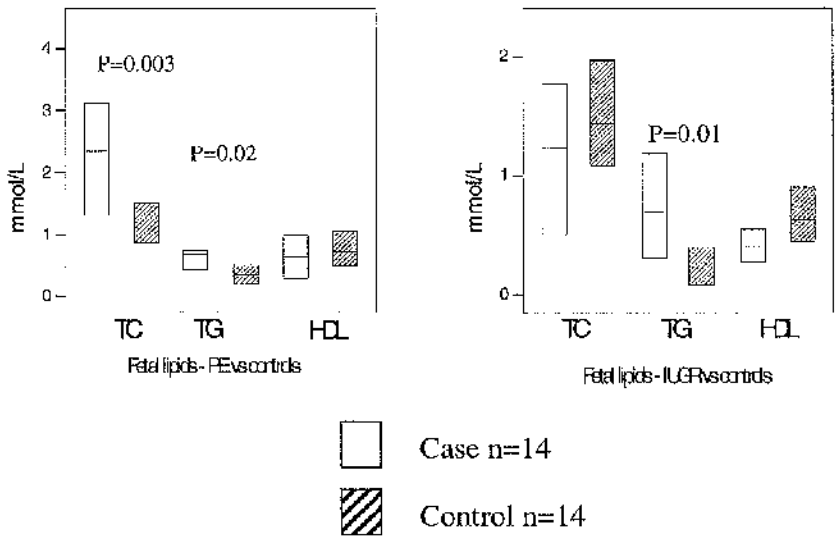


Figure 4-10. Fetal lipids in PET, IUGR and control pregnancies.

Data shown is adjusted for mode of delivery. All values are mean and 95% confidence intervals. Statistical analysis was performed using a 2-sample *t*-test. Log transformed data was used for statistical analysis of fetal total cholesterol (TC) and triglyceride (TG).

4.2.3.5 Fetal PON-1 activity in PET and IUGR and uncomplicated pregnancies.

The fetal PON-1 samples were the same as those used in the fetal lipid cohort. The fetal venous cord blood concentrations of PON-1 were not different in the fetal group compared with their mothers (p>0.07 in all groups). In PET, there was no significant difference between log fetal PON-1 activity between cases [1.55 (0.42) log nmol/min/ml] and controls [1.73 (0.53) log nmol/min/ml, p=0.45]. There was also no difference in fetal log PON-1 activity in IUGR subjects [1.61 (0.43) log nmol/min/ml] compared to controls [1.61 (0.52) nmol/min/ml, p=1.00] (Figure 4-11).

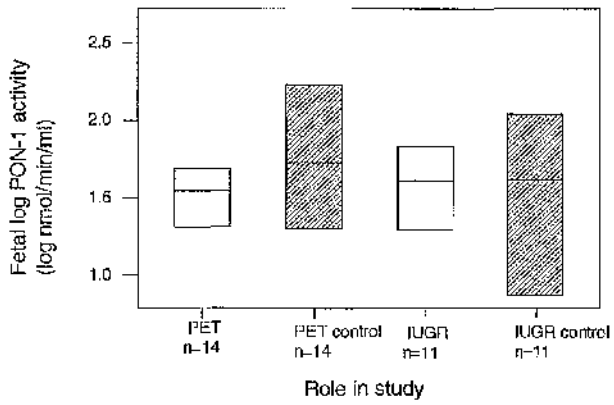


Figure 4-11. Fetal log paraoxonase-1 (PON-1) activity by role in study.

Data was log transformed to achieve normality, and is unadjusted. Log transformed data is shown to allow graphical representation. All values are mean and 95% confidence intervals. Statistical analysis was performed using a 2-sample *t*-test.

With respect to fetal genotype, in PET there was no difference in PON-1 activity based on L55M or Q192R genotype when compared with controls (table 4-7). However, small sample size must be taken into account when analysing this data.

	PET	PET control	p	IUGR	IUGR control	p
L-carrier	1.55 (0.42)	1.88 (0.40)	0.14	1.61 (0.43)	1.80 (0.36)	0.44
MM	*	0.87	N/A	*	0.87	N/A
Q-carrier	1.56 (0.44)	1.73 (0.53)	0.49	1.61 (0.43)	1.61 (0.52)	1.00
RR	1.46	*	N/A	*	*	N/A

Table 4-7. Fetal log paraoxonase-1 (PON-1) activity (log nmol/min/ml) based on fetal genotype (L55M and Q192R).

Statistical analysis was performed using ANOVA. *n=0.

4.2.4 Associations between maternal and fetal lipids and between maternal and fetal PON-1 activity.

There were no correlations between fetal and maternal TC, TG or HDL-C in the PET or IUGR groups. There was no association between fetal or maternal lipids and fetal birthweight or placental weight in any of the groups. Similarly, HDL-C and TG were not correlated within any of the fetal groups.

There were no correlations between fetal and maternal log PON-1 activity in the PET or IUGR groups. There were no associations between maternal or fetal log PON-1 activity and maternal or fetal lipids in the PET group, PET controls or the IUGR control groups. There were no correlations between the groups and neither maternal nor fetal log PON-1 activity was associated with TG or HDL levels in any of the groups. In the PET group, there was a positive association between log maternal PON-1 activity and fetal weight ($r=0.90$, $p<0.001$), birth weight centile ($r=0.44$, $p=0.05$) (figure 4-12) and placental weight ($r=0.74$, $p=0.002$) (figure 4-13A). The latter correlation was not seen in the IUGR group ($r=0.13$, $p=0.71$) (figure 4-13B) or in the total controls ($r=0.31$, $p=0.09$) (figure 4-13C).

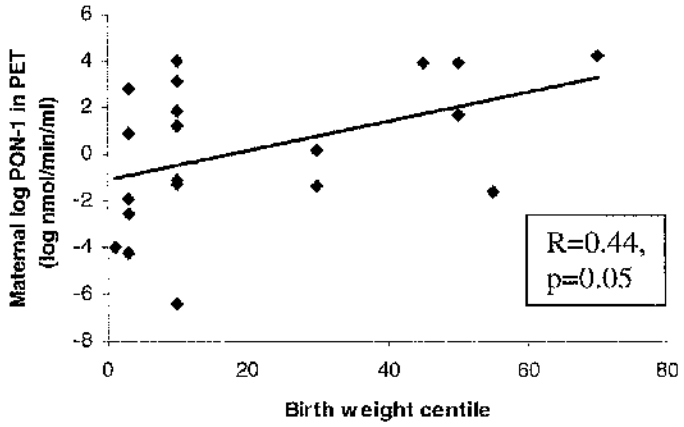


Figure 4-12. Association of maternal log paraoxonase-1 (PON-1, n=23) with birth weight centile in the PET group.

Statistical analysis was performed using Pearson's coefficient of correlation, on data adjusted for gestational age at sampling.

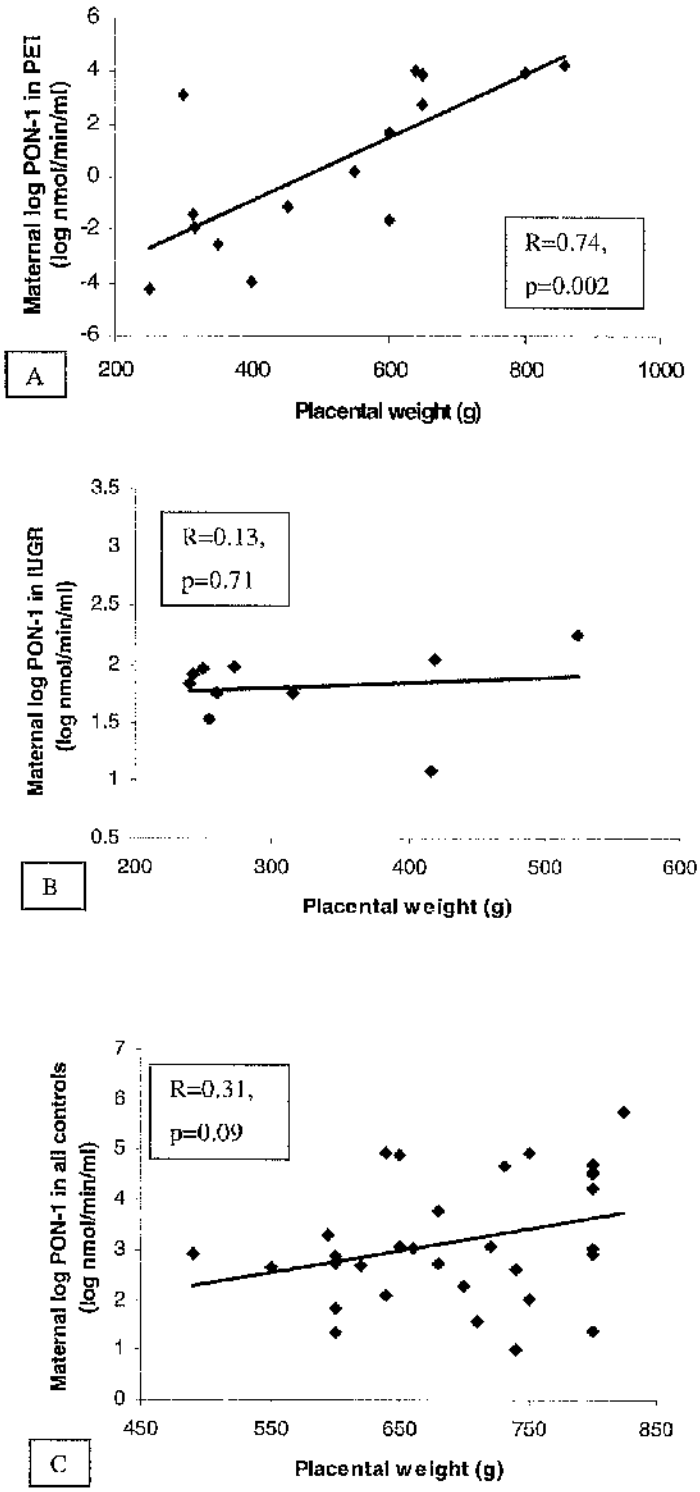


Figure 4-13 A, B, C. Association of maternal log paraoxonase-1 (PON-1) with placental weight (g) in, A – PET, B - IUGR and C – all controls.

Statistical analysis was performed using Pearson's coefficient of correlation, on data adjusted for gestational age at sampling.

4.3 Discussion

This is the first report to describe fetal lipid concentrations in pregnancies complicated by PET. Pre-eclamptic pregnancies demonstrated an elevated fetal log TC concentration, with resulting raised fetal TC/HDL-C ratio, and a significantly raised fetal log TG level. With respect to IUGR pregnancies, a significantly elevated fetal log TG level has been demonstrated. These latter findings are consistent with those of previous studies (Jones *et al* 1999). The marked effects on fetal lipids observed in this study were apparent despite the minimal effects on maternal lipids from the same pregnancy, and there were no correlations between maternal and fetal lipids in the PET or IUGR groups.

In the present study, lower lipid levels in fetal compared with maternal blood were confirmed (Averna *et al* 1991, Kilby *et al* 1998, Neary *et al* 1995), as was the predominance of HDL-C. A detailed understanding of lipoprotein metabolism in the fetus remains undetermined. It is possible that HDL-C may be involved in the transfer of cholesterol from the placenta to the fetal circulation after interaction with the ABCA1 cholesterol efflux transporter. This is speculative, as to date ABCA1 has not been studied in detail in the placenta, although it is highly expressed in this tissue (Langmann *et al* 1999). Pregnancies were not matched for mode of delivery; it has been suggested previously that method of delivery does not affect fetal lipid levels (Lane and McConathy 1983). However, in the present study of fetal lipids in uncomplicated pregnancies, it was demonstrated that fetal lipids were significantly affected by mode of delivery, using ANOVA (figure 1) ($p<0.001$). The lipids levels were highest in possibly the most stressful situation – emergency LUSCS. Thus, all fetal data was adjusted to account for the effect of mode of delivery.

Although not significant in this study, a trend was demonstrated towards the maternal hypertriglyceridaemia (Hubel *et al* 1996, Kaaja *et al* 1995, Sattar *et al* 1997a) and elevated LDL-C levels (Belo *et al* 2002a, Lorentzen and Henriksen 1998, Ogura *et al* 2002, Sattar *et al* 1997a) previously reported in PET, compared to control subjects. This study did not confirm the previously reported maternal dyslipidaemia of IUGR (Sattar *et al* 1999a). Obesity is associated with dyslipidaemia (Reaven 1988), and abdominal-visceral fat correlates with increased plasma levels of triglyceride and reduced HDL levels (Kissebah and Krakower 1994). BMI is a risk factor for PET (Sattar *et al* 2001) and because of the clear influence of BMI on plasma lipids in pregnancy (Ramsay *et al* 2002), sample groups were matched case by case for maternal BMI in this study. Different methods of correction

for BMI may explain some of the differences between the present observations of maternal lipids and those of earlier studies.

This study observed that in uncomplicated pregnancies, median maternal PON-1 levels are lower than the non-pregnant UK median (112.0nmol/min/ml vs 215 nmol/min/ml, Mackness, personal communication). This may reflect the increased atherogenic risk seen in pregnancy (Sattar and Greer 2002) and may contribute to the proposed association between PET and future risk of CVD. These findings are in conflict with those of Roy and colleagues (Roy *et al* 1994) who demonstrated an increase in PON-1 activity in pregnant subjects. This discrepancy may again be attributed to different methods of correction for BMI, as leanness is associated with increased oxidative capacity. In the study by Roy *et al* cases and controls were reported to have comparable weight and height, although data regarding BMI was unavailable for comparison between the 28 week and the 32 week groups and BMI calculation was observed to vary by >2kg/m^2 between the 28 week pregnant group and the non-pregnant group. In the current study, there was no comparison with non-pregnant women as there was in the data from Roy and colleagues, therefore findings are purely observational with respect to comparisons with the non-pregnant UK median.

PON-1 activity remained constant with advancing gestation in the longitudinal study of uncomplicated pregnancy, although PON-1 values were significantly reduced by gestational age at sampling in the control group from the 3rd trimester. This may indicate that in uncomplicated pregnancy PON-1 activity is reduced significantly only in the later stages of pregnancy, which may indicate a role in the dyslipidaemia and oxidation seen at this stage of pregnancy. Because activity remains constant with advancing gestation between the trimesters, PON-1 activity may not play a direct role in the metabolic alterations or oxidative stress seen in normal pregnancy as gestation advances but may be involved in the long-term increased risk of CVD because of its significant reduction at the later stages of the 3rd trimester. Data in the PON-1 PET/control study were corrected for the effect of gestational age at sampling, and it is interesting to note that data uncorrected for this potential confounder would magnify the reduction in PON-1 activity in PET compared with controls.

Although numbers were small, this study was consistent with the observation that being a MM homozygote for the L55M PON-1 polymorphism is associated with a trend towards lower PON-1 activity when compared to being an L-carrier (Humbert *et al* 1993). MM homozygosity may be associated with increased oxidative stress and potential CVD risk.

The current study also observes that RR homozygosity for the Q192R PON-1 polymorphism is associated with higher PON-1 activity in the 1st and 2nd trimesters of uncomplicated pregnancies (p=0.02 both groups), with a trend towards the same in the 3rd trimester (p=0.07). This data supports previous research describing higher PON-1 activity in the R-carriers (Mackness *et al* 1996) and may indicate that in uncomplicated pregnancy, RR homozygosity is related to increased protection from oxidative stress. However, recent studies in the non-pregnant state have highlighted a paradox surrounding the Q192R polymorphism and PON-1 activity and risk of CVD. The Q alloenzyme is more efficient at protecting LDL from oxidation (Mackness *et al* 2002), and the R polymorphism has been more closely associated with CHD than the Q polymorphism. It is interesting to note that although the R allele is considered to confer higher paraoxonase activity, measured using paraoxon as a substrate, the Q alloenzyme is more efficient at protecting LDL from oxidation. Thus differing substrate specificity of the PON-1 alleles may explain this paradox (Davies *et al* 1996) (Furlong *et al* 1989).

This is the first report of maternal PON-1 activity in both PET and IUGR pregnancies. In PET, a lower maternal PON-1 activity was demonstrated compared to control subjects (p=0.001), independent of genotype. This confirms findings published during the time of writing this thesis by Kumru and colleagues (Kumru *et al* 2004), who have recently described significantly lower maternal PON-1 activity, independent of genotype, in severe PET compared to uncomplicated pregnancy. This group proposed a role for PON-1 in the pathogenesis of PET. Together these findings would support the proposal that oxidative stress is a component of PET (Hubel 1999) and that markers of oxidative stress are altered in PET (Chappell *et al* 2002) (Akyol *et al* 2000). There was no difference in maternal PON-1 activity in IUGR pregnancies compared with controls (p=0.49) in this study, which may provide further evidence that PON-1 has a role in the pathogenesis of PET via oxidative stress rather than placental pathology.

In this study, it was considered that sample sizes in the genotype subgroups were too small to draw any firm conclusions and data are purely observational. However, in general, the reduction in maternal PON-1 activity seen in PET persisted in both L-carrier and MM homozygote groups of the L55M polymorphism but did not persist in the Q-carriers or RR homozygotes of the Q192R polymorphism. There were no differences in maternal PON-1 activity based on L55M or Q192R polymorphism in IUGR and no differences based on either polymorphism in the fetal group.

In the PET group, increased PON-1 activity was associated with increased fetal weight, birth weight centile and placental weight. These associations were not observed in the IUGR and control uncomplicated pregnancies. This may indicate that in a situation where the placenta is poorly functioning and there is an abnormal maternal metabolic response, as in PET, it is the subjects with highest maternal PON-1 activity who deliver the largest babies, as the anti-oxidant properties of PON-1 act to allow optimal placental growth and thereby optimal fetal nutrition. This may help to explain why the fetus is small in IUGR, where the placenta is also poorly functioning, as the maternal metabolic response does not act to provide the fetus with optimal growth. There were no differences observed in fetal PON-1 activity between offspring from PET subjects and controls or between offspring from IUGR subjects and controls, independent of fetal genotype. In this study, no conclusions have been made regarding the observations of fetal PON-1 activity based on genotype as sample sizes are too small. In these observations (see table 4-7), there were no rare homozygotes in each of the groups for either one or both polymorphisms. Further studies would analyse potential correlations between markers of oxidative stress such as isoprostanes and PON-1 activity in pregnancy, and would study the effects of genotype in much larger cohorts.

This study has a number of limitations. Non-fasting samples were used to facilitate the collection of both maternal and fetal bloods from the same pregnancy, which may affect TG levels only. This was particularly pertinent to sampling of our cases where emergency delivery (within 24 hours) is common. Similarly, in PET and IUGR studies, it is difficult to control for gestational age at sampling and most previous studies have not done this. In this group, we found that gestational age at sampling had no significant effect on maternal or fetal lipids and therefore we did not statistically adjust the lipid data for gestational age at sampling. However, maternal PON-1 activity was affected by gestational age at sampling in the 3rd trimester samples, and thus data was adjusted correspondingly in the PET and IUGR case controls study. In previous reports, authors have either accounted (Kilby *et al* 1998, Sattar *et al* 1997a, Sattar *et al* 1999a) or not (Belo *et al* 2002b, Kaaja *et al* 1995, Ogura *et al* 2002) for gestational age. This may explain some of the discrepancies regarding maternal lipids in the literature. One study has demonstrated an effect of fetal gender on lipid levels (Loughrey *et al* 2000) where female neonates have significantly lower triglyceride concentrations than males ($p<0.001$) and non-significant tendencies towards higher concentrations of LDL and HDL. However, in our study, fetal gender had no influence on fetal lipid profile in the newborns from uncomplicated pregnancies.

The data obtained supports the hypothesis that maternal lipid alterations in PET and in IUGR pregnancies will result in disturbances in the fetal lipids. It is possible that the increased supply of maternal lipids in these conditions would result in changes in mass lipid transfer across the placenta, leading to the fetal lipid alterations. However, it is interesting to note that fetal lipids can be greatly altered by PET and IUGR when their mothers' lipids are minimally changed, and that in our study the pattern of dyslipidaemia in the offspring does not match that in the mother. This implies that fetal lipids are not determined merely by circulating concentrations of maternal lipid available for placental uptake.

The alterations in the fetal lipid profile observed may be due to changes in trans-placental transport. The human placenta is known to express lipoprotein receptors in high amounts (Jensen *et al* 1989, Wittmaack *et al* 1995), and the LDL receptor plays an important role in the uptake of maternal plasma lipoproteins for placental steroid metabolism (Stepan *et al* 1999). It has recently been demonstrated that human first trimester and term trophoblast cells express SR-BI (an HDL receptor), and this could serve as an efficient route for supplying cholesteryl esters from maternal lipoproteins to fetal tissues (Wadsack *et al* 2003). However, there is a paucity of data explaining how these receptors are regulated throughout healthy pregnancy and in pathological pregnancies. Maternal triglyceride does not cross the placental barrier, but TG in maternal lipoproteins can be hydrolysed by placental lipase and the resulting fatty acids transferred across the placenta by fatty acid binding proteins. The up-regulation of these mechanisms might increase fatty acid transport across the placenta, thus supplying the fetal liver with substrate for TG synthesis (Haggarty 2002, Herrera 2002). On reaching the fetal circulation the non-esterified fatty acids can be transported to the fetal liver for triglyceride synthesis (Haggarty 2002).

Pathological or adaptive changes in placental transport may play an aetiological role in the fetal lipid alterations in PET and IUGR. Pre-eclampsia and IUGR have devastating effects on placental function that might easily influence lipid transport. The changes in fetal lipids may be an appropriate physiological response to an adverse *in-utero* environment. Transport mechanisms may be up regulated to compensate for oxidative and structural damage to the placenta (Demir *et al* 1994, Laskowska-Klita *et al* 2001). Interestingly, Stepan and colleagues (Stepan *et al* 1999) described a significantly higher LDL receptor mRNA expression relative to actin in IUGR when compared with term and pre-term pregnancies ($p<0.05$) and explained this as a compensatory mechanism for the lower circulating LDL concentrations in women with IUGR. Although we did not find reduced TC in mothers with IUGR, we did find an increase in fetal log TC levels from PET

pregnancies, and increased fetal log TG levels from PET and from IUGR pregnancies. This could potentially be due to up-regulation of placental lipoprotein receptor expression or activity in PET and IUGR. Changes in placental fatty acid transport could additionally be attributed to increases in lipase or fatty acid binding protein activity, once again as a compensatory mechanism to supply the fetus with an energy source via increased TG levels. It is possible that compensatory mechanisms differ between IUGR and PET. If the alterations in fetal lipids are to be ascribed to changes in placental transport then correlations between indices of placental function and fetal lipids might be expected. Although in the uncomplicated group, placental weight correlated with fetal log TG, log TC and the cholesterol/HDL ratio, there was no association between placental weight and fetal lipid levels in the PET or IUGR groups. However, placental weight is a relatively crude measurement of placental function and more specific markers such as PAI-1/PAI-2 (Chappell *et al* 1999) may be useful.

Alternatively, fetal lipids may be altered in PET and IUGR because of an incomplete development or immaturity of the biochemical pathways and processes involved in fetal TG metabolism. This may be apparent in complicated pregnancies either because of the earlier gestational age at delivery or due to a developmental 'sparing effect' diverting nutrients to key organ development under conditions where growth is restricted. The former possibility is inconsistent with the lack of association of fetal lipids with gestational age at delivery. It has been reported that small for gestational age have impaired utilisation of circulating triglycerides, consistent with peripheral adipose depletion (Jones *et al* 1999), and this may be particularly evident in PET and IUGR fetuses especially when growth is restricted.

Alterations in fetal lipids in PET and IUGR pregnancies could be attributed to an acute fetal stress response due to mode of delivery, or a chronic response secondary to poor placental perfusion. Since our fetal lipid findings were adjusted for mode of delivery, it is unlikely that the short-term immediate stress of labour or traumatic delivery should account for the lipid aberrations seen in our study. Long-term stress may alter fetal production of hormones such as cortisol with consequent effects on lipid metabolism. Future studies to examine any association between markers of fetal stress and fetal lipid levels may be worthwhile.

Although the future impact of altered lipid levels in neonates resulting from PET and IUGR pregnancies is unknown, it is interesting to note that neonatal VLDL-C and LDL-C concentrations were predictive of levels in the offspring at the age of thirteen (Fonnebo *et*

al 1991). In light of the weight of evidence supporting fetal programming and the results presented herein, future studies should now investigate lipid and other cardiovascular risk factors in offspring from PET pregnancies. It is concluded that fetal lipids are altered in pregnancies complicated by PET and IUGR. These findings have potential implications for the future cardiovascular health of the offspring and highlight the need for research into placental lipid transport in healthy and pathological pregnancy.

5 Inflammation and insulin resistance in uncomplicated pregnancy and in PET and IUGR

5.1 Introduction

In the Introduction (chapter 1), the generalised maternal inflammatory response (section 1.2.3.4) and state of insulin resistance (section 1.3.2) seen in uncomplicated pregnancy have been detailed along with the exaggerated inflammation and insulin resistance of PET. Elevated levels of TNFα, IL-6, VCAM-1, ICAM-1, E-, P- and L-selectins and PECAM-1, (Acar *et al* 2001, Bretelle *et al* 2001, Carr *et al* 2001, Greer *et al* 1994, Lyall *et al* 1994, Vince *et al* 1995, Zeisler *et al* 2001) in the peripheral blood in PET, along with neutrophil activation in the peripheral circulation in PET (Greer *et al* 1989) and in IUGR (Johnston *et al* 1991) (Sabatier *et al* 2000) have also been discussed. Elevated CRP levels (though not independently of BMI) have been demonstrated in the 1st trimester of pregnant women who later develop PET (Wolf *et al* 2001). Data is conflicting regarding fetal inflammatory markers in PET. One study demonstrated no differences in the circulating fetal concentrations of ICAM, VCAM and E-selectin between normal pregnancies and pregnancies complicated by PET (Krauss *et al* 1998). Aliefendioglu *et al* observed elevated maternal and fetal sICAM in PET pregnancies compared to controls (Aliefendioglu *et al* 2002). There are no reports of fetal inflammatory markers in IUGR pregnancies.

Four key inflammatory markers will be studied in this chapter; CRP, TNFα, IL-6 and IL-10, along with the hormone leptin and the nuclear receptor PPARγ which are associated with obesity and insulin resistance.

CRP is an acute-phase reactant marker of inflammation and tissue damage in the body that is synthesised by the liver. CRP is considered to act as a scavenger and is responsible for the clearance of membranes and their nuclear antigens (Du Clos 1996). Levels of CRP correlate significantly with features of the metabolic syndrome including indices of adiposity, hyperinsulinaemia and insulin sensitivity index, hypertriglyceridaemia, and low HDL cholesterol (Festa *et al* 2000, Forouhi *et al* 2001, Yudkin *et al* 1999) which are also features of PET. Alterations in CRP levels are predictive of future CVD risk (Packard *et al* 2000, Pradhan and Ridker 2002, Ridker *et al* 2000) and of the development of diabetes in middle-aged men, independent of established risk factors (Freeman *et al* 2002). CRP levels

are elevated in the serum of obese pregnant women compared with lean subjects (Ramsay *et al* 2002).

TNFα is a pro-inflammatory cytokine derived from macrophages and lymphocytes (Kupferminc *et al* 1994) and is involved in immunoregulation, modulation of cell growth and differentiation, coagulation, endothelial cell function and insulin resistance (Beutler and Cerami 1987, 1989, Fasshauer *et al* 2004, Le and Vilcek 1987). This cytokine is elevated in peripheral blood in normal pregnancy and increases from the 1st to the 3rd trimester (Freeman *et al* 2004). TNFα is further increased in PET (Vince *et al* 1995). It has been suggested that hypoxia stimulates a 2-fold increase in TNFα production in villous explants from human placenta (Benyo *et al* 1997). Intermittent perfusion of the placenta secondary to reduced trophoblast invasion as in PET, causes increased placental secretion of TNFα and this contributes to the activation of maternal endothelial cells that characterise PET (Hung *et al* 2004). However, recent data from villous explants demonstrated no significant increase in protein or mRNA expression of TNFα from normal or PET pregnancies (Benyo *et al* 2001). Thus there is conflicting evidence in the literature to suggest that the increased plasma levels of TNFα are a result of increased placental production in advancing pregnancy and in PET. This conflict may suggest that sources other than the placenta contribute to the elevated circulating levels of this cytokine (Benyo *et al* 2001).

Elevated maternal plasma and amniotic fluid levels of TNFα have also been associated with IUGR (Heyborne *et al* 1992), although there is debate in the literature as to whether inflammatory mediators are elevated in maternal plasma in IUGR. It is possible that these are not elevated because there is no maternal metabolic response to this condition, or there is no release of 'factor X' from the placenta or because there is a degree of maternal resistance to its effects (Johnson *et al* 2002). Activated adipocytes may provide an additional source of TNFα (Mohamed-Ali *et al* 1998) in uncomplicated pregnancy and in pregnancies complicated by PET or IUGR. This adipose production may account for the lack of increase of this cytokine from the 1st to the 3rd trimester after correction for BMI (Freeman *et al* 2004), as it is recognised that adipose tissue of obese individuals expresses increased amounts of TNFα compared to the adipose tissue from lean subjects (Fried *et al* 1998, Hotamisligil *et al* 1993). Human TNF-α inhibits human leptin secretion by cultured human adipocytes collected from the subcutaneous fat of pregnant women, suggesting an autocrine or paracrine regulation of leptin secretion in human adipose tissue *in vivo* (Yamaguchi *et al* 1998).

IL-6 is an inflammatory cytokine that is involved in cell-to-cell signaling within the immune system and is produced by many cell types including macrophages, adipocytes and skeletal muscle (Greenberg and McDaniel 2002, Kishimoto 1989). This cytokine also has a role in glucose and insulin metabolism (Fernandez-Real *et al* 2000, Senn *et al* 2002, Stouthard *et al* 1996). IL-6 is elevated from the 1st to the 3rd trimester in women who later develop PET (Freeman *et al* 2004, Vince *et al* 1995) although adjusting for BMI and smoking status attenuated the latter observation. IL-6 protein (Jauniaux *et al* 1996) and mRNA (Stephanou *et al* 1995) are present in cyto- and syncytiotrophoblast cells in 1st trimester trophoblast cells. Synthesis has also been documented in the normal human placenta (Kameda *et al* 1990) and term placenta has been shown to spontaneously secrete IL-6 *in vitro* (Turner *et al* 2002). However, the hypoxia that stimulated an increase in TNFα production from villous explants had no effect on IL-6 production in human placentae (Benyo *et al* 1997). Further, as for TNFα, data from villous explants demonstrated no significant increase in protein or mRNA expression of IL-6 from normal or PET pregnancies (Benyo *et al* 2001). In fact, IL-6 production has been shown to decrease in placental explants from PET pregnancies (Kauma *et al* 1995), and Yin and colleagues (Yin *et al* 1998) demonstrated that although plasma IL-6 levels increase in pregnant hypertensive patients compared with normotensive women, placental mRNA expression was reduced. This data may suggest that placental production of IL-6 does not account for the increase in plasma IL-6 in PET, and maternal adipose tissue may be considered as another source (Mohamed-Ali *et al* 1998). Several *in vivo* studies have revealed that IL-6 is secreted from subcutaneous adipose tissue in the non-pregnant state (Mohamed-Ali *et al* 1997, Orban *et al* 1999). Adipose tissue of obese individuals expresses increased amounts of IL-6 compared to the adipose tissue from lean subjects (Fried *et al* 1998), and high circulating levels of IL-6 and TNFα are found in patients with hyperinsulinaemia. It is important to note that elevated levels of IL-6 and leptin have been reported in obese pregnant women (Ramsay *et al* 2002).

IL-10 is an anti-inflammatory cytokine and a natural immuno-suppressant of TNFα (Edwards-Smith *et al* 1999). IL-10 is a Th2 cytokine, which acts as a potent inhibitor of Th 1 effector mechanisms (Fiorentino *et al* 1989, Fiorentino *et al* 1991, Mosmann 1994). It is predominantly expressed in B-lymphocytes, and is involved in the terminal differentiation of B cells into plasma cells. IL-10 exhibits predominantly inhibitory effects on inflammatory reactions (Lalani *et al* 1997). A predominance of IL-10 and other Th2 cytokines has been suggested to be compatible with a successful pregnancy (Lin *et al* 1993). Human plasma IL-10 is increased from the 1st to the 3rd trimester in uncomplicated

pregnancy (Freeman *et al* 2004). However, there is debate in the literature as to whether this cytokine is reduced in the serum of PET subjects hence contributing to the inflammation associated with this disease (Benian *et al* 2002, Freeman *et al* 2004, Hennessy *et al* 1999). Human placental tissue from uncomplicated pregnancies has been shown to express IL-10 (Bennett *et al* 1997, Bennett *et al* 1999, Cadet *et al* 1995, Roth *et al* 1996). Placental expression of IL-10 has been shown to increase in healthy pregnancy with gestation, but is down-regulated at term and after parturition, perhaps demonstrating the inflammatory nature of labour (Hanna *et al* 2000). Human term placental tissue expresses high levels of IL-10 mRNA suggesting that cells that produce IL-10 and that are associated with the placenta may play a role in preventing rejection of the fetal allograft by the mother (Cadet *et al* 1995). IL-10 has been highlighted as a regulator of placental morphogenesis, acting to retard expansion of the placental labyrinth and to modify the architecture of the maternal blood sinuses (Roberts *et al* 2003). Reduced decidual IL-10 mRNA expression has been described in association with IUGR (Hahn-Zoric *et al* 2002) and reduced placental IL-10 levels have been observed in PET (Hennessy *et al* 1999) and in cultured trophoblast cells from PET pregnancies as determined by ELISA (Rein *et al* 2003). This is consistent with the theory that IL-10 has an important anti-inflammatory role at the maternal-fetal interface in pregnancy and is reduced in situations of impaired placentation (Marjono *et al* 2002). However, data are conflicting. Rinehart and colleagues have demonstrated an increase in placental production of IL-10 mRNA in PET (Rinehart *et al* 1999). To date there have been no studies of IL-10 production by subcutaneous adipose tissue in uncomplicated pregnancy or pregnancies complicated by PET or IUGR.

Leptin is a hormone produced abundantly by adipose tissue (Ogawa *et al* 1995, Zhang *et al* 1994) and is involved in energy and the regulation of metabolism. Its role in the regulation of food intake and body weight by acting as an endocrine signal to the hypothalamus is clearly established (Friedman and Halaas 1998). Leptin is also produced by the placental trophoblast (Masuzaki *et al* 1997) and high circulating levels occur during pregnancy, predominantly secondary to placental production, but also secondary to up-regulation of adipose leptin production and production of leptin-binding proteins (Anderson and Ren 2002). Maternal serum leptin levels are elevated in normal pregnancy, especially in the 2nd trimester, then rapidly decrease and return to normal after delivery when the influence of the placenta is removed (Anderson and Ren 2002, Hardie *et al* 1997). It has been proposed that production of leptin by the placenta may play a role in the regulation of placental and fetal growth (Masuzaki *et al* 1997). It has recently been demonstrated in a longitudinal prospective case-control study that serum leptin concentrations were significantly higher in PET compared to low-risk women, after correction for BMI (Chappell *et al* 2002). Leptin

has been shown to be significantly higher in PET and obese pregnant subjects compared to lean pregnant women (Ramsay *et al* 2002) and leptin levels correlated independently with both CRP and fasting insulin levels. Thus leptin may be a marker of insulin resistance in pregnancy. Both plasma leptin levels and placental leptin mRNA are higher in women with PET (Laivuori *et al* 2000, Mise *et al* 1998) and fall to normal after delivery. In contrast, Bersinger and colleagues (Bersinger *et al* 2002) found no differences in leptin levels from healthy term and PET placental extracts using ELISA. Jakimiuk and colleagues (Jakimiuk *et al* 2003) have demonstrated that placental leptin mRNA production correlates with cord blood concentrations of leptin, and growth restricted fetuses at term have been shown to have significantly lower cord blood concentrations of leptin than appropriately grown fetuses for gestation. Because leptin is expressed abundantly in the adipose tissue, lower cord blood concentrations of leptin in growth restricted fetuses compared with appropriately grown fetuses may suggest that adipose tissue is a major source of fetal leptin production (Pighetti *et al* 2003).

The nuclear receptor PPARγ is also involved in energy, the regulation of metabolism and insulin action as well as inflammation. A role for PPARs, both in the non-pregnant via effects on inflammation and insulin resistance, and also in healthy and complicated pregnancies has been discussed in Chapter 3.

Both the placenta and maternal adipose tissue produce markers of inflammation and insulin resistance and data suggest that elevations in these markers seen with advancing gestation and in PET may not simply be secondary to increased placental production, but possibly due to up-regulation in adipocytes. Since BMI is a risk factor for PET, it is proposed that changes in IL-6, TNFα, IL-10 and PPARγ expression in PET could be secondary to increased fat mass or to a more 'active' adipose tissue in addition to, or instead of, increased placental production (figure 5-1). Another potential source of cytokines is lymphocytes, but this material was not available in the present study. As it is well established that increases in maternal plasma leptin levels in pregnancy are predominantly secondary to placental production, placental leptin was examined as a positive control. It is expected that placental production of leptin will be increased in PET and/or IUGR compared to controls.

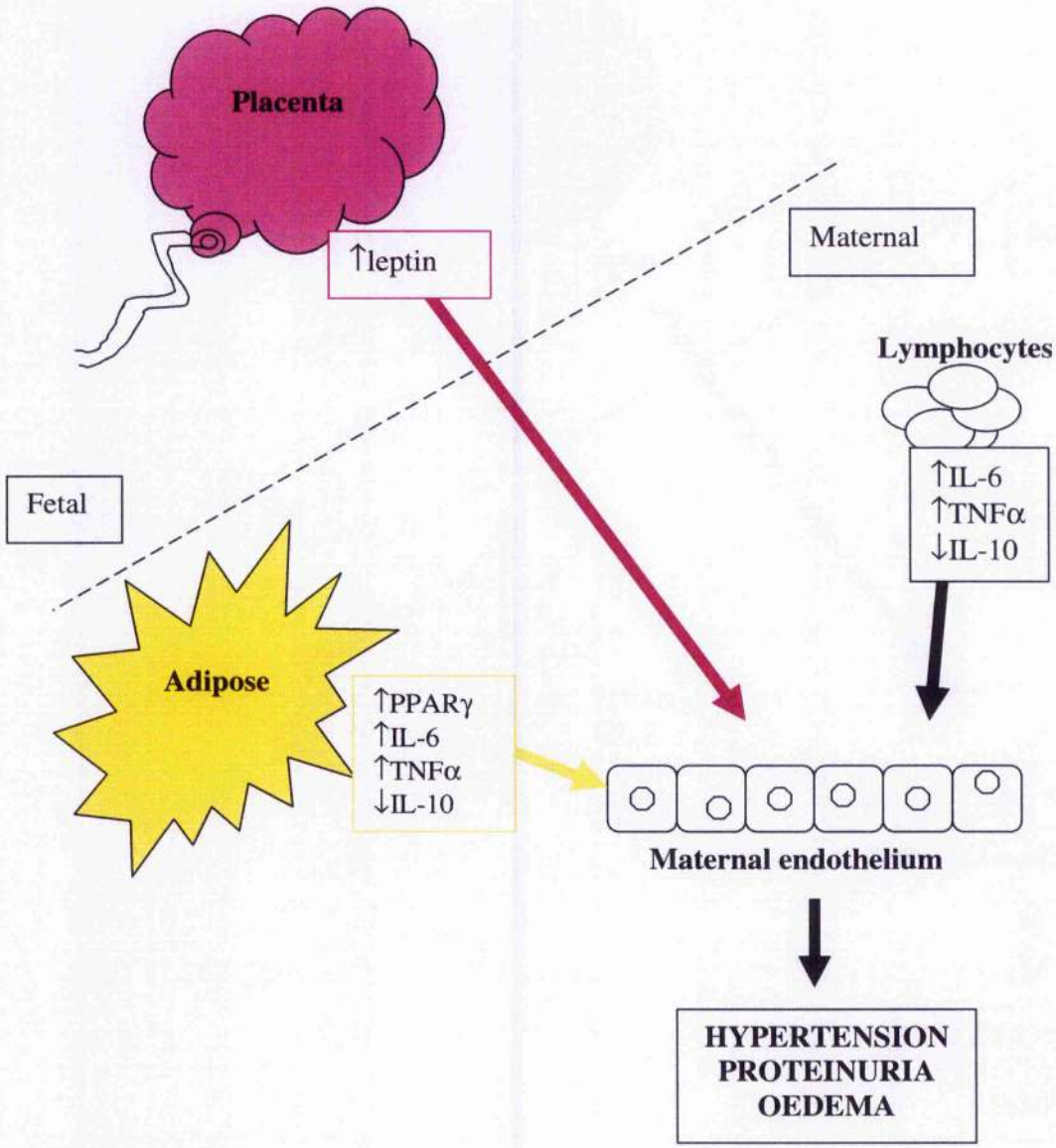


Figure 5-1. Proposed production of inflammatory and insulin resistance mediators in uncomplicated pregnancy and in PET.

The aims of this chapter were;

- To determine plasma levels of maternal and fetal inflammatory markers and markers of insulin resistance (IL-6, IL-10, CRP, TNFα, leptin) in 3rd trimester

uncomplicated pregnancies and in pregnancies complicated by PET or IUGR in a case control study.

- To determine mRNA expression of IL-6, IL-10, TNFα, PPARγ and leptin in human placenta (n=6 per group) and maternal subcutaneous adipose tissue (n=6 per group) mRNA, using quantitative real-time PCR (TaqMan) from uncomplicated, PET and IUGR pregnancies in a case control study.

- To determine protein expression of these inflammatory and insulin resistance markers using western analysis.

- To determine any associations between tissue expression and corresponding plasma levels of each of the inflammatory markers or markers of insulin resistance, in order to highlight potential tissue sources of production. PPARγ tissue values were compared with plasma HDL, TG and the inflammatory markers as surrogate markers of PPARγ function.

5.2 Results

5.2.1 Plasma analyses

5.2.1.1 Maternal and fetal plasma inflammatory markers in uncomplicated pregnancies

Baseline characteristics for eighty-one subjects from uncomplicated pregnancies are shown in table 4-1, as for the plasma lipids. Plasma inflammatory markers for these 81 subjects are demonstrated in table 5-1 below.

Maternal VCAM, TNFα and IL-10 were not related to maternal age, BMI (<25 kg/m^2 or >25 kg/m^2), smoking status, parity or gestational age at sampling (range 32-42 weeks), using linear regression analysis or ANOVA. However, maternal ICAM was associated with smoking status (p=0.006, r^2=8.6%) (figure 5-2) and gestational age at sampling (p=0.03, r^2=5.1%). Maternal log IL-6 was associated with gestational age at sampling (p<0.001, r^2=16.8%) and maternal log CRP was associated with BMI (p=0.001, r^2=12.3%) (figure 5-3). Maternal log leptin was also associated with maternal BMI (p<0.001, r^2=18.6%) (figure 5-4) and parity (p=0.003, r^2=9.6%).

Inflammatory markers	n=81
Log VCAM	2.57 (0.08)
(log ng/mL)	[371.54 (1.20)]
ICAM (ng/mL)	187.03 (59.66)
Log TNFα	0.32 (0.22)
(log pg/mL)	[2.09 (1.66)]
Log IL-6	0.32 (0.22)
(log pg/mL)	[2.09 (1.66)]
IL-10 (pg/mL)	1.37 (0.90)
IL-6/IL-10	3.61 (10.05)
Log CRP	0.60 (0.40)
(log mg/L)	[3.98 (2.51)]
Log Leptin (ng/mL)	1.43 (0.29)
	[26.91 (1.95)]

Table 5-1. Maternal inflammatory markers from uncomplicated pregnancies.

All values are mean and standard deviation (S.D.). Log transformed data was used for maternal VCAM, TNFα, IL-6, CRP and leptin, and is presented as mean (S.D.) and [geometric mean (S.D.)].

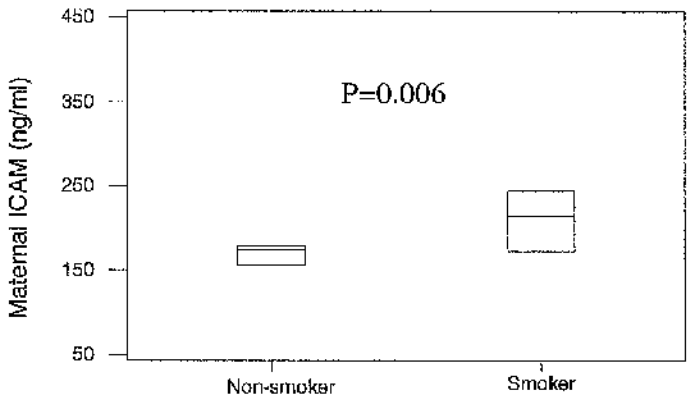


Figure 5-2. Maternal ICAM levels by smoking status.

Data is presented as mean and 95% CI. Statistical analysis was performed using linear regression analysis.

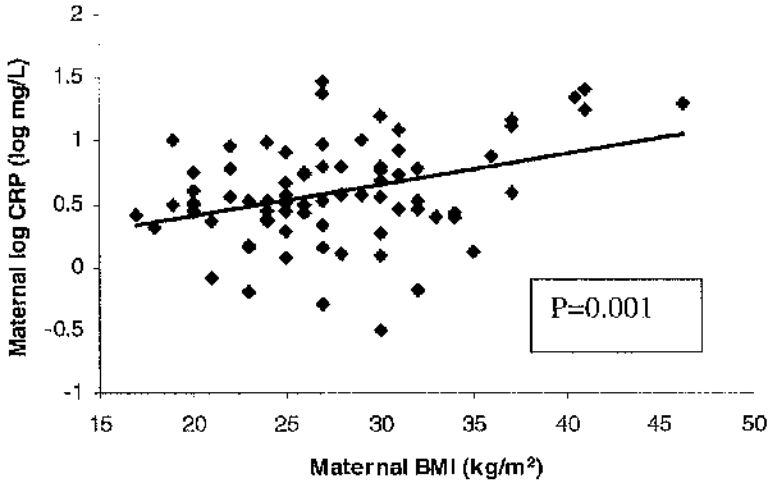


Figure 5-3. Association of maternal body mass index (BMI) with maternal log CRP. Statistical analysis was performed using linear regression analysis.

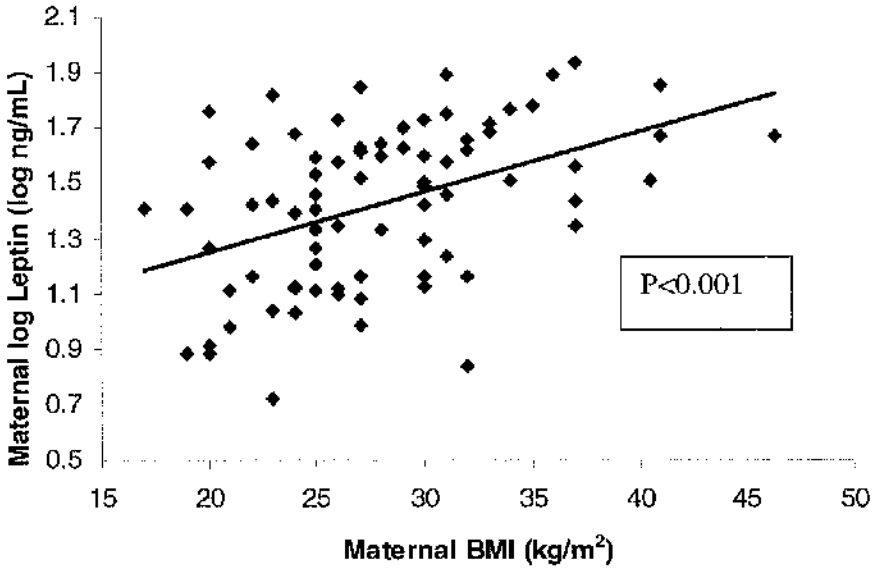


Figure 5-4. Association of maternal body mass index (BMI) with maternal log leptin. Statistical analysis was performed using linear regression analysis.

Fetal inflammatory markers (table 5-2) were available from forty-one of these uncomplicated pregnancies, and were unaffected by maternal BMI, age, smoking status, gestational age at delivery or gender, except fetal leptin which was associated with maternal BMI ($p=0.04$, $r^2=8.9\%$). Maternal BMI and fetal birth weight centile were weakly correlated in this cohort ($r=0.27$, $p=0.02$).

Fetal plasma	n=41
Fetal VCAM (ng/mL)	834.1 (215.2)
Fetal log ICAM	2.13 (0.13)
(log ng/mL)	[134.09 (1.35)]
Fetal TNFα (pg/mL)	2.89 (1.57)
Fetal log IL-6	0.75 (0.44)
(log pg/mL)	[5.62 (2.75)]
Fetal IL-10 (pg/mL)	1.93 (1.96)
Fetal log IL-6/IL-10	0.59 (0.47)
	[3.89 (2.95)]
Fetal log CRP	-1.35 (0.43)
(log mg/L)	[0.04 (2.69)]
Fetal log Leptin (log ng/mL)	0.87 (0.46)
	[7.41 (2.88)]

Table 5-2. Fetal inflammatory marker concentrations from uncomplicated pregnancies. All values are mean and standard deviation (S.D.). Log transformed data was used for fetal ICAM, IL-6, IL-6/IL-10, CRP and leptin, and is presented as mean (S.D.) and [geometric mean (S.D.)].

However, all fetal inflammatory markers apart from IL-10 and leptin differed significantly (ANOVA, $p<0.007$) between types of delivery; emergency lower uterine Caesarean section (LUSCS), elective LUSCS, vaginal delivery or assisted delivery (figure 5-5). IL-10 demonstrated a trend towards differing by mode of delivery ($p=0.08$).

Babies delivered by emergency LUSCS had the highest levels of ICAM, TNFα, IL-10, CRP and leptin. Fetal log ICAM was positively correlated with fetal weight ($r=0.39$, $p=0.01$), placental weight ($r=0.50$, $p=0.001$) (figure 5-6) and birth weight centile ($r=0.36$, $p=0.03$) in this group. Fetal log leptin was positively correlated with fetal weight ($r=0.70$, $p<0.001$), birth weight centile ($r=0.59$, $p<0.001$) and placental weight ($r=0.60$, $p<0.001$) (figure 5-7).

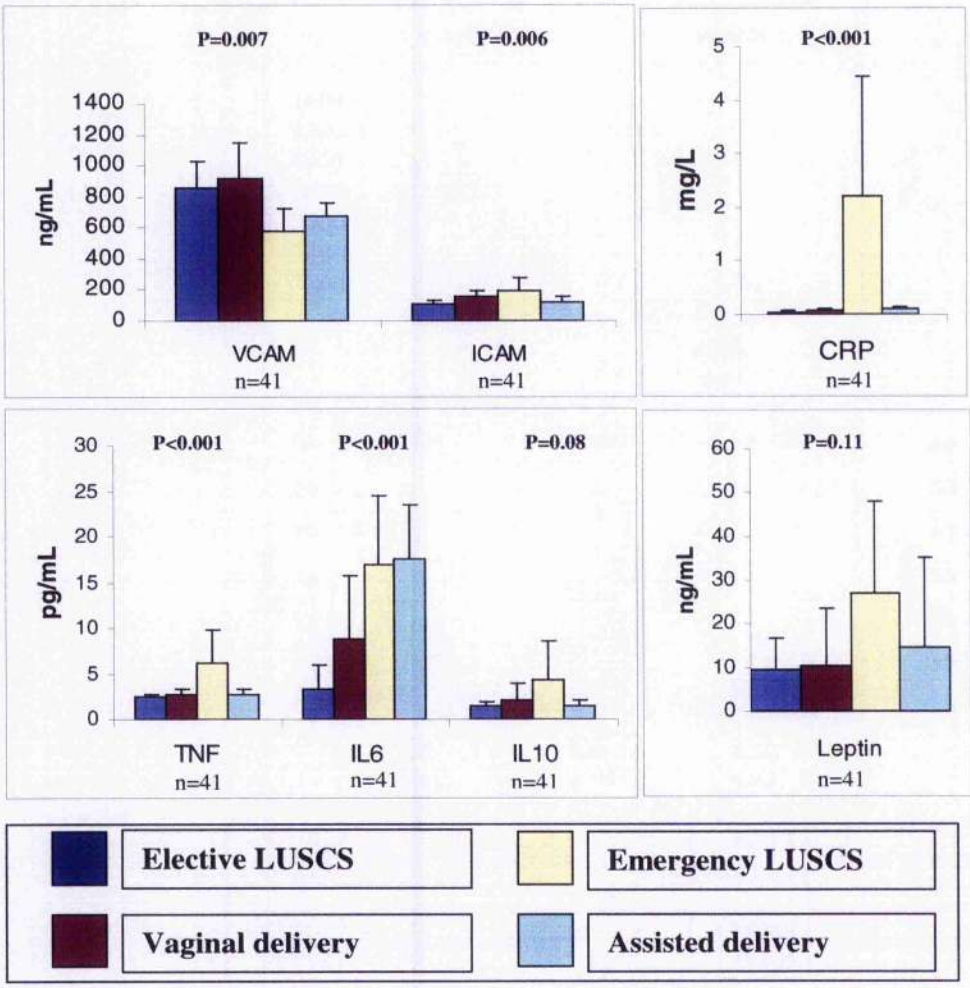


Figure 5-5. Fetal inflammatory markers (n=41) by mode of delivery.

Raw data is shown. Statistical analysis was performed using a one-way ANOVA, using log-transformed data for fetal ICAM, IL-6, CRP and leptin. Data are presented as mean and SD. LUSCS-lower uterine Caesarean section. Assisted delivery- forceps or Ventouse.

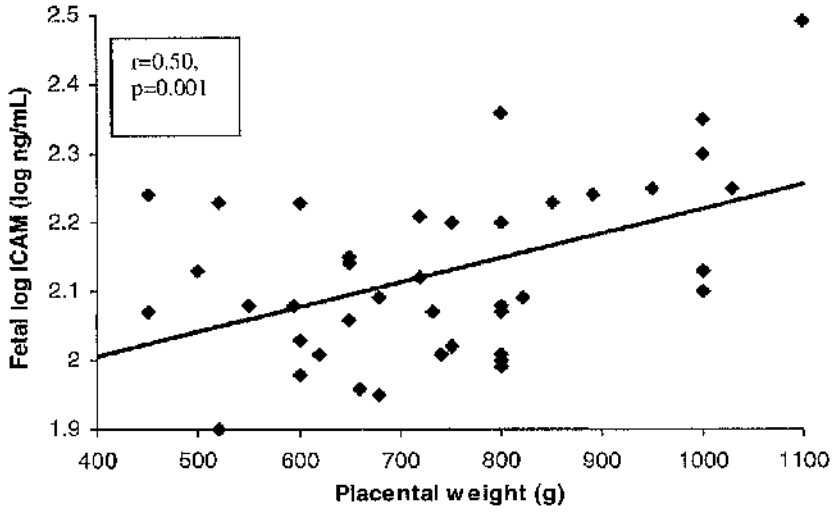


Figure 5-6. Association of fetal log ICAM with placental weight.
Statistical analysis was performed using Pearson's coefficient of correlation.

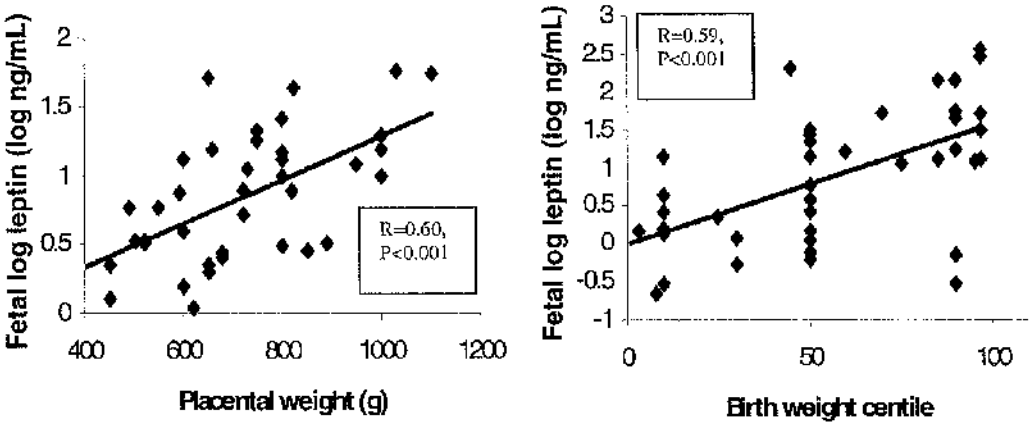


Figure 5-7. Association of fetal log leptin with placental weight and birth weight centile.
Statistical analysis was performed using Pearson's coefficient of correlation.

Fetal TNFα was also associated with placental weight (r=0.37, p=0.02). Maternal log VCAM was positively correlated with fetal weight (r=0.33, p=0.004), placental weight (r=0.35, p=0.003) (figure 5-8) and birth weight centile (r=0.22, p=0.06).

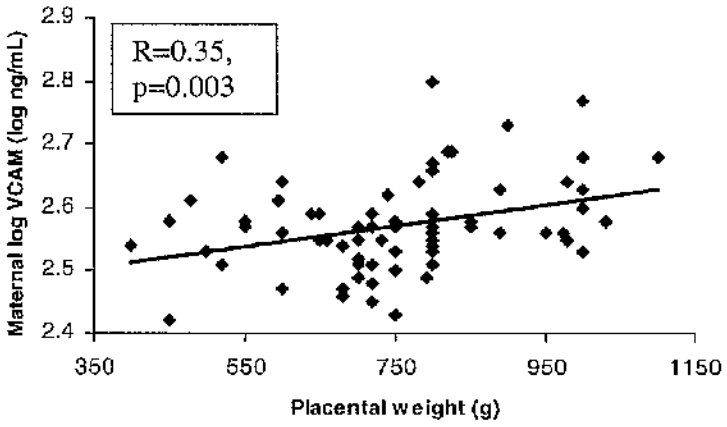


Figure 5-8. Association of maternal log VCAM with placental weight.
Statistical analysis was performed using Pearson's coefficient of correlation.

Fetal TNFα was significantly elevated in male offspring [2.12 (2.40) pg/mL] compared to female offspring [1.20 (0.32) pg/mL, p=0.01]. This observation was related to mode of delivery as there were no differences in TNFα levels in female offspring based on mode of delivery (p=0.48, ANOVA). There were no female offspring delivered by emergency LUSCS (see figure 5-5). There was a significant difference in the male values based on mode of delivery with the significant difference being elevated TNFα levels in those delivered by emergency LUSCS (17%) as demonstrated in figure 5-5.

5.2.1.2 Maternal and fetal characteristics in pregnancies complicated by PET and IUGR

For the analysis of plasma inflammatory markers in complicated pregnancy, cases were matched for age, parity and BMI with controls. Characteristics of the matched groups are shown in table 4-6 as this is the same dataset as that for the plasma lipid analyses. Consequently, maternal characteristics are the same as those described in Chapter 4, section 4.2.3.1.

We have demonstrated that in our control population, maternal ICAM is affected by smoking status and gestational age at sampling, maternal IL-6 is affected by gestational age at sampling and that maternal leptin and CRP are affected by BMI. For this reason, data presented have been corrected for these confounders by adjustment in a General Linear Model. In our population, fetal VCAM, log ICAM, TNFα, log IL-6, log IL-6/IL-10 and log CRP levels were all significantly affected by mode of delivery (figure 5-5) and so data adjusted for mode of delivery are reported.

5.2.1.3 Maternal inflammatory profile in PET, IUGR and uncomplicated control pregnancies

Plasma inflammatory markers in PET, IUGR and controls are demonstrated in table 5-3.

	PET			IUGR		
	Case n = 23	Control n = 23	p	Case n = 17	Control n = 17	p
Log VCAM (log ng/mL)	2.58 (0.07) [380 (1.17)]	2.56 (0.07) [363 (1.17)]	0.28	2.50 (0.08) [316 (1.20)]	2.55 (0.07) [355 (1.17)]	0.05
ICAM (ng/mL)	152 (73.8)	221 (169.0)	0.10	203 (92.9)	226 (125.0)	0.57
Log TNF (log pg/mL) TNF geom. mean	0.30 (0.20)	0.40 (0.25)	0.14	0.38 (0.31)	0.44 (0.20)	0.51
Log IL6 (log pg/mL) IL6 geom mean	0.63 (0.81)	0.43 (0.37)	0.28	0.42 (0.51)	0.38 (0.37)	0.80
IL10 (pg/mL)	3.99 (7.46)	1.56 (0.74)	0.15	1.59 (1.57)	1.91 (1.42)	0.55
IL6/IL10 ratio	2.58 (2.36)	4.06 (8.07)	0.42	2.08 (0.90)	1.74 (0.93)	0.29
Log CRP (log mg/L)	0.74 (0.82) [5.50 (6.61)]	0.54 (0.60) [3.47 (3.98)]	0.34	0.74 (0.60) [5.50 (3.98)]	0.47 (0.52) [2.95 (3.31)]	0.16
Log leptin (log ng/mL)	1.62 (0.42) [41.7 (2.63)]	1.48 (0.44) [30.2 (2.75)]	0.27	1.31 (0.64) [20.4 (4.37)]	1.39 (0.51) [24.6 (3.24)]	0.67

Table 5-3. Maternal plasma inflammatory markers in pregnancies complicated by PET or IUGR.

All values are mean and standard deviation (S.D.). Log transformed data was used for maternal VCAM, TNFα, IL-6 and CRP, and is presented as mean (S.D.) and [geometric mean (S.D.)]. Results are independent of maternal age, smoking status, BMI, parity and gestational age at sampling. Statistical analysis was performed using 2-sample *t*-test.

In PET subjects, plasma levels of maternal inflammatory markers were similar to those found in controls (Table 5-3). In the IUGR group, there was a lower maternal log VCAM of borderline significance [2.50 log ng/mL (0.08 log ng/mL), geometric (geom.) mean 316ng/mL (1.20 ng/mL)] compared to control subjects [2.55 log ng/mL (0.07 log ng/mL), geom. mean 355 ng/mL (1.17 ng/mL), p=0.05].

5.2.1.4 Fetal inflammatory profile in PET, IUGR and uncomplicated pregnancies

The fetal cord blood concentrations of VCAM and IL-6 were significantly higher than in their mothers' blood ($p<0.001$ both groups). There was a trend towards higher fetal TNFα compared with maternal TNFα ($p=0.09$). Maternal plasma concentrations of CRP and ICAM were significantly higher compared with the fetal concentrations ($p<0.001$ both groups). IL-10 levels were not different between maternal and fetal samples. For analysis, all fetal plasma inflammatory markers with the exception of IL-10 and leptin were adjusted for the effect of mode of delivery using a General Linear Model. In the venous cord serum of pregnancies complicated by PET ($n = 14$), fetal log CRP was significantly elevated [-1.00 log mg/L (0.58 log mg/L), geom. mean 0.10 mg/L (3.80 mg/L)] compared to controls [-1.63 log mg/L (0.64 log mg/L), geom. mean 0.02 mg/L (4.37 mg/L), $p=0.01$]. There were no other differences in fetal inflammatory markers between cases and controls (figure 5-9). In IUGR pregnancies ($n = 11$), a significantly elevated concentration of fetal TNFα [3.01 pg/mL (1.19 pg/mL)] was noted, compared to the control group ($n = 11$) [2.04 pg/mL (0.59 pg/mL), $p = 0.03$] (figure 5-10). There were no differences in fetal VCAM, log ICAM, log IL-6, IL-10, IL-6/IL-10 ratio, CRP or leptin between the groups.

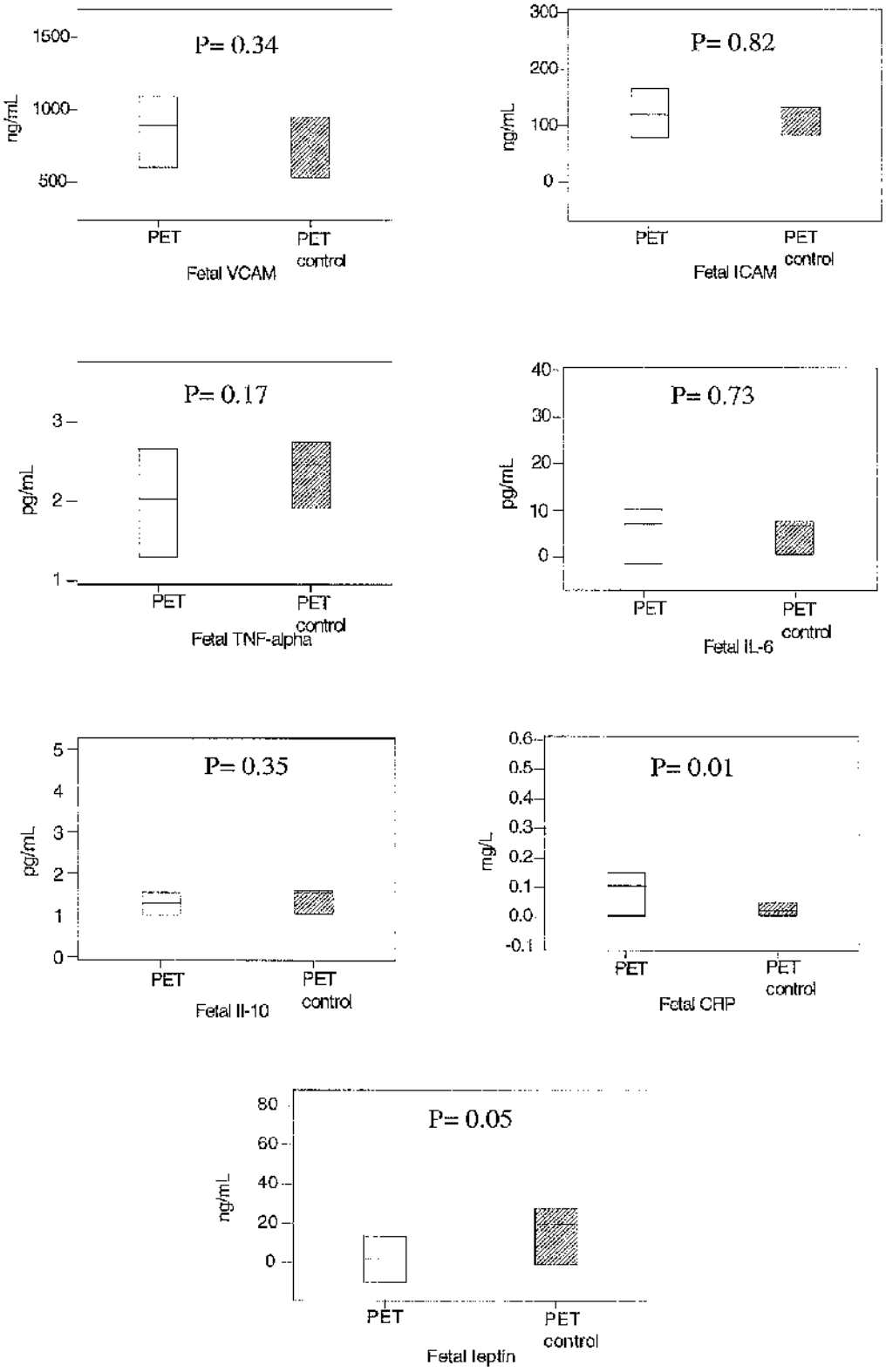


Figure 5-9. Fetal inflammatory markers in PET (n=14) and control (n=14) pregnancies.

Data shown is adjusted for mode of delivery for all except IL-10 and leptin. Leptin values are adjusted for maternal BMI. All values are mean and 95% confidence intervals. Statistical analysis was performed using a 2-sample *t*-test. Log transformed data was used for statistical analysis of fetal ICAM, IL-6, CRP and leptin.

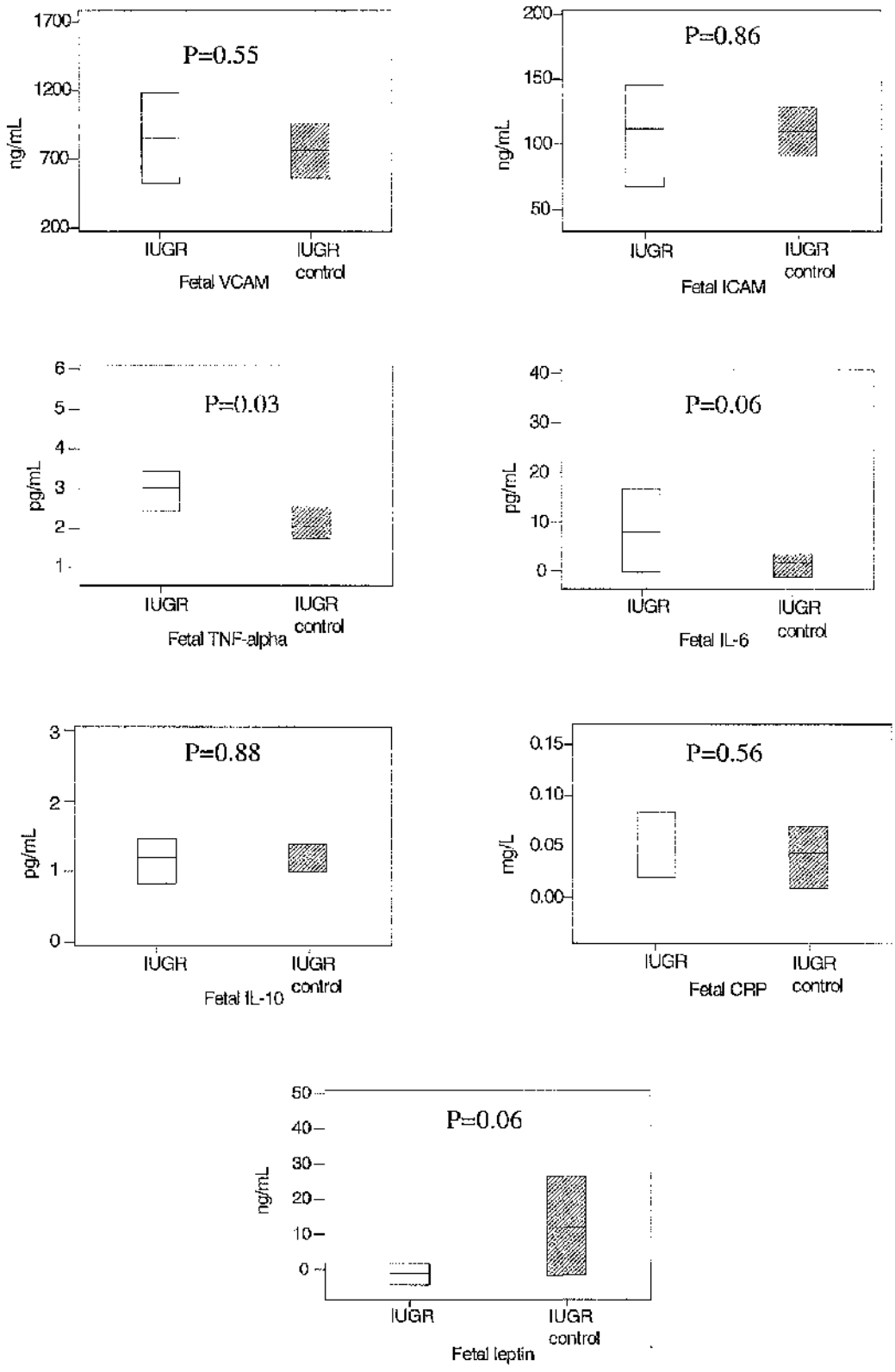


Figure 5-10. Fetal inflammatory markers in IUGR (n=11) and control (n=11) pregnancies.

Data shown is adjusted for mode of delivery for all except IL-10 and leptin. Leptin values are adjusted for maternal BMI. All values are mean and 95% confidence intervals. Statistical analysis was performed using a 2-sample *t*-test. Log transformed data was used for statistical analysis of fetal ICAM, IL-6, CRP and leptin.

5.2.1.5 Associations between maternal and fetal plasma inflammatory markers

There were no correlations between fetal and maternal VCAM, ICAM, TNFα, IL6 or IL10 in the combined control group. However, in this group, fetal leptin correlated with maternal plasma leptin levels (r=0.62, p=0.002) (figure 5-11). In controls, maternal log leptin was associated with placental weight (r=0.39, p=0.03) and fetal log leptin correlated with placental weight (r=0.59, p<0.001), birthweight centile (p=0.58, p=0.005) (figure 5-12), and fetal weight (r=0.61, p=0.003). This data confirms the results in the larger cohort (n=81) demonstrated in figure 5-7.

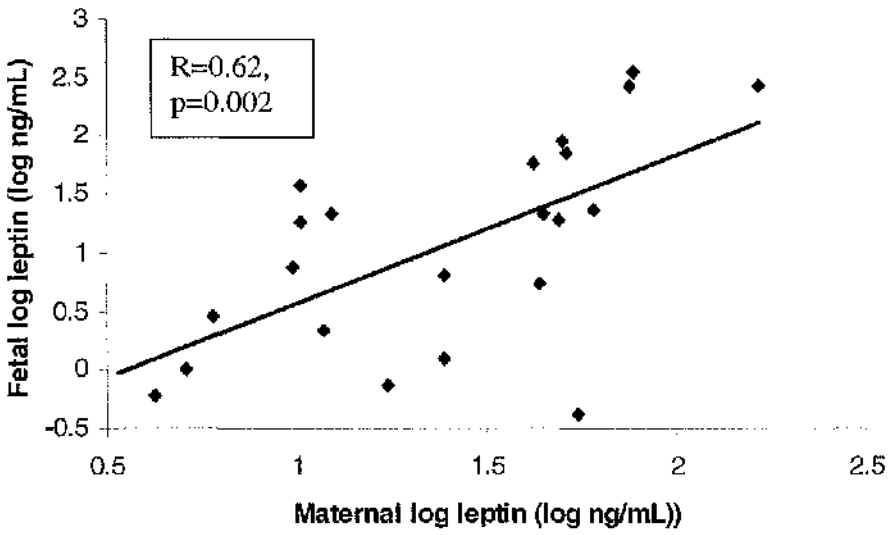


Figure 5-11. Association of maternal log leptin with fetal log leptin in control subjects. Statistical analysis was performed using Pearson's coefficient of correlation.

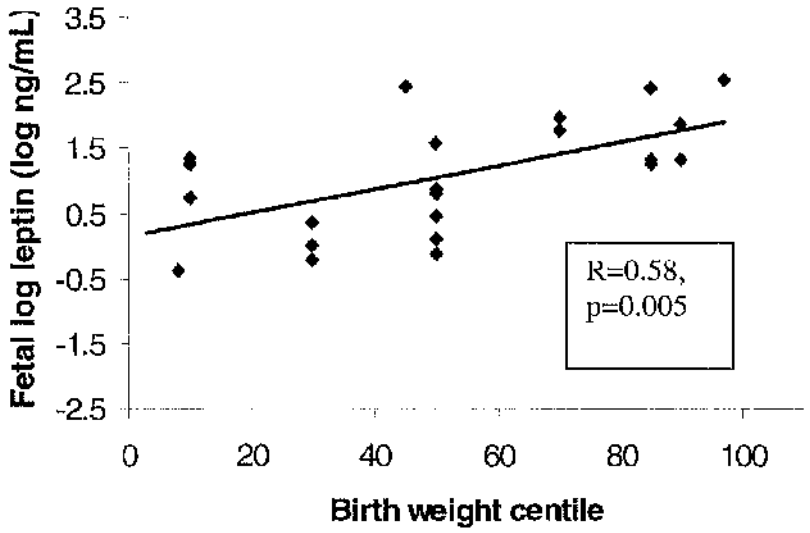


Figure 5-12. Association of fetal log leptin with birth weight centile in controls. Statistical analysis was performed using Pearson's coefficient of correlation.

In the PET group, there were no correlations between fetal and maternal VCAM, ICAM, TNFα, IL6, IL10 or leptin. Specifically, although fetal CRP levels were significantly increased in PET pregnancies, fetal CRP was not associated with maternal CRP or with fetal weight, birthweight centiles or placental weight in this group.

In the IUGR group, although maternal VCAM was reduced in IUGR pregnancies, maternal VCAM was not associated with fetal VCAM levels or with fetal weight, birthweight centiles or placental weight. There were no other correlations between maternal or fetal inflammatory markers in this group. However, in this group, maternal log IL6 correlated with fetal weight (r=-0.70, p=0.004) (Figure 5-13). This association was not seen in the PET (r=0.11, p=0.63) and control groups (r=0.04, p=0.78) (figure 5-13). There was no correlation between maternal log IL-6 and birth weight centiles (r=-0.11, p=0.70) in the IUGR group.

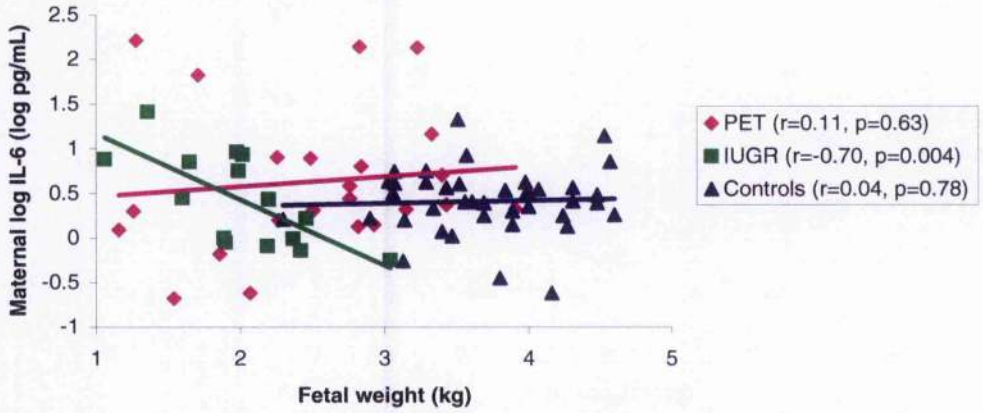


Figure 5-13. Association of maternal log IL-6 and fetal weight in IUGR group, showing lack of association in the PET and control groups.

Statistical analysis was performed using Pearson's coefficient of correlation.

5.2.2 Tissue analyses

Baseline characteristics for 6 PET subjects, 6 IUGR subjects and 12 corresponding controls are shown in table 5-14. Cases and controls were matched for age, parity, BMI and smoking status.

Characteristic	PET			IUGR		
	Case (n=6)	Control (n=6)	p	Case (n=6)	Control (n=6)	p
Age (years)	29.0 (27.0-33.5)	30.0 (27.0-33.0)	1.00	28.0 (26.8-33.0)	29.5 (27.0-32.3)	0.87
BMI (kg/m^2)	25.5 (23.0-30.3)	26.0 (24.8-30.3)	0.75	27.5 (24.0-35.5)	27.5 (23.8-35.5)	1.00
Primigravidae n (%)	5 (83%)	3 (50%)	0.22	3 (50%)	4 (67%)	0.56
Smokers n (%)	1 (17%)	2 (33%)	0.51	3 (50%)	2 (33%)	0.56
Gestation at sampling (weeks)	34 (32-35)	39.5 (37.3-41.0)	0.05	35.5 (32.8-37.8)	40.0 (39.0-41.0)	0.02
Gestation at delivery (weeks)	34 (32-35)	39.5 (37.3-41.0)	0.05	35.5 (32.8-37.8)	40.0 (39.0-41.0)	0.02
Placental weight (g)	334 (299-413)	695 (50.5-763)	0.05	285 (248-417)	741 (645-800)	0.005
Fetal weight (kg)	1.63 (1.25-2.12)	3.75 (2.92-4.38)	0.04	1.82 (1.47-2.00)	3.78 (3.40-4.38)	0.005
Birth weight centile	3 (2.5-15)	60 (38-86)	0.03	3 (1-7.8)	77.5 (40-86.3)	0.007
Fetal sex (%)	17% male 83% female	50% male 50% female	0.22	50% male 50% female	67% male 33% female	0.56

Table 5-4. Subject characteristics in pregnancies complicated by PET or IUGR and controls.

All values are median and interquartile (IQ) range. Statistical analysis was performed using Mann-Whitney U test for continuous variables, and chi-square for categorical variables.

In comparison to the larger cohort of women with complicated pregnancies and their controls (table 4-6), this subset is comparable and was considered to be a good representation of the larger group, with similar characteristics in each group.

5.2.2.1 Leptin mRNA expression in subcutaneous adipose tissue and placentae from pregnancies complicated by PET or IUGR

In PET subjects, relative to the 18s control gene, there were no differences in subcutaneous (SC) adipose tissue mRNA expression of leptin between cases [leptin:18s ratio, median 2805 (IQ range 2188-5792)] and controls [leptin:18s ratio, median 2836 (IQ range 1388-3813), p=0.47]. However, placental mRNA expression of leptin was significantly elevated [median 18315 (IQ range 3385-32060)] compared to control subjects [median 1369 (IQ range 430-4211), p=0.03] (figure 5-14).

Similarly, in IUGR, there were no differences detected in SC adipose tissue expression of leptin between IUGR cases [median 2918 (IQ range 1654-6160)] and controls [median 3034 (IQ range 1935-3813), p=1.00]. However, placental mRNA expression was significantly higher [median 9663 (IQ range 4080-16979)] than in controls [median 857 (IQ range 273-2034), p=0.005] (figure 5-14).

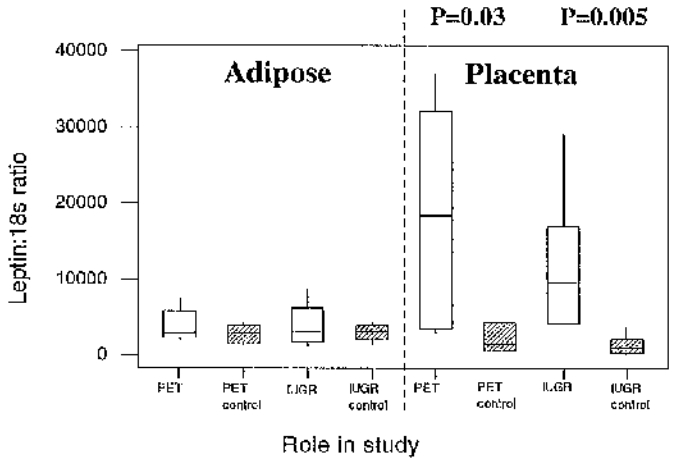


Figure 5-14. Leptin:18s ratio in adipose and placental tissue of PET, IUGR and control subjects (n=6 per group).

Statistical analysis was performed using Mann-Whitney U-test, and data is presented as median and IQ range. All values are $x10^{-5}$.

In control subjects, leptin expression related to 18s, appears to be within a similar range in both placenta and adipose tissue (figure 5-14).

5.2.2.2 PPARγ mRNA expression in SC adipose tissue and placentae from pregnancies complicated by PET or IUGR

Subcutaneous adipose tissue mRNA expression of PPARγ was not different between PET cases [PPARγ:18s ratio, median 574 (IQ range 368-918)] and controls [median 505 (IQ range 433-824), p=0.69], nor between IUGR cases [median 401 (IQ range 220-512)] and controls [median 490 (IQ range 291-824), p=0.34] (figure 5-15).

In PET, relative to the 18s control gene, placental mRNA expression of PPARγ was not different between cases [PPARγ:18s ratio, median 71 (IQ range 32-322)] and controls [median 54 (IQ range 27-82), p=0.34]. In IUGR, there was a trend towards elevated placental PPARγ expression in cases [median 152 (IQ range 55-594)] compared with controls [median 33 (IQ range 21-109), p=0.07] (figure 5-15).

Related to the 18s gene, PPARγ expression appears to be greater in adipose tissue compared to placental tissue (figure 5-15).

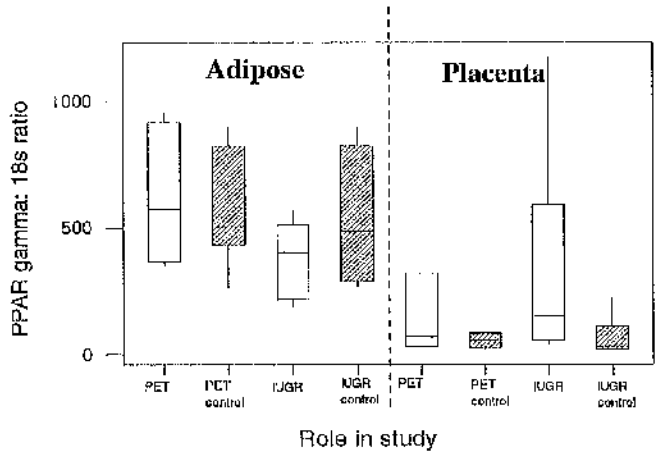


Figure 5-15. PPARγ:18s ratio in adipose and placental tissue of PET, IUGR and control subjects (n=6 per group).

Statistical analysis was performed using Mann-Whitney U-test, and data is presented as median and IQ range. All values are $x10^{-5}$.

5.2.2.3 TNFα mRNA expression in SC adipose tissue and placentae from pregnancies complicated by PET or IUGR

There were no differences in SC adipose tissue mRNA expression of TNFα between PET cases [TNFα:18s ratio, median 24 (IQ range 16-36)] and controls [median 47 (IQ range 36-55), p=0.09]. There were also no differences in placental mRNA expression of TNFα between PET subjects [median 50 (IQ range 24-97)] and controls [median 122 (IQ range 64-162), p=0.17] (figure 5-16).

In IUGR, there were no differences detected in either SC adipose tissue [IUGR median 37 (IQ range 8-81) vs control median 49 (IQ range 42-77), p=0.69] or placental [IUGR median 87 (IQ range 51-166) vs control median 122 (IQ range 64-151), p=0.69] mRNA expression of TNFα between cases and controls (figure 5-16).

Related to 18s, TNFα tissue expression appears greater in the placenta than in the adipose tissue in all groups (figure 5-16).

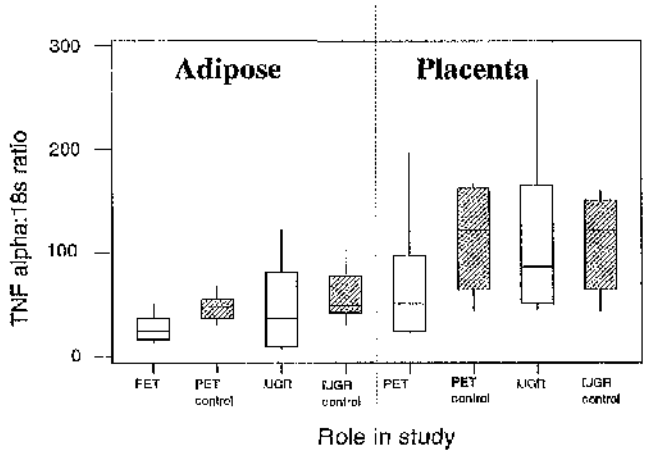


Figure 5-16. TNFα:18s ratio in adipose and placental tissue of PET, IUGR and control subjects (n=6 per group).

Statistical analysis was performed using Mann-Whitney U-test, and data is presented as median and IQ range. All values are $x10^{-5}$.

5.2.2.4 IL-6 mRNA expression in SC adipose tissue and placentae from pregnancies complicated by PET or IUGR

In PET, there were no significant differences between SC adipose tissue mRNA expression of IL-6 between PET subjects [IL-6:18s ratio, median 9 (IQ range 3-17)] and controls [median 16 (IQ range 7-35), p=0.23]. Similarly, placental mRNA expression of IL-6 was not significantly different between PET cases [median 14 (IQ range 4-24)] and control subjects [median 11 (IQ range 5-17), p=0.87] (figure 5-17).

In IUGR, there were also no significant differences detected in adipose tissue [IUGR median 4 (IQ range 2-24) vs control median 15 (IQ range 7-30), p=0.13] or placental [IUGR median 18 (IQ range 5-29) vs control median 8 (IQ range 3-15), p=0.30] mRNA expression between cases and controls (figure 5-17).

Related to 18s, both adipose and placental tissue expression of IL-6 appear similar in all groups (figure 5-17).

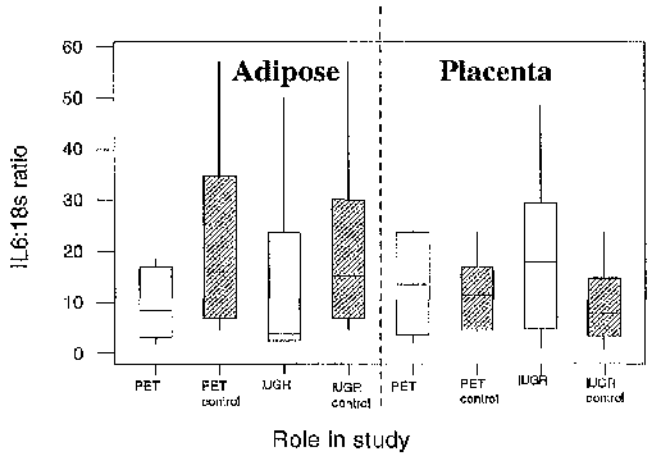


Figure 5-17. IL-6:18s ratio in adipose and placental tissue of PET, IUGR and control subjects (n=6 per group).

Statistical analysis was performed using Mann-Whitney U-test, and data is presented as median and IQ range. All values are $x10^{-5}$.

5.2.2.5 IL-10 mRNA expression in SC adipose tissue and placentae from pregnancies complicated by PET or IUGR

No differences were detected between SC adipose tissue expression in PET [IL-10:18s ratio, median 55 (IQ range 33-78)] compared with controls [median 72 (IQ range 58-85), p=0.23], or between the placental mRNA expression of IL-10 in PET [median 12 (IQ range 9-16)] compared with controls [median 22 (IQ range 10-32), p=0.23] (figure 5-18).

In IUGR, there were no significant differences in SC adipose tissue mRNA expression between IUGR subjects [median 88 (IQ range 19-168)] and controls [median 71 (IQ range 58-116), p=0.94], or in placental IL-10 mRNA expression between cases [median 16 (IQ range 14-46)] and controls [median 18 (IQ range 10-32), p=0.81] (figure 5-18).

Tissue expression of IL-10 appears to be higher in adipose tissue than in placental tissue, in all groups (figure 5-18).

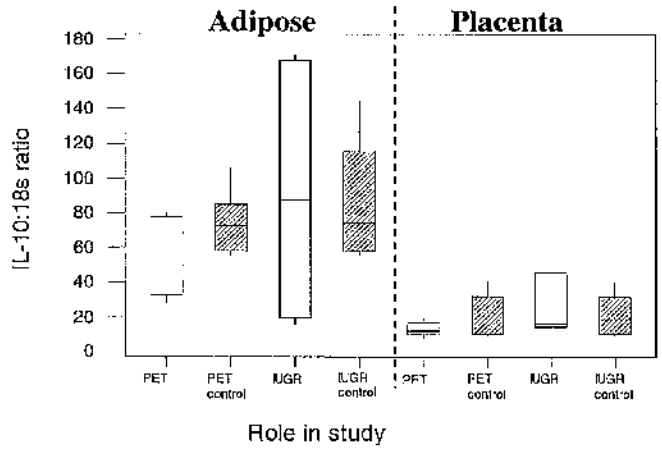


Figure 5-18. IL-10:18s ratio in adipose and placental tissue of PET, IUGR and control subjects (n=6 per group).

Statistical analysis was performed using Mann-Whitney U-test, and data is presented as median and IQ range. All values are $x10^{-5}$.

5.2.2.6 Associations between adipose, placental and plasma levels of inflammatory markers in PET, IUGR and controls.

In the combined control groups (n=7 different samples), adipose tissue expression of IL-10 was associated with maternal plasma IL-10 levels (r=0.96, p<0.001) (figure 5-15). This was not demonstrated in the PET or IUGR groups (figure 5-19).

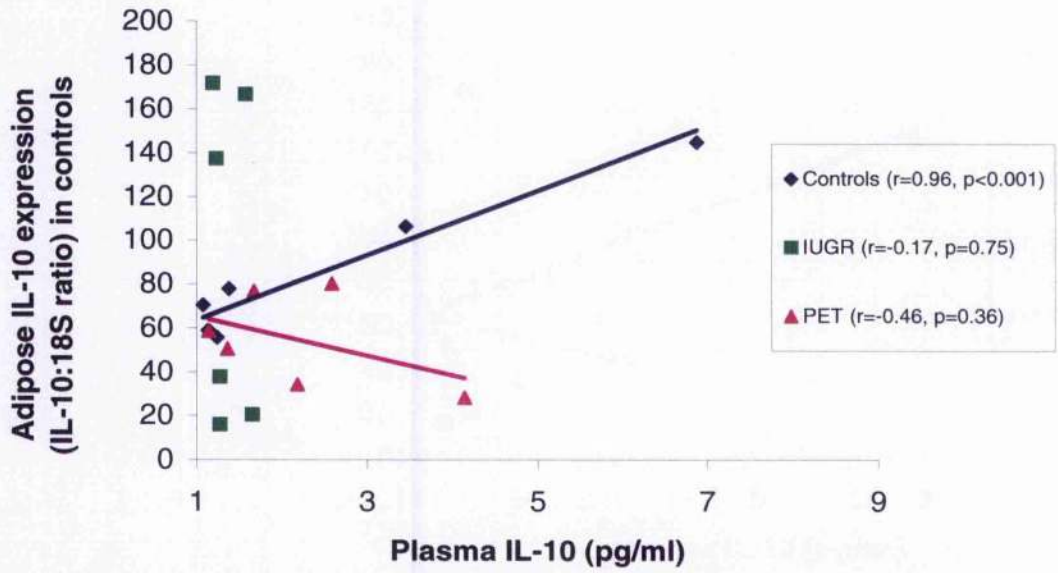


Figure 5-19. Association of adipose IL-10 and maternal plasma IL-10 levels in controls. Statistical analysis was performed using Pearson's coefficient of correlation.

There were no other correlations between adipose expression of the inflammatory markers and their corresponding maternal plasma levels in the controls. In the combined control group, there were no correlations between adipose expression of leptin and TNFα, or between adipose or placental expression of TNFα or leptin and plasma levels of TNFα.

In the combined control group, placental PPARγ expression correlated positively with fetal HDL levels (r=0.92, p=0.004) (figure 5-20). There was a trend towards a negative correlation between placental PPARγ expression and maternal HDL levels (r=-0.70, p=0.08) (figure 5-20). Placental PPARγ expression also correlated with fetal plasma IL-10 (r=0.82, p=0.002).

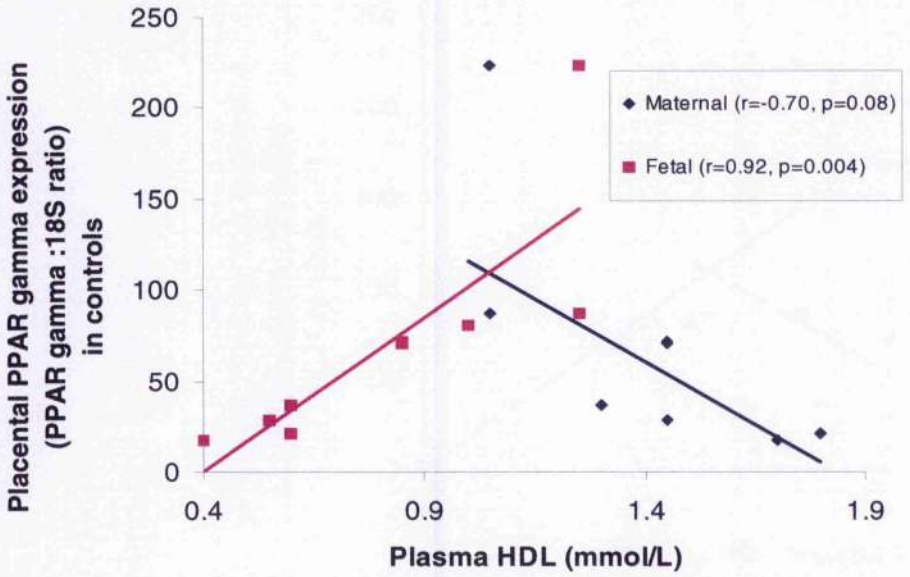


Figure 5-20. Association of placental PPARγ expression with maternal and fetal plasma HDL levels.

Statistical analysis was performed using Pearson's coefficient of correlation.

Placental leptin production was associated with fetal plasma HDL levels (r=0.80, p=0.03) (figure 5-21). There were no other correlations between placenta or adipose expression of inflammatory markers and corresponding fetal plasma levels.

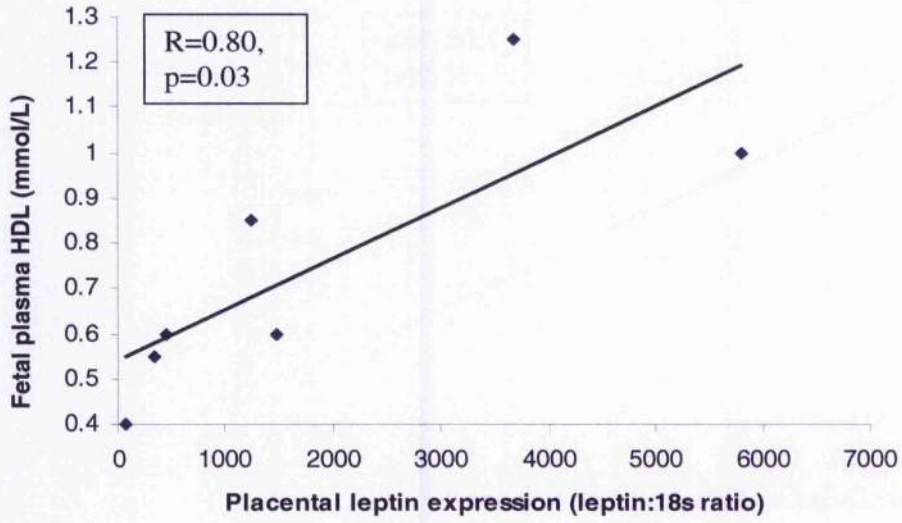


Figure 5-21. Association of fetal plasma HDL levels with placental leptin expression in controls.

Statistical analysis was performed using Pearson's coefficient of correlation.

In the PET group, there were no correlations between adipose tissue expression of the inflammatory markers and their corresponding plasma levels. Similarly, none of the placental inflammatory markers were associated with their corresponding plasma levels. Specifically, in PET, placental leptin was not associated with plasma levels despite the increases seen in placental leptin production. In the PET group, adipose IL-6 mRNA correlated with adipose IL-10 mRNA (r=0.96, p=0.002) (figure 5-22).

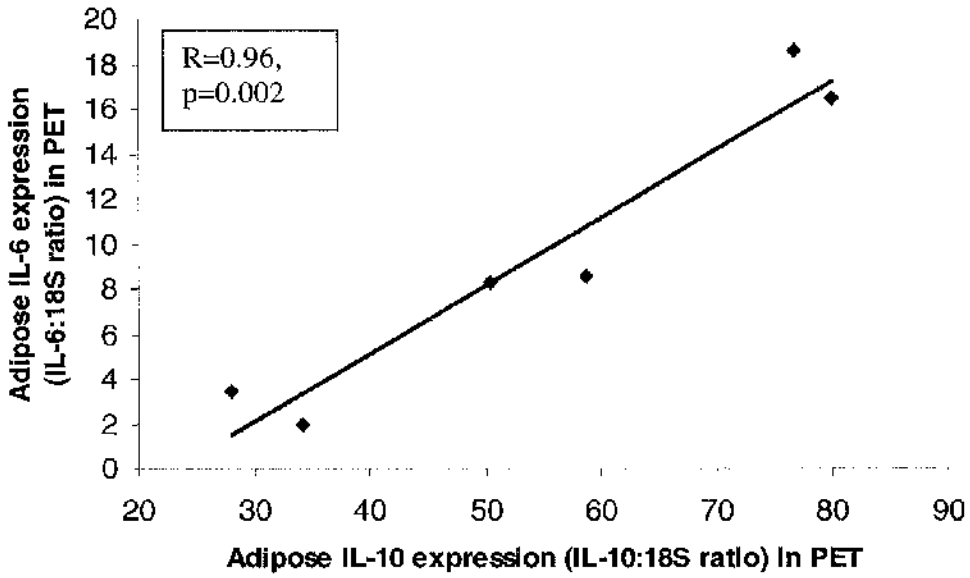


Figure 5-22. Association between IL-6 and IL-10 adipose tissue expressions in PET. Statistical analysis was performed using Pearson's coefficient of correlation.

In PET, there were no correlations between adipose or placental tissue expression of inflammatory markers or leptin, and corresponding fetal plasma levels. In IUGR, there were no correlations between adipose or placental tissue expression of the inflammatory markers or leptin and their corresponding plasma levels.

5.2.3 Protein expression of markers of inflammation and insulin resistance in placentae from pregnancies complicated by PET or IUGR

Within the timescale limitations of my thesis, I was unable to confirm adipose and placenta mRNA results with protein quantification of the inflammatory and insulin resistance markers by western analysis in this study. Using the methods described in Chapter 2 (2.5.2), which was a successful working technique in the PPAR study (Chapter 3), all recombinant protein positive controls and molecular weight markers were demonstrated in the films, but none of the samples were visible (figure 5-23). Further work using tissue homogenates and ELISA detection is underway.

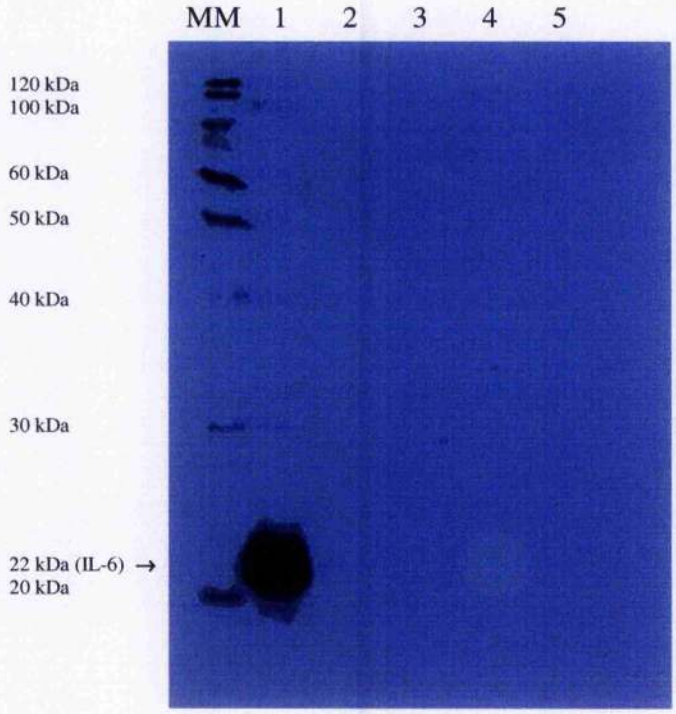


Figure 5-23. IL-6 protein expression from placenta and adipose tissue - an example of unsuccessful western analyses.

Note +ve control, but no band for any of the samples. MM = molecular weight markers, lane 1 – positive control (100ng recombinant IL-6), lane 2 – placental tissue 50μg, lane 3 – placental tissue 100μg, lane 4 – adipose tissue 50μg, lane 5 – adipose tissue 100μg.

PPARγ western results were obtained using our previous method (chapter 3). There were no correlations between the PPARγ western results and the PPARγ mRNA results, using the combined groups (n=24, r=-0.15, p=0.58) (figure 5-24).

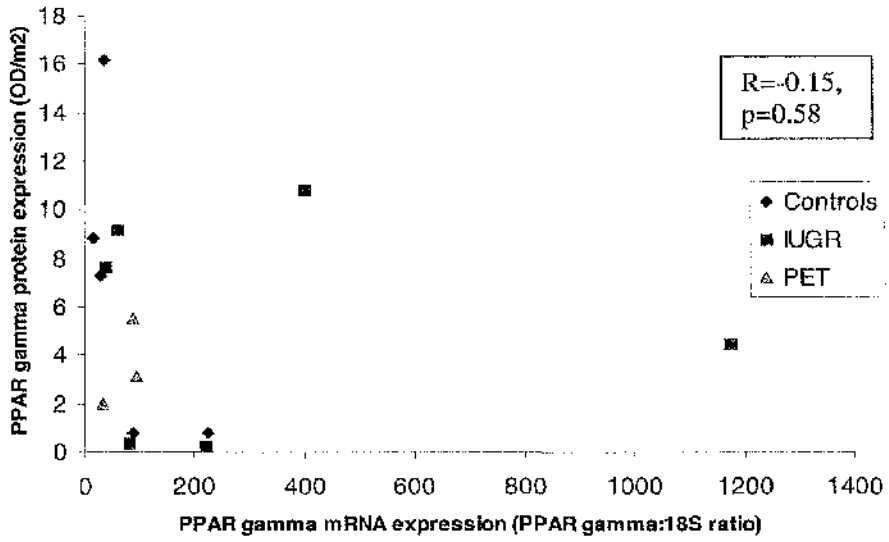


Figure 5-24. PPARγ mRNA versus protein expression in combined groups (PET, IUGR and controls).

There was no overall association demonstrated. Statistical analysis was performed using Pearson's coefficient of correlation.

5.3 Discussion

This chapter confirms the established relationship between maternal BMI and inflammation, demonstrated by the association of maternal CRP levels with BMI (figure 5-3). This is the first report to describe fetal cord plasma inflammatory markers in pregnancies complicated by IUGR. An increase in fetal plasma TNFα levels in IUGR pregnancies and an increase in fetal CRP levels in PET compared to control subjects was observed, despite minimal alterations in maternal inflammatory markers in these conditions. The reduction in maternal VCAM in IUGR is small and of borderline significance and may therefore not be of any physiological relevance. However, it is interesting to note that in the uncomplicated pregnancy group, increasing levels of maternal VCAM were associated with increasing fetal weight, placental weight and birth weight centiles, suggesting that there may be a connection between maternal plasma VCAM levels and offspring size. With the exception of IL-10 and leptin, all fetal inflammatory markers differed based on mode of delivery. However, the elevated levels of fetal CRP in PET and TNFα in IUGR were independent of this effect. In this study, the

previously observed maternal inflammatory changes were not reproduced. This is likely due to the careful matching of the groups for BMI, the similar smoking status in both cases and controls and the adjustment for confounders such as gestational age at sampling in the present study.

Fetal CRP levels were not correlated with maternal values suggesting that this is a predominantly fetal response to PET. In fetal cord blood, the increase in the acute phase reactant CRP in PET pregnancies may be secondary to a compensatory increased production by the fetal liver in response to a 'stressful' *in-utero* environment. This increase may also be attributed to the placental pathology seen in this condition, as the uterine vasculature does not undergo adequate physiological remodelling in PET and reduced placental perfusion may result in a generalised production of placental and fetal cytokines. Alternatively, the elevated fetal CRP in PET may be a response to short-term stress in the 3rd trimester due to the maternal metabolic deregulation seen in PET, explaining why this response is confined to offspring from PET pregnancies.

The increased fetal plasma TNFα seen in IUGR pregnancies may also be attributed to the placental dysfunction, either resulting from increased placental production or from increased fetal systemic production in response to the abnormal placentation. A lack of association between maternal and fetal plasma TNFα levels supports this. Increased levels of fetal TNFα in offspring from IUGR pregnancies may be a result of longer-term *in-utero* stress leading to increased production of this cytokine. It is possible that the increased levels of fetal TNFα in IUGR is a chance observation, although the concomitant borderline increase in fetal plasma IL-6 levels would argue against this. One might speculate that the inflammatory responses are different in PET and IUGR because the degree of stress experienced by the fetus is different. Perhaps IUGR fetuses are exposed to a chronic long-term stress throughout pregnancy whereas PET fetuses respond to a more acute stress in the 3rd trimester as the maternal clinical syndrome develops. In this study, there were no differences in either placental or adipose production of TNFα between IUGR and controls. Trophoblast cells are known to produce TNFα (Hunt *et al* 1996) and although placental TNFα mRNA production was not significantly increased in PET or IUGR, its expression was higher in the placenta than the adipose tissue in all groups. However, these data suggest that the increased fetal plasma TNFα in IUGR pregnancies may originate from another source. It is recognised that there is neutrophil activation in the peripheral circulation in IUGR and PET (Johnston *et al* 1991, Sabatier *et al* 2000). It may be possible that activated macrophages within the placental bed or the fetal circulation, which are

known to produce TNFα (Wang and Walsh 1996), are responsible for the elevation of fetal TNFα demonstrated in response to the *in-utero* environment in IUGR.

A negative relationship was demonstrated between maternal plasma IL-6 levels and fetal weight in the IUGR group, which was not seen in PET or uncomplicated pregnancies. The association between increasing levels of maternal plasma IL-6 and decreasing fetal weight in IUGR pregnancies may be interpreted as an effect of increasing maternal insulin resistance on fetal weight as has been suggested previously (Hattersley and Tooke 1999) rather than an inflammatory response. In common with the discrepant results within the literature (Al-Othman *et al* 2001, Benyo *et al* 1997, Benyo *et al* 2001, Kauma *et al* 1995, Yin *et al* 1998), we found no difference in tissue IL-6 expression in IUGR and PET and no clear tissue source of IL-6 was demonstrable. This indicates that both the adipose and placenta may produce this cytokine in a complex interrelated manner in pregnancy and peripheral activated lymphocytes may also contribute.

Adipose tissue appeared to produce more IL-10 per cell in all groups studied based on the IL10:18s ratio, compared to placental tissue in this study (see figure 5-18) suggesting that adipose is indeed the predominant tissue source of this anti-inflammatory cytokine in pregnancy, as hypothesised. In the control group, adipose IL-10 expression correlated with plasma IL-10 levels, which strengthens the suggestion that adipose tissue is the primary source of plasma IL-10 in uncomplicated pregnancies, although this association was not evident in pregnancies complicated by PET or IUGR. This may indicate that although adipose tissue produces IL-10 in all pregnancies, there is no relationship with the plasma levels in the complicated pregnancies because the complex maternal metabolic interactions result in overall lower plasma levels of a cytokine involved in reducing inflammation, specifically IL-10, as suggested by figure 5-19. We observed no differences in placental IL-10 mRNA expression between cases and controls. In PET pregnancies, adipose IL-6 and IL-10 expressions were directly related (figure 5-22). This may reflect the more 'active' state of the adipose tissue in PET leading to a general increase in production of adipocyte-derived cytokines, whether pro- or anti-inflammatory. Previous reports have suggested a reduction in placental IL-10 expression in PET or IUGR pregnancies (Hahn-Zoric *et al* 2002, Hennessy *et al* 1999), or conversely an increase in placental IL-10 expression in PET (Rinehart *et al* 1999).

This study confirms the relationship between maternal BMI and plasma leptin levels (Ramsay *et al* 2002). This relationship, described by Ramsay and colleagues (Ramsay *et al* 2002) ($r=0.73$, $p<0.005$), indicates that although a large percentage of maternal plasma

leptin is produced by the adipose tissue, there could potentially be other sources. Fetal leptin was reduced in PET and IUGR, although this failed to reach statistical significance (p=0.05 and p=0.06 respectively). However, this was observed despite increased placental mRNA expression of leptin in these conditions. This suggests that placenta is not the main source of fetal leptin, and is supported by previous data suggesting that growth restricted fetuses have lower cord blood concentrations of leptin than appropriately grown fetuses for gestation (Jakimiuk *et al* 2003). Since PET and IUGR fetuses are small in this study and are thus likely to have small adipose tissue depots, these data would be consistent with fetal adipose tissue being the main source of fetal leptin, as has been previously proposed (Pighetti *et al* 2003) (see figure 5-25 below). In the control group, fetal leptin levels are correlated with birth weight centile and fetal weight, which would be consistent with this hypothesis. An association between maternal and fetal leptin levels was observed in the present study, in uncomplicated pregnancy, and this would be consistent with a 'fatter' mother resulting in a correspondingly fatter baby and thus adipose mass would be correlated. This is supported by the weak correlation between maternal BMI and birth weight centile in the cohort under study (r=0.27, p=0.02). These anthropometric measures are inadequate measures of adipose mass and further studies on maternal determinants (including fat mass) of neonatal body composition are required. It should be noted that there are *in vitro* data indicating that over 90% of leptin synthesised in the placenta may be delivered to the maternal circulation (Linnemann *et al* 2000).

The present data has confirmed previous literature demonstrating that there is an increase in placental leptin mRNA expression in PET (Laivuori *et al* 2000) and IUGR (Lepercq *et al* 2003) pregnancies compared with controls. The observed increase in placental leptin production in PET and IUGR may be a response intended to provide the fetus with adequate 'fuel' supplies, despite the abnormal placentation seen in these situations. A relationship between both placental leptin mRNA expression and placental PPARγ mRNA expression with fetal plasma HDL levels was also observed within the group of uncomplicated controls. This is interesting because it suggests a link between leptin and PPARγ in the placenta and fetal lipid stores. It has been observed in the literature that leptin induces the HDL receptor SR-BI in mouse liver (Lundasen *et al* 2003) and also induces the system A amino acid transporter in human placental villous fragments (Jansson *et al* 2003). It could be speculated that placental leptin up-regulation in PET and IUGR is a compensatory mechanism in order to optimise nutrient transfer in these conditions of placental insufficiency (figure 5-25).

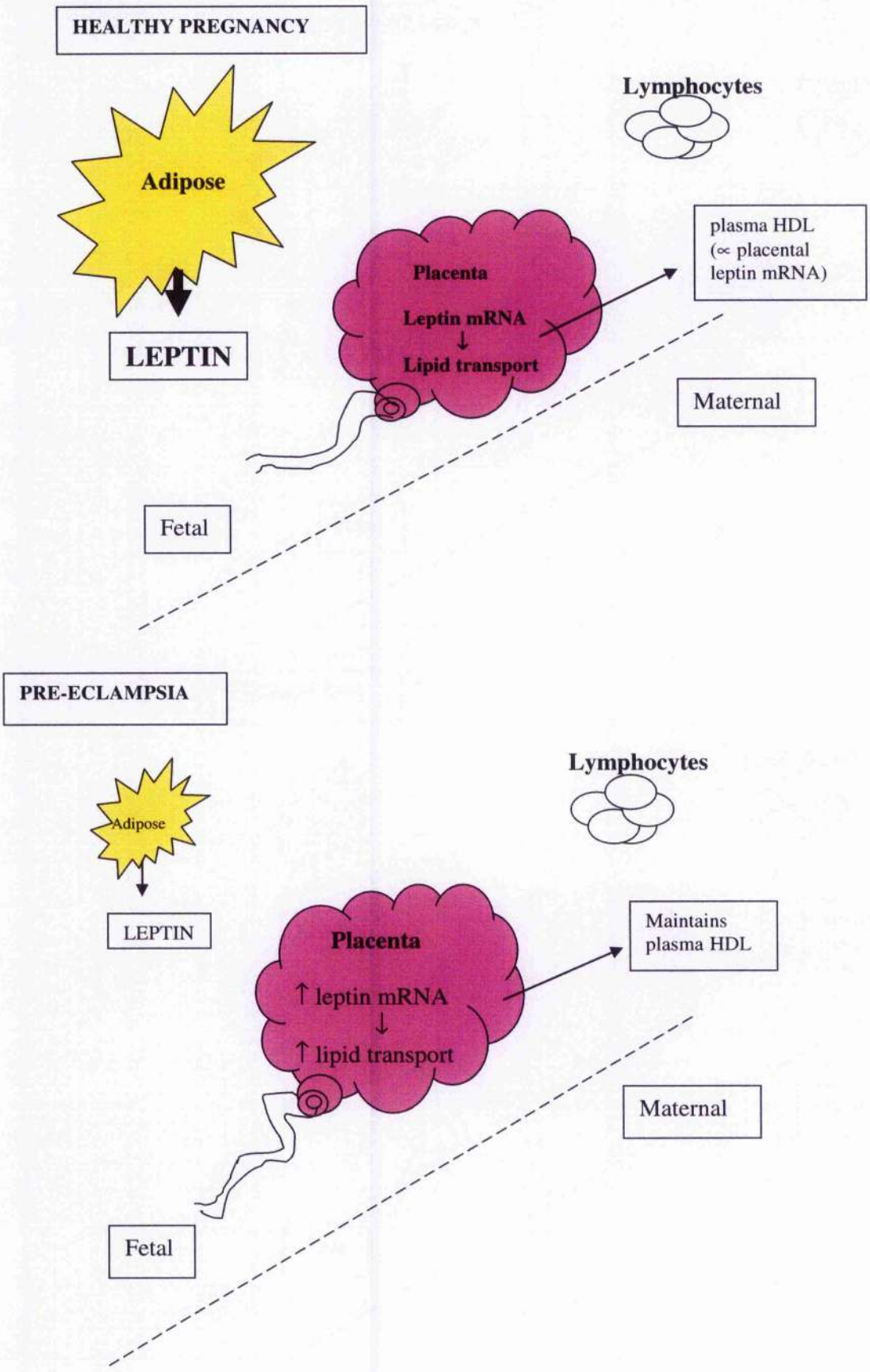


Figure 5-25. Proposed mechanisms for maternal and fetal leptin production and relation to plasma HDL, in PET and uncomplicated pregnancies.

The discovery of leptin receptors in the placenta (Bodner *et al* 1999) suggests that leptin could function through autocrine or paracrine mechanisms. PPARγ has important roles in lipid metabolism but has not yet been directly linked to up-regulation of transport mechanisms in the placenta. The negative correlation between placental PPARγ mRNA expression and maternal plasma HDL in the control group might suggest the involvement of this nuclear receptor in placental lipid transport.

This study has a number of limitations. A power calculation was performed in this study, which demonstrated that there was at least 80% power to detect a 50% difference in expression with a standard deviation (SD) of 30%, with a sample size of 6 per group. In the analyses, SDs were higher than 30% indicating that this study was underpowered. Extending the placental and adipose tissue studies may produce more robust data with smaller standard deviations. Maternal SC adipose tissue was obtained at time of Caesarean section on the assumption that this tissue would provide inflammatory data reflecting the long-term maternal state. However, it may be more prudent to collect visceral adipose tissue as this may provide data on the longer-term inflammatory state of the mother and further studies should be performed to compare present results with those of the visceral fat. There were no results available to report regarding the protein expression of the inflammatory markers and insulin resistance mediators in this study. It is possible that a different method of protein extraction or concentration may achieve improved results. The Trizol method was used in this study, with acetone precipitation for the concentration of protein, and was a success for the larger PPAR molecules. The cytokines, however, are smaller molecules and thus results may be obtained by using tissue homogenate in buffer with a protease inhibitor 'cocktail' to extract the cytokine proteins. There was no association between mRNA and protein expression of PPARγ in the combined groups in this study. This may be due to small numbers studied and results may be more robust if separate groups were studied in greater numbers. However, this result demonstrates the importance of performing western analyses on our tissue. In this study, each placenta was sampled in a random fashion, as in chapter 2, section 2.2.1. It was considered that multiple biopsies taken in a systematic fashion and then randomised would be warranted in view of the highly variable cytokine expression demonstrated within a single placenta (Benyo *et al* 2001).

From this data, which concentrates on very complex interrelated metabolic interactions, it is speculated that the key change observed in this study is an apparent elevation of placental leptin in PET and IUGR. It is also proposed that up-regulation of placental leptin

might lead to increased placental nutrient transport. The data presented suggest that fetal plasma levels of leptin are derived from adipose tissue and that further studies of maternal and offspring fat mass are required. Future studies on the placental and SC adipose expression of leptin related to plasma leptin and other peripheral blood markers of inflammation and insulin resistance may help to understand the role of pregnancy in aetiology of cardiovascular disease. It may also be prudent to examine visceral adipose tissue expression of such markers as these may provide data on more long-term changes in metabolism.

6 Erythrocyte membrane fatty acid status In healthy, PET and IUGR pregnancies

6.1 Introduction

Essential fatty acids and their longer chain more unsaturated derivatives play a major role in maternal nutrition and fetal development during pregnancy. The n6 fatty acids [e.g arachidonic acid (AA, 20:4n6)] have strong pro-inflammatory effects and the n3 fatty acids [e.g eicosapentaenoic acid (EPA, 20:5n3)] are less active or even exhibit anti-inflammatory effects. The essential fatty acids α-linolenic acid (18:3n3) and linoleic acid (18:2n6), are unsaturated long carbon chain molecules that are obtained mainly from plants as mammals are unable to introduce double bonds into the n3 or n6 positions (Dyerberg 1986). These essential fatty acids (EFAs) are metabolised by chain elongation and desaturation to long chain polyunsaturated fatty acids (PUFAs) containing 20 or more carbon atoms in their n6 (e.g AA) and n3 families [e.g., EPA and docosahexaenoic acid (DHA, 22:6n3)] (figure 6-1). The endogenous synthesis of DHA from α-linolenic acid is a slow process (Li *et al* 1999a) although it has been suggested that women may posses a greater capacity for α-linolenic acid conversion than men (Burdge and Wootton 2002). Desaturases are key enzymes in the regulation of unsaturated fatty acid biosynthesis. The Δ6-desaturase enzyme is involved in the bioconversion of 18:2 into 18:3 and 24:4 into 24:5 in the n6 series, and of 18:3 into 18:4 and 24:5 into 24:6 in the n3 series (Voss *et al* 1991). The Δ5-desaturase enzyme is involved in the last step of AA biosynthesis from dihomo-γ-linolenic acid (20:3n6) (Rodriguez *et al* 1998) (figure 6-1). Both Δ5 and Δ6-desaturases are highly dependent on nutritional and hormonal factors (Brenner 1989, p.45-79).

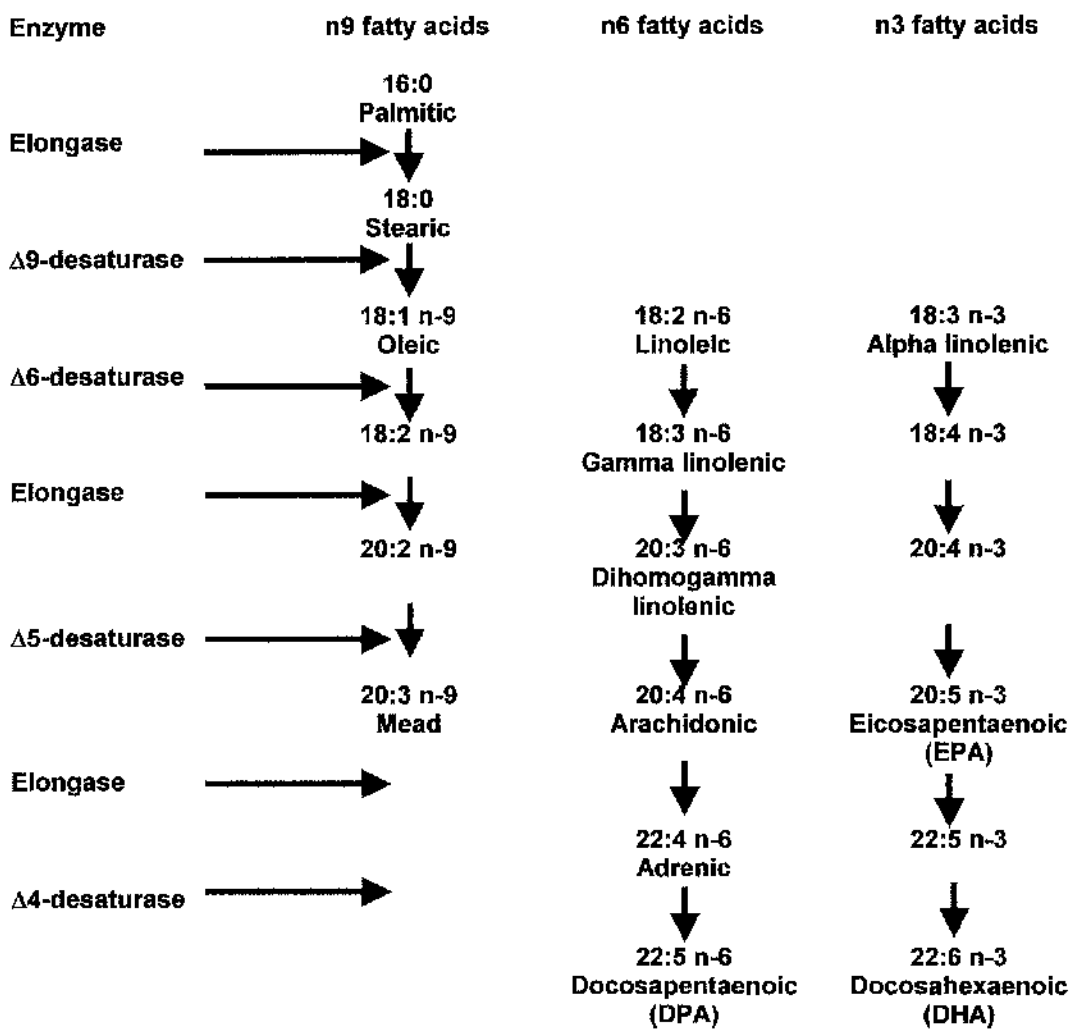


Figure 6-1. Major polyunsaturated fatty acids in the n-6 and n-3 classes.

Chemical formulae are shown for each of the fatty acids. Derived from Borkman *et al* (Borkman *et al* 1993).

Little consistent information is available with respect to the course of maternal fatty acid status during uncomplicated pregnancy. Few comparable studies have been conducted in terms of study design and populations observed and often only a few select fatty acids (FAs) are reported. Maternal nutritional requirements are increased during pregnancy and n3 polyunsaturated fatty acids (PUFAs) are required for energy and for accretion by the fetus (Makrides and Gibson 2000). Studies have demonstrated lower relative amounts of long chain polyunsaturated fatty acids (LCPUFAs) (Al *et al* 1995, Holman *et al* 1991, Rump *et al* 2001) throughout gestation. Montgomery and colleagues (Montgomery *et al* 2003) have shown that maternal plasma and erythrocyte DHA status is maximal in mid-trimester and declines to term. Al and colleagues (Al *et al* 1995) described an increase in total amounts of FAs during pregnancy with relative reductions in AA and DHA, despite a

temporary increase until 18 weeks' gestation in the latter. This group also observed that maternal DHA, an important component of the developing fetal central nervous system, decreases not only with gestation but also with parity and is significantly lower in multigravid women than primigravidae at 16, 22 and 32 weeks of pregnancy and after delivery (Al *et al* 1997). However, this reduction with parity is no longer apparent at 1-year post-partum (van den Ham *et al* 2001), indicating normalisation within this time in the maternal plasma and erythrocyte membranes. The reduction of certain fatty acids during pregnancy may highlight a role for supplementation of fatty acids during pregnancy for optimal fetal development.

The maternal LCPUFA status during pregnancy is critical in determining essential fatty acid status in the newborn (Connor *et al* 1996, Sattar *et al* 1998a, van Houwelingen *et al* 1995). Comparison of the LCPUFA concentration in the maternal and fetal plasma indicate a much higher concentration of AA and DHA in all the major lipid classes in the fetal compared to the maternal blood (Berghaus *et al* 2000, Haggarty 2002, Hoving *et al* 1994, Otto *et al* 1997). Ruyle and colleagues also showed lower levels of 18:2 and 18:3n3 FAs in fetal plasma and erythrocyte membranes compared to maternal (Ruyle *et al* 1990). The essential fatty acids α-linoleic acid (18:2n6) and linolenic acid (18:3n3) are important for optimal growth (Innis 1991, Rump *et al* 2001). These fatty acids are converted to LCPUFAs such as DHA and AA. All of the n-3 and n-6 fatty acids required by the fetus for development have to cross the placenta (Haggarty 2002). It has been demonstrated in a Dutch birth cohort (Rump *et al* 2002) that umbilical cord blood phospholipid γ-linolenic acid and dihomo-γ-linolenic acid concentrations are negatively related to insulin concentrations, body fatness, pro-insulin and leptin at 7 years of age. High insulin concentrations were found in children with a low birth weight and a low γ-linolenic acid concentration at birth.

All fatty acids can be transported across the lipid bilayer of the syncytiotrophoblast by simple diffusion and partition (Haggarty 2002). The fatty acid transport protein FAT/CD36 and the fatty acid binding protein FATP, which facilitate the placental transfer of the fatty acids, have been identified on both the microvillous and basal membranes of the placenta (Campbell *et al* 1998a). A placenta-specific protein p-FABPpm has also been demonstrated on the microvillous membrane only (Campbell *et al* 1998a). This placenta-specific protein has higher affinities and binding capacities for AA and DHA compared with linoleic acid and oleic acids, unlike FATP and FAT/CD36 (Campbell *et al* 1998b). There are also cytoplasmic binding proteins, H-FABP and L-FABP, within the

syncytiotrophoblast (Campbell *et al* 1998a). Because there are fatty acid binding proteins on both the basal and microvillous membranes of the placenta, transport is bi-directional between the maternal and fetal circulations (Haggarty 2002). The increase in surface area and blood flow along with the appearance of the terminal villi with advancing gestation are associated with an increase in fetal fat deposition (Haggarty 2002). There are multiple mechanisms by which the placenta could generate the observed LCPUFA gradient between the maternal and fetal circulation. These include selectivity by placental lipases for the release of LCPUFAs from TG, placental synthesis of AA and DHA resulting in higher concentrations of these LCPUFAs in the fetal circulation and metabolism of fatty acids within the fetus itself. These fatty acids may not be available to the placenta for re-uptake in esterified form if lipase activity and lipoprotein receptors are only found on the microvillous membrane (Haggarty 2002). The placenta may regulate its own fatty acid substrate supply via the action of placental leptin on maternal adipose tissue (Haggarty 2002).

Studies are conflicting with regard to the maternal and neonatal FA status in pregnancies complicated by PET or IUGR. Studies of PET and IUGR pregnancies often omit the effect of important confounders such as BMI. Leanness is associated with increased oxidative capacity and unsaturation of membranes in skeletal muscle. So by not controlling for BMI, these studies may have produced conflicting results regarding FA status. It has been demonstrated that maternal serum phospholipids have higher proportions of AA in PET subjects compared to controls (Ogburn *et al* 1984). It has been suggested that high intakes of PUFAs in the first half of pregnancy may independently increase the risk for PET (Clausen *et al* 2001). One prospective study has shown that in pregnancy-induced hypertension (PIH), altered essential fatty acid status in the form of higher levels of DHA in umbilical plasma phospholipid was a late phenomenon (beyond 32 weeks' gestation and post-partum) and concluded that these changes were not likely to be involved in the pathogenesis of the disease (Al *et al* 1995). Another group described an increase in unsaturation of maternal serum phospholipids and concluded that this might aid placental transfer of LCPUFAs (van der Schouw *et al* 1991). Both groups described a relatively unaltered neonatal essential fatty acid status in offspring born to mothers with PIH. Some studies have reported reduced levels of LCPUFAs in the umbilical vessels in PET compared to normotensive women, and have attributed this to the abnormal placentation and insufficiency seen in this condition (Velzing-Aarts *et al* 1999). Lower concentrations of essential fatty acids have been described in the erythrocyte membranes, plasma phospholipids and walls of the umbilical artery of low birth weight neonates (Crawford *et al* 1989). In addition, fetal growth restriction has been associated with an increase in

arachidonic acid in placenta and in umbilical artery, and this was significantly higher in asymmetric than symmetric fetal growth restriction (Matorras *et al* 2001). However, once again, data is conflicting. Some studies have reported a reduction in DHA concentration in the umbilical vein of growth restricted fetuses (Felton *et al* 1994), whereas others have described higher concentrations of DHA in the basal membrane of the placenta in these growth restricted fetuses (Powell *et al* 1999), and some have demonstrated no alteration in AA or DHA in cord in small for gestational age infants (Percy *et al* 1991). Interestingly, recent data has shown a negative correlation between birth weight and eicosapentaenoic acid (EPA) concentrations (Grandjean *et al* 2001). Experimental evidence also suggests that a high intake of n-3 PUFA may cause a reduction in fetal birth weight in rats (Clarke *et al* 1988, Olsen *et al* 1990).

Because maternal fatty acid status is considered to decline during pregnancy, there is much interest in dietary intervention. Dietary n3 fatty acid supplementation is known to reduce the incidence and severity of inflammatory disorders, cardiovascular diseases and some cancers (Rose 1997, Suchner and Senftleben 1994) by decreasing the production of prostaglandin E_2 (PGE_2) (Hamilton *et al* 1999). Numerous studies have observed the effects of fatty acid supplementation in pregnancy on the maternal and fetal erythrocyte membrane fatty acid (EMFA) composition. For example, fish oil supplementation with n-3 fatty acids (56% DHA and 28% EPA) from 20 weeks of pregnancy is an effective means of enhancing n-3 fatty acid status of both mothers and neonates (Dunstan *et al* 2004), with a concomitant reduction in AA status. Recent work (Montgomery *et al* 2003, Sanjurjo *et al* 2004) has shown that DHA supplementation (200 mg/d) in the last trimester of pregnancy results in an increase in maternal plasma levels of DHA, although Sanjurjo and colleagues could not demonstrate a corresponding increase in the fetus (Sanjurjo *et al* 2004). One group has demonstrated that supplementation with DHA (33 or 133 mg from eggs) from 24-28 weeks' gestation until delivery, in a mostly black population, actually increases duration of gestation when DHA intake was increased during the last trimester. Interestingly, de Groot *et al* have demonstrated that supplementation with α-linolenic acid from 14 weeks' gestation until delivery neither prevented the decreases in maternal plasma DHA and AA concentrations nor promoted neonatal plasma DHA and AA status (de Groot *et al* 2004). It is possible that any additional DHA produced from the supplemented α-linolenic acid is transferred directly to the fetus and is thus not evident in maternal plasma. There have been indications in the literature that high intakes of n3 LCPUFAs during pregnancy may increase birth weight and duration of gestation (Helland *et al* 2001), in contrast to experimental evidence suggesting a reduction in intrauterine growth with a high

intake of n-3 PUFA (Clarke *et al* 1988, Olsen *et al* 1990). With respect to complicated pregnancy, results of randomised clinical studies suggest that n3 LCPUFA supplementation during pregnancy does not affect the indices of PIH and PET (D'Almeida *et al* 1992, Onwude *et al* 1995). As for the effects of supplementation on fetal FA status, some studies have found a beneficial effect of n3 PUFA supplementation on fetal FA status (Agostoni *et al* 1995, Birch *et al* 1998, Carlson *et al* 1996, Makrides *et al* 1995), whereas others have reported no effect (Auestad *et al* 1997, Innis *et al* 1997, Jensen *et al* 1997, Nielsen and Jensen 1997).

Insulin resistance is a metabolic disturbance that is central to the development of a variety of prevalent diseases including non-insulin dependent diabetes mellitus (NIDDM), various dyslipidaemias, adult obesity, hypertension, heart disease (Bjorntorp 1991, Reaven 1988, 1993) and the metabolic complications of pregnancy, in particular pre-eclampsia (PET). The state of insulin resistance seen in uncomplicated pregnancy and also in PET was discussed in the introduction (Chapter 1, section 1.3.2).

The euglycaemic clamp is considered by many to be the gold standard for determining insulin sensitivity (Saccomani *et al* 1996). However, this test is labour intensive and difficult to perform in pregnant subjects and this has lead to the use of simpler methods of measuring insulin sensitivity. There are no validated measures of insulin resistance for use in pregnancy. Previous studies have employed fasting glucose and insulin levels or levels after glucose administration as indicators of insulin sensitivity (Solomon and Seely 2001) and have projected results based on non-pregnant data. Insulin resistance has been estimated by the calculation of the homeostasis model assessment (HOMA) index of insulin resistance despite this test being validated only in the non-pregnant state (Matthews *et al* 1985). It is often difficult to obtain fasting samples in pregnancy studies, especially in PET or IUGR, where patients are often admitted on an emergency basis for immediate delivery. For these reasons, this and other studies often aim to observe other potential measures of insulin sensitivity in the pregnant state.

Insulin is an important regulator of serum sex hormone binding globulin (SHBG) concentration that works by inhibiting its synthesis in hepatocytes. Low SHBG levels are associated with increased insulin resistance and hyperinsulinaemia (Bartha *et al* 2000, Haffner 1996). SHBG has been suggested as a useful diagnostic tool for evaluating insulin resistance and cardiovascular risk (Pugeat *et al* 1996). In non-pregnant subjects, SHBG correlates inversely with glucose tolerance (Goodman-Gruen and Barrett-Connor 1997), insulin levels (Haffner *et al* 1988) and insulin resistance as determined by the

euglycaemic-hyperinsulinaemic clamp (Sherif *et al* 1998). Two prospective studies have demonstrated that reduced SHBG levels are associated with increased risk of future type II diabetes in otherwise healthy women (Haffner *et al* 1993, Lindstedt *et al* 1991). Pregnant women with gestational diabetes have lower SHBG levels than healthy controls (Bartha *et al* 2000). In uncomplicated pregnancy, SHBG levels rise steadily until the 3rd trimester reaching a peak that is 4-6 times that of non-pregnant values (Kerlan *et al* 1994, O'Leary *et al* 1991, Wolf *et al* 2002). It has been demonstrated that first trimester SHBG levels are significantly lower in nulliparous women who subsequently develop PET compared with normotensive women, and that this association between early pregnancy insulin resistance and development of PET is strengthened in lean compared to obese women (Wolf *et al* 2002). SHBG values are reliable in the non-fasting state (Key *et al* 1990) and exhibit minimal diurnal variation (Hamilton-Fairley *et al* 1995).

Insulin resistance and adult obesity are associated with relatively low proportions of polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA), in muscle membrane structural lipid. Skeletal muscle plays a major role in insulin-stimulated glucose uptake and whole body energy expenditure (Helge *et al* 1998). Studies in both animal and human models have shown a relationship between skeletal muscle membrane structural lipid (phospholipid) and measures of insulin resistance (Borkman *et al* 1993, Storlien *et al* 1991, Vessby *et al* 1994). A higher percentage of more saturated fatty acids (FAs) in muscle membrane phospholipid is associated with insulin resistance, whereas a higher percentage of long-chain polyunsaturated fatty acids (LCPUFAs) is associated with insulin sensitivity (Baur *et al* 1999).

It has been demonstrated that erythrocyte membrane fatty acid (EMFA) composition is a reasonable index of muscle DHA, total n-3 PUFA and the n-3/n-6 PUFA ratio (Baur *et al* 2000). Similarly, for a range of fatty acids (16:0, 18:2n-6, 20:4n-6, 20:5n-3, and 22:5n-3), EMFA composition has been shown to have close associations with that of muscle membranes (Felton *et al* 2004). This group also demonstrated relationships between EMFA concentrations and insulin sensitivity; highly insulin sensitive male subjects (measured using the intravenous glucose tolerance test [IVGTT]), had decreased EMFA concentrations compared with normal/low insulin sensitivity subjects, and Δ6 and Δ5 desaturase activities were higher and lower respectively, compared with low/normal insulin sensitivity subjects (Felton *et al* 2004). However, Di Marino and colleagues (Di Marino *et al* 2000) have shown that compared with erythrocyte membranes, muscle membranes show a significantly higher proportion of omega-6 polyunsaturated fatty acid ($P < 0.001$) and lower saturated fatty acid ($P < 0.001$), monounsaturated fatty acid ($P <$

0.001), and omega-3 PUFA ($P < 0.001$). From these results, this group concluded that erythrocyte and muscle membrane phospholipid fatty acids are significantly different, and that data on muscle membranes could not be extrapolated on the basis of measures of erythrocyte phospholipid fatty acid composition.

In this current study, EMFA concentrations were used to identify individual fatty acid status. Skeletal muscle biopsies were considered too invasive for use in pregnancy and as discussed earlier, there are no validated tests of insulin sensitivity in pregnancy. Although there is controversy regarding whether EMFA concentrations are indicative of skeletal muscle fatty acid status, it was considered that the red blood cells would at very least provide us with an indication of overall fatty acid status and allow us to compare maternal and fetal values.

The hypotheses were 3-fold;

1. That plasma markers of insulin resistance, inflammation and dyslipidaemia would increase with advancing gestation and be further exaggerated in pregnancies complicated by PET and/or IUGR.

2. That there would be a reduction in LCPUFAs as pregnancy advanced and an increase in a saturated fatty acid profile, again further exaggerated by PET and/or IUGR.

3. That changes seen in fatty acid status throughout gestation or in complicated pregnancy would relate to the plasma markers of insulin resistance, inflammation and dyslipidaemia.

Thus the objectives were;

- To determine maternal plasma markers of insulin resistance (fasting insulin, glucose and HOMA where fasting samples were available in the longitudinal study, and SHBG, non fasting insulin, glucose and HOMA where fasting samples were not obtained in the case control study), inflammation (ICAM, VCAM, IL-6, IL-10, CRP) and dyslipidaemia (TC, TG, VLDL, LDL, IIDL) in a prospective longitudinal study of each trimester of uncomplicated pregnancy (n=47 each group) and in a case control study of pregnancies complicated by PET (n=23) and IUGR (n=17).

- To determine the availability of EMFAs in the prospective longitudinal study group, as an observational study of the fatty acid status throughout gestation in normal pregnancy, and to determine the availability of the EMFAs in the case control study of pregnancies complicated by PET, a recognised state of insulin resistance (Sattar and Greer 1999), or by IUGR, where the maternal response to abnormal placentation is absent.

- To relate the plasma markers of insulin resistance, inflammation and dyslipidaemia to any changes observed in EMFAs in both study groups.

6.2 Results

6.2.1 Uncomplicated pregnancy.

6.2.1.1 Maternal characteristics in uncomplicated pregnancies

Baseline characteristics for forty-seven subjects from uncomplicated pregnancies are shown in table 6-1.

Using third trimester values, maternal log 14:0 ($p=0.03$, $r^2=8.9\%$), log 20:0 ($p=0.02$, $r^2=10.2\%$), 22:4n6 ($p=0.03$, $r^2=7.7\%$), log 24:0 ($p=0.02$, $r^2=9.3\%$) and 24:1n9 ($p=0.01$, $r^2=2.1\%$) were significantly associated with maternal BMI (univariate regression analysis). Maternal 18:3n6 ($p=0.03$, $r^2=8.0\%$) and 22:5n3 ($p=0.04$, $r^2=6.7\%$) were associated with maternal age (univariate regression analysis). Maternal 22:0 ($p=0.03$), total n3 ($p=0.006$) and log n6/n3 ($p=0.002$) were affected by smoking status (ANOVA). Maternal log 20:3n3 ($p=0.03$) was affected by parity, maternal log 22:5n6 ($p=0.02$, $r^2=9.0\%$) and 22:6n3 ($p=0.05$, $r^2=6.2\%$) were affected by gestational age at sampling, and maternal log 20:1n9 was affected by parity ($p=0.03$) and gestational age at sampling ($p=0.02$, $r^2=10.0\%$), all tested using univariate regression analysis or ANOVA. For statistical analysis, all data was adjusted for these effects using a general linear model in the PET, IUGR and control study, and for all effects except for gestational age at sampling in the longitudinal study. Markers of inflammation and lipids were adjusted as previously (Chapters 4 & 5) in both studies. Maternal log glucose was adjusted for the effect of maternal age and gestational age at sampling, and log insulin for gestational age at sampling, using the fasting subjects within the same cohort used for the inflammatory and lipid data previously described (n=61).

6.2.2 Longitudinal study

6.2.2.1 Maternal characteristics in a longitudinal study.

The forty-seven subjects studied in each trimester of uncomplicated pregnancy are the same cohort as that used for the data normalisation in section 6.2.1.1, and baseline characteristics are thus demonstrated in table 6-1.

6.2.2.2 Markers of insulin resistance, inflammation and dyslipidaemia in a longitudinal study.

All subjects were within normal glucose control as demonstrated by a fructosamine test, thus excluding any latent diabetes. In uncomplicated pregnancy, log SHBG ($p<0.001$), fasting log glucose ($p=0.003$), fasting log insulin ($p<0.001$) and HOMA ($p=0.003$) were all significantly increased as gestation advanced. Similarly, log IL-6 ($p=0.002$) was increased as gestation advanced, although log sCRP was unchanged ($p=0.64$). Maternal TC, log TG, VLDL and LDL were all significantly increased with advancing gestation ($p<0.001$ in all groups). However, log HDL was unchanged ($p=0.72$) (table 6-2).

6.2.2.3 Maternal EMFA profile in a longitudinal study.

For observational purposes, all erythrocyte membrane fatty acids tested in each trimester are shown in table 6-3.

Characteristic	N=47
Age (years)	28.7 (5.0)
BMI (kg/m^2)	28.4 (6.1)
Primigravidae n (%)	24 (51%)
Smokers n (%)	18 (36%)
Gestation at sampling (weeks)	35.3 (1.34)
Gestation at delivery (weeks)	40.0 (1.60)
Vaginal delivery n (%)	37 (79%)
Placental weight (g)	770.0 (184.7)
Fetal weight (kg)	3.63 (0.59)
Birth weight centile	56.7 (33.2)
Fetal sex	20 female (43%)
n (%)	27 male (57%)

Table 6-1. Maternal characteristics from uncomplicated pregnancies (data normalisation cohort and longitudinal study group).

Data shown are mean and S.D.

	1st trimester (n=47)	2nd trimester (n=47)	3rd trimester (n=47)	P
Log SHBG (log nmol/L)	2.31 (0.09) [204.17 (1.23)]	2.41 (0.10) [257.04 (1.26)]	2.41 (0.06) [257.04 (1.15)]	<0.001
Fasting log glucose (mmol/L)	0.66 (0.11) [4.57 (1.29)]	0.73 (0.09) [5.37 (1.23)]	0.72 (0.08) [5.25 (1.20)]	0.003
Fasting log insulin (log mU/L)	1.22 (0.37) [16.60 (2.34)]	1.54 (0.35) [34.67 (2.24)]	1.57 (0.36) [37.15 (2.29)]	<0.001
HOMA	5.59 (7.29)	11.89 (10.53)	13.02 (12.50)	0.003
Log IL-6 (log pg/mL)	0.06 (0.33) [1.15 (2.14)	0.14 (0.27) [1.38 (1.86)]	0.27 (0.24) [1.86 (1.74)]	0.002
Log CRP (log mg/L)	0.67 (0.44) [4.68 (2.75)]	0.66 (0.44) [4.57 (2.75)]	0.59 (0.40) [3.89 (2.51)]	0.64
TC (mmol/L)	4.59 (0.76)	5.90 (0.89)	6.06 (0.94)	<0.001
Log TG (log mmol/L)	0.11 (0.16) [1.29 (1.45)]	0.36 (0.14) [2.29 (1.38)]	0.45 (0.14) [2.82 (1.38)]	<0.001
VLDL (mmol/L)	0.40 (0.23)	0.68 (0.28)	0.78 (0.30)	<0.001
LDL (mmol/L)	2.77 (0.68)	3.78 (0.80)	3.89 (0.90)	<0.001
Log HDL (log mmol/L)	0.14 (0.08) [1.38 (1.20)]	0.15 (0.08) [1.41 (1.20)]	0.13 (0.09) [1.35 (1.23)]	0.72

Table 6-2. Markers of insulin resistance, inflammation and dyslipidaemia in a longitudinal study of uncomplicated pregnancies.

Statistical analysis was performed using repeated measures ANOVA, and data is presented as mean and S.D. Log transformed data was used for maternal SHBG, glucose, insulin, IL-6, CRP, TG and HDL, and is presented as mean (S.D.) and [geometric mean (S.D.)].

Fatty acids (% total)	1st trimester (n=47)	2nd trimester (n=47)	3rd trimester (n=47)	p
12:0	0	0	0	*
14:0	0.61 (0.27)	0.63 (0.30)	0.62 (0.26)	0.94
14:1n7	0.00 (0.03)	0.02 (0.10)	0.01 (0.05)	0.49
16:0	21.86 (2.19)	22.03 (2.41)	22.14 (1.39)	0.69
16:1n7	0.86 (0.23)	1.01 (0.27)	0.99 (0.23)	0.006
17:0	0.35 (0.19)	0.31 (0.22)	0.33 (0.21)	0.80
17:1n7	0.04 (0.11)	0.04 (0.11)	0.07 (0.13)	0.40
18:0	15.71 (1.41)	14.78 (1.47)	14.48 (1.27)	<0.001
18:1n6	0	0	0	*
18:1n9	14.46 (1.72)	14.82 (2.08)	14.79 (1.67)	0.59
18:1n7	0	0	0	*
18:2n6	8.58 (1.05)	8.69 (1.58)	8.90 (0.92)	0.45
18:3n6	0.16 (0.17)	0.11 (0.15)	0.11 (0.14)	0.15
18:3n3	0.19 (0.15)	0.33 (0.20)	0.29 (0.18)	0.001
20:0	0.72 (0.14)	0.70 (0.19)	0.62 (0.14)	0.006
20:1n9	0.60 (0.13)	0.65 (0.24)	0.56 (0.12)	0.09
20:2n6	0.38 (0.16)	0.41 (0.22)	0.41 (0.17)	0.34
20:3n9	0	0	0	*
20:3n6	1.83 (0.41)	2.03 (0.51)	1.95 (0.43)	0.11
20:4n6	13.67 (2.52)	12.72 (2.23)	12.84 (1.45)	0.07
20:3n3	0.20 (0.23)	0.19 (0.22)	0.16 (0.20)	0.25
20:5n3	0.80 (0.45)	0.85 (0.46)	0.71 (0.41)	0.32
22:0	1.72 (0.41)	1.56 (0.35)	1.49 (0.37)	0.01
22:1n9	0.17 (0.24)	0.14 (0.24)	0.17 (0.23)	0.80
22:2n6	0.04 (0.15)	0.01 (0.07)	0.01 (0.05)	0.66
22:4n6	2.70 (0.75)	2.68 (0.79)	2.84 (0.70)	0.53
22:3n3	0.37 (0.32)	0.39 (0.27)	0.41 (0.33)	0.73
22:5n6	0.54 (0.22)	0.67 (0.22)	0.68 (0.20)	0.003
22:5n3	2.01 (0.41)	2.01 (0.49)	2.07 (0.31)	0.66
24:0	3.20 (0.71)	3.30 (0.52)	3.28 (0.54)	0.51
22:6n3	3.29 (0.92)	3.79 (0.97)	3.85 (0.69)	0.004
24:1n9	4.92 (0.75)	5.12 (0.98)	5.21 (0.80)	0.23
% saturated FAs	46.47 (3.66)	45.61 (2.90)	45.31 (2.28)	0.16
% monounsaturated FAs	20.44 (1.74)	21.19 (1.86)	21.16 (1.64)	0.06
% PUFAs	33.10 (4.23)	33.21 (3.88)	33.51 (2.41)	0.85
Total n9 FAs	19.42 (1.62)	19.97 (1.73)	19.94 (1.53)	0.18
Total n7 FAs	1.03 (0.29)	1.22 (0.38)	1.21 (0.32)	0.006
Total n6 FAs	27.00 (3.68)	26.45 (3.07)	26.82 (2.11)	0.67
Total n3 FAs	6.14 (1.22)	6.78 (1.27)	6.69 (0.91)	0.02
% unsaturated FAs	53.54 (3.66)	54.39 (2.90)	54.69 (2.28)	0.16
UI	147.95 (16.66)	148.92 (16.28)	149.56 (10.54)	0.87
Average CL	18.56 (0.17)	18.56 (0.21)	18.58 (0.15)	0.85
C20-22 FAs	29.25 (4.09)	29.08 (4.49)	29.01 (3.07)	0.95
Total n6/n3	4.54 (0.93)	4.04 (0.80)	4.07 (0.59)	0.004
Delta 5 desaturase	7.79 (1.94)	6.58 (1.63)	6.94 (1.70)	0.004
Delta 6 desaturase	0.20 (0.04)	0.25 (0.28)	0.20 (0.05)	0.23
Delta 9 desaturase	0.93 (0.13)	1.02 (0.19)	1.04 (0.14)	0.003
Elongase	0.65 (0.05)	0.61 (0.08)	0.59 (0.04)	<0.001

Table 6-3. All fatty acids tested (%) in each trimester of uncomplicated pregnancy.

Raw data is shown, and statistical analysis was performed on log transformed data for 12:0, 14:0, 16:0, 17:0, 17:1n7, 18:0, 18:1n9, 20:0, 20:1n9, 20:2n6, 20:3n3, 20:5n3, 22:2n6, 22:3n3, 22:5n6, 24:0, %monounsaturated FAs, total n9, total n6/n3, delta 6 and delta 9 desaturases. Statistical analysis was performed on square root of total n7. Statistical analysis was performed on data independent of maternal age, BMI, smoking status, parity, as described in section 6.2.1.1.Statistical analysis was performed using repeated measures ANOVA, and data are presented as mean and S.D. FA = fatty acid, UI = unsaturation index (the average number of double bonds per fatty acid residue multiplied by 100), CL = chain length.

Of particular interest, 18:0 was significantly reduced with advancing gestation [T1 15.71 (1.41), T2 14.78 (1.47), T3 14.48 (1.27)%, p<0.001, repeated measures ANOVA], and this difference was most notable in early pregnancy, between the 1st and 2nd trimesters (p=0.002) (figure 6-2).

The results show that 12:0, 18:1n6, 18:1n7 and 20:3n9 values are negligible in each trimester of uncomplicated pregnancy. The % α-linolenic acid (18:3n3) was significantly different between the trimesters [T1 0.19 (0.15), T2 0.33 (0.20), T3 0.29 (0.18)%, p=0.001, repeated measures ANOVA], with a peak value in the 2nd trimester, and the significant difference between 1st and 2nd trimesters (p<0.001) (figure 6-3).

In this longitudinal observational study, EPA (20:5n3) was not significantly altered as gestation advanced [T1 0.80 (0.45), T2 0.85 (0.46), T3 0.71 (0.41)%, p=0.32), repeated measures ANOVA]. However, DHA (22:6n3) significantly increased as gestation advanced [T1 3.29 (0.92), T2 3.79 (0.97), T3 3.85 (0.69)%, p=0.004, repeated measures ANOVA], with the significant increase seen between the 1st and 2nd trimesters (p=0.01), and thereafter demonstrating a plateau between the 2nd and 3rd trimesters (p=0.75) (figure 6-4).

The n6 fatty acid, AA (20:4n6) was not significantly altered as gestation advanced in uncomplicated pregnancy [T1 13.67 (2.52), T2 12.72 (2.23), T3 12.84 (1.45)%, p=0.07, repeated measures ANOVA].

The percentage of saturated FAs (p=0.16), monounsaturated FAs (p=0.06), unsaturated FAs (p=0.16), polyunsaturated FAs (PUFAs) (p=0.85), the unsaturation index (UI) (p=0.87) and the total percentage of the C20-22 FAs (p=0.95) were not altered significantly as gestation advanced. Similarly, the percentage of the total n6 FAs was not altered with advancing gestation (p=0.67). However, the percentage of the total n3 FAs was altered significantly between the trimesters [T1 6.14 (1.22), T2 6.78 (1.27), T3 6.69 (0.91)%, p=0.02], with a significant increase from the 1st to the 2nd trimester (p=0.02) (figure 6-5), and the total n6/n3 ratio was consequently significantly altered as gestation advanced [T1 4.54 (0.93), T2 4.04 (0.80), T3 4.07 (0.59)%, p=0.004].

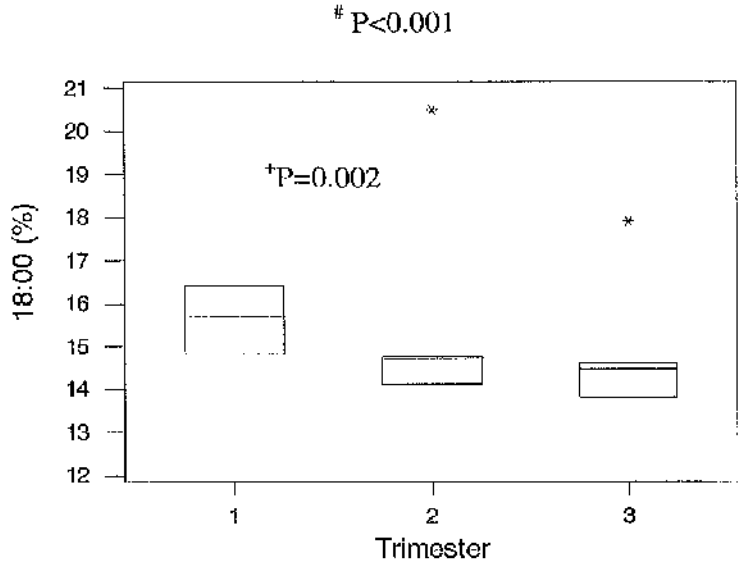


Figure 6-2. % 18:0 (stearic acid) in each trimester of uncomplicated pregnancy (*n*=47 per trimester).

Statistical analysis was performed on log transformed data, using repeated measures ANOVA (#) between the trimesters, with post hoc Students *t*-test (+) on 1st vs 2nd trimester, and is shown as mean and 95% CI. * = outlier.

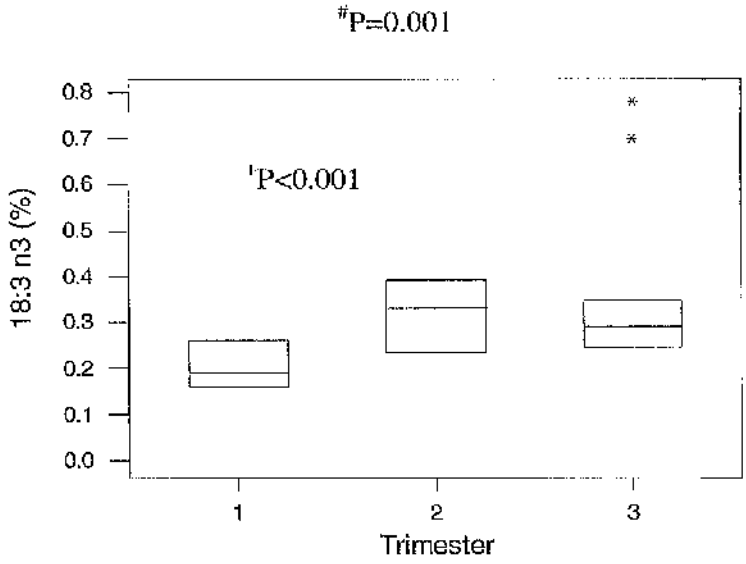


Figure 6-3. %18:3n3 (α-linolenic acid) in each trimester of uncomplicated pregnancy (n=47 per trimester).

Statistical analysis was performed using repeated measures ANOVA (#) between the trimesters, with post hoc Students *t*-test (+) on 1st vs 2nd trimester, and is shown as mean and 95% CI. *= outlier.

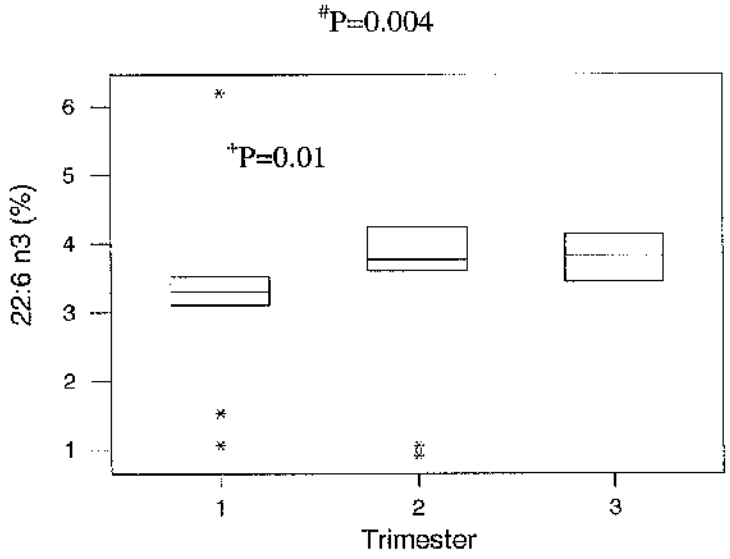


Figure 6-4. % 22:6n3 (DHA) in each trimester of uncomplicated pregnancy (n=47 per trimester).

Statistical analysis was performed using repeated measures ANOVA (#) between the trimesters, with post hoc Students *t*-test (+) on 1st vs 2nd trimester, and is shown as mean and 95% CI. *= outlier.

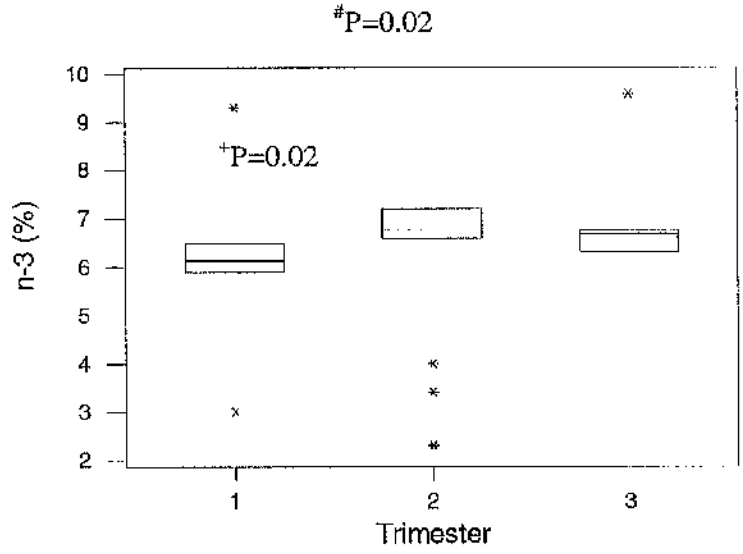


Figure 6-5. % total n3 FAs in each trimester of uncomplicated pregnancy (n=47 per trimester).

Statistical analysis was performed using repeated measures ANOVA (#) between the trimesters, with post hoc Students *t*-test (+) on 1st vs 2nd trimester, and is shown as mean and 95% CI. *= outlier.

The ratio of 20:4n6/20:3n6, used to estimate delta 5 desaturase activity, was significantly altered with advancing gestation [T1 7.79 (1.94), T2 6.58 (1.63), T3 6.94 (1.70), p=0.004], with a significant reduction in activity from the 1st to the 2nd trimester (p=0.02) (figure 6-6). Delta 9 desaturase activity was also altered throughout the trimesters (T1 0.93 (0.13), T2 1.02 (0.19), T3 1.04 (0.14), p=0.003], with a significant increase in activity in the 2nd trimester compared to the 1st (p=0.01). Elongase activity (18:0/16:0 ratio) was significantly reduced as gestation advanced [T1 0.65 (0.05), T2 0.61 (0.08), T3 0.59 (0.04), p<0.001], with a significant reduction in the 2nd trimester compared with the 1st (p=0.003) (figure 6-7).

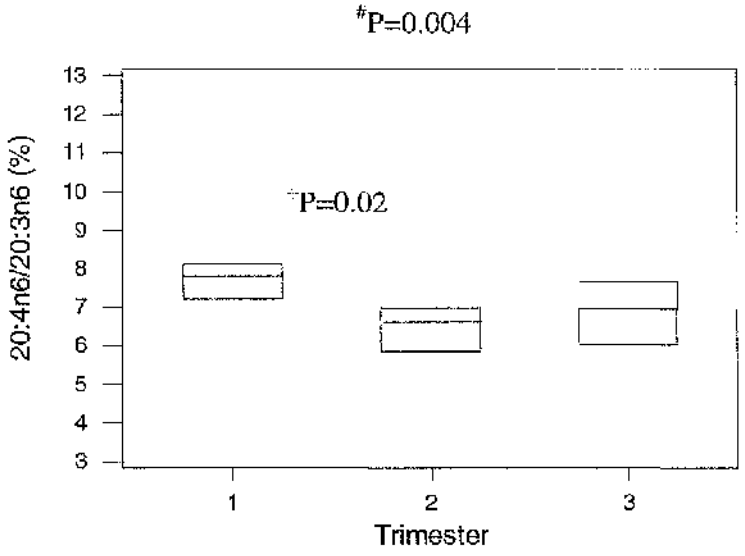


Figure 6-6. % 20:4n6/20:3n6 (delta 5 desaturase activity) in each trimester of uncomplicated pregnancy (n=47 per trimester).

Statistical analysis was performed using repeated measures ANOVA (#) between the trimesters, with post hoc Students *t*-test (+) on 1st vs 2nd trimester, and is shown as mean and 95% CI.

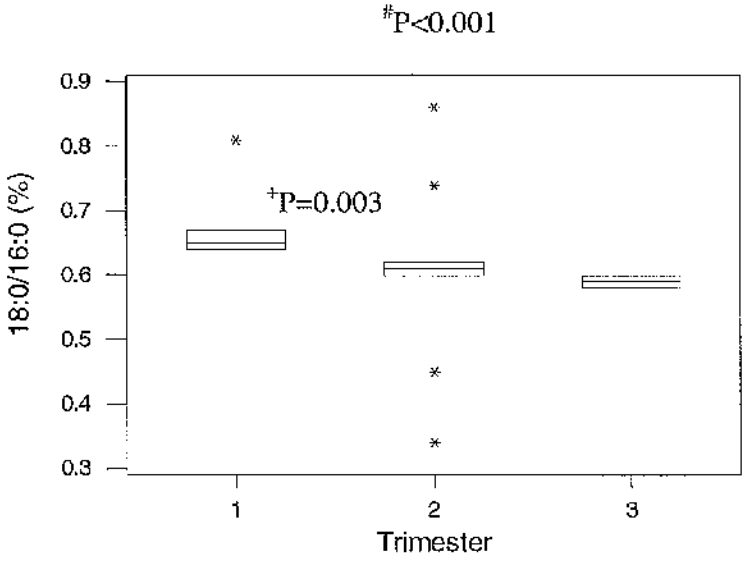


Figure 6-7. % 18:0/16:0 (elongase activity) in each trimester of uncomplicated pregnancy (n=47 per trimester).

Statistical analysis was performed using repeated measures ANOVA (#) between the trimesters, with post hoc Students *t*-test (+) on 1st vs 2nd trimester, and is shown as mean and 95% CI. = outlier.

6.2.2.4 Associations between markers of insulin resistance, inflammation and dyslipidaemia and EMFA profiles in uncomplicated pregnancy.

Because a large number of individual fatty acids are reported, it was decided to use <0.005 as a level of significance for discussion, to limit chance associations.

6.2.2.4.1 1st trimester

There were no significant correlations ($p<0.005$) between maternal plasma markers of insulin resistance, dyslipidaemia or inflammation and the fatty acids in the 1st trimester.

6.2.2.4.2 2nd trimester

In the 2nd trimester, maternal plasma CRP correlated negatively with 18:0 (r=-0.43, p=0.003) (figure 6-8).

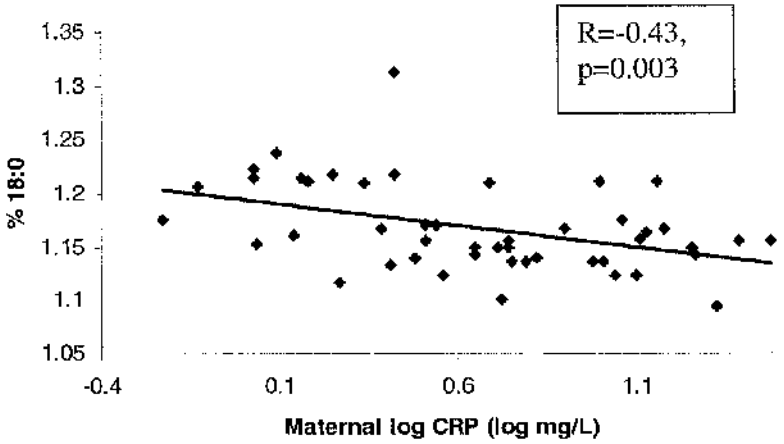
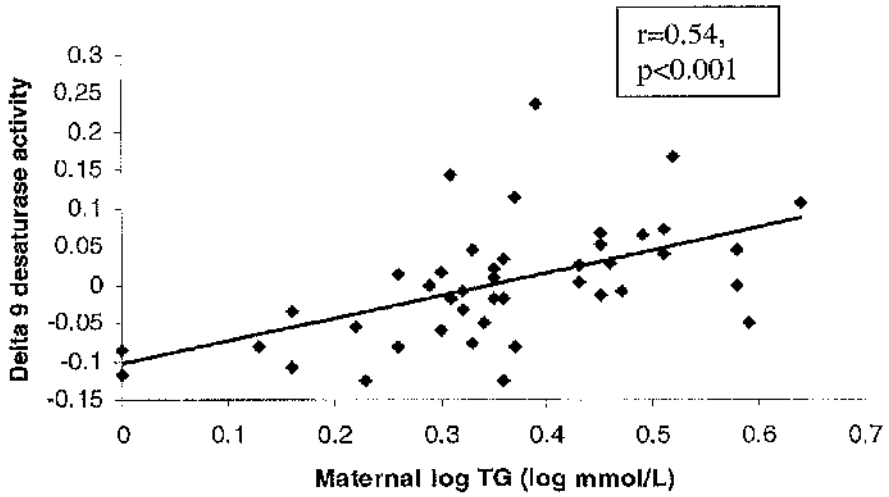


Figure 6-8. Association between maternal log CRP and % 18:0 (stearic acid) in the 2nd trimester.

Statistical analysis was performed using Pearson's coefficient of correlation, on log transformed data for 18:0.

TG levels were negatively associated with elongase activity (r=-0.50, p=0.001), and



positively correlated with delta 9 desaturase activity (r=0.54, p<0.001) (figure 6-9).

Figure 6-9. Association between maternal log TG and delta 9 desaturase activity (18:1n9/18:0) in the 2nd trimester.

Statistical analysis was performed using Pearson's coefficient of correlation, on log transformed data for delta 9 desaturase.

There were no significant correlations between markers of insulin resistance and fatty acids in the 2nd trimester.

6.2.2.4.3 3rd trimester

Fasting insulin correlated with 22:6n3 (DHA) (r=0.39, p=0.008) (figure 6-10) in the 3rd trimester.

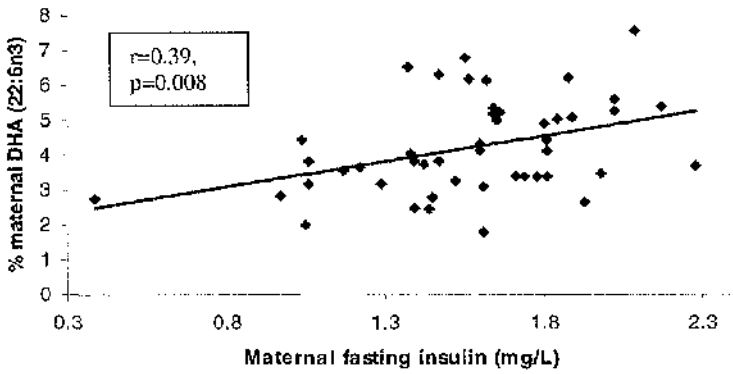


Figure 6-10. Association between maternal fasting log insulin and DHA (22:6n3) in the 3rd trimester.

Statistical analysis was performed using Pearson's coefficient of correlation.

Maternal TG levels were associated with the total percentage of monounsaturated FAs (r=0.61, p<0.001) and delta 9 desaturase activity (r=0.67, p<0.001) (figure 6-11), and correlated negatively with elongase (r=-0.45, p=0.002).

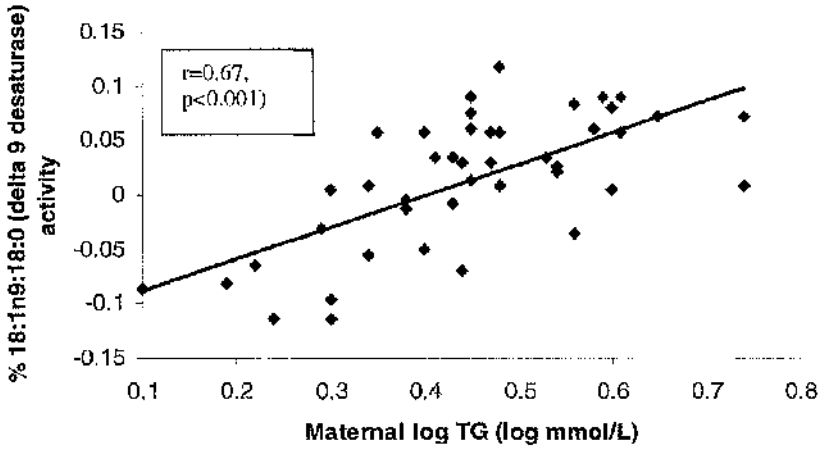


Figure 6-11. Association between maternal log TG and delta 9 desaturase activity (18:1n9/18:0) in the 3rd trimester.

Statistical analysis was performed using Pearson's coefficient of correlation, on log transformed data for delta 9 desaturase.

There were no associations between any of the FAs and the inflammatory markers or between the EPA/AA ratio (as an inflammatory index) and the inflammatory markers in the 3rd trimester.

6.2.3 PET and IUGR case control study.

6.2.3.1 Maternal characteristics in pregnancies complicated by PET and IUGR.

For the analysis of complicated pregnancy, cases were matched for age, parity, BMI and smoking habit with controls. Characteristics of the matched groups are as for the lipid and inflammatory data, shown in table 4-6, in section 4.2.3.1.

6.2.3.2 Markers of insulin resistance, inflammation and dysiipidaemia in PET, IUGR and control subjects.

Markers of inflammation and dyslipidaemia in PET and IUGR pregnancies are demonstrated in chapters 4 (section 4.2.3.1, table 4-11) and 5 (section 5.2.1.2, table 5-3). To summarise, there were no differences in markers of inflammation in PET compared with controls, and maternal cholesterol levels were significantly elevated in PET (p=0.04). In IUGR, there were no differences in lipids between cases and controls, and there was a trend towards reduced maternal log VCAM in IUGR (p=0.05). Although samples were not taken under fasting conditions in this group, in PET and controls, there were no differences in log SHBG [PET 2.40 (0.12) vs controls 2.45 (0.14), p=0.21], log glucose [PET 0.81 (0.23) vs controls 0.73 (0.19), p=0.24], log insulin [PET 1.16 (0.94) vs controls 1.28 (0.87), p=0.68] and HOMA [PET 10.6 (19.1) vs controls 10.4 (19.1), p=1.00]. Similarly, in IUGR and controls, there were no differences in log SHBG [IUGR 2.39 (0.14) vs controls 2.40 (0.12), p=0.69], log glucose [IUGR 0.71 (0.12) vs controls 0.78 (0.15), p=0.14] and HOMA [IUGR 3.49 (3.34) vs controls 8.20 (10.20), p=0.11]. However, there was a trend towards reduced log insulin in IUGR subjects [0.91 (0.53)] compared to controls [1.37 (0.69), p=0.05) (Table 6-4). All subjects were in normal glucose control as demonstrated by a fructosamine test.

	PET n=23	Controls n=23	p	IUGR n=17	Controls n=17	p
Log SHBG (log nmol/L)	2.40 (0.12) [251.19 (1.32)]	2.45 (0.14) [281.84 (1.38)]	0.21	2.39 (0.14) [245.47 (1.38)]	2.40 (0.12) [251.19 (1.32)]	0.69
Log glucose (log mmol/L)	0.81 (0.23) [6.46 (1.70)]	0.73 (0.19) [5.37 (1.55)]	0.24	0.71 (0.12) [5.13 (1.32)]	0.78 (0.15) [6.03 (1.41)]	0.14
Log insulin (log mU/L)	1.16 (0.94) [14.45 (8.71)]	1.28 (0.87) [19.05 (1.55)]	0.68	0.91 (0.53) [8.13 (3.39)]	1.37 (0.69) [23.44 (4.90)]	0.05
HOMA	10.60 (19.10)	10.60 (19.10)	1.00	3.49 (3.34)	8.20 (10.20)	0.11

Table 6-4. Markers of insulin resistance in PET, IUGR and control pregnancies.

Statistical analysis was performed using Student's t-test, and data is presented as mean and S.D. Log transformed data was used for maternal SHBG, glucose and insulin and is presented as mean (S.D.) and [geometric mean (S.D.)].

6.2.3.3 Maternal EMFA profile in PET, IUGR and uncomplicated pregnancies.

For observational purposes, all erythrocyte membrane fatty acids tested in the 3rd trimester are demonstrated in table 6-5.

Results demonstrate that 12:0, 18:1n6, 18:1n7 and 20:3n9 fatty acids are negligible, as in the longitudinal study cohort. However, in this arm of the study, in PET, IUGR and controls, 14:1n7, 22:1n9 and 22:2n6 fatty acids were also negligible.

Of interest, there was a trend towards increased % 14:0 in PET compared with controls [PET 1.13 (0.33) vs controls 0.94 (0.33)%, p=0.05] (figure 6-12), and also in % 16:0 [29.73 (3.58) vs 27.37 (4.63)%, p=0.05] (figure 6-13) and 18:1n9 [16.73 (2.55) vs 15.23 (2.41)%, p=0.05] (figure 6-14).

Fatty acids (% total)	PET (n=23)	PET Controls (n=23)	p	IUGR (n=17)	IUGR Controls (n=17)	p
12:0	0	0	*	0	0	*
14:0	1.13 (0.33)	0.94 (0.33)	0.05	0.96 (0.40)	1.04 (0.32)	0.89
14:1n7	0	0	*	0	0	*
16:0	29.73 (3.58)	27.37 (4.63)	0.05	26.57 (3.40)	28.02 (3.68)	0.25
16:1n7	1.31 (0.55)	1.04 (0.42)	0.07	1.15 (0.55)	1.03 (0.46)	0.48
17:0	0.43 (0.28)	0.48 (0.22)	0.80	0.49 (0.22)	0.48 (0.22)	0.30
17:1n7	0.07 (0.15)	0.10 (0.17)	0.59	0.08 (0.16)	0.10 (0.16)	0.74
18:0	19.33 (4.00)	20.61 (4.08)	0.28	22.01 (8.07)	20.71 (3.44)	0.73
18:1n6	0	0	*	0	0	*
18:1n9	16.73 (2.55)	15.23 (2.41)	0.05	14.65 (2.67)	15.31 (2.16)	0.39
18:1n7	0	0	*	0	0	*
18:2n6	7.00 (1.33)	6.79 (1.78)	0.65	6.38 (1.55)	6.97 (2.04)	0.35
18:3n6	0.11 (0.16)	0.10 (0.15)	0.85	0.10 (0.15)	0.16 (0.17)	0.25
18:3n3	0.26 (0.23)	0.31 (0.34)	0.54	0.25 (0.19)	0.35 (0.18)	0.13
20:0	0.77 (0.22)	0.84 (0.31)	0.67	0.96 (0.46)	0.84 (0.29)	0.46
20:1n9	0.55 (0.23)	0.42 (0.29)	0.90	0.39 (0.26)	0.37 (0.26)	0.46
20:2n6	0.10 (0.19)	0.12 (0.20)	0.76	0.13 (0.21)	0.10 (0.16)	0.48
20:3n9	0	0	*	0	0	*
20:3n6	1.41 (1.10)	1.16 (0.54)	0.33	1.09 (0.62)	1.07 (0.40)	0.90
20:4n6	5.77 (2.85)	6.46 (3.81)	0.49	6.47 (3.11)	6.42 (3.63)	0.96
20:3n3	0.65 (0.47)	0.49 (0.30)	0.79	0.46 (0.33)	0.50 (0.28)	0.80
20:5n3	1.65 (0.71)	1.82 (0.60)	0.85	1.56 (0.94)	1.70 (0.53)	0.37
22:0	0.09 (0.23)	0.24 (0.57)	0.27	0.31 (0.98)	0.07 (0.14)	0.36
22:1n9	0	0	*	0	0	*
22:2n6	0	0	*	0	0	*
22:4n6	0.93 (0.73)	1.37 (0.98)	0.10	1.25 (0.79)	1.22 (0.88)	0.92
22:3n3	0.11 (0.16)	0.14 (0.19)	0.79	0.13 (0.19)	0.12 (0.15)	0.45
22:5n6	0.10 (0.17)	0.13 (0.21)	0.27	0.14 (0.18)	0.19 (0.21)	0.60
22:5n3	0.53 (0.50)	0.81 (0.67)	0.12	0.78 (0.63)	0.79 (0.69)	0.94
24:0	4.45 (1.40)	5.35 (2.14)	0.14	5.97 (2.72)	4.94 (1.59)	0.26
22:6n3	1.53 (1.05)	1.98 (1.30)	0.34	1.96 (1.66)	2.03 (1.41)	0.96
24:1n9	5.24 (1.12)	5.70 (1.39)	0.22	5.75 (2.08)	5.48 (1.29)	0.63
% sat	58.04 (6.26)	57.86 (7.76)	0.93	59.20 (9.61)	58.16 (7.27)	0.73
% mono	22.83 (2.73)	21.49 (2.71)	0.11	21.07 (3.63)	21.28 (2.67)	0.73
% poly	19.13 (6.06)	20.63 (7.94)	0.47	19.73 (8.01)	20.54 (7.66)	0.77
Total n9 FAs	21.30 (2.73)	20.21 (2.99)	0.20	19.69 (3.80)	20.02 (2.80)	0.65
Total n7 FAs	1.53 (0.63)	1.28 (0.47)	0.36	1.38 (0.61)	1.26 (0.56)	0.55
Total n6 FAs	14.85 (4.83)	15.59 (6.57)	0.67	15.06 (5.70)	15.56 (6.57)	0.81
Total n3 FAs	4.28 (1.40)	5.04 (1.69)	0.11	4.97 (2.59)	4.67 (1.50)	0.66
% unsat	41.96 (6.26)	42.14 (7.76)	0.93	40.80 (9.61)	41.84 (7.27)	0.73
UI	87.75 (23.54)	94.93 (30.56)	0.38	93.30 (33.57)	91.82 (28.46)	0.89
Average CL	18.02 (0.23)	18.21 (0.30)	0.02	18.24 (0.28)	18.14 (0.25)	0.27
C20-22	16.38 (4.23)	18.81 (6.46)	0.15	19.01 (5.58)	17.91 (5.25)	0.56
Log n6/n3	3.72 (1.47)	3.15 (0.96)	0.07	3.44 (1.58)	3.13 (0.90)	0.52
Delta 5	5.29 (2.94)	5.66 (2.31)	0.65	6.21 (2.37)	6.14 (2.56)	0.94
Delta 6	0.19 (0.17)	0.16 (0.07)	0.31	0.15 (0.08)	0.15 (0.08)	0.27
Delta 9	0.92 (0.25)	0.78 (0.23)	0.10	0.74 (0.24)	0.77 (0.18)	0.51
Elongase	0.59 (0.14)	0.69 (0.14)	0.03	0.76 (0.33)	0.67 (0.09)	0.29

Table 6-5. All fatty acids tested (%) in PET, IUGR and control pregnancies.

Raw data is shown, and statistical analysis was performed on log transformed data for 12:0, 14:0, 16:0, 17:0, 17:1n7, 18:0, 18:1n9, 20:0, 20:1n9, 20:2n6, 20:3n3, 20:5n3, 22:2n6, 22:3n3, 22:5n6, 24:0, % monounsaturated FAs, total n9, total n6/n3, delta 6 and delta 9 desaturases. Statistical analysis was performed on square root of total n7. Statistical analysis was performed on data independent of maternal age, BMI, smoking status, parity, as described in section 6.2.1.1. Statistical analysis was performed using ANOVA, and data are presented as mean and S.D. FA = fatty acid, UI = unsaturation index, CL = chain length.

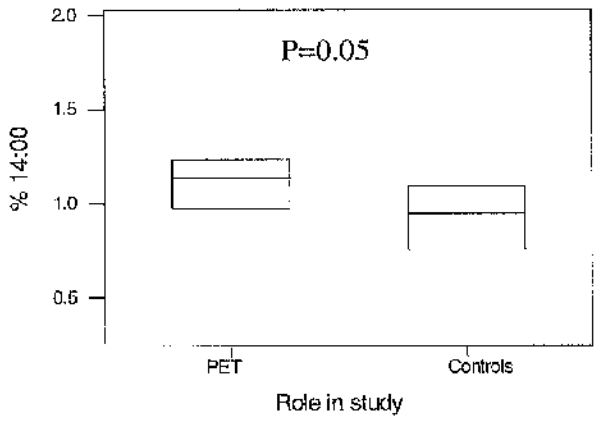


Figure 6-12. % 14:0 FA in PET (n=14) and control (n=14) pregnancies.

Raw data is shown, presented as mean and 95% CI, and statistical analysis was performed using Students t-test on log transformed data.

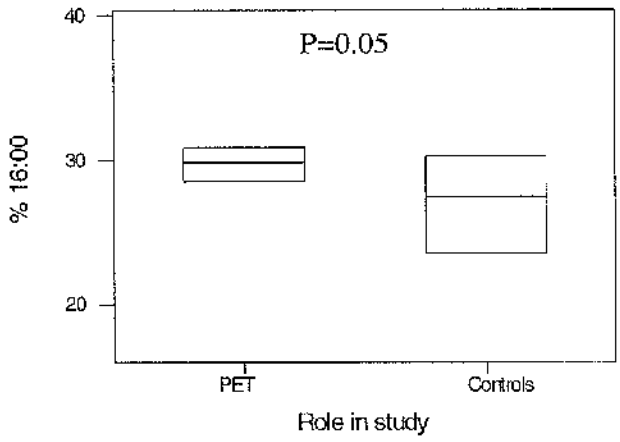


Figure 6-13. % 16:0 (palmitic acid) FA in PET (n=14) and control (n=14) pregnancies.

Raw data is shown, presented as mean and 95% CI, and statistical analysis was performed using Students t-test on log transformed data.

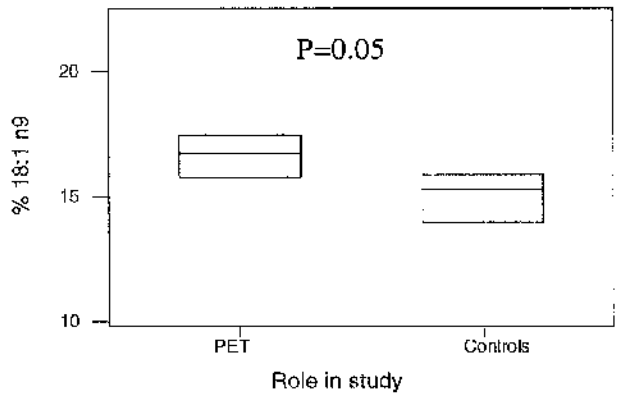


Figure 6-14. % 18:1n9 (oleic acid) FA in PET (n=14) and control (n=14) pregnancies. Raw data is shown, presented as mean and 95% CI, and statistical analysis was performed using Students t-test on log transformed data.

In the PET group, the average chain length was significantly reduced compared with controls [PET 18.02 (0.23) vs controls 18.21 (0.30)%, p=0.02] (figure 6-15). Similarly, elongase activity was significantly reduced in PET compared with controls [PET 0.59 (0.14) vs controls 0.69 (0.14)%, p=0.03] (figure 6-16).

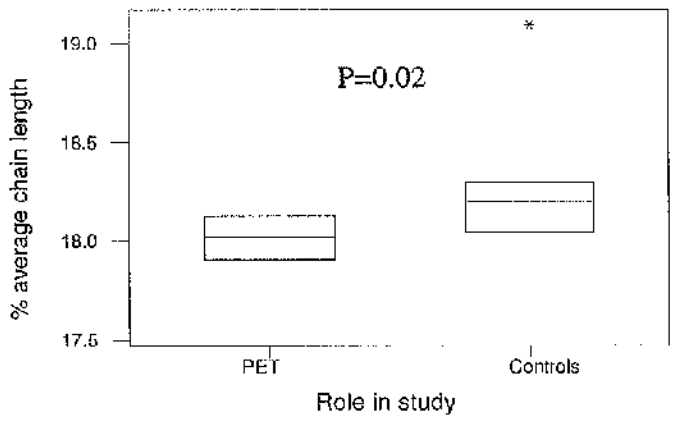


Figure 6-15. % average chain length (CL) in PET (n=14) and control (n=14) pregnancies.

Raw data is shown, presented as mean and 95% CI, and statistical analysis was performed using Students t-test. * = outlier.

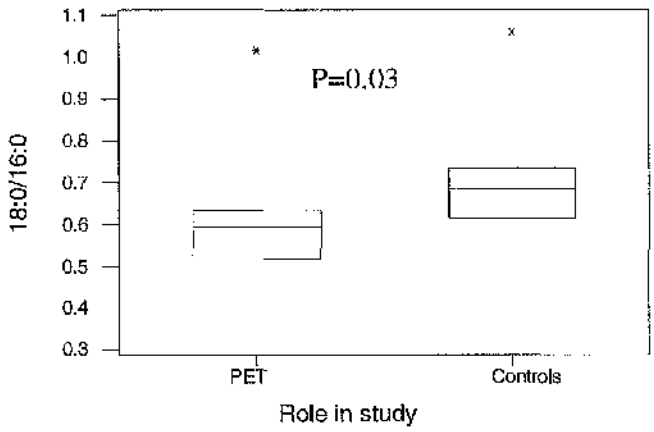


Figure 6-16. Elongase activity (18:0/16:0) in PET (n=14) and control (n=14) pregnancies. Raw data is shown, presented as mean and 95% CI, and statistical analysis was performed using Students t-test. * = outlier.

There were no differences in any of the fatty acids or indices tested, between IUGR pregnancies and controls.

6.2.3.4 Associations between markers of insulin resistance, inflammation and dyslipidaemia and EMFA profiles PET, IUGR and control subjects.

The fatty acids studied in this section were those that were altered in the PET group (14:0, 16:0, 18:1n9, average chain length and elongase activity), and those that demonstrated associations with plasma markers in the 3rd trimester longitudinal study group (22:6n3, % monounsaturated FAs, delta 9 desaturase activity and elongase activity).

In the PET group, maternal TC and LDL were negatively associated with elongase activity (r=-0.46, p=0.03 and r=-0.50, p=0.02 respectively) (figure 6-17). No other associations were observed.

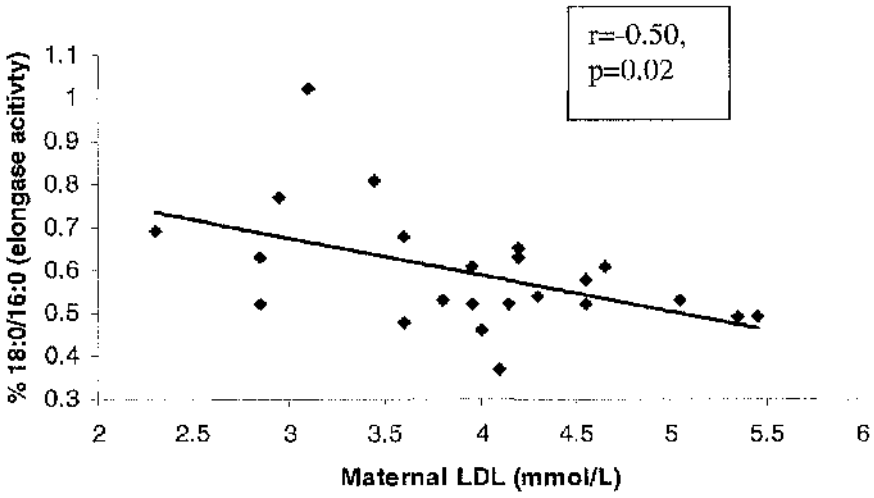


Figure 6-17. Association between maternal LDL and elongase activity (18:0/16:0) in the PET group.

Statistical analysis was performed using Pearson's coefficient of correlation.

In IUGR pregnancies, there were no correlations between any of the markers of insulin resistance, inflammation or dyslipidaemia and the fatty acids tested.

Similarly, in the combined control group (n=40), there were no correlations between any of the markers of insulin resistance, inflammation or dyslipidaemia and the fatty acids tested.

6.3 Discussion

This chapter has produced descriptive evidence of the EMFA profile as gestation advances in a large cohort of uncomplicated pregnancies, independent of potential confounders such as maternal BMI, smoking status, parity and gestation at sampling. Data in this study suggest fasting markers of insulin resistance (glucose, insulin and HOMA) are increased as gestation advances, and that the acute inflammatory marker of inflammation, IL-6, is elevated with advancing gestation. Increases in TC, TG, VLDL and LDL are also demonstrated with advancing gestation. These data support the recognised dyslipidaemia, state of inflammation and insulin resistance seen in uncomplicated pregnancy (see

introduction). In this study, SHBG levels were increased from the 1st to the 2nd trimester, in agreement with previous data (Kerlan *et al* 1994, O'Leary *et al* 1991, Wolf *et al* 2002).

It has been demonstrated in this study that the percentage of log 18:0 was reduced as pregnancy advanced. Because the fetus requires the maternal transfer of LCPUFAs, this depletion in the saturated FA 18:0 may be secondary to its utilisation in order to produce more of these LCPUFAs for optimal fetal development. An increase in the amount of α-linolenic acid (18:3n3) from the 1st to the 2nd trimester was also demonstrated. This n3 FA is important for fetal growth and greater levels in mid-trimester are likely to be secondary to improved dietary intake in this study group, which consequently allows increased conversion to DHA (22:6n3) for placental transfer to the fetus. In this study, an increase in the production of DHA was observed from the 1st to the 2nd trimester, with a platcau in production thereafter. This data supports the mid-trimester peak in production noted by Montgomery and colleagues (Montgomery *et al* 2003) in a prospective study of maternal blood samples taken at 15 and 28 weeks and at delivery. The early increase in maternal DHA is likely to indicate mobilisation of maternal stores to facilitate preferential transfer to and accumulation by the fetus (Al *et al* 1995). However, this data does not support previous findings, suggesting that production actually decreases thereafter until term (Al *et al* 1995, Montgomery *et al* 2003). However, the mean gestation at sampling in the 3rd trimester in this study was 35.3 weeks (see table 6-1), and it is possible that placental transfer to the fetus is not maximal until later in the 3rd trimester, when a decline would then be noted in DHA. Different methods of correction for BMI may account for this difference in pattern of maternal DHA in the 3rd trimester; Montgomery and colleagues (Montgomery *et al* 2003) used subjects 'similar in anthropometry' but did not adjust for BMI, as was carried out in the present study. Similarly, in the study by Al *et al*, subjects were not matched for BMI. At very least, the 'plateau' demonstrated in DHA in the 3rd trimester in this study indicates that maternal levels cannot be sustained and that fetal accretion occurs at the expense of maternal stores of DHA. Although production of AA (20:4n6) was not significantly altered as gestation advanced, a trend towards a reduction in the 2nd trimester compared to the 1st trimester was noted [T1 13.67 (2.52) vs T2 12.72 (2.22), p=0.06]. This data supports the previous findings of Al and colleagues (Al *et al* 1995) who demonstrated relative reductions in AA during pregnancy, and indicates placental transfer to the fetus.

The total percentage of all saturated FAs, unsaturated FAs, monounsaturated FAs and PUFAs were unchanged during uncomplicated pregnancy. This overall balance in EMFA status as pregnancy advances may indicate that production of precursor FAs is altered to

counteract any reductions in FAs that are secondary to the placental transfer to the fetus, thereby keeping the overall state relatively stable. This may also indicate that, in our study group, maternal intake of essential FAs is adequate to prevent observed declines in PUFAs. In this West of Scotland population, an increase in total n3 FAs was observed between the 1st and 2nd trimesters, which may be interpreted as a maternal response to the inflammatory state of advancing gestation, to provide the n3 beneficial functions within the vascular system, as an anti-inflammatory mediator and to provide the fetus with n3 FAs.

In this population, elongase activity was observed to decrease with advancing gestation, reflected in the reduction in 18:0 production from 16:0. A generalised reduction of elongase would result in reduced proportions of long chain and polyunsaturated fatty acids. This down-regulation of an enzyme involved in the addition of carbon atoms would result in a shorter-chain and more saturated fatty acid profile and may be considered to reflect the state of insulin resistance seen with advancing gestation. Delta 9 desaturase activity increased from the 1st to the 2nd trimester, which may be a response to the reduction observed in 18:0, via a maternal metabolic drive to produce more LCPUFAs by converting 18:0 to the more unsaturated 18:1n9. A reduction in delta 5 desaturase activity was demonstrated in the 2nd compared with the 1st trimester of pregnancy. This could be a consequence of reduced availability of the relatively unsaturated precursors of the delta 5 desaturase enzyme, secondary to the reduced elongase activity. Again, this may reflect the insulin resistant state of advancing gestation.

One of the limitations of this study is the uncertainty regarding the optimal time at which to compare erythrocyte membrane fatty acid composition with plasma markers of insulin resistance. In this study, plasma samples were compared with membrane composition in the same trimester, although it may be prudent to consider examining plasma values in one trimester with erythrocyte membrane composition at the subsequent trimester (or vice versa), as there will inevitably be some delay in the influence of the plasma values on the membrane composition. For example, Wolf and colleagues (Wolf *et al* 2002) found a relationship between 1st trimester SHBG levels and the development of PET in the 3rd trimester. In this study, repeated tests were performed on the individual samples, and a p value of <0.005 was considered significant in the longitudinal study, to reduce the possibility of chance associations. However, it must be considered that these two limitations may have an influence on the reported associations. For example, in the 2nd trimester, an increase in the inflammatory marker CRP was related to a reduction in 18:0. This finding may be related to chance but could also be interpreted as a result of an increased production of longer chain more unsaturated fatty acids, which seems counter-

intuitive. This result may also simply indicate that in the 2nd trimester, 18:0 is a poor indicator of inflammation in the pregnant state. However, increasing TG levels were associated with a reduction in elongase activity. This is an interesting result as the recognised dyslipidaemia of pregnancy is evident by the 2nd trimester and this increase in TG levels is associated with a reduction in an enzyme that may be considered to be associated with increased insulin sensitivity. It may be considered also, that elongase is a reasonable marker of insulin resistance and dyslipidaemia in the 2nd trimester of pregnancy. Elongase was not correlated with HOMA levels in the 2nd trimester, which may highlight the problems associated with measures of insulin resistance in pregnancy, but which may also be related to the aforementioned uncertainty of timing of comparisons. One might speculate that a better comparison may be made between 2nd trimester HOMA levels and 3rd trimester elongase activity. A systematic examination of relationships between plasma markers and fatty acids between the trimesters is yet to be performed.

The positive association between maternal fasting insulin levels and the anti-inflammatory n3 FA DHA in the 3rd trimester is another counter-intuitive finding, as it is recognised that insulin resistance is associated with relative reductions in PUFAs including DHA in the non-pregnant state, as discussed in the introduction. From this data, it is possible that this relationship is not easily demonstrable in erythrocyte membranes in pregnancy, or that it is simply associated with small sample size. The lack of association between the FAs and inflammatory markers may suggest that FA status is not a good predictor of inflammation in the 3rd trimester of pregnancy. The correlations between TG and increasing proportions of monounsaturated fatty acids and a reduction in elongase activity further demonstrates the dyslipidaemia of pregnancy reflected in fatty acid status.

In the case control study of pregnancies complicated by PET or IUGR, an elevation of the relatively saturated fatty acids 14:0, 16:0 and 18:1n9 was demonstrated in PET, as proposed. This is likely to reflect the exaggerated state of insulin resistance seen in PET and it should be considered that these fatty acids might be useful as markers of insulin resistance in the 3rd trimester of pregnancy. It is interesting to note that, although not significant, 16:0 and 18:1n9 were increased from the 1st to the 2nd trimester in the longitudinal study. These findings further support the suggestion that these fatty acids might identify subjects at increased risk of the complications of exaggerated insulin resistance in pregnancy, in particular PET, and may suggest that the changes are already demonstrated in the maternal erythrocyte membranes by the 2nd trimester. The significant reduction in average fatty acid chain length seen in PET may indicate decreased production of the intermediate fatty acids from which the beneficial LCPUFAs are produced.

Therefore, average chain length may be considered to be a good marker for insulin resistance (Baur *et al* 1999). Similarly, the reduction in elongase activity demonstrated in PET suggests an accumulation of saturated fatty acids in the membranes of these subjects. Interestingly, elongase is significantly reduced with gestation in uncomplicated pregnancy, and thus its activity may be considered as a good marker for PET, related to insulin resistance. The erythrocyte lifespan is approximately 90 days, and therefore elongase could be considered to be a good research tool for PET risk, reflecting the insulin resistance status of the previous trimester.

As well as the previously discussed limitations, in this study, PET and IUGR subjects were not fasted at time of blood sampling and therefore HOMA was unreliable as a marker for insulin resistance. SHBG was used in this group as a marker of insulin resistance as values are know to be reliable in the non-fasting state (Key *et al* 1990). However, we were unable to demonstrate any significant changes in SHBG levels between either PET or IUGR pregnancies and their controls. Although an association between first trimester SHBG levels and PET independent of BMI has been described (Wolf *et al* 2002), and SHBG correlates inversely with insulin resistance in the non-pregnant state (Sherif *et al* 1998), it is possible that more accurately matching for BMI will have some influence on SHBG variation between PET and control pregnancies because of the effect of obesity on insulin sensitivity. However, it should also be considered that the samples were obtained between 35.8 and 39.6 weeks' gestation for the PET, IUGR and controls (table 4-6), and it has been observed that 37 weeks' gestation is a time when the physiological insulin resistance of normal pregnancy peaks and thus may reduce the difference in SHBG levels between normotensive and PET women. It is also important to note that studies of dietary fat and erythrocyte FA composition confirm that erythrocyte FA content may accurately reflect dietary fat intake for the preceding seven days (Romon *et al* 1995) or longer (von Schacky *et al* 1985). Therefore, differences in this study and in others may be reflected in the different diets consumed by the differing populations. It is also important to note that because data is expressed as % of total fatty acids in this and in other studies, an increase in one fatty acid will cause one or more of the others to decrease by default, which may make it difficult to interpret the driving source of such alterations.

In summary, this chapter has demonstrated the fatty acid profiles of pregnant women throughout gestation in our population and in pregnancies complicated by PET or IUGR. It has been possible to highlight elongase activity as a potential research marker for PET, via insulin resistance, demonstrable by the 2nd trimester in the non-fasting state. This marker may adequately reflect the fatty acid status of the preceding trimester. Further work should

be directed towards prospective observations of the fatty acids and enzyme activities, relating them to diet and the development of such disease states. Erythrocyte membrane fatty acid status is a controversial marker in the literature for state of insulin resistance, and it is known that adipose tissue obtained by needle biopsy is also an informative method for obtaining data on fatty acid profiles from humans (Cunnane *et al* 1999). Further work is underway to observe differences in FA status from different tissues within the same population, including SC adipose, umbilical cord and placenta.

7 The contribution of genetic variation at genes coding for inflammatory mediators to the risk of PET

7.1 Introduction

For many years, a genetic contribution to PET onset has been considered, and it is recognised that the condition is likely to develop through an interaction of maternal, fetal and environmental factors (multi-factorial inheritance). A family clustering effect is apparent in PET. In women who had been eclamptic, the rate of PET was found to be higher in sisters (37%), daughters (26%) and granddaughters (16%) than in daughters-in-law (6%) (Chesley and Cooper 1986). A maternal susceptibility locus for PET has been reported on chromosome 2 (Arngrimsson *et al* 1999, Moses *et al* 2000). Many studies have observed the contribution of variation at different genes to the risk of PET although with no clear conclusions (Arngrimsson *et al* 1993, Currie *et al* 2002, Lachmeijer *et al* 2001, Livingston *et al* 2001). The association between PET and risk of CVD may be secondary to maternal genetic factors which are not only linked to PET but also CVD (Irgens *et al* 2001). Primigravidity is a recognised risk factor for PET, although the mechanisms for this are not clear (Roberts and Cooper 2001). The protective effect of a previous pregnancy does not exclude a potential paternal genetic contribution to PET risk. As discussed in the introduction chapter, PET is characterised by defective placental implantation, and because the placenta is fetal in origin, it may be hypothesised that paternal genes are involved in placental development (Haig 1993). Polymorphism studies have highlighted both a maternally and paternally transmitted genetic predisposition to PET (Zusterzeel *et al* 1999, Zusterzeel *et al* 2002). Current literature would suggest that the causes of PET are heterogeneous, and there seems to be both a maternal and a paternal genetic predisposition to the condition, although the mode of inheritance is not known.

Because of the role of PPARγ in adipocyte differentiation, glucose and lipid homeostasis, insulin resistance and trophoblast differentiation, it is clearly a candidate gene for cause or association for the metabolic complications of pregnancy, especially pre-eclampsia (PET). The PPARγ gene is located on chromosome 3p25 (Beamer *et al* 1997), and has been cloned and characterised (Fajas *et al* 1997). Variations of the PPARγ gene may affect the function of PPARγ (Valve *et al* 1999). A number of genetic variants in the PPARγ gene have been identified. Although the tissue distributions vary for different PPARγ isoforms,

they are all expressed in adipose tissue and regulate adipocyte differentiation (Fajas *et al* 1997, Flier 1995, Tontonoz *et al* 1994, Wang *et al* 1999). PPARγ polymorphisms include the highly prevalent Pro12Ala polymorphism in PPARγ2, which is associated with reduced transcriptional activity of PPARγ *in vitro* and a 25% reduced risk for type 2 diabetes, probably mediated by increased insulin sensitivity in response to free fatty acids (Stefan *et al* 2001, Stumvoll and Haring 2002). The Pro12Ala mutation is the result of a CCA-to-GCA (Proline to Alanine) missense mutation in codon 12 of exon B of the PPARγ gene. This exon encodes the NH_2-terminal residue that defines the adipocyte-specific PPARγ2 isoform. The Pro/Ala genotype has also been associated positively (Beamer *et al* 1998, Cole *et al* 2000, Lindi *et al* 2001, Meirhaeghe *et al* 2000, Valve *et al* 1999) and negatively (Deeb *et al* 1998) with obesity measures, although some studies have reported no such associations (Evans *et al* 2000, Hara *et al* 2000, Mancini *et al* 1999, Mori *et al* 1998). Evans and colleagues (Evans *et al* 2000) observed that healthy male patients who were AA homozygotes had significantly higher serum leptin concentrations (p=0.001), but they concluded that variation in the PPARγ gene is unlikely to play a major role in the development of morbid obesity, despite the strong genetic component of risk of becoming obese (Chagnon *et al* 1997). Beamer *et al* found that the A allele of the P12A polymorphism was associated with a higher BMI in obese American Caucasians (Beamer *et al* 1998). Another group (Lindi *et al* 2001) has shown that the A allele is associated with a tendency to gain weight over time, in Finnish non-diabetic subjects in a 10-year follow-up study. It has been considered that inconsistencies in the literature regarding the association with obesity may be explained by the finding that the A allele was associated with lower BMI in non-obese subjects and higher BMI in obese subjects (Ek *et al* 1999). Because the natural ligands for the PPARγ receptor may include fatty acids, another possibility is gene: nutrient interaction; evidence has indicated that BMI is higher in A allele carriers than in P homozygotes when dietary polyunsaturated: saturated fat ratio is low (Luan *et al* 2001). It has been reported that the Pro12Ala substitution in PPARγ2 is associated with lower lipoprotein lipase activity in vivo, thus providing a new target for the analysis of coronary artery disease (Schneider *et al* 2002).

The silent C161T substitution at exon 6 of the PPARγ gene is associated with raised circulating leptin levels in obese individuals (Meirhaeghe *et al* 1998). The C161T base change has also been associated with a reduced risk for coronary artery disease, particularly evident among patients of CT heterozygosity (p = 0.0045) (Wang *et al* 1999). Since PPARγ agonists promote trophoblast differentiation and such cells increase leptin secretion, PPARγ may be an important signal for fuel supply to the fetus. A reduction in

PPARγ transcription and its target genes may be part of the molecular mechanism to accelerate maternal fat catabolism to meet fetal nutritional demands in late gestation, via elevations in fasting insulin levels (Krempler *et al* 2000). The observations that the C161T polymorphism of the PPARγ gene is associated with raised leptin levels in obese individuals (Meirhaeghe *et al* 1998) and that leptin and PPARγ mRNA levels correlate in adipose tissue of obese subjects (Krempler *et al* 2000) confirm that PPARγ is an important regulator of leptin production in non- pregnant individuals and that PPARγ mRNA expression is inversely associated with cardiovascular risk factors. The relationship between PPARγ activation and leptin secretion has not been studied in pregnancy.

The association of leptin with inflammation and fasting insulin levels has been discussed in Chapter 5. This data, along with the observations in the literature that leptin levels increase with gestation and are elevated further in PET independent of BMI (introduction, chapter 5), indicates that variation at the genes encoding for leptin may be considered as candidate genes for PET risk. The leptin gene is located on chromosome 7q31. The link between elevated serum leptin and obesity, a known risk factor for PET, has also been discussed previously. Recently a common polymorphism in the promoter region of the leptin gene (G2548A), which influences leptin expression and therefore also influences adipose secretion levels of the hormone has been described (Heo *et al* 2002, Hoffstedt *et al* 2002). Mutations in the leptin gene are associated with defective leptin production and severe obesity in both animal models (Zhang *et al* 1994) and humans (Montague *et al* 1997, Strobel *et al* 1998). The G2548A genetic variant in the 5' region of the leptin (LEP) gene is associated with a difference in BMI reduction following a low calorie diet in overweight women (Mammes *et al* 1998), and has also been associated with extreme obesity in women (Li *et al* 1999b). Raised leptin levels have also been associated with an increase in blood pressure in the animal model (Casto *et al* 1998, Dunbar *et al* 1997, Shek *et al* 1998), independent of obesity. The direct effect of leptin on blood pressure (Casto *et al* 1998, Dunbar *et al* 1997, Shek *et al* 1998) and on obesity makes it a potential candidate gene for hypertension and cardiovascular disease. The highly polymorphic tetranucleotide repeat polymorphism in the 3'-flanking region of the leptin gene (leptin 3'tet) has been shown to have a marginal effect on obesity (Shintani *et al* 1996) and to be associated with hypertension independent of obesity (Shintani *et al* 2002), with a higher frequency of I/I genotypes in hypertensive subjects. To date, these polymorphisms have not been studied in PET.

The inflammatory marker IL-10 may be reduced in the serum of PET subjects thereby contributing to the inflammation associated with the disease (Conrad *et al* 1998, Ellis *et al* 2001, Hennessy *et al* 1999) as discussed in Chapter 5, making this another potential candidate gene for PET risk. The IL-10 gene is located on chromosome 1q31-q32. The promoter regions of the IL-10 gene contains three biallelic polymorphisms at positions –1082 (A/G), -819 (T/C) and –592 (A/C) base pairs from the transcription start site, which produces three different haplotypes, GCC, ACC and ATA (Crawley *et al* 1999, Edwards-Smith *et al* 1999, Turner *et al* 1997b). These mutations are associated with a reduction in IL-10 protein in concanavalin A-stimulated peripheral blood mononuclear cells (Turner *et al* 1997b). The polymorphism at nucleotide position –1082 is thought to have an influence on IL-10 plasma levels (Lauten *et al* 2002). Patients homozygous for the IL-10 G allele have been shown to express high IL-10 plasma levels (Edwards-Smith *et al* 1999). IL-10 genotype has been associated with liver, heart and renal transplant rejection (Bathgate *et al* 2000, Middleton *et al* 1998, Sankaran *et al* 1999, Turner *et al* 1997a) and IL-10 gene promoter polymorphisms have been implicated in evaluating the severity of several inflammatory diseases (Crawley *et al* 1999, Helminen *et al* 1999, Mozzato-Chamay *et al* 2000).

The aims of this study were;

- To study variation at genes encoding for molecules involved in the metabolic pathways of inflammation and insulin resistance i.e. IL-10 (A-1082G, C-819T, C-592A), leptin (G-2548A, and 3' tetranucleotide repeat) and PPARγ (C161T and Pro12Ala), which may confer susceptibility to development of PET. A case (n=130) control (n=260) study was performed, and cases were matched for age, parity and BMI.

- To determine whether any of the polymorphisms that affect PET risk in our population are associated with maternal plasma levels of markers of inflammation in PET or controls, in baseline 1st trimester bloods (n=34) and in 3rd trimester bloods (n=56).

- To determine whether the polymorphisms affect any short-term pregnancy-induced changes in the plasma inflammatory markers (1st to 3rd trimester change) in PET and controls (n=34 per group).

- To determine whether the polymorphisms, which affect PET risk in our population affect any of the maternal plasma inflammatory markers 20 years after the index pregnancy in PET or controls (n=36 per group).

7.2 Results

7.2.1 Genetic predictors of PET

As discussed in the methods chapter (section 2.1.4), PET and control samples were collected from the same West of Scotland population attending the Princess Royal Maternity Hospital (formally Glasgow Royal Maternity Hospital) as described in sections 2.1.1 and 2.1.2, and samples were supplemented from an archival collection, described in detail in Hypertension (Freeman *et al* 2004). The different sample numbers in each arm of this study is due to the combination of different study groups. No data regarding booking blood pressure is demonstrated in the combined group (table 7-1), as there was insufficient data available due to the combination of the different study groups.

Maternal baseline characteristics are reported in table 7-1. Cases and controls were matched for age and parity. PET cases had higher BMI and fewer smokers than controls, and offspring from PET pregnancies had significantly lower birth weight centiles compared to controls.

Characteristic	PET (n=130)	Controls (n=260)	P
Age (yrs)	26.6 (5.9)	26.6 (5.8)	0.92
BMI (kg/m^2)	25.3 (4.5)	23.9 (4.2)	0.01
Primigravidae n (%)	107 (82.3%)	213 (81.9%)	0.93
Smokers n (%)	25 (19.2%)	88 (33.8%)	0.003
Birth weight centile	30.7 (27.8)	50.7 (29.9)	<0.001

Table 7-1. Subject characteristics in PET and controls.

Statistical analysis was performed using Students *t*-test for continuous data and chi-square test for categorical data. Data is presented as mean and S.D.

The allele frequencies for IL-10 A-1082G, IL-10 C-189T, IL-10 C-592A, Leptin G-2548A, Leptin 3'tet, PPARγ C161T and PPARγ Pro12Ala polymorphisms within the control group, are shown in table 7-2.

Polymorphism	Controls (n=260)
IL-10 A-1082G	Common (G) 0.56
	Rare (A) 0.44
IL-10 C-189T	Common (C) 0.77
	Rare (T) 0.23
IL-10 C-592A	Common (C) 0.78
	Rare (A) 0.22
Leptin G-2548A	Common (G) 0.52
	Rare (A) 0.48
Leptin 3'tet	Common (II) 0.58
	Rare (I) 0.42
PPARγ C161T	Common (C) 0.87
	Rare (T) 0.13
PPARγ Pro12Ala	Common (P) 0.89
	Rare (A) 0.11

Table 7-2. Allele frequencies of each polymorphism in the control group.

Genotype frequencies in the total group, cases and controls were in Hardy-Weinberg equilibrium for each of the polymorphisms and are shown in table 7-3. Examples of each polymorphism PCR gel are shown in figure .

Frequency	Number (%)		Univariate Analysis		
	Case	Control	Odds Ratio	Confidence Interval	P-value
IL-10 A-1082G					
GG	43 (34)	87 (34)	1.00	referent value	
AG	55 (43)	110 (43)	1.01	0.62 – 1.65	0.96
AA	29 (23)	57 (22)	1.03	0.58 – 1.83	0.92
IL-10 C-819T					
CC	81 (64)	157 (61)	1.00	referent value	
CT	39 (31)	84 (33)	0.90	0.57 – 1.43	0.66
TT	6 (5)	17 (7)	0.68	0.29 – 1.80	0.44
IL-10 C-592A					
CC	83 (65)	161 (62)	1.00	referent value	
CA	40 (31)	83 (32)	0.93	0.59 – 1.48	0.77
AA	5 (4)	14 (5)	0.69	0.24 – 1.99	0.49
Leptin G-2548A					
GG	38 (30)	71 (28)	1.00	referent value	
AG	61 (48)	122 (48)	0.93	0.57 – 1.54	0.79
AA	28 (22)	63 (25)	0.83	0.46 – 1.50	0.54
Leptin 3'tet					
II/II	27 (21)	93 (36)	1.00	referent value	
I/II	73 (57)	113 (44)	2.23	1.32 – 3.74	0.002
I/I	28 (22)	53 (21)	1.82	0.97 – 3.41	0.06
PPARγ C161T					
CC	89 (71)	194 (75)	1.00	referent value	
CT	34 (27)	62 (24)	1.20	0.73 – 1.95	0.47
TT	3 (2.4)	2 (0.8)	3.27	0.54 – 19.9	0.15*
PPARγ Pro12Ala					
PP	98 (78)	203 (78)	1.00	referent value	
AP	23 (18)	57 (22)	0.84	0.49 – 1.44	0.88
AA	4 (3.2)	0 (0)	N/A	N/A	0.012*

Table 7-3. Genotype frequencies and univariate analysis of IL-10, leptin and PPARγ polymorphisms in cases and controls.

Statistical analysis was performed using a chi-squared test apart from * Fisher's Exact test.

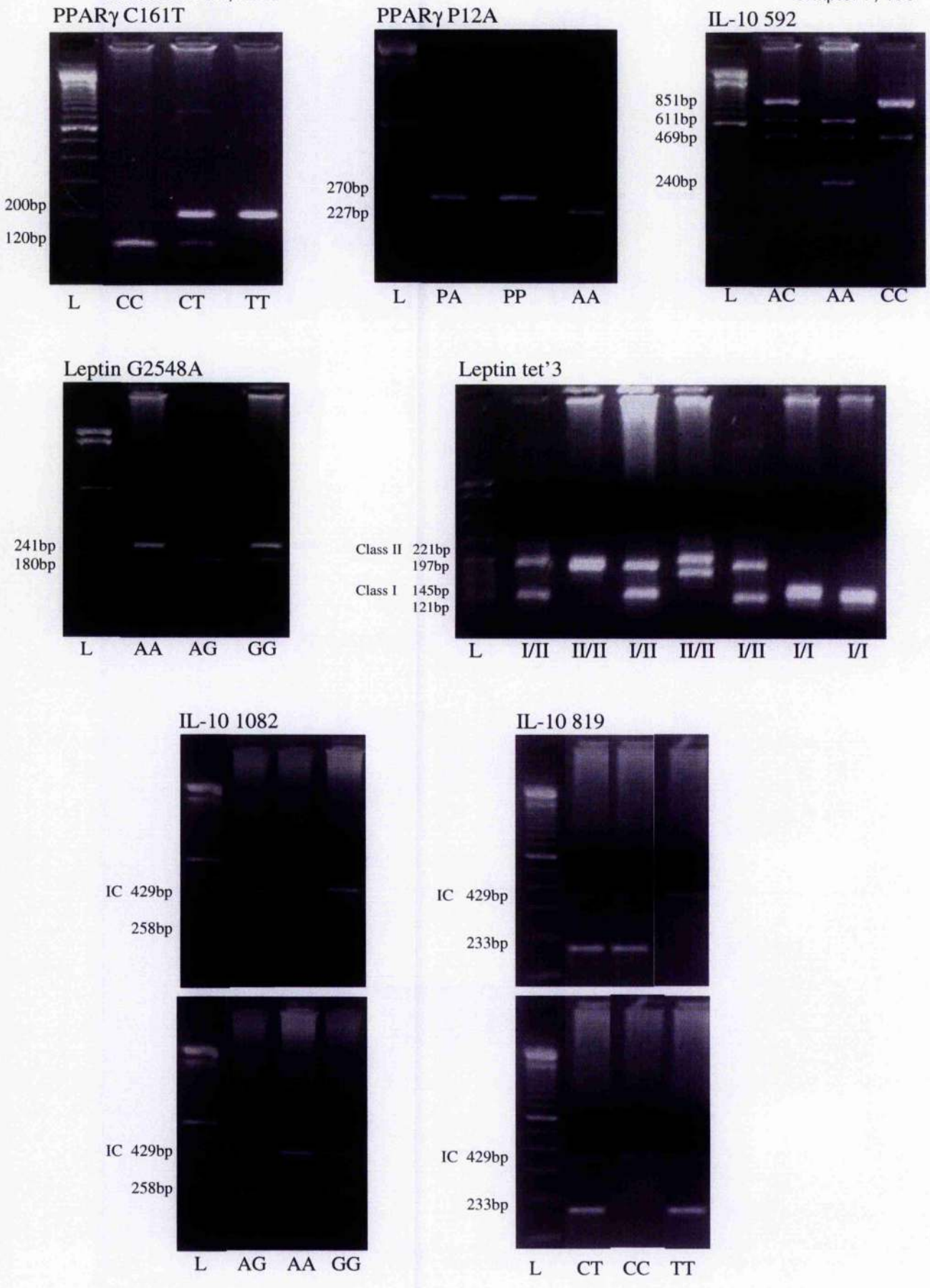


Figure 7-1. Examples of each genotyping PCR gel. IC = internal control for ARMS PCR.

Using univariate analysis, the leptin 3' tetranucleotide repeat (leptin 3'tet) and the PPARγ Pro12Ala were found to be significantly associated with the development of PET (table 7-3). Carriers of the small repeat size of the leptin 3'tet polymorphism were 2.2 times more likely to develop PET (95% confidence interval 1.32 – 3.74, p=0.002). Homozygotes for the A allele of the PPARγ Pro12Ala polymorphism were at increased risk of developing PET (p=0.012) with all of the AA homozygotes being in the PET group.

7.2.2 Baseline maternal plasma inflammatory markers in relation to the PPARγ Pro12Ala and Leptin 3'tet polymorphisms in PET and controls

7.2.2.1 Subject characteristics

First trimester inflammatory markers were available in a subset of 34 individuals per group from the larger cohort in 7.2.1 (Table 7-1), and baseline characteristics are shown in table 7-4.

Characteristic	PET (n=34)	Control (n=34)	p
Age (years)	27.7 (4.47)	28.2 (4.35)	0.66
BMI (kg/m^2)	26.0 (4.33)	24.3 (4.59)	0.15
Booking BP (systolic, mmHg)	113 (10)	110 (12)	0.36
Booking BP (diastolic, mmHg)	69 (9)	66 (10)	0.11
Gestation at sampling (weeks)	10.7 (2.2)	10.4 (2.3)	0.57
Smokers n (%)	7 (21%)	16 (47%)	0.03
Primigravidae n (%)	23 (62%)	24 (71%)	0.79
Birth weight centile	34.7 (28.0)	51.2 (33.5)	0.03

Table 7-4. Subject characteristics in PET and controls in 1st trimester study.

Statistical analysis was performed using Students *t*-test for continuous data and chi-square test for categorical data. Data is presented as mean and S.D. BP = blood pressure.

There were no differences in age, booking BMI, gestation at sampling, booking systolic or diastolic blood pressure or parity. As expected, the PET group had significantly fewer smokers (p=0.03) and offspring had significantly lower birth weight centiles at delivery (p=0.03). Subject characteristics were similar to those in the larger cohort (table 7-1).

7.2.2.2 Pro12Ala PPARγ polymorphism and 1st trimester maternal plasma inflammatory markers.

Because the Ala homozygotes were uncommon, all analyses were conducted comparing Pro homozygotes with Ala allele carriers.

There were no significant differences in first trimester maternal plasma levels of ICAM, VCAM, IL-6, IL-10, TNFα or CRP in common homozygotes (PP) or rare-allele carriers (PA/AA) in either PET or control groups (tables 7-5 and 7-6).

PET group	PA/AA (n=6)	PP (n=26)	P
ICAM (ng/mL)	200.3 (28.0)	197.8 (94.2)	0.91
VCAM (ng/mL)	310.0 (82.1)	284.6 (62.8)	0.50
IL-6 (pg/mL)	1.35 (0.35)	1.69 (2.32)	0.92
TNFα (pg/mL)	1.47 (1.15)	1.41 (1.05)	0.70
IL-10 (pg/mL)	1.28 (1.03)	1.88 (1.74)	0.63
CRP (mg/L)	4.56 (3.30)	4.00 (3.85)	0.15

Table 7-5. Baseline maternal plasma inflammatory markers in common homozygotes and rare-allele carriers of the Pro12Ala PPARγ polymorphism, in PET.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP, and data are presented as mean and SD.

Control group	PA/AA (n=12)	PP (n=21)	P
ICAM (ng/mL)	212.8 (73.8)	180.0 (51.1)	0.19
VCAM (ng/mL)	310.8 (78.7)	283.1 (51.4)	0.29
IL-6 (pg/mL)	1.06 (0.57)	1.31 (0.87)	0.31
TNFα (pg/mL)	1.34 (0.86)	2.53 (2.65)	0.60
IL-10 (pg/mL)	1.05 (1.29)	2.19 (1.74)	0.15
CRP (mg/L)	2.79 (2.58)	3.30 (2.69)	0.73

Table 7-6. Baseline maternal plasma inflammatory markers in common homozygotes and rare-allele carriers of the Pro12Ala PPARγ polymorphism, in controls.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP, and data are presented as mean and SD.

7.2.2.3 Leptin 3'tet polymorphism and 1st trimester maternal plasma inflammatory markers.

There were no significant differences in first trimester maternal plasma levels of ICAM, VCAM, IL-6, IL-10, TNFα or CRP in common homozygotes (II/II), heterozygotes (I/II) or rare homozygotes (I/I) in the PET group (table 7-7).

PET group	I/I (n=4)	I/II (n=22)	II/II (n=8)	p
ICAM (ng/mL)	284.00 (220.84)	192.09 (42.76)	168.25 (25.44)	0.06
VCAM (ng/mL)	268.75 (120.51)	296.59 (58.15)	271.88 (51.13)	0.55
IL-6 (pg/mL)	1.45 (0.55)	1.81 (2.52)	1.34 (0.47)	0.96
TNFα (pg/mL)	2.05 (2.12)	1.31 (1.13)	1.98 (0.77)	0.28
IL-10 (pg/mL)	2.60 (2.09)	1.60 (1.69)	1.94 (1.24)	0.46
CRP (mg/L)	4.35 (2.49)	4.16 (4.16)	4.06 (2.57)	0.64

Table 7-7. Baseline maternal plasma inflammatory markers in common homozygotes, heterozygotes and rare homozygotes of the Leptin 3'tet polymorphism, in PET.

Statistical analysis was performed using ANOVA, on log transformed data for IL-6, TNFα, IL-10 and CRP.

However, in the control group, first trimester levels of maternal plasma VCAM were significantly different between the groups (p=0.04) (table 7-8 and figure 7-1); maternal VCAM was significantly higher in I/I allele carriers compared with II/II allele carriers (p=0.05, *post hoc* students *t* test).

Control group	I/I (n=4)	I/II (n=20)	II/II (n=9)	P
ICAM (ng/mL)	163.00 (27.76)	195.00 (66.30)	198.00 (62.22)	0.61
VCAM (ng/mL)	366.25 (54.98)	285.50 (46.96)	277.78 (79.97)	0.04
IL-6 (pg/mL)	1.43 (0.85)	1.24 (0.87)	1.11 (0.59)	0.78
TNFα (pg/mL)	1.80 (1.58)	1.79 (2.03)	2.91 (2.86)	0.69
IL-10 (pg/mL)	1.87 (1.97)	1.66 (1.45)	2.09 (2.16)	0.90
CRP (mg/L)	3.52 (3.43)	3.57 (2.79)	1.94 (1.58)	0.70

Table 7-8. Baseline maternal plasma inflammatory markers in common homozygotes, heterozygotes and rare homozygotes of the Leptin 3'tet polymorphism, in controls.

Statistical analysis was performed using ANOVA, on log transformed data for IL-6, TNFα, IL-10 and CRP.

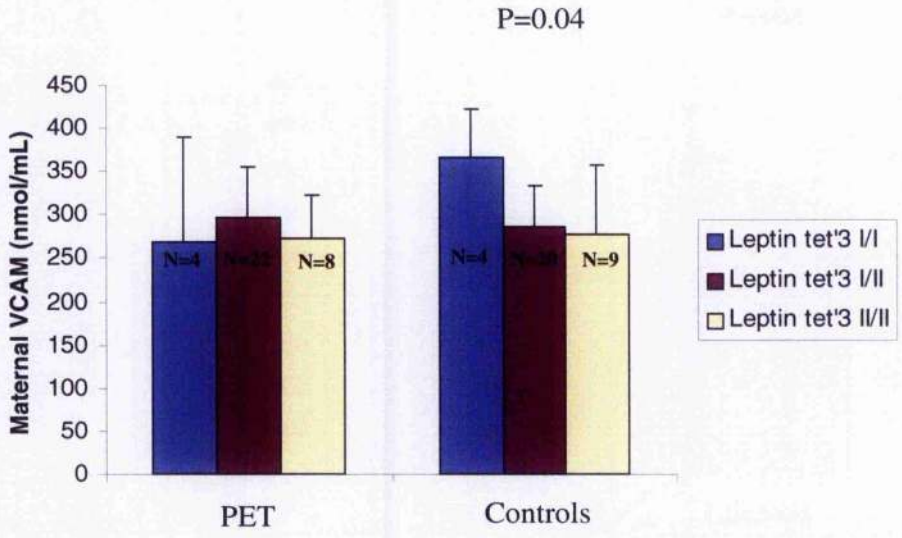


Figure 7-2. Maternal plasma VCAM levels in PET and control subjects, in common homozygotes, heterozygotes and rare homozygotes of the Leptin 3'tet polymorphism.

Statistical analysis was performed using ANOVA, on log transformed data for IL-6, TNFα, IL-10 and CRP.

7.2.3 Third trimester maternal plasma inflammatory markers in relation to the PPARγ Pro12Ala and Leptin 3'tet polymorphisms in PET and controls

7.2.3.1 Subject characteristics

Third trimester inflammatory markers were available in a subset of 56 individuals per group from the larger cohort in 7.2.1 (Table 7-1), and baseline characteristics are shown in table 7-9.

Characteristic	PET (n=56)	Control (n=56)	P
Age (years)	28.37 (5.40)	28.61 (5.300	0.81
BMI (kg/m^2)	26.78 (4.58)	25.48 (5.09)	0.17
Booking BP (systolic, mmHg)	133 (27)	118 (17)	0.001
Booking BP (diastolic, mmHg)	83 (18)	68 (11)	<0.001
Gestation at sampling (weeks)	34.40 (5.05)	32.18 (4.29)	0.01
Smokers n (%)	11 (20%)	22 (39%)	0.03*
Primigravidae n (%)	39 (70%)	41 (73%)	0.68*
Birth weight centile	29.6 (26.2)	51.6 (31.9)	<0.001

Table 7-9. Subject characteristics in PET and controls in 3rd trimester study.

Statistical analysis was performed using Students *t*-test for continuous data and chi-square test for categorical data*. Data is presented as mean and S.D.

There were no significant differences in maternal age, booking BMI, or parity. Gestation at sampling, which was performed immediately prior to delivery, was significantly later in the PET group (p=0.01). Booking systolic and diastolic blood pressure was significantly elevated in the PET group (p=0.001 and p<0.001 respectively). As expected, the PET group had significantly fewer smokers (p=0.03) and offspring had significantly lower birth weight centiles (p<0.001). Again, subject characteristics were similar to those in the larger cohort (table 7-1).

7.2.3.2 Pro12Ala PPARγ polymorphism and 3rd trimester maternal plasma inflammatory markers.

In the PET group, maternal plasma CRP levels were significantly higher among the rare allele carriers (PA/AA) than in the common homozygotes (p=0.04) (table 7-10 & figure 7-2). There were no other significant differences in plasma inflammatory markers in this group (table 7-10).

PET group	PA/AA (n=14)	PP (n=43)	P
ICAM (ng/mL)	198.9 (32.3)	199.9 (66.2)	0.94
VCAM (ng/mL)	343.0 (73.1)	315.9 (76.7)	0.25
IL-6 (pg/mL)	3.51 (3.30)	4.05 (4.76)	0.90
TNFα (pg/mL)	2.02 (0.68)	2.30 (2.18)	0.59
IL-10 (pg/mL)	4.22 (8.96)	2.59 (1.78)	0.86
CRP (mg/L)	7.27 (6.28)	6.41 (6.04)	0.04

Table 7-10. Third trimester maternal plasma inflammatory markers in common homozygotes and rare-allele carriers of the Pro12Ala PPARγ polymorphism, in PET.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP, and data are presented as mean and SD.

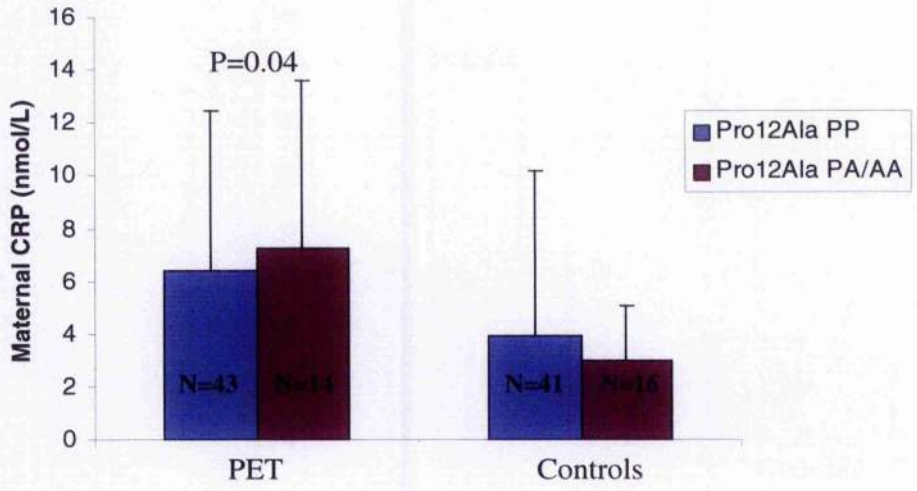


Figure 7-3. Maternal plasma CRP levels in PET and control subjects and controls, in common homozygotes and rare allele carriers of the PPARγ Pro12Ala polymorphism.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP.

In the control group, maternal plasma IL-10 levels were significantly reduced among the rare allele carriers (PA/AA) compared with the common homozygotes (p=0.02) (table 7-11 & figure 7-3). There were no other significant differences in plasma inflammatory markers in this group (table 7-11).

Control group	PA/AA (n=16)	PP (n=41)	P
ICAM (ng/mL)	209.1 (74.3)	173.8 (43.6)	0.09
VCAM (ng/mL)	331.2 (73.4)	310.6 (89.7)	0.39
IL-6 (pg/mL)	1.93 (0.97)	2.75 (3.56)	0.66
TNFα (pg/mL)	2.13 (1.46)	2.99 (2.98)	0.74
IL-10 (pg/mL)	1.53 (1.34)	2.50 (2.22)	0.02
CRP (mg/L)	2.99 (2.06)	3.90 (3.48)	0.49

Table 7-11. Third trimester maternal plasma inflammatory markers in common homozygotes and rare-allele carriers of the Pro12Ala PPARγ polymorphism, in controls.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP, and data are presented as mean and SD.

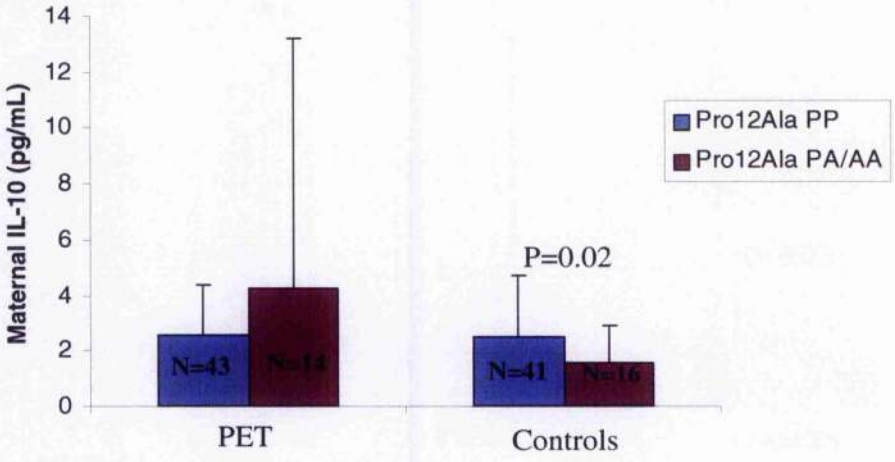


Figure 7-4. Maternal plasma IL-10 levels in PET and control subjects, in common homozygotes and rare allele carriers of the PPARγ Pro12Ala polymorphism.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP.

7.2.3.3 Leptin 3'tet polymorphism and 3rd trimester maternal plasma inflammatory markers.

In the PET group, there were no significant alterations in maternal plasma inflammatory markers in different allele carriers of the leptin 3'tet polymorphism (table 7-12).

PET group	I/I (n=13)	I/II (n=31)	II/II (n=12)	P
ICAM (ng/mL)	194.83 (51.81)	192.62 (49.59)	219.30 (84.09)	0.42
VCAM (ng/mL)	354.42 (67.32)	309.31 (75.51)	308.55 (83.62)	0.18
IL-6 (pg/mL)	3.03 (1.31)	4.56 (5.47)	3.01 (2.71)	0.48
TNFα (pg/mL)	2.53 (1.90)	2.05 (2.13)	2.70 (1.01)	0.12
IL-10 (pg/mL)	4.29 (9.26)	2.76 (1.92)	2.31 (1.81)	0.42
CRP (mg/L)	7.70 (6.46)	7.04 (6.37)	4.98 (4.93)	0.35

Table 7-12. Third trimester maternal plasma inflammatory markers in common homozygotes, heterozygotes and rare homozygotes of the Leptin 3'tet polymorphism, in PET.

Statistical analysis was performed using ANOVA, on log transformed data for IL-6, TNFα, IL-10 and CRP, and data are presented as mean and SD.

Similarly, in the control group, there were no significant alterations in maternal plasma inflammatory markers in different allele carriers of the leptin 3'tet polymorphism (table 7-13).

Control group	I/I (n=9)	I/II (n=29)	II/II (n=19)	P
ICAM (ng/mL)	177.35 (45.13)	182.30 (47.83)	190.93	0.83
VCAM (ng/mL)	309.57 (39.31)	314.66 (85.93)	323.61 (101.29)	0.92
IL-6 (pg/mL)	2.98 (2.48)	2.06 (1.54)	3.01 (4.72)	0.48
TNFα (pg/mL)	1.63 (0.77)	2.60 (2.55)	3.50 (3.20)	0.36
IL-10 (pg/mL)	1.70 (1.68)	2.46 (2.39)	2.12 (1.65)	0.26
CRP (mg/L)	3.71 (3.15)	3.75 (3.62)	3.45 (2.46)	0.83

Table 7-13. Third trimester maternal plasma inflammatory markers in common homozygotes, heterozygotes and rare homozygotes of the Leptin 3'tet polymorphism, in controls.

Statistical analysis was performed using ANOVA, on log transformed data for IL-6, TNFα, IL-10 and CRP, and data are presented as mean and SD.

7.2.4 First to third trimester changes in maternal plasma inflammatory markers in relation to the PPARγ Pro12Ala and Leptin 3'tet polymorphisms in PET and controls

7.2.4.1 Subject characteristics

First and third trimester samples were available for plasma inflammatory markers in 34 individuals, using the same subset as that used for the first trimester (baseline) study in section 7.2.2.1, and thus demographics are the same as those in section 7.2.2.1 and table 7-4.

The difference between 1st and 3rd trimester values of each of the inflammatory markers was calculated by subtraction of the 1st trimester sample from the 3rd trimester sample.

7.2.4.2 Pro12Ala PPARγ polymorphism and 1st to 3rd trimester changes in maternal plasma inflammatory markers.

In PET subjects, the change in IL-6 from the 1st to the 3rd trimester demonstrated a trend towards increased levels in the common homozygotes (PP) compared to the rare allele carriers (PA/AA) (p=0.05) (table 7-14 and figure 7-4).

PET group	PA/AA (n=6)	PP (n=28)	P
ICAM (ng/mL)	9.7 (17.6)	4.0 (75.5)	0.73
VCAM (ng/mL)	-23.3 (43.9)	9.2 (89.0)	0.21
IL-6 (pg/mL)	0.52 (0.66)	1.51 (2.10)	0.05
TNFα (pg/mL)	0.52 (0.46)	0.74 (1.51)	0.53
IL-10 (pg/mL)	0.43 (1.29)	0.85 (1.85)	0.53
CRP (mg/L)	0.83 (4.69)	1.63 (5.67)	0.73

Table 7-14. Maternal plasma inflammatory marker changes from the 1st to the 3rd trimester in common homozygotes and rare-allele carriers of the Pro12Ala PPARγ polymorphism, in PET.

Statistical analysis was performed using Students *t* test on raw data, and data are presented as mean and SD.

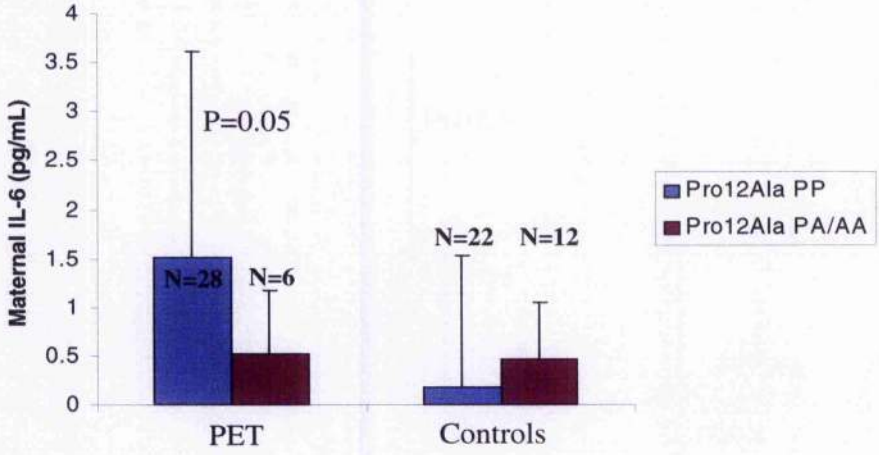


Figure 7-5. Maternal plasma IL-6 levels in PET and control subjects, in common homozygotes and rare allele carriers of the PPARγ Pro12Ala polymorphism.

Statistical analysis was performed using Students *t* test.

In the control group, there were no significant differences in 1st to 3rd trimester changes in the plasma inflammatory markers based on the PPARγ Pro12Ala polymorphism (table 7-15).

Control group	PA (n=12)	PP (n=22)	P
ICAM (ng/mL)	-14.5 (38.5)	2.2 (38.1)	0.24
VCAM (ng/mL)	-10.0 (71.8)	-24.8 (82.7)	0.60
IL-6 (pg/mL)	0.46 (0.59)	0.17 (1.36)	0.41
TNFα (pg/mL)	0.60 (1.28)	0.73 (1.86)	0.81
IL-10 (pg/mL)	0.69 (0.96)	0.71 (1.73)	0.97
CRP (mg/L)	0.15 (1.49)	0.33 (3.77)	0.85

Table 7-15. Maternal plasma inflammatory marker changes from the 1st to the 3rd trimester in common homozygotes and rare-allele carriers of the Pro12Ala PPARγ polymorphism, in controls.

Statistical analysis was performed using Students *t* test on raw data, and data are presented as mean and SD.

7.2.4.3 Leptin 3'tet polymorphism and 1st to 3rd trimester changes in maternal plasma inflammatory markers.

In PET subjects, changes in IL-10 levels from the 1st to the 3rd trimester differed significantly between the groups (p=0.02, ANOVA) (table 7-16); the difference was most notable between the I/I allele carriers [-1.38 (1.84) pg/ml] and the I/II carriers [1.15 (1.43) pg/ml, p=0.08, *post hoc* students *t* test].

PET group	I/I (n=4)	I/II (n=22)	II/II (n=8)	P
ICAM (ng/mL)	-54.50 (147.71)	6.00 (31.60)	27.75 (77.65)	0.13
VCAM (ng/mL)	16.25 (127.17)	-0.23 (87.13)	-0.63 (27.57)	0.93
IL-6 (pg/mL)	2.13 (1.78)	0.88 (1.14)	1.99 (3.23)	0.25
TNFα (pg/mL)	-0.03 (0.53)	0.76 (1.60)	0.79	0.77
IL-10 (pg/mL)	-1.38 (1.84)	1.15 (1.43)	0.81 (1.75)	0.02
CRP (mg/L)	3.63 (5.36)	1.55 (5.22)	1.09 (6.86)	0.75

Table 7-16. Maternal plasma inflammatory marker changes from the 1st to the 3rd trimester in common homozygotes, heterozygotes and rare homozygotes of the Leptin 3'tet polymorphism, in PET.

Statistical analysis was performed using ANOVA on raw data, and data are presented as mean and SD.

In the control group, there were no significant differences in 1st to 3rd trimester changes in the plasma inflammatory markers, based on the Leptin 3'tet polymorphism (table 7-17).

Control group	I/I (n=4)	I/II (n=20)	II/II (n=9)	P
ICAM (ng/mL)	0.50 (36.42)	0.10 (43.19)	-14.67 (28.90)	0.63
VCAM (ng/mL)	-73.75 (34.25)	-7.00 (76.80)	-22.78 (89.86)	0.30
IL-6 (pg/mL)	-0.20 (0.99)	0.38 (1.38)	0.18 (0.50)	0.71
TNFα (pg/mL)	-0.65 (1.54)	0.86 (1.80)	0.89 (1.14)	0.23
IL-10 (pg/mL)	0.93 (0.92)	0.69 (1.62)	0.66 (1.49)	0.96
CRP (mg/L)	-1.43 (1.82)	0.38 (3.80)	0.75 (1.18)	0.50

Table 7-17. Maternal plasma inflammatory marker changes from the 1st to the 3rd trimester in common homozygotes, heterozygotes and rare homozygotes of the Leptin 3'tet polymorphism, in controls.

Statistical analysis was performed using ANOVA on raw data, and data are presented as mean and SD.

7.2.5 Inflammatory markers 20 years after pregnancy in relation to the PPARγ Pro12Ala and Leptin 3'tet polymorphisms in PET and controls

7.2.5.1 Subject characteristics

Subject index pregnancy characteristics for the long-term study are shown in table 7-18. Cases and controls were selected to match, as a group, for current age and parity. All women at index pregnancy were primigravid, and no differences were observed in booking BP between cases and controls. PET women at index pregnancy had significantly higher BMI, lower birth weight centile, higher gestational age at sampling and lower gestational age at delivery. At recall, there were no significant differences in age, time since index pregnancy, BMI, parity or smoking status.

Characteristic		PET (n=36)	Controls (n=36)	P
Index pregnancy characteristics				
Index age (yrs)		24.86 (5.39)	24.86 (3.90)	1.00
Booking BMI (kg/m^2)		23.09 (3.92)	21.30 (1.68)	0.02
Booking BP (systolic)		124 (15)	119 (15)	0.20
Booking BP (diastolic)		81 (2)	76 (11)	0.08
Primigravidae n (%)		36 (100%)	36 (100%)	n/a
Gestation at sampling (weeks)		14.09 (4.24)	10.44 (4.91)	0.01
Smokers n (%)		9 (25%)	10 (27.7%)	0.79
Birth weight centile		36.0 (32.7)	58.6 (33.2)	0.009
Gestation at delivery (weeks)		35.44 (3.61)	39.11 (2.96)	<0.001
Recall characteristics				
Current age (years)		44.75 (6.00)	44.64 (3.22)	0.92
Time elapsed since index pregnancy (years)		19.89 (3.11)	19.66 (3.87)	0.78
Current BMI (kg/m^2)		27.00 (4.69)	26.00 (3.65)	0.32
Parity n (%)	1	9 (25%)	10 (28%)	0.79
	2	15 (42%)	16 (45%)	
	>2	10 (28%)	10 (28%)	
Smokers n (%)		8 (22%)	5 (14%)	0.36

Table 7-18. Subject characteristics for long-term study.

Statistical analysis was performed using Students *t*-test for continuous data and chi-square test for categorical data. Data is presented as mean and S.D.

7.2.5.2 Pro12Ala PPARγ polymorphism and maternal plasma inflammatory markers 20 years after pregnancy.

In the PET group, there were no significant differences in any of the maternal plasma inflammatory markers, based on PPARγ Pro12Ala genotype, 20 years remote from pregnancy (table 7-19).

PET group	PA/AA (n=5)	PP (n=29)	P
ICAM (ng/mL)	427.0 (235.0)	357.0 (133.0)	0.55
VCAM (ng/mL)	363.2 (85.4)	421.0 (152.0)	0.25
IL-6 (pg/mL)	3.18 (3.85)	2.62 (2.49)	1.00
TNFα (pg/mL)	0.88 (0.50)	1.26 (1.00)	0.32
IL-10 (pg/mL)	0.78 (0.43)	1.30 (1.40)	0.43
CRP (mg/L)	2.54 (4.28)	3.22 (4.51)	0.55

Table 7-19. Maternal plasma inflammatory markers in PET group by PPARγ Pro12Ala polymorphism, 20 years remote from index pregnancy.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP. Data are presented as mean and SD.

In the control group, maternal plasma ICAM levels were significantly increased in the common homozygotes (PP) compared with the rare allele carriers (PA/AA) (table 7-20 and figure 7-5). Otherwise, there were no significant differences in maternal plasma inflammatory markers, based on PPARγ Pro12Ala genotype, 20 years remote from pregnancy.

Control group	PA/AA (n=5)	PP (n=30)	P
ICAM (ng/mL)	217.2 (40.5)	282.0 (124.0)	0.04
VCAM (ng/mL)	387.7 (68.3)	337.6 (89.2)	0.20
IL-6 (pg/mL)	3.10 (2.32)	1.84 (1.02)	0.33
TNFα (pg/mL)	0.76 (0.20)	1.13 (0.78)	0.07
IL-10 (pg/mL)	1.10 (0.49)	2.93 (8.96)	0.49
CRP (mg/L)	1.95 (2.11)	1.64 (1.78)	0.90

Table 7-20 Maternal plasma inflammatory markers in controls, by PPARγ Pro12Ala polymorphism, 20 years remote from index pregnancy.

Statistical analysis was performed using Students *t* test, on log transformed data for IL-6, TNFα, IL-10 and CRP. Data are presented as mean and SD.

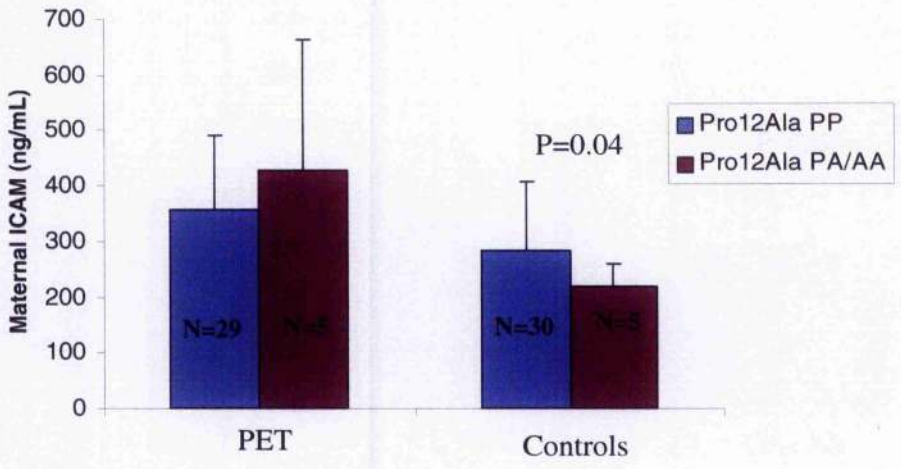


Figure 7-6. Maternal plasma ICAM levels in PET and controls, based on Pro12 Ala genotype, 20 years remote from pregnancy.

Statistical analysis was performed using Students *t* test, and data are presented as mean and SD.

7.2.5.3 Leptin 3'tet polymorphism and maternal plasma inflammatory markers 20 years after pregnancy.

In the PET group, maternal plasma VCAM levels differed significantly between the different genotypes (p=0.03, ANOVA) (table 7-21 & figure 7-6). The greatest difference was between the I/I genotype and the I/II genotype (p=0.11, *post hoc* Students *t* test). Maternal plasma TNFα levels were also significantly different between the groups (P=0.03, ANOVA) (table 7-21 & figure 7-7). In this case, TNFα levels were highest among the I/I genotype, and the greatest difference was noted between the I/I and the II/II genotype (p=0.06, post hoc Students *t* test).

PET group	I/I (n=7)	I/II (n=21)	II/II (n=6)	P
ICAM (ng/mL)	321.6 (116.9)	399.7 (123.2)	306.1 (241.9)	0.27
VCAM (ng/mL)	529.0 (221.8)	367.9 (92.7)	432.9 (128.8)	0.03
IL-6 (pg/mL)	2.94 (3.11)	2.76 (2.77)	2.22 (2.05)	0.81
TNFα (pg/mL)	2.04 (1.74)	1.06 (0.46)	0.73 (0.38)	0.03
IL-10 (pg/mL)	2.30 (2.51)	0.94 (0.54)	0.98 (0.73)	0.31
CRP (mg/L)	5.33 (7.08)	2.26 (3.15)	3.52 (4.35)	0.33

Table 7-21 Maternal plasma inflammatory markers in PET, by Leptin 3'tet polymorphism, 20 years remote from index pregnancy.

Statistical analysis was performed using ANOVA, on log transformed data for IL-6, TNFα, IL-10 and CRP. Data are presented as mean and SD.

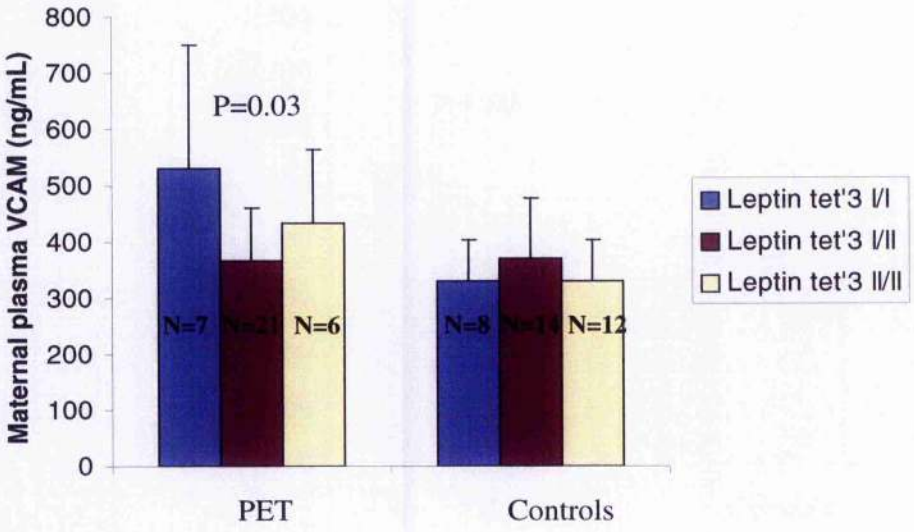


Figure 7-7. Maternal plasma VCAM in PET and controls, by Leptin 3'tet polymorphism, 20 years remote from index pregnancy.

Statistical analysis was performed using ANOVA and data are presented as mean and SD.

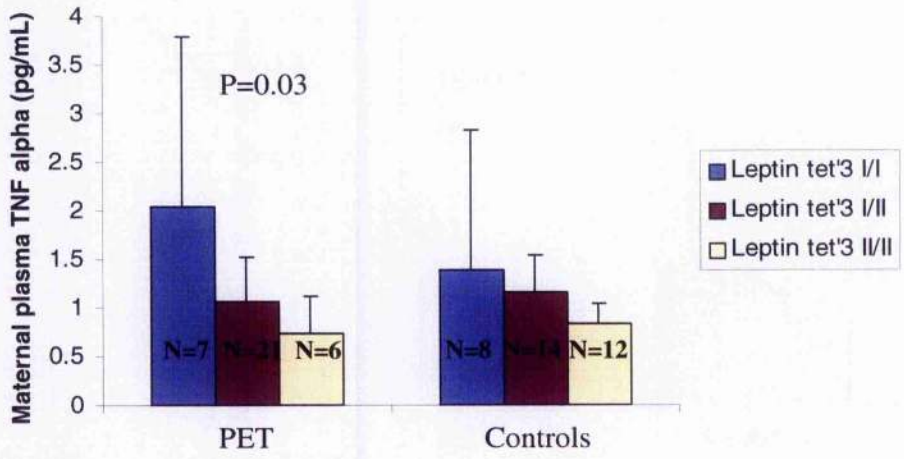


Figure 7-8. Maternal plasma TNFα in PET and controls, by Leptin 3'tet polymorphism, 20 years remote from index pregnancy.

Statistical analysis was performed using ANOVA and data are presented as mean and SD.

In the control group, there were no significant differences in maternal plasma inflammatory markers between genotypes, based on the Leptin 3'tet polymorphism, 20 years remote from pregnancy (table 7-22).

Control group	I/I (n=8)	I/II (n=14)	II/II (n=12)	P
ICAM (ng/mL)	227.0 (70.7)	307.2 (155.5)	267.7 (89.7)	0.32
VCAM (ng/mL)	328.88 (75.50)	368.93 (107.02)	329.43 (72.36)	0.45
IL-6 (pg/mL)	2.01 (1.77)	2.31 (1.44)	1.65 (0.78)	0.33
TNFα (pg/mL)	1.38 (1.44)	1.16 (0.38)	0.83 (0.20)	0.13
IL-10 (pg/mL)	1.23 (0.59)	1.40 (1.43)	5.17 (14.13)	0.86
CRP (mg/L)	1.06 (1.56)	1.58 (1.40)	2.35 (2.27)	0.21

Table 7-22 Maternal plasma inflammatory markers in controls, by Leptin 3'tet polymorphism, 20 years remote from index pregnancy.

Statistical analysis was performed using ANOVA, on log transformed data for IL-6, TNFα, IL-10 and CRP. Data are presented as mean and SD.

7.3 Discussion

The main findings of this study were an association between the leptin 3' tetranucleotide repeat, and the PPARγ Pro12Ala with the development of PET. Carriers of the small repeat size of the leptin 3'tet polymorphism (I/II) were 2.2 times more likely to develop PET (95% confidence interval 1.32-3.74, p=0.002). This data is in agreement with findings of a small study (n=40 PET, n=39 controls) produced within another population, which were published during the writing of this thesis (Muy-Rivera *et al* 2004). Homozygotes for the A allele of the PPARγ Pro12Ala polymorphism were at increased risk of developing PET (p=0.012), all AA homozygotes being in the PET group. There were no strong associations between the presence of the IL-10, PPARγ C161T or leptin G2548A polymorphisms and PET risk.

This study demonstrates an increased PET risk with the PPARγ Pro12 Ala mutation (Pro to Ala). The frequency of the AA genotype was markedly higher in patients with PET than in the normotensive pregnant subjects. It is possible that in pregnancy, the P12A polymorphism is associated with reduced transcriptional activity of PPARγ, in keeping with the *in vitro* data (Stumvoll and Haring 2002). However, this is not consistent with the observed reduced risk for Type 2 diabetes secondary to increased insulin sensitivity (Stefan *et al* 2001), independent of BMI. It has also been shown that the P12A genotype has no effect on 1st trimester plasma inflammatory markers although in the 3rd trimester, around the time of PET manifestation, there was an association between Ala 12 allele carriers and increasing levels of CRP in the PET group. The mechanisms by which the PPARγ Ala12

allele could contribute to PET risk are unknown. However, this association may suggest a link between A-allele carriers and inflammation in increasing risk of PET in our population. CRP is an acute phase reactant and thus it is possible that subjects with the P12A mutation demonstrate an exaggerated 3rd trimester acute inflammatory response to pregnancy, which manifests as PET. Based on this theory, in A-allele carriers, development of PET through the inflammatory effects of PPARγ rather than its insulin sensitising function may account for the increased PET risk despite reduced Type 2 diabetes risk as discussed previously. However, the AA genotype has been associated both positively (Beamer *et al* 1998, Cole *et al* 2000, Lindi *et al* 2001, Meirhaeghe *et al* 2000, Valve *et al* 1999) and negatively (Deeb *et al* 1998) with obesity measures, and since BMI is a recognised risk factor for PET, adiposity may also account for the associations. For example, the relationship between the A allele and elevated BMI in an American population (Beamer *et al* 1998) may account for the association between A allele carrier status and increased risk of PET in the present study, via increased adiposity or related to weight gain in pregnancy. The evidence that BMI is higher in A allele carriers than P homozygotes when the dietary polyunsaturated: saturated fat ratio is low (Luan *et al* 2001), is important in this West of Scotland population who are known to have a diet high in saturated fat. This dietary association may also account for increased PET risk related to A allele carrier status in our PET study group. In this study, it should be considered that results might be affected by BMI differences between groups. The effects of the P12A polymorphism on adipose tissue metabolism should be studied further in both lean and obese pregnant subjects because of this variation based on BMI. Results in this study show that the association between A-allele carriers and increased CRP levels in PET is no longer apparent 20 years remote from pregnancy, suggesting that this genetic influence may not be important in CVD risk and may not explain the association between PET and CVD risk.

In this study, the genetic association of a microsatellite polymorphism in the leptin gene (leptin 3'tet) with PET was studied. The frequency of the class I/class II genotype was markedly higher in patients with PET than in the normotensive pregnant subjects ($p=0.002$), and the I/I genotype demonstrated a trend towards increased frequency in the same group ($p=0.06$). Previous data suggests that there is an increased association of the I/I genotype with hypertension in the non-pregnant subject, which is independent of obesity (Shintani *et al* 2002). Support is given to this suggestion by a report on transgenic skinny mice, which described that chronic hyperleptinaemia lead to a significant elevation of BP without obesity (Aizawa-Abe *et al* 2000). In the present study, BMI differed between case and control groups. However, if the association between the I/I genotype and high blood

pressure is independent of adiposity in the non-pregnant state, then obesity may not account for the link between the leptin gene polymorphism (in particular, I-allele carrier status) and increased risk of PET. However, this leptin polymorphism may still confer increased susceptibility to obesity, which could account for the associations with PET risk. Other metabolic parameters, including inflammation, insulin resistance and hyperleptinaemia may be considered as links between the I/II allele carrier status and PET. To investigate the role of inflammation as a possible connection in the association of the leptin gene polymorphism with PET, maternal plasma markers of inflammation were studied in the 1st and 3rd trimesters. In PET, circulating levels of the anti-inflammatory marker IL-10 were reduced from the 1st to the 3rd trimester in subjects with the I/I genotype (p=0.02), whereas in the I/II and II/II carriers, IL-10 levels increased with advancing gestation. This result, not observed in the control group, may indicate a role for inflammation based on I allele carrier status in the development of PET. Interestingly, in subjects 20 years remote from a PET pregnancy, circulating levels of VCAM and TNFα were significantly different based on Leptin 3'tet genotype. The VCAM levels were significantly reduced in the group who had PET (the I/II group) compared with the other genotypes. This may indicate that in subjects at increased risk of PET, leptin genotype has little influence on vascular function remote from pregnancy. In the PET group, 20 years after the index pregnancy, TNFα levels also differed between the different genotypes, with highest circulating levels in the I/I group. This finding may indicate a role for inflammation based on genotype in both PET and CVD, via the effects of TNFα. Because of the reports on the direct effects of leptin on blood pressure (Aizawa-Abe *et al* 2000, Casto *et al* 1998, Shek *et al* 1998), future work should concentrate on comparisons of circulating leptin levels based on genotype in PET. The leptin 3'tet polymorphism is located in the 3'-untranslated region of the leptin gene, and this polymorphism may also affect the expression of the leptin gene, perhaps influencing potential *cis*-acting regulatory elements. Differences in local expression of the leptin gene could account for the association with PET, and further studies are required to clarify this possibility.

The association of leptin and PPARγ polymorphisms with risk of developing PET is potentially very important. As with all genetic association studies, it is essential to confirm the observation in an independent study population, a limitation of the present study. Associations within one population are subject to the confounding effects of environmental factors. However, our group have arranged to analyse, blind, a PET and control collection from an American population, in collaboration with Dr Carl Hubel, Magee Women's Research Institute, University of Pittsburgh. Should these results be confirmed, these data

would strengthen the hypothesis that there is a role for the leptin and PPARγ genes in the aetiology of PET. Case-control studies such as this one are at risk of producing false positive results due to differences in ethnicity, as allele frequencies of many genes differ markedly between racial groups (Plummer and Morgan, 2003 p225-235). However, by collaborating with the Pittsburgh group, this issue is addressed, as the latter population has an approximately 20% black population, which is significantly different to our completely Caucasian dataset. Genetic studies are consistently performed on limited study numbers and lack statistical power. The small number of observations regarding rare homozygotes for the Pro12Ala variant may also limit analyses. Genotyping of greater numbers of samples is required to confirm our findings. However, this is an understudied population, which adds strength to this study.

In summary, this work describes the potentially very important association between P12A PPARγ and leptin 3'tet polymorphisms in PET risk. These associations may exert their influence via metabolic effects rather than on PET development directly and demonstrate an attractive target for studies on the metabolic mechanisms of PET. Inflammation may play a role in the development of PET based on PPARγ genotype through transcriptional activity of this nuclear receptor but future studies should concentrate on associations with obesity, P12A genotype and pregnancy, in order to establish the role of adiposity. Furthermore, the reported association between PPARγ P12A substitution and lower lipoprotein lipase activity *in vivo* (Schneider *et al* 2002) should be analysed more closely, perhaps looking at plasma lipid levels based on P12A genotype which may provide another potential mechanism for the metabolic effects of PPARγ on PET risk. The leptin 3'tet polymorphism may exert its effect on PET through local expression of the leptin gene or though control of circulating levels of leptin, and further population based studies should concentrate on these possibilities.

In conclusion, the P12A and leptin 3'tet polymorphism may have a role in the aetiology of the metabolic abnormalities associated with the development of PET. Further genetic research may lead to the identification of women at high risk for PET who may subsequently be targeted for appropriate antenatal care, and may ultimately provide insight into the prevention of the condition.

8 Discussion

Pre-eclampsia is a multi-system disorder particular to pregnancy and is characterised by widespread endothelial damage and dysfunction, resulting in hypertension due to vasoconstriction, proteinuria attributable to glomerular damage and oedema secondary to increased vascular permeability. Despite a decline in severe morbidity associated with hypertensive disorders of pregnancy in developed countries, little progress has been made towards the understanding of the pathophysiology, prediction and prevention of PET over the last 40 years. Hypotheses regarding such pathophysiology include inflammatory disease, endothelial derived factors, placental ischaemia, genetic predisposition, and immune response (Brosens 1977, Redman *et al* 1999, Roberts *et al* 1989). It is recognised that PET and IUGR are conditions that have complex underlying mechanisms involving a spectrum of exaggerated disturbances in maternal metabolism, and that the trigger for PET comes from within the placenta as the condition is resolved with delivery. Furthermore, PET shares many risk factors with CVD and recent data suggests that PET may be associated with future maternal CVD (Irgens *et al* 2001, Sattar and Greer 2002).

Studies into PET often concentrate on the role of a single molecule, surrogate risk marker or candidate gene in the aetiology of the condition. For example, recent studies have focused on the proposed increase in placental production of the soluble fms-like tyrosine kinase 1 (sFlt1) receptor (Maynard *et al* 2003), which captures free vascular cell endothelial growth factor (VEGF, an angiogenic growth factor) and have proposed that this receptor may be involved in the development of PET. Such studies have proposed that strategies designed to normalize circulating free VEGF levels might be expected to halt progression of the disease (Luttun and Carmeliet 2003). However, much less attention is focused on the relationship between the proposed factors and the metabolic complications arising in the mother and, because of the multi-factorial nature of PET, it seems more likely that a combination of multiple factors underlies the condition. Although there is abundant data concerning the metabolic pathways and vascular function in the non-pregnant individual, there is little such data with respect to pregnancy and PET. This would indicate that research should focus on the combination of metabolic aberrations that occur in this condition.

This thesis has concentrated on the hypothesis that PET is a 'metabolic syndrome' that develops in pregnancy. PET shares many risk factors with CVD and the similarities between the metabolic syndrome (a range of metabolic abnormalities associated with

insulin resistance) and PET has lead to the proposal that PET is in fact metabolic syndrome of pregnancy. The list of predisposing factors for PET such as obesity, dyslipidaemia and hypertension, are also risk factors for other endothelial disorders including CVD. Women are more likely than men to have multiple risk factors for coronary heart disease (Lewis *et al* 1998), and for these reasons this thesis has concentrated on key mediators involved in the metabolic syndrome, in relation to PET, including PPAR receptor expression, lipoprotein metabolism, insulin resistance, inflammation and genes involved in PET risk.

PPAR receptor expression is interesting in relation to the metabolic complications of pregnancy as this family of nuclear receptors control some of the pathways involved in the metabolic syndrome, including adipocyte differentiation, the regulation of glucose and lipid homeostasis, fatty acid metabolism and insulin action. Animal and human models have highlighted a role for the PPARs in pregnancy. PPARγ (Barak *et al* 1999, Kubota *et al* 1999) and PPARδ (Barak *et al* 2002, Ding *et al* 2003) are involved in placentation, and PPARα, δ and γ and RXRα expression have been demonstrated in cyto- and syncytiotrophoblast cells (Fournier *et al* 2002, Tarrade *et al* 2001a, Tarrade *et al* 2001b, Wang *et al* 2002), in trophoblastic disease and hydatidiform molar pregnancies (Capparuccia *et al* 2002), and in a choriocarcinoma cell line (JEG-3) and in fetal endothelial cells (Schaiff *et al* 2000, Waite *et al* 2000). The results of this thesis have confirmed the localisation of PPARs within the placenta, in uncomplicated pregnancies and those complicated by PET or IUGR, and have demonstrated that PPARδ expression is up-regulated between the 1st and 3rd trimester indicating a role for this nuclear receptor in placental development. More importantly, evidence is provided that PPARδ, γ and RXRα expression is unaltered in PET and IUGR, suggesting that changes in total placental PPAR expression are not involved in the pathophysiology of these conditions. However, it must be considered that the present study concentrated on the expression of these receptors and did not observe receptor activation or ligand binding within the placenta. It is possible that activation of these nuclear receptors via changes in circulating activator ligands might be altered in PET or IUGR, as suggested recently by Waite and colleagues (Waite *et al* 2005). This may be an interesting area of future research, as it is recognised that some lipids and fatty acids (e.g. polyunsaturated fatty acids) (Kliewer *et al* 1997) act as PPARγ agonists. It is possible that as yet unidentified factors in pregnant serum, such as oxidised lipids, may act as PPARγ agonists. For these reasons, PPARs may still have a role in PET or IUGR in the production of the metabolic derangements seen in these conditions, rather than via altered expression.

In chapter 4, the relationship between maternal and fetal plasma lipid metabolism and paraoxonase-1 (PON-1) activity was observed in PET and IUGR pregnancies. Despite the minimal alterations in maternal lipid profile in the complicated pregnancies in this chapter, I agree with the general hypothesis that demonstrates an increased atherogenic lipid profile in PET. However, my data suggests that these clear changes previously described may be in some way related to adiposity as subjects were matched strictly in the present study for BMI. However, this is difficult to assess as other factors including disease severity, populations studied and small study numbers may play a role. Larger cohorts with control groups adequately matched for BMI should attempt to address whether this dyslipidaemia is related to obesity or an independent factor.

The fetal lipid profile described in this chapter is more interesting and novel. It has been hypothesised that maternal metabolic disturbances may result in similar alterations in the fetus. These changes in fetal metabolism may be short-term *in-utero* alterations in response to the altered placental transfer of maternal nutrients, although it should also be considered that these metabolic aberrations might persist in the long-term. Barker and colleagues have suggested that fetal adaptation to inadequate intra-uterine nutrition, secondary to either reduced placental function or inadequate maternal diet, results in a physiological programming of the fetus. This may result in insulin resistance and the metabolic syndrome to compensate for the sub-optimal intra-uterine environment although this compensatory effect may ultimately result in an increased risk of coronary heart disease later in life (Barker 1994). Low birthweight is also associated with insulin resistance, hypertension, and non-insulin-dependent diabetes (NIDDM). Since low birth weight has been associated with an increased risk of coronary heart disease in both mother and offspring in adult life (Barker *et al* 1989, Eriksson *et al* 1999, Leon *et al* 1998), it is likely that there is a common underlying mechanism. It has also been proposed that genetically determined insulin resistance results in impaired insulin-mediated growth in the fetus as well as insulin resistance in adult life (Hattersley and Tooke 1999) and that low birthweight and cardiovascular disease may be different manifestations of the same insulin-resistant genotype. It has been considered that the abnormal vascular development during fetal life is a result of genetic insulin resistance, which is also responsible for the increased risk of hypertension and vascular disease (Hattersley and Tooke 1999). If fetal growth restriction at birth is used as a surrogate for poor intra-uterine fetal growth, it may be postulated that maternal or fetal metabolic parameters, including dyslipidaemia or insulin resistance, may affect fetal growth. Catalano and colleagues have described a vicious circle of maternal insulin resistance and obesity and subsequent increased risk of the offspring developing adolescent obesity and type 2 diabetes, as a result of fetal

macrosomia or overgrowth (Catalano *et al* 2003). However, despite these hypotheses, no specific mechanisms regarding fetal metabolic disturbances in relation to maternal metabolism have been developed, and so the fetal lipid data produced in this chapter provides important progress in the fetal origins of adult disease and have potential implications for the future cardiovascular health of the offspring.

I have also provided evidence in this thesis to suggest that the anti-oxidant PON-1 has reduced activity in pregnancies complicated by PET, which supports the theory that oxidative stress plays a role in the aetiology of this condition as well as in atherosclerosis. That I have reported an association between PON-1 activity and increasing fetal weight, birth weight centiles and placental weight in the PET group alone, adds support to the theory that the maternal metabolic response supports optimal fetal nutrition and development in the face of abnormal placentation and dyslipidaemia. Together these data suggest that PON-1 plays a role in the 'metabolic syndrome' that is PET and strengthens the proposed association between PET and CVD.

The inflammatory mediators involved in the maternal metabolic alterations seen in healthy pregnancy and pregnancies complicated by PET and IUGR are discussed in chapter 5. It is widely recognised that in normal pregnancy a systemic inflammatory response is evoked and it is proposed that PET arises in response to an extreme response with decompensation. Despite this, in the context of this thesis, the maternal inflammatory response was only minimally altered in complicated pregnancies compared with uncomplicated controls. Once again, it is proposed that the effect of adiposity may account for such extensive maternal inflammation demonstrated in PET pregnancies within the literature, as adipocytes are complex metabolically active cells, which release inflammatory mediators into the maternal circulation. Most previous research has not taken BMI into account when analysing such data and adiposity itself is a major risk factor for PET. However, as for the lipid data, it is conceded that there is some degree of exaggerated maternal inflammatory response in PET and that the lack of alteration in this study may be secondary to disease severity or sample size. However, the key finding within this chapter is the fetal inflammatory responses seen in offspring from mothers with PET or with IUGR, despite the minimal maternal metabolic changes. Once more, the fetal response may be attributable to intra-uterine adaptation and programming or to a genetic pro-inflammatory state, and has implications for the future health of not only the mother but also her child. However, it must also be considered that the fetal inflammatory responses seen in PET and IUGR are related to a stress response secondary to poor placentation, and it is proposed that longer-term *in-utero* stress in IUGR results in elevated fetal levels of

TNFα, while a short-term, more acute 3rd trimester stress response to maternal metabolic aberrations results in elevated levels of fetal CRP in PET. The main observation of these two chapters was that maternal metabolic derangement is associated with fetal metabolic derangement although this has not been studied mechanistically. Further work into this relationship may provide insight into the effect of the fetal situation in the development of CVD.

Data produced in this thesis indicates that adipose tissue may be the main source of fetal leptin. The associations reported between the tissue and plasma metabolic parameters suggest that placental expression of leptin may have an effect on lipid transport to the fetus. These results highlight the benefits of studying global metabolic maternal and fetal parameters of plasma and tissue in PET and IUGR rather than concentrating on specific molecules or markers, which may be potentially related to these conditions.

The features of PET, including hypertension, dyslipidaemia and endothelial cell dysfunction, are all features of the insulin resistance syndrome (Reaven *et al* 1996) seen in atherosclerosis. The lack of validated measures of insulin resistance in pregnancy is discussed in chapter 6. Not only are tests such as the euglycaemic clamp, fasting insulin, glucose and HOMA calculation not validated in the pregnant state, but also they are difficult to perform within a realistic clinical situation. For example, fasting samples are often difficult to obtain in pregnancy, and this problem is exacerbated in emergency situations such as PET or IUGR when a first presentation may be associated with immediate need for delivery. This presents problems in obtaining good markers of insulin resistance for use in the research setting, and in interpreting such data. Important research over the last decade has demonstrated an association between insulin resistance and skeletal muscle and erythrocyte membrane fatty acid lipid composition. I have proposed that this metabolic association may be extended to include PET, and was interested in the erythrocyte membrane fatty acid composition as a potential research marker of PET, via effects on insulin resistance. The erythrocyte itself has a lifespan of approximately 90 days, and it was considered that the fatty acid composition might provide a reasonable index of insulin resistance from the preceding trimester.

It was demonstrated in this study, that there was an increase in the more saturated fatty acids 14:0 and 16:0 in red blood cell membranes in PET compared with uncomplicated pregnancy. This work also provides evidence that elongase enzyme activity is reduced throughout gestation in uncomplicated pregnancy and is reduced in PET compared to uncomplicated pregnancy as hypothesised. These findings all demonstrate a shorter-chain,

more saturated erythrocyte membrane fatty acid profile in PET, which may be interpreted as increased insulin resistance. This is a particularly important finding as it may highlight elongase activity as a potential research marker of insulin resistance in the pregnant non-fasting state. I was able to report a comprehensive erythrocyte membrane fatty acid profile within the longitudinal and cross sectional studies, including the proportions of all of the fatty acids produced throughout pregnancy, including those that are negligible. This work provides a firm basis on which future research may build. This study agrees with the general pattern of the main fatty acids reported in the literature in uncomplicated pregnancy, in particular DHA and AA, and also demonstrates a relationship between maternal plasma triglyceride levels and a reduction in elongase activity. This relationship strengthens the suggestion that elongase may be a good marker for the metabolic aberrations of pregnancy, including dyslipidaemia and insulin resistance, beyond the 1st trimester.

There is no doubt that there is a familial component to the development of PET. Large epidemiological studies have demonstrated that there is an increased risk associated with first-degree relatives of affected women. Many trials involving genome-wide screening and identification of candidate genes have been performed, but these tend to concentrate on genes involved in regulation of blood pressure and placentation. Recently, more attention has been directed at the study of genes involved in the underlying processes involved in PET, including inflammation and endothelial activation. In this thesis, I have observed PET risk associated with genes involved in the maternal metabolic processes, which have an aetiological role in PET, and which are also involved in the pathophysiology of CVD. The PPARγ Pro12Ala and C161T polymorphisms were studied because of the function of PPARs in regulation of metabolism and adipocyte differentiation, and their relationship to CVD and the metabolic syndrome. The IL-10 genes, A-1082G, C-819T and C-592A were examined because of the relationship between inflammation and PET and CVD, and the leptin 3'tet and G2548A polymorphisms were observed due to the association of leptin with metabolic regulation and energy. It has been reported in this work that the PPARγ Pro12Ala and the Leptin 3'tet polymorphisms predict PET risk within our population. These associations are interesting because the link between PET risk and genes involved in metabolism give strength to the theory that PET is the metabolic syndrome of pregnancy. It is also important to realise that these genes are involved in prediction of type 2 diabetes and CVD risk, further highlighting the relationship between PET and CVD. However, it is important to confirm genetic studies within a secondary population. Similarly, it should be remembered that the role of these genes in PET is unclear. It is not known whether the

PPARγ Pro12 Ala polymorphism affects the amount of PPARγ expressed or whether it is function of the receptor that is altered. It seems likely that a polymorphism found within the coding area of such a gene would exert its effect on the way in which the receptor functions. However, if the polymorphism affects, for example, ligand binding at the receptor, then the expression of the receptor is not as important as its ability to function in conditions such as PET and IUGR. Further work into PPARγ receptor function may help to clarify the role of the polymorphism, possibly looking prospectively at macrophage function with respect to PPARγ ligand binding and adipocyte lipolysis in trophoblast culture studies in relation to genotype.

The leptin 3'tet polymorphism was also associated with PET risk in this study. Maternal plasma leptin levels are increased in uncomplicated pregnancy, possibly secondary to placental production or increased adipose production. This is most likely a physiological response in pregnancy to control placental and fetal growth and to regulate energy and metabolism. In this study, maternal leptin genotype is assessed and therefore placental derived leptin is irrelevant as this is fetal in origin. If maternal leptin genotype were of importance, one would assume that it is related to maternal adipose leptin production. As I have previously suggested, maternal leptin by its association with maternal BMI may mediate risk of PET.

I have suggested previously that placental leptin may have an important role in increasing placental transport of lipids. In this case, the fetal leptin genotype would be of paramount importance. To some degree, maternal genotype will reflect fetal genotype and it is possible that a weak association of maternal genotype with risk of PET may be reflecting a stronger association with fetal genotype. It is unknown whether the maternal or fetal genotype is paramount for the development of PET. Ideally, both genotypes should be measured in conjunction. The leptin 3'tet polymorphism is located in the 3'flanking region of the gene, and therefore does not directly affect the leptin coding region. However, regulatory elements in adjacent areas may well affect gene transcription rates or alternatively the leptin 3'tet polymorphism may be in linkage dysequilibrium with an unknown gene that also affects PET. It is important to note the potential effects of both of these polymorphisms on obesity, and the link between adiposity and PET risk. Further work would confirm these findings in a larger population, precisely matched for BMI.

The metabolic consequences of PET and perhaps IUGR affect both mother and offspring, in the short and long term. The data produced in the context of this thesis aims to concentrate on these conditions as multi-system metabolic syndromes, rather than

employing the reductionist approach of studying one molecule or gene, which is inappropriate for multi-factorial disease. The results produced provide robust data based on an attempt to reveal the underlying systemic abnormality that results in the abnormal maternal response. The three main hypotheses regarding the development of metabolic disturbance include insulin resistance, endothelial dysfunction and oxidative stress. However, it is unlikely that these mechanisms will function independently, rather that they form a complex group of inter-related processes, governed phenotypically by an underlying genetic influence. For example, conditions associated with insulin resistance, including polycystic ovarian syndrome (PCOS) and obesity, are also associated with an increased risk of PET and CVD later in life. This suggests that insulin resistance may be considered a reasonable marker of PET, but as for the metabolic syndrome, it is not known whether the insulin resistance is the cause of these conditions or an effect of the altered metabolic state associated with another underlying pathology, for example, obesity.

Recent data from Sattar and Greer (Sattar *et al* 2003) suggests that the link between PET and CVD may be explained by the presence of pre-pregnancy metabolic mediators, and it has been proposed that advancing age or even pregnancy stimulates a metabolic stressor, which steps up the baseline level of risk of CVD. The data produced within this thesis has attempted to study the overall effects of these potential 'metabolic stressors' and to describe possible markers for these metabolic changes, for example via fatty acids and enzyme activities involved in their metabolism. This is a particularly sound example, as these molecules and enzymes are not simply static molecules, but provide a metabolic 'picture' of the preceding three months. I have preliminary data, not included in this thesis, to suggest that fatty acid changes in the mother may have an effect on those of the fetus, and this data implicates insulin resistance in maternal *and* fetal metabolic abnormalities.

Why is this work relevant to the expanding field of PET research? The metabolic syndrome, CVD and PET are all associated with obesity, a major health problem within Western society. There is abundant evidence regarding the effects of obesity on metabolism, and these effects are similar although perhaps less obvious in metabolic complications of pregnancy. As the obesity epidemic increases, so does the incidence of deranged metabolic function and CVD risk, and considering the similarities, the incidence of PET seems sure to follow suit. Metabolic derangement not only affects maternal cardiovascular health, but is also likely to have long-term implications for the offspring. This thesis presents robust data highlighting obesity-related problems in pregnancy and provides insight into potential preventative measures, protecting the future health of the nation. Identification and modification of pre-pregnancy risk factors, such as obesity, may

aid the primary prevention of PET. If insulin resistance underlies PET, then one may contemplate pre-pregnancy or antenatal therapy with such interventions as lifestyle advice or even insulin sensitising agents. Metformin is an insulin sensitising agent used commonly in the treatment of PCOS and results demonstrate an associated increase in fertility in this cohort (Barbieri 2003). More recently, the insulin sensitising PPARγ agonists are being employed for this condition, and results on incidental fertility are sure to be reported in due course. I have provided evidence that PPARs may have a role in placental function, and it should be considered that PPARγ agonists might be used for the primary prevention of the underlying insulin resistance of PET in the future. Based on my thesis, other such therapies might include dietary fish oils, with recent interest in this area of research increasing. Furthermore, as oxidative stress is linked to the metabolic syndrome and to PET, ongoing large multi-centre anti-oxidant trials in PET may produce interesting results for potential therapeutic measures.

This thesis should stimulate future work into the effects on the offspring delivered of mothers with the metabolic complications of pregnancy, in particular PET and IUGR, providing valuable information regarding the future health and CVD risk of our society. Such data should include anthropometric measures to help elucidate further the associations between birth characteristics and subsequent risk of CVD. The content of this thesis reports important data regarding maternal fatty acid status, and large prospective adequately powered clinical trials should now be conducted, concentrating on possible predictive capacity of 1st and/or 3rd trimester fatty acids or enzyme activities for the development of PET. It may be interesting to examine the relationship between erythrocyte fatty acid composition and that of other tissues, in particular, adipose tissue which is implicated in PET and CVD. Similarly, this thesis reveals the paucity of data regarding placental lipoprotein transport, and provides data which suggests that transfer of lipids across the placenta may be altered in the metabolic complications of pregnancy, which may in turn have implications for the future health of the offspring. Studies should now concentrate on demonstrating the mechanisms of placental transport of lipids, and any alterations in these mechanisms in pregnancies complicated by PET. It may also be prudent to consider conducting larger population studies on PON-1 activity in PET, based on genotype, in an attempt to determine whether PON- activity alone is more important than genotype related alterations. Finally, it was not possible to determine protein expression of placental and adipose inflammatory mediators within the time scale of this work, and it seems important to follow-up on this, as no direct correlation was noted between available protein expression of placental PPARγ and TaqMan data which may be related to the small

groups studied. Because of the problems encountered during the western analyses, it was considered that the molecular weights of the inflammatory marker proteins may be too small to use Trizol extraction, and it may be necessary to use placental homogenates or ELISA methods to further develop a successful technique.

In conclusion, this data provides further evidence that multiple metabolic alterations occur in PET and IUGR, and demonstrates that these changes are not only confined to the mother but are also evident in the fetus. These perturbations in fetal lipids and inflammatory markers may be relevant to fetal programming of adult vascular disease. Although PPAR expression is unaltered in PET, these receptors may still have an influence on the actiology of the condition through genetic effects on metabolism, as the PPARγ P12A polymorphism appears to be related to increased risk. A potential research marker for PET, elongase enzyme activity, has been described and this has its action via insulin resistance. Adipose tissue has been highlighted as a potential source of inflammatory mediators, and further large studies should examine the adipocytes of women with PET to determine the origin of elevated fatty acids and leptin levels. It seems likely from this data that multiple interacting metabolic processes are involved in the aetiology of PET and that these processes are directly related to the development of CVD later in life. An understanding of the genetic and metabolic mechanisms involved in PET may inform strategies for identification and intervention in individuals at risk. Metabolic disorders have mulitfactorial origins in which both genetic and environmental factors are thought to be involved (Kahn 1994), and work produced by this thesis demonstrates the importance of looking at PET as a metabolic disorder rather than searching for a single candidate gene or molecule to account for aetiology.

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