Pre-eclampsia; early prediction and long-term consequences

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Summary

Approximately one in ten pregnant women will have their blood pressure recorded above normal at some point during their pregnancy. Pre-eclampsia, the most common hypertensive disorder of pregnancy, affects around 5% of all first time mothers, and is an important cause of foetal and maternal morbidity and mortality worldwide. Efforts to diagnose the condition have been hampered by inability to predict which women are likely to be affected. Multiple pathways are known to be involved in its pathogenesis, and several screening tests have been suggested for its early prediction. None, however, have been sensitive or specific enough to have come into routine medical practice.

The work contained in this thesis describes a study which was designed to detect biochemical and clinical markers that could improve ability to predict pre-eclampsia. Over 3900 women were recruited in early pregnancy at four maternity clinics across the West of Scotland; baseline characteristics and information on past medical and obstetric history were obtained. Women were followed up throughout their pregnancy, and information on deliveries obtained from hospital databases. One-hundred and eighty of these women, who had multiple risk factors for pre-eclampsia, attended for further sampling and vascular assessment at gestational weeks 16 and 28.

The primary aim of the overall study was to examine whether a proteomic strategy could be used to identify patterns of peptides in urine that detect pre-eclampsia in the first and second trimesters. Using samples from healthy pregnant and non-pregnant women I was able to describe the normal human urinary proteome in pregnancy. By comparing these pregnancy-associated peptides between women who went on to develop pre-eclampsia and matched controls, I was able to identify a pattern of peptides, characterised by collagen fragments, fibrinogen and uromodulin that accurately predicted pre-eclampsia at week 28. No such markers were identified in the first trimester samples.

A further aim of the overall study was to identify early pregnancy plasma markers that could help to identify women destined to develop pre-eclampsia.

By examining samples from early pregnancy I was able to demonstrate that the angiogenic markers soluble endoglin and placental growth factor are already altered at week 12-16 in women who go on to develop pre-eclampsia. Using a multi-marker approach, I also showed that E-Selectin, an adhesion molecule expressed on endothelial cells which controls interaction between circulating leukocytes and the endothelium, is higher at week 12-16 in women who go on to develop pre-eclampsia. Experiments using samples from later pregnancy, alternative analysis techniques and samples from an independent study population all helped to confirm these novel findings.

Endothelial dysfunction is known to play a key role in the development of pre-eclampsia, contributing to the hypertension, proteinuria and oedema seen in affected women. In the risk factor cohort I used vascular function studies to examine whether they supplied additional information to aid in risk stratification. Peripheral arterial tonometry, a novel non-invasive tool for the assessment of microcirculatory endothelial function, was examined in 180 women at gestational weeks 16 and 28. Reactive hyperaemia index (RHI), a measure of endothelial dysfunction calculated from vascular response to arm blood-flow occlusion, did not correlate with maternal factors such as age, BMI and blood pressure. Further, RHI did not help to identify which women would go on to develop pre-eclampsia, when examined at either week 16 or 28. I found that PAT score was negatively correlated with baseline digital pulse amplitude, suggesting that in later pregnancy, when women are more vasodilated, PAT and other techniques which rely on flow-mediated dilatation are less likely to be reliable.

I used pulse wave analysis, a well-established method for measuring arterial stiffness and central pressures, to determine whether it supplied additional information about pre-eclampsia risk. This technique has been previously reported to predict pre-eclampsia in early pregnancy. In this cohort of high risk women, no difference was seen at either week 16 or 28 between those who would go on to develop pre-eclampsia and those who would have normotensive pregnancies.

Although blood pressure and proteinuria return to normal after pre-eclampsia, evidence has emerged the condition has long-lasting implications; women with a

history of pre-eclampsia have an increased risk of cardiovascular disease later in life, suffering stroke or myocardial infarction more frequently than women who had a healthy pregnancy. Conventional risk factors are thought to contribute, but do not fully explain this increased risk. I carried out further vascular function studies in women after pre-eclamptic pregnancy, to examine whether they had ongoing detectable endothelial dysfunction and arterial stiffness. At 6-9 months post-natally, affected women had lower baseline digital pulse amplitude but no other evidence of persistent vascular dysfunction.

Taken together, these data provide information about a number of markers that may improve understanding of the pathophysiological mechanisms underlying pre-eclampsia. As well as potentially improving the early prediction of disease, this work represents a highly topical area for further studies. While vascular function analysis does not appear to provide additional information on top of risk factors, these studies also provide useful information on vascular physiology in high-risk pregnancies.

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Author's declaration

The experimental design of the work presented in this thesis was that of the author and his supervisors, Dr Christian Delles and Professor Anna Dominiczak. All experimental work was carried out by myself with the exception of the proteomic analysis (carried out by the staff of Mosaiques Diagnostics, Hannover, Germany), the ELISA biomarker measurements and the brachial artery ultrasound studies (both performed by Miss Lesley Anderson, University of Glasgow), and a proportion of the peripheral arterial tonometry (PAT) recordings (performed by Miss Laura Rooney, Clinical Research Facility, Western Infirmary, Glasgow).

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been previously submitted for a higher degree.

David Martin Carty

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List of abbreviations

ACOG	American College of Obstetrics and Gynecology
AIx	Augmentation Index
AMH	Ayrshire Maternity Hospital
ANCA	Anti-neutrophil cytoplasmic antibodies
AP	Augmentation pressure
AU	Arbitrary units
AUC	Area under the curve
BMI	Body Mass Index
CAD	Coronary artery disease
CAM	Cell adhesion molecule
CCD	Charge coupled device
CE	Capillary Electrophoresis
CEMACH	Confidential enquiry into maternal and child health
CI	Confidence interval
CKD	Chronic kidney disease
CSF	Cerebro-spinal fluid
Da	Dalton
EGF	Epidermal growth factor
ESRD	End-stage renal disease
FMD	Flow-mediated dilatation
GROW	Gestation related optimal weight
GSH	Glutathione
GSSG	Oxidised glutathione
HbA1C	Glycosylated haemoglobin
HELLP	Haemolysis, elevated liver enzymes, low platelets
HOMA	Homeostatic model assessment
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
IMT	Intima-media thickness
ISSHP	International society for the study of hypertension in pregnancy

IUGR	Intra-uterine growth restriction
LC	Liquid chromatography
LR	Likelihood ratio
MALDI	Matrix-assisted laser desorption / ionisation
MCP	Monocyte chemotactic protein
MS	Mass Spectrometry
OGTT	Oral glucose tolerance test
OR	Odds ratio
PAT	Peripheral arterial tonometry
PI	Pulsatility index
PIGF	Placental growth factor
PIP	Proteomics in Pre-eclampsia
PRMH	Princess Royal Maternity Hospital
QMH	Queen Mother's Hospital
RHI	Reactive hyperaemia index
RI	Resistance Index
ROC	Receiver operating characteristic
RR	Relative risk
SCOPE	Screening for pregnancy end-points study
SELDI	Surface-enhanced laser desorption / ionisation
sENG	Soluble Endoglin
sFLT-1	Soluble FMS-like tyrosine kinase 1
SGH	Southern General Hospital
SHBG	Sex-hormone binding globulin
SNP	Single nucleotide polymorphisms
SVM	Support vector machine
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1. Introduction

1.1 Introduction

The French physician François Boissier de Sauvages de Lacroix in 1739 was the first to coin the term eclampsia, which he described as an acute form of convulsion, and contrasted with the chronic condition now known as epilepsy (1). He is credited with establishing the first methodical nosology for disease, and described several species of the genus eclampsia, one of which was *eclampsia parturientum*. Given the dramatic nature of the condition, it is perhaps surprising that more is not written in historic literature about eclampsia. This may be explained by the fact that delivery was historically the domain of the midwife: doctors, who in those days were exclusively men, were excluded from the scene.

Proteinuria in the context of eclamptic seizures was first described in 1840, and high blood pressure as recorded by sphygmographic tracings shortly after. By 1894 it was reported that high blood pressure and proteinuria could occur in pregnant women, without eclamptic seizures, and the term pre-eclampsia was born (2).

Approximately 10% of pregnant women will have their blood pressure recorded above normal at some point during pregnancy. Pre-eclampsia, which is now known to be a multi-system disorder of pregnancy, complicates 2-8% of pregnancies in the Western world and 5% of first time mothers (3). Although an important issue in the developed world, the effect of pre-eclampsia and eclampsia in the developing world is staggering; 10-15% of maternal deaths are attributed to pre-eclampsia, of which 99% occur in the developing world (4). The number of maternal deaths from pre-eclampsia is equivalent to the loss of 170 jumbo jets full of pregnant women each year.

The cardinal features of pre-eclampsia are hypertension and proteinuria occurring after 20 weeks' gestation in women who were not previously known to

be hypertensive. Most women with the condition are symptom free, a fact that contributes to the difficult and often delayed diagnosis. Women with more severe forms of pre-eclampsia often complain of headache, abdominal pain and visual disturbance, and can suffer complications to virtually all the body's systems, as summarised in table 1.1. The disease does not just affect the mother; a third of babies born to women with pre-eclampsia will be born premature, and a quarter will be growth restricted.

System	Complication
Central nervous system	Eclampsia (seizures)
	Cerebral haemorrhage
	Cerebral oedema
	Retinal oedema
	Cortical blindness
Renal system	Renal tubular necrosis
	Renal cortical necrosis
Respiratory system	Pulmonary oedema
	Laryngeal oedema
Hepatic system	Disseminated intravascular coagulation
	Haemolysis
Liver	Haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome
	Jaundice
Placenta	Placental abruption
	Placental infarction
Baby	Growth restriction
	Preterm delivery
	Death

Table 1-1 Complications of pre-eclampsia. Adapted from Duley et al. (4)

Even with all the advances in modern medicine, the exact cause of pre-eclampsia remains unknown, and the only cure is delivery of the foetus and placenta. In addition, clinicians have no effective way of predicting which women are likely to develop the condition. Identifying high risk women is an

important aim; modern obstetric care places emphasis upon the primary care setting for expectant women, a marker which identified high risk women would allow for closer supervision, most likely in the secondary care setting. Such a marker would also facilitate recruitment for trials of potential therapeutic agents, for accurate diagnosis and for timely intervention whenever problems develop.

In this chapter I will examine the pathophysiology of pre-eclampsia, potential roles for its prediction, and I will discuss long-term consequences associated with the condition.

1.1.1 Diagnostic Criteria

One of the pitfalls in pre-eclampsia research over the years has been differences in diagnostic criteria used worldwide. A number of different terms and systems are used; some are more detailed than others, some are more inclusive than others, and in some cases the same term has been used to describe different disorders by different authors. To combat this, a working group of the International Society for the Study of Hypertension in Pregnancy (ISSHP) published a Consensus statement in 2000, which is now the most commonly used set of criteria in the literature (5).

In brief, in the consensus statement hypertension in pregnancy is split into 4 main categories; gestational hypertension, pre-eclampsia, chronic hypertension (essential or secondary) and pre-eclampsia superimposed on chronic hypertension. Gestational hypertension is defined as de novo arterial hypertension (systolic BP ≥ 140 mmHg and / or diastolic BP ≥ 90 mmHg on 2 occasions > 6 hours apart) occurring after gestational week 20, which returns to normal post-partum. Pre-eclampsia is defined as gestational hypertension *plus* proteinuria, in turn defined as ≥300 mg/24 hrs, protein: creatinine ratio ≥ 30 mg/mmol or if neither are available dipstick analysis of ≥ + (which “is often but not always associated with 300 mg/24 hrs”). Chronic hypertension is hypertension diagnosed before the 20th gestational week *or* de novo hypertension which fails to settle post-partum. Pre-eclampsia superimposed on chronic hypertension is defined as the appearance of de novo proteinuria starting after gestational week 20. A sudden increase in blood pressure or

proteinuria, or the appearance of thrombocytopenia or deranged transaminases are said to be suggestive but not diagnostic of superimposed pre-eclampsia.

Despite this general agreement, many questions remain about how the disease should be classified. Some classifications define pre-eclampsia as pregnancy-induced hypertension in association with evidence of multi-organ dysfunction; proteinuria, *or* other complications such as renal insufficiency, liver disease, neurological problems, haematological disturbance, or intra-uterine growth restriction (3). Further definitions separate proteinuric from non-proteinuric pre-eclampsia; proteinuric pre-eclampsia is reported to carry a worse prognosis than non-proteinuric pre-eclampsia, which is in turn reported to carry a worse prognosis than gestational hypertension alone (6).

Further questions remain over whether "early onset" (usually defined as that occurring before 34 weeks' gestation) and "late onset" variants of pre-eclampsia are the same disease, or whether they have completely different pathological mechanisms. How to define "severe" and "mild" pre-eclampsia is another matter for debate. A further complicating factor is that the association of pre-eclampsia with preterm delivery, small for gestational age (SGA) babies, or both, appear to have different consequences, particularly for the mother's future cardiovascular health (7).

For the purposes of this thesis, the diagnostic criteria defined by the ISSHP, but requiring at least ++ proteinuria on Dipstick analysis will be used throughout.

1.1.2 Pathogenesis of pre-eclampsia

Although the cause of pre-eclampsia remains largely unknown, the pathogenesis is thought to occur in two main phases. The first phase begins in the placenta, while the second stage is characterised by an abnormal maternal endothelial response, resulting in the hypertension, proteinuria and oedema that characterise the condition.

1.1.2.1 Placental phase

The placenta is well recognised as having a key role in the development of pre-eclampsia. This is known to be the case since pre-eclampsia occurs only during pregnancy, it resolves after delivery of the placenta, and it can occur in the absence of a viable foetus, for example in molar pregnancies. Blood supply to the placenta is via the spiral arteries, branches of the uterine arteries; placental development is a closely regulated process which is essential for normal foetal development.

The spiral arteries are remodelled in pregnancy in several stages, beginning at around the time of implantation. Remodelling transforms the arteries from low-flow, highly resistant vessels into the high-flow, low resistance vessels which are vital for normal placental development (Figure 1.1). Impaired remodelling of the spiral arteries is considered to be a key factor in the pathogenesis of pre-eclampsia (8). In pre-eclampsia, disturbance of spiral artery remodelling may occur as early as the time of implantation, offering a potential explanation for the fact that women with a history of sub-fertility or early miscarriage are at increased risk of the condition (8).

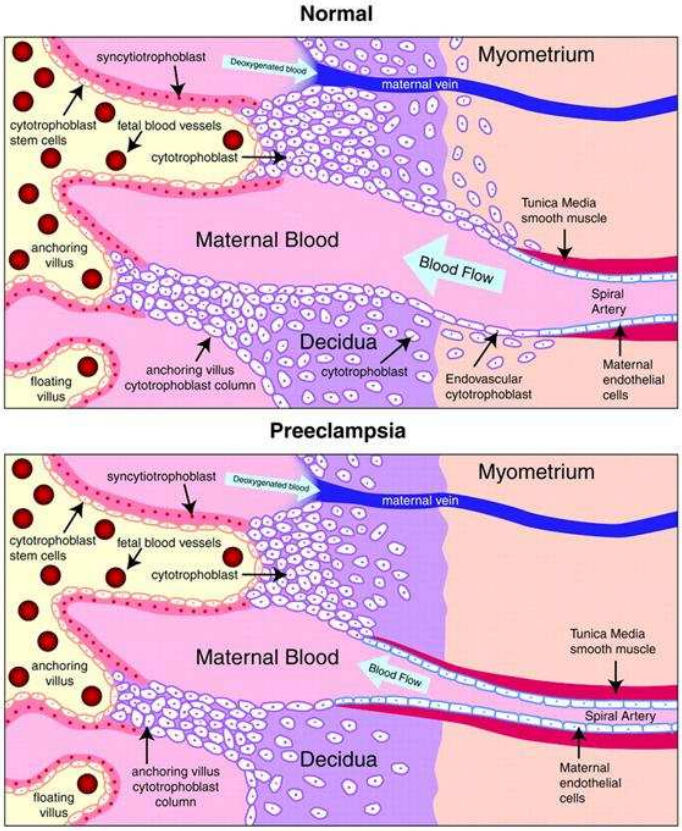

Figure 1-1 Cytotrophoblasts invading the maternal spiral arteries transforming them from high-flow, low-resistance vessels in normal pregnancy (top) and impaired remodelling in pre-eclampsia (bottom). From Powe et al. (9)

Intervillous flow, characterised by the appearance of connecting channels between the spiral arteries and the blastocyst, begins at 7-8 weeks' gestation. Following this, the cytotrophoblastic cells of the developing placenta invade the decidual segments of the spiral arteries at around 10-12 weeks' gestation, and then the myometrial segments, at around 15-16 weeks' gestation. The trophoblast then invades both the endothelium and the highly muscular tunica media of the maternal spiral arteries.

In pre-eclampsia the cytotrophoblast invades the decidual portion of the spiral arteries, but invasion of the myometrial segments is impaired; the spiral arteries remain narrow, and blood supply to the foetus is restricted. The effects of this on the foetus become more significant as pregnancy progresses, since the uterine vasculature is unable to keep up with the increased amount of blood and nutrients necessary for foetal development.

What causes the impaired development of the uteroplacental circulation in pre-eclampsia remains unknown, and is a subject of much debate. Vascular, environmental and genetic factors all appear to play a role (10). Maternal natural killer (NK) cells are now thought to have an important role in these early stages of disease development. NK cells are the main maternal immune cells in the endometrium prior to implantation, and are have their main role in regulation of placental development. Interaction between maternal NK cells and foetal major histocompatibility complex (MHC) antigens may represent an initial step. Particular combinations of maternal NK cells and foetal MHC-C genotypes are linked to impaired placental development, and are reported to be associated with an increased risk of miscarriage and pre-eclampsia (11). Ongoing studies examining the interaction between NK cells and foetal gene expression may help to improve understanding of the vital initial stages in pre-eclampsia development.

Reduced perfusion of the placenta from the abnormal remodelling of the spiral arteries appears to be related to impaired placental development. In keeping with this theory, conditions associated with vascular insufficiency, including hypertension, diabetes, systemic lupus erythematosis (SLE) and renal disease all increase the risk of abnormal placentation and pre-eclampsia (12).

Hypoperfusion of the developing placenta results in placental ischaemia; placental pathological findings indicative of ischaemia include atherosis, fibrinoid necrosis, thrombosis and placental infarction. The typical placental pathological appearances are not seen in all women with pre-eclampsia, but their presence does appear to correlate with disease severity (13).

The interface between the placental and maternal components of pre-eclampsia development is thought to occur when the under-perfused, ischaemic placenta releases a variety of factors into the maternal circulation (14).

1.1.2.2 Maternal response

The second phase of pre-eclampsia development is characterised by exaggerated maternal endothelial activation and a pro-inflammatory state compared to normal pregnancy (15). Placental hypoxia leads to oxidative stress, destruction of syncitial architecture, and release of components from the intervillous space into the maternal circulation. The trophoblastic debris in the maternal circulation includes syncytiotrophoblastic membrane microparticles, and factors arising from the syncytiotrophoblast including soluble endoglin (sENG), and the soluble form of the vascular endothelial growth factor (VEGF) receptor, (sFLT-1). These and other as yet unknown factors lead to production of inflammatory cytokines in the maternal circulation, endothelial dysfunction and increased vascular reactivity. Loss of integrity of the maternal endothelium contributes to reversal of the physiological vascular changes in pregnancy, and subsequent hypertension, proteinuria and oedema (Figure 1.2).

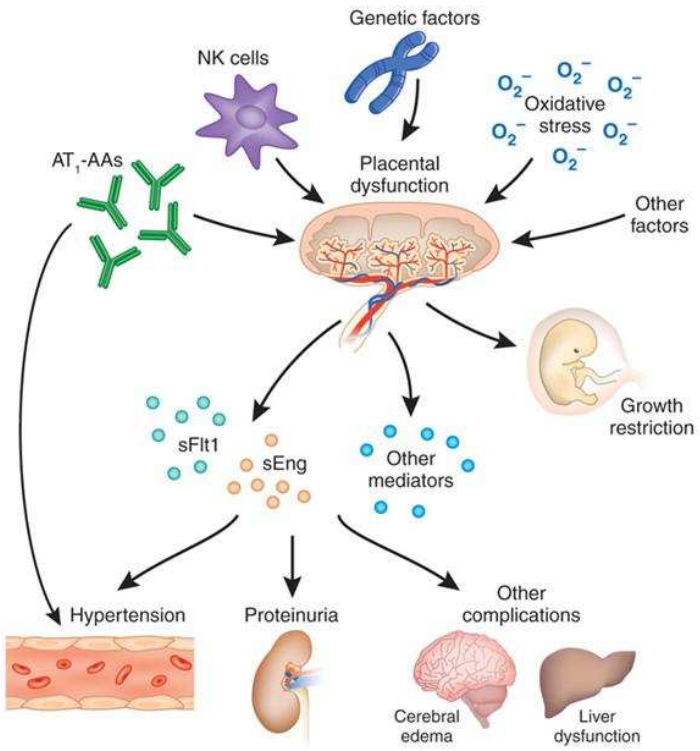

Figure 1-2 Current understanding of pathophysiology of pre-eclampsia. From Parikh et al. (16)

1.1.2.3 Animal studies

One of the key aspects that has hindered progress in pre-eclampsia research is the lack of an accurate animal model to further study the disease. The disease appears to be unique to humans; although case reports have described conditions in pregnant animals such as seizures or characteristic renal lesions in association with elevated blood pressure, there have been no reports that have been similar enough to the human disease. Blood pressure tends to improve in pregnancy in rat models of hypertension (17), while renal function tends to improve in pregnant rats with various types of renal disease (18).

A number of models have been used over the years to attempt to reproduce pre-eclampsia, including uteroplacental ischaemia, nitric oxide synthase inhibition, adriamycin nephropathy and antagonism of angiogenesis (19). Each has improved understanding of certain aspects of pre-eclampsia, but in most studies the blood pressure did not improve after delivery, and the pathological appearances were seen both in the pregnant and non-pregnant animals.

More recent animal models have been able to more closely replicate the human syndrome. The inbred mouse strain, BPH/5, is one such model. These mice develop a maternal and foeto-placental syndrome that resembles pre-eclampsia in humans, including development of late-gestational hypertension, proteinuria, renal glomerular lesions and endothelial dysfunction. These appearances are paralleled by alterations in angiogenic factors, which are known to be associated with the human disease (20). A further model has been developed in which mice are deficient in the complement component C1q; these animals also display multiple features resembling pre-eclampsia. One recent study using this model has also reported a potential role for pravastatin in reducing anti-angiogenic factors and blood pressure in affected animals (21).

Finding a consistent definition for pre-eclampsia has proved difficult enough in humans, given the variation in presenting symptoms and signs that are seen. Any animal studies would have to fulfil all the criteria used in human diagnostic definitions; otherwise there would always be uncertainty as to how applicable the data would be to humans. Development of an animal model that recreates the full clinical spectrum of pre-eclampsia remains an important goal, and it is

hoped that such studies will provide a unique opportunity to examine the exact cause of molecular changes in angiogenic factors in pre-eclampsia.

1.1.3 Maternal Risk Factors

Although accurate prediction of pre-eclampsia remains difficult, there are a number of maternal risk factors which can be easily assessed in early pregnancy that are known to be associated with an increased risk of developing pre-eclampsia.

1.1.3.1 Nulliparity

The most common of the pre-eclampsia risk factors is nulliparity, defined as never having previously given birth to a viable foetus; nulliparity has been shown to almost triple the risk of pre-eclampsia (12). The protective effect of having had a previous birth is lost, however, when a subsequent pregnancy is conceived with a new partner, or when there is a long interval between pregnancies. This had led to the theory that prior exposure to paternal antigens has a protective role against pre-eclampsia. This theory was supported by a study of nulliparous women, whereby women with previous termination of pregnancy with the same partner as the index pregnancy were nearly half as likely to develop pre-eclampsia as women who had a previous termination with a different partner (22). Reduced exposure to paternal antigen, by limited exposure to their sperm is also a risk factor for pre-eclampsia. Women who conceive after a short period of sexual relations, or by alternative techniques such as non-partner donor insemination or intracytoplasmic sperm injection (ICSI) are also at increased risk of disease (10).

1.1.3.2 Obesity

Obesity is another important risk factor for pre-eclampsia; increased body mass index or increased abdominal circumference before pregnancy or in early pregnancy are well established risk factors for the condition (23). The maternal risk of pre-eclampsia increases with increasing degree of obesity, which persists

after accounting for other potential confounding factors (24). This is likely to be related to the altered metabolic state associated with marked obesity rather than the obesity itself. Maternal obesity results in alteration of the plasma lipid profile with higher serum triglyceride and VLDL cholesterol, and lower HDL-cholesterol concentrations than those observed in lean pregnant women. This pattern of dyslipidaemia is similar to that of the "metabolic syndrome" described in the non-pregnant population (25). Obesity is also associated with chronic low-grade inflammation, a feature common to many of the other risk factors for the condition.

1.1.3.3 Diabetes

The association of pre-gestational diabetes and pre-eclampsia is well recognised, and women with a history of diabetes have an up to 4-fold increased risk of development of pre-eclampsia compared to the general population. Recent data from both the UK and the USA suggest that 0.5 - 0.75% of pregnant women have pre-existing type 1 or type 2 diabetes (26). In keeping with the general population, rates of gestational, type 1 and type 2 diabetes in pregnant women rose significantly between 1994 and 2004 in all age groups (27). The increase in rates of type 2 diabetes in pregnant women has been the most striking, and is likely to be related to increased rates of obesity.

Diabetes in pregnancy increases the risk of poor maternal and neonatal outcomes. As well as the increased risk of pre-eclampsia, the condition is also associated with elevated risk of pregnancy loss, maternal infection, polyhydramnios, premature labour and failure to progress in the first or second stage of labour. Foetal and neonatal complications associated with diabetes include congenital malformation, macrosomia, respiratory distress syndrome, hypoglycaemia and jaundice. Further, the babies born to diabetic mothers have been shown in long-term follow up studies to be at increased risk of future obesity and type 2 diabetes themselves (26).

Improving glycaemic control in early pregnancy is associated with a reduced risk of miscarriage and congenital malformations, but it has been less clear whether rates of pre-eclampsia are similarly affected. In a study of 290 pregnant women with type 1 diabetes, Temple et al. (28) reported that $HbA1_c$, an indicator of

long-term glycaemic control, at week 12 and week 24 was strongly associated with pre-eclampsia on univariate analysis. In this study, however, pre-pregnancy care to target pre-conceptual glucose control, and improved glucose levels in very early pregnancy were not associated with a difference in rates of pre-eclampsia.

Gestational diabetes, the onset or first recognition of glucose intolerance in pregnancy, is also dramatically increasing in incidence in the Western world. Until recently there has been a lack of consensus on how the condition should be diagnosed or treated, and in particular it has not been clear how aggressively blood glucose should be monitored or lowered. A recent study designed to investigate the use of metformin and / or insulin in gestational diabetes examined the influence of baseline oral glucose tolerance test (OGTT) results, $HbA1_c$ and both fasting and post-prandial capillary glucose levels on a variety of maternal outcomes (29). Diagnostic OGTT results (from the time of diagnosis of gestational diabetes, at mean gestation 30 weeks) did not predict outcomes, but the key finding was that capillary glucose levels were strongly and independently related to the primary outcome composite of neonatal outcomes, pre-eclampsia and frequency of large for gestational age (LGA) babies. Pre-eclampsia itself was most strongly associated with elevated post-prandial glucose levels, and since fluctuating glucose levels are thought to contribute to endothelial dysfunction in non-pregnant patients with diabetes (30), this is perhaps not surprising.

1.1.3.4 Ethnicity

Pre-eclampsia rates vary significantly around the world; whether this is related to different diagnostic criteria, or whether there are truly differences between different ethnic populations remains uncertain. Further, whether both maternal and paternal ethnicity play a role in determining pre-eclampsia risk is also unclear. A retrospective American cohort study of 127,000 low-risk pregnant women reported that rates of pre-eclampsia were higher among African-American women (5.2%, OR 1.41, 95% CI 1.25-1.62), and lower amongst Latina (4.0%, OR 0.9, 95% CI 0.84-0.97) and Asian women (3.5%, OR 0.79, 95% CI 0.72-0.88) compared to white women (31). Paternal ethnicity followed a similar

pattern, with highest rates in African-American fathers, and lowest rates in Asian fathers. When maternal and paternal ethnic discordance were examined, the overall rate of pre-eclampsia was higher among mothers whose ethnicity differed from the father.

1.1.3.5 Relative risk of individual risk factors

Established risk factors associated with pre-eclampsia, along with relative risk and 95% confidence interval are shown in table 1 below.

Risk Factor	Relative Risk (95% CI)
Nulliparity	2.91 (1.28 to 6.61)
Previous pre-eclampsia	7.19 (5.85 to 8.83)
Age > 40 yrs	1.96 (1.34 to 2.87)
BMI ≥ 35 kg/m^2	1.55 (1.28 to 1.88)
Twin pregnancy	2.93 (2.04 to 4.21)
Diastolic BP > 80 at booking	1.38 (1.01 to 1.87)
Pre-existing diabetes	3.56 (2.54 to 4.99)
Family history (mother or sister)	2.90 (1.70 to 4.93)
Antiphospholipid antibody	9.72 (4.34 to 21.75)

Table 1-2 Maternal risk factors for pre-eclampsia. Adapted from Duckitt et al. (12)

In addition to these well-established risk factors, others have more recently been reported to be associated with pre-eclampsia. Asthma, another condition characterised by chronic inflammation, has been implicated; in a study of 650 asthmatic and 1000 non-asthmatic pregnant women, women with moderate to severe asthma symptoms had an increased risk of developing pre-eclampsia, regardless of treatment, compared to those without symptoms (32). In addition, a history of coronary heart disease in the pregnant woman's father has been recently reported as conferring an almost 2-fold increased risk of pre-eclampsia (3).

The problem with using these factors to stratify pre-eclampsia risk is that many of them are extremely common in the pregnant population. Further, the majority of these risk factors are non-modifiable. The relationship between different risk factors is also uncertain; for example it is not known whether a multiparous woman with high BMI remains at increased risk of pre-eclampsia if her previous pregnancies were uncomplicated.

As women in modern Western society begin to have children at a later stage in life, and as the population as a whole becomes more obese, these risk factors will become less useful in risk stratification. There is now therefore more of a clinical need than ever to identify clinical or biochemical parameters that will help to inform clinicians which women are at increased risk of developing pre-eclampsia.

1.2 Factors that may reduce risk of pre-eclampsia

Although there is no effective treatment for pre-eclampsia other than delivery of the baby and placenta, several potential therapeutic agents have been studied over the years, which are summarised below.

1.2.1 Aspirin

Low-dose aspirin, used almost universally in cardiovascular disease, has been studied extensively for its role in preventing pre-eclampsia. The rationale for its use is that impaired trophoblastic invasion in early pregnancy is thought to lead to activation of platelets and the clotting system, leading to an imbalance of the thromboxane A2 / prostacyclin ratio. Aspirin treatment in pregnancy inhibits synthesis of platelet-derived thromboxane A2, without affecting prostacyclin production, potentially helping to prevent the development of pre-eclampsia (33).

Initial studies using aspirin in the 1980s examined small numbers of very high-risk women (women with a history in a previous pregnancy of severe pre-eclampsia with foetal growth restriction or foetal death) and reported

significant reductions in incidence of pre-eclampsia (34) and in levels of thromboxane A2 (35). These initial promising results were not fully reproducible; a subsequent larger study, including 1100 women at medium and high risk failed to show any significant benefit of aspirin therapy in preventing pre-eclampsia or other adverse pregnancy outcomes (36).

Several other studies have shown a small and non-significant benefit of aspirin use in preventing pre-eclampsia. Two systematic meta-analyses in recent years have therefore pooled data from these studies. Examining 59 (37) and 31 (38) randomised trials respectively, a modest but consistent benefit was seen with aspirin in risk of pre-eclampsia (RR 0.90, 95% CI 0.84 to 0.97) , preterm delivery (RR 0.90, 95% CI 0.83 to 0.98) and of serious pregnancy outcomes (RR 0.90 95% CI 0.85 to 0.96) (38). Results were similar regardless of dose of aspirin used and gestation at initiation of therapy. Subgroup analysis failed to demonstrate exactly which groups of women were most likely to benefit.

Further studies have therefore attempted to elucidate exactly which groups of "at risk" women benefit from aspirin use. Nulliparity, or having a first baby, is one of the most important risk factors for pre-eclampsia, and is the most common. A study which randomised over 3000 healthy nulliparous women to low dose aspirin or placebo reported a relative risk of 0.7 for pre-eclampsia in the aspirin group (95% CI 0.6 to 1.0)(39). The reduction in risk, however, was highest in women with an initial blood pressure in the upper end of the normal range, which may have confounded results. Further, there was a small but significant excess of placental abruption, the most common cause of late pregnancy bleeding, in the aspirin group. A further study using uterine artery Doppler at 23 weeks' gestation to identify high risk women failed to show any reduction in pre-eclampsia incidence with aspirin (18% in aspirin group vs 19% in control group, p=0.6) (40).

The American College of Obstetrics and Gynaecology produced a clinical management guideline for pre-eclampsia in 2002, but the authors were unable to reach a consensus on which women should be treated, and at what stage of gestation they should be treated (41). Reflecting this uncertainty, one study reported that in 2399 women at "high-risk" of the condition (defined as having at least one risk factor), aspirin use varied throughout different regions of the

United Kingdom from 8% to 49% (42). Of the women who did develop pre-eclampsia, only 33% had been on aspirin at any point during their pregnancy.

More recent guidelines from the UK's National Institute for Health and Clinical Excellence (NICE) recommend aspirin prophylaxis from 12 weeks' gestation in high-risk women, who are in turn defined as those with chronic hypertension, diabetes, chronic kidney disease and those with autoimmune conditions such as systemic lupus erythematosis (43). Aspirin prophylaxis is also recommended in women with 2 or more "moderate" risk factors (nulliparity, age >40 years, BMI >35 kg/m^2, family history of pre-eclampsia and twin pregnancies). Aspirin is safe in the second and third trimesters of pregnancy (44) but its safety in the first trimester remains unknown. Given that the underlying pathophysiological mechanisms begin early in the first trimester, further research examining the safety of aspirin in the first few weeks of pregnancy is merited.

1.2.2 Heparin

An alternative therapeutic modality that has been studied in the prevention of pre-eclampsia is the anticoagulant heparin. The placenta in pre-eclampsia displays several features of ischaemia, including villous infarcts and decidual necrosis. Thrombophilias, coagulation disorders associated with thrombosis, are increasingly associated with pre-eclampsia pathogenesis; the antiphospholipid syndrome is associated with a 9 fold increase in relative risk of pre-eclampsia (12), and in a study of 172 pregnant women with a history of pre-eclampsia in a previous pregnancy, 60 (35%) were found to have a thrombophilic defect. These women also had an increased risk of recurrence of pre-eclampsia in a subsequent pregnancy (47% vs 26%, p=0.01) (45).

A small number of studies have examined the use of heparin in preventing pre-eclampsia. One study reported that in women with previous pre-eclampsia but without an inherited thrombophilia, the risk of severe pre-eclampsia or other placental mediated outcomes (birthweight <5th centile, major placental abruption) fell from 24% in the control group to 5.5% in the heparin group (46). When pre-eclampsia was studied alone, however, the results were non-

significant. Other studies examining heparin administration either alone or in combination with aspirin have been too small to be applicable to the general pregnant population.

1.2.3 Calcium supplementation

Several reports over the years have suggested that calcium supplementation can reduce the incidence of pre-eclampsia (47) ; indeed as far back as 1952 it was reported that Ethiopian women, who had a high dietary intake of calcium, had a low prevalence of pre-eclampsia (48). Its use is not recommended in the healthy pregnant population (47), but whether calcium supplementation is beneficial in selected high-risk groups of pregnant women remains uncertain.

To examine this, a Cochrane database systematic review was published in 2006 (49). 12 studies including over 15,000 pregnant women, comparing calcium supplementation of 1g with placebo, were included in the analysis. The authors reported an overall relative risk in the calcium supplement group of pre-eclampsia of 0.48 (95% CI 0.33 to 0.69). In high-risk women (defined as those with previous pre-eclampsia, teenagers, those with increased sensitivity to Angiotensin II and those with pre-existing hypertension) the relative risk was 0.38 (95% CI 0.21 to 0.68). The promising results of this meta-analysis, however, were reported by the authors to be potentially confounded by the inclusion of several small trials with a high proportion of women with low dietary calcium intake.

The importance of the baseline characteristics of the pregnant women being studied was demonstrated further in the calcium to prevent pre-eclampsia (CPEP) trial. Over 4500 healthy nulliparous women, at 13 to 21 weeks' gestation, were randomised to either 2g of calcium daily or placebo. Between the two groups there was no significant difference in rates of pre-eclampsia (6.9% in the calcium group, 7.3% in the placebo group), gestational hypertension, small for gestational age (SGA) babies, preterm deliveries, or foetal or neonatal deaths. It therefore remains uncertain whether calcium supplementation in those with a normal dietary intake is beneficial; a recent systematic review by the US Food and Drug Administration (FDA) concluded that a relationship between calcium

supplementation and gestational hypertension or pre-eclampsia was unlikely (50).

1.2.4 Anti-oxidants

1.2.4.1 Vitamins C&E

A number of studies have been performed in recent years to examine the role of antioxidants in the prevention of pre-eclampsia. Oxidative stress is thought to play a role in the development of pre-eclampsia, and markers of oxidative stress have been reported to be elevated in the circulation of affected women (51).

The antioxidants vitamins C and E, both relatively cheap and widely available, have been extensively studied in this context. In an initial study, high-risk women (those with abnormal uterine artery Doppler studies or a previous history of the condition) were randomised to either 1g of Vitamin C and 400 iu of Vitamin E per day, or placebo, from 16-22 weeks' gestation until delivery. Markers of endothelial activation (Plasminogen-activator inhibitor 1, PAI-1) and placental dysfunction (PAI-2) were measured monthly until delivery. The authors reported a 21% reduction in the PAI-1 /PAI-2 ratio during gestation; the rate of pre-eclampsia in the placebo group was 17% compared with 8% in the treatment group (52). Following these promising results, the same group went on to carry out a much larger multicentre study, known as the Vitamins in Pre-eclampsia (VIP) study (53). Over 2400 women were randomised to take either Vitamin C and E (in the same doses as the previous study) or placebo; incidence of pre-eclampsia was 15% in the treatment group and 16% in the control group. Rather than showing any benefit, in fact the treatment group had a higher incidence of low birthweight babies (28% vs 24% in the placebo group, p=0.023) leading to some concern about the safety of such agents.

A World Health Organisation (WHO) study examined high-risk women with low socio-economic and nutritional status from centres in Peru, India, Vietnam and South Africa, women thought to be the most likely to benefit from vitamin supplementation. Following the same protocol as the VIP study, this paper also reported that vitamin supplementation did not prevent against pre-eclampsia, eclampsia, gestational hypertension, or any other maternal outcome. Further,

there was no difference between the groups in preterm deliveries or low birth weight babies (54). A further study which examined over 10,000 unselected low-risk nulliparous women again showed no difference in rates of pregnancy-induced hypertension, pre-eclampsia or adverse neonatal outcomes between those treated with vitamins C & E and controls (51).

As mentioned above, women with diabetes are at two to four times increased risk of developing pre-eclampsia compared to the general population. Those with complications related to diabetes such as diabetic nephropathy are at further increased risk; indeed the presence of microalbuminaemia in early pregnancy is associated with a four-fold increase in risk of pre-eclampsia (55). Given that type 1 diabetes is associated with increased oxidative stress and depletion of antioxidants, it seems logical that these women could benefit from antioxidants such as Vitamins C and E during pregnancy. In a large double blinded randomised controlled trial, over 700 pregnant women with type 1 diabetes were treated with either vitamins C and E or placebo. No difference was seen between the 2 groups in rates of pre-eclampsia, gestational hypertension, low birthweight deliveries, or in PAI-1 / PAI-2 ratio (56).

Overall, these studies provide evidence of the lack of effectiveness of Vitamins C and E for the prevention of pre-eclampsia, when started in the first and second trimesters, in both high-risk and low-risk pregnant women. Whether they could help to prevent pre-eclampsia if given in earlier pregnancy, or pre-conceptually, remains unclear.

1.2.4.2 Fish oils

The relatively low incidence of pre-eclampsia and preterm delivery in populations with a fish-based diet such as inhabitants of Greenland and the Faroe Islands have led to several studies examining the potential role of fish oil supplements in preventing pre-eclampsia (57). Fish oils are rich in long-chain polyunsaturated fatty acids, precursors to the 3-series prostaglandins, and have been shown to modulate vascular and inflammatory effects via down-regulation of the thromboxane A2 pathway (57). The Fish Oil Trials In Pregnancy (FOTIP) study randomised 386 pregnant women with a history of hypertension in a previous pregnancy to either fish oils or olive oil at gestational week 20. A

further 79 women who had developed hypertension in the current pregnancy were also randomised at 24-30 weeks' gestation in a "treatment" arm. No effect was seen on either incidence or development of hypertension (58). A Cochrane database meta-analysis of 6 trials of 2755 women similarly found no beneficial effects of fish oil supplementation (57).

1.2.5 Cigarette smoking

There can be little doubt that cigarette smoking has an adverse effect on pregnancy. As well as the maternal association with chronic lung disease, preterm delivery and placental abruption, smoking is associated with an increased risk of foetal complications such as stillbirth, intra-uterine growth restriction (IUGR), placenta praevia and spontaneous abortion. Paradoxically, however, smoking has been consistently shown over the years to be associated with a reduced risk of pre-eclampsia; smokers were shown in a systematic review of the literature to have a 32% lower incidence of pre-eclampsia compared to non-smokers (59).

The GOPEC (Genetics of Pre-Eclampsia) consortium analysed over 1000 women with moderate to severe pre-eclampsia in a multi-centre study, and carried out a sub-analysis to analyse the relationship between smoking and pre-eclampsia. 9% of the women with pre-eclampsia admitted to being current smokers, compared to an estimate of 27% in the general pregnant UK population (60). In women with pre-eclampsia, smokers had a 2-fold increased risk of requiring delivery at less than 34 weeks' gestation, of delivering babies with a birthweight below the 3rd centile, or of any adverse outcome (60). In addition the risk of developing eclamptic seizures was increased five-fold in smokers compared to non-smokers. These data would appear to suggest therefore that smokers are less likely to develop pre-eclampsia than non-smokers, but that when they do, they develop a more severe form of the disease. The question remains; why are smokers less likely than non-smokers to develop pre-eclampsia? One theory proposed by the GOPEC consortium was that foetal demands in later pregnancy trigger the onset of pre-eclampsia, and since smokers tend to have smaller babies, they may put the placenta under less strain and so lessen the risk of pre-eclampsia (60).

The “protective” effects of smoking appear not to extend to chewed tobacco. In a large epidemiological study of over 600,000 Nordic women, Wikström et al. (61) concluded that the use of Swedish snuff, a smokeless form of tobacco, was not associated with a reduction in rates of pre-eclampsia. They suggested that it was not the constituents of tobacco such as nicotine that were protective, but rather it was combustion products from smoking, such as carbon monoxide (CO) that influenced the reduced rates. At the time of trophoblastic implantation, the uterus is relatively hypoxic, and it has been previously proposed that CO increases effective trophoblastic invasion in the first few weeks of pregnancy (62). CO also has been shown to contribute to utero-placental blood flow, and to reduce the inflammatory response at the spiral arterioles, both mechanisms known to contribute to pre-eclampsia. Further, smoking causes a down-regulation of endothelial sensitivity to activating signals (22), and so chronic endothelial injury caused by chronic smoking may in fact protect the mother from the endothelial dysfunction caused by pre-eclampsia.

A further interesting observation from the Swedish population study was that change in tobacco habits during pregnancy also appeared to affect pre-eclampsia rates. Women who reported tobacco use at the first antenatal visit but not at 30-32 weeks’ gestation did not have reduced rates of pre-eclampsia, whereas those who reported no tobacco use initially, but were smoking at 30-32 weeks’ gestation did (61). This may imply that smoking in the second half of pregnancy is necessary for reducing the risk of pre-eclampsia.

Any study examining the effects of smoking in pregnancy relies on accurate self-reporting of smoking rates, and this may be a source of bias in these studies. Regardless of whether or not smoking protects against pre-eclampsia, it is clear that any strategies that help to reduce smoking rates in pregnancy must remain a public health priority.

1.2.6 Lifestyle measures

Up until relatively recently, it was recommended that women increase their calorific intake and reduce physical activity during pregnancy. Current

guidelines, however, advocate regular exercise in pregnancy; as well as helping to prevent long term obesity, one of the perceived beneficial effects is a reduction in risk of pre-eclampsia. Exercise is well known to reduce risk of hypertension in non-pregnant subjects, while markers of insulin resistance and endothelial dysfunction known to be elevated in pre-eclampsia are also reduced by physical activity. Exercise could in theory, however, also be associated with an increased risk of pre-eclampsia; exercise may be associated with increased oxidative stress (63), one of the potential pathophysiological mechanisms for the development of pre-eclampsia. As a result there remains conflicting advice from health-care professionals about exercise during pregnancy, and some women remain concerned about the potentially harmful effects of vigorous exercise on the foetus.

A study reported in 2003 retrospectively examined 201 women with pre-eclampsia and 383 women with normotensive pregnancies (64). Women were given a structured questionnaire about type, duration, frequency and intensity of exercise both during pregnancy, and in the year preceding the pregnancy. The authors reported an overall 35% reduced risk (95% CI 0.43 to 0.99) of pre-eclampsia in women who took regular exercise in the first 20 weeks of pregnancy, compared to inactive women (64). This risk reduction remained significant when corrected for age, BMI, parity, smoking and race. The risk of pre-eclampsia was inversely related to the frequency and intensity of exercise. An additional finding was that women who had taken regular exercise in the year before pregnancy had a similar risk reduction to those who exercised during pregnancy (OR 0.67, 95%CI 0.42 to 1.08) (64).

Other studies have, however, been less convincing about the beneficial effects of exercise in pregnancy. A prospective Scandinavian study, using a questionnaire at gestational week 14-22, reported an overall reduction in risk of pre-eclampsia with exercise of 21% (95% CI 0.65-0.96). This effect was most marked in women with a BMI within the normal range, and no beneficial effect of exercise was seen with pregnancy in those with a BMI>30 kg/m^2, leading authors to suggest that the beneficial effects of exercise in pregnancy only applied to the non-obese population (65). A further prospective study, using a telephone interview in the first trimester, did not show any overall protective benefit of exercise in the first trimester. In contrast, authors reported that

women with more intensive exercise (more than 270 minutes per week) had an increased risk of severe forms of pre-eclampsia (63). A prospective study in which women were randomised to exercise using a home-based cycling programme from 20 weeks' gestation to delivery was recently reported. Women who exercised regularly delivered babies with lower birth weight which remained significant after correction for sex and gestational age at delivery. In contrast to the authors' original hypothesis, no difference was seen between the 2 groups in blood markers of insulin resistance, gestation at delivery, or maternal BMI (66).

A review of the literature in this field led to American College of Obstetrics and Gynaecology (ACOG) recommendations that in the absence of medical or obstetric complications pregnant women partake in 30 minutes of moderate exercise daily (67). Similarly, NICE guidelines recommend that "beginning or continuing a moderate course of exercise is not associated with adverse outcomes." (68) Further large-scale prospective studies looking at both foetal and maternal outcomes are required before clinicians can give definitive guidance about optimal exercise duration and type during pregnancy.

1.2.7 Bariatric surgery

While the benefits and risks of exercise in pregnancy remain uncertain, there can be no doubt that obesity should be avoided both in pregnancy and in those considering pregnancy. A third of all women of reproductive age are obese (69), and obesity is an important risk factor for pre-eclampsia, as well as for other complications of pregnancy including gestational diabetes, preterm delivery and congenital anomalies.

Bariatric surgery has emerged in the last decade as the single most effective treatment for obesity. Surgical procedures, including the Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding, work mainly by restricting calorific intake via a smaller stomach reservoir, and have been reported to have enormous beneficial effects on blood pressure, cholesterol and diabetes (70).

Since bariatric surgery is a relatively new technique, there is limited experience of bariatric surgery in young women of reproductive age, and little is known about the effects of such surgery on pregnancy-related complications. A recent study used retrospective analysis of insurance claim forms to examine whether pre-eclampsia and other hypertensive disorders were affected by bariatric surgery. 585 women who had undergone bariatric surgery for obesity were included; 269 had delivered a baby prior to surgery and 316 had had their surgery prior to pregnancy (71). Women with surgery prior to pregnancy had rates of pre-eclampsia and eclampsia of 3% compared to 15% those with surgery after pregnancy ($p<0.001$), lower rates of pregnancy induced hypertension (2.5% vs 13%, $p<0.001$), and lower rates of pre-eclampsia superimposed on chronic hypertension (1% vs 12% $p>0.001$).

As the global obesity epidemic spreads, progressively more morbidly obese women will be considering pregnancy. The American College of Obstetrics and Gynaecology recently recommended that bariatric surgery should be considered pre-pregnancy in women with a BMI > 40, and in those with a BMI > 35 in association with co-morbidities including chronic hypertension (72). Future studies will determine whether the beneficial effects of such surgery on mothers and their children will be long-standing.

1.3 Screening tests for pre-eclampsia; clinical studies

There have been many screening tests evaluated in the literature over the years for predicting pre-eclampsia; these have been comprehensively reviewed in a World Health Organisation publication (73). A further summary of predictive tests, including a review of preventative interventions and economic modelling has been published by the UK National Institute for Health Research (74). The most promising biochemical and clinical parameters that have been studied will be discussed here.

1.3.1 Uterine artery Doppler studies

The advent of ultrasound has revolutionised the practice of obstetrics in the last 50 years, by offering a window to the womb through which the anatomic structures of the foetus can be evaluated. The more recent addition of Doppler flow studies of the maternal and foetal vessels have provided further useful information, allowing assessment of the physiology of the foeto-maternal unit. Doppler studies are non-invasive, acceptable to patients, and can be carried out at the same time as a detailed anomaly scan, and as such have been studied extensively for their role in screening for adverse foetal and maternal outcomes.

1.3.1.1 Doppler studies in normal pregnancy

In the non-pregnant state there is a rapid rise and fall in uterine artery flow velocity during systole and a "notch" in the descending waveform in early diastole. As discussed in section 1.1.2, remodelling of the spiral arteries in early pregnancy is an important step in regulating and maintaining placental perfusion. The reduction in resistance of the spiral arteries as a result of remodelling in pregnancy can be reflected in uterine artery Doppler studies, by a high diastolic velocity with continuous flow throughout diastole, and the loss of the diastolic "notch" at 20 to 24 weeks' gestation (figure 1.3.) (75). These changes can be quantified by demonstrating changes in the resistance index (RI, maximum – minimum velocity / maximum velocity) and the pulsatility index (PI, maximum – minimum velocity / mean velocity) of the uterine vessels which begin to decrease in normal pregnancy from between 8 and 18 weeks' gestation (75).

1.3.1.2 Doppler studies in pre-eclampsia

In pre-eclampsia the remodelling of the spiral arteries is impaired; the spiral arteries maintain their muscular elastic coating, and impedance to blood flow persists. This pathological resistance to placental flow can be detected by Doppler studies of the maternal uterine vessels, offering the potential to detect women at risk not only of pre-eclampsia, but also of intra-uterine growth restriction. The majority of research has focussed either on an elevation in RI or PI using percentile cut-off values, or by persistence of the diastolic "notch."

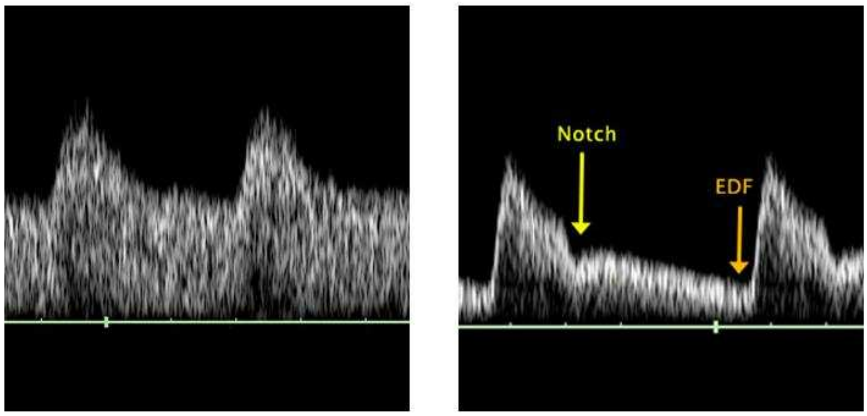

Figure 1-3 Week 28 uterine artery Doppler studies at week 28 in a woman with a normal pregnancy (left) and in a women with pre-eclampsia showing persistent "notch" and low flow at end-diastole (right). From Doppler in Obstetrics, Nicolaides et al.

Abnormal Doppler studies in both the first and second trimesters have been reported to be associated with pre-eclampsia. Abnormalities are detectable as early as 12 weeks' gestation (8); for women with abnormal first trimester testing the likelihood ratio (LR) for development of pre-eclampsia is approximately 5, while normal Doppler studies carry an LR of 0.5 (75). Although this relationship persists into the second trimester, the optimal timing for performing these studies remains uncertain.

The use of uterine artery Doppler was perhaps best summarised in a recent comprehensive meta-analysis. Cnossen et al. (76) reviewed 74 uterine artery Doppler studies including nearly 80,000 pregnant women, of whom 2,500 developed pre-eclampsia. The majority of Doppler indices had poor predictive characteristics, but this varied with patient group studied and severity of disease. For all women the overall risk of pre-eclampsia was best predicted by an elevated second trimester PI accompanied by persistent bilateral uterine artery notching. For women deemed on the basis of risk factors to be at low-risk, positive likelihood ratio (+LR) was 7.5, and negative likelihood ratio (-LR) was 0.59. For women at high risk of developing pre-eclampsia, +LR was 21, and -LR was 0.82.

As a result of these findings the authors concluded that Doppler studies were more accurate for prediction of future pre-eclampsia when performed in the second trimester rather than the first trimester. Since the false positive rate was relatively high, they felt that the increased anxiety and expense associated with abnormal results could not justify their use for screening in low-risk women.

The majority of studies using uterine artery Doppler studies to predict pre-eclampsia have been performed in large centres with significant experience and expertise in their use. It remains to be seen whether these findings are applicable in smaller centres, or in the developing world where they would be more likely to be clinically useful. The tests are also relatively time-consuming, taking around 20 minutes to perform (75), which may preclude their use in the general pregnant population. The combination of these studies with maternal risk factors, or with analysis of serum markers such as anti-angiogenic factors (77) may ultimately be more clinically useful.

1.3.2 Blood pressure measurement

Although blood pressure measurement is clearly essential for the detection of hypertensive disorders of pregnancy, it remains to be seen whether measurement of blood pressure in the first or second trimesters can predict which women will develop problems later on. Increased diastolic blood pressure is associated with an increased risk of pre-eclampsia (12), but studies using assessment of blood pressure in the first or second trimester for prediction of pre-eclampsia have reported false-positive rates ranging from 7 - 52% and detection rates ranging from 8 – 93% (78). It is likely that these differences relate to differences in the populations studied, different techniques used to measure blood pressure, and different definitions of pre-eclampsia. A meta-analysis of 34 studies evaluated the role of systolic pressure (SBP), diastolic pressure (DBP), mean arterial pressure (MAP) and increase in blood pressure during the first or second trimester to predict pre-eclampsia in low and high-risk women (79). MAP was shown to be superior to SBP, DBP and increase in blood pressure between the first and second trimester in predicting pre-eclampsia;

second trimester MAP of ≥90 mmHg was associated with a positive likelihood ratio of 3.5 for pre-eclampsia and a negative likelihood ratio of 0.46.

Ambulatory blood pressure monitoring (ABPM) allows multiple readings to be taken using automated blood pressure devices, in a non-clinic environment. As such this may provide a better estimate of true blood pressure, since it is not confounded by the "white-coat" effect (80). Few studies have examined ABPM in the context of pre-eclampsia prediction, and the majority of these are suboptimal (81). One study which examined over 1100 women who had 24 hour ABPM between 18 and 24 weeks' gestation did not find ABPM to be a useful predictor of hypertension later on in pregnancy (80), since absolute differences in ABPM measurements were small, and overlap between hypertensive and normotensive women was high.

1.3.3 Sympathetic nervous system activation

Since vascular tone is largely determined by the sympathetic nervous system, it has been proposed that sympathetic vasoconstrictor activity may be important in regulating the increase in peripheral resistance seen in pre-eclampsia. Indirect evidence of this association is provided by methyldopa, a drug which acts by reducing central sympathetic outflow, which was previously the drug of choice for hypertensive disorders of pregnancy. Plasma catecholamines have also been reported to be elevated in pre-eclampsia (82), but these are insensitive markers of sympathetic nervous system activity, and their levels are influenced by several different factors.

More recent studies have used alternative techniques to measure sympathetic nervous system activity in pregnancy and pre-eclampsia. Schobel et al. (83) measured postganglionic action potentials in sympathetic nerve-fibres innervating vessels in skeletal muscle of women with pre-eclampsia. They found that sympathetic discharge to skeletal muscle was elevated in women with pre-eclampsia compared to women with normal pregnancies, and normalised after delivery. Age-matched non-pregnant women with essential hypertension did not have such increases in sympathetic activity. It seems likely therefore that the increase in vascular resistance in pre-eclampsia is due at least in part to an increase in sympathetic vasoconstrictor activity. Any method for its

measurement in pregnancy, however, is unlikely to be feasible in large clinical studies of pregnant women.

1.3.4 Studies of endothelial function

The vascular endothelium is thought to be responsible for many of the physiological changes in normal pregnancy such as reduction in blood pressure and peripheral resistance, changes which are largely thought to be regulated by nitric oxide. Pre-eclampsia is associated with vasoconstriction of a number of maternal beds, and dysfunction of the endothelium is thought to have a key role in its pathogenesis. Detection of endothelial dysfunction remains difficult; the gold standard for measurement of endothelial function is angiographic response to intra-arterial injection of acetylcholine. This method is clearly too invasive to be used in large clinical studies, while the majority of other methods of assessment of endothelial function cannot be safely used in pregnancy. Less invasive methods for the detection of endothelial dysfunction that are safe in pregnancy and applicable in large scale studies are therefore required.

1.3.4.1 Brachial artery ultrasound

Measuring the change in brachial artery diameter as a response to increased flow (flow-mediated dilatation, FMD) using high-resolution ultrasound has emerged as a reliable non-invasive method for detection of endothelial dysfunction. FMD after a 5 minute period of occlusion with a blood pressure cuff has been shown to depend mainly on nitric oxide release (84), and endothelial dysfunction measured using this technique has been shown to correlate with response noted on coronary angiography after acetylcholine injection (85). Pre-test preparation is important, since a number of factors can affect FMD measurement including caffeine, alcohol, temperature and the stage of women's menstrual cycle.

Normal pregnancy has been reported to be associated with an increase in FMD of the brachial artery of 38%, an increase which is apparent from at least 10 weeks' gestation (84). Baseline brachial artery diameter and volumetric flow also increases significantly in a linear fashion, in keeping with the generalised

vasodilatation seen in pregnancy. From around 32 weeks, FMD falls significantly, and by 6 weeks post-partum levels fall to non-pregnant values (86).

If FMD is to be useful as a screening tool for pre-eclampsia, it is important to understand the effect that maternal risk factors for pre-eclampsia have upon results. Kinzler et al. (87) examined 28 high risk women (7 with pre-gestational diabetes, 4 with chronic hypertension, 6 with twins, and 11 with a history of pre-eclampsia in a previous pregnancy), as well as 44 low risk women, of whom 11 were nulliparous. They found no difference in FMD after 5 minutes occlusion between the first and second trimesters, in either high or low-risk women. High-risk women had a significantly reduced brachial artery diameter change compared to low-risk women in both trimesters. Women with pre-gestational diabetes and chronic hypertension had significant reductions in response compared to low-risk women, while women with twin pregnancies had an increased response compared to women with singleton pregnancies. When low-risk women were analysed alone, nulliparous women had increased response compared to multiparous women. Potential limitations of this study were that it did not include non-pregnant controls, that high-risk women were older than those at low-risk, and that women who went on to develop pre-eclampsia were not analysed separately.

FMD has been reported to be reduced in women at the time of diagnosis of pre-eclampsia compared to normotensive women (88), but it is unclear whether this is a cause or a consequence of the disease. Few studies have examined the ability of brachial artery FMD to predict future pre-eclampsia. Garcia et al. (89) examined 506 Colombian women at gestational week 22, of whom 14 subsequently developed pre-eclampsia. The women who went on to develop pre-eclampsia had a lower FMD (13.4% ± 4.3 vs 18.2% ± 7.2, p=0.022) than those with normal pregnancies.

1.3.4.2 Ex-vivo studies

Further evidence for the relationship between endothelial dysfunction and pre-eclampsia has been provided by measurement of FMD using ex-vivo organ bath

studies of small arteries. Cockell et al. (90) examined small arteries dissected from subcutaneous fat samples obtained during Caesarean section and gynaecological surgery. Arteries from women with pre-eclampsia, women with normotensive pregnancy, and non-pregnant healthy controls were of similar internal diameter, and constricted appropriately in response to addition of noradrenaline. Arterial response to increments of intraluminal flow was then recorded; vessels from normotensive women showed substantial flow-induced relaxation, while those from non-pregnant women and from women with pre-eclampsia demonstrated modest constriction. The authors concluded that flow-induced shear stress is part of the physiological stimulus to vasodilatation in normal pregnancy, and that failure of this vasodilatation can contribute to the hypertension which is seen in pre-eclampsia.

1.3.5 Studies of arterial stiffness

Normal pregnancy is associated with a number of changes to the maternal circulation, with an increase in heart rate and cardiac output and a corresponding reduction in vascular resistance and arterial blood pressure. In contrast pre-eclampsia is characterised by an increase in vascular resistance and generalised vasoconstriction (91). The traditional measurement of peripheral blood pressure using sphygmomanometry means that much of the information contained in the shape of the arterial waveform is lost; it may be that assessment of central pressures and arterial stiffness can provide further information about risk of development of pre-eclampsia.

1.3.5.1 Pulse wave analysis

Although the arterial pulse waveform has been used as an assessment tool since the end of the 19th century, it is only more recently that technological advances have allowed detailed recording and analysis of the waveforms for clinical use. Applanation tonometry is one such technique that allows non-invasive assessment of the various components of the pulse wave, which is simple, validated, non-invasive and reproducible (92).

1.3.5.2 Physiology

Left ventricular ejection pumps blood into the arterial tree, creating a pulse pressure wave that travels forward in the arterial system. Once the wave reaches areas of bifurcation or other areas of impedance mismatch, a retrograde (reflected) waveform is initiated (93). The shapes of the arterial pulse waveforms vary at different sites, related to both varying elastic qualities along the arterial tree and to wave reflection. The wave form at the proximal aorta is important since it is the blood pressure profile here rather than the peripheral blood pressure which determines left ventricular afterload and coronary artery blood flow. The contour and amplitude of the pressure waveform are influenced by large artery pulse wave velocity (PWV); with compliant arteries and slow PWV, reflected waves return to the central aorta in diastole, augmenting DBP and subsequently coronary blood flow. On the contrary when arteries are less compliant and PWV is faster, the reflected waves return earlier and augment central SBP, increasing left ventricular workload and compromising coronary blood flow (94).

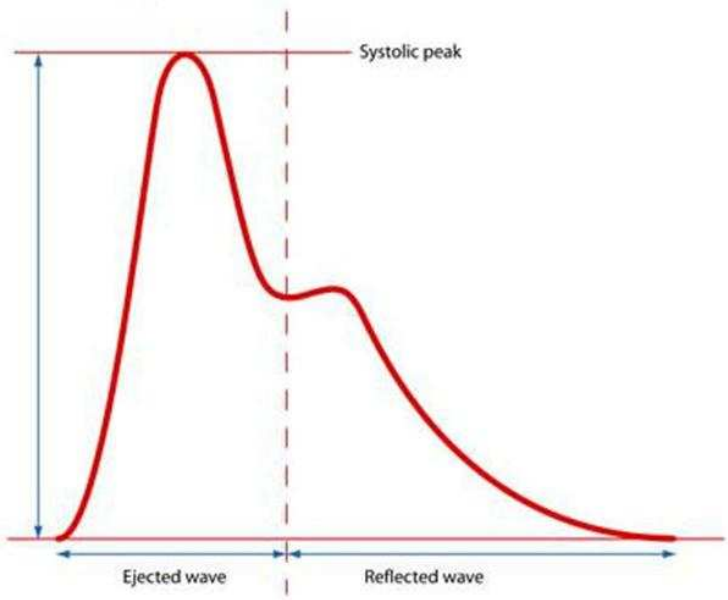

Figure 1-4 Typical arterial pulse waveform showing ejected wave and reflected wave

Pulse wave analysis (PWA) recordings are generated from pulse pressure waveforms obtained with applanation tonometry. When recorded at the radial

artery the waveform is calibrated to the brachial BP (measured using an oscillometric machine). The SphygmoCor software uses a generalised transfer function to derive central aortic waveforms from those acquired from the peripherally acquired arterial waveform.

The characteristics of the transfer function generated by SphygmoCor are determined by the physical properties of the arterial system, such as arterial diameter, wall elasticity, wall thickness and the condition of the peripheral vascular beds. Not all brachial vasculature is identical in all adults, and it would be expected that there are differences in the overall transfer function among individual subjects. More recently, however, the transfer function has been validated under different conditions including age, disease, medication and state of vasodilatation (95), supporting its use in the generalised population. The transfer function has not, however, been validated for use in pregnancy. Millasseau and colleagues have previously suggested that similar information on central pressure wave reflection can be obtained directly from the radial pulse and radial AIx without use of a generalised transfer function, and so it may be that such generalised transfer functions are not necessary (96).

From the generated central aortic waveform central blood pressure values and the augmentation index (AIx) can then be calculated. The AIx is the proportion of central PP that results from arterial reflection and is a commonly used measure of arterial stiffness. A typical central pressure wave is shown in figure 1.5.

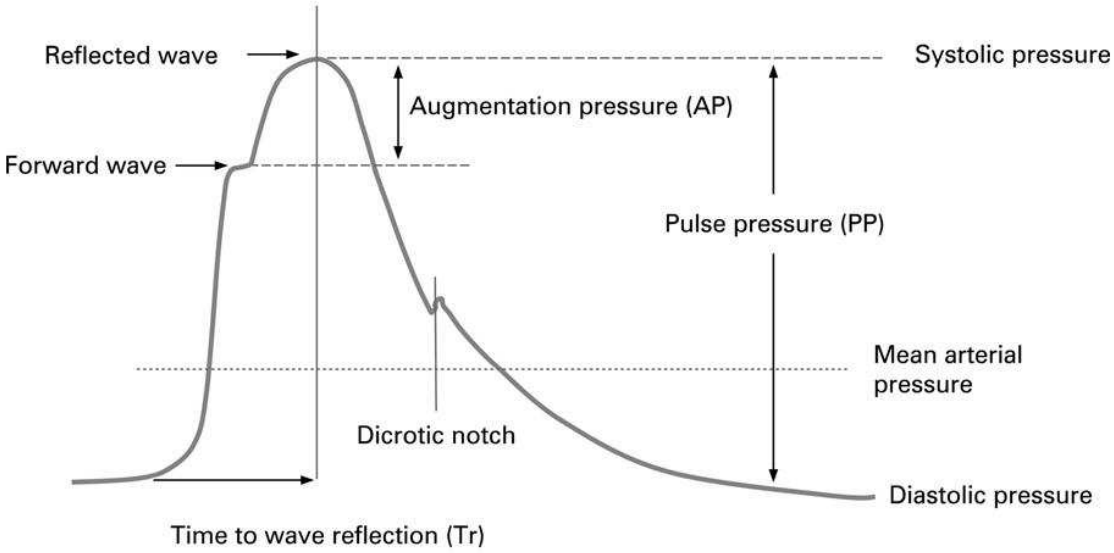

Figure 1-5. An aortic pulse waveform as produced by the SphygmoCor system from applanation tonometry of the radial artery. Augmentation pressure is the difference between the systolic peak (forward wave) and first systolic inflection (reflected wave) pressures. This difference divided by the pulse pressure generates the augmentation index, from Mills et al. (97)

PWA is widely used as a measurement of arterial stiffness, although the use of a generalised transfer function for estimation of central pressures has led to some criticism of this technique (98). AIx is affected by changes in heart rate; an increase in heart rate shortens the duration of systole. As a result the reflected wave reaches the advancing wave in diastole rather than systole, resulting in reduced augmentation of the advancing wave and reduced AIx. Many PWA software systems therefore standardise AIx for a heart rate of 75 beats per minute (AIx75).

1.3.5.3 PWA in cardiovascular disease

Arterial stiffness is increasingly recognised as an important technique in the assessment of cardiovascular disease, since aortic stiffness and arterial pulse wave reflection are key determinants of central systolic and diastolic pressures

(98). In addition, arterial stiffness as assessed by PWA has been shown to be associated with multiple traditional risk factors for cardiovascular disease; both AIx and augmentation pressure (AP) are independent risk markers for premature coronary artery disease (99). In patients with end stage renal failure, AIx is a highly predicative indicator of cardiovascular mortality (100). As with the other vascular parameters derived using applanation tonometry, AIx is sensitive to modulations of NO bioactivity. Inhibition of basal NO synthesis, with intra-arterial infusion of L-N^G-monomethyl arginine (L-NMMA), has been shown to lead to a dose dependent increase in mean arterial pressure, peripheral vascular resistance, and both aortic and systemic arterial stiffness (101).

As well as being used in the assessment of cardiovascular disease, the possibility of targeting arterial stiffness with therapeutic strategies has been a topic of recent interest. Increased arterial stiffness may be reversible; one study reported that smoking cessation is associated with an improvement in parameters of arterial stiffness, returning to levels found in non-smokers after 10 years of cessation (102). The effect of different blood pressure agents on AIx has also been examined in a study in which 59 patients with untreated isolated arterial hypertension were randomised to receive either an angiotensin converting enzyme (ACE) inhibitor, a thiazide diuretic, a beta-blocker or a calcium channel blocker. Although all 4 drugs led to a similar reduction in blood pressure, beta blockers did not reduce pulse pressure. Only calcium channel blockers led to a reduction in AIx, while beta blockers appeared to have a deleterious effect (103).

It may be, therefore, that PWA can aid in the stratification of patients with hypertension and cardiovascular disease; this potential was highlighted by the inclusion of PWA in the 2003 guidelines from the European Society of Hypertension (ESH) on the management of arterial hypertension (104).

1.3.5.4 PWA in pre-eclampsia

It is well established in the non pregnant population that arterial stiffness is associated with traditional risk factors for cardiovascular disease. Given that

pre-eclampsia and cardiovascular disease share a number of common mechanisms, several studies have examined the relationship between arterial stiffness and pre-eclampsia. In non-pregnant women, AIx decreases during the luteal phase of the menstrual cycle, and rises at the beginning of the menstrual cycle (105). AIx is lower in pregnant women compared to non-pregnant controls, and within pregnancy AIx decreases further, reaching its nadir at mid-pregnancy and rising towards term (106-108). This fall is thought to be related to NO-mediated vasodilatation.

Several studies have shown that, at term, women with pre-eclampsia have increased AIx, indicating increased arterial stiffness, compared to non-pregnant controls (91,109,110). Women with pre-eclampsia have a higher AIx compared to women with gestational hypertension without proteinuria, an increase that persists after correction for blood pressure (110). No difference has been reported between women with pre-eclampsia at term and those with pre-eclampsia requiring preterm delivery (105), nor has a difference been reported between different ethnic groups studied (95).

Although these studies may help our understanding of vascular haemodynamics in pre-eclampsia, in order to be a clinically useful test for its prediction these abnormalities would need to be detectable prior to the onset of clinical disease. Due in part to the rarity of pre-eclampsia, few longitudinal studies have been performed. One study examined PWA in 210 women with singleton pregnancies at gestational week 11-14, at the initial antenatal hospital “booking” visit. Of them, 14 (6.7%) developed pre-eclampsia and 196 remained normotensive throughout pregnancy. AP and AIx75 were significantly elevated in the women who went on to develop pre-eclampsia compared to normotensive controls, while the 3 women who developed severe pre-eclampsia had a higher AIx75 than those with mild disease (95). For a false positive rate of 11%, pulse wave analysis predicted 79% of women who went on to develop pre-eclampsia and 88% of those who developed severe disease. The relatively high incidence of pre-eclampsia in the study cohort, and the low numbers of affected women mean that these findings may not be applicable to the general pregnant population; this area merits further research.

1.4 Screening tests for pre-eclampsia; biomarkers

1.4.1 Angiogenic factors

As research in the field of pre-eclampsia progresses, much of the attention in recent years has been focused on peptides related to angiogenesis. Angiogenesis, the development of new blood vessels from existing endothelium, is essential for normal placental development. Two of the angiogenic growth factors, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are thought to contribute to normal trophoblastic proliferation and implantation (111), and it has been hypothesised that an imbalance in levels of these growth factors has a crucial role in pre-eclampsia. As normal pregnancy progresses, maternal VEGF expression is reduced (112), but placental levels of mRNA encoding VEGF have been shown to be much lower in women with pre-eclampsia compared to controls (113). Similarly, maternal plasma levels of PlGF have been reported to be significantly reduced in the second trimester in women who went on to develop pre-eclampsia compared to controls (114). The use of anti-VEGF antibodies for systemic treatment in cancer is associated with a dose dependant increase in rates of hypertension and proteinuria (115), which may further indicate the role of these factors in the development of pre-eclampsia. Many recent studies have therefore concentrated on factors which antagonise VEGF and PlGF, to assess their role in the development of pre-eclampsia. Two of the most extensively studied peptides, which are produced by the placenta, are soluble FMS-like tyrosine kinase (sFLT-1) and soluble endoglin.

1.4.1.1 Soluble FMS-like Tyrosine Kinase

Soluble FMS-like tyrosine kinase (also known as soluble VEGF receptor 1 or sFLT-1) is a secreted splice variant of FLT-1. It binds to and neutralises the angiogenic actions of VEGF and PlGF (116), and is thought to be one of the key peptides involved in the development of pre-eclampsia. Maternal serum levels of sFLT-1 have been shown to be elevated in women with pre-eclampsia compared to

normotensive controls (117-119), to correlate with disease severity (119), and to decrease markedly following delivery (117). It has also been reported that levels of sFLT-1 are increased in women during their first pregnancy (an important risk factor for development of pre-eclampsia) when compared to multiparous women (120). A series of studies by Maynard et al. (121) revealed that mRNA of sFLT-1 is up-regulated in the placenta of women with pre-eclampsia, leading to increased systemic levels. Furthermore, these authors demonstrated that when a recombinant adenovirus encoding sFLT-1 was injected into pregnant rats, hypertension and proteinuria, as well as glomerular endotheliosis, one of the typical pathological lesions seen in pre-eclampsia, were observed.

Further evidence for the placental origin of the elevated sFLT-1 was provided by Staff et al. (122) Given that "delivery" includes both the foetus and the placenta, the group investigated whether foetal as well as maternal levels of sFLT-1 were elevated in pre-eclampsia. They found that although foetal levels of sFLT-1 (measured in cord blood) are elevated in pre-eclampsia, the maternal serum levels were 29-fold higher, and concluded that there was no substantial foetal contribution to the elevated circulating maternal sFLT-1 levels seen in pre-eclampsia. Further evidence for the role of sFLT-1 in pre-eclampsia is that levels have also been reported to be increased in women with pre-eclampsia superimposed upon systemic lupus erythematosis (123) and glomerulonephritis (124).

1.4.1.2 sFLT-1 and smoking

As discussed in section 1.2.5, smokers appear to be relatively protected against pre-eclampsia, and the reasons for this remain poorly understood. Smoking is associated with reduced levels of sFLT-1 in men and in non-pregnant women (125,126). It has been hypothesised, therefore, that reduced levels of sFLT-1 in smoking pregnant women would put them at lower risk of pre-eclampsia, since sFLT-1 is thought to be a key factor in the development of the condition. To examine this relationship Kamareinen et al. (127) compared third trimester plasma sFLT-1 levels and first trimester placental sFLT-1 levels (obtained at termination of pregnancy) between pregnant smokers and non-smokers; no significant differences between the two groups was found.

1.4.1.3 Effects of anti-hypertensives on sFLT-1 levels

Another mechanism that has been proposed for altering placental production of sFLT-1 is through the use of anti-hypertensive agents. Carr et al. (128) examined whether anti-hypertensive therapy with the beta-blocker atenolol could affect sFLT-1 levels throughout pregnancy. In a longitudinal study, women who were at high risk for pre-eclampsia (defined by previous pre-eclampsia, obesity, mid-trimester hypertension or chronic hypertension) and had high cardiac output as measured by Doppler studies were treated with atenolol, while women without risk factors and with normal cardiac output were defined as low-risk for pre-eclampsia, and were not treated. The authors had previously reported that such treatment was associated with a reduction both in incidence of pre-eclampsia and in levels of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) (129), and retrospectively examined sFLT-1 levels in the treatment and control groups. In keeping with the TNF-α levels, the authors reported that the rise in sFLT-1 with increased gestation was blunted in the treatment group when compared to controls. In addition, cardiac output and mean arterial pressure were reduced in those receiving atenolol. Potential limitations to this study were that it was not a randomised trial and there was neither a placebo nor an active control group. Further, given that there is no convincing evidence that continuing anti-hypertensive agents in pregnant women with chronic hypertension has any effect on incidence of pre-eclampsia (130), it is clear that further work is required in this area.

1.4.1.4 Soluble Endoglin

The other anti-angiogenic peptide implicated in the pathogenesis of pre-eclampsia is soluble endoglin (sEng). Endoglin, a co-receptor for transforming growth factors β1 and β3 (131) is highly expressed on endothelial cell membranes and syncytiotrophoblasts. Mutations in the gene encoding Eng are the underlying cause of hereditary haemorrhagic telangiectasia, a genetic condition characterised by AV malformations, epistaxis and telangiectasiae

(116). In normal pregnancy, levels of sEng fall between the first and second trimesters but in women who go on to develop pre-eclampsia this reduction is blunted (132). Consistent with studies involving sFLT-1, it has also been demonstrated that levels of sEng are elevated in the serum of pregnant women with pre-eclampsia, correlate with disease severity and fall after delivery (116). A promising discovery in terms of predicting the condition was that levels of sEng are elevated several weeks before the development of clinical symptoms in women who developed pre-eclampsia; furthermore, in patients who developed pre-term pre-eclampsia the serum sEng levels are elevated (approximately 2-fold) as early as gestational weeks 17-20 (133). These findings have led researchers to examine sFLT-1 and sENG levels in the first trimester in women who subsequently developed pre-eclampsia. Rana et al. (132) reported that levels of sEng and sFLT-1 were elevated in the serum of pre-eclamptic women at 17-20 weeks' gestation when compared to controls, but reported that the levels at 11-13 weeks were similar between cases and controls. Baumann et al. (134) in contrast reported elevated levels of both sEng and sFLT-1 in the first trimester in samples from gestational week 11 to 14.

The molecular mechanisms regulating the release of sFLT-1 and sENG remain poorly understood. This is an important area of research, since mechanisms to block their release may help to delay or even prevent the onset of pre-eclampsia, improving both maternal and foetal outcomes. It has previously been shown that trophoblastic cells produce VEGF, PIGF and sFLT-1. Li et al. (135) demonstrated that sFLT-1 production by trophoblastic cells was increased, while PIGF levels were decreased when the cells were exposed to hypoxic conditions. They also reported an increased level of lipid peroxidase production, a marker of oxidative stress, in these cells. This led them to propose that hypoxic placental conditions in the placenta and subsequent oxidative stress lead to alterations in trophoblastic production of VEGF, PIGF and sFLT-1.

1.4.1.5 Haem Oxygenase pathway

Further work in this area has explored the role of the haem oxygenase (HO) pathway. Haem oxygenase, an enzyme that catalyses the degradation of haem,

has 3 known isoforms: HO-1 is induced by a wide variety of stimulants known to be associated with oxidative stress, HO-2 responds to glucocorticoid stimuli, while HO-3 has little known enzymatic activity. Previous studies by Ahmed et al. (136) have shown that HO-1 is anti-inflammatory, and provides a defence against oxidative stress. Adenoviral over expression of HO-1 leads to reduced VEGF-induced sFLT-1 production in endothelial cells, while inhibitors of HO-1 cause an increase in its production (136).

The same group carried out further work examining the relationship between sFLT-1 and the HO pathway, in particular looking at the role of the cholesterol-lowering HMG-CoA reductase inhibitors, more commonly known as statins. As well as their role in reducing cholesterol, statins have been shown to have anti-inflammatory actions in conditions as diverse as rheumatoid arthritis (137) and kidney transplant rejection (138). When human umbilical vein endothelial cells (HUVECs) from women with pre-eclampsia were incubated with simvastatin, HO-1 was upregulated while sFLT-1 release was reduced. When cells were incubated with the anti-oxidants vitamins C and E, there was no effect on sFLT-1 and sENG release. This finding was perhaps not surprising, since as discussed in section 1.2.4 these vitamins do not appear to have any role in preventing pre-eclampsia.

This work has led to the proposal that statins may have a role in the prevention of pre-eclampsia in high-risk women. Statins are contra-indicated in pregnancy because early studies revealed skeletal malformations in rat-foetuses exposed to large doses. A recent observational study however, did not find an increase in rates of congenital anomalies in women who took statins in the first trimester of pregnancy (139). The role of statins in pre-eclampsia prevention is the basis of an ongoing trial, “Statins to ameliorate early onset pre-eclampsia” or “STAMP.” This study, funded by the Medical Research Council and co-ordinated by the University of Birmingham, will recruit women with pre-eclampsia at between 24 and 33 weeks’ gestation. Women will be randomised to pravastatin 40 mg or placebo, to be continued until delivery. The primary outcome will be maternal sFLT-1 levels at 48 hours post-randomisation, and secondary outcomes include a variety of maternal and foetal outcomes describing severity of disease. The trial aims to complete recruitment by December 2011.

1.4.1.6 HO-1 and carbon monoxide

In addition to exogenous sources such as smoking and exhaust fumes, carbon monoxide (CO) is generated in cells by the catalytic breakdown of free haem by HO-1. CO and CO-releasing molecules have been reported to lower sFLT-1 and sENG production in both endothelial cells and placental organ cultures (136), which potentially provides a molecular explanation for the lower levels of sFLT-1 and sENG noted in smokers during pregnancy. Additional observations that women with pre-eclampsia exhale more CO than those with normal pregnancies, and that HO-1 expression decreases as the severity of pre-eclampsia increases (140) lend weight to the argument that this pathway plays an important role in pre-eclampsia development.

1.4.2 Uric acid

Hyperuricaemia was first reported to be elevated in pre-eclampsia in 1917 (141), and it remains one of the most common blood tests used in assessment of the condition in clinical practice in the UK. Uric acid is a marker of oxidative stress, tissue injury and renal dysfunction, and several studies have reported a positive correlation between elevated maternal serum uric acid levels and adverse pregnancy outcomes (142). In view of this the National Heart, Lung and Blood Institute (NHLBI) in the United States recommend measurement of serum uric acid in high-risk women with normal blood pressure (143).

A systematic review of the literature, however, examining 18 studies of 4000 pregnant women, consistently observed poor performance of uric acid in predicting various maternal and foetal outcomes (142). Furthermore, a study designed to examine the impact of the uricosuric agent Probenacid in lowering uric acid in 40 women with pre-eclampsia did not demonstrate any effect on maternal blood pressure, nor on any other foetal or maternal outcomes (141). It therefore appears that while uric acid may be of value in the detection of pre-eclampsia, it is not useful in the early prediction of disease.

1.4.3 Placental Protein 13

Placental protein 13 (PP-13), a 32-kDa dimer protein highly expressed in the placenta, is involved in placental implantation and maternal vascular remodelling (144). During normal pregnancy levels of PP-13 gradually increase, but abnormally low levels of PP-13 have been reported at gestational weeks 11-13 (145) and weeks 9-12 (146) in women who went on to develop pre-eclampsia and foetal growth restriction compared with controls. In women who subsequently developed pre-eclampsia, second and third trimester PP-13 levels have also been reported to be independently associated with intra-uterine growth restriction and preterm delivery (147).

Combining maternal serum PP-13 levels with uterine artery Doppler studies early in pregnancy may further improve ability to predict more severe forms of pre-eclampsia. Nicolaides et al. (144) reported that women who went on to develop early onset pre-eclampsia associated with preterm delivery had a higher median uterine artery pulsatility index and a lower median serum PP-13 in the first trimester when compared to controls. Thus they concluded that for a 90% detection rate of the condition, using serum PP-13 for all women and Doppler studies in the 14% at highest risk, a false-positive rate of 6% could be achieved.

1.4.4 Pregnancy-Associated Plasma Protein A

Pregnancy-Associated Plasma Protein A, (PAPP-A) is a large and highly glycosylated protein complex produced by the developing trophoblast (148), which is used in many centres as a marker for Down's syndrome. It has been shown to be responsible for the cleavage of insulin-like growth factor (IGF) binding proteins, which are inhibitors of IGF action, in several biological fluids (149). PAPP-A was first reported to be altered in the plasma of pre-eclamptic women nearly 30 years ago (150). More recent studies have shown that although reduced first trimester serum levels of PAPP-A are associated with pre-eclampsia, levels are also reduced in women with other pregnancy-related complications such as ante-partum haemorrhage and stillbirth (151-153). Further, it has been suggested that PAPP-A is more useful as a marker of foetal

growth restriction than of pre-eclampsia (154). Spencer et al. (155) described only a small increase in likelihood ratio of developing pre-eclampsia with decreasing levels of PAPP-A, and suggested that, similarly to PP-13, sensitivity could be improved by combining with uterine artery Doppler studies.

1.4.5 Sex hormone binding globulin

Insulin resistance has long been implicated in the pathogenesis of pre-eclampsia. Carbohydrate metabolism is known to be altered in women with pre-eclampsia, while fasting insulin levels have also been shown to be elevated prior to the onset of disease (156). Furthermore, as mentioned in section 1.1.3, type 1, type 2 and gestational diabetes are all well-established risk factors for the condition (157). Normal pregnancy is characterised by increased insulin secretion by the pancreatic β cells, and, following initially increased insulin sensitivity, there follows a progressive increase in insulin resistance throughout the second and third trimesters (158).

Sex-hormone binding globulin (SHBG) is a glycoprotein produced by the liver which binds circulating oestrogens and testosterone. Production of SHBG is inhibited by insulin; low levels of SHBG are associated with elevated insulin and as a result several studies have used low SHBG levels as a marker of insulin resistance in both cardiovascular disease (159) and in pre-eclampsia (156,157,160). One study looking at first trimester (mean gestation 10.6 weeks) SHBG levels in 45 nulliparous women who went on to develop pre-eclampsia found that levels were significantly reduced when compared with controls (157). In contrast, however, a further study looking at SHBG levels at gestational weeks 10-14 in 103 women who went on to develop pre-eclampsia, 64 women who developed hypertension without proteinuria and 400 controls with normotensive pregnancies found no significant difference between cases and controls (156). This retrospective study included multiparous women, which may have confounded results. A further study reported no difference in SHBG levels at

either gestational weeks 17 or 33 between 29 women who went on to develop pre-eclampsia and 142 controls (160).

1.4.6 Adiponectin

Although obesity is associated with increased overall body fat, it is the distribution of body fat rather than its total amount which is the major determinant of morbidity related to obesity (161). Adipocytokines, derived from adipose tissue include adiponectin, leptin, and tumour-necrosis factor-α, and are produced preferentially by visceral rather than subcutaneous fat. Adiponectin levels are reduced in obesity, and its levels are inversely correlated with insulin resistance; high concentrations have been reported to be protective against the development of type 2 diabetes (162). Adiponectin is thought to have a protective role on the vasculature, by reducing levels of adhesion molecules such as ICAM-1, E-Selectin and VCAM-1 (161).

Given the role of insulin resistance in the pathogenesis of pre-eclampsia it has been hypothesised that visceral fat accumulation during pregnancy may induce dysregulation of adipocytokines, contributing to the development of the condition. As a result adiponectin levels have been studied for their role in pre-eclampsia prediction. D'Anna et al. (163) examined first trimester serum adiponectin levels in 36 women who subsequently developed pre-eclampsia, of whom 16 had early onset disease, and 36 controls. They found that levels were significantly lower in cases than in controls, and that levels were significantly different between those who developed early-onset and late-onset disease. Further studies have shown that adiponectin levels correlate negatively with BMI and with weight gained during pregnancy, and positively with flow-mediated dilatation (161). Further, serum levels of adiponectin have also been reported to correlate with sEng levels in women with established disease (164).

In contrast, however, Ramsay et al. (165) found that serum adiponectin levels in the third trimester were in fact higher in women with pre-eclampsia compared with controls, a finding which has been confirmed elsewhere (166), and has led to speculation that adiponectin forms part of the physiological response to pre-

eclampsia by improving insulin sensitivity later in pregnancy (166). The role of adipocytokines in disease prediction in pre-eclampsia therefore remains uncertain.

1.4.7 Apolipoprotein E

A further mechanism by which pre-eclampsia has been postulated to develop is via abnormal lipid metabolism associated with oxidative stress. Women with pre-eclampsia have an abnormal lipid profile, with elevated concentrations of triglyceride-rich lipoproteins, which may contribute to endothelial dysfunction (167). Apolipoprotein E is a major constituent of very low-density lipoproteins (VLDLs) whose role involves modifying inflammatory responses, and removal of excess cholesterol from the circulation via regulation of hepatic uptake (168). The Apolipoprotein E (ApoE) gene on Chromosome 19 has 3 common isoforms, which translate into 3 alleles of the gene: e2, e3 and e4. ApoE e4 is associated with familial Alzheimer's disease, whilst both e2 and e4 have been associated with abnormally high triglyceride and VLDL levels (169). It has been postulated that Apolipoprotein E levels and polymorphisms of its gene are associated with an increased risk of pre-eclampsia. Nagy et al. (170) found a higher incidence of ApoE e2 amongst women with pre-eclampsia at the time of disease diagnosis compared to controls. Makkonen et al. (171) studied 133 women with pre-eclampsia, and in contrast found that none of the ApoE alleles were over represented when compared with controls, findings that have been confirmed elsewhere (168). Elevated Apolipoprotein E levels have been reported in a study of serum proteomic analysis in women with established pre-eclampsia, but not before the onset of clinical symptoms (172), and as such it seems unlikely to have a role in disease prediction.

1.4.8 Inhibin A and Activin A

Many studies have been reported using Inhibin A and Activin A as predictors of pre-eclampsia. Both are glycoproteins, are members of the transforming growth factor β family, and during pregnancy are largely released by the feto-placental subunit (173). Inhibin A has an important endocrine role in the negative

feedback of gonadotrophins, while Activin A is thought to have activity in various biological tissues. In normal pregnancy, concentrations of both hormones rise in the third trimester, and levels have been shown to be elevated approximately 10-fold in women with severe pre-eclampsia compared to controls (174). Second trimester levels of Inhibin A have been reported to be elevated in both serum (175) and amniotic fluid (176) in women who went on to develop severe pre-eclampsia, and when measured at term, serum levels have been shown to correlate with pre-eclampsia severity (177). In addition, urinary Activin A and Inhibin A levels have also been found to be elevated in women with pre-eclampsia, as have uterine vein levels (178).

Second trimester levels of both Inhibin A and Activin A have been reported to add significant prognostic information when measured in women with abnormal uterine artery Doppler studies (179). Davidson et al., (180) however, found that although second trimester levels of Activin A were elevated in women who went on to develop pre-eclampsia, Inhibin A levels were not different between cases and controls, findings confirmed by D'Anna et al. (181). Studies using first trimester Inhibin A (182) have also shown a low predictive value.

1.4.9 Markers of inflammation

As outlined in section 1.1.2, it is generally accepted that pre-eclampsia is a disease occurring in 2 main stages; the "placental stage" occurring as a result of a poorly-developed utero-placental blood supply, and the "maternal stage" which arises from a systemic maternal inflammatory response. Several markers of inflammation and the inflammatory cascade have therefore been studied in pre-eclampsia, both to explore their role in pathogenesis, and to examine whether they are useful predictors of disease.

1.4.9.1 CRP

C-reactive protein (CRP) is a marker of the acute phase of the systemic inflammatory response; levels are elevated within hours of detection of inflammatory stimuli. Several large prospective studies have reported that,

when measured by highly-sensitive assay, CRP is an independent predictor of future cardiovascular events (183) and of all-cause mortality (184). Normal pregnancy is characterised by a mild systemic inflammatory response, which begins during the luteal phase of the menstrual cycle before implantation, and develops as pregnancy progresses. In keeping with this, CRP levels have been reported to be elevated in normal human pregnancy from as early as 4 weeks' gestation (185).

In pre-eclampsia a similar inflammatory response occurs, but is of far greater intensity. CRP in the third trimester is elevated in women with pre-eclampsia compared to those with normal pregnancies, and levels have been shown to correlate with disease severity (186). The potential role of CRP measurement in the early prediction of pre-eclampsia has been less well defined. For example, Garcia et al. (89) reported elevated levels at 22 weeks' gestation in women who subsequently developed pre-eclampsia, while Teran et al. (187) reported no difference when levels were checked at gestational week 16. One possible explanation for this discrepancy is that CRP levels in pregnancy have been shown to vary between different racial groups, with higher levels in black women compared to Caucasian women (188).

1.4.9.2 Adhesion Molecules

As has been mentioned above, disruption of the vascular endothelium plays a key role in the development of pre-eclampsia. Adhesion molecules support the adherence of leucocytes to endothelial cells and the subsequent migration of leucocytes as part of the inflammatory response. Expression of these molecules on the endothelial surface is a tightly regulated process, and increased serum levels of adhesion molecules are thought to indicate the presence of endothelial dysfunction (189). Specific forms of adhesion molecules mediate specific steps of the leucocyte-endothelial cell interaction, and the concentration of each of these molecules is thought to reflect the degree of activation of a specific cell type. Elevations in P-selectin reflect platelet activation, elevations in E-Selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) reflect endothelial cell activation and changes in L-selectin are indicative of leucocyte activation (190).

Normal pregnancy is associated with alterations in adhesion molecule levels; elevation in P-Selectin, reduction in L-Selectin, and no change in E-Selectin, VCAM-1 or ICAM-1 have been reported compared to non-pregnant controls (190). Several studies have examined levels of adhesion molecules at delivery in women with pre-eclampsia; compared to normotensive controls, significant elevation in P-Selectin (189,190), E-Selectin, (189,190) VCAM-1 (189-192) and significant reduction in L-Selectin (190) have been reported. Further studies using placental biopsies have shown that spiral artery endothelial cells stain positive for ICAM-1 and VCAM-1, but that there is no difference in their levels between women with pre-eclampsia and normotensive controls (193).

As with any other biomarker for pre-eclampsia, to be clinically useful elevated levels would have to be detectable prior to the onset of clinical disease. Chavarria et al. (194) examined pregnant women at 20 weeks' gestation and reported elevated levels of E-Selectin, P-Selectin and ICAM-1, and lower levels of L-Selectin and VCAM-1 in women who subsequently developed pre-eclampsia. In a separate longitudinal study of 70 pregnant women of whom 20 later developed pre-eclampsia, P-Selectin levels were reported to be elevated as early as week 10-14 in affected women (195).

Research in this field has been somewhat limited by inconsistent results, and by lack of comparison with non-pregnant controls. Although adhesion molecules are thought to have a role in the development of the endothelial dysfunction associated with the condition, none have been specific enough to be used routinely. An improved understanding of adhesion molecule levels in normal pregnancy is required before they can be considered as useful predictors of hypertensive disorders.

1.4.9.3 Cytokines

The failure of the uterine vasculature to remodel in early pregnancy and subsequent placental hypoperfusion is thought to lead to the generation of cytotoxic factors that circulate and injure the maternal circulation. Among these factors are inflammatory cytokines, upregulated by the placenta. In keeping with adhesion molecules, several of these cytokines have been studied for their role in the development of pre-eclampsia.

Interleukin 6 (IL 6) and tumour necrosis factor alpha (TNF α) have been shown in several studies to be elevated in women with pre-eclampsia when measured at delivery,(196,197) while elevated levels of Interleukin-10 (IL 10) have also been reported (198). Placental levels of these and other pro-inflammatory cytokines have not, however, been found to be different between pre-eclamptic women and women with normotensive deliveries, suggesting that tissues other than the placenta may contribute to their production (196). These findings are in keeping with the theory that there are separate placental and maternal responses underlying pre-eclampsia.

1.4.10 Aldosterone

Aldosterone, a mineralocorticoid synthesised from cholesterol in the zona glomerulosa of the adrenal cortex, has emerged in recent years as a key cardiovascular hormone. In addition to regulation of fluid and electrolyte balance and blood pressure, it is now known to exert its effects on a number of different non-adrenal sites including the heart and the central nervous system. Up to 15% of patients with essential hypertension have inappropriate regulation of aldosterone (199), and the hormone is thought to play an important role in determining cardiovascular risk.

Aldosterone concentrations rise in the luteal phase of the menstrual cycle, and if conception occurs, its levels increase significantly (around 20-fold) throughout pregnancy towards term (200). Normal pregnancy is characterised by marked vasodilatation, and by an increase in extracellular and intracellular volume of around 6-7 litres (201) ; the increased aldosterone concentrations are thought to facilitate this volume expansion, ensuring adequate utero-placental perfusion. Much of the increased mineralocorticoid activity in pregnancy is related to increased production of deoxycorticosterone (DOC), produced from progesterone. Levels of the glucocorticoid cortisol are also markedly increased throughout normal pregnancy. The developing placenta is protected from high levels of cortisol, however, by the enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11- βHSD-2), which converts cortisol into the inactive cortisone. As a result cortisol is virtually absent in normal placentas (200).

In pre-eclampsia, the balance of cortisol and aldosterone regulation is altered; 11- βHSD-2 activity is reduced, and as a result trophoblasts are exposed to increased concentrations of cortisol. Aldosterone levels are also substantially reduced in pre-eclampsia, and this reduction is paralleled by a reduction in plasma volume expansion and placental growth (202).

Given the key role that cortisol and aldosterone play in blood pressure regulation in the non-pregnant population, these findings have led to the suggestion that genetic variations in their production may contribute to the development of pre-eclampsia. Shojaati et al. (202) reported in 2004 that some women with pre-eclampsia carry genetic mutations that lead to decreased activity of aldosterone synthase (*CYP11B2*), resulting in elevated levels of aldosterone precursors but reduced and inefficient production of aldosterone itself. Supporting this theory, increased aldosterone concentrations, measured as urine tetra-aldosterone excretion, are associated with lower maternal blood pressure in pregnancy and with larger, healthier neonates (203). Further work has shown that aldosterone increases proliferation of cultured human trophoblasts, an effect reversed in animal studies by the aldosterone antagonist spironolactone and by high-dose glucocorticoids (200).

This work suggests therefore that uncompromised aldosterone production can contribute to an uneventful pregnancy outcome. Diminished aldosterone production, due to genetic variations in the *CYP11B2* gene, or for other reasons, in the face of increased progesterone availability may therefore reduce the compensatory potential for shifts in circulating volume. The practical consequences of these findings are unclear; tailored follow up during pregnancy based upon *CYP11B2* genotype could be one possibility. Given its role in essential hypertension, aldosterone may also help to explain the increased cardiovascular risk seen in women with a history of pre-eclampsia.

1.5 Genetic influences

There are clearly genetic factors involved in the development of pre-eclampsia. As discussed in section 1.1.3, women with a family history of pre-eclampsia in

their mother or sister are at nearly 3 times higher risk of development of the condition compared to those without a family history (12); the role of paternal genes remains uncertain. A Swedish study examining over 700,000 pregnancies from 240,000 sibling pairs reported a 35 % contribution of maternal genes to the risk of development of pre-eclampsia. The foetal genes, with similar contribution of genetic effects from both parents, were responsible for 20% of the risk (204). In a further Swedish family study in which 2.8% of women studied were affected by pre-eclampsia, odds ratios of 3.3 (95% CI 3.0-3.6) for sisters and 2.6 (95% CI 1.6-4.3) for daughters of those affected were reported, compared to odds ratios of 1.4 (95% CI 0.9-2.2) for maternal half-sisters and 1.0 (95% CI 0.6 to 1.6) for paternal half-sisters of those affected. Full sisters of women with pre-eclampsia were also reported to be at increased risk of pregnancy-induced hypertension without proteinuria, with a reported odds ratio of 2.5 (95% CI 2.2 to 2.8) (205).

From these and other epidemiological studies there is clearly evidence of a genetic component to the development of pre-eclampsia, and several modes of inheritance have been suggested. One of the problems with research in this field, however, is the rarity of the condition. The magnitude of genetic influence in disease is often assessed using twin studies; the rarity of pre-eclampsia has meant that very few twin studies have been possible. The Swedish group did report on 928 monozygotic twin pairs, reporting a substantial increase in risk of pre-eclampsia (odds ratio 33.6, 95% CI 7.8 to 145) in the identical twins of those affected (205).

Several candidate genes have been proposed as having a role. Genes encoding elements of the renin-angiotensin system, inherited thrombophilias, synthesis of the vasorelaxant endothelial nitric oxide synthase (eNOS) and tumour necrosis factor alpha (TNFα) have featured amongst the most common genes studied (206). Linkage studies examining the genetic contribution to pre-eclampsia have mainly implicated loci on Chromosome 2 (207-209), although loci on Chromosomes 4, 7,9 and 10 have also been studied (206,210-213). The majority of studies, however, have examined different populations and have had inconsistent results.

The Genetics of Pre-eclampsia (GOPEC) consortium attempted to further elucidate the role of genetic influences in pre-eclampsia. Seven candidate genes which had previously been reported as conferring susceptibility to pre-eclampsia were extensively studied in over six-hundred Caucasian women with strictly defined pre-eclampsia. These women and their families were genotyped at 28 single-nucleotide polymorphisms (SNPs): none of the SNPs or haplotypes achieved statistical significance (214). It is hoped that more recent advances in genotyping technology will facilitate genome-wide association studies (GWAS) in pre-eclampsia which may result in novel candidate genes for the disorder. Results from studies into other polygenic disorders such as coronary artery disease and diabetes have been promising (Wellcome Trust Case Control Consortium (WTCCC) 2007)(215), and pre-eclampsia is being investigated as part of the latest phase of the consortium's studies (WTCCC 3). It is clear, however, that investigators have to follow strict rules to avoid false positive and underpowered negative results (216).

1.6 Proteomics

1.6.1 Introduction

Proteomics is the study of the proteome, the protein complement of the genome. The terms "proteome" and "proteomics" were coined in the mid-1990s to mirror the terms "genome" and "genomics," which describe the entire collection of genes in an organism.

Proteomics is defined as the "knowledge of the structure, function and expression of all proteins in the biochemical or biological contexts of all organisms" (217), and has emerged in recent years as an important tool for early detection of disease. Traditional definitions of disease usually rely on description of the observed symptoms and signs, often without full understanding of the pathophysiology underlying the disease process. For complex conditions such as pre-eclampsia and other cardiovascular diseases, the use of single "markers" to diagnose, stage and assess prognosis of disease is

perhaps unrealistic. The majority of previous pre-eclampsia research has been driven by pathways that are known to be involved in the development of the condition, and one advantage of proteomics-based research is that many of these mechanisms can be integrated, developing a “multimarker” approach. In addition, since proteomics is a “hypothesis-generating” research tool, it is likely that this line of investigation will open new avenues for potential biomarker discovery, and for new diagnostic and preventative measures.

Recent technological advances have allowed for a shift towards multi-parametric “omic” based approaches for biomarker discovery. All classes of biological compounds can be studied; genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) are all important areas of research.

The human genome contains around 20,000 genes; each gene can be subject to differential splicing, translation and post-translational modification. As a result each gene can code for at least 10 times as many proteins, all of which can determine different cellular functions. It can be said, therefore, that while genomics explains one’s disposition to disease, proteomics gives a more accurate reflection of proteins expressed and therefore of exact disease processes at the time of study (218).

1.6.2 Choice of sample type

Clinical proteomic research can be carried out using any type of human tissue, bodily fluid or cultured cells. Samples of tissue are the optimal way in which to detect pathological changes, but large scale studies of human tissue will always be limited by the invasive measures necessary to obtain them. Bodily fluids such as blood, cerebrospinal fluid (CSF), ejaculate and urine have all been used for proteomic research; substances in bodily fluids contain a huge amount of information that can be used to monitor the well-being of an organism.

Polypeptides in all bodily fluids are prone to proteolytic degradation; for example peptides in human serum have been shown to be exposed to high

proteolytic activity immediately upon clotting (219). For that reason the Human Proteome Consortium (HUPO) recommend the use of plasma rather than serum for proteomic analysis (220). Although proteolytic activity in plasma is significantly less than serum, there is still sufficient activity to render accurate proteomic analysis difficult.

Urine may represent a more stable platform for proteomic research. As well as being available non-invasively in large volumes, the lack of proteolytic activity means that urine peptides are stable enough for accurate proteomic analysis. Urine contains a rich source of information relating to the function of several internal organs. The peptide composition of the urine depends on a number of factors: glomerular filtration, absorption in the proximal tubules and lysosomal proteolytic activity at the brush border. Peptides present in the urine may therefore provide a signature for particular pathological processes (221).

Urine can be stored for several years at -20°C without significant alterations in the proteome (222). Further studies have shown that the urine proteome does not undergo significant changes when stored for 3 days at 4°C or for 6 hours at room temperature (223).

One of the key considerations with proteomic research of bodily fluids is sample preparation. The presence of compounds such as albumin in urine, or lipids and carbohydrates in blood can cause bias and artefact in proteomic results. A key element of proteomic research is minimising variability in sample collection, preparation and analysis. For the purpose of this study a number of steps were used to minimise variability, and are outlined in Chapter 2.

1.6.3 Different proteomic platforms

Several different platforms for proteomic analysis have been developed over the last few decades, which will be briefly described in the following paragraphs.

1.6.3.1 2-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) separates proteins on 2 dimensions, their isoelectric point and molecular mass. Proteins are then treated with a proteolytic in-gel digest, the gel is extracted and mass spectrometry (MS) is used to analyse the resultant peptide fragments. 2-DE provides high resolution protein separation and identification, and is most useful for comparative analysis of proteins. Since 2-DE is not an automated process, a potential drawback is that it is too time-consuming for analysis of large numbers of proteins simultaneously.

1.6.3.2 Liquid Chromatography – Mass Spectrometry

Liquid chromatography (LC) is a powerful method of fractionation, which separates large numbers of analytes on an LC column with high sensitivity. For further analysis, sequential separation using different matrices can be performed in independent steps, generating large amounts of information. The main limitation of LC-MS is the length of time (days) taken for analysis of a single sample. In addition, large proteins (>10 kD) have to be cleaved by a protease before analysis.

When data from tryptic digests were analysed using both 2-DE and LC-MS, different proteins were detected by the 2 techniques (223). Both techniques, either independently or together can therefore analyse proteins with high sensitivity, but neither is suitable for the comparative analysis of large numbers of samples.

1.6.3.3 SELDI-Mass Spectrometry

Surface-enhanced laser desorption / ionisation (SELDI) uses different hydrophilic surfaces to selectively adsorb proteins while the unbound proteins are washed away. A matrix is then added to the surface which crystallises with the sample peptides. Binding to the matrix surface separates the proteins making subsequent MS analysis less complex. The aim of SELDI is to bind a small but well-defined fraction of proteins to the surface, depending on the pH, concentration and salt content. SELDI has the capacity to analyse multiple samples in a short time, but reproducibility of peptide binding (and therefore

subsequent MS analysis) appears to be low (218). Further, since much of the biological sample is eliminated during preparation, comparing data sets for different samples is limited.

1.6.3.4 Capillary Electrophoresis – Mass Spectrometry

Capillary electrophoresis separates proteins in a single step based on their migration through a buffer-filled capillary. The separated proteins are delivered from the end of the capillary into a mass-spectrometer in a small stream of liquid by nano-ion spray. CE-MS has the advantage over other techniques that it provides fast separation with high resolution, it uses inexpensive capillaries instead of expensive LC columns and it is compatible with the majority of buffers and analytes. CE-MS enables the generation of comparable high-resolution data sets, allowing the rapid analysis of large numbers of biological samples. As with LC, a drawback of CE is that it is not ideally suited for the analysis of large proteins, which are removed with ultrafiltration prior to CE-MS analysis.

1.6.4 Clinical applications for urinary proteomics

1.6.4.1 Kidney disease

Identification of patients with chronic kidney disease (CKD) at an early disease stage, and early diagnosis of specific kidney diseases without requirement for renal biopsy is one area of clinical research in which urine proteomics may have an important role. To date proteomic studies have determined disease-specific urine peptide patterns for IgA-associated nephropathy (224), ureteropelvic junction obstruction (225) and ANCA-associated vasculitis (226).

Peptide patterns specific to diabetic nephropathy have also been described, which can predict the development of diabetic nephropathy before microalbuminaemia can be detected (227). As well as early detection of renal changes, urine proteomics may also be a useful way to monitor treatment of diabetic renal disease; one study showed that treatment of macroalbuminuric diabetic patients with the angiotensin-receptor blocker candesartan for 2 months led to significant changes in the urine proteome (228).

1.6.4.2 Coronary artery disease

Current methods for detection of significant coronary artery disease (CAD) rely almost entirely on coronary angiography, an invasive procedure which is associated with a risk of bleeding, coronary artery perforation and stroke. Non-invasive detection of CAD is therefore a further area studied in the context of proteomics. Using CE-MS technology, Zimmerli et al. (221) reported a set of 15 urine peptides that defined a characteristic CAD signature panel, which had a sensitivity of 98% (95% CI 88.7-99.6) and a specificity of 83% (95% CI 51.6-97.4). This study used patients with severe CAD on coronary angiography, comparing them with controls recruited from a health club with no symptoms suggestive of CAD or other vascular disease. In a further study which reflected the "real-life" situation faced by clinicians, urine and plasma proteomics of patients with acute chest pain and cardiovascular risk factors were examined; all patients subsequently underwent coronary angiography to confirm or exclude significant coronary artery disease. Using a combination of 17 peptides the authors reported a sensitivity for diagnosing CAD of 81% (95% CI 60-93) and a specificity of 92% (95% CI 62-99) (229). The majority of urinary peptides associated with CAD originated from collagen, reflecting its central role in the pathophysiology of atherosclerosis. Plasma proteomics did not accurately discriminate between patients with CAD and those without, perhaps related to latent protease activity in plasma (219). Further evidence for the dynamic nature of the proteomic patterns associated with coronary disease is that treatment with the angiotensin receptor-blocker irbesartan can lead to significant changes in the pattern, with many peptides changing towards levels seen in the healthy proteomic signature (230).

1.6.4.3 Other conditions

Improvements in the early detection of cancers are a further vitally important area of medical research. The majority of studies using urinary proteomics to improve early detection of malignancies have focused unsurprisingly on the renal tract; disease specific urine peptide profiles have been reported for bladder cancer, renal cancer and for differentiation between prostate cancer and benign

prostatic hyperplasia (226). Given that peptides in the urine reflect the protein constituents of the plasma, diseases distal to the renal tract may also be identifiable in the urine proteome. For example, urinary proteomic biomarkers for bowel cancer (231), lung cancer (232) and graft-versus-host disease after bone marrow transplantation have all been described (233).

1.7 Proteomics studies in pre-eclampsia

1.7.1 Gel electrophoresis studies

1.7.1.1 Serum studies

While the field of proteomics research in renal disease is fairly well established, there have been few studies in reproductive medicine. An early study by Watanabe et al. (234) used 2-dimensional gel electrophoresis to examine maternal serum from 6 patients with established pre-eclampsia associated with foetal growth restriction, and 6 matched women with normal pregnancies. Over-expressed spots on 2-D GE were then identified using MALDI-TOF mass spectrometry, Western blot analysis and searches of a protein database. Using these techniques pre-eclamptic women were found to over-express clusterin, a protein which induces cholesterol efflux from lipid laden macrophages and is associated with vascular disease, renal disease and oxidative stress. The group then validated the finding in 80 pre-eclamptic women, and found that clusterin levels were significantly higher (1.62 ± 0.46 vs 1.30 ± 0.46 times the reference level) than in 80 matched women with normal pregnancies.

1.7.1.2 Plasma studies

A further study using 2-D GE techniques for protein separation was reported by Wang et al. (235) who examined plasma in 11 women with pre-eclampsia and 11 controls. Using mass spectrometry to identify peptides, they were able to demonstrate a significant reduction in pre-eclamptic women in H-ficolin and L-ficolin, factors thought to play a role in innate immunity. These findings were then validated in plasma samples from a separate cohort of 20 patients.

Although these studies potentially introduce new pathways for pre-eclampsia research, they highlight some of the limitations in pre-eclampsia research to date; samples were taken at time of disease rather than before it, and sample numbers were small.

A more recent study used differential gel eletrophoresis (DIGE) to examine the plasma proteome at 20 weeks' gestation in a cohort of 27 women who went on to develop pre-eclampsia with appropriately grown babies, 12 who developed pre-eclampsia in association with foetal growth restriction and 57 controls who had normal pregnancies (236). Protein spots of interest were then identified with liquid-chromatography / mass spectrometry (LC-MS). The authors found that 36 moderate to high-abundance proteins were differentially expressed in pre-eclamptic women at 20 weeks, prior to the onset of clinical disease. The proteins identified are involved in lipid metabolism, the complement cascade, inflammation and haem scavenging, all mechanisms thought to be involved in pre-eclampsia. Of interest, 18 of the proteins identified overlapped with those identified in a previous study reporting the role of HDL cholesterol in cardiovascular disease. The authors also reported elevated levels of ApoA-1, the predominant lipoprotein in HDL cholesterol, clusterin and fibrinogen. As well as being a large, clinically useful study, these findings also provide an interesting insight into the link between pre-eclampsia and cardiovascular disease later in life.

1.7.1.3 Placenta studies

The placenta is thought to be the source of many of the circulating factors which cause maternal endothelial damage in pre-eclampsia. Placental tissue can be easily obtained at delivery, and as a result placental tissue has been used in proteomic studies to elucidate further information about the condition. In a relatively small study with 5 affected women, 2-dimensional gel electrophoresis (2D-GE) was used to generate biomarker patterns that characterised pre-eclampsia, and peptides were identified using TOF mass spectrometry. Pre-eclampsia was associated with elevated levels of Apolipoprotein A1, Interleukin-8 and altered expression of coagulation factors and anti-oxidants.

1.7.2 LC-MS studies

1.7.2.1 Plasma studies

As discussed in section 1.6.3, gel-free techniques for proteomic analysis such as LC-MS have the advantage over gel electrophoresis that they are less labour intensive, and several samples can be analysed simultaneously. A recent proof of concept study described the use of iTRAQ (isobaric tagging for relative and absolute quantification) in association with LC-MS for proteome analysis. Blankley et al. (237) examined 23 women with pre-eclampsia at the time of disease, and 23 gestation matched controls. In the pre-eclamptic women elevated levels of several peptides were reported, including PAPP-A, SHBG, endoglin and pregnancy-specific β-1 glycoprotein, all of which have featured in previous pre-eclampsia research (237). Although these findings are encouraging, it is clear that demonstrating elevations of these and other peptides prior to disease onset would be far more clinically useful.

1.7.3 SELDI-TOF studies

1.7.3.1 Urine studies

Since renal pathology is a hallmark of pre-eclampsia, and since the level of proteinuria is often related to disease severity, it may be that proteomic analysis of the urine will help point to novel mechanisms in this area of research.

To date only one study has been published using urinary proteomics in pre-eclampsia research. Buhimschi et al. (238) studied urine from 284 women, of whom 29 had mild pre-eclampsia, 31 had severe pre-eclampsia and 28 had pre-eclampsia superimposed on chronic hypertension. Urine samples were taken at the time of disease onset; proteomic profiles for pre-eclampsia were generated using surface-enhanced laser desorption / ionisation, and peptides were then identified using tandem mass spectrometry. The proteomic profile for pre-eclampsia was then validated in a separate cohort of 225 pregnant women, and was compared with other markers of disease severity including sFLT-1 : PlGF ratio and albumin : creatinine ratio.

The proteomic signature profile for pre-eclampsia was largely characterised by fragments of serpin peptidase inhibitor (SERPINA) 1 and albumin. SERPINA1 is an abundant plasma protein, which has a role in the inhibition of neutrophil elastase, trypsin and pancreatic elastase. Elevated SERPINA1 levels were shown to be most strongly associated with severe pre-eclampsia requiring early delivery; furthermore placental levels were also found to be elevated in women with pre-eclampsia compared to those of women with normal deliveries. This work therefore demonstrated that a urine proteomic "fingerprint" made up of SERPINA1 and albumin fragments can diagnose pre-eclampsia, and can differentiate severe forms of the disease from milder variants. As well as being a potential biomarker it may be that fragments of SERPINA1 have a role in the pathogenesis of pre-eclampsia. Future studies with larger numbers of pre-eclamptic women followed longitudinally throughout pregnancy are clearly required to determine whether this is a clinically useful biomarker.

One factor relating to urinary proteomics is that whole proteins do not generally pass unfiltered into the urine, and that it is only peptides that can be detected. Identification of the parent proteins requires further mass-spectrometry based sequencing techniques.

1.7.3.2 Plasma studies

SELDI-TOF mass spectrometry has also been used to identify plasma biomarkers for pre-eclampsia. In a pilot study, Myers et al. (239) identified 26 women at 18-20 weeks' gestation as being high risk based on uterine artery Doppler studies. Plasma was taken at 26 weeks' gestation; protein profiles were examined using Ciphergen protein chip technology, which uses SELDI-TOF mass spectrometry to detect protein binding to a biochip array. 18 of the women subsequently developed pre-eclampsia and 8 had a normal pregnancy outcome; 5 proteins were found to be significantly elevated in the affected women, although the technology did not facilitate identification of the proteins.

1.7.3.3 Amniotic fluid studies

Another bodily fluid that has been studied in reproductive proteomic research is amniotic fluid. Amniotic fluid, sampled using amniocentesis, is used in certain circumstances to obtain information about fetal genetic health. One study used SELDI-TOF mass spectrometry to examine the amniotic fluid proteome in 18 women with pre-eclampsia, 7 with chronic hypertension, and 16 women with normotensive pregnancies (240). Amniotic fluid samples were taken either at the time of Caesarean section, or were taken during clinically indicated amniocentesis. Two discriminatory proteomic peaks were identified; one which differentiated women with pre-eclampsia and chronic hypertension from controls was identified as proapolipoprotein A-1, a prohormone for apolipoprotein A-1. The other, which differentiated women with pre-eclampsia from normotensive controls, was a peptide of unknown functionality called SBB142. Given that polymorphisms of the apolipoprotein E gene have been postulated to cause pre-eclampsia (241), it is of interest that a further member of the apolipoprotein family was identified in this study as being associated with pre-eclampsia. The invasive nature of amniocentesis, however, and the associated risk of miscarriage, mean that this technique is not feasible for large scale studies.

1.7.3.4 Cerebrospinal fluid

Neurological complications such as seizures (eclampsia), headaches and stroke are associated with severe variants of pre-eclampsia; the aetiology of the neurological manifestations remains unclear. To further examine this, Norwitz et al. (242) examined the cerebrospinal fluid (CSF) in 7 women with severe pre-eclampsia, 8 with mild pre-eclampsia and 7 normotensive controls. CSF, which was obtained at the time of spinal anaesthesia prior to delivery, was analysed using SELDI-TOF mass spectrometry, while gel digests, Western blot analysis and immunoassays were used to identify the protein biomarkers. Total CSF protein and leucocyte concentrations were not different between the 3 groups, but a protein profile made up of α- and β- chains of haemoglobin distinguished severe pre-eclampsia from mild disease and normal controls. In addition women with

severe pre-eclampsia were found to have nanomolar amounts of free haemoglobin in the CSF, as measured by spectrophotometry. The authors speculated that the free haemoglobin in the CSF may act not just as a marker for pre-eclampsia, but may also contribute to the neurological manifestations commonly seen in these women.

1.8 Metabolomics

In complement to studies examining the human genome and proteome, metabolomics, the study of the human metabolome has emerged as a further important research tool in recent years. The low molecular weight chemicals (metabolites) studied are the final downstream product of gene expression, and have been described as the "the unique chemical fingerprints that specific cellular processes leave behind" (243).

In common with proteomics, studies of the human metabolome can be carried out on routine samples of urine, plasma or serum and require minimal specialist preparation of samples. Metabolomics (also referred to as metanomics or metabonomics) has been used to characterise signature chemical profiles for cardiovascular disease (244), for Alzheimer's disease (245), and for hypertension (246).

Initial studies using plasma from women with established pre-eclampsia identified 8 metabolic peaks that were elevated in women with pre-eclampsia, which were then validated in a separate cohort of women and identified using mass spectrometry (247). Among the metabolites identified were uric acid, a marker which is used clinically to assess severity of disease, and 2-oxoglutarate, a marker for oxidative stress.

A more recent study using metabolomics was reported using plasma samples from the "Screening for Pregnancy Endpoints" (SCOPE) study cohort (248). In this prospective international study, nulliparous women with no history of hypertension, renal disease or diabetes were recruited at gestational week 14-16. In a nested case control design, samples from 60 cases and controls were

analysed in a “discovery” phase, and samples from 39 cases and 40 controls were analysed in a “validation” phase. Using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), 45 unique metabolic “peaks” for pre-eclampsia were identified, of which 14 were used to generate a multivariate predictive model, with an area under the receiver operator characteristics curve (AUC) of 0.94. The 45 molecules came from 11 clear metabolite classes, including fatty acids, lipids, steroids, pophyrins and phospholipids. The 14-metabolite model was reported by the authors to compare favourably with other studies using anti-angiogenic peptides, and ongoing studies from the same group will attempt to validate the findings in different ethnic populations. Other metabolomic studies in pre-eclampsia have been confined to studies of placental tissue from women with established pre-eclampsia (249), but in keeping with proteomics these studies may help to explain some of the underlying mechanisms in this disease.

1.9 Pre-eclampsia and future maternal health

Evidence has mounted in recent years that pre-eclampsia is not just a disease of pregnancy, but that the condition has important implications for future maternal health, in particular cardiovascular health. Pre-eclampsia and cardiovascular disease share many risk factors such as diabetes, obesity and underlying hypertension. In addition several proposed pathological mechanisms such as oxidative stress, endothelial dysfunction and insulin resistance are common to both conditions (figure 1.6). It is not known, however, whether pre-eclampsia itself, or underlying maternal genetic and pathophysiological risk factors, or a combination of both contribute to future cardiovascular disease. In this section the evidence for the long-term implications of pre-eclampsia, the evidence for the causes of it, and potential future areas of research will be discussed.

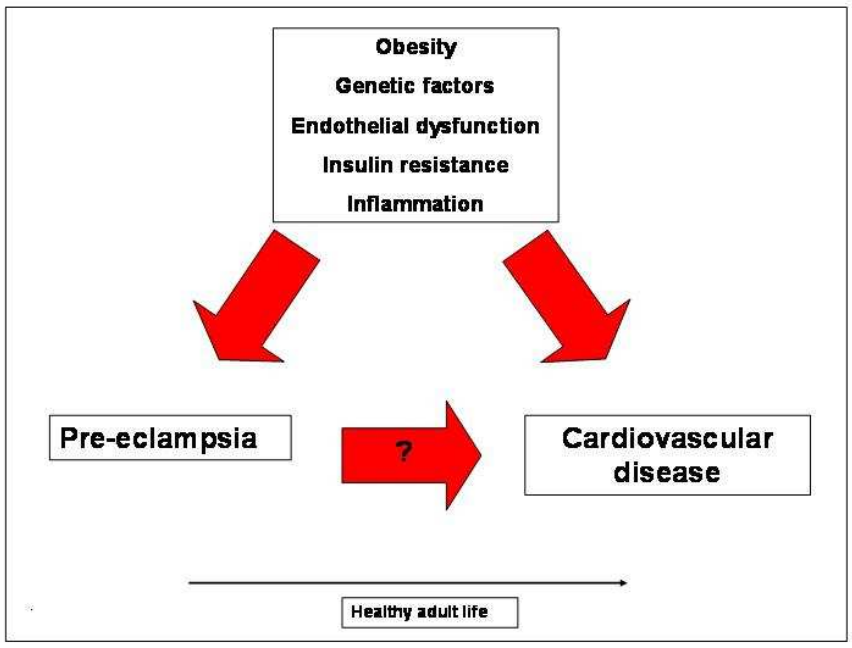

Figure 1.6. From Carty et al. (250)

1.9.1 Epidemiological data

A link between pre-eclampsia and future maternal hypertension was first suggested 50 years ago (251), but it has not been generally accepted until fairly recently that pre-eclampsia had long term implications for maternal health. As has been mentioned in section 1.1, different diagnostic criteria for pre-eclampsia are used worldwide, making accurate comparison of epidemiological studies difficult. Further, there can be huge variation in onset, severity and duration of disease.

Despite these obstacles, the linkage of birth records with registers of morbidity and hospitalisation has allowed several recent studies to examine the relationship between pre-eclampsia and future maternal health. In a systematic review of the literature and meta-analysis, Bellamy et al. (252) examined the relationship between pre-eclampsia, cardiovascular diseases and cancer. When women with previous pre-eclampsia were compared with women who had not developed the condition, relative risks (95% confidence intervals (CI)) of 3.70 (2.70 to 5.05) for hypertension, 2.16 (1.86 to 2.52) for ischaemic heart disease,

and 1.81 (1.45 to 2.27) for stroke were reported, with follow-up of 14 yrs, 11 yrs and 10 yrs, respectively. There was no difference seen in rates of breast cancer, or of cancers in general, between those with and without previous pre-eclampsia. In several of the studies analysed, the risk of future cardiovascular disease appeared to relate to disease severity. For example, one study found that women with previous pre-eclampsia associated with preterm delivery and low birth-weight babies had a 7 fold increase (95% CI 3.3 to 14.5) in death or hospitalisation from ischaemic heart disease at 15-19 years follow-up. Preterm delivery, independent of other co-morbidities, was associated with a 1.8 fold (95% CI 1.3 to 2.5) increase in risk of future ischaemic heart disease (7).

Studies in recent years have also examined the relationship between pre-eclampsia and future maternal renal disease. It has previously been shown that women have an increased incidence of microalbuminuria, a potential marker of both renal damage and cardiovascular disease, when examined 3-5 years after a pre-eclamptic pregnancy (253). This would appear to suggest either that these women have underlying previously unrecognised renal disease, or that pre-eclampsia has had an adverse effect on the future kidney function.

In a Norwegian study, researchers used birth registers and renal registers to examine the relationship between pre-eclampsia and future end-stage renal disease (ESRD)(254). The group had previously shown that, compared with women with normal deliveries of babies of birth-weight >2.5kg, women with a history of pre-eclampsia in the first pregnancy have an increased risk of requiring kidney biopsy (for either proteinuria >1g / 24 hrs or serum creatinine >150 µmol/L with suspicion of renal parenchymal disease) in future life (255). In a study examining over 550,000 women who delivered between 1967 and 1991, women with previous pre-eclampsia in their first pregnancy were found to be 4.7 times (95% CI 3.6 to 6.1) more likely to develop ESRD than those with uncomplicated pregnancies. The risk of ESRD was reported to be higher in those who had pre-eclampsia in more than one pregnancy, and in those who had an uncomplicated first pregnancy and were affected in later pregnancies. In keeping with cardiovascular studies, the risk of ESRD was also found to be higher in those affected by pre-eclampsia in association with intra-uterine growth restriction (255).

The typical histopathological changes seen in pre-eclampsia are of glomerular endotheliosis, characterised by fibrin deposition, endothelial swelling and loss of capillary space (256). These changes had been previously thought to resolve after pregnancy, but it has been proposed that the characteristic renal changes are more long-standing. One study examining the nature of the association between pre-eclampsia and renal disease examined 127 middle-aged women aged between 45 and 65 with chronic kidney disease who had undergone renal biopsy. Of them, 32 (25%) had a history of pre-eclampsia. The most common renal lesion seen in the patients with previous pre-eclampsia was of focal glomerular sclerosis, which was unique to women with previous pre-eclampsia. All other causes of chronic renal failure including IgA nephropathy were commonly seen in both groups, indicating that these syndromes are less likely to be related to pre-eclampsia (257).

Although the relative risk for developing ESRD is low, these studies do provide an insight into future maternal renal health, and may assist in future disease screening and prevention.

1.9.2 Insulin resistance

As mentioned in section 1.4.5, insulin resistance is a mechanism thought to be implicated in the pathogenesis of pre-eclampsia, which could potentially help to explain the link with future cardiovascular disease. During normal pregnancy, there is a progressive increase in insulin production from the beta cells of the pancreas, with a corresponding reduction in insulin sensitivity in the second and third trimesters (258). Women with pre-eclampsia have a further reduction in insulin sensitivity as measured by euglycaemic hyperinsulinaemic clamp studies, an abnormality which persists for up to 3 months post delivery (259). Girouard et al. (260) examined women at an average of 7.8 years after an index pregnancy complicated by pre-eclampsia and gestational hypertension, to assess whether they were more insulin resistant than those with uncomplicated pregnancies. Women with previous gestational hypertension or pre-eclampsia had a higher apolipoprotein B : A1 ratio, higher leptin and insulin levels, and lower adiponectin and LDL cholesterol levels compared to controls, indicating insulin resistance. In addition these women had higher insulin resistance as measured by

homeostasis model assessment (HOMA2). When women with pre-eclampsia or gestational hypertension alone were analysed, these results were less convincing. In addition the women with previous pre-eclampsia or gestational hypertension had a higher BMI at follow-up, which may have confounded results.

Libby et al. (261) examined the relationship between pre-eclampsia and future risk of type 2 diabetes both in the mothers and their children. The study, examining more than 7,000 women for a mean of 45 years after an index pregnancy found no difference in death rates between women with previous pre-eclampsia and those with uncomplicated pregnancies. Women with previous pre-eclampsia had an increased risk of developing type 2 diabetes with adjusted odds ratio of 1.37 (95% CI 1.12 to 1.75). In the offspring, the risk of type 2 diabetes was highest in those with a low birth-weight, and was not related to maternal pre-eclampsia. A potential limitation of this study, however, was that the presence or absence of gestational diabetes was not recorded, which may have influenced the number of women going on to develop future type 2 diabetes.

Further studies have examined the relationship between pre-eclampsia and future diabetes in the mothers. Callaway et al. (262) used questionnaires to study self-reported diabetes in over 3600 women at 21 years after delivery. They found that women with previous hypertensive diseases of pregnancy (including both gestational hypertension and pre-eclampsia) had a 2-fold increase (95% CI 4.42 to 2.91) in the risk of future diabetes compared to those with normal pregnancies. In another study using either laboratory results or prescriptions for diabetes medications to confirm future diabetes, over 2,000 women with previous pre-eclampsia and 29,000 women without pre-eclampsia were examined. They reported an adjusted hazard ratio of 1.82 (95% CI 1.26 to 2.62) for future diabetes in the pre-eclampsia group, at a median follow-up of 8.2 years (263). These studies confirm that increased surveillance for development of diabetes should be considered in women with a history of pre-eclampsia.

1.9.3 Vascular function studies

As has been mentioned in section 1.3.4, endothelial dysfunction is thought to have a key role in the pathophysiology of pre-eclampsia, and is known to contribute to the pathogenesis of hypertension and cardiovascular disease. Flow-mediated dilatation has been used in many studies as a measure of endothelial function, and has been shown to be impaired in women with pre-eclampsia compared to those with normal pregnancies (90,264). Women with HELLP syndrome, a severe variant of pre-eclampsia which is characterised by haemolysis, deranged liver function and thrombocytopenia, did not have such impairment, suggesting that this syndrome may be characterised by different cardiovascular adaptive processes (264).

A number of studies have evaluated women to determine whether they have ongoing endothelial dysfunction after a pre-eclamptic pregnancy. The largest study of its type examined 78 women with a single episode of pre-eclampsia, 35 women with recurrent episodes, and 48 women with uncomplicated pregnancies, at a median of 3 years post-partum. Women with previous pre-eclampsia had reduced flow mediated dilatation, indicating endothelial dysfunction, while glyceryl trinitrate (GTN) induced vasodilatation, which is endothelium-independent was not different between the two groups. Endothelial dysfunction was more marked in those with a history of recurrent pre-eclampsia, a finding which was not explained by traditional risk factors such as maternal obesity or hypertension (265). Further studies using plethysmography to assess forearm blood flow have demonstrated ongoing endothelial dysfunction at 6-12 months (266) and at one year (267) after a pre-eclamptic pregnancy. A study examining women 5-6 years after a pre-eclamptic pregnancy found impaired response in forearm blood flow to low and high doses of the endothelium-independent vasodilator sodium nitroprusside and the endothelium-dependant vasodilator acetylcholine, when compared to women with previous uncomplicated pregnancy (268). A longer-term study using laser Doppler perfusion studies and examining response to vasodilators as a measure of endothelial function reported persistent abnormalities at 15-25 years after a pre-eclamptic pregnancy compared to women with previous uncomplicated pregnancies (269).

As discussed in section 1.3.5, pulse wave analysis has also been reported as a technique both for early prediction of pre-eclampsia and for cardiovascular disease. Few studies, however, have examined the use of pulse wave analysis to examine women after pre-eclamptic pregnancy. In one study in which women were examined 5 years post-natally, no difference was found in augmentation index between women who had pre-eclampsia and those who had normal pregnancies (109). This study may have been limited, however, by the exclusion of women on anti-hypertensive therapy. Whether pulse wave analysis provides additional information on cardiovascular risk in previously pre-eclamptic women remains to be seen, and it is clear that further studies are required in this area.

1.9.4 Cardiac data

Normal pregnancy is associated with a 50% increase in cardiac output, which is mediated by an expansion of plasma volume, generalised vasodilatation and a reduction in total peripheral resistance. The development of pre-eclampsia is associated with alterations in the maternal vasculature, with reduced cardiac output and elevated peripheral resistance at the time of onset of symptoms. There are few longitudinal studies examining echocardiographic abnormalities in women who subsequently develop pre-eclampsia. Abnormalities do, however, appear to develop prior to the onset of clinical symptoms, with elevated cardiac output and signs of hyperdynamic circulation reported as early as week 10-14, and a subsequent fall in cardiac output and rise in peripheral resistance at time of diagnosis (270).

Women with a history of pre-eclampsia have been reported to have echocardiographic evidence of diastolic dysfunction in a subsequent pregnancy (271), and to have impaired cardiac response to exercise (272). There is, however, very little information in the literature regarding the longer term cardiac consequences of pre-eclampsia. Imaging studies using cardiac echocardiography, or more sophisticated techniques such as magnetic resonance imaging (MRI) are required in women years after a pre-eclamptic pregnancy. Such studies may help to further explain the relationship between pre-eclampsia

and future cardiovascular disease, leading to potential screening and therapeutic opportunities.

1.9.5 Carotid artery ultrasonography

Carotid artery ultrasound is a further imaging modality that has been used to determine cardiovascular risk after pre-eclampsia. Ultrasound-based measurement of carotid artery intima-media thickness (IMT) and detection of atherosclerotic plaque are useful measures of preclinical atherosclerosis, and of progression of atherosclerotic disease in non-pregnant populations. IMT measurement is non-invasive, reliable and simple to perform, and has been reported as a risk factor for future development of ischaemic heart disease and stroke.

Increased IMT of the femoral artery (but not the carotid arteries) has been reported in women one year after a pre-eclamptic pregnancy compared to controls (273), but the long-term significance of this finding remains uncertain. One study examined carotid artery IMT and plaque volume at thirty years after a pre-eclamptic pregnancy, a time-point when one might expect a clinically detectable difference. The study reported increased atherosclerotic plaque volume in women with a history of pre-eclampsia, but that IMT was not different between cases and controls (274). On logistical regression analysis, age and a history of pre-eclampsia were independent risk factors for development of atherosclerotic plaque. Whether this imaging modality can help to further determine future cardiovascular risk in affected women, however, remains uncertain.

1.9.6 Biomarkers

In keeping with other inflammatory markers, CRP levels are elevated in normal human pregnancy (275), and as mentioned in section 1.4 have been reported to be elevated in women with pre-eclampsia when compared to women with uncomplicated pregnancies (276), although this has not been a consistent finding (275).

Given the relationship between CRP and cardiovascular disease, several studies have examined whether CRP and other inflammatory markers remain elevated in later life in women with a history of pre-eclampsia. A group in Iceland examined post-menopausal women 30 years after they had eclampsia, the severe variant of the pre-eclampsia syndrome characterised by seizures (277). They reported elevated CRP levels in women with previous eclampsia when compared to controls, which remained significant after correction for smoking status, BMI, hormone replacement therapy (HRT) use and current age. Studies looking at women with pre-eclampsia alone, however, have been less convincing. One group examined inflammatory markers in women 20 years after a pregnancy complicated by pre-eclampsia. They reported a significantly higher ratio of pro-inflammatory cytokine IL-6 to anti-inflammatory cytokine IL-10 in women with previous pre-eclampsia, but levels of CRP were not statistically different between the 2 groups, and there was no difference in TNF-α levels (278). A further study looking at women six years after a pre-eclamptic pregnancy similarly found no difference in CRP levels when compared to those with a history of normal pregnancies (279), while another group reported that the elevated CRP in previously pre-eclamptic women lost statistical significance after correction for BMI (260).

As has been mentioned in section 1.4, many other biomarkers are associated with pre-eclampsia, but few have reported persistent abnormalities in the years after pre-eclamptic pregnancies. One group examined intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-Selectin, as well as leptin, fasting insulin and glycosylated haemoglobin ($HbA1_c$) in a cohort of women 15-25 years after a pre-eclamptic pregnancy. Compared to women matched for BMI, smoking and time since index pregnancy, they reported significantly elevated levels of VCAM-1 ($p=0.038$), ICAM-1 ($p=0.002$) and $HbA1_c$ ($p=0.004$), with a trend towards elevated insulin ($p=0.08$) levels in the formerly pre-eclamptic group (280). Although these studies may emphasise the role of insulin resistance and endothelial dysfunction in these women's future cardiovascular disease, it is again unclear whether it is the pre-eclampsia itself or the underlying risk factors predisposing to cardiovascular disease that cause the abnormalities.

1.10 Aims of this thesis

Although improvements in obstetric and neonatal care have led to a reduction in morbidity and mortality from pre-eclampsia, the ability of clinicians to accurately predict the condition in earlier pregnancy has not improved significantly. The overall aim of this work is to design a large prospective study to identify clinical and biochemical markers which might help to improve our ability to predict pre-eclampsia.

The first hypothesis that this thesis will test is that proteomics can be used to develop a disease specific peptide pattern that predicts pre-eclampsia before the onset of clinically detectable disease. As discussed, in a multi-factorial condition such as pre-eclampsia identifying a single biomarker for predicting disease is perhaps unrealistic. To test this hypothesis I will use urinary proteomics to try to identify novel pathways and mechanisms that might lead to novel biomarkers for disease prediction.

The second hypothesis that this thesis will test is that levels of inflammatory markers and anti-angiogenic peptides are already altered in the first trimester in women who go on to develop pre-eclampsia. Many of the proposed biomarkers for pre-eclampsia are raised at the time of onset of hypertension and proteinuria, a time-point where the only real option available to obstetricians is delivery of the baby. To test this hypothesis I will examine a panel of inflammatory cytokines, adhesion molecules and anti-angiogenic biomarkers in a mixed parity general obstetric population: to examine whether they are elevated in the first trimester, what happens to their levels throughout pregnancy, and how are their levels affected by maternal risk factors such as obesity and increasing maternal age.

The third hypothesis that this thesis will test is that vascular function studies can be used to predict pre-eclampsia before the onset of hypertension and proteinuria. Endothelial dysfunction and arterial stiffness underpin many of the pathophysiological mechanisms behind pre-eclampsia, but the optimal method for their measurement remains unknown. Since such methods would have to be

safe in pregnancy, well tolerated and applicable in large scale studies, to test this hypothesis I will focus on pulse wave analysis and peripheral arterial tonometry, techniques which are simple to perform and are user-independent. Further, since techniques such as these are unlikely to be feasibly used in all pregnant women, I will focus on women known, based upon risk factor profile to be at increased risk, to see whether vascular function testing helps to further discriminate between those likely to be affected.

The fourth hypothesis that this thesis will test is that vascular function remains abnormal after delivery in pregnancy affected by pre-eclampsia. To test this hypothesis I will examine the use of these vascular function analysis techniques, after both pre-eclamptic and unaffected pregnancies, to identify whether they might help to explain why they are more likely to develop cardiovascular disease in the future.

Chapter 2

Materials and methods

2. Materials and methods

2.1 Summary

This chapter provides a description of the general protocols of the clinical techniques used in the studies described in this thesis.

2.2 Ethical approval

All studies were approved by the West Glasgow Ethics committee. Patient information sheets and consent forms are enclosed in Appendix 1-3.

2.3 Recruitment of pregnant women

2.3.1 Description of maternity care in Glasgow & Ayrshire

Prior to January 2010, maternity services in Greater Glasgow were served by 3 main maternity units: the Queen Mother's Hospital (QMH), the Southern General Hospital (SGH) and the Princess Royal Maternity Hospital (PRMH), with approximately 3500, 3000 and 4500 annual deliveries respectively. The Calder report of 2006 (http://www.scotland.gov.uk/Topics/Health/NHS-Scotland/17273/CalderGroup/mbs) recommended that the number of maternity units in Glasgow be reduced from 3 to 2. The decision was therefore made to extend maternity services at the Southern General Hospital, with plans to ultimately build a new hospital incorporating adult, maternity and paediatric services on one site. In January 2010 the Queen Mother's Hospital closed, with pregnant women from its catchment areas choosing to attend either the Southern General Hospital or the Princess Royal Maternity Hospital.

In August 2006 the new Ayrshire Maternity Unit (AMU) opened at the Crosshouse Hospital campus, Kilmarnock. This hospital replaced maternity services at

Ayrshire Central Hospital, Irvine, and in keeping with service changes in Glasgow allowed incorporation of adult, maternal and paediatric services on a single site.

2.3.2 Routine antenatal care

Routine antenatal care for pregnant women in Glasgow and Ayrshire is as follows. Following a positive pregnancy test in the community, women visit their General Practitioner (GP) for a confirmatory urine human chorionic gonadotrophin (HCG) test, and if this is positive they are subsequently referred to secondary care for a "booking visit." Depending on their history and location, women are either referred to community midwife-led maternity units, or to their local maternity hospital. In general, women with previous medical or obstetric problems are seen at hospital, while those deemed to be at low-risk are referred to the community units. The booking visit, normally at 12-16 weeks gestation, consists of an ultrasound scan which assesses the viability and number of foetuses, as well as measuring the crown-rump length to assess gestational age (281). Women with a non-viable foetus are referred to the perinatal team for counselling. Those with a continuing pregnancy are then seen by a midwife, who takes a detailed medical, family and obstetric history, takes routine urine and blood collections, and discusses delivery options. Women then have the opportunity to meet their Consultant, who will be in charge of their overall care, providing an opportunity to discuss specific concerns about the pregnancy, as well as to discuss delivery options.

Maternity services in the UK are currently in a state of change. Instead of hospital based care, there has been an emphasis in recent years of multidisciplinary community-based care, in order to emphasise the "normality" of pregnancy. A "hub and spoke" system is coming into practice in Scotland. Women who are deemed to be medium or high risk are seen at the "hub" units, where a full range of antenatal services, in-patient services and perinatal services are available. Women who are deemed to be low risk are seen at the "spoke" units, which are midwife led, with an emphasis on birth planning, breast feeding and education. Within the areas included in this study, the "hubs" were the main obstetric centres at the Southern General Hospital, the Princess Royal Maternity Hospital, the Queen Mother's Hospital and Ayrshire Maternity Unit.

Much of the work in this thesis is directed towards finding clinical or biochemical "markers" for early prediction of pre-eclampsia. Such tests would be useful in this modern design of maternity care; women found to be "low-risk" would be cared for in the "spoke" units, while those at higher-risk would be seen in the hospitals.

2.3.3 Initial recruitment

Women were recruited for the "Proteomics in Pre-eclampsia" (PIP) study at the initial antenatal (booking) visit, at gestational week 10-18. An invitation letter, a patient information sheet and a consent form were sent to all expectant women along with their appointment for their booking visit at the maternity units at QMH, PRMH, SGH and AMU. At the booking visit women were invited to participate in the study by either myself or a study nurse. Invitation letters, consent forms and patient information sheets are included in Appendix 1-3. All women aged over 16 yrs were invited to participate. Women who were unable to give informed consent, due to language barriers or learning difficulties, were not included in the study. In addition, since this was largely a study aiming to identify early pregnancy markers, women whose ultrasound appearances were in keeping with a gestational age of over eighteen weeks were not included.

Informed consent was obtained, and in addition to samples taken for routine clinical purposes, a sample of blood (five millilitres whole blood, EDTA tube, Vacutainer system) and urine (mid-stream, fresh, universal container) were obtained. Samples were stored in a fridge at 4°C, and at the end of the clinic were transferred refrigerated to the BHF Glasgow Cardiovascular Research Centre. Immediately on arrival samples of urine were separated into three 1ml aliquots and were labelled and stored at -70°C. Blood samples were centrifuged at 4°C, at 3000 revolutions per minute (RPM) for 10 minutes. 3 x 0.5 ml aliquots of plasma were then aliquoted, labelled and stored at -70°C. The remaining blood was then stored at 4°C for DNA extraction.

The following clinical information was collected from women agreeing to take part in the study: age, gestational age according to crown-rump length (weeks),

height (m), weight (kg), BMI (kg/m^2), blood pressure after 5 minutes seated (mmHg) and dipstick urinalysis findings. In addition the following background information was obtained: parity, history of pre-eclampsia in a previous pregnancy (yes / no), history of diabetes (yes / no), history of kidney problems (yes / no), history of hypertension (yes / no), number of years since last pregnancy (years), family history (mother or sister) of pre-eclampsia (yes / no), smoking status (yes / no), presence of a multiple pregnancy (yes / no), history of antiphospholipid syndrome (yes / no), or history of autoimmune disease (yes / no). Information was recorded on a study data collection form, which is included in Appendix 4. Pregnant women were allocated a study code *N*-00001, where *N* was the first letter of the hospital in which they were recruited (Q=Queen Mother's Hospital, P=Princess Royal Maternity Hospital, X=Ayrshire Maternity Hospital, and S=Southern General Hospital), followed by their study number. In addition women were asked if they would be willing to participate in further studies at gestational weeks 16 (visit 2) 28 (visit 3) and post-natally (visit 4).

Pregnant womens' details (name, date of birth, hospital number and community health information (CHI) number) were entered into a Microsoft Access database along with patient study code. Only the lead research nurse and I had access to this database. In a separate Microsoft Access database the patient study code was used as an identifier and all clinical information was then entered. Both databases were password protected and double encrypted.

2.3.4 "Risk-factor" group

A number of risk factors are known to be associated with an increase in risk of developing pre-eclampsia, and are summarised in Chapter 1. In brief, nulliparity (having the first baby), a family history of pre-eclampsia (mother or sister,) age over 35 yrs or under 20 years, a history of pre-eclampsia in a previous pregnancy, body mass index >30 kg/m^2, and a history of diabetes, hypertension, renal and autimmune disease are all implicated. Also known to be at increased risk are those with a multiple pregnancy and those who have a gap of more than ten years since their last pregnancy. Women with 2 or more of these risk factors were initially identified based on information given at the booking visit. These women were contacted by telephone by me and invited to attend the BHF

Glasgow Cardiovasular Research Centre for additional studies at gestational week 16 (14-18), week 28 (26-30,) and at 6-9 months post-natally.

In addition, a cohort of healthy nulliparous non-pregnant women were recruited to the study to act as “controls.” These women, aged between eighteen and forty years, were recruited using advertising posters in the University of Glasgow. Study protocols were exactly the same as those described for the pregnant women, except that these women were only seen on one occasion.

Finally, a cohort of women recruited for the study who went on to develop pre-eclampsia, but who had not been seen for vascular function studies at gestational week 16 and 28, were invited for vascular function studies at 6-9 months post-natally. A flowchart outlining the study is shown in figure 2.1 below.

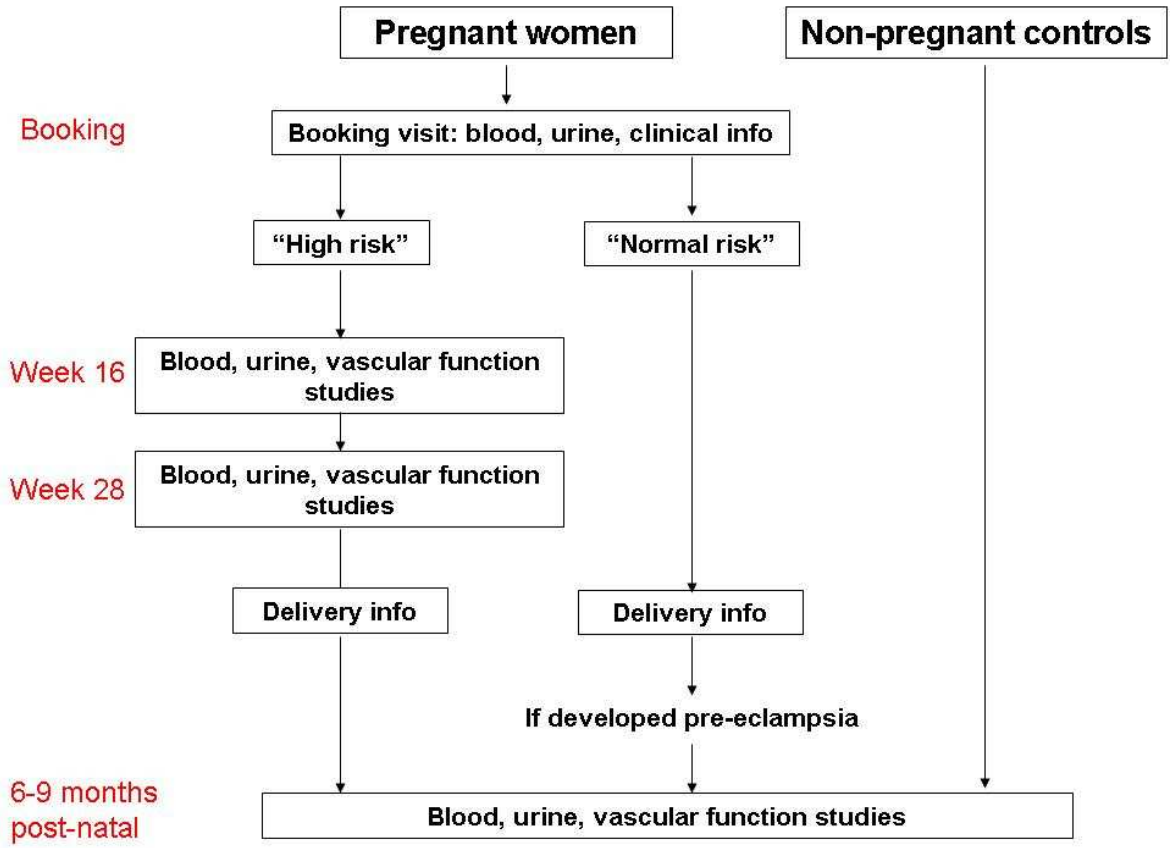

Figure 2-1 Flowchart describing overall study design

2.4 Vascular Function Sub-studies

2.4.1 Study protocol

Women who agreed to attend for vascular function studies were sent an information sheet, a consent form and a map by post. All women were seen in the afternoon at the British Heart Foundation Glasgow Cardiovascular Research Centre. Appointments were on Mondays, Tuesdays and Wednesdays, at 1300, 1430 or 1600 hrs. Women were asked to abstain from alcohol, caffeine and cigarettes on the day of the study. All studies were carried out in a dedicated room which was quiet, with minimal artificial lighting, and with temperature controlled between 22-24°C.

Following written informed consent, body weight and height were measured with women in light clothes and without shoes to the nearest 0.5kg of weight and the nearest 0.5cm of height. Exactly the same equipment was used for all studies, and the weighing scales (Seca, Germany) were calibrated regularly. Body mass index (BMI, kg/m^2) was calculated as:

$BMI = weight\ (kg) / height\ (m)^2$

Blood pressure was measured after 5 minutes seated using the Omron MX2 automated device, which was calibrated regularly by the University of Glasgow's Department of Medical Physics. If blood pressure was elevated, this was communicated back to the woman's General Practitioner or midwife, and if necessary women were referred directly to hospital for assessment. Throughout the course of the study a total of 3 pregnant women were referred to the hospital Day Care Unit for assessment: 2 because of hypertension, and 1 because of concern about reduced foetal movements.

A 12-lead ECG was performed with the woman lying supine. Any significant abnormalities were reported to the woman's General Practitioner.

2.4.2 Urine samples

A sterile container was used to collect a mid-stream sample. The sample was then transferred to a universal container, while a Dipstick was used to analyse the urine remaining in the sterile container. Results of Dipstick measurement and blood pressure were then forwarded to the woman's General Practitioner, along with a letter informing them of their participation in the study. If glycosuria was detected, women were referred to their midwife for repeat sampling and oral glucose tolerance testing if required. Two pregnant women were referred to the hospital day care unit because of glycosuria.

Urine samples in the universal container were then transferred to the laboratory. Urine was separated into three 1ml aliquots, which were frozen at -70°C.

2.4.3 Blood samples

Samples were taken from the antecubital fossa of the non-dominant arm using the Vacutainer system. The non-dominant arm was chosen since it was the dominant arm that would be used for blood pressure cuff inflation during the subsequent endothelial function studies. A standard tourniquet was used and the following samples were obtained:-

- 7 mls in a Lithium Heparin tube
- 7 ml in an EDTA tube
- 7 ml in a serum tube
- 2.5 ml in a PAXgene blood RNA tube

The RNA tube contained an additive that stabilises the gene transcription profile by reducing RNA degradation and minimising gene induction. In view of this the blood was collected for the RNA tube last. The tourniquet was released as soon as blood started to flow into the RNA tube, and the tube was inverted 8-10 times immediately after blood collection.

On arrival of the samples in the laboratory, 50µl of whole blood was removed from the EDTA tube to a vial on ice for subsequent glutathione (GSH) analysis. 100µl was removed from the EDTA tube to a vial on ice, to which was added 10µl of M2VP scavenger, for subsequent oxidised glutathione (GSSG) analysis. The RNA blood tube was kept at room temperature for a minimum of 2 hours and a maximum of 72 hours before processing. All other blood samples were then centrifuged at 2700 rpm, at 4°C, for 10 minutes.

Samples were then aliquoted as follows:-

- EDTA tube
    - 2 x 500µl plasma

    - 1 x 200µl plasma
    - 1 x 150µl plasma
- Lithium Heparin tube
    - 2 x 1ml plasma
    - 2 x 100µl plasma
- Serum tube
    - 3 x 500µl serum
- Whole blood
    - 50µl for GSH analysis
    - 100µl for GSSG analysis

All samples were then labelled using the study code and stored at -70°C.

2.5 Laboratory studies

2.5.1 Cytokines and adhesion molecules

2.5.1.1 Sample collection

Samples were collected and spun as outlined above, stored at -70°C and thawed prior to analysis. For all measurements batch analysis was performed to minimize inter assay variability. Each sample from a woman who went on to develop pre-eclampsia (case) was matched for age, body mass index (BMI) and parity with 2 controls; cases and their controls were analysed during the same batch.

2.5.1.2 Biochip technology

Analysis was performed using Evidence Investigator Technology (Randox Laboratories Ltd). This system uses biochips measuring 9x9 mm, each of which is supplied pre-fabricated with an array of discrete test regions deposited in exactly pre-defined co-ordinates on the surface of the biochip. This allows simultaneous analysis of multiple parameters. When analytes are present in the patient sample they attach to the surface-bound specific ligands, and the concentration of each analyte can be determined relative to the calibrators of known concentration.

2.5.1.3 Biochip carrier

The biochip carrier has nine separate reaction wells arranged in a 3x3 format (figure 2.2). Each reaction well contains a biochip, secured in the base of the well, each of which accommodates a single patient sample. Carrier trays facilitate handling of 6 carriers at a time.

The multi-biochip carrier is supplied with a handle to facilitate biochip handling during the assay procedure, and to minimise human contact with the biochip surface.

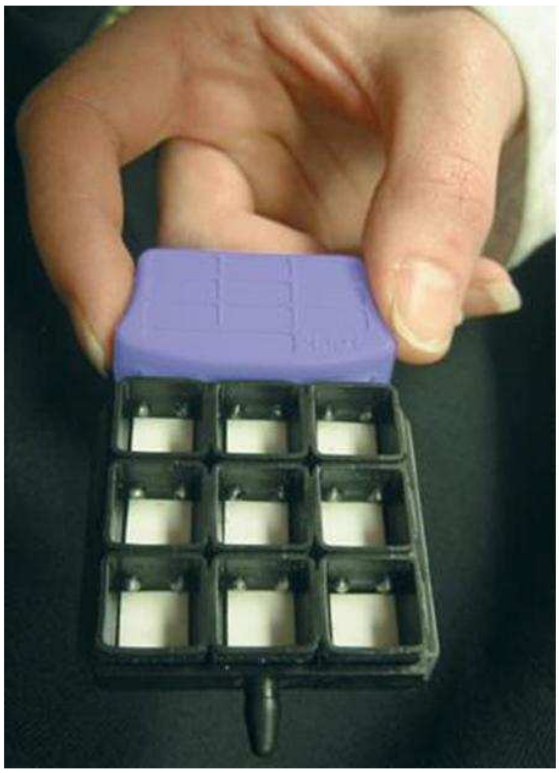

Figure 2-2. Biochip carrier with 9 reaction wells.

2.5.1.4 Adhesion molecules array protocol

To each well 225µl assay diluent and 25µl of sample or standard was added. The carrier well was incubated at 37°C for 60 minutes at 370 rpm. Following this 50µl conjugate was added, and the carrier was incubated for a further 60 minutes at 37°C at 370rpm. Liquid was then decanted and each well was then washed with buffered saline; 2 quick washes and 4 x 2 minute soaks. Following this 250µl signal reagent mix was added to each well. Samples were then kept at room temperature and protected from light with tin foil for 2 minutes, prior to imaging.

The following analytes were measured using this protocol: VCAM-1, ICAM-1, E-Selectin, P-Selectin and L-Selectin.

2.5.1.5 Cytokine & Growth factors profile protocol

To each well 225µl assay buffer and 100µl of sample or standard was added. The carrier was incubated at 37°C for 60 minutes at 370 rpm on the thermoshaker,

covered, and then further incubated for 16-20 hours at 2-8°C at 0rpm. The liquid was then decanted, and the wells were washed with buffered saline; 2 quick washes and 4 x 2 minute soaks. 300µl conjugate was then added to each reaction well, and the carrier was then incubated at 37°C for 60 minutes at 370 rpm. Liquid was then decanted, and wells were again washed with buffered saline; 2 quick washes and 4 x 2 minute soaks. Liquid was decanted, 250µl signal reagent was added, and the wells were incubated at room temperature for 2 minutes, protected from light, prior to imaging.

The following analytes were measured using this protocol: Interleukins -1α, -1ß, -2, -4, -6, -8, -10, VEGF, TNF-α, IFN-γ, MCP-1, EGF.

2.5.1.6 Chemiluminescent technology

Chemiluminescence is the production of light via a chemical reaction. It is used in immunassays to determine the level of analyte present in a sample. The "Evidence Investigator" system uses a chemiluminescent substrate with horseradish peroxidise (HRP) label for the detection of antibodies or analytes bound to the biochip surface. The intense signal output enables the detection of picogram quantities of antigen or antibody. The signal reagent used in these experiments comprises a 1:1 mixture of luminol / enhancer solution and a peroxide solution.

The reaction mechanism is based on oxidation and reduction reactions, which results in the formation of free radical components and the emission of light. HRP reacts with peroxide to form an intermediate compound. This compound then reacts with luminol to form the luminol radical, which itself decomposes with emission of light. Enhancer molecules regenerate free radicals, which aid the chemiluminescent reaction.

2.5.1.7 Chemiluminescence detection

Light emitted from the chemiluminescent reactions taking place at the discrete test reactions on the surface of the biochip is detected and quantified with a charge coupled device (CCD) camera. This camera simultaneously records the light emitted from each of the discrete test regions on each of the nine biochips

in the biochip carrier. The CCD is made up of a collection of tiny light-sensitive diodes which convert photons of light into electrons which produce electrical charge. Each diode or photosite is sensitive to light; the brighter the light that hits a photosite the greater the electrical charge that accumulates. The degree of light emission can therefore be quanitified based on the strength of the electrical signal generated.

2.5.2 Enzyme linked immunosorbent assays

2.5.2.1 Assay principles

Enzyme linked immunosorbent assays (ELISAs) employ the quantitative sandwich antibody principle. Plasma (or other appropriate medium) samples containing the antigen of interest and standards of known concentration are pipetted into a plate of test wells which are coated with primary antibody to the antigen. After incubation sufficient to allow >95% of the antigen to bind the primary antibody, the wells are washed to remove any unbound material. An enzyme-linked polyclonal antibody, specific for the antigen of interest, is then added to each well. After incubation time to allow end-point binding of the secondary antibody to the captured antigen, the plate is washed to remove excess unbound conjugate antibody.

A substrate for the specific enzyme on the secondary antibody is then added, and colour develops in proportion to the amount of antibody bound in the initial step. An acid solution is then added to stop the reaction at a point where the standard curve added is detectable and linear. The colour change in each well is then measured photometrically using an appropriate wavelength on a plate reader (Multi Scan EX, Thermo Electron Corporation, UK). The plate reader is programmed to draw a standard curve and infer the concentrations of the plasma samples from this curve using each relevant optical density (OD). An example of a standard curve obtained for an ELISA is shown in figure 2.3. All R&D systems standard operating procedures can be procured from links on the website:
http://www.rndsystems.com/product_detail_objectname_elisa_assay_product.aspx

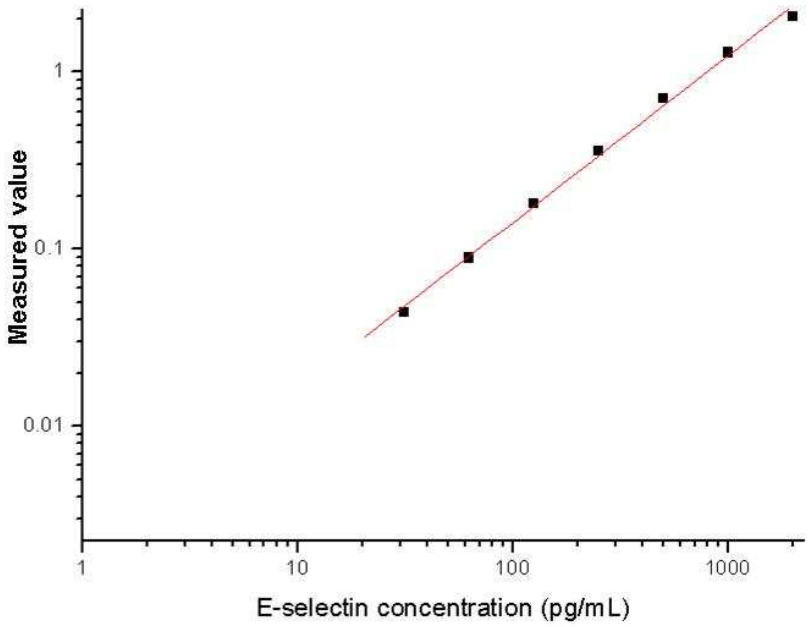

Figure 2-3 Example of a standard curve obtained for E-Selectin ELISA comparing measured values with known concentrations of standard test solutions

2.5.2.2 sFLT-1 (sVEGF-r1)

Soluble FMS-like tyrosine kinase-1 (also known as serum VEGF receptor-1) was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK).The manufacturers claim the assay is sensitive to 1.5pg/ml. The reported intra-assay CV is 2.6-3.2%, and the reported inter-assay CV is 5.5-9.8%.

2.5.2.3 sENG measurement

Soluble endoglin (sEng) was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK.) The manufacturers claim that the assay is

sensitive to 0.007 ng/ml. The reported intra-assay CV is 2.8-3.2%, and the reported inter-assay CV is 6.3-6.7%%.

2.5.2.4 PlGF measurement

Placental Growth Factor (PlGF) was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK). The manufacturers claim that the assay is sensitive to less than 7 pg/ml. The reported intra-assay CV is 3.6%, and the reported inter-assay CV is 11%.

2.5.2.5 E-Selectin measurement

E-Selectin was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK). The manufacturers claim that the assay is sensitive to less than 0.009 ng/ml. The reported intra-assay CV is 5.2-6.6%, and the reported inter-assay CV is 7.3-8.7%.

2.5.3 Urinary Proteomics

2.5.3.1 Sample preparation

Each pregnant woman recruited to the study had 3 x 1000 µl aliquots of urine stored at -20°C at the BHF Glasgow Cardiovascular Research Centre. Samples were stored for up to 3 years before analysis. All proteomic analysis was performed at Mosaiques Diagnostics, Hannover, Germany, and samples were transferred on dry ice to Germany via courier. Previous studies at Mosaiques have shown no significant storage-related degradation in samples stored for 10 years compared to those stored for 6 weeks (222).

Shortly before proteomic analysis, each 0.7 ml aliquot of urine was thawed and diluted with 0.7ml of 2 mol/L urea and 20 mmol/l NH_4OH containing 0.02% SDS. To remove proteins with a high molecular mass such as albumin, samples were then filtered using a Centrisart ultracentrifugation filter device (Sartorius, Goettingen, Germany) with a 20 kDa molecular weight cut-off. Centrifugation

was carried out at 3000g, until 1.1 ml of filtrate was obtained. The filtrate was then applied onto a PD-10 desalting column (GE Healthcare Bio-Sciences, Uppsala, Sweden), pre-equilibrated with 0.01% NH_4OH in HPLC-grade H_2O, to remove urea and salts, and to enrich the low molecular-weight peptides present. Finally, samples were lyophilised in a freeze-drier (Speed-Vac RVC 2-18/Alpha 1-2, Christ, Osterode a.H, Germany) and stored at 4°C suspended in high performance LC-grade H_2O until CE-MS analysis.

2.5.3.2 Capillary electrophoresis separation

Peptides were first separated based on differential migration through a liquid filled capillary column. For capillary electrophoresis (CE) separation of urine sample peptides, the P/ACE MDQ system (Beckan Coulter, Fullerton, USA) was used. A 90 cm length, 75µm inner diameter fused-silica capillary was first rinsed with running buffer (30% methanol, 0.5% formic acid) for 3 minutes. The urine sample was then injected into the capillary for 99 seconds with 1 psi positive pressure, resulting in an injection of approx 700 nl. The separation was then performed by applying a charge of +30kV at the inlet of the capillary, resulting in a current of approximately 13µA. The temperature was kept constant at 35°C for the entire length of the capillary. After each sample analysis, the capillary was rinsed first with 0.1M NaOH, then for 5 minutes with water, then with running buffer.

Figure 2-4. Capillary electrophoresis (left), microTOF mass spectrometer (middle) and computer for data acquisition (right).

2.5.3.3 Mass spectrometry

The capillary electrophoresis instrument was coupled online with a micro time-of-flight (TOF) mass spectrometer (Bruker Daltronic, Bremen, Germany.) The electrospray ionisation interface (Agilent technologies, Palo-Alto, Ca, USA) was grounded, and the potential was set to -4 to -4.5 kV. Acquisition of data was controlled automatically by the capillary-electrophoresis program through close-contact relays. Mass spectra were accumulated every three seconds over a range of mass:charge (m:z) ratio of 350-3000, and the mean was then calculated.

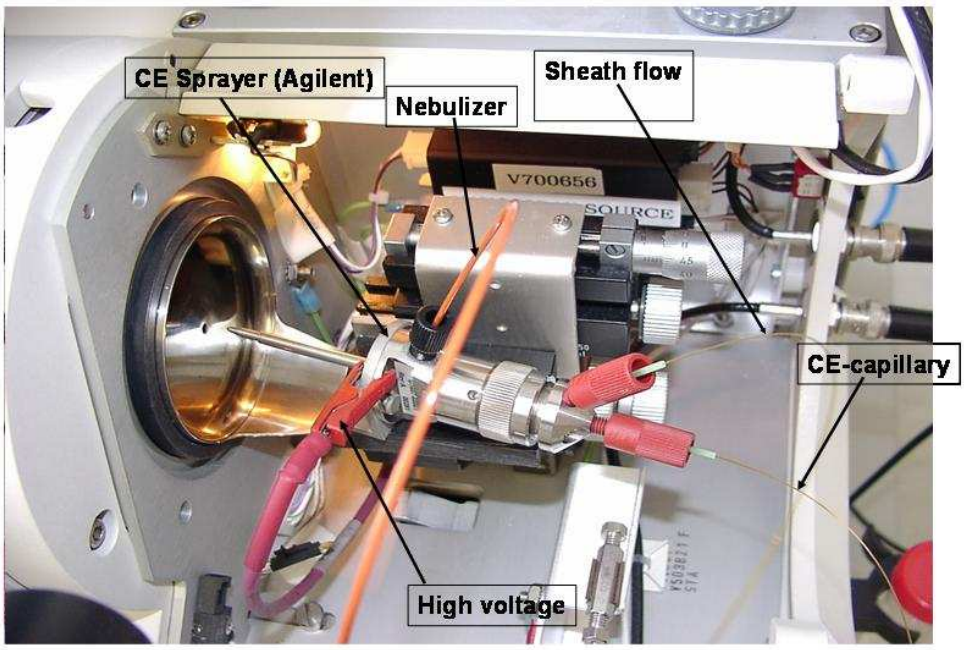

Figure 2-5. Interface of capillary and mass spectrometer

2.5.4 Data Processing

Each CE-MS run provides a large amount of information, with over 1000 individual spectra for each sample. A number of steps are therefore required in order to accurately process the data.

2.5.4.1 Deconvolution

Data is analysed using a specific software tool, Mosaiques-Visu. Initially, CE/MS peaks are detected using a signal / noise (S/N) ratio of eight. The charge of each peak is then calculated, based on isotopic distributions and conjugated masses. The Mosaiques-Visu software then deconvolutes the data, so that mass spectral ion peaks from the same molecule at different charge states are recorded as a single mass.

2.5.4.2 Calibration

MS data needs to be normalised to correct for analytical issues such as signal suppression, and biological issues, for example state of hydration of the patient

studied. Migration time and ion signal intensity (amplitude) are normalised based on 29 peptides that serve as internal standards. These peptides are the result of normal biological processes, and do not appear to be affected by age, sex or disease state. Normalisation using these “housekeeping” peptides has been shown to be as accurate as absolute peptide quantification using urinary quantification or stable isotope labelled synthetic marker analogues (282).

2.5.4.3 Annotation

The resulting peak list characterises each peptide by its molecular mass (in daltons), CE migration time and signal intensity, providing a unique identification mark. Data are then entered into a Microsoft SQL database, which allows further analysis and comparison with other samples. Mass spectrometry peaks from different samples were presumed identical if mass deviation is less than 100 ppm, and the migration time deviation is less than 5 mins.

2.5.5 Derivation of classification factor

The information about the multiple peptide patterns associated with pre-eclampsia was then transformed into a single number, or classification factor, allowing analysis of the different peptide levels in different patient groups. Classification factors were generated using the MosaCluster software (225); using a support-vector-machine (SVM) based mathematical model, MosaCluster displays data points (urine samples) from n proteomic markers as an n-dimensional vector, and attempts to separate them in an n-dimensional hyperplane. The resultant classification factor was calculated for each patient sample from the distance and direction of its vector to the separating mathematical hyperplane.

2.5.6 Sequencing

Candidate biomarkers were sequenced using LC-MS/MS analysis using instruments with electron transfer dissociation (ETD) capability (283-285). Mass spectrometry data were searched against the IPI human database using the Open Mass Spectrometry Search Algorithm (OMSSA; http://pubchem.ncbi.nlm.nih.gov/omssa), using an e-value cut-off of 1.00E-2.

All matched sequences were manually validated. All sequences obtained from human urine can be assessed at the Mosaiques human urine proteome database (http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front_content.php?idcat=257).(286)

2.5.7 Statistical methods and sample classification

Estimates of sensitivity and specificity were calculated based on tabulating the number of correctly classified samples. Confidence intervals (95% CI) calculated using MedCalc version 8.1.1.0 (MedCalc Software, Mariakerke, Belgium). The reported unadjusted p-values were calculated using the natural logarithm-transformed intensities and the Wilcoxon Rank-Sum Test. Statistical adjustment for multiple testing was performed using the false discovery rate (FDR) adjustments of Benjamini and Hochberg (287).

2.5.8 Definition of biomarkers

2.5.8.1 Training set

In order to identify potential biomarkers, urine samples from women who went on to develop pre-eclampsia (cases) were compared with samples from women who had normotensive pregnancies (controls). Women were matched for age, BMI and parity. Peptides that were present in at least 75% of the samples in either group (cases or controls) were identified as potential biomarkers, and were further evaluated using receiver operator characteristics (ROC) statistics. The amplitude distribution of CE-MS data peptides present in the samples was used as the ROC variable, and the affiliation to a diagnostic group (cases or controls) was used as the classification variable. The obtained area under the ROC curve for a particular peptide was used as a measure of its discriminatory potential. An initial list of potential biomarker candidates was then further refined using the Mann-Whitney test, with $p \leq 0.05$ being indicative of significance. Model establishment and sample classification were performed

using a linear classification algorithm. This algorithm generates a classification model based on peptides that are most able to discriminate between cases and controls. In general models consisted of fewer peptides than samples, in order to avoid over-fitting.

2.5.8.2 Test set

Using the peptides identified, the ability of the model to differentiate between cases and controls was then evaluated using a blinded assessment of urine samples, from women who went on to develop pre-eclampsia (cases) and women who had normotensive deliveries (controls).

2.5.9 Statistical analysis

All of the statistical analyses for pregnant women's characteristics and clinical data were performed using the SPSS software package (SPSS inc., Chicago, USA). Normality of data distribution was assessed using the Kolmogorov-Smirnov test and manual inspection of Q-Q plots. Data were expressed as mean ± standard deviation (SD) if normally distributed, or as median ± interquartile range if their distribution was not normal. For continuous variables, differences between the groups were evaluated using the unpaired Student's *t* test if data was normally distributed, or Mann-Whitney *U* test for variables that were not normally distributed. Fisher's exact test was used for comparison of categorical variables. A p-value of <0.05 was considered significant.

Sensitivity and specificity were calculated based on tabulating the number of correctly classified samples. Confidence intervals (95% CI) were calculated in MedCalc (MedCalc for Windows 8.1.1.0, Medcalc Software, Mariakerke, Belgium). The ROC plot was obtained by plotting all sensitivity values (true positive fraction) on the *y* axis against their equivalent (1 − specificity) values (false positive fraction) for all available thresholds on the *x* axis (MedCalc Software).

2.6 Clinical studies

2.6.1 Pulse Wave Analysis

Pulse wave analysis was carried out using the SphygmoCor (Atcor Medical, West Ryde, Australia) system. Pregnant women lay supine in a dedicated room, temperature controlled at 22-24°C. The SphygmoCor device consists of a pencil shaped applanation tonometer (Millar instruments, Houston, Texas, USA), which was placed over the radial artery of the dominant hand, with the wrist extended. The wrist was used since the radial artery is easily accessible in the majority of individuals. The radial artery was gently compressed with the tip of the manometer at the site of maximal pulsation. The radial artery recording was calibrated with the blood pressure measured at the brachial artery, since the blood pressure is practically identical in both vessels. The probe was connected to a laptop computer upon which data was collected directly.

The supporting software applies a generalised transfer function to the radial artery waveform which is used to derive the aortic waveform. The generalised transfer function is described in Chapter 1. From the aortic waveform the augmentation pressure (AP) and the augmentation index (AIx) were calculated. The AP is defined as the height of the late systolic peak above the inflection point on the waveform. The AIx is defined as the AP expressed as a percentage of the aortic pulse pressure (PP). AIx is affected by heart rate; with a faster heart rate the duration of systole is shortened. This leads to the reflected wave meeting the advancing wave in diastole, rather than the usual systole. This in turn leads to reduced augmentation of the advancing wave, or a reduced AIx with a faster heart rate. Since there is a linear relationship between heart rate and AIx, the AIx was standardised to a heart rate of 75 bpm (AIx-75).

After a waveform was established, sequential waveforms over a 9 second period were recorded, and the proprietary software created an average arterial waveform. The recordings were included if they passed certain quality controls; if systolic or diastolic variability between waveforms exceeded 5% then the reading were discarded, and if the amplitude of the waveform was below a

certain threshold the readings were excluded. In addition, the SphygmoCor system has its own quality control, or “operator index” which is displayed on screen. An operator index of ≥80% is deemed acceptable, 100% is the best achievable. Fifty PWA recordings performed by me were selected at random and were reviewed by an observer employed by SphygmoCor, to ensure the standard of recordings was acceptable. A typical aortic pulse waveform is shown in figure 2.6 below.

Figure 2-6. Aortic waveform generated from SphygmoCor system.

PWA was recorded until 3 separate acceptable recordings had been recorded for each study visit. All PWA studies were carried out by me. The mean of the 3 recordings was used for analysis.

2.6.2 Endothelial Function assessment

PAT (peripheral arterial tone) measurement is a non-invasive technique for assessment of endothelial function which is safe and well-tolerated in pregnancy, is user-independent, and may be applicable in large clinical studies.

PAT technology captures a beat-to-beat plethysmographic recording of the finger arterial pulse wave amplitude (PWA) with pneumatic probes. PAT probes, which are fitted to the index fingers of both hands, exert a uniform pressure field around the entire surface of the distal phalynx, measuring pulsatile volume changes. The inflated probes apply a counter-pressure of approximately 70mmHg to the finger tips, avoiding venous distension which could distort results. Probes are attached by flexible tubing to isolated volume reservoirs which buffer pressure changes within the probes. These pressure changes are then filtered, amplified and diplayed on screen. Women were asked to switch off mobile telephones, and not to move or speak during the recordings.

PAT recordings were performed using the EndoPAT 2000 system (Itamar medical, Caesarea, Israel) after a period of at least twenty minutes rest, in order to allow women to acclimatise to their surroundings. This is particularly important in the winter months, when study participants are relatively peripherally vasoconstricted. In practical terms this meant that women would have their ECG, followed by pulse wave analysis, and then PAT assessment. After 10 minutes of baseline recordings at rest, a blood pressure cuff (Hokanson SC12, Bellevue, USA) was inflated around the dominant arm to either 60 mmHg above the systolic blood pressure or at least 200 mmHg, whichever was higher, with a maximum inflation to 300mmHg. The signal in the study arm was amplified to 20,000 arbitrary units (au), to ensure that the blood supply was fully occluded. This was constantly monitored during the occlusion to ensure that there was no visible pulsatile waveform, with the cuff being inflated a further 20 mmHg if there was any breakthrough. After exactly 5 minutes the cuff was immediately deflated, which induced flow-mediated reactive hyperaemia, which was recorded over a further 5-10 minutes. The contralateral (non-dominant) arm remained uncuffed and acted as a “control” to correct for any systemic artefact, for example temperature changes. The test therefore lasted for approximately 20 minutes.

Figure 2-7. PAT typical clinical set-up.

The magnitude of flow-mediated hyperaemia was then calculated as a measure of endothelial function. Pulse wave amplitude in the minute beginning exactly 60 seconds after release of the blood pressure cuff was compared with a 210 second period prior to occlusion. The use of the 1 minute period beginning exactly 1 minute after cuff release is based on data showing that this time-point provides the closest correlation with coronary artery endothelial function on ROC (Receiver Operator Characteristic) curve (288). To correct for systemic factors including arm ischaemia itself this ratio was corrected for readings in the uncuffed left arm. The result of the test is the reactive hyperaemia index, (RHI) a measure of endothelial function. The majority of PAT recordings were performed by myself, the remainder were performed by a research nurse trained by myself. All PAT studies were reviewed by myself; PAT measurements were analysed using proprietary software in an automated, operator-independent manner.

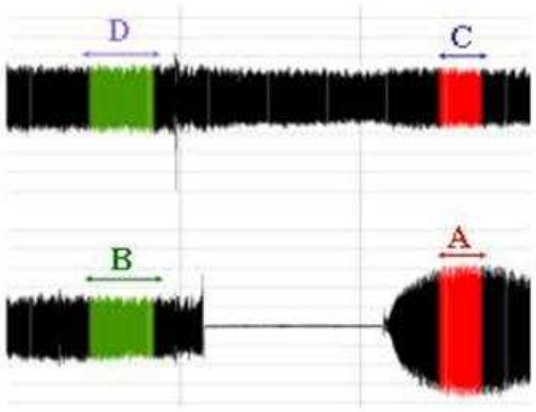

Figure 2-8. RHI was calculated as pulse amplitude following occlusion (A) / pulse amplitude before occlusion (B) in the study arm, divided by the same ratio in the control hand, (A/B) / (C/D).

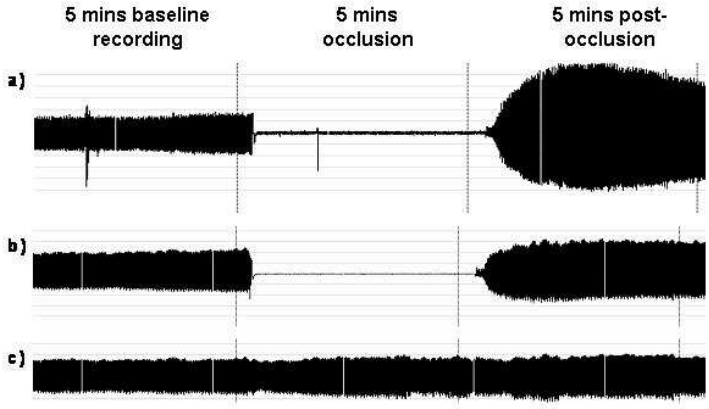

Figure 2-9. PAT recording with 5 minutes of baseline recording, 5 minutes of arm occlusion and 5 minutes of post-occlusion recording. (a) is a subject with normal reactive hyperaemia, (b) is a subject with impaired reactive hyperaemia while (c) is the control arm which corrects for systemic factors.

In addition the Endo-PAT 2000 device was also used to calculate a peripheral augmentation index (AIx). Using an automated computerised algorithm, peak volume and inflection points were identified on the pulse waveforms. Peripheral AIx was calculated as the ratio of the difference between the early and late systolic peaks of the waveform relative to the early peak (P2-P1 / P1), expressed as a percentage.

The following measurements were then recorded in the database: reactive hyperaemia index (RHI), Augmentation Index (AIx) and baseline pulse amplitude in both the study arm and the control arm.

2.6.3 Brachial artery FMD

Doppler ultrasound was used to measure flow-mediated dilatation (FMD) at the brachial artery. Following 5 minutes lying supine after the PAT examination, the brachial artery was identified and scanned longitudinally at 5 to 10 cm proximal to the antecubital fossa using an 8.0 MHz linear array transducer (Acuson 8L5 system, Sequioa 512, Siemens). Baseline measurements of artery diameter and peak flow velocity were obtained at end diastole. Vessel diameter was measured using ultrasonic callipers to assess the distance between the anterior and posterior interface between the media and adventitia (m-line). Electrocardiographic recordings were made throughout the study, and images were triggered on the R wave.

Baseline scanning was recorded for 5 minutes; a pneumatic blood pressure cuff was then inflated to 250mmHg on the forearm distal to the artery. The cuff was released after 5 minutes and the artery was then scanned for a further 5 minutes to record the effects of reactive hyperaemia.

The study was recorded and analysed offline with Vascular Research Tools (Medical imaging Applications LLC, USA). FMD was expressed as the percentage change in the arterial diameter pre and post cuff occlusion, relative to the baseline diameter. All recordings were performed by the same experienced investigator.

2.7 Delivery outcomes

2.7.1 Diagnostic criteria

Diagnostic criteria for pre-eclampsia are described in Chapter 1. In order to fulfil diagnostic criteria for pregnancy-induced hypertension, women had to have 2 or more blood pressure readings of ≥140 mmHg systolic and ≥90 mmHg diastolic at least 6 hrs apart, but before the onset of labour, along with proteinuria as outlined in Chapter 1. Those with hypertension arising before 20 weeks gestation were classified as having chronic hypertension. Women with positive urinalysis in the context of urinary infection were not included.

2.7.2 Delivery outcomes

The methods used for achieving accurate outcomes of pre-eclampsia varied between the different maternity units involved in the study. I used the following methods to ensure that I obtained delivery information on the maximal possible number of women in the study.

2.7.2.1 Labour wards

In all hospitals participating in the study a poster was placed in the labour ward, inviting midwives to use a patient label sticker to identify all women admitted with hypertension in pregnancy. These stickers were collected at the end of each month, were checked against study databases, and notes were requested for all highlighted women who had been recruited for the study.

2.7.2.2 Day care units

Each hospital has a “day care” area, which is where women attend when they are referred from the community for assessment of pregnancy related problems. In general the majority of women who develop hypertension at any stage in pregnancy are referred to the day care unit. Here, they are assessed by an experienced midwife, and are referred to a doctor if necessary. Posters advertising the study were placed in the day care unit, and for women with hypertension, a sticker with hospital number was attached to the poster. Posters

were checked against study databases, and case notes for all identified women who had been recruited into the study were requested.

2.7.2.3 Induction books

The majority of women who develop hypertension and pre-eclampsia which cannot be adequately controlled medically will have induction of labour or Caesarean section arranged. Each labour ward has an “induction book” which has details of all women requiring induction of labour or Caesarean section arranged. I analysed each of these induction books monthly, and took details of all women who required induction or Caesarean section for hypertension, pre-eclampsia or intra-uterine growth restriction. These lists were checked against the study databases, and case notes were requested for each woman who had been recruited into the study.

2.7.2.4 Databases

Different hospitals have different systems of database where details of deliveries are recorded. QMH, SGH and AMU have systems through which all delivery reports are uploaded onto a centralised database. These allow for a search each month for all women with a mention of hypertension, pre-eclampsia or intra-uterine growth restriction in their delivery summary. All such women were then checked against the study database, and case notes were reviewed if the mentioned women had been recruited for the study.

2.7.2.5 Record of delivery

A record of delivery is generated for each delivery in each hospital. For each woman recruited to the study, the following information was extracted from the record of delivery: gestation at delivery, sex of baby, weight of baby (grams), mode of delivery, presence of any medical problems including hypertension and diabetes, and blood pressure post-partum. Case notes were reviewed by myself for all women in the study who had developed hypertension before, during or after delivery.

2.7.2.6 Case note analysis

I analysed the case notes in detail of each woman who was said to have had hypertension, pre-eclampsia or intra-uterine growth restriction. Diagnostic criteria as described in Chapter 1 were used to identify true cases of pre-eclampsia, those with isolated pregnancy-induced hypertension, and those without either condition, for example those women who only had hypertension during labour.

For the purposes of these experiments, each woman who went on to develop pre-eclampsia was matched with at least 2 "controls" who had uncomplicated pregnancies. Cases were matched with controls as closely as possible for age, body mass index, parity and gestational age at time of booking. Women who developed any degree of hypertension or gestational diabetes were not included as controls. Women who had their baby before 36 weeks gestation for any reason were not included, since it could be argued that they could have gone on to develop pre-eclampsia had their pregnancy gone to term. Similarly women who had babies under the 10^{th} corrected birth centile (which is indicative of intra-uterine growth restriction, a hallmark of hypertensive disorders) or those with babies bigger than the 90^{th} corrected centile (in keeping with hyperglycaemic disorders) were also not eligible to be used as controls.

2.7.2.7 Corrected centile charts

It has long been argued that "normal values" for birthweight and foetal growth need to be locally applicable, and cannot necessarily be transferred from one country to another. Such reference values need to reflect physiological variation in maternal height and weight, ethnic origin and sex of the baby (once known).

The West Midlands Perinatal Research Institute have developed corrected centile charts based upon the principles of the gestation related optimal weight (GROW) program. Coefficients for each of the factors mentioned above have been derived and validated for maternity population datasets in the UK (N=40,000), Sweden (N=400,000), New Zealand (N=5,000), Australia (N=12,500) and the United States (N=35,000) (289).

Microsoft Excel (2003) based calculators were used to calculate corrected birthweight centiles. The following data were entered into the calculator:-

- Maternal height (cm)
- Maternal weight (kg)
- Parity
- Maternal ethnicity
- Parity at beginning of pregnancy
- Baby sex
- Birthweight
- Baby sex

These corrected centile charts have been used in several international studies, and have demonstrated that physiological parameters appear to affect growth similarly in different countries and continents (290). The group now aim to add to the existing data by examining these coefficients in different populations in different geographic areas. In particular the effect of social deprivation (which was not included in the PIP study) is important, since it is known to affect perinatal mortality rates.

2.8 Overall study numbers

At the time of the final study analysis 3919 women who had been recruited to the study had delivered. Delivery information was available for 3631 women (93%); after exclusion of women who had miscarriages or terminations at ≤ 20 weeks' gestation, information was available on 3592 women who had live births. Demographics of women at booking are shown in table 2.1. The reconfiguration of maternity services within Glasgow, and in particular the closure of the Queen Mother's hospital in January 2010, is likely to have contributed to the incomplete delivery information. Figure 2.10 outlines the overall recruitment rates at the various sites.

Of the women on whom delivery information was available, a total of 83 (2.3%) developed pre-eclampsia, while 69 (1.9%) women developed gestational hypertension without significant proteinuria. Delivery information from women at each of the maternity hospitals in the study is shown in table 2.1.

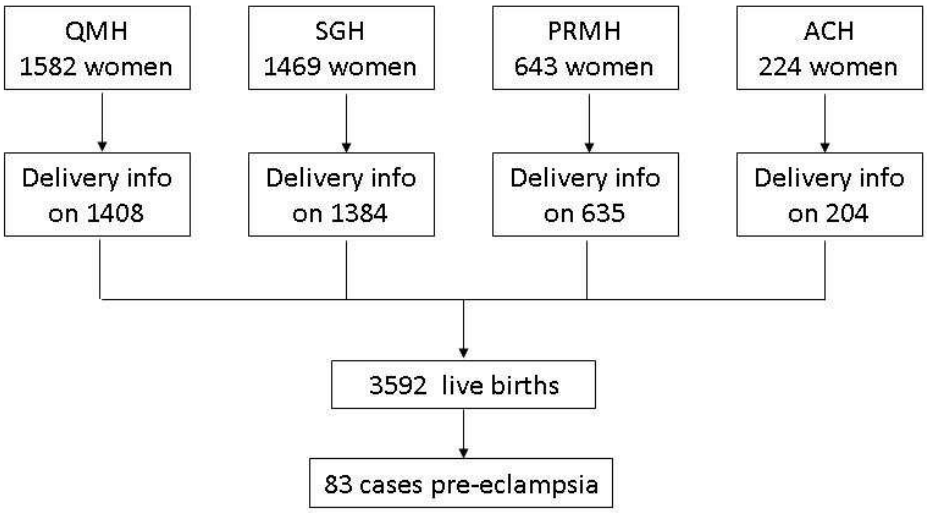

Figure 2-10. Breakdown of pregnant women recruited at each centre

	Total (3919)	QMH (1582)	SGH (1469)	PRMH (643)	ACH (224)
Age (years)	30.1 (6.0)	30.4 (6.0)	29.9 (5.9)	30.8 (6.0)	28.0 (5.8)
Ethnicity					
Caucasian	3552 (91%)	1452 (92%)	1273 (87%)	603 (94%)	223 (99.6%)
Non-Caucasian	367 (9%)	130 (8%)	196 (13%)	40 (6%)	1 (0.004%)
Height (cm)	163 (12)	164 (11)	162 (12)	162 (15)	164 (8)
Weight (kg)	69.9 (16)	69.4 (15)	70.1 (16)	70.2 (17)	71.1 (17)
BMI (kg/m^2)	26 (5)	25 (5)	26 (5.6)	26.5 (6)	26.8 (6)
Nulliparous	1788 (46%)	754 (48%)	688 (47%)	255 (40%)	90 (37%)
Smoking	534 (14%)	251 (16%)	118 (8%)	105 (16%)	60 (27%)
Previous pre-eclampsia	106 (2.7%)	31 (1.9%)	40 (2.7%)	32 (5%)	3 (1.3%)
FH of pre-eclampsia	150 (3.8%)	43 (2.7%)	68 (4.6%)	33 (5%)	6 (2.7%)
Previous hypertension	101 (2.5%)	52 (3%)	34 (2.3%)	13 (2%)	2 (1%)
Previous renal disease	12 (0.003%)	7 (0.004%)	5 (0.003%)	0 (0%)	0 (0%)
Gestation at booking	13.4 (1.7)	13.3 (1.8)	13.6 (1.7)	12.6 (1.5)	12.5 (1.4)
SBP at booking (mmHg)	111 (28)	111 (34)	113 (13)	114 (15)	109 (10)
DBP at booking (mmHg)	67 (12)	67 (13)	67 (11)	71 (11)	64 (7)

Table 2-1. Demographics of pregnant women at booking visit. FH= family history.

	Total (3919)	QMH (1582)	SGH (1469)	PRMH (643)	ACH (224)
Delivery information available	3631 (93%)	1408 (89%)	1384 (94%)	635 (99%)	204 (91%)
Live births	3592 (92%)	1392 (88%)	1375 (94%)	623 (97%)	202 (90%)
Stillbirth / termination < 20 weeks	24 (0.006%)	8 (0.006%)	6 (0.004%)	8 (0.01%)	2 (0.008%)
Stillbirth > 20 weeks	15 (0.004%)	8 (0.006%)	3 (0.002%)	4 (0.006%)	0 (0%)
Pre-eclampsia	83 (2.3%)	38 (2.7%)	32 (2.3%)	10 (1.6%)	3 (1.5%)
Pregnancy-induced hypertension	69 (2%)	28 (2%)	24 (1.7%)	7 (1.1%)	10 (4.9%)
Eclamptic fits	2 (0.0002%)	0 (0%)	1 (0.0006%)	1 (0.002%)	0 (0%)
Gestational diabetes	22 (0.005%)	8 (0.005%)	5 (0.003%)	8 (1.2%)	1 (0.004%)
Gestation at delivery	39.5 (2.6)	39.5 (2.7)	39.7 (2.2)	39.2 (3.2)	39.7 (2.2)
Caesarean section	942 (26%)	321 (23%)	372 (27%)	187 (29%)	62 (28%)
Birthweight (grams)	3396 (599)	3416 (633)	3370 (570)	3412 (588)	3390 (581)
Corrected centile	46.1 (30.4)	47.1 (30)	44 (30.5)	49 (30.7)	42.4 (31)

Table 2-2. Delivery information of women recruited to PIP study.

	Pre-eclampsia (n=83)	No pre-eclampsia (3548)	p-value
Age (years)	28.7 (6)	30.2 (6)	**0.03**
Ethnicity			
Caucasian	78	2967	
Non-Caucasian	5	362	0.21
Height (cm)	163 (12)	163 (12)	0.9
Weight (kg)	76 (16)	69.8 (16)	**0.001**
BMI (kg/m^2)	28 (6)	26 (5)	**<0.001**
Nulliparous	69 (83%)	1718 (52%)	**<0.001**
Smoking	9 (11%)	525 (16%)	0.28
Previous pre-eclampsia	2 (2.4%)	104 (3%)	1.0
FH of pre-eclampsia	9 (11%)	141 (4%)	**0.009**
Previous hypertension	5 (6%)	96 (3%)	**0.04**
Previous renal disease	0 (0%)	12 (0.003%)	1.0
Gestation at booking	13.6 (2)	13.4 (2)	0.3
SBP at booking (mmHg)	117 (20)	111 (28)	**0.04**
DBP at booking (mmHg)	73 (14)	67 (12)	**<0.001**

Table 2-3. Comparison of demographics at booking between women with and without pre-eclampsia. FH=family history

2.8.1 Discussion

At booking, women who went on to develop pre-eclampsia were younger, had a higher BMI, were more likely to be nulliparous and were more likely to have a family history of pre-eclampsia than those who did not develop the condition. Systolic and diastolic blood pressure was already higher at booking in women who went on to develop pre-eclampsia. There was no difference in ethnicity or in smoking rates between affected and unaffected women.

These findings are in largely in keeping with the literature, since nulliparity, increased BMI, blood pressure in early pregnancy, and a family history in the mother or sister, are all known to be associated with an increased risk of development of pre-eclampsia (12). Smoking is associated with a reduced risk of

pre-eclampsia; in this cohort there was no significant difference between affected and unaffected women. I relied, however, on self-reported smoking rates, which can often be inaccurate. Women who developed pre-eclampsia were also younger than those who remained normotensive; this goes against what one would expect, since increased age is reported to be associated with an increased risk of pre-eclampsia, with the risk increasing from the age of 34 onwards (12).

	Pre-eclampsia (n=83)	No pre-eclampsia (n=3548)	p-value
Gestation at delivery (wks)	37.3 (3.6)	39.6 (2.5)	<0.001
Birthweight (g)	2918 (790)	3408 (589)	<0.001
Corrected centile	36.5 (31)	46.3 (30)	0.009
Caesarean Section	33 (40%)	953 (29%)	0.007

Table 2-4. Comparison of delivery information between women with and without pre-eclampsia

Delivery information again confirmed that women who developed pre-eclampsia were more likely to have delivered at an earlier gestation, to have had smaller babies and to have been delivered by Caesarean section.

Women who attended for booking at QMH or SGH were asked if they would be willing to attend for vascular function studies and further sampling at gestational week 16 and 28. Those who were willing to participate in further studies and who and had 2 or more traditional risk factors for pre-eclampsia were then contacted by telephone and invited to attend. 214 women with risk factors attended at week 16, of whom 180 (84%) attended again at week 28. Analysis of vascular function studies was restricted to the 180 women who attended at gestational weeks 16 and 28. Women were invited to attend at 6-9 months post-natally, of whom 80 attended.

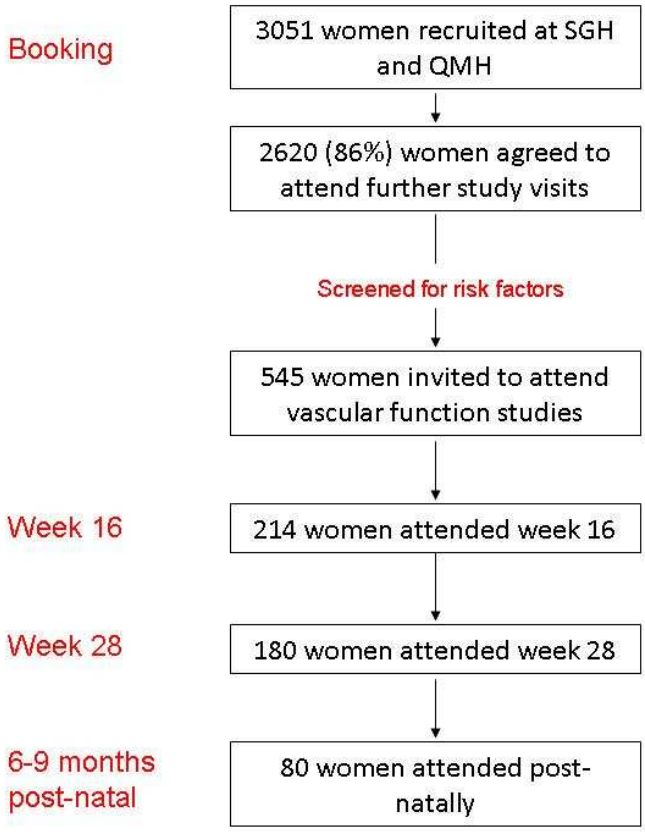

Figure 2-11. Flowchart depicting women recruited for vascular function studies and sampling

Chapter 3

A study of urinary proteomics for the prediction of pre-eclampsia

3. A study of urinary proteomics for the prediction of pre-eclampsia

3.1 Introduction

As discussed in Chapter 1, the prediction of pre-eclampsia before the onset of clinically detectable disease remains a challenge. The use of medications to delay or even prevent the onset of disease has been reported widely; several potential therapeutic agents have been studied, but inability to screen for high-risk women has limited the number of proper randomised controlled trials performed. To be most likely to be effective, such agents would have to be started before 20 weeks gestation; discriminating affected pregnancies from unaffected pregnancies is not yet possible.

It has long been proposed that toxins in the maternal circulation contribute to the development of pre-eclampsia; the traditional term pre-eclamptic toxaemia, or abbreviation PET reflects this. Several biochemical markers have been proposed for the early prediction of pre-eclampsia, but none have yet been sensitive or specific enough to be adopted for use in routine clinical practice (81). The pathogenesis of pre-eclampsia is complex, with an interaction occurring between impaired placentation and a maternal response to factors in the maternal circulation that culminates in the clinical manifestations of the disease. Several studies support the theory that the under-perfused placenta releases circulating factors (291), and that the maternal response includes endothelial cell dysfunction, vasoconstriction and activation of inflammatory and coagulation pathways (236).

To date, the identity of the majority of these circulating markers remains unknown; syncytiotrophoblast debris and angiogenesis related factors such as vascular-endothelial growth factor-1 (VEGF) and its receptor (VEGFr-1 or sFLT-1) have been reported as being involved (121), but many others remain uncharacterised. The use of a “hypothesis-generating” strategy such as proteomics may lead to the discovery of these markers, leading to the discovery

of novel pathways and mechanisms that could themselves be targets for potential therapeutics.

Proteomics, the large-scale study of peptides and proteins, holds promise for the discovery of new biomarkers for pre-eclampsia, and as discussed in section 1.6.2 urine appears to be a stable platform for proteomic-based research. Renal pathology is one of the hallmarks of the condition, and the degree of proteinuria is said to reflect the severity of disease (292). In keeping with serum, urinary levels of anti-angiogenic peptides have been shown to be altered in women who went on to develop pre-eclampsia (293,294). In contrast to many other human tissues, urine is relatively stable against proteolytic degradation (229); freezing urine samples at -70°C for several years has been shown to have little or no impact on the proteome (222). As a filtrate of blood, urine contains an abundance of information about the functioning of many internal organs, and the appearance in the blood of certain proteins may result in their appearance in the urine, either as intact proteins or as peptide fragments. Normal urine contains small amounts of intact albumin, as well as much larger quantities of low-molecular-weight albumin fragments. Although most antibody-based or other assays detect the intact form of albumin, the majority fail to detect smaller fragments of albumin. Since mass spectrometry can be used to measure and characterise these small peptide fragments, it could prove to be a useful tool for identifying predictive biomarkers. As discussed in section 1.6.4, urinary proteomic biomarkers have been validated for the early diagnosis of various diseases, including coronary artery disease (221,229), diabetes (295) and diabetic nephropathy (296). As well as providing potentially clinically useful biomarkers, these studies have given insight to novel pathophysiological mechanisms.

In this study, therefore, capillary electrophoresis / mass spectrometry (CE / MS) based technology was used to examine the urinary proteome in pregnancy. Once the changes in the urinary proteome in pregnancy had been determined, a further aim was to identify a urinary proteomic peptide pattern that could be used to accurately predict which women are likely to go on to develop pre-eclampsia.

3.2 Methods

3.2.1 Patient recruitment

The Proteomics in Pre-eclampsia (PIP) study protocol is described in detail in chapter 2.The study was designed primarily to investigate urinary proteomics for the ability to predict pre-eclampsia, and was extended to include blood markers and vascular function studies which form the bulk of this thesis. In brief, pregnant women were recruited at their initial antenatal hospital visit (gestational week 12-16) at the Southern General Hospital, the Queen Mother's Hospital and the Princess Royal Maternity Hospital, Glasgow, and Ayrshire Maternity Hospital, Kilmarnock. Written informed consent was obtained either by myself or by a midwife.

Information about past medical history, past obstetric history, family history and risk factors for pre-eclampsia were obtained and recorded on a data collection form (Appendix 4). Blood pressure was recorded after 5 minutes seated with an automated device and body mass index was calculated. Blood and urine samples were also obtained.

In order to examine the peptide patterns later in pregnancy, a cohort of women with ≥2 traditional risk factors for pre-eclampsia were invited for further sampling at gestational weeks 16 and 28, and at 6-9 months post-natally, as outlined in Chapter 2.

For the purpose of this initial urinary proteomics study, the first 2500 women with singleton pregnancies who had been recruited into the study were included. Delivery information, obtained from hospital databases and labour ward delivery books, was available on 2407 women (95%); those who had foetal losses <20 weeks gestation (n=22) were not included, leaving 2385 women in the final analysis. Case notes and patient hand-held records were reviewed by myself for every woman who developed hypertension at any stage during pregnancy, to ensure accurate diagnosis of pre-eclampsia. Women with a history of hypertension outwith pregnancy, diabetes and renal disease were excluded from the proteomic analysis study. Although women with twin pregnancies were

included in the overall PIP study, these women were not included in this proteomics study. Overall, 45 (1.9%) of the 2385 women who delivered >20 weeks gestation developed pre-eclampsia (cases); these women were matched in a nested case-control design with 90 women who had uncomplicated pregnancies (controls). Cases and controls were matched as closely as possible for age, body mass index at booking and gestational age at sampling; controls were women who delivered appropriately grown healthy babies at ≥37 weeks' gestation with no antenatal obstetric or medical complications. Samples from 4 controls were unsuitable for proteomic studies, leaving 86 controls for the final analysis. Subject characteristics are shown in table 3.1.

	Pre-eclampsia n=45	Controls n=86	*p*-value
Maternal Characteristics			
Age (years)	29 (5)	28 (5)	0.686
Ethnicity			
Caucasian	42 (93%)	81 (94%)	1.00
Other	3 (7%)	5 (6%)	
Body mass index (kg/m^2)	29 (5)	29 (5)	0.763
Nulliparous	39 (87%)	55 (64%)	0.007
Smoker	4 (9%)	21 (24%)	0.04
Previous pre-eclampsia	1 (2%)	6 (7%)	0.42
Family history of pre-eclampsia (mother or sister)	8 (17%)	7 (8%)	0.14
At Booking			
Systolic blood pressure (mmHg)	121 (10)	114 (12)	0.002
Diastolic blood pressure (mmHg)	76 (9)	69 (10)	0.001
Proteinuria (≥++ on dipstick)	1 (2%)	2 (2%)	1.00
Gestation at sampling (wks)	13.8 (1.6)	13.7 (1.3)	0.606
Pregnancy Outcome			
Highest systolic blood pressure (mmHg)	165 (11)	125 (12)	<0.001
Highest diastolic blood pressure (mmHg)	101 (8)	71 (9)	<0.001
Caesarean section	20 (44%)	10 (12%)	<0.001
Gestation at delivery (wks)	38.3 (2)	40 (1.4)	<0.001
Birthweight (g)	3155 (621)	3558 (416)	<0.001
Customised birthweight centile	39.4 (30)	48.5 (23)	0.09
Small for gestational age (<10th customised birthweight centile)	12 (27%)	3 (3%)	<0.001

Table 3-1. PIP study patient characteristics. Shown as mean (SD) or number (% of total).

132 of the women with at least two risk factors for pre-eclampsia (12) attended for further sampling at gestational week 28 as outlined in Chapter 2. Of them, 18 (13.6%) developed pre-eclampsia, and were matched for age and body mass index at sampling to 17 controls who had normotensive deliveries, delivering

appropriately grown babies after 37 weeks gestation. Patient characteristics of the women in the sub-study are shown in Table 3.2.

	Pre-eclampsia n=18	Controls n=17	*p*-value
Maternal Characteristics			
Age (years)	30 (4)	29 (4)	0.62
Ethnicity			
Caucasian	16 (89%)	17 (100%)	0.49
Other	2 (11%)	0 (0%)	
Body mass index (kg/m^2)	30 (5)	30 (4)	0.75
Nulliparous	16 (89%)	9 (53%)	0.03
Smoker	1 (6%)	4 (24%)	0.18
Previous pre-eclampsia	1 (6%)	5 (29%)	0.09
Family history of pre-eclampsia (mother or sister)	8 (44%)	4 (24%)	0.29
At Week 28			
Systolic blood pressure (mmHg)	130 (10)	126 (9)	0.17
Diastolic blood pressure (mmHg)	80 (8)	73 (6)	0.006
Proteinuria (≥+ on dipstick)	2 (11%)	1 (6%)	1.0
Gestation at sampling (wks)	28.2 (1.4)	28.6 (1.5)	0.37
Pregnancy Outcome			
Highest systolic blood pressure (mmHg)	159 (9)	126 (10)	<0.001
Highest diastolic blood pressure (mmHg)	100 (6)	73 (7)	<0.001
Caesarean section	4 (22%)	3 (17%)	1.0
Gestation at delivery (wks)	39.3 (2)	40.5 (1)	0.02
Birthweight (g)	3277 (419)	3722 (468)	0.001
Customised birthweight centile	34.4 (24)	45 (19)	0.16
Small for gestational age (<10th customised birthweight centile)	4 (22%)	0 (0%)	0.10

Table 3-2. PIP study : patient characteristics for "risk factor" sub-group

3.2.2 Validation in an independent study cohort

Further studies were then performed to validate the biomarker patterns in an independent cohort of pregnant women. Early pregnancy urinary samples were obtained from the "Screening for Pregnancy Endpoints" (SCOPE) study, an international cohort study of healthy nulliparous women with singleton pregnancies (297). Between November 2004 and October 2008, 3234 women were recruited into the SCOPE study in Auckland, New Zealand and Adelaide, Australia. Women were interviewed, examined and specimens obtained at 20±1 weeks gestation. Women were followed prospectively throughout pregnancy with outcome data collected by research midwives. Pregnancy outcome data was available on 99% (n=3196). After exclusion of women with foetal losses before 22 weeks' gestation (n=26) and women not attending the 20 week interview (n=69), the base population for the study comprised 3101 women (96% of recruits). Of the 3101 women, 175 (5.6%) developed pre-eclampsia, of whom 158 (5.1%) delivered after 34 weeks' gestation. For the current study, SCOPE cases (n=50) were randomly selected from the late onset pre-eclampsia subgroup and controls (n=50) from women with uncomplicated pregnancies (n=1767), defined as no antenatal obstetric or medical complications with delivery of an appropriately grown healthy baby at 37 or greater weeks' gestation. Urinary samples from gestational week 20 were used for proteomic analysis, with 1 control specimen excluded for technical reasons. SCOPE study patient characteristics are shown in table 3.3.

	Pre-eclampsia n=50	Controls n=49	*P*-value
Maternal Characteristics			
Age (years)	26 (6)	26 (6)	0.93
Ethnicity			
Caucasian	41 (82%)	42 (86%)	0.62
Other	9 (18%)	7 (14%)	
Body mass index (kg/m^2)	27 (6)	25 (6)	0.12
Gravidity			
1	37 (76%)	42 (84%)	0.04
≥2	12 (24%)	7 (16%)	
Smoker	5 (10%)	7 (14%)	0.51
At 20 weeks gestation			
Systolic blood pressure (mmHg)	114 (10)	109 (10)	0.01
Diastolic blood pressure (mmHg)	68 (10)	64 (7)	0.05
Gestation at blood sampling (wks)	20.1 (0.7)	20.1(0.6)	0.86
Pregnancy Outcome			
Systolic blood pressure (mmHg)	148 (15)	118 (10)	<0.0001
Diastolic blood pressure (mmHg)	95 (9)	70 (9)	<0.0001
Caesarean section	18 (36%)	9 (18%)	0.05
Gestation at delivery (wks)	38.5 (1.6)	40.3 (1.0)	<0.0001
Birthweight (g)	3090 (616)	3609 (407)	<0.0001
Customised birthweight centile	40 (18, 66)	55 (40, 74)	0.02
Small for gestational age (<10th customised birthweight centile)	9 (18%)	0 (0%)	0.003

Table 3-3. SCOPE study patient characteristics.

Diagnosis of pre-eclampsia was in keeping with the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (5); hypertension (systolic BP >140 or diastolic BP >90, on 2 occasions > 6 hours apart,) in association with proteinuria (defined as ≥ 300mg / 24 hrs or ++ on dipstick analysis).

3.2.3 Sample handling

Study protocols are outlined in Chapter 2. In brief, urine samples (mid-stream, universal container) were taken at the morning out-patient clinic (PIP) or study interview (SCOPE), were anonymised, given a study code, and were stored in a refrigerator at 4°C for up to 4 hours. Samples were then transferred refrigerated to the research centre, separated and aliquoted. Samples were stored at -70°C, and thawed immediately prior to proteomic analysis.

Capillary electrophoresis / mass spectrometry (CE/MS) proteomic analysis was performed as outlined in Chapter 2. To determine the urinary proteome in normal pregnancy, samples from 17 healthy pregnant women with uncomplicated pregnancies were compared to samples from 67 apparently healthy, non-pregnant female volunteers from Hannover medical school. In a "discovery phase" a training set of potential biomarkers was then developed using samples from 20 women who had gone on to develop pre-eclampsia and 20 matched women who had uncomplicated pregnancies. In a validation phase the training set of discriminatory peptides was then analysed on a test set of a further 40 blinded samples from 20 cases and 20 controls.

3.3 Results

3.3.1 Urine proteome in healthy pregnancy

As a proof of principle, the urinary proteome of healthy pregnant women (who did not develop pre-eclampsia, n=17) was compared to that of healthy non-pregnant female volunteers (n=67). As shown in Figure 3.1, clear differences between these two cohorts were seen. On examination of the datasets, 284 peptides that were significantly altered between the two groups were identified.

The pregnancy-associated urinary peptides were then evaluated in a second set of independent samples; 248 urine samples from pregnant women that were included in the study and 141 healthy non-pregnant controls. Support vector machine (SVM) classification based on the 284 peptides revealed 98.6%

specificity and 98.4% sensitivity in the independent dataset. All pregnancy associated peptides are listed in Appendix XI.

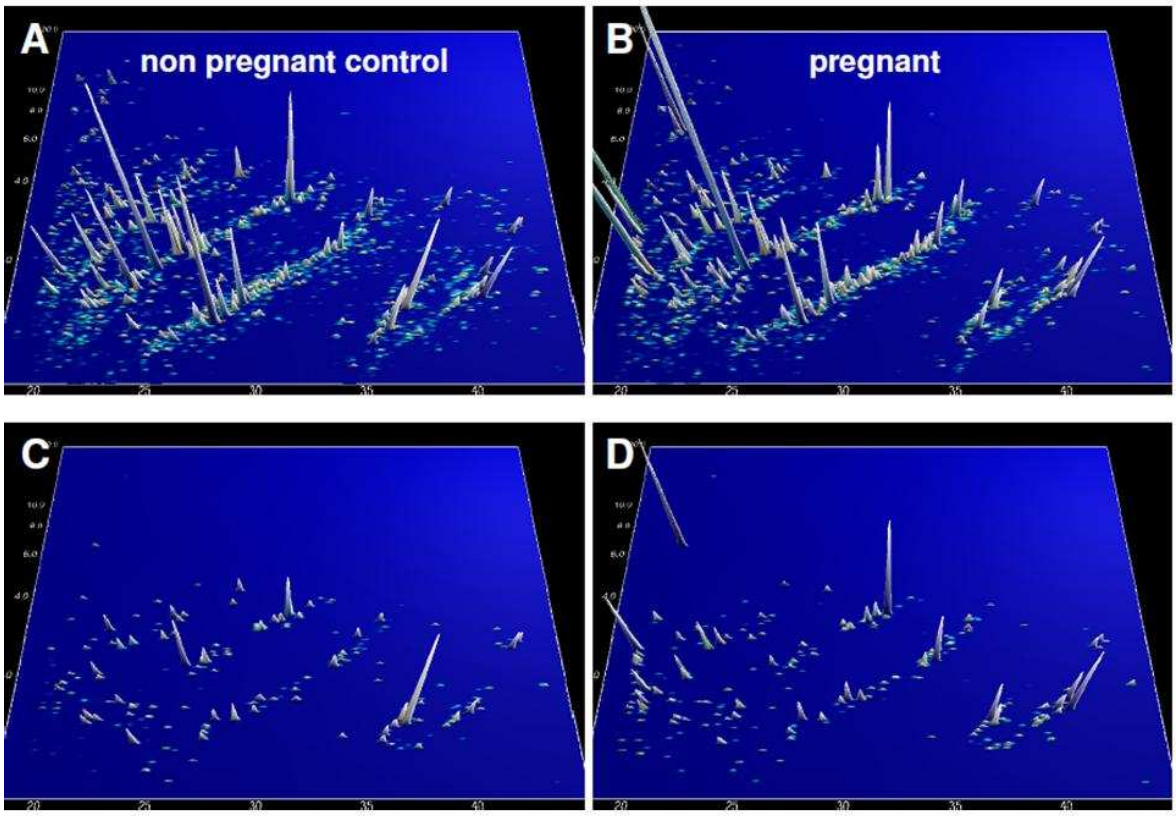

Figure 3-1. Averaged urinary proteome in non-pregnant controls (a) and pregnant women at gestational week 12-16 (b). x-axis shows CE migration time (secs), y-axis molecular mass (Da), and z-axis signal intensity. Discriminatory peptides are shown between non-pregnant controls (c) and pregnant women at gestational week 12-16 (d)

3.3.2 Biomarkers for prediction of pre-eclampsia at Week 28

After having established clear differences in the urinary proteome between pregnant and non-pregnant women, further studies were performed to investigate whether biomarkers for the prediction of pre-eclampsia could be detected in the urinary proteome at gestational week 28. From the cohort of women with ≥2 risk factors for pre-eclampsia, 18 samples from women who subsequently developed pre-eclampsia (cases) and 17 samples from women who had normotensive pregnancies (controls) were examined (figure 3.2). After adjustment for multiple testing, the 10 biomarkers most significantly associated with pre-eclampsia were identified. A model containing these 10 biomarkers

differentiated between cases and controls (p<0.001). A biomarker model was then generated which included an additional 40 peptides which were nominally significant between cases and controls. This more stable model enabled classification of the samples with 100% sensitivity and specificity, even when evaluated using complete take-one-out cross-validation. Based upon this model a classification factor was calculated as outlined in Chapter 2; classification factor was 1.032 ± 0.411 in women who subsequently developed pre-eclampsia (cases) compared to -1.038±0.432 (*P*<0.001, figure 3.3) in those whose pregnancy continued without developing pre-eclampsia.

The peptides which were associated with pre-eclampsia include collagen fragments, fibrinogen and uromodulin. Full details of the peptides are shown in table 3.4.

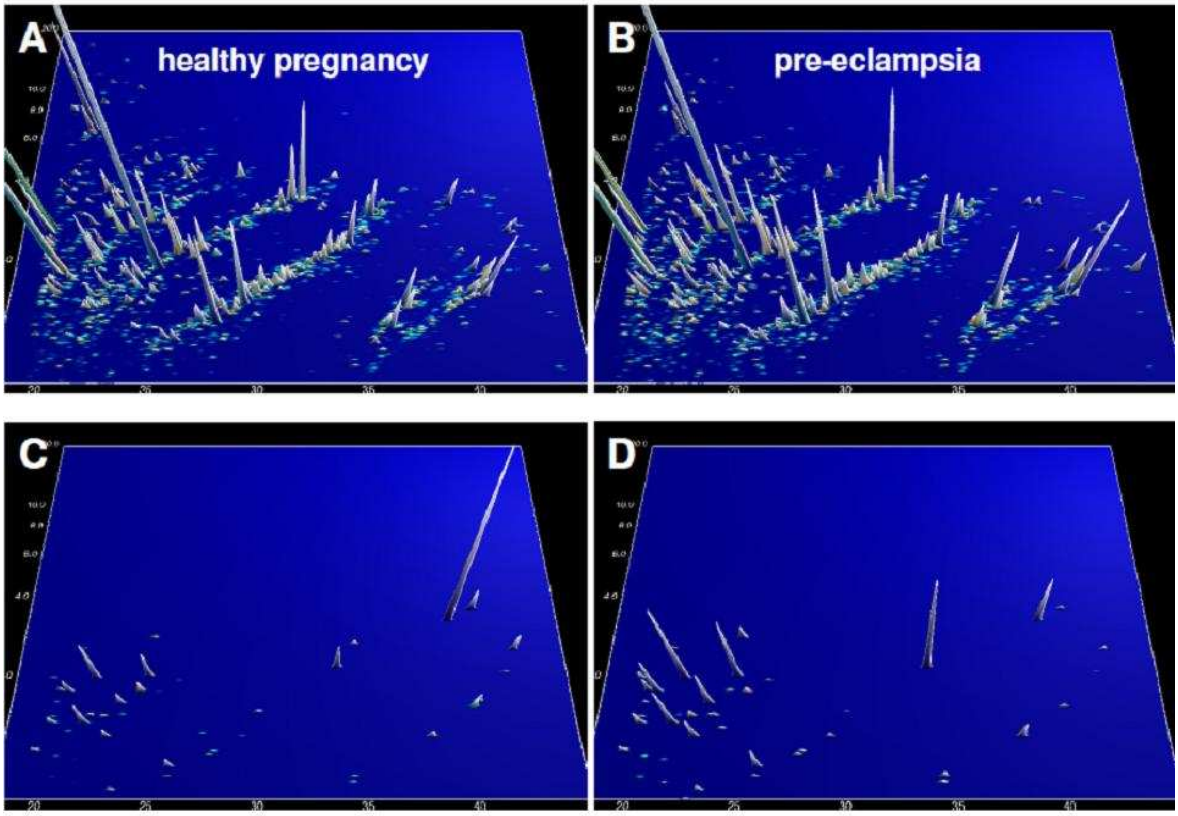

Figure 3-2 Averaged urinary proteome for pregnant controls at gestational week 28 (a) and women who subsequently developed pre-eclampsia (b). x-axis shows CE migration time (secs), y-axis molecular mass (Da), and z-axis signal intensity. Discriminatory peptides are shown for pregnant controls (c) and for women who subsequently developed pre-eclampsia (d).

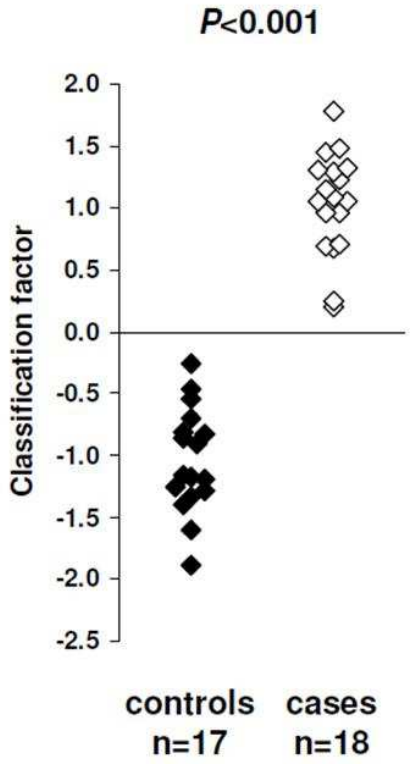

Figure 3-3 Classification of pre-eclampsia specific urinary biomarkers. Translation of the pre-eclampsia specific urinary polypeptide signature (figure 3.2) into a classification factor demonstrates significant difference between women at gestational week 28 who subsequently developed pre-eclampsia (cases; open diamonds) and women who had normotensive pregnancies (controls; filled diamonds). Sensitivity and specificity were 100% with positive numbers predicting high risk for pre-eclampsia.

This 50-marker panel was then examined to determine whether it could be used to detect pre-eclampsia at an earlier point in pregnancy. Using samples from the PIP study (gestational week 12-16) 45 women who subsequently developed pre-eclampsia were matched for age and body mass index at booking to 86 controls who had normotensive pregnancies. Using the pre-eclampsia pattern generated from week 28, the model differentiated between those who went on to develop pre-eclampsia (classification factor -0.567±0.372) and those who had uncomplicated pregnancies (classification factor -0.721±0.439, *P*=0.048) (Figure 3.4a).

In a second step the classification factors were examined in samples from women with risk factors who had provided urine samples both at weeks 12-16

and 28. Classification factor decreased slightly from gestational week 12-16 to 28 in those women who were sampled at both times and had a normal pregnancy (n=16; from -0.647±0.437 to -1.024±0.433, *P*=0.043; Figure 3.5a) whereas it markedly changed towards positive scores in those women who subsequently developed pre-eclampsia (n=16; from -0.392±0.383 to 1.070±0.383, *P*<0.001; Figure 3.5b).

In a third step, week 20 urine samples were analysed from 99 women (50 cases, 49 controls) from the SCOPE study. In this cohort there was no significant difference between cases (classification factor, -0.755±0.533) and controls (-0.724±0.418, *P*=0.73; Figure 3.4b), suggesting that the biomarkers identified at week 28 do not reliably predict pre-eclampsia when analysed earlier in pregnancy. Finally, the samples from gestational week 12-16 and 20 were analysed in order to find whether any other biomarker patterns could be used to accurately differentiate between cases and controls, and thereby to predict pre-eclampsia, at an earlier time in pregnancy. No such early predictive biomarker patterns were identified.

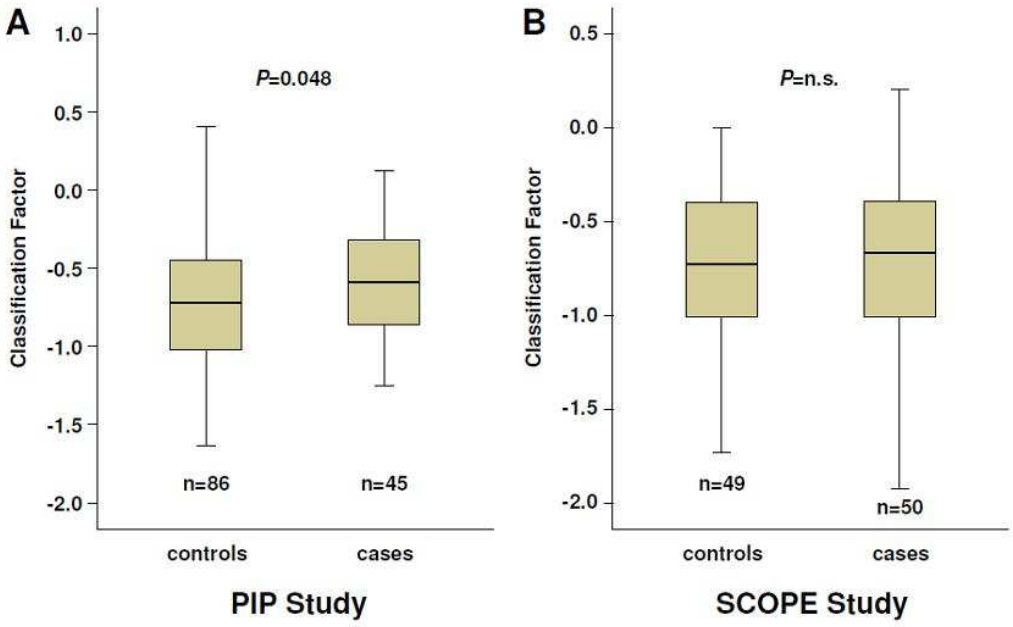

Figure 3-4 The classification factor was different between controls and women with future pre-eclampsia at gestational week 12-16 (PIP study; panel A) but not in an independent study cohort at gestational week 20 (SCOPE study; panel B).

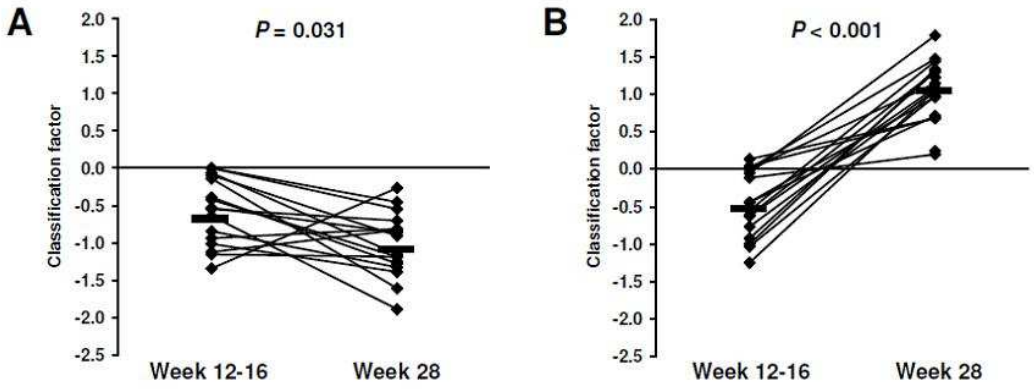

Figure 3-5 Panel A shows changes in classification factor from gestational week 12-16 to 28 in normotensive pregnancy (n=16; A) and in women who subsequently develop pre-eclampsia (n=16; B). Horizontal lines indicate mean values.

3.4 Discussion

In this study CE-MS technology was used to examine the urinary proteome in pregnancy, and to attempt to identify a urinary proteomic signature that can be used in early and mid pregnancy to predict pre-eclampsia. At gestational week 28, a urinary peptide pattern was identified, characterised by breakdown products of fibrinogen, collagen and uromodulin, which could differentiate women who subsequently developed pre-eclampsia. Given that the majority of women in this study were diagnosed with pre-eclampsia after 36 weeks' gestation, this set of biomarkers may prove to be a helpful aid in the diagnosis of late-onset pre-eclampsia.

Although the peptide pattern was used to differentiate between cases and controls at gestational week 12 to 16, it did not differentiate between cases and controls in an independent cohort of pregnant women at gestational week 20. The magnitude of overlap between cases and controls at 12 to 16 weeks, and the failure to replicate these findings in another cohort, preclude the use of this urinary peptide pattern as an early screening test. The different findings in the 2 cohorts may result from differences in gestational age at sampling (20 weeks rather than 28 weeks) and study populations: SCOPE comprised healthy

nulliparous women, whereas PIP was a mixed-parity general obstetric population.

The performance of this urinary peptide pattern at 28 weeks to discriminate women who subsequently developed pre-eclampsia from those with an uncomplicated pregnancy is similar to or better than that reported with serum FMS-like tyrosine kinase-1 (sFLT-1) and placental growth factor (PGF) in the second trimester for early-onset pre-eclampsia (298-300). The discrimination seen with the urinary peptide pattern at 28 weeks is more in keeping with the diagnostic performance of the angiogenic biomarkers when measured in women after they have presented with symptoms or signs of pre-eclampsia (301).

The failure of "classical" circulating biomarkers to accurately predict pre-eclampsia (81), and the inconsistent results obtained from genetic studies (302) have led to an increase in recent years in "systems-medicine" based approaches such as proteomics. As discussed in chapter 1.7, previous proteomic research in pre-eclampsia has yielded mixed results. Amniotic fluid (240), cerebro-spinal fluid (242) and placental tissue (303) have all been used for proteomic studies in pre-eclampsia, using samples taken from women undergoing amniocentesis, spinal anaesthesia, and Caesarean section respectively. All have reported peptide patterns that can accurately differentiate between women with pre-eclampsia and women with normal pregnancies, but in keeping with most other studies in the field, samples were taken at the time of delivery rather than before it when such patterns may be clinically useful. Further, the invasive sampling required means that these media could never be applicable in large-scale studies, nor in routine clinical practice.

Plasma has been studied more extensively. Examining plasma from women at the time of diagnosis of pre-eclampsia, Blankley et al. (237) identified discriminatory peptides for pre-eclampsia included endoglin, pregnancy-associated plasma protein A (PAPP-A) and sex hormone binding blobulin (SHBG), all of which have been reported to be altered in pre-eclampsia. In a separate study Blumenstein et al. (236) examined the plasma proteome at gestational week 20 in 39 women from the SCOPE cohort who subsequently developed pre-eclampsia. They found 36 moderate-highly abundant proteins that were differentially expressed in women who went on to develop pre-eclampsia

compared to controls. Among these were proteins involved in lipid metabolism including Apolipoprotein A1, fragments of collagen and fibrinogen, and complement precursors.

As discussed in section 1.6, the complexity of the plasma proteome, and the relatively high proteolytic activity of human blood mean that proteomic analysis of plasma and serum is potentially unreliable (218). To date, one other study has used urinary proteomics to identify disease-specific proteomic biomarkers for pre-eclampsia. As outlined in section 1.7.3, Buhimschi et al. (238) identified fragments of serpin peptidase inhibitor 1 (SERPINA-1) and albumin as biomarkers for pre-eclampsia. Although the peptide pattern allowed discrimination of severe from mild forms of pre-eclampsia, there was considerable overlap in peptide patterns with women with chronic hypertension. Further, patient numbers in their study were low, with only 19 women studied longitudinally during their pregnancy, of whom 3 developed pre-eclampsia.

The differentially expressed polypeptide sequences between cases and controls identified in the current study are not only of potential clinical use but point towards biological processes that are altered during the development of pre-eclampsia. Changes in extracellular matrix are a feature of all vascular diseases and are represented by a number of differentially expressed collagen fragments in the peptide pattern. These findings are not only in line with plasma proteome data by Blumenstein et al.(236), but also with previous studies of urinary proteomic markers of chronic kidney disease (304) and coronary artery disease (221,305). These conditions share some of the features of pre-eclampsia including endothelial dysfunction and inflammation and may therefore also be expected to share some of the characteristic proteomic markers with pre-eclampsia. Fibrinogen leads to endothelial cell activation, which is another hallmark of the pathogenesis of pre-eclampsia and also features as a biomarker of pre-eclampsia in the study by Blumenstein et al. (236). Differential expression of uromodulin sequences in urine between cases and controls is not surprising since uromodulin, or Tamm-Horsfall protein, is the most abundant protein in normal urine and could therefore simply indicate non specific changes in the composition of urine in parallel to the disease process. The *UMOD* gene locus, however, has been found to be associated with glomerular filtration rate in genome-wide association studies (306) indicating a potential functional role of

uromodulin in the development of cardiovascular diseases. More recently, a genome-wide association study demonstrated that rs13333226 in the promoter region of *UMOD* is associated with hypertension, independently of renal function (307). These data on uromodulin, as a key player in renal disease and hypertension, could point towards subclinical vascular and renal damage in the early stage of pre-eclampsia that are indicated by differential urinary expression of uromodulin fragments and warrant further investigation.

In this proteomic approach, analysis was restricted to peptides and proteins with a molecular mass >800 Da, whose concentrations were at least 2-fold different between cases and controls. Whereas typical pregnancy-related hormones such as oestrogen or progesterone and their respective metabolites can be detected in urine using mass spectrometry, the sample preparation and the data evaluation used in this study excluded these and many other smaller metabolites. This analytical approach should therefore be regarded as complementary to other approaches for the identification of diagnostic and predictive markers. The findings outlined here require validation in larger predictive and diagnostic studies to define their potential role in clinical care.

Mass	CE_time	Sequence	Protein name	Start AA	Stop AA	Swissprot name
3657.665	40.71091					
3292.541	39.42096					
2841.256	24.53535	ERGEAGIpGVpGAkGEDGKDGSpGEpGANG	Collagen alpha-1 (III) chain	448	477	CO3A1_HUMAN
2674.217	34.58338					
2658.271	19.47609	DEAGSEADHEGTHSTKRGHAKSRPV	Fibrinogen alpha chain	605	629	FIBA_HUMAN
2587.195	21.09996					
2570.19	42.56018					
2196.993	33.68827					
2117.032	42.00351					
2048.927	24.46324					
2030.912	21.85225	EGSpGRDGSpGAkGDRGETGP	Collagen alpha-1 (I) chain	1021	1041	CO1A1_HUMAN
2019.876	19.75444					
2014.898	21.90602	EGSpGRDGSpGAKGDRGETGP	Collagen alpha-1 (I) chain	1021	1041	CO1A1_HUMAN
1968.9	25.96413					
1817.694	20.23435					
1812.786	24.13738					
1807.809	20.64857					
1795.793	24.99647					
1668.805	40.46556					
1652.698	20.12811					
1640.581	23.24178					
1594.762	40.21545	pGpSGLPGLPGpPGPPGP	Collagen alpha-3 (IX) chain	141	158	CO9A3_HUMAN
1540.772	29.86551	SGSVIDQSRVLNLGP	Uromodulin	589	603	UROM_HUMAN
1495.684	23.3582					
1484.666	23.56837					
1482.666	22.46624					
1474.658	20.05341					
1417.635	20.02529					
1407.602	21.61461					
1405.635	20.13912	DGPpGRDGQpGHKG	Collagen alpha-2 (I) chain	933	946	CO1A2_HUMAN

1353.532	23.96295					
1319.584	20.88625					
1294.601	27.2447					
1270.503	38.0709					
1268.565	29.1139					
1263.543	22.72857					
1154.576	19.52543					
1138.586	19.50684					
1135.49	27.82094					
1101.537	27.61594					
1091.482	20.51256					
1071.494	21.43072					
1016.445	25.78512	ApGDKGESGPS	Collagen alpha-1 (I) chain	777	787	CO1A1_HUMAN
949.219	34.33332					
945.4161	25.7221					
942.4489	20.46245					
906.1788	34.26189					
903.4102	21.58346					
858.3934	23.2367	SpGEAGRpG	Collagen alpha-1 (I) chain	522	530	CO1A1_HUMAN

Table 3-4 List of peptides associated with pre-eclampsia at week 28

Chapter 4

A study of plasma biomarkers for the prediction of pre-eclampsia

4. Early pregnancy plasma biomarkers for the prediction of pre-eclampsia

4.1 Introduction

Pre-eclampsia has major implications for maternal and foetal health worldwide; despite decades of research it remains difficult to predict which women are likely to be affected. As discussed in section 1.1, several maternal characteristics including nulliparity, obesity, blood pressure and age at conception are associated with an increased risk of developing the condition, but no clinical or biochemical markers exist that can be used in early pregnancy to accurately risk-stratify women. Several biomarkers are reported to be elevated at the time of disease, but few have been shown to be elevated before diagnosis, a time-point when they could be clinically useful (308).

Although the diagnosis of pre-eclampsia is usually made after 30 weeks of gestation, the underlying pathophysiology begins much earlier. The disease development is described as occurring in several stages: the early stages involve primarily the placenta, and only the final stages, characterised by hypertension and proteinuria, are clinically detectable (309). The search for predictive biomarkers has focused largely on placental factors, in particular anti-angiogenic peptides, which are released into the maternal circulation. As discussed in Chapter 1, elevated levels of soluble fms-like tyrosine kinase-1 (sFLT-1) and soluble endoglin (sENG) have been reported in women with pre-eclampsia, both at diagnosis (310) and, crucially, prior to the onset of clinically detectable disease (134,311,312). Neither these nor other biomarkers, however, have crossed the boundary to routine clinical practice.

The syndrome of pre-eclampsia is characterised by impaired placentation, maternal endothelial dysfunction and inflammation, arising from both the oxidatively stressed and ischaemic placenta, and from the excessive maternal systemic inflammatory response. This excessive response is thought to be predominantly Th1-cell mediated, in contrast to the largely Th-2 mediated response which is associated with normal pregnancy, and which may help to protect the foetus from the maternal immune system (313). Associations of several inflammatory markers with pre-eclampsia have been investigated: tumour necrosis factor alpha (TNF-α), interleukin 1-α and 1-β, interleukin-6 and interleukin-8 have all been reported to be elevated in the blood of women with pre-eclampsia (314). Adhesion molecules also play a key role in the inflammatory response, regulating the adherence of leucocytes to endothelial cells and the migration of leucocytes into perivascular tissue. Cellular forms of these molecules can be detected in the blood, and their levels are altered dramatically in conditions such as sepsis, acute coronary syndromes and rheumatoid arthritis (190). Circulating concentrations of the adhesion molecules P-Selectin (189,190), vascular cellular adhesion molecule-1 (VCAM-1; (191,315)), E-Selectin (189,190,316) and inter-cellular adhesion molecule-1 (ICAM-1; (190)) are reported to be elevated in women with established pre-eclampsia, indicating endothelial dysfunction. Few studies have examined these molecules in longitudinal studies throughout pregnancy. Inconsistent perturbations in their concentrations have been reported at 18-20 weeks of gestation among women who subsequently developed pre-eclampsia (194,278,317), while one study reported elevated P-Selectin concentrations at week 10-14 in 20 affected women (195). These hypothesis-generating findings require further study.

In this study, therefore, a panel of circulating inflammatory cytokines and adhesion molecules were examined in the first trimester of pregnancy, and were screened for those that could be developed into clinically useful predictive markers for pre-eclampsia.

4.2 Methods

4.2.1 Patient recruitment

The Proteomics in Preeclampsia (PIP) study is described in detail in Chapter 2. At the time of this analysis delivery information was available on 2600 women who had been recruited at their initial antenatal hospital (booking) visit, of whom 49 (1.9%) developed pre-eclampsia. These women were matched as closely as possible for age, body-mass index and gestational age at booking with 74 control women who had normotensive deliveries and delivered appropriately grown babies after 37 weeks' gestation. Patient characteristics are shown in Table 4.1.

	Cases (n=49)	**Controls (n=74)**	***P*-value**
Maternal age (yrs)	27.5±5.2	27.9±5.4	0.7
Gestation at booking (weeks)	13.7±1.9	13.6±1.2	0.64
BMI (kg/m^2)	28.8±5.5	29.0±5.0	0.83
SBP (mmHg)	120±12	114±11	0.01
DBP (mmHg)	74±9	69±9	0.01
Nulliparous	43 (88%)	54 (73%)	0.07
Previous pre-eclampsia	1 (2%)	3 (4%)	1.00
Family history of preeclampsia	7 (14%)	4 (5%)	0.11
Twin pregnancy	2 (4%)	1 (1%)	0.56
Smoker	6 (12%)	14 (19%)	0.45
PlGF (pg/mL)	19.8 [10.4]	30.0 [20.2]	<0.001
sFLT-1 (pg/mL)	1766±609	1705±550	0.58
sENG (ng/mL)	5.56 [3.9]	5.39 [2.3]	0.025
Gestation at delivery (weeks)	37.8±2.5	39.9±2	<0.001
Caesarean Section	26 (53%)	12 (16%)	<0.001
Birthweight (grams)	2938±734	3575±420	<0.001

Table 4-1. Characteristics at booking of 49 women who developed pre-eclampsia (cases) and 74 women who had normotensive pregnancies (controls). Data are expressed as mean ± standard deviation, or as n (percentage of total.) Non-normally distributed data are expressed as median [inter-quartile range].

As outlined in Chapter 2, women from the PIP study with 2 or more traditional risk factors for pre-eclampsia were invited for further sampling at gestational weeks 16 and 28. 180 women attended at gestational week 16 and 28; 17 (9.4%) of these women developed pre-eclampsia, 7 (3.9%) developed pregnancy-induced hypertension, while the remainder had normotensive deliveries. For the this study, plasma samples of the first 11 cases and 39 women matched as closely as possible for age, BMI and parity who had normotensive deliveries (controls) were examined (table 4.2). Of these 50 women, 44 attended for further sampling 6-9 months post-natally (6 cases, 38 controls).

	Cases (n=11)	**Controls (n=39)**	***P*-value**
Maternal age (yrs)	27.8±4.4	33.5±4.9	0.001
BMI (kg/m^2)	31.3±5.5	27.5±5.7	0.059
SBP (mmHg)	127±9	123±14	0.469
DBP (mmHg)	79±6	76±12	0.395
Nulliparous n (%)	10 (91%)	14 (36%)	0.002
Previous pre-eclampsia (n%)	1 (9%)	9 (23%)	0.423
Family history of pre-eclampsia n (%)	5 (45%)	6 (15%)	0.005
Twin pregnancy n (%)	1 (9%)	0 (0%)	0.22
Smoker	1 (9%)	7 (18%)	0.67
Gestation at delivery (weeks)	39.1±1.8	40.3±1.3	0.018
Caesarean Section n (%)	5 (45%)	5 (13%)	0.03
Birthweight (g)	3284±469	3755±492	0.007

Table 4-2. Characteristics at booking of women sampled at gestational week 16 and 28. Data are expressed as mean ± standard deviation, or as n (percentage of total.) Non-normally distributed data are expressed as median [inter-quartile range]. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Having identified a potential biomarker in this study cohort, a further aim was to confirm the findings in an independent validation cohort. For this purpose, samples were made available from the Glasgow Outcome, Activated protein C resistance and Lipid (GOAL) study. This prospective pregnancy study, carried out in Glasgow between May 1997 and May 1999 was designed to examine the impact

of Factor V Leiden mutations on vascular complications of pregnancy. Over 4200 pregnant women were included in the study; recruitment protocols have been described in detail elsewhere (318). Women were sampled at their first antenatal hospital visit (7-16 week gestation) and again in the third trimester (between 27 and 40 weeks of gestation). Patient characteristics were broadly similar to the PIP study, with a median maternal age of 29 (25-32) and an overall incidence of pre-eclampsia of 1.7% (318). Sixty-four first trimester (gestational week 7-16) plasma samples (33 cases and 31 controls matched for age and parity) and 58 third trimester (gestational week 27-34) plasma samples (28 cases, 30 controls) were re-analysed for the current study.

4.2.2 Circulating biomarkers

Blood samples were obtained from the antecubital fossa into an EDTA tube (PIP) or sodium citrate tube (GOAL). Samples were stored at room temperature for less than one hour, transferred refrigerated to the research centre, and centrifuged at 3000 rpm for 10 minutes. The resultant plasma was stored at -70°C and defrosted immediately prior to analysis.

Plasma levels of VCAM-1, ICAM-1, P-Selectin, E-Selectin and L-Selectin, Interleukins 1-α, 1-ß, 2, 4, 6, 8 and 10, vascular endothelial growth factor (VEGF), tumour necrosis factor alpha (TNF-α), interferon-γ, monocyte chemotactic protein-1 (MCP-1) and epidermal growth factor (EGF) were determined by immunoassay using multi-analyte biochip array technology (Evidence Investigator, Randox Laboratories). Protocols and quality control procedures are described in Chapter 2.

Soluble FMS-like tyrosine kinase-1 (sFLT-1), soluble endoglin (sENG), placental growth factor (PlGF) and E-selectin levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R+D Systems, Abingdon, UK) as outlined in Chapter 2. All samples were measured in duplicate, and mean values were used for statistical analysis. Intra-assay coefficients of

variation were 3.0%, 8.0%, 3.8%, 3.7% and 2.9% for sFLT-1, PlGF, sEng, E-selectin (PIP study) and E-selectin (GOAL study), respectively.

4.2.3 Statistical analysis

Statistical analysis was performed using SPSS (Version 15.0; SPSS Inc., Chicago IL, USA). Data are expressed as mean ± standard deviation or as number (percentage of total). The Kolmogorov-Smirnov test was used to evaluate the data distribution. Non-normally distributed data were log-transformed prior to analysis, and mean values were compared using the unpaired two tailed Student's *t*-test. Variables that remained non-parametric after log-transformation are expressed as median [inter-quartile range] and were compared using the Mann-Whitney *U*-test. A probability value of $P<0.05$ was considered significant.

4.3 Results

4.3.1 PIP booking visit samples: screening experiments

Controls were similar to cases in respect of matched variables (Table 4.1). As expected, women who subsequently developed pre-eclampsia already had higher systolic and diastolic blood pressure at booking, going on to deliver babies of lower birthweight at an earlier gestation and with a higher rate of Caesarean delivery (Table 4.1). To further confirm that this study cohort was in keeping with other studies in the field, circulating concentrations of sENG, sFLT-1 and PIGF from the booking visit were measured in women who went on to develop pre-eclampsia and in women who remained normotensive. As expected, plasma levels of PIGF were significantly lower ($P<0.001$) in women who went on to develop pre-eclampsia, while levels of sENG were significantly higher ($P=0.025$) in affected women (figure 4.1). At booking, sFLT-1 levels were not different between the 2 groups; when sFLT-1 levels were measured at gestational week 28, however, levels were higher in affected women (1979±1118 *vs* 1230±624 pg/ml; $P=0.003$).

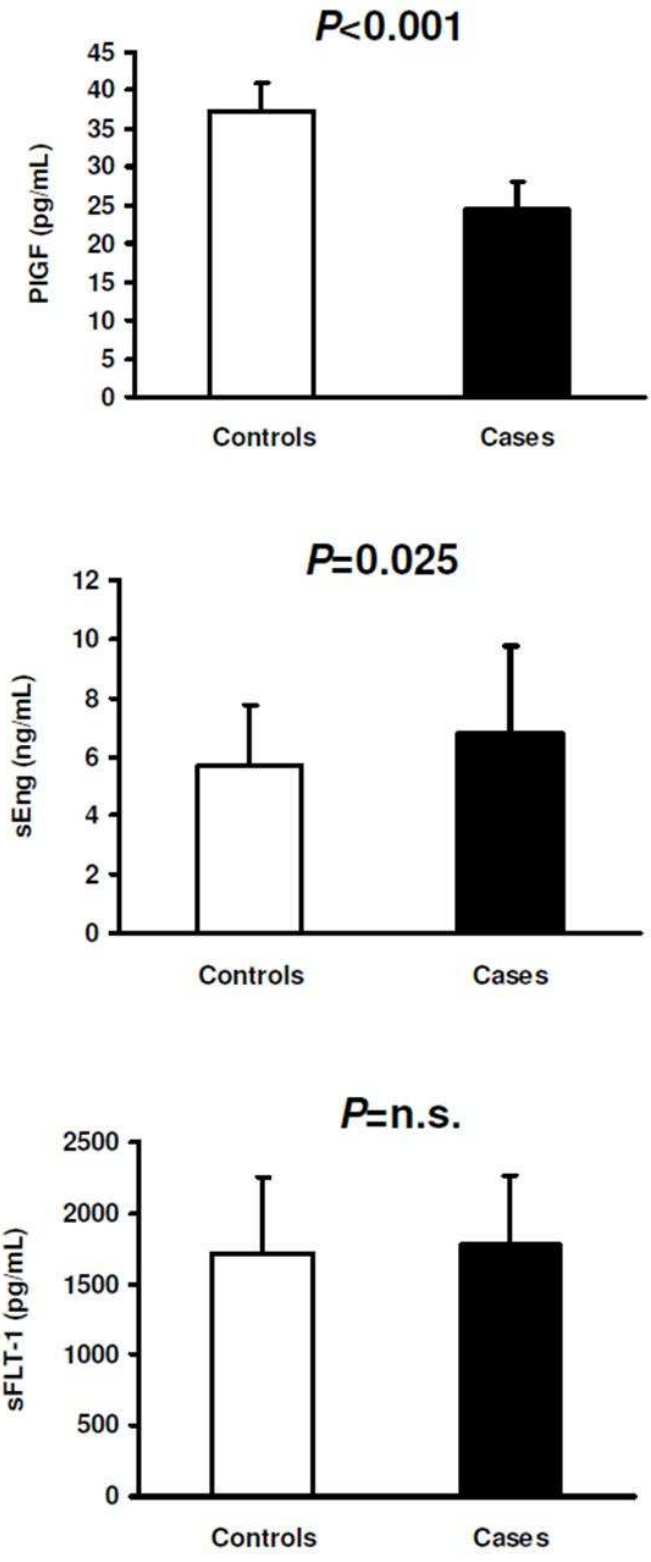

Figure 4-1 Levels of PlGF, sENG and sFLT-1 at the booking visit in the overall (low risk) population. Mean first trimester concentrations in women who developed preeclampsia (cases) and women who had normotensive pregnancies (controls) are displayed.

Multimarker assays of inflammatory markers and cytokines were then performed comparing the 49 cases and 74 controls (Table 4.1). E-Selectin levels were significantly higher (*P*=0.022) in women who went on to develop pre-eclampsia, compared to controls (figure 4.2). There were no significant differences between cases and controls in levels of any of the other biochemical markers (all *P*>0.05, table 4.3). When analysed using a receiver operating characteristic (ROC) curve, the area under the curve (AUC) for E-Selectin was 0.653. Using the median E-Selectin level (12.5 ng/ml) as a cut-off, a sensitivity of 68% and a specificity of 58% for the detection of pre-eclampsia was determined.

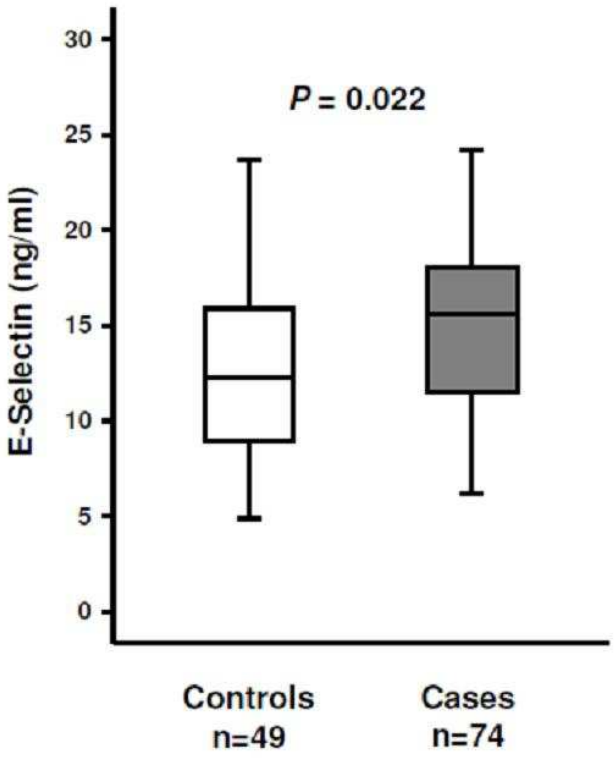

Figure 4-2 First trimester E-Selectin concentrations in the overall (low risk) population in women who developed preeclampsia (cases) and women who had normotensive pregnancies (controls). Boxplots indicate median, interquartile range and range.

	Cases (n=49)	Controls (n=74)	*P*-value
VCAM-1 (ng/mL)	512±87	487±101	0.22
ICAM-1 (ng/mL)	254±75	248±61	0.70
E-Selectin (ng/mL)	15.1±4.9	12.9±4.5	0.02
P-Selectin (ng/mL)	93.7±21	96.8±25	0.52
L-Selectin (ng/mL)	1381±234	1318±288	0.27
IL-2 (pg/mL)	1.27 [0.68]	1.18 [0.65]	0.75
IL-4 (pg/mL)	1.58 [0.69]	1.55 [0.54]	0.59
IL-6 (pg/mL)	0.84 [0.51]	0.86 [0.55]	0.89
IL-8 (pg/mL)	2.09 [1.02]	2.13 [0.96]	0.48
IL-10 (pg/mL)	0.76 [0.35]	0.69 [0.32]	0.25
VEGF (pg/mL)	3.71 [1.28]	3.94 [1.08]	0.93
Interferon-γ (pg/mL)	0.45 [0.3]	0.38 [0.5]	0.83
TNF-α (pg/mL)	1.82 [0.61]	1.69 [0.53]	0.33
IL-1 α (pg/mL)	0.08 [0.06]	0.09 [0.07]	0.27
IL-1 ß (pg/mL)	0.92 [0.78]	0.79 [0.77]	0.33
MCP-1 (pg/mL)	81.8 [55.9]	96.9 [46.3]	0.41
EGF (pg/mL)	5.43 [9.52]	9.4 [13.7]	0.12

Table 4-3 Comparison of circulating levels of adhesion molecules and inflammatory cytokines at gestational week 12-16 in cases and controls. Normally distributed data are expressed as mean ± standard deviation, non-normally distributed data are expressed as median [inter-quantile range].

4.3.2 PIP longitudinal study

In order to investigate the consistency of E-selectin as a marker of risk of pre-eclampsia throughout pregnancy, further cytokine and growth factor assays were measured in the women with risk factors for pre-eclampsia who had been sampled longitudinally throughout pregnancy. Plasma samples from gestational weeks 16 and 28 (11 cases and 39 controls) and from 6-9 months post-natally (6 cases and 38 controls) were examined. In keeping with the findings from the booking samples, E-Selectin levels were elevated at both time-points (week 16: 13.6±5.4 vs 10.6±3.3 ng/mL, *P*=0.032; week 28: 14.4±5.6 *vs* 10.7±3.5 ng/mL,

P=0.010), while there was no difference between cases and controls when measured post-natally (11.6±3.9 *vs* 12.5±3.8 ng/mL, *P*=0.596; figure 4.3). None of the other inflammatory markers were significantly different between cases and controls at gestational weeks 16 or 28, or post-natally (table 4.4)

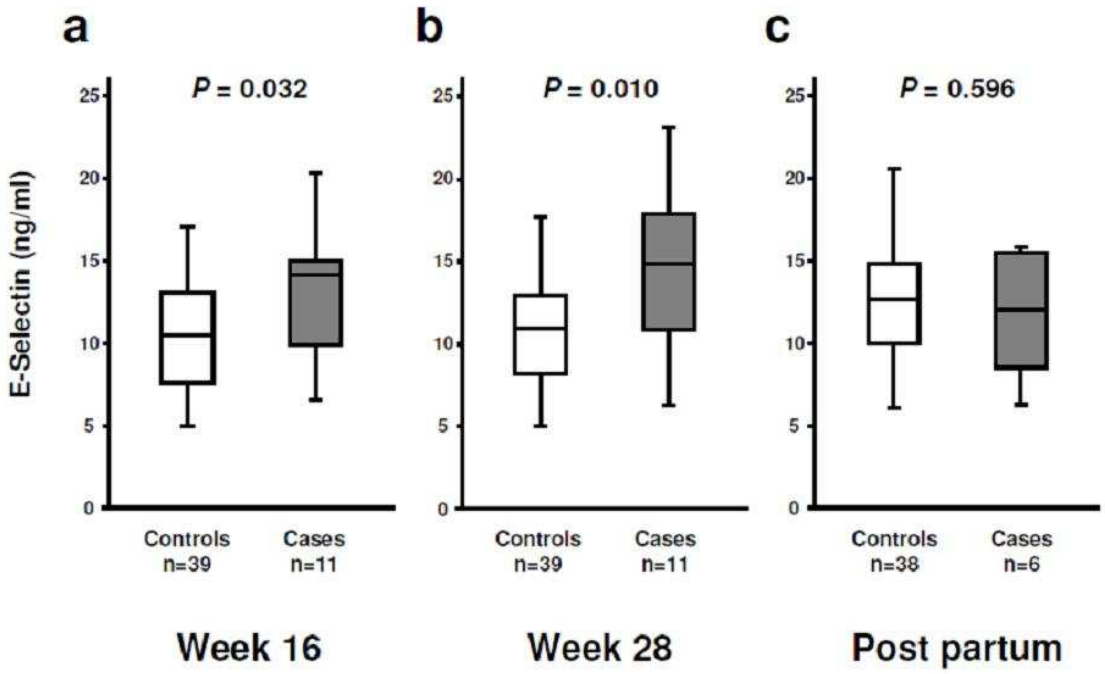

Figure 4-3 E-Selectin concentrations in the risk factor (high risk) population at (a) gestational week 16, (b) week 28, and at (c) 6 months post-natally in women who developed pre-eclampsia (cases) and women who had normotensive pregnancies (controls). Boxplots indicate median, interquartile range and range.

4.3.3 Replication using a singleplex ELISA

In order to minimise any limitations in sensitivity that are inherent from multiplex detection assays, these findings were replicated using a commercial E-Selectin specific ELISA. Samples from gestational week 12-16 that had already been measured with the multi-analyte biochip array were reanalysed with the ELISA. There was a strong correlation between data from both methods (r=0.715, *P*<0.001) although ELISA data were 4.1-fold lower than multi-analyte biochip array data. ELISA data were therefore corrected by multiplying levels by 4.1 and the agreement between the two assays subsequent to this adjustment is demonstrated in a scatterplot and a Bland-Altman plot (Figure 4.4)

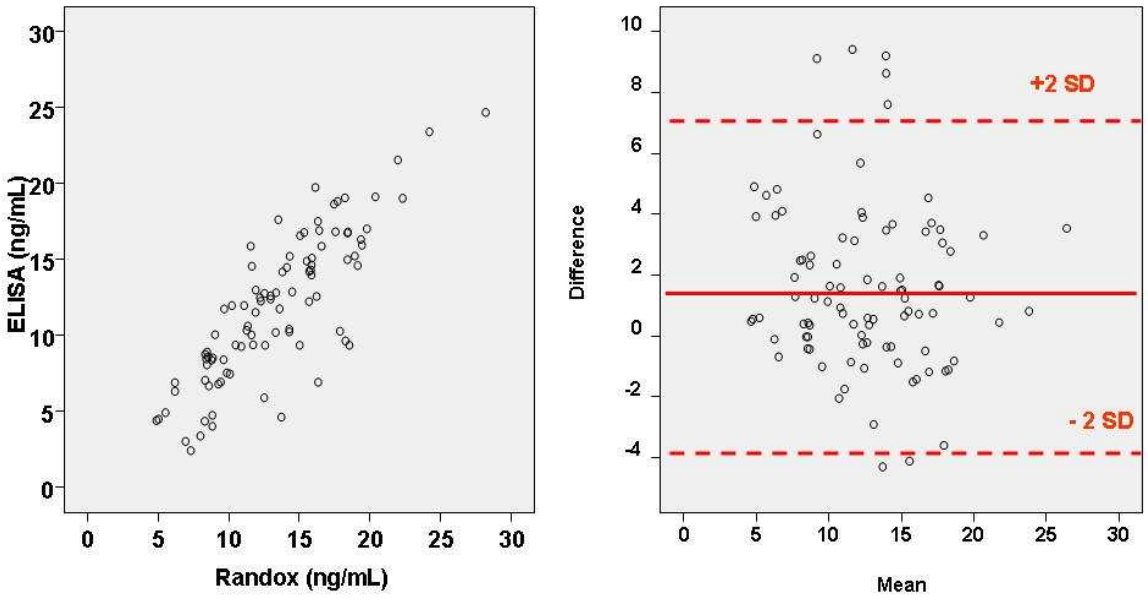

Figure 4-4 Left: Scatterplot showing correlation of E-Selectin measured using Randox assay (x-axis) and ELISA assay (y-axis.) There was a strong correlation between data from both methods (r=0.715, P<0.001) Right: Bland-Altman plot demonstrating the agreement between E-Selectin concentrations when measured using the 2 techniques.

4.3.4 GOAL: Validation in an independent study cohort

Findings from the PIP plasma samples were then validated in an independent study cohort. Using the GOAL study cohort, first trimester samples from 33 cases and 31 controls, and third trimester samples from 28 cases and 30 controls were analysed using the E-Selectin specific ELISA. When measured in the first trimester, plasma E-Selectin levels were not different in women who went on to develop pre-eclampsia compared to controls (3.0±1.0 *vs* 2.7±1.3 ng/mL, *P*=0.371; Figure 4.5a). When measured in the third trimester, E-Selectin levels were higher in the women who were subsequently affected compared to controls (3.9±1.9 *vs* 3.0±1.6 ng/mL, *P*=0.048; Figure 4.5 b).

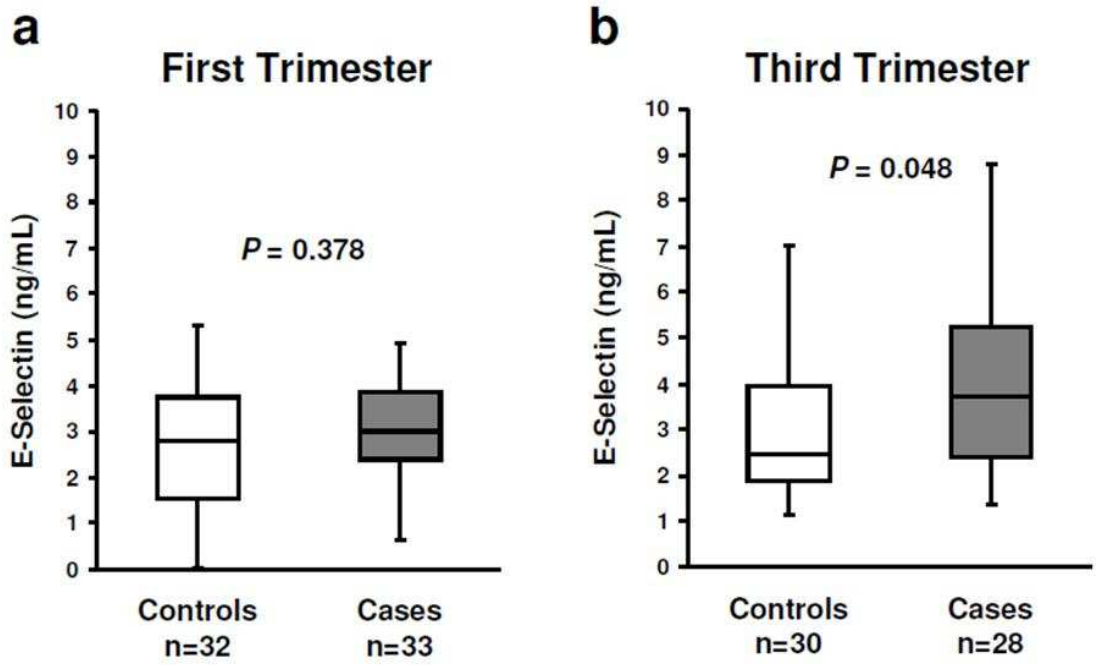

Figure 4-5 : E-Selectin concentrations in the GOAL (mixed risk factor status) study cohort in (a) first and (b) third gestation samples, in women who developed preeclampsia (cases) and women who had normotensive pregnancies (controls). Boxplots indicate median, interquartile range and range.

4.4 Discussion

Given that pre-eclampsia is a condition characterised by widespread inflammation (319), the hypothesis for this study was that screening for a large number of inflammatory and adhesion markers using a multi-analyte biochip

array would lead to identification of the markers with the strongest association with pre-eclampsia. Despite this, circulating concentrations of all inflammatory cytokines and growth factors, and the majority of soluble cell adhesion molecules in early pregnancy were not different at booking between affected and unaffected women. The multiplex assays did, however, demonstrate that levels of E-Selectin were significantly higher in women who went on to develop pre-eclampsia compared to controls. These findings were confirmed by re-analysing using an ELISA technique, although multiplex assays were 4.1 fold higher than ELISA levels. This difference is perhaps explained by the fact that there is no international standard for quantifying E-selectin concentrations, and so different manufacturers will have different in-house processes and will use different standards for calibrating their assay method. This in itself is not a problem when comparing assays as long as they have good agreement, as well as good correlation as outlined by Bland & Altman (320). The strong agreement observed further suggests that the antibodies used by the manufacturers are likely to be recognising similar antigens and not distinct isoforms of the soluble E-selectin fragment. Further verification of the elevated E-selectin levels in pre-eclampsia was provided using samples from the same cohort of women at later time points during pregnancy, and from third trimester (but not first trimester) samples from an independent cohort.

E-Selectin is an 11 kD cell-surface glycoprotein, and is a member of the selectin family involved in mediating the interaction of circulating leucocytes with the vascular endothelium. It is expressed exclusively on endothelial cells, in response to cytokines including TNF-α and Interleukin-1 (189). Elevated soluble E-Selectin levels are indicative of endothelial cell activation and damage, and given that endothelial dysfunction can explain many of the clinical characteristics of the condition (321), it is perhaps not surprising that elevated soluble E-Selectin levels are associated with pre-eclampsia. E-Selectin concentrations have been previously reported to be elevated at the time of delivery in women with both mild and severe pre-eclampsia (189), and to be elevated at week 20 in women who went on to develop the condition (194). This study has demonstrated that elevated E-selectin is detectable at an earlier point in pregnancy, possibly as early as the end of the first trimester. This may suggest an aetiological role, or that expression and cleavage of the molecule reflects

early pathophysiological changes leading to pre-eclampsia. It is clear, however, that E-Selectin levels alone would not be a useful predictor of pre-eclampsia; the area under the ROC curve for E-Selectin was inferior to that recently demonstrated using a model of clinical risk factors for predicting pre-eclampsia in the SCOPE cohort described in Chapter 3 (3).

Strengths and limitations of this study should be considered. Many of the studies designed to find biomarkers for predicting pre-eclampsia in early pregnancy have rigid protocols where women attend at certain time-points during pregnancy. In reality, women attend their healthcare professional at a variety of time-points, depending on the regularity of their normal menstrual cycle, on local protocols, and on pressures upon midwifery and ultrasound services. One advantage of the PIP study is that the "real-life" situation of antenatal care has been reflected, by including all women up to 18 weeks of gestation.

In keeping with the literature, elevated circulating levels of sENG (134) and reduced circulating PlGF levels (311) were seen in women who went on to develop pre-eclampsia compared to controls. sFLT-1 levels (310) were not different between the 2 groups at booking, but when measured in third trimester samples were higher in affected women. These results suggest that PIP is an externally comparable cohort to test predictive markers for pre-eclampsia.

A potential limitation of this paper is that both the PIP and the GOAL studies were performed in Glasgow, a city which has some of the highest rates of cardiovascular disease in Europe. E-Selectin polymorphisms have been reported to be associated with essential hypertension (322) and coronary artery disease (323), while E-Selectin levels are also associated with a variety of risk factors for cardiovascular disease (324). It is possible therefore that increased E-selectin levels do not specifically explain the pathophysiology of pre-eclampsia in these cohorts but rather lead to higher blood pressures and thereby trigger the initiation of a process that eventually causes pre-eclampsia. In this study as in many other studies (12), both systolic and diastolic blood pressures were already slightly but significantly higher at booking in women who subsequently developed pre-eclampsia compared to those with uncomplicated pregnancies.

How best to diagnose pre-eclampsia, and how to define severe forms of the disease remain a matter of debate. The majority of women in this study had late-onset, milder variants of pre-eclampsia, with only 9 having more severe, early onset (diagnosed at <37 weeks' gestation) disease. Previous studies have reported that levels of angiogenic factors correlate with severity of disease (325), and one might expect the same of E-Selectin levels. Bretelle et al. (326) reported that E-Selectin levels at term were elevated in normotensive women with small for gestational age (SGA) babies, as well as in those with pre-eclampsia. Further studies, comparing E-Selectin levels in women with early and late-onset disease, and in women with pre-eclampsia with SGA babies would be beneficial.

A further limitation is the first trimester findings from the PIP study were not replicated in first trimester samples from the GOAL study. There are a number of factors which could account for this: first trimester samples from the GOAL study were taken as early as gestational week 7, which is earlier than in PIP and the majority of other studies, and may be before the early pathophysiological changes of pre-eclampsia can be detected. Another factor may be that quality of GOAL samples was suboptimal due to a storage time of up to 13 years.

Finally, out of the many biomarkers analysed with the multi-analyte biochip array only E-selectin levels were different between cases and controls. This may be because this method of measurement was not sensitive enough to detect differences between cases and controls, or because there is truly no difference in levels at this early stage in pregnancy. The fact that the findings were confirmed both in later stages of pregnancy, and in an independent study cohort, suggest that the elevated E-Selectin levels in affected women were not a false positive finding related to multiple testing. These data highlight a potential functional role of soluble E-Selectin in the development of pre-eclampsia, or reflect E-selectin being a biomarker of underlying pathophysiological processes. Further epidemiological and basic science studies are required to confirm and expand on these findings.

	Week 16			Week 28			Post-natal		
	Cases (n=11)	Controls (n=39)	*P*	Cases (n=11)	Controls (n=39)	*P*	Cases (n=6)	Controls (n=38)	*p*
VCAM-1 (ng/mL)	471±69	491±110	0.58	505±82	476±97	0.38	558±98	507±105	0.27
ICAM-1 (ng/mL)	214±78	239±50	0.21	235±93	239±52	0385	180±41	230±60	0.06
E-Selectin (ng/mL)	13.6±5.4	10.6±3.3	0.03	14.4±5.6	10.7±3.5	0.01	11.6±3.9	12.5±3.8	0.60
P-Selectin (ng/mL)	115±33	112±44	0.85	123±38	123±38	0.99	116±33	124±55	0.75
L-Selectin (ng/mL)	1166±131	1158±177	0.89	993±127	966±133	0.55	1255±81	1257±175	0.98
IL-2 (pg/mL)	2.73 [2.84]	1.54 [1.29]	0.35	1.34 [0.27]	1.34 [0.69]	0.89	1.6 [5.5]	1.89 [2.6]	0.79
IL-4 (pg/mL)	1.54 [0.7]	1.62 [0.89]	0.45	1.67 [1.26]	1.49 [0.46]	0.68	2.1 [2.1]	1.71 [1]	0.16
IL-6 (pg/mL)	0.69 [0.29]	0.82 [1.04]	0.5	1.15 [0.48]	1.1 [1.21]	0.89	1.39 [0.73]	0.93 [0.81]	0.17
IL-8 (pg/mL)	2.38 [2.08]	2.38 [1.24]	0.75	2.28 [0.99]	2.46 [1.75]	0.36	3.39 [1.98]	4.5 [3.0]	0.16
IL-10 (pg/mL)	0.81 [0.72]	0.78 [0.42]	0.91	0.7 [0.18]	0.7 [0.34]	0.3	0.68 [0.21]	0.79 [0.46]	0.32
VEGF (pg/mL)	5.08 [4.13]	4.42 [1.83]	0.98	4.89 [4.18]	5.17 [3.3]	0.48	24.5 [24]	22 [19]	0.81
IFN-γ (pg/mL)	0.55 [0.43]	0.58 [0.63]	0.83	0.62 [0.4]	0.59 [0.8]	0.99	0.54 [2.4]	0.88 [1.1]	0.66
TNF-α (pg/mL)	1.43 [0.61]	1.61 [0.83]	0.27	1.6 [0.6]	1.73 [0.6]	0.64	2.3 [0.48]	2.23 [0.8]	0.42
IL-1 α (pg/mL)	0.12 [0.04]	0.1 [0.1]	0.61	0.13 [0.2]	0.11 [0.1]	0.49	0.15 [0.19]	0.17 [0.14]	0.96
IL-1 ß (pg/mL)	0.7 [1.62]	0.86 [1.0]	0.86	0.77 [0.77]	0.95 [0.99]	0.39	1.17 [1.12]	1.1 [1.3]	0.89
MCP-1 (pg/mL)	89.6 [45]	84.3 [41.6]	0.56	68.3 [30.8]	71.6 [34]	0.84	120 [31]	130 [99]	0.43
EGF (pg/mL)	18.6 [29.6]	12.7 [21.2]	0.45	12.2 [17.5]	21 [25]	0.57	15.2 [21]	20.1 [44]	0.61

Table 4-4 Circulating concentrations of adhesion molecules and inflammatory cytokines in women studied longitudinally throughout pregnancy. Normally distributed data are expressed as mean ± standard deviation, non-normally distributed data are expressed as median [inter-quantile range].

Chapter 5

A study of peripheral arterial tonometry (PAT) for the assessment of endothelial function in pregnancy

5. A study of peripheral arterial tonometry (PAT) for the assessment of endothelial function in pregnancy

5.1 Introduction

Pregnancy is associated with a number of physiological vascular changes, including an increase in cardiac output and a reduction in peripheral resistance and blood pressure (106). These changes, which are evident as early as 5 weeks gestation (90), are reported to be largely governed by the vascular endothelium. Long considered to be an inert barrier to elements contained within the blood, the endothelium is now known to have a number of physiological roles, acting as a biological interface between blood and the tissues, and modulating growth, haemostasis, vascular tone and inflammation throughout the circulatory system.

Although pre-eclampsia is recognised as having its origins in the placenta (327), it is thought that many of the clinical manifestations of the condition including hypertension, proteinuria and oedema are caused by dysfunction and mal-regulation of the maternal endothelium (265). Detection of endothelial dysfunction could therefore represent a potential area for the early prediction of pre-eclampsia, which could in turn lead to earlier diagnosis, closer monitoring of affected women, and intervention where indicated.

Several studies have demonstrated the relationship between endothelial dysfunction and pre-eclampsia (89,90,265,328), but the majority have been restricted to in-vitro experiments involving endothelial cells or vessels from maternal or foetal tissues. These and other techniques for assessment of the endothelium involving infusion of vasoactive substances are clearly too invasive to be used either in large studies of pregnant women, or in routine clinical practice.

A non-invasive technique for the measurement of endothelial function, ultrasonic measurement of flow-mediated dilatation (FMD) at the brachial artery, has been reported extensively. As discussed in section 1.3, previous studies have shown enhanced FMD as normal pregnancy progresses (86), with the highest dilatation in the third trimester (329). FMD measurement using this technique has been reported to be significantly reduced, suggesting endothelial dysfunction, in women with pre-eclampsia compared to women with normotensive pregnancies (88).

The use of ultrasound to measure FMD, however, requires specific training, and is dependent upon the skill of the operator, which may preclude its use in widespread clinical practice. An alternative technique for assessment of endothelial function which has emerged in recent years is the assessment of peripheral arterial tone (PAT). This technique assesses peripheral microvascular endothelial function by measuring changes in digital pulse volume during reactive hyperaemia. The reactive hyperaemia index (RHI) generated is highly correlated with ultrasound-based FMD (330), and also with coronary artery endothelial function on angiography following infusion of acetylcholine (288). Data from the Framingham cohort have demonstrated an independent relationship between RHI and multiple traditional and metabolic cardiovascular risk factors (331). In contrast to ultrasound based techniques the assessment of endothelial function by PAT requires minimal specific training, and therefore may be applicable in large clinical studies (332).

The aim of this study was to evaluate endothelial function as assessed by PAT at different time-points during pregnancy. I assessed the ability of PAT studies in early pregnancy to predict hypertensive disorders in later pregnancy, and examined the relationship between RHI and risk factors for pre-eclampsia. Further, I examined how endothelial function scores derived from PAT correlate with those derived from flow-mediated dilatation, and examined whether abnormalities persisted after pregnancy.

5.2 Methods

5.2.1 Patient recruitment

The overall Proteomics in Pre-eclampsia (PIP) study is described in detail in Chapter 2. A subset of 180 pregnant women with ≥2 risk factors for pre-eclampsia were invited to attend for study visits at gestational weeks 16 and 28, and at 6-9 months post-partum for further sampling and vascular function studies. Of this subset, 17 (9.4%) women developed pre-eclampsia, 7 (3.9%) developed pregnancy-induced hypertension (PIH) without significant proteinuria, and 156 (87%) remained normotensive throughout pregnancy. Demographics of the women in the vascular function study are shown in table 5.1, those of the overall study cohort are described in Chapter 2.

	Pre-eclampsia or PIH (n=24)	**Normotensive (n=156)**	**p-value**
Age (years)	30.7±5.1	33.1±5.2	0.03
BMI at booking(kg/m^2)	30.3±5.8	27.8±5.6	0.04
SBP at booking (mmHg)	126±10	120±12.6	0.06
DBP at booking (mmHg)	79±7	74±9.3	0.007
Smoking n(%)	1 (4%)	12 (7.6%)	1.0
Nulliparous n(%)	20 (83%)	67 (43%)	<0.001
Ethnicity (% non-Caucasian)	2 (8.3%)	9 (5.8%)	0.64
Pre-eclampsia in previous pregnancy n (%)	2 (8.3%)	33 (21%)	0.17
Family history of pre-eclampsia n (%)	9 (37.5%)	21 (13.5%)	0.007
Gestation at delivery (wks)	39.3±2	39.9±1.6	0.098
Birthweight (grams)	3295±520	3601±509	0.007

Table 5-1. Demographics of women studied at gestational week 16 and 28. Data are expressed as mean ± standard deviation, or as number (% of total).

5.2.2 Post delivery studies

Of the 180 women studied at gestational weeks 16 and 28, 80 attended again at 6-9 months post-natally, of whom 8 had developed pre-eclampsia, 4 had

developed PIH, and 68 had remained normotensive throughout. A further 15 women, who had been recruited to the study at booking and went on to develop pre-eclampsia, but who had not attended for vascular function studies during pregnancy, also attended at 6-9 months post-natally. There was therefore post-natal data available on 96 women, 23 of whom had developed pre-eclampsia, 4 of whom had developed PIH, and 69 who had remained normotensive. Demographics of the women studied post-natally are shown in table 5.2.

	Pre-eclampsia or PIH (n=27)	**Normotensive (n=69)**	**p-value**
Age (yrs)	31.2±4.5	35.6±4.9	<0.001
BMI (kg/m^2)	28.8±7.4	27.5±5.5	0.346
SBP (mmHg)	127±13	124±13	0.385
DBP (mmHg)	83±10	78±11	0.074
Smoking (%)	1 (3.7%)	6 (8.7%)	0.67
Ethnicity (% non-Caucasian)	1 (3.7%)	4 (5.8%)	1.0
Family history of pre-eclampsia (%)	8 (29.6%)	9 (13%)	0.074

Table 5-2 Demographics of women studied post-natally. Data are expressed as mean ± standard deviation, or as number (% of total).

Pre-eclampsia and pregnancy-induced hypertension (PIH) were defined in keeping with the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (5) as described in section 1.1.

5.2.3 PAT recording

Peripheral arterial tone (PAT) was recorded using the EndoPAT 2000 device (Itamar Medical, Caesarea, Israel) as described in Chapter 2. In brief, the system comprises pneumatic finger probes which assess digital volume changes accompanying pulse waves. A blood pressure cuff was placed on the dominant arm (study arm) while the other arm acted as a control. PAT probes were placed on the index fingers of each hand for continuous recording of the PAT signal. After a 10 minute acclimatisation period, the blood pressure cuff was inflated to suprasystolic pressures for 5 minutes, then released, while PAT recording continued for a further 5 minutes.

PAT data were analysed using proprietary software in an operator-independent manner. Reactive hyperaemia index (RHI) was calculated as described in Chapter 2. All recordings were performed in the same temperature-controlled room by myself or by a research nurse trained by myself.

5.2.4 Brachial artery flow-mediated dilatation

Brachial artery ultrasound was used to measure flow-mediated dilatation as outlined in Chapter 2. 16 consecutive women attending for vascular function studies at week 16 and 28 were examined on the same day as PAT assessment. Of them, one woman developed pre-eclampsia and one developed pregnancy-induced hypertension; the remaining 14 remained normotensive. In brief, after baseline brachial artery recordings for 5 minutes, a blood pressure cuff was inflated on the forearm distal to the artery. The cuff was released after 5 minutes and the artery was then scanned for a further 5 minutes to record the effects of reactive hyperaemia. The study was recorded and analysed offline using proprietary software. Flow-mediated dilatation (FMD) was expressed as the percentage change in the arterial diameter pre and post cuff occlusion, relative to the baseline diameter. All FMD recordings were performed by the same experienced investigator, Miss Lesley Anderson.

5.2.5 Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normality of data distribution. Normally distributed data are expressed as mean ± standard deviation, non-normally distributed data are expressed as median [IQR]. Data were compared using the Students t-test for normally distributed data, the Mann-Whitney U-test for non-normally distributed data and Fisher's exact test for categorical variables. Univariate correlations were performed using Pearson's correlation. A p-value of <0.05 was considered significant. Statistical analysis was performed using the statistical package SPSS version 15.0 (SPSS inc., Chicago, Illinois, USA).

Since EndoPAT 2000 has only rarely been reported for use in pregnancy, it was not possible to perform a formal power calculation prior to the study. The

original aim was to examine 200 pregnant women with ≥ 2 risk factors at both gestational weeks 16 and 28, with an expected "drop-out" rate of 10%. Recruitment for the vascular function sub-study therefore continued until 180 women had been examined at both week 16 and 28. Based upon the sample size, the standard deviations, and an anticipated 10% incidence of pre-eclampsia or PIH in this cohort, the study was 80% powered to detect a difference of 0.43 in RHI between women who would go on to develop hypertensive disorders and those who would remain normotensive.

5.3 Results

5.3.1 Cases vs controls

Women who went on to develop PIH or pre-eclampsia (cases) were younger than those with normotensive deliveries (controls). Although systolic blood pressure was not significantly different between the two groups at booking, cases already had a higher diastolic blood pressure (79±7 vs 74±9 mmHg, p=0.007) at week 16 than controls (table 5.1).

In a first step, reactive hyperaemia index (RHI) was compared between women who went on to develop pre-eclampsia or PIH and women who remained normotensive. No significant difference was seen between the two groups at either gestational week 16 (1.78 [0.59] vs 1.81 [0.94], p=0.69) or week 28 (1.46 [0.32] vs 1.52 [0.29], p=0.27) (table 5.3).

There was no relationship between RHI and either birth weight or gestation at delivery when examined at either week 16 or week 28.

	Week 16			Week 28		
	Cases (n=24)	Controls (n=156)	p-value	Cases (n=24)	Controls (n=156)	p-value
RHI	1.78 [0.59]	1.81 [0.94]	0.69	1.46 [0.32]	1.52 [0.29]	0.27
Baseline pulse amplitude (AU) occluded arm	547 ±333	579 ±376	0.69	981±470	1040±426	0.53
Baseline pulse amplitude (AU) control arm	491±306	535±355	0.57	902±425	963±422	0.51

Table 5-3. PAT recordings at gestational week 16 and 28. Data are expressed as mean ±SD (normally distributed data) and median [inter-quartile range] (non-normally distributed data). AU = arbitrary units.

5.3.2 Relationship between RHI and maternal risk factors

5.3.2.1 Week 16

There was no relationship between RHI at week 16 and maternal age, BMI, systolic blood pressure, parity, smoking status, or total number of risk factors for pre-eclampsia. There was, however, a significant inverse correlation between diastolic blood pressure and RHI (r=-0.165, p=0.02) (figure 5.1).

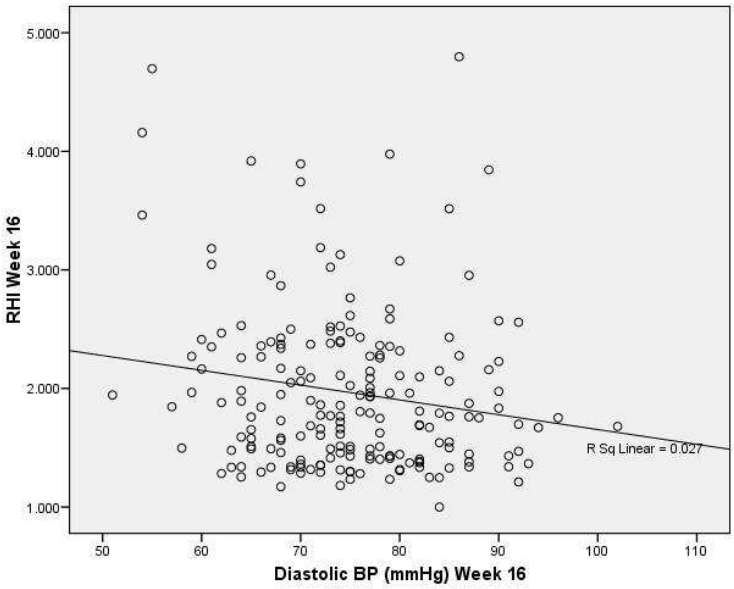

Figure 5.1. Scatterplot depicting the relationship between RHI and diastolic blood pressure at gestational week 16.

Baseline pulse amplitude was not related to maternal age, BMI, smoking status, diastolic blood pressure, parity or total number of risk factors, but was higher in women with a higher systolic blood pressure (r=0.142, p=0.049) (figure 5.2).

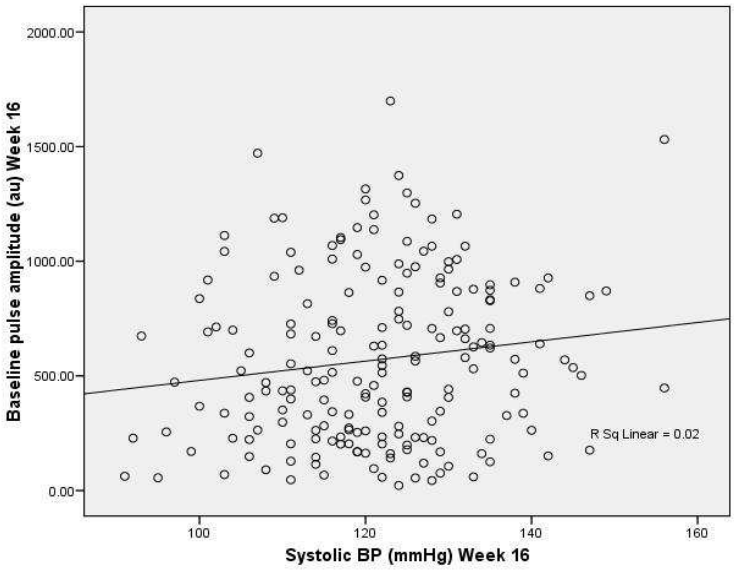

Figure 5.2. Scatterplot depicting relationship between baseline pulse amplitude and systolic blood pressure at gestational week 16.

Pregnant women with a history of pre-eclampsia in a previous pregnancy were then examined to determine whether vascular function was altered in their subsequent pregnancy. When compared to pregnant women without a history of the condition no difference was seen in RHI or baseline pulse amplitude between the two groups.

5.3.2.2 Week 28

At gestational week 28, there was no relationship between RHI and maternal age, BMI, systolic BP, diastolic BP, smoking status, parity or total number of risk factors.

Baseline pulse amplitude was higher in women with a higher BMI (r=0.188, p=0.009) (figure 5.3), and in women with a higher systolic blood pressure (r=0.187, p=0.009) (figure 5.4), but was not related to age, smoking, parity, diastolic blood pressure or total number of risk factors.

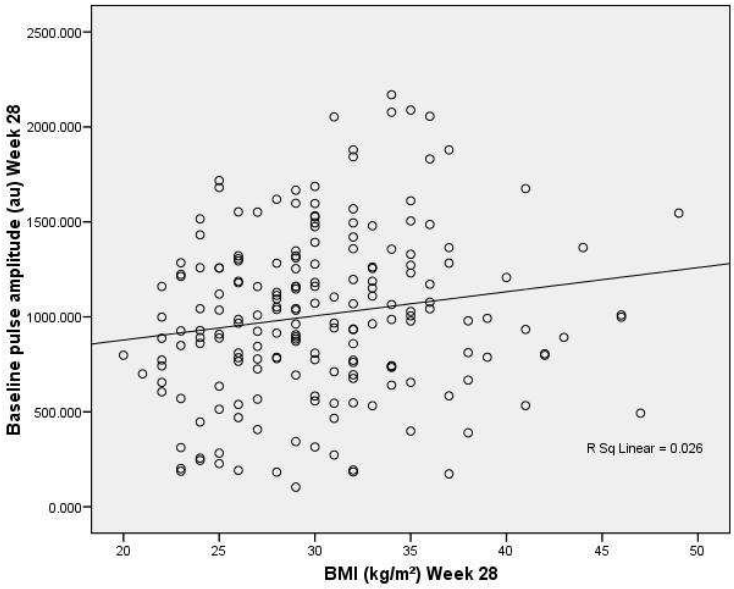

Figure 5.3 Scatterplot depicting relationship between baseline pulse amplitude and BMI at gestational week 28.

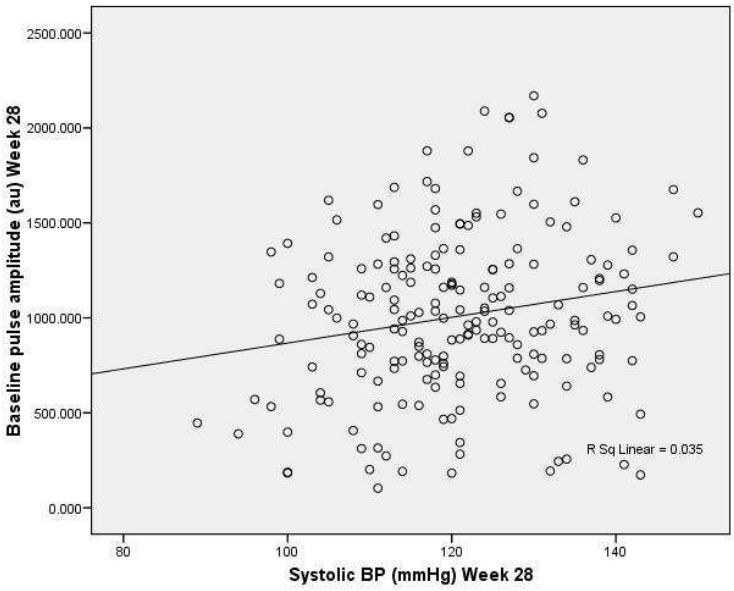

Figure 5.4 Scatterplot depicting relationship between baseline pulse amplitude and systolic blood pressure at gestational week 28.

5.3.3 Relationship between RHI and baseline pulse amplitude

In the study cohort overall, RHI at gestational week 28 was significantly lower when compared to week 16 (1.51 [0.32] vs 1.78 [0.89], p<0.001, figure 5.5a) suggesting an apparent deterioration in endothelial function with advanced pregnancy. This deterioration occurred both in women who went on to develop pre-eclampsia or PIH (1.46 [0.32] week 28 vs 1.78 [0.59] week 16, p=0.028) and in women who had normotensive pregnancies (1.52 [0.29] week 28 vs 1.81 [0.94] week 16, p<0.001).

Further analysis revealed that the baseline pulse amplitude was significantly higher in both arms, indicative of vasodilatation at week 28 compared to week 16 (1009±434 AU week 28 vs 570±364 AU week 16 study arm, 934±422 AU week 28 vs 526±342 AU week 16 control arm, p<0.001, figure 5.5b).

Baseline pulse amplitude was then compared with RHI. There was a significant inverse correlation between RHI and baseline pulse amplitude, with lower RHI in women with higher blood flow at baseline. This was true both at gestational week 16 (r=-0.326, p<0.001) and at week 28 (r=-0.329, p<0.001).

5.3.4 Comparison with FMD

In order to determine whether the reduction in endothelial function score in later pregnancy was restricted to the PAT device, FMD scores using brachial artery ultrasound were compared between 16 pregnant women at gestational week 16 and 28. In keeping with the findings using PAT there was a significantly lower percentage change in artery diameter at gestational week 28 compared to gestational week 16 (7.46% ±1.83% vs 9.42% ±2.22%, p=0.009; figure 5.5c). In addition, baseline brachial artery diameter was higher at week 28 compared to week 16 (3.73mm ±0.17mm vs 3.30mm ±0.29mm, p<0.001; figure 5.5d), indicating vasodilatation with advanced pregnancy. RHI measured using PAT and FMD measured using brachial artery ultrasound were correlated at gestational week 28 (*r*=0.49, p=0.03), but not significantly at week 16 (*r*=0.47, p=0.085).

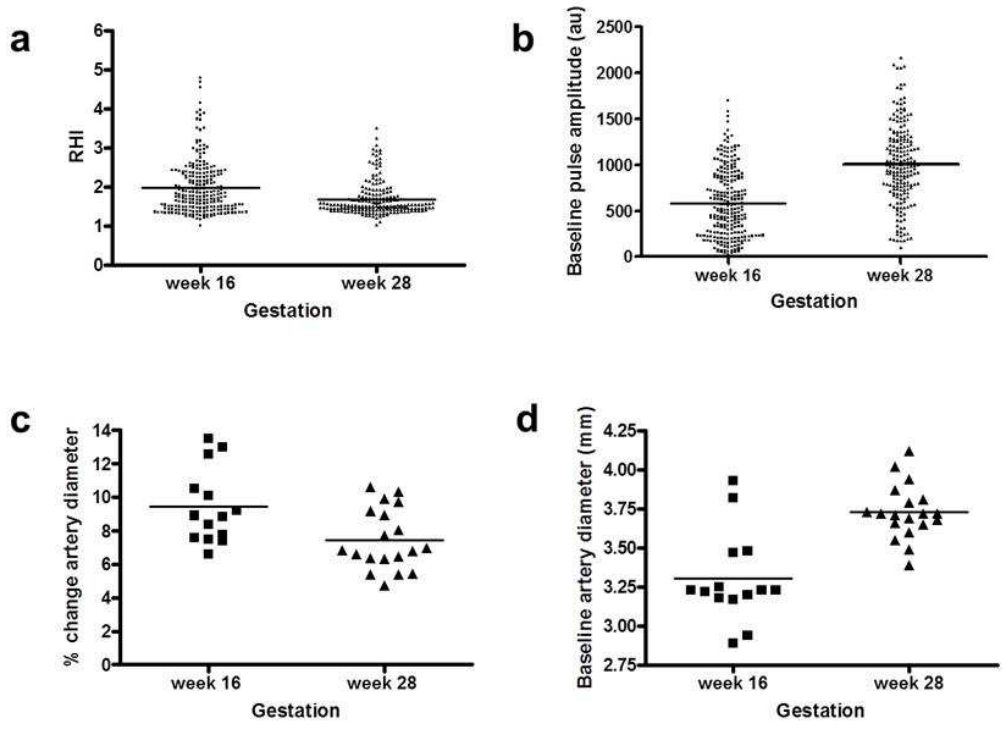

Figure 5.5 (a) Comparison of RHI between gestational weeks 16 and 28 in overall study cohort. (b) Comparison of baseline pulse amplitude between gestational weeks 16 and 28 in overall study cohort. (c) Comparison of FMD percentage between gestational weeks 16 and 28 in 16 pregnant women. (d) Comparison of brachial artery diameter at baseline in 16 women.

5.3.5 Post-natal data

Endothelial function was then examined at 6-9 months following pregnancy. 27 women who had developed pre-eclampsia or PIH were compared with 69 women who had normotensive, uncomplicated pregnancies. RHI at 6-9 months post-natally was not different between cases and controls (2.09 [0.76] vs 1.92 [1.07], p=0.28) (Figure 5.6a). Baseline pulse wave amplitude, however, was significantly higher in controls compared to cases (583±333 AU vs 411±248 AU, p=0.017 study arm, 535±297 vs 404±229, p=0.042 control arm) (Figure 5.6b).

	Pre-eclampsia or PIH (n=27)	**Normotensive (n=69)**	**p-value**
RHI	2.09 [0.76]	1.92 [1.07]	0.297
Pulse amplitude (AU) study arm	411±248	583±333	0.007
Pulse amplitude (AU) control arm	404±229	535±297	0.042

Table 5-4. PAT recordings in women studied post-natally. Data expressed as mean ± standard deviation (normally distributed data) and median [inter-quartile range] (non-normally distributed data).

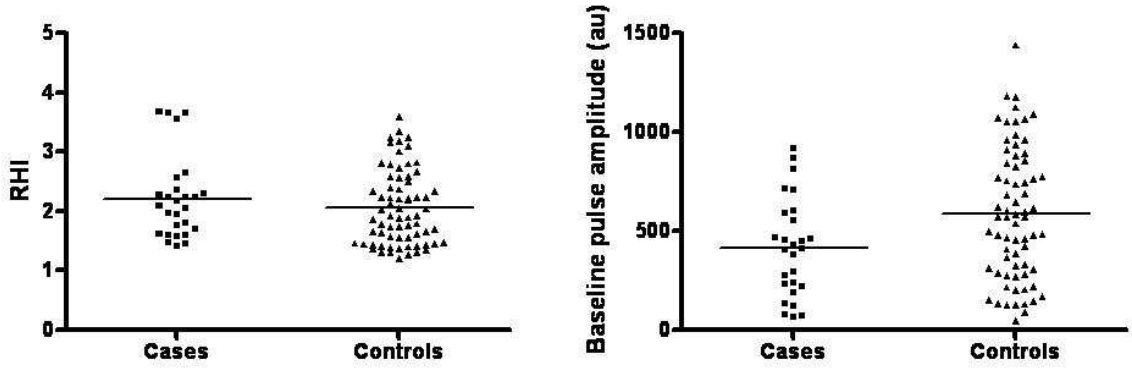

Figure 5.6 [a] RHI at 6-9 months post-natally in women with recent pre-eclampsia (cases) and women with normotensive pregnancies (controls). [b] Baseline pulse amplitude post-natally in cases and controls.

5.4 Discussion

In this study PAT was used to examine endothelial function during and after pregnancy in a cohort of women with risk factors for pre-eclampsia. At both gestational week 16 and 28, there was no difference in reactive hyperaemia index between women who would go on to develop hypertensive disorders, and those who would go on to have normotensive pregnancies. Previous studies using brachial artery ultrasound have reported increased FMD in pregnancy compared to non-pregnant controls, a finding which is thought to be secondary to increased nitric oxide (NO) production (333). It is less certain what happens to FMD in later pregnancy: although some studies have reported increased FMD after 30 weeks gestation (329,334), others have reported a reduction in the final few weeks of pregnancy (87). Using PAT an apparent deterioration in endothelial function was seen between gestational week 16 and 28, both in women who went on to develop pre-eclampsia or PIH and in those who remained normotensive. RHI at all stages of pregnancy was lower than in healthy non-pregnant women studied as part of the Framingham cohort. When the data were evaluated further there was a marked increase in baseline pulse amplitude in keeping with vasodilatation, suggesting that in later pregnancy, women were

less able to further vasodilate from baseline. Similar data using the more established brachial artery ultrasound technique suggest that this is a common feature with techniques which analyse flow-mediated dilatation, and not limited to the EndoPAT system.

There was not a clear correlation between PAT and FMD recordings. While this may be due to the relatively small number of women (16) studied using both techniques, it may also be the case that the 2 techniques are measuring different parameters; FMD measures shear-mediated changes in large artery (brachial) function, while PAT measures hyperaemic response in small vessels. The position of the cuff may also be of importance; with FMD the cuff is placed distal to the vessel being studied, whereas with PAT studies the cuff is proximal to the vessels being studied, and the fingertips are “downstream” of the local tissue injury caused by cuff inflation.

FMD has been reported to be reduced in women at the time of diagnosis of pre-eclampsia compared to normotensive women (88), but it is unclear whether this is a cause or a consequence of the disease. Few longitudinal studies have examined ability of brachial artery FMD during pregnancy to predict future pre-eclampsia. One study in which 506 women were examined at mean gestational week 22 reported that those who went on to develop pre-eclampsia (n=14) had a lower FMD than those with normal pregnancies (89). Only one study has been reported in the literature using PAT to examine endothelial function in women with pre-eclampsia. Yinon et al. (335) examined 17 women at the time of diagnosis or pre-eclampsia (mean gestation 32 weeks) and compared them with 25 women with normotensive pregnancies. The group reported reduced RHI in women with pre-eclampsia compared to controls, and a positive correlation between RHI and birthweight. Women with pre-eclampsia were, however, studied at a later point in pregnancy than women with normal pregnancies, a factor that I have shown may have influenced their capacity for further vasodilatation from baseline, and may have confounded results.

A growing body of evidence has emerged in recent years that women with pre-eclampsia are at increased risk of future development of hypertension, renal disease and coronary artery disease (252). One potential mechanism that links pre-eclampsia and cardiovascular disease in later life is maternal endothelial

dysfunction. In keeping with this theory, a number of studies have reported the use of brachial artery ultrasound to demonstrate impaired endothelial function in women with a history of pre-eclampsia. One study, examining women with a history of pre-eclampsia at 3 years post-partum reported reduced flow-mediated dilatation compared to women with a history of normotensive pregnancy, findings that were not explained by the presence of maternal risk factors (265). Further studies using this technique have reported ongoing endothelial dysfunction when measured at 6-12 months (266) and at one year (267) after a pre-eclamptic pregnancy. In the present study, using PAT to examine endothelial function at 6-9 months after pregnancy, there was no difference in RHI between those who had pre-eclampsia or PIH and those who had normotensive pregnancies. Women with a history of pre-eclampsia or PIH did, however, have reduced baseline pulse amplitude during baseline recordings. This feature, suggesting reduced vasodilatation after an affected pregnancy, may represent an early change in vascular function, and merits further investigation.

In this study both women with pre-eclampsia and women with PIH without significant proteinuria were included together in the analysis. As is discussed in section 1.1, how best to diagnose pre-eclampsia and how to differentiate between the different forms of the disease is a matter of significant debate. When women with PIH alone were excluded from the analysis results were not affected. Many diagnostic criteria do not require significant proteinuria for diagnosis of pre-eclampsia (3). Further, in a recent study examining predictors of adverse foetal and maternal outcomes in women with pre-eclampsia it was reported that altering the diagnostic criteria to include women without significant proteinuria made no difference to analysis (336).

In conclusion, RHI measured using PAT during pregnancy did not identify which women were at risk of going on to develop pre-eclampsia or PIH. I found that PAT and brachial artery ultrasound FMD, methods which rely upon flow-mediated dilatation to assess endothelial function, are less likely to be reliable when used in later pregnancy when women are more vasodilated. Women with a history of pre-eclampsia are at increased risk of future cardiovascular disease (7); further studies using these techniques may aid in explaining the mechanisms underlying this risk.

Chapter 6

A study of pulse wave analysis: assessment of a novel technique for the prediction of pre-eclampsia

6. Pulse wave analysis: assessment of a novel technique for the prediction of pre-eclampsia

6.1 Introduction

The importance of blood pressure measurement during pregnancy has been firmly established, but blood pressure alone is not sensitive enough when measured in early pregnancy to be of value in the prediction of pre-eclampsia. Current medical practice relies upon the measurement of peripheral systolic and diastolic blood pressure; it may be that analysis of other components of the pulse wave and estimation of central pressures can provide further understanding of cardiac and vascular physiology. Pulse wave analysis (PWA) is one such technique, and can be used to estimate central pressures and augmentation index (AIx), an estimate of arterial stiffness. Pulse wave analysis with applanation tonometry has been used extensively in non-pregnant populations to demonstrate arterial stiffness in patients with hypertension, diabetes mellitus and hyperlipidaemia (106). Further, increased AIx using PWA has been shown to be a strong independent risk factor for premature cardiac disease (99).

Normal pregnancy is associated with a number of important changes to the maternal vasculature, including a rise in heart rate, intravascular volume and cardiac output, and a corresponding reduction in vascular resistance. Studies using PWA to examine maternal wave reflections and arterial stiffness in pregnancy have reported lower central systolic and diastolic blood pressures, and lower AIx compared to the non-pregnant state (106,107). These changes are evident from the first trimester, reach their nadir in mid-pregnancy, and rise to pre-pregnancy levels in the third trimester.

Pre-eclampsia is thought to be characterised by an aberrant maternal cardiovascular adaptation to pregnancy (337), and a small number of studies have used applanation tonometry to examine arterial stiffness in women with pre-eclampsia. Compared to women with normotensive pregnancies, women

with pre-eclampsia have been reported to have significantly elevated AIx, indicating arterial stiffness, when examined at the time of diagnosis of pre-eclampsia (91,109,110,338). Results, however, have not been consistent, with one more recent study demonstrating no significant difference in AIx between women with pre-eclampsia and those with normotensive pregnancies (337).

In keeping with many other methods for the prediction of pre-eclampsia, in order to be clinically useful the alterations in pulse wave characteristics would have to be evident prior to the onset of clinically detectable disease. Further, to justify the increased expense, training and time associated with the studies, they would have to supply additional information on top of the maternal risk factor profile. One longitudinal study has been reported, using applanation tonometry to examine 210 pregnant women at 11-14 weeks gestation. The authors reported increased augmentation pressure (AP) and AIx in women who subsequently developed pre-eclampsia compared to those who had normotensive pregnancies, and that both AP and AIx corrected for heart rate (AIx 75) were significantly negatively correlated with birth weight and with gestation at delivery (95).

In this longitudinal prospective study, PWA was used to examine characteristics of the pulse waveform in a cohort of pregnant women both during and after pregnancy. These women had been selected from the overall study cohort because of the presence of ≥ 2 traditional risk factors for pre-eclampsia. Further, to examine the effects of pregnancy itself, characteristics of the pulse waveform of pregnant women were compared with non-pregnant women.

6.2 Methods

6.2.1 Recruitment

Pregnant women were recruited at their initial antenatal hospital appointment, as described in Chapter 2. Women with 2 or more traditional risk factors for pre-eclampsia (12) were invited for vascular function studies at gestational weeks 16, 28 and at 6-9 months post-natally. Women with chronic hypertension,

diabetes, and renal disease were not included in the study. A total of 180 pregnant women were studied at gestational week 16 and 28; 17 (9.4%) developed pre-eclampsia, 7 (3.9%) developed pregnancy induced hypertension without significant documented proteinuria (PIH) and 156 women had normotensive pregnancies. Characteristics of the women studied longitudinally throughout pregnancy are shown in table 6.1.

	Pre-eclampsia or PIH (n=24)	Normotensive (n=156)	p-value
Age (years)	30.7±5.1	33.1±5.2	0.03
BMI at booking(kg/m^2)	30.3±5.8	27.8±5.6	0.04
SBP at booking (mmHg)	126±10	120±12.6	0.06
DBP at booking (mmHg)	79±7	74±9.3	0.007
Smoking (%)	1 (4%)	12 (7.6%)	1.0
Nulliparous (%)	20 (83%)	67 (43%)	<0.001
Ethnicity (% non-Caucasian)	2 (8.3%)	9 (5.8%)	0.64
Pre-eclampsia in previous pregnancy (%)	2 (8.3%)	33 (21%)	0.17
Family history of pre-eclampsia (%)	9 (37.5%)	21 (13.5%)	0.007
Gestation at delivery (wks)	39.3±2	39.9±1.6	0.098
Birthweight (grams)	3295±520	3601±509	0.007

Table 6-1. Demographics of women studied at gestational week 16 and 28. Data are expressed as mean ± standard deviation, or as number (% of total).

Of the women who attended at weeks 16 and 28, 81 attended at 6-9 months post-natally, of whom 8 had developed pre-eclampsia, 4 had developed pregnancy-induced hypertension without proteinuria, and 69 had remained normotensive. A further 15 women, who had been recruited to the original study at booking and had developed pre-eclampsia, but who had not attended for vascular function studies during pregnancy, were also seen at 6-9 months post-natally. Demographics of women studied post-natally are shown in table 6.2. 30 health nulliparous non-pregnant women, recruited using advertising posters in the University of Glasgow, were examined using the same protocols.

	Pre-eclampsia or PIH (n=27)	Normotensive (n=69)	p-value
Age (yrs)	31.2±4.5	35.6±4.9	<0.001
BMI (kg/m^2)	28.8±7.4	27.5±5.5	0.346
SBP (mmHg)	127±13	124±13	0.385
DBP (mmHg)	83±10	78±11	0.074
Smoking (%)	1 (3.7%)	6 (8.7%)	0.67
Ethnicity (% non-Caucasian)	1 (3.7%)	4 (5.8%)	1.0
Family history of pre-eclampsia (%)	8 (29.6%)	9 (13%)	0.074

Table 6-2 Demographics of women studied post-natally. Data are expressed as mean ± standard deviation, or as number (% of total).

Pre-eclampsia definition was in keeping with the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (5); systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg on 2 occasions >6 hours apart, in association with proteinuria (≥ 300 mg/24 hrs, protein : creatinine ratio ≥ 30 mg/mmol, or if neither were available, ++ or more on dipstick measurement). Pregnancy-induced hypertension (PIH) was defined using the same blood pressure criteria, but without significant documented proteinuria. Delivery information was obtained from labour ward databases; casenotes for all women who developed pre-eclampsia or gestational hypertension were reviewed by myself. All women gave written informed consent, and the study was approved by the West Glasgow Research Ethics Committee.

6.2.2 Pulse wave analysis

Pulse wave analysis was performed using the SphygmoCor device (Atcor medical, West Ryde, Australia), as described in Chapter 2. In brief the radial artery was gently compressed with the tip of a pencil-shaped tonometer at the site of maximal pulsation. The tonometer contains a micro-manometer which provides an accurate recording of the pressure within the radial artery. The aortic pressure waveform was derived from the peripheral waveform using a validated

generalised transfer function. The augmentation index (AIx), a composite measure of systemic arterial stiffness and wave reflection amplitude, central systolic, diastolic and pulse pressures, and augmentation pressure (AP) were calculated using the integrated software. Since AIx is influenced by heart rate (109), AIx was standardised to a heart rate of 75 beats per minute (AIx-75). Waveforms over an 8 second period were recorded, and the SphygmoCor software created an average arterial waveform which was used for the analysis. The system has an internal quality control index, and readings were discarded if amplitude was too low or if variability between waveforms was too high. All studies were performed by myself until three satisfactory waveforms were produced; mean values were used in the subsequent analysis.

6.3 Results

6.3.1 Comparison between non-pregnant women and pregnant women at gestational week 16

Non-pregnant controls were younger, and had a lower BMI than pregnant women at gestational week 16 (table 6.3). In keeping with the vascular changes expected in pregnancy, pregnant women had a higher resting heart rate than non-pregnant controls (77±9 bpm vs 66±12 bpm, $p<0.001$). Brachial systolic and diastolic blood pressure was not different between the 2 groups. Peripheral and central AIx, AP and central systolic and diastolic pressures were not significantly different between pregnant women at gestational week 16 and non-pregnant women.

	Non-pregnant (n=30)	Gestational week 16 (n=180)	p-value
Age (years)	28.3±5.9	32.6±5.3	<0.001
BMI (kg/m^2	23.9±3.5	28±5.6	<0.001
SBP (mmHg)	120±10	121±12	0.542
DBP (mmHg)	77±7	75±10	0.284
Heart rate (bpm)	66±12	77±9	<0.001
Peripheral AIx (%)	55.9±11.8	52.9±15	0.305
Central SBP (mmHg)	105±9	106±13	0.722
Central DBP (mmHg)	78±8	76±9	0.394
Central PP (mmHg)	27.7±5.4	30.1±8.9	0.154
Central AP	2.38±3.1	2.25±3.8	0.863
Central AIx (%)	8.3±10	6.7±12	0.463
Central AIx-75 (%)	4.5±12	7.8±12	0.159

Table 6-3 Comparison of PWA characteristics between non-pregnant controls and pregnant women at gestational week 16.

6.3.2 Comparison between Sphygmocor and EndoPAT- derived augmentation index

There was a significant correlation between AIx derived from EndoPAT (as described in Chapter 5) and AIx derived from Sphygmocor, both at gestational week 16 (r=0.523, p<0.001) and week 28 (r=0.629, p<0.001). Bland Altman plots demonstrating the relationship between the 2 methods of assessment of augmentation index are shown in figure 6.1.

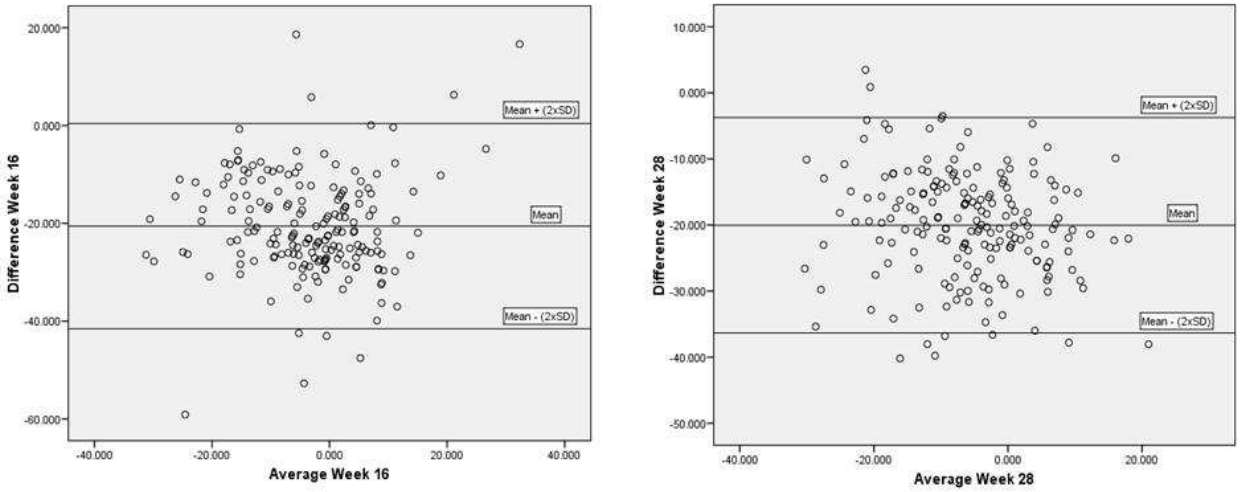

Figure 6.1. Bland Altman plots demonstrating the relationship between augmentation index as measured by EndoPAT and SphygmoCor at gestational week 16 (left) and 28 (right).

6.3.3 Correlation of PWA parameters with maternal risk factors

At gestational week 16, AIx-75 was significantly correlated with both brachial systolic (r=0.226, p=0.002) and diastolic (r=0.373, p<0.001) blood pressures, maternal age (r=0.366, p<0.001) and maternal BMI (r=0.169, p=0.021) (figures 6.2, 6.3). AIx-75 was not related to smoking status (8.4±12.6 smokers vs 7.4±11.5 non-smokers, p=0.8) or ethnicity (8.9±9.2 non-Caucasian vs 7.8±11.8 Caucasian, p=0.76), but was lower in nulliparous women (3.77±11.2 nulliparous vs 11.88±10.8 parous, p<0.001), reflecting their younger age.

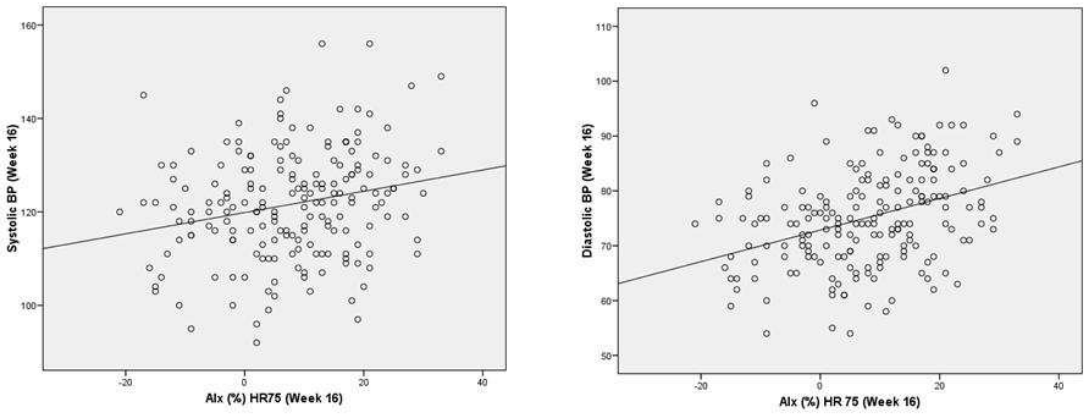

Figure 6.2 Scatterplots demonstrating relationship between AIx-75 and systolic blood pressure (left) and diastolic blood pressure (right).

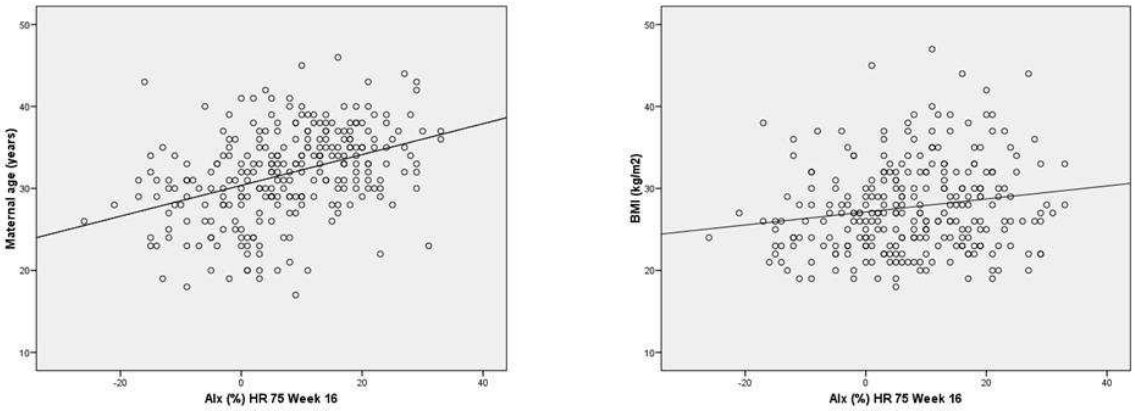

Figure 6.3 Scatterplots demonstrating relationship between AIx-75 and maternal age (left) and body mass index (right).

At gestational week 16, women with a history of pre-eclampsia in a previous pregnancy had a higher AP (3.5±2.7 vs 1.9±3.9, p=0.008), PAIx (59.5±13 vs 51.4±15, p=0.003) and central AIx 75 (11.3±11.1 vs 7.0±11.7, p=0.046) compared to those without a history of the condition. Systolic blood pressure was not different, but diastolic blood pressure was higher (78±10 vs 74±9, p=0.03) in women with a history of previous pre-eclampsia.

At gestational week 28, women with a history of previous pre-eclampsia again had a higher AP (2.94±3.4 vs 0.96±3.6, p=0.003), PAIx (59.0±16 vs 47.8±17, p<0.001) and central AIx 75 (13.8±11 vs 6.9±11.6, p=0.001). These findings were independent of maternal age, maternal BMI and maternal systolic and diastolic blood pressure, which were not different between the two groups.

6.3.4 Comparison of women who developed pre-eclampsia or PIH and controls

6.3.4.1 Overall study cohort

As expected, heart rate rose from week 16 to 28 in both cases and controls (table 6.4). At gestational week 16, women who went on to develop pre-eclampsia or PIH had a higher brachial DBP than those who remained normotensive (table 6.4). Central DBP was also higher in cases at week 16, but there was no difference in central SBP, central pulse pressure, augmentation pressure, nor in peripheral or central AIx between cases and controls.

At gestational week 28, brachial systolic and diastolic pressures were significantly higher in women who went on to develop pre-eclampsia or PIH, but there was no difference in central systolic, diastolic or pulse pressure, augmentation pressure, or peripheral or central augmentation index between cases and controls.

When the longitudinal changes from gestational week 16 to 28 were compared between cases and controls, there was a significant difference in systolic blood pressure change, (p=0.02), but not in any determinants of pulse wave analysis (table 6.4).

	Week 16			Week 28		
	Cases (n=24)	Controls (n=156)	p-value	Cases (n=24)	Controls (n=156)	p-value
Brachial SBP (mmHg)	126±10	120±13	0.061	131±10 †	120±12	<0.001
Brachial DBP (mmHg)	79±7	74±10	0.007	80±7.4	72±8.5	<0.001
Heart rate (bpm)	79±8	77±9	0.492	82±21	84±12	0.546
Aortic SBP (mmHg)	110±9	106±13	0.119	108±25	103±15	0.178
Aortic DBP (mmHg)	81±7	75±9	0.007	79±18	74±11	0.052
Aortic PP (mmHg)	29±6	30.1±9	0.588	28.8±8.5	29.1±9.7	0.901
Augmentation Pressure	2.65±3.1	2.25±3.9	0.639	1.45±3.2	1.37±3.8	0.853
Peripheral AIx (%)	54.7±13	52.8±15	0.568	49.7±17	49.8±17	0.987
Central AIx (%)	8.17±10	6.56±12	0.544	3.26±9.7	3.81±12.1	0.837
Central AIx-75 (%)	10.0±9.2	7.7±12.1	0.390	9.14±9.3	8.35±12	0.936

Table 6-4. Comparison of PWA parameters between cases and controls at gestational week 16 and 28. † represents a significant difference between cases and controls in longitudinal changes from week 16 to 28.

6.3.4.2 Women studied at week 16, 28 and post-natally

Of the women who were studied both during and after pregnancy, 12 developed pre-eclampsia or PIH, and 69 remained normotensive throughout. There was no significant difference in PAIx, CAP or CAIx-75 between week 16 and week 28 in either cases or controls. When women were examined post-natally, however, compared to week 28 there was a significant elevation in AP (5.67±3.1 vs 1.83±3.8, p=0.001 cases , 6.82±4.3 vs 1.44±3.4, p<0.001 controls), in PAIx (66.6±9 vs 53.8±14.6, p=0.003 cases, 71.7±15 vs 52.6±14.9, p<0.001 controls) and in AIx-75 (14.3±10.5 vs 8.8±11.3, p=0.043 cases, 16.95±10.7 vs 9.2±12.2, p<0.001 controls), figure 6.4.

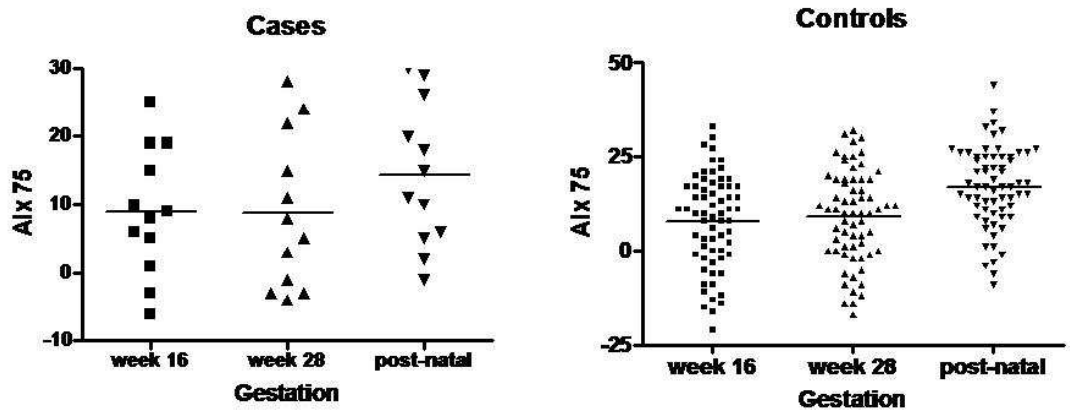

Figure 6.4 Comparison of augmentation index corrected for heart rate (AIx-75) in cases (left) and controls (right) at week 16, week 28 and post-natally.

6.3.5 Correlation with birth outcomes

Women who developed pre-eclampsia or PIH delivered babies of smaller birthweight with a higher rate of operative delivery than those who remained normotensive (table 6.1). It has previously been reported that both augmentation index and augmentation pressure correlate with birthweight and gestation at delivery (95). In this cohort there was a significant negative correlation between AIx 75 at week 16 and gestation at delivery (P=-0.163,

p=0.033), with higher augmentation index in women who delivered at an earlier gestation. There was, however, no relationship between any of the other parameters of PWA and either birthweight or gestation at delivery, when recorded at either week 16 or week 28.

6.3.6 Post-natal data

27 women who had developed pre-eclampsia or PIH were then compared with 69 women who had normotensive deliveries at 6-9 months post-natally. Women who had developed pre-eclampsia or PIH were younger, with a trend towards a higher post-natal diastolic blood pressure than those who had normotensive deliveries (table 6.5). There was no difference between the 2 groups in central systolic diastolic pressure, AP, or peripheral or central augmentation index.

	Pre-eclampsia or PIH (n=27)	Normotensive (n=69)	p-value
Age (yrs)	31.2±4.5	35.6±4.9	<0.001
BMI (kg/m²)	28.8±7.4	27.5±5.5	0.35
SBP (mmHg)	127±13	124±13	0.39
DBP (mmHg)	83±10	78±11	0.07
Central SBP (mmHg)	114±12	112±13	0.46
Central DBP (mmHg)	83±8.7	79±10.3	0.07
Central PP (mmHg)	31.1±8.3	33.2±7.6	0.26
Augmentation Pressure	5.7±3.6	6.8±4.2	0.24
Peripheral AIx (%)	67.9±12.6	71.7±14.9	0.25
Central AIx (%)	18.1±8.7	19.7±10.5	0.47
Central AIx (%) HR 75	15.4±8.7	16.9±10.5	0.50

Table 6-5 Comparison of PWA parameters at 6-9 months post-natally between women who had pre-eclampsia or PIH (cases) and women who had normotensive pregnancies (controls).

6.4 Discussion

Understanding the relationship between arterial stiffness and pre-eclampsia may help to improve our understanding of the pathogenesis of this important condition. In this study, pulse wave analysis was examined longitudinally throughout pregnancy, at 6-9 months post-natally, and in healthy non-pregnant controls. Pregnant women were pre-selected on the basis of risk factors for pre-eclampsia because any technique used to predict pre-eclampsia in early pregnancy would have to be capable of differentiation in such a "high-risk" population group. Further, pre-selecting women on the basis of risk factors is perhaps more realistic for clinical practice, since it would not be feasible logistically or financially to carry out testing such as PWA in all pregnant women.

Previous studies have reported reduced augmentation pressure and augmentation index throughout normal pregnancy (106-108), with changes evident from the first trimester. In this study, there were no differences in any of the components of pulse wave analysis when comparing pregnant women at gestational week 16 with non-pregnant controls. This discrepancy is likely to be in part because the non-pregnant women in this study were significantly younger and had a lower BMI - even allowing for the expected weight gain during pregnancy (339)- than the pregnant women. A further potential factor affecting pulse wave characteristics is the phase of the menstrual cycle. This was not recorded in the non-pregnant controls nor the women studied post-natally; it has been previously reported that the menstrual cycle has a significant effect on pulse wave analysis, with lower AIx in the luteal phase (105). When women who had been examined during pregnancy were examined again post-natally, augmentation pressure and both peripheral and central AIx were higher than during pregnancy.

It has long been generally accepted that blood pressure falls in normal pregnancy, beginning from 12-14 weeks, reaching a trough at around 24 weeks, and rising in the third trimester. These findings, based on large observational studies dating back to the 1960s (340) have been the basis for clinical guidelines

and for classification of hypertensive disorders (341), and as reported in Chapter 1 have led to the theory that a lack of “drop” in mid-trimester blood pressure is an independent risk factor for pre-eclampsia. In this study there was no significant alteration in blood pressure between gestational week 16 and week 28 in either cases or controls, other than a rise in systolic blood pressure in the women who went on to develop pre-eclampsia. In keeping with this observation, a recent study performed in pregnant Caucasian women with singleton uncomplicated pregnancies in fact reported a significant rise in both systolic and diastolic blood pressures during pregnancy, with no mid-trimester dip (342). The authors speculated that these findings may have been because women in the Western world are generally more obese than in the past, and are having children at a later age than previously. It may be that “normal” ranges for blood pressure during pregnancy need to be revised, and this area merits further investigation.

A further aim of this study was to examine the ability of pulse wave analysis to predict pre-eclampsia in early pregnancy. Khalil et al. (95) examined pregnant women at gestational week 11-14, and using applanation tonometry, demonstrated elevated augmentation pressure and augmentation index at this early stage in women who went on to develop pre-eclampsia. In the current study, at week 16, women who went on to develop pre-eclampsia or PIH already had a higher, but within normal limits, brachial blood pressure compared to those who had normotensive pregnancies. In contrast to the findings of Khalil et al., there was no difference in AP or AIx between cases and controls at this early time-point. Further studies at gestational week 28 again did not differentiate women destined to develop pre-eclampsia or gestational hypertension from controls. The reason for this difference is not clear; the study of Khalil et al. had a particularly high incidence of pre-eclampsia (6.7% compared to 2.3% in the overall PIP study population) which in turn was felt by the authors to be related to the high Afro-Caribbean population studied. Previous studies which demonstrated elevated AIx in women with pre-eclampsia were not fully adjusted for maternal age, BMI and brachial blood pressure (337), all factors which I have here shown to have a significant effect on AIx, and which may contribute to the discrepancy seen.

Women with pre-eclampsia are at increased risk of future development of cardiovascular disease (252). Since endothelial dysfunction has been reported in women after a pre-eclamptic pregnancy, and since arterial stiffness is associated with cardiovascular disease, it seems plausible that increased arterial stiffness would be seen in women after a pre-eclamptic pregnancy. In keeping with this theory, AIx at 7 weeks post-partum has been reported to be elevated in women with pre-eclamptic pregnancies, despite a normalisation in blood pressure (105). Whether the differences in AIx are longer lasting remains uncertain; one study in which women were examined at a mean of 5 years post delivery did not report any difference in any measurement of pulse wave analysis between women who had pre-eclampsia and those who had normotensive pregnancies (109). In the current study, in which women were examined at 6-9 months after delivery, there was similarly no difference between affected and unaffected women.

One potential limitation of this technique in the assessment of pregnant women is the use of a generalised transfer factor to calculate central pressures. Although the transfer factor has been validated in a wide range of different patient groups, ages, diseases and drugs, it has not been specifically validated for use in pregnancy. Further studies, examining normal values for different stages in pregnancy, and assessing the applicability of the generalised transfer factor during pregnancy are indicated. A further potential limitation of the current study is that pulse wave velocity (PWV) was not examined. Further information about arterial wave reflection can be gained from examining aortic (carotid to femoral) and brachial (carotid to brachial) pulse wave velocity, and this technique has previously been used to demonstrate increased pulse wave velocity in women with established pre-eclampsia compared to controls (337). Studying PWV is, however, more time consuming, more invasive, and requires further training and expertise than PWA alone, and so may not be applicable in large scale studies for screening for pregnancy complications.

As in Chapter 5, in this PWA study both women with pre-eclampsia and women with PIH without significant proteinuria were included together in the analysis. When women with PIH alone were excluded from the analysis results were not affected.

In summary, in this study of women with risk factors for pre-eclampsia, pulse wave analysis did not provide additional information beyond brachial blood pressure and maternal risk factor profile about risk of future development of pre-eclampsia, whether analysed at gestational week 16 or 28. There was no significant difference in blood pressure or in pulse wave analysis characteristics between week 16 and week 28. In contrast to other literature in the field, significant differences were not seen between pregnant women at gestational week 16 and non-pregnant controls, nor was there any difference post-natally between cases and controls.

Although PWA did not help to differentiate between cases and controls during pregnancy, it is uncertain whether it could be used to aid the early detection of severe disease, early onset disease, or adverse foetal or maternal outcomes. Further longitudinal studies examining its role in predicting long-term cardiovascular disease in affected women are also merited.

Chapter 7

General discussion and conclusions

7. General discussion & conclusions

7.1 Introduction

It is clear that rather than just being proteinuric gestational hypertension, pre-eclampsia is a complex multi-system disease characterised by exaggerated systemic inflammation and endothelial dysfunction. The condition remains a challenge for doctors, midwives, expectant mothers and scientists alike. The only known cure for pre-eclampsia is delivery; in women who develop pre-eclampsia before term the only options available to obstetricians are supportive and temporising measures until delivery is safe (336). The maternal and foetal short and long-term outcomes for such management, however, remain uncertain, with few evidence-based guidelines available. It is evident, therefore, that any method which could improve early detection of the disease, and which could help to predict adverse foetal and maternal outcomes, would have the potential to be of enormous clinical benefit.

The overall aim of the work described in this thesis was to attempt to identify clinical and biochemical markers that could lead to an improvement in our ability to predict pre-eclampsia in early pregnancy. To do this I designed and set up a large prospective study, in which women were recruited in the first trimester, and were followed longitudinally throughout their pregnancy. To attempt to examine what happens to these markers throughout pregnancy, I recruited a cohort of women with established risk factors such as nulliparity, obesity and a family history of pre-eclampsia, to carry out further sampling and vascular function studies throughout their pregnancy. Finally, since women with a history of pre-eclampsia are known to be at increased risk of future cardiovascular disease, I examined these women after their pregnancy to determine whether they had detectable vascular dysfunction that might contribute to their increased long-term cardiovascular risk.

7.1.1 Proteomic studies

Given the multi-factorial nature of the disease, and the key role that the kidney plays in its pathogenesis, urinary proteomics was felt to represent a promising area for identification of novel predictive biomarkers. As outlined in Chapter 3, capillary electrophoresis / mass spectrometry techniques were used to successfully define, for the first time, the human urinary proteome in pregnancy. In addition, this work led to the identification of a pattern of peptides that were altered at gestational week 28 in women who subsequently developed pre-eclampsia. It was disappointing not to be able to identify a proteome pattern that could be used to predict the condition at an earlier point in pregnancy, since such a "late" biomarker may be of limited benefit in clinical practice. I was able to demonstrate, however, that the pre-eclampsia related peptides identified at week 28 were not present at an earlier point in pregnancy. Further, I demonstrated that their levels are dynamic, with a clear elevation in their urinary concentration between week 12-16 and week 28 in affected women, and no real difference in women who were unaffected. These dynamic changes suggest that such peptide patterns do not exist, or at least cannot be detected using these methods at earlier stages in pregnancy.

The potential clinical relevance of the predictive biomarker pattern at week 28 should not be overestimated, since these data have yet to be validated in further cohorts of pregnant women. Proteomic analysis was only carried out in samples from the first 2500 women recruited to the PIP study; it may be possible to validate findings in the subsequent 1500 women in the study. Further studies using independent study cohorts, and complementary techniques such as metabolomics (248), and transcriptomics (343), may help with future research in this area.

7.1.2 Plasma biomarkers

A second main focus of this study was to attempt to identify plasma biomarkers that could improve early prediction of pre-eclampsia. Much of the work performed in this field in recent years has focussed on anti-angiogenic peptides, in particular soluble FMS-like tyrosine kinase 1 (sFLT-1) and soluble Endoglin

(sENG), and the role that they play in disease development. I was able to demonstrate that at booking, women who went on to develop pre-eclampsia already had altered circulating concentrations of the anti-angiogenic peptide sENG, and PGF, the factor that it inhibits. Circulating concentrations of sFLT-1, the peptide most studied in the recent literature, were not different between cases and controls at booking, but were significantly higher at week 28 in affected women. One advantage of this study is that, in keeping with the real life situation, women were sampled at their initial antenatal hospital visit which was at any point between 10 and 18 weeks of gestation, rather than at a fixed gestational time-point as in the majority of other studies. Despite this, the alterations in concentrations remained consistent, and may further help to identify at-risk women.

Inflammation, arising from both the developing placenta and the maternal vasculature, is known to play a key role in the pathogenesis of pre-eclampsia. In view of this I also used early pregnancy plasma samples to examine a panel of adhesion molecules and inflammatory markers, to determine whether any could be utilised as pre-eclampsia biomarkers, in order to improve early prediction. Of the inflammatory biomarkers studied, I was able to show that E-Selectin levels were significantly elevated at booking in women who went on to develop pre-eclampsia. To ensure that this was not a chance finding related to multiple testing, I also showed that circulating E-Selectin levels were elevated in affected women both at week 16 and at week 28. When tested in an independent study cohort, levels were again shown to be elevated at a gestational time-point well before the onset of clinically detectable disease. E-Selectin is released exclusively from endothelial cells, and since endothelial dysfunction is known to be an important step in disease development, this work suggests a novel role for E-Selectin in pre-eclampsia pathogenesis.

7.1.3 Vascular function studies

A further focus of the PIP study was to examine the ability of non-invasive vascular function phenotyping to predict future pre-eclampsia. Using peripheral arterial tonometry (PAT) at gestational weeks 16 and 28, I was unable to detect

any difference in endothelial function between women who would go on to develop pre-eclampsia and those who would have normotensive pregnancies. I was able to show that PAT score was negatively correlated with fingertip blood flow during baseline recordings, which suggests that PAT and other similar techniques may be of limited utility in pregnancy, when women are more vasodilated. I also used pulse wave analysis to examine augmentation index, a marker of arterial stiffness as a predictor of future pre-eclampsia. Although a previous study had shown increased augmentation index in early pregnancy in women who went on to develop pre-eclampsia (95), this study had been performed in a cohort of women with particularly high rates of pre-eclampsia. In the PIP cohort, with a disease incidence of around 2.3%, I was able to demonstrate that there was no significant difference between affected and unaffected pregnancies, suggesting that this is unlikely to be a useful test for pre-eclampsia detection in the first and second trimesters.

7.2 Diagnostic criteria discussion

7.2.1 Blood pressure thresholds

A recurring theme throughout this thesis is that definitions of pre-eclampsia have varied throughout the years and continue to be different in different studies and in different parts of the world. Historically, emphasis was placed upon diastolic blood pressure rather than systolic, and so was over-represented in classifications of hypertension both during and outwith pregnancy. The initial international classification and definition of the hypertensive disorders of pregnancy compiled by Davey et al. in 1988 (344) reflected this, requiring a diastolic blood pressure of 90 mmHg on two occasions, or a single diastolic blood pressure of 110 mmHg to make the diagnosis. Further complicating the classification of hypertensive disorders in pregnancy is the fact that pregnant women are mainly looked after by obstetricians rather than general physicians, whose classifications of hypertension can often be different. “Normal” blood pressure levels vary significantly at different time-points throughout pregnancy, causing further uncertainty. Some pre-eclampsia classifications have therefore

suggested that changes in blood pressure levels between the first and third trimesters, with or without proteinuria, rather than absolute blood pressure levels are more important for determining maternal and foetal outcomes (345).

7.2.2 Proteinuria thresholds

The level of proteinuria required to define pre-eclampsia is another area which has differed in different classifications of the disease. Traditional diagnostic criteria relied upon 24 hour urinary collections, which were well adhered to since the majority of affected women were in-patients in hospital. In the out-patient or community setting where most women are now looked after, however, compliance with 24 hour urinary collection is poor. The International Society for Study of Hypertension in Pregnancy (ISSHP) guidelines require a protein : creatinine ratio of ≥30 mg/mmol or a Dipstick analysis of at least + proteinuria, in the absence of infection. Some classifications, however, do not require any proteinuria for diagnosing pre-eclampsia. The SCOPE study, an ongoing international prospective study which is outlined in Chapter 3 uses the Australasian criteria which consist of pregnancy-induced hypertension (as defined above), plus a multisystem complication, which can be significant proteinuria, or renal insufficiency, liver test abnormalities, neurological complications or haematological complications.

The presence of proteinuria, however, does appear to be significant in terms of foetal and maternal outcomes. One recent Australasian study compared outcomes in proteinuric and non-proteinuric pre-eclampsia over an 18 year period (6). They reported that proteinuric pre-eclampsia was associated with a higher incidence of perinatal mortality, preterm delivery and severe hypertension than non-proteinuric pre-eclampsia. There are a number of women who develop gestational hypertension with liver failure, renal failure or foetal growth restriction, but do not have significant proteinuria. What diagnosis should be given to these women, and how they should be classified, remains uncertain.

7.2.3 Disease severity thresholds

How to define severe pre-eclampsia is a further area in which classifications vary worldwide. Early onset disease is often considered to be a criterion for severe disease, which is in turn defined as disease detected at <34 weeks' gestation in Canada and <35 weeks in the USA (8). Other features which appear in diagnostic classifications for disease severity include heavy proteinuria (3-5 grams per day or >1g / litre), signs of maternal end-organ dysfunction, abnormal maternal laboratory testing (including HELLP syndrome), elevated uric acid levels, and foetal morbidity or mortality. Definitions of severe hypertension also differ, with systolic blood pressure over 160 mmHg in UK and Australasian classifications, over 170 mmHg in American and Canadian studies, and diastolic blood pressures over 110 mmHg being defined as severe.

One of the reasons for the lack of agreement is that none of the classification systems have been independently assessed for their ability to identify which women and foetuses are at highest risk of adverse outcomes. One recent study was designed to do this, and described the development and validation of an outcome and prediction model fullPIERS (Pre-eclampsia Integrated Estimate of RiSk) (336). In this study, a number of variables assessed at the time of admission to hospital with pre-eclampsia were examined for their ability to predict fatal or life-threatening maternal outcomes. In a collaborative international study over 2000 women with pre-eclampsia were included, of whom 13% developed associated adverse maternal or foetal outcomes. Of the variables assessed, early gestational age at diagnosis, the presence of hypoxia, chest pain or dyspnoea, and elevations in serum creatinine and liver enzymes were all associated with adverse outcomes, both within 48 hours of admission, and within 7 days of admission. Interestingly, blood pressure levels themselves did not appear to predict adverse maternal or foetal outcomes.

7.2.4 Treatment thresholds

The management of pre-eclampsia revolves around close observation of affected women, delivery of the foetus when possible, and therapeutic strategies to attempt to reduce foetal and maternal complications. Which women should be

treated with blood pressure lowering medications, and how they should be treated also remains uncertain. Management guidelines tend to be based on practice patterns which have been established over the years, rather than clinical trials with clearly-defined end-points. There are many systematic reviews of the subject, but as discussed above the different diagnostic criteria, different methods for measuring blood pressure, and the presence or absence of underlying conditions such as chronic hypertension limit their usefulness.

Since prescription of anti-hypertensive therapies do not appear to affect the underlying disease process (which is initiated in the placenta), and since blood pressure levels do not appear to influence foetal or maternal outcomes (336), the main indication for treating high blood pressure in pregnancy is to help to prevent stroke. A systolic blood pressure above 160 mmHg is associated with a significantly increased risk of cerebral haemorrhage (346), and as a result many clinicians advocate treatment above this level. In a recent Confidential Enquiry into Maternal and Child Health (CEMACH) report, intracranial haemorrhage accounted for 12 of the 18 deaths associated with pre-eclampsia (42). Reflecting the inconsistency in treatment throughout the UK, the Vitamins in Pregnancy (VIP) trial of high risk women described in section 1.2.4 reported that a third of women with recorded sustained systolic hypertension >160 mmHg had received no anti-hypertensive treatment (42).

Although most would advocate treatment in women with associated symptoms such as cardiac or neurological decompensation, it is unclear whether women with “moderate” hypertension or those with isolated diastolic hypertension should be treated. To further explore this, a Cochrane systematic review examined the use of anti-hypertensive therapies in women with mild-moderate hypertension in pregnancy, which was in turn defined as systolic blood pressure of 140-169 mmHg or diastolic blood pressure of 90 to 109 mmHg, with or without significant proteinuria (347). Examining 46 trials of over 4200 affected women, the authors reported that use of antihypertensive therapy was associated with a significant reduction in development of severe hypertension, (RR 0.5, 95% CI 0.41 to 0.61), but that there was no significant effect on rates of development of pre-eclampsia. One of the concerns of treating women with mild-moderate hypertension is the potential to compromise foetal wellbeing, since lowering systemic blood pressure could in theory reduce placental blood flow. The

Cochrane meta-analysis did not reveal any effect on foetal or neonatal death rates, nor on rates of small for gestational age (SGA) babies, or preterm deliveries. The target to which blood pressure should be lowered, and how rapidly the blood pressure should be reduced remains uncertain.

7.3 Disease pathogenesis

As outlined in Section 1.1 there are two main phases in the development of pre-eclampsia: the “placental” phase characterised by impaired placental development, placental ischaemia and oxidative stress, and the “maternal” phase characterised by maternal endothelial dysfunction and inflammation. Factors released by the placenta, including the anti-angiogenic peptides and syncytiotrophoblastic debris, appear to provide a link between the two main phases.

The strength of association between the 2 main stages of pre-eclampsia, however, may be different between different individuals. Whether pre-eclampsia is of early or late onset appears in turn to depend upon whether or not the placenta is small and under-developed (8). Although thought to be crucial to the development of pre-eclampsia, not all women with impaired placental development will develop hypertensive problems. Similarly, women with normally developed placentas can develop pre-eclampsia. In these women it is likely that underlying maternal cardiovascular and metabolic factors contribute to set off a cascade of maternal and placental inflammation and oxidative stress, resulting in late-onset pre-eclampsia (8). In this study, largely because of the numbers involved, I included all women with pre-eclampsia in the analysis whether their disease was of early or late onset, or whether they had growth-restricted babies or not. It may in fact be the case that early onset (placental) and late onset (maternal) pre-eclampsia are 2 different conditions. Further research will determine whether or not methods for reducing the excessive inflammatory and oxidative stress (for example with statins or metformin) and improving endothelial function can improve foetal and maternal outcomes.

7.4 PIP study

Pre-eclampsia is said to affect between 3-8% of all pregnancies in the Western world, and around 5% of all first-time mothers. One of the surprising aspects of this study was that the incidence was lower, at 2.3% overall. A number of reasons may account for this discrepancy. First, as outlined in Section 1.1 the diagnostic criteria used in this study are more stringent than those used in some other studies, requiring sustained blood pressure recordings over 140 mmHg systolic and 90 mmHg diastolic on at least 2 occasions, with significant proteinuria, occurring after 20 weeks' gestation. Further, in this study only a small number of women with chronic hypertension, diabetes and renal disease were included, since they were usually already attending dedicated "high-risk" antenatal clinics. The main theme underlying the PIP study was to identify which women were at increased risk of going on to develop pre-eclampsia, with the implication being that these women would be more closely monitored throughout their pregnancy. Women with diabetes, pre-existing hypertension and renal disease are already known to be at high risk of pre-eclampsia; as a result they are already closely monitored throughout, and a marker identifying them as high-risk would not add to their management.

Another reason behind the relatively low incidence seen may be that the population of the study was largely Caucasian, with a low number of black women, who as discussed in Chapter 1 are at increased risk of the condition. Rates of ethnicity varied in this study: in the Southern General Hospital 13% of women were non-Caucasian (of whom the majority were of South Asian ethnicity) whilst in Ayrshire only 1 woman included in the study was non-Caucasian. Cigarette smoking was another potentially important factor; smokers are known to be at lower risk of developing pre-eclampsia compared to non-smokers (59), and it may be that the relatively high rates of smoking (albeit self-reported and potentially inaccurate) in this study were partially protective against the development of hypertensive problems.

Despite this, the incidence of pre-eclampsia was similar to that reported in the Glasgow Outcome APCR and Lipid (GOAL) study, described in Chapter 4. This study, carried out in the same city and with broadly similar patient demographics and disease definitions, reported an incidence of pre-eclampsia of 1.7%. This again highlights the importance of definitions of pre-eclampsia that are agreed internationally, allowing accurate comparison of different studies.

7.5 Future cardiovascular health

An emerging concept in pre-eclampsia, as outlined in Chapters 1, 5 and 6, is the associated risk of future maternal hypertension, ischaemic heart disease, stroke, diabetes and renal disease. The studies describing this long-term risk are, however, largely observational, employing linkage analysis of birth records with morbidity and mortality databases. The mechanisms determining the increased cardiovascular risk remain poorly understood. As part of this study I examined vascular function in women at 6-9 months after pregnancy. I was unable to detect a difference in endothelial function or arterial stiffness between women whose pregnancies had been complicated by pre-eclampsia, and those who had normotensive pregnancies. While the peripheral arterial tonometry (PAT) technique and its reliance on baseline pulse amplitude may help to explain this, it is of interest that other more established techniques for determining cardiovascular risk including carotid ultrasound measurement of intima media thickness (IMT) have also not been shown to be different between affected and unaffected women (274). There remain other unanswered questions. Nearly all the studies describing the relationship between pre-eclampsia and future cardiovascular disease have been performed in Northern Europe, in largely Caucasian populations. Pre-eclampsia is more common in other racial groups (31), and in particular in the developing world (4), and whether these women are also at increased risk of future cardiovascular disease is not yet known. It is also uncertain whether pregnancy induced hypertension without significant proteinuria is associated with the same risk of future cardiovascular disease.

The longer term implications of pre-eclampsia do not appear to be restricted to mothers; previous observational studies have reported elevated systolic and / or diastolic blood pressures in the offspring of affected women when examined in childhood and adolescence. A recent prospective study looked at this relationship in more detail, and examined the offspring of 205 women who had pre-eclampsia, 1118 who had pregnancy-induced hypertension and 5345 normotensive mothers at 9 years of age (348). Offspring of women with a history of pre-eclampsia or gestational hypertension had an increased SBP and DBP compared to offspring of normotensive women, a finding which was independent of obesity or other lifestyle factors. The association of pre-eclampsia with elevated offspring blood pressure was felt to be at least partly related to the association with low birth weight and preterm delivery.

7.5.1 Opportunities for intervention

Rather than being a discrete condition, pre-eclampsia appears to represent the extreme end of a maternal systemic response engendered by pregnancy itself (349) and has been described as the “metabolic syndrome” of pregnancy (23). As a risk factor for future cardiovascular disease it is largely ignored by clinicians - mainly because of poor understanding of the long-term implications, and because of poor communication between obstetric and primary care teams (350).

The importance of a history of pre-eclampsia has, however, been recognised by its inclusion alongside traditional risk factors such as smoking and family history in the 2011 American Heart Association guidelines for prevention of cardiovascular disease in women (351). Although the short-term cardiovascular risk for women with pre-eclampsia is low, it has been suggested that affected women should be closely monitored after pregnancy, starting as early as 12 months post partum (352). It is important that these women avoid obesity and smoking, and that lipids, blood pressure and glucose are monitored, with early intervention where indicated. For many women, pregnancy will be the only time they seek medical attention between childhood and retirement; it is important that clinicians make the most of the information about future health that can be gained at this time.

7.5.2 Recurrence in subsequent pregnancies

Many of the modern methods for risk-stratifying pregnant women rely on "secondary prevention" of pre-eclampsia: women who have previously had the disease are closely monitored throughout pregnancy. It is clear, however, that using a history of previous pre-eclampsia to identify high-risk women is inadequate. In the PIP study, 83% of the women who developed pre-eclampsia were nulliparous; of the 35 women with a history of pre-eclampsia in a previous pregnancy who were considered "high-risk" and invited for further vascular function studies, pre-eclampsia recurred in only 2 (5.7%). In this study, however, it was not possible to check case records from previous pregnancies for accuracy of diagnosis, and since the gestation at which pre-eclampsia had developed in previous pregnancies was not always available this information was not included in the overall analysis. The risk of recurrence of pre-eclampsia is known to be lower in women with pre-eclampsia who delivered at term compared to those who had required early delivery (353), so this information may have been relevant. Population database studies have reported recurrence rates of pre-eclampsia of around 15% (354) but again these do not include gestation at diagnosis, and rely on routinely collected delivery data which can often be inaccurate.

The Vitamins in Pre-eclampsia (VIP) study mentioned in section 1.2.4 recruited women who were at high-risk of developing pre-eclampsia, and as part of this separately examined 500 women who had developed pre-eclampsia (in the pregnancy immediately preceding the index pregnancy) and had required delivery before 37 completed weeks' gestation (355). In these women pre-eclampsia recurred in 23%; risk of recurrence was highest in those who had required delivery at less than 34 weeks' gestation, in non-Caucasian women, those with higher blood pressure at booking, and in those with detectable proteinuria at booking. Even in women who remained normotensive in the subsequent pregnancy, those with a history of previous pre-eclampsia had a higher risk of delivering smaller babies at an earlier gestation than those without a history of previous pre-eclampsia. The risks of recurrent pre-eclampsia are not

confined to pregnancy; women who have had pre-eclampsia in more than one pregnancy appear to be at higher cardiovascular risk, and have been shown to be at higher risk of future development of hypertension (356), and of end-stage renal disease (254) than those with single episodes.

7.6 Conclusions

The work described in this thesis reports on a number of studies designed to improve early prediction of pre-eclampsia. Despite the huge amount of research done in the field, it remains difficult to predict the condition early, and the vast majority of women are only diagnosed when all the manifestations of the condition are evident, by which time treatment options are limited.

In routine clinical practice we currently screen for several conditions in early pregnancy such as syphilis, HIV and Rhesus iso-immunisation that have a far lower incidence than pre-eclampsia. It could be argued that screening for hypertensive disorders should be given a higher priority, and is the single most important aspect of antenatal care (357). Why has our ability to predict pre-eclampsia not improved? As medical students we are taught that pre-eclampsia has one cause (pregnancy) and one cure (delivery) and it may be that some clinicians see pre-eclampsia prediction as a futile and pointless exercise.

The reports of confidential enquiries into maternal deaths over the years have consistently identified deficiencies in care related to pre-eclampsia: in 46% of maternal deaths and 65% of foetal deaths, different management could reasonably have expected to alter the outcome (357). Many of the problems relate to community based care, in particular inadequate checking of blood pressure and proteinuria, and inadequate communication with hospital-based staff. If the care of pregnant women is to continue to be moved into the community setting, it is clear that the identification of at-risk women will have to improve.

Although proteomics based, and other laboratory biomarkers are not yet sensitive or specific enough to accurately predict the condition, there are many maternal risk factors that can easily be assessed in early pregnancy. A predictive model based upon these risk factors was recently shown to be associated with an

area under the ROC curve of 0.71 for disease prediction (3), but in current clinical practice much of the information about risk factors is collected and documented but not acted upon.

Education of pregnant women also has to take a prominent role. Mothers and midwives often take a healthy pregnancy outcome for granted, and do not understand the potential for pregnancy related problems. Many pregnant women do not understand why they are having their blood pressure taken (357), while 80% of affected women have no idea of the “danger” signs associated with the condition (358). Further work in pre-eclampsia research must establish generalised guidelines for diagnosis and classification of severity, to improve early prediction and to allow the development of proper evidence based guidelines for treatment and prevention. Pre-eclampsia is a potentially devastating condition, and it is hoped that future research and resources will help to improve the care of affected women.

Appendix I; Main study invitation letter

BHF Glasgow Cardiovascular Research Centre
PIP Study Team

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Dear Madam,

We would like to tell you about a research project we are carrying out at Glasgow University. The aim of the research project is to find out more information about a condition called pre-eclampsia - in which pregnant women can develop high blood pressure. In particular we hope, as a result of the research project, to be able to predict easier which women are at risk of developing the condition.

We will be asking all pregnant women to donate samples of urine and blood that may be analysed later to help us with our research. These samples will be taken at your booking visit, as well as the routine blood and urine tests that the midwives take. Please take the time to read the enclosed Patient Information Leaflets and consent form, and if you have any further questions do not hesitate to contact us.

Best wishes,

PIP (Proteomics In Pre-eclampsia) Study Team

Appendix II; Main patient information sheet

Invitation

You are being invited to take part in a research study (Proteomics In Pre-eclampsia, PIP study.) Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Pre-eclampsia is a condition in which pregnant women can develop high blood pressure. It affects approximately 5% of all pregnancies, and in rare cases can cause problems for the mother or baby. Although it is a fairly common condition, doctors do not yet fully understand the causes of the condition. In this study we aim to do detailed analysis of the urine and blood of pregnant women, looking especially at the patterns of proteins (proteomics). It is hoped that this information will help us to work out which women are at risk of developing pre-eclampsia, and to find out more about the condition.

Why have I been chosen to take part?

All pregnant women who have decided to have their baby at the Queen Mothers Hospital, Southern General Hospital, and Princess Royal Maternity Hospital will be invited to take part in this study. The study will run for one year.

Do I have to take part?

No. It is up to you to decide whether or not to take part: participation is completely voluntary. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will be involved if I decide to take part?

At your booking visit the midwife will be taking samples of blood. If you decide to take part in the study we will take an extra sample of blood from you (about 1 teaspoonful), which will be stored, and may be analysed at a later date. You will also give a sample of urine to your midwife at your booking visit. We will take a small sample of this urine which will be stored in our laboratory, and we may use the sample at a later date to look at the proteins in the urine.
In some women we would like to carry out further tests on the blood vessels, and we may invite you to visit the clinical investigations unit at the BHF Glasgow Cardiovascular Research Centre for these tests. These tests are simply a research tool, and do not give us any further information about your pregnancy. They can, however, tell us more about how the blood vessels work in pregnancy, and we hope this will increase our understanding of pre-eclampsia. We will only be able to carry out these additional tests in a small number of women, and we will contact you by telephone to arrange the tests if we think you could take part.

What are the risks of taking part in this research?

Blood and urine samples are taken for clinical purposes at your booking visit so that there is no extra risk associated with this research. The amount of blood taken does not place you at any risk. This study will not affect your health, travel, life or any other insurance cover.

What are the benefits of taking part?

There is no benefit to you in taking part in this study, as we will simply be taking samples of your blood and urine. It is hoped, however, that the information that we get from this study will help us to find out more about pre-eclampsia, and will help with the care of pregnant women in the future.

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All samples will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital or the Clinical Investigation Unit will have your name and address removed so that you cannot be recognised from it.

What will happen to any samples I give?

You will donate blood and urine samples for research purposes. Some examinations on these samples will be done straight away. Other examinations will be done at a later stage when we collect more samples from other patients. We will also store some of the samples in our laboratory, so that we can perform additional tests in the future if required. This is why we enclose another information sheet and consent form allowing us to look a t a wider range of pregnancy-related conditions in the future. The samples are treated as "gift"; this means you will not be entitled to any future financial reimbursement related to this study and related research.

Genetic testing

We know that the risk of pre-eclampsia also depends on the genetic makeup. We will therefore examine the genes that may be related to pre-eclampsia. The genetic tests are for research only and do not look at specific changes of your genetic makeup. These tests do not have any immediate clinical relevance. In addition, like all other tests in this study, this is done after removing your name from the sample so that researchers cannot race the results back to you.

What will happen to the results of the research study?

The results of the research study will be stored on a computer database and are likely to be published in medical journals. Reports or publications resulting from the study will not contain any personal details. The research doctor will provide a copy of the results on request.

Who is organising and funding the research?

The research is organised by the BHF Glasgow Cardiovascular Research Centre, University of Glasgow, in collaboration with Obstetric Units at the Queen Mother's Hospital, Southern General Hospital, and Princess Royal Maternity Hospital. The study is funded by charities and the Scottish Government and researchers will not receive any payment for conducting this research.

Contact for Further Information

Should you have any further questions please feel free to call Dr David Carty, Dr Christian Delles or Prof. Anna Dominiczak at the BHF Glasgow Cardiovascular Research Centre, telephone 0141 330 4565 or 0141 330 5420.

Appendix III; Main PIP study consent form.

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Title of Project:
Proteomics in Pre-eclampsia study (PIP)

Name of Researcher:
Prof. Anna F. Dominiczak

Reference: 07/S0709/79
Version: 1.1
Date: 03/10/2007

Patient Identification Code for this Study: | | – | | | | | | – | |

I agree to take part in the above project.

Please initial box

- I confirm that I have read and understand the information sheet dated 03/10/2007 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
- I understand that sections of any of my medical notes and data collected during the study, may be looked at by members of the research team, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
- I agree to donate samples of blood and urine, for the purposes of the research project. I understand that samples will be examined for genes and proteins that may be related to pre-eclampsia or other pregnancy-related conditions. ☐
- I agree to my GP being informed of my taking part in this study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

1 for participant; 1 for researcher

Appendix IV; PIP Study Data Collection Form

Patient Name:		**Date:**	**Nurse Initials:**
Study Number		**Parity:**	
Hospital Number		**Gestation at Booking:**	
Address		**EDD:**	
		Height: **Weight:**	
		Currently Smoking: **Yes** ☐ **No** ☐	
Mobile/Home Phone		**Blood sample obtained:** **Yes** ☐ **No** ☐	
DOB		**Urine sample obtained:** **Yes** ☐ **No** ☐	

Agree to sub-study contact? Yes ☐ No ☐

Primigravida…….. ☐

Age ≥ 40……..... ☐

BMI > 25……..... ☐

Previous pre-eclampsia……... ☐

10 years or more since last pregnancy……... ☐

Multiple pregnancy……..... ☐

BP at booking - > 130 systolic or > 80 diastolic……... ☐

Diabetes…… ☐

Kidney disease…….. ☐

Antiphospholipid syndrome…….. ☐

Autoimmune disease……. ☐

Proteinuria at booking……... ☐

Family history of pre-eclampsia (mother or sister)……... ☐

History of hypertension……. ☐

Appendix V; PIP sub-study data collection form

Patient Demographics

Study Code	
Date of Birth	
Patient Initials	
Number of Weeks Gestation	
Examination Date	

Consent Obtained ☐

Clinical Examination

Height (cm)	
Weight (kg)	
BMI	
Blood Pressure (seated) using OMRON machine	
Heart Rate	

Blood Samples

Type of Sample Required	Obtained
5mls in purple top	☐
7mls in orange top	☐
5mls in green top	☐

Urine Samples

Sample obtained for proteomics	☐
Dipstick test	Comment:

Endothelial Function test using Endo-pat 2000

RHI	
AI	
HR	

Pulse Wave Analysis carried out ☐ ECG carried out ☐

If relevant, arrange time for next visit ☐

Please attach:

Completed consent form ☐ ECG trace ☐

PWA printout ☐ Endo-pat 2000 printout ☐

Appendix VI; Invitation letter for sub-study

BHF Glasgow Cardiovascular Research Centre
PIP Study Team

UNIVERSITY
of
GLASGOW

###Name###
###Address Line 1###
###Address Line 2###
###Address Line 3###
###Address Line 4###

###Post Code###

Dear Ms ###Name###,

Further to our telephone conversation, thank you for agreeing to participate in the second stage of our research project. We would like you to come on:-

DATE:

TIME:

Enclosed you will find a flier, with a map that has details of how to get to our research centre, and an information leaflet that has further details about the tests. We will provide you with refreshments if needed. The tests will take around one hour in total. Please contact us if you have any questions about the tests.

Best wishes,

Proteomics in Pre-eclampsia (PIP) Study Team.

Appendix VII; Consent form for vascular function substudy

UNIVERSITY of GLASGOW

Title of Project:
Proteomics in Pre-eclampsia study (PIP)

Name of Researcher:
Prof. Anna F. Dominiczak

Reference: AB/115252/1
Version: PIP-VF.1.0
Date: 30/08/2007

Patient Identification Code for this Study: | | – | | | | | | – | |

I agree to take part in the above project.

Please initial box

- I confirm that I have read and understand the information sheet dated 30/08/2007 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
- I understand that this is a substudy of the PIP Study and that all of my rights as outlined in the main study also apply to the substudy. My consent to participating in the main study will be attached to this form. ☐
- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
- I understand that sections of any of my medical notes and data collected during the study, may be looked at by members of the research team, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
- I agree to donate samples of blood and urine, for the purposes of the research project und to undergo tests to look at the function of my blood vessels. I understand that samples will be examined for genes and proteins that may be related to pre-eclampsia. ☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature

Appendix VIII; Patient information sheet for vascular function substudy

Invitation

You are being invited to take part in the second part (substudy) of our research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The purpose of our study is to find markers in blood and urine that will help us to work out which women are at risk for developing pre-eclampsia. You have already given us a blood and urine sample at your booking visit. We have chosen a subset of women from the booking visit to carry out a few more tests. These tests will help us to further narrow down the markers that we hope to find in blood and urine. They will also tell us how meaningful these markers are

Why have I been chosen to take part?

Pre-eclampsia has many causes, many of which we do not yet fully understand, and it can be very difficult to predict which women will get it. There are certain factors which can make pregnant women slightly more likely than average to get pre-eclampsia. These factors include women who are having their first baby, women who are over 35 years old, women with a family history of the condition, and women who have certain medical conditions such as high blood pressure. When you booked in at the hospital you gave the midwife information about your medical and family history, and it is from this information that you have been chosen to take part in the study.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be asked again to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will be involved if I decide to take part?

If you decide to take part, we will invite you to come to our research centre for a 45 minutes appointment. We will invite you to come twice - one visit shortly after your booking visit, and again when you are around 28 weeks pregnant. On each occasion we will carry out the following tests:

- We would like to measure your blood pressure, using a machine which is similar to ones used at your GP practice.
- We would like to take another sample of urine, which will be stored in our lab, and may be analysed along with the urine sample you gave at the booking visit.
- We would like to take a sample of blood (about 1½ tablespoonful) which will be stored in our lab.

- We would like to examine the blood vessels in your wrist. This examination involves assessment of your pulse with a pencil-like probe. It is carried out at the artery at your wrist and takes 1 to 2 minutes. We can then look at the pulse waveform, which gives us useful information about the blood vessels in pregnant women.
- We would like to attach a small probe to your index finger, which measures the blood flow. We will then inflate a blood pressure cuff for 5 minutes, and then deflate it and continue to monitor the blood flow to the finger. This test takes about 20 minutes, and can give us useful information about the endothelium, which is the inner layer of blood vessels.

What are the risks of taking part?

We will take blood from the vein in your arm which in rare cases results in a small bruise. The amount of blood taken for this research does not place you at any risk. The other tests are "non-invasive", that is that probes are only attached to your skin. Assessment of your pulse waveform has no specific risks or side-effects. Measurement of blood flow in your finger may lead to some numbness in your arm during the test, which will disappear when the cuff is deflated. A small bruise at your forearm may result from the cuff but will disappear within one or two days.

What are the benefits of taking part?

There is no benefit to you in taking part in this study, as we will simply be taking samples of your blood and urine. The blood vessel tests which we will do are research investigations, and do not tell us whether or not you are likely to get pre-eclampsia, or any other pregnancy-related problems. It is hoped, however, that the information that we get from this study will help us to find out more about pre-eclampsia, and will help with the care of pregnant women in the future.

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All samples will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital or the Clinical Investigation Unit will have your name and address removed so that you cannot be recognised from it.

What will happen to any samples I give?

You will donate blood and urine samples for research purposes. Some examinations on these samples will be done straight away. Other examinations will be done at a later stage when we collect more samples from other patients. We will also store some of your samples for up to 10 years to perform additional tests if required. The samples are treated as "gift"; this means you will not be entitled to any future financial reimbursement related to this study and related research.

Contact for Further Information

Should you have any further questions please feel free to call Dr David Carty, Dr Christian Delles or Prof. Anna Dominiczak at the BHF Glasgow Cardiovascular Research Centre, telephone 0141 330 4565 or 0141 330 5420.

Appendix IX; Patient information sheet for non-pregnant control group

Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The purpose of our study is to improve our ability to predict which women are at risk of developing pre-eclampsia. As part of this study we have been studying the function of the inner layer of blood vessels (endothelium) in pregnant women at different time-periods in their pregnancy. We are also carrying out these studies in women who are not pregnant, to examine how the function of blood vessels differs between pregnant and non-pregnant women.

Why have I been chosen to take part?

We are inviting women are not pregnant, who are aged between 18 and 45 years to take part in the study.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be asked again to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will be involved if I decide to take part?

If you decide to take part, we will invite you to come to our research centre for a 45 minutes appointment. We will carry out the following tests:

- We would like to measure your blood pressure, using a machine which is similar to ones used at your GP practice.
- We would like to take a sample of urine, which will be stored in our lab.
- We would like to take a sample of blood (about 1½ tablespoonful) which will be stored in our lab.
- We would like to examine the blood vessels in your wrist. This examination involves assessment of your pulse with a pencil-like probe. It is carried out at the artery at your wrist and takes 1 to 2 minutes. We can then look at the pulse waveform, which gives us useful information about the blood vessels.
- We would like to attach small probes to your index finger, which measure the blood flow. We will then inflate a blood pressure cuff around one arm for 5 minutes, and then deflate it and continue to monitor the blood flow to the finger. This test takes about 20 minutes, and can give us useful information about the endothelium, which is the inner layer of blood vessels.

What are the risks of taking part?

We will take blood from the vein in your arm which in rare cases results in a small bruise. The amount of blood taken for this research does not place you at any risk. The other tests are "non-invasive", that is that probes are only attached to your skin. Assessment of your pulse waveform has no specific risks or side-effects. Measurement of blood flow in your finger may lead to some numbness in your arm during the test, which will disappear when the cuff is deflated. A small bruise at your forearm may result from the cuff but will disappear within one or two days.

What are the benefits of taking part?

There is no benefit to you in taking part in this study, as we will simply be taking samples of your blood and urine. The blood vessel tests which we will do are research investigations, and do not tell us whether or not you are likely to develop problems if you were to become pregnant. It is hoped, however, that the information that we get from this study will help us to find out more about pre-eclampsia, and will help with the care of pregnant women in the future.

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All samples will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital or the Clinical Investigation Unit will have your name and address removed so that you cannot be recognised from it.

What will happen to any samples I give?

You will donate blood and urine samples for research purposes. Some examinations on these samples will be done straight away. Other examinations will be done at a later stage when we collect more samples from other patients. We will also store some of your samples for up to 10 years to perform additional tests if required. The samples are treated as "gift"; this means you will not be entitled to any future financial reimbursement related to this study and related research.

Contact for further Information

Should you have any further questions please feel free to call Dr David Carty, Dr Christian Delles or Prof. Anna Dominiczak at the BHF Glasgow Cardiovascular Research Centre, telephone 0141 330 4565 or 0141 330 5420.

Appendix X; Further information sent to substudy participants

Dear .....

Thank you for agreeing to take part in the PiP study. An appointment has been made for you at the BHF Glasgow Cardiovascular research centre on ..... at .....

Transport will / will not be organised by us.

If you are arriving with your own transport please see the map attached for directions.

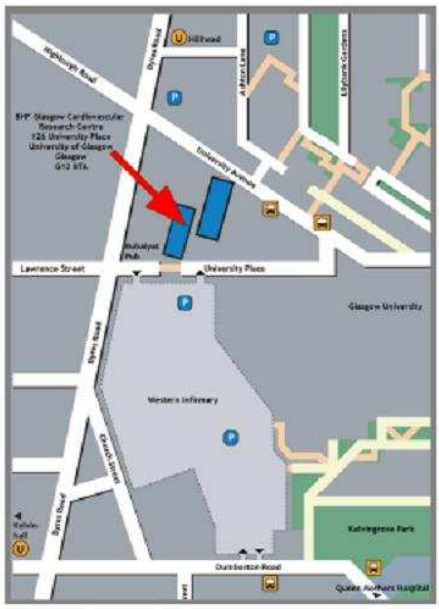
When you arrive please report to reception. Someone will then take you through to the clinic area.

The visit should last no more than 1 hour.

Kind Regards

The PiP Study Team

For appointment queries or to change your appointment please contact the research team on 0141 330 4558 or 0141 330 3008.



PiP

Proteomics In Pre-eclampsia

British Heart Foundation

UNIVERSITY of GLASGOW

BHF Glasgow Cardiovascular Research Centre
126 University Place
University of Glasgow

Version 1.0 - 30/08/2007

BHF Glasgow Cardiovascular Research Centre
126 University Place
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Glasgow
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Appendix XI. List of pregnancy-associated peptides.

protID	Mass	CE_t	Sequence	Protein name	Swissprot name
4845	900.2692	43.6572			
5675	911.4349	25.87627	DGKTGPpGPA	Collagen alpha-1 (I) chain	CO1A1_HUMAN
11282	980.4996	22.40554			
11413	981.5851	24.79552	VLNLGPITR	Uromodulin	UROM_HUMAN
14478	1040.475	25.05015	SpGPDGKTGPp	Collagen alpha-1 (I) chain	CO1A1_HUMAN
14906	1050.477	26.92478	MGPRGPpGPpG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
16915	1083.504	21.67677			
18448	1108.534	20.38335			
20457	1138.586	19.50684			
20749	1141.511	26.05729			
21294	1153.399	36.63236			
21365	1154.512	25.64497	PpGEAGKpGEQG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
21637	1155.454	36.9503			
21747	1157.537	37.44405	GPPGPpGppGPPS	Collagen alpha-1 (I) chain	CO1A1_HUMAN
21893	1159.577	20.64463			
23356	1179.52	37.4916			
23467	1181.485	36.99772			
23968	1191.517	36.17672			
24168	1195.479	37.50522			
24442	1199.473	37.69087			
24510	1200.538	24.14255			
25429	1217.529	35.78057			
25893	1223.571	19.39261			
25932	1223.57	20.18306			
26092	1224.711	24.76768			
26326	1229.476	36.56928			
26431	1231.488	39.56649			
26630	1234.563	27.37202	ApGDRGEPGPpGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
27229	1245.551	21.62387			

27340	1247.482	39.60707			
28103	1257.443	33.9189			
28306	1260.605	27.4268			
28839	1270.503	38.0709			
29279	1276.398	35.92331			
30263	1294.488	39.46778			
30575	1297.582	27.36504	SpGSpGPDGKTGPp	Collagen alpha-1 (I) chain	CO1A1_HUMAN
30593	1297.591	22.08441	ApGKNGERGGpGGp	Collagen alpha-1 (III) chain	CO3A1_HUMAN
31626	1314.348	36.15068			
33209	1339.602	27.48587	SpGERGETGPpGPA	Collagen alpha-1 (III) chain	CO3A1_HUMAN
33840	1352.779	24.60145			
33973	1353.656	25.63162			
34186	1358.38	36.46108			
34432	1363.428	36.33749			
34724	1366.622	27.4376	pPGEAGKpGEQGVp	Collagen alpha-1 (I) chain	CO1A1_HUMAN
34766	1367.643	38.88257	PpGPpGPpGPPGTPV	Collagen alpha-1 (XVIII) chain	COIA1_HUMAN
35201	1375.572	38.27861			
35204	1375.605	23.245			
35424	1380.639	23.83049			
35912	1389.601	39.02101			
35979	1390.442	36.94585			
36156	1392.623	21.75213			
36566	1401.384	36.55667			
36759	1405.606	39.03535			
36965	1408.62	28.99544			
36988	1408.66	39.13381	GPPGppGPpGPPGPPS	Collagen alpha-1 (I) chain	CO1A1_HUMAN
37650	1422.545	37.5732			

37785	1423.638	20.16397			
37903	1424.662	39.29955	GLPGPpGPpGSFLSN	Collagen alpha-1 (XVII) chain	COHA1_HUMAN
37949	1425.587	22.31776			
38021	1426.667	38.94155	GLpGLPGPpGPpGpPG	Collagen alpha-3 (IX) chain	CO9A3_HUMAN
38174	1428.391	36.74784			
38605	1435.659	28.83627	SpGSPGPDGKTGPpGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
38752	1438.449	36.76394			
38798	1438.667	27.87549	GLpGTGGPpGENGKpG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
38910	1440.562	24.30044	DEAGSEADHEGTHS	Fibrinogen alpha chain	FIBA_HUMAN
39275	1445.618	37.35901			
39322	1446.636	39.42789			
40091	1449.641	21.85606			
40737	1462.616	39.42141			
41431	1466.659	21.8713			
41514	1467.807	24.68522			
41972	1478.614	39.30267			
42304	1485.674	23.76807	DGQpGAKGEpGDAGAK	Collagen alpha-1 (I) chain	CO1A1_HUMAN
43442	1507.738	40.02385	VGPpGPpGPpGPPGPPS	Collagen alpha-1 (I) chain	CO1A1_HUMAN
43642	1510.565	39.0518			
43678	1510.676	19.51616			
43691	1510.682	20.16625			
44633	1523.841	29.75377	VIDQSRVLNLGPIT	Uromodulin	UROM_HUMAN
44718	1525.479	37.16451			
45857	1549.687	20.21417			
45980	1552.498	37.21467			

46542	1561.448	36.95663			
46554	1561.686	40.62815			
46880	1567.702	20.19208	GSEADHEGTHSTKRG	Fibrinogen alpha chain	FIBA_HUMAN
46961	1569.654	39.31398			
47367	1576.6	26.37432			
47855	1576.743	19.50785	YKRKANDESNEHS	Osteopontin	OSTP_HUMAN
48008	1577.687	40.084			
48162	1580.886	24.84996			
48176	1580.879	23.8674	IDQSRVLNLGPITR	Uromodulin	UROM_HUMAN
48540	1587.656	39.46728			
48580	1588.706	30.15033	TGLSMDGGGSPKGDVDP	Sodium/potassium-transporting ATPase gamma chain	ATNG_HUMAN
48651	1590.442	37.39939			
49248	1593.75	39.57015			
50075	1611.695	29.4784			
50212	1613.819	23.98601	VGGGEQPPPAPAPRRE	Xylosyltransferase 1	XYLT1_HUMAN
50633	1620.671	29.49355			
50904	1624.546	37.72626			
51328	1632.709	40.14696			
51678	1633.757	19.52845			
51804	1634.799	29.71747	SpGNIGPAGKEGPVGLpG	Collagen alpha-2 (I) chain	CO1A2_HUMAN
51875	1635.786	40.44105	VGPpGPpGPpGPPGPPSAG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
52096	1638.736	19.60149			
52100	1638.728	20.22988	AGSEADHEGTHSTKRG	Fibrinogen alpha chain	FIBA_HUMAN

52189	1640.581	23.24178			
52507	1646.795	21.88543			
52609	1648.727	22.82574			
52769	1649.727	22.64299			
53216	1654.782	23.12638	SpGEAGRpGEAGLpGAKG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
53957	1669.689	21.46473	DEAGSEADHEGTHSTK	Fibrinogen alpha chain	FIBA_HUMAN
54525	1680.752	30.02747	GLpGTGGPpGENGKpGEp	Collagen alpha-1 (III) chain	CO3A1_HUMAN
54687	1684.666	31.75269			
54688	1684.671	30.65638			
54846	1687.536	37.78593			
54976	1689.742	40.60201			
55756	1700.721	29.93699	GmpGSpGGpGSDGKpGPpG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
56011	1705.731	40.43673			
56325	1712.771	30.99943			
58222	1752.783	19.61447			
59056	1767.781	19.81004			
59100	1768.826	20.77489			
59430	1776.855	21.4446			
60975	1812.786	24.13738			
61039 61048	1813.715 1813.778	31.69499 40.65807			
61221	1817.694	20.23435			
61573	1825.786	20.13497	DEAGSEADHEGTHSTKR	Fibrinogen alpha chain	FIBA_HUMAN
61753	1829.752	40.83957			
61899	1833.844	27.08198			

62044	1836.791	31.14372			
62226	1840.836	41.17953			
63391	1864.807	20.42988			
64170	1880.895	43.9095			
64431	1885.651	38.81996			
65368	1901.823	43.82548			
66161	1916.768	20.32244			
66240	1917.938	31.77883			
66656	1927.914	19.51821	DkGETGEQGDRGIkGHRG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
67951	1950.851	35.76881			
69769	1991.941	22.04607			
69979	1996.786	20.9757			
70361	2006.857	33.43506			
70413	2007.945	22.10222	DGESGRpGRpGERGLpGPpG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
70485	2009.879	32.29178			
70999	2021.903	33.23276			
73177	2062.933	26.57903	DAGApGAPGGKGDAGApGERGPpG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
73246	2063.932	21.94861	NGDDGEAGKpGRpGERGPpGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
73434	2067.818	20.62077			
73628	2070.022	19.3539			
75410	2100.013	19.48241	PpGKAGEDGHpGKPGRpGERG	Collagen alpha-2 (I) chain	CO1A2_HUMAN
76423	2117.96	27.7093	DGQPGAKGEPGDAGAKGDAGPPGp	Collagen alpha-1 (I) chain	CO1A1_HUMAN
77679	2148.015	25.28724	QGLpGTGGPpGENGKpGEPGpKG	Collagen alpha-1 (III) chain	CO3A1_HUMAN

79737	2187.987	27.17073			
79786	2188.999	26.88501	ADGQPGAKGEPGD AGAKGDAGPpGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
81196	2210.954	33.60553	NGApGNDGAKGDA GApGApGSQGApG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
81457	2216.03	33.83058	IGPpGPAGApGDKG ESGPSGPAGPTG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
81607	2219.007	19.65068	DEAGSEADHEGTH STKRGHAK	Fibrinogen alpha chain	FIBA_HUMAN
82026	2226.992	26.27752	GNSGEpGApGSKG DTGAKGEpGPVG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
83107	2244.066	19.49733			
83441	2248.968	33.69466			
84126	2256.973	33.5541			
84164	2257.869	35.92739			
84363	2261.995	27.12127	GADGQpGAKGEPG DAGAKGDAGPpGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
85020	2276.023	27.23118	ADGQPGAKGEpGD AGAKGDAGPpGPA	Collagen alpha-1 (I) chain	CO1A1_HUMAN
85503	2286.115	19.41889			
85761	2292.019	27.28272	ADGQpGAKGEpGD AGAKGDAGPpGPA	Collagen alpha-1 (I) chain	CO1A1_HUMAN
85896	2294.983	33.51783			
86426	2306.034	19.53317	DEAGSEADHEGTH STKRGHAKS	Fibrinogen alpha chain	FIBA_HUMAN
86677	2308.017	27.33705	ADGQpGAKGEpGD AGAKGDAGppGPA	Collagen alpha-1 (I) chain	CO1A1_HUMAN
86951	2314.012	33.65553			

87012	2315.102	19.41716			
87341	2319.995	34.29261			
87619	2325.995	33.76476			
88052	2334.985	33.6041			
88282	2339	34.00721			
88622	2343.131	19.45944			
90651	2385.054	33.94877			
91542	2407.092	27.67152	LDGAKGDAGPAGPKGEpGSpGENGApG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
91855	2414.154	19.57439			
92350	2423.02	34.02705			
92841	2430.098	28.32809	ADGQPGAKGEpGDAGAKGDAGPpGPAGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
93227	2442.074	34.09673			
93361	2445.098	28.23779	mASDASHALEAALEQMDGIIAGTK	Liprin-beta-2; N-term.	LIPB2_HUMAN
93417	2446.092	28.37261	ADGQpGAKGEpGDAGAKGDAGpPGPAGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
94308	2471.155	34.77354	TGPIGPpGPAGApGDKGESGPSGPAGPTG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
94674	2480.06	34.12281			
94807	2483.121	27.56515	AGQDGRpGPpGppGARGQAGVmGFpG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
94948	2487.125	28.27413	GADGQPGAKGEpGDAGAKGDAGPpGPAGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
97257	2539.1	34.45086			

97463	2544.128	28.25992			
97506	2545.12	28.20161	GPpGADGQpGAKGEpGDAGAKGDAGpPGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
97653	2548.286	35.15708			
97736	2551.152	34.71513			
98089	2559.18	19.40742	DEAGSEADHEGTHSTKRGHAKSRP	Fibrinogen alpha chain	FIBA_HUMAN
98596	2563.147	21.21006			
98660	2564.15	22.97496	GApGQNGEpGGKGERGApGEKGEGGPpG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
99021	2570.19	42.56018			
100255	2596.233	34.89552			
100767	2608.127	42.23556			
102725	2642.214	27.69602	QGpPGPSGEEGKRGPNGEAGSAGPPGppG	Collagen alpha-2 (I) chain	CO1A2_HUMAN
102924	2647.199	23.46703	NRGERGSEGSPGHPGQPGPpGPpGApGP	Collagen alpha-1 (III) chain	CO3A1_HUMAN
103080	2650.173	34.95789			
104307	2666.176	35.03149			
104376	2668.25	41.97426			
104786	2679.197	23.52889	NRGERGSEGSPGHpGQpGppGpPGAPGP	Collagen alpha-1 (III) chain	CO3A1_HUMAN

104954	2682.143	22.49183			
105352	2695.198	23.52252	NRGERGSEGSpGHpGQpGppGPPGAPGp	Collagen alpha-1 (III) chain	CO3A1_HUMAN
105744	2705.16	35.12898			
106667	2726.283	42.93932			
108119	2756.268	35.24034			
108327	2761.315	21.49226	ERGSPGpAGPKGSpGEAGRpGEAGLpGAKG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
108574	2764.214	42.62656			
109164	2777.316	21.5225	ERGSpGPAGpKGSpGEAGRpGEAGLpGAKG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
110052	2799.085	25.06936			
110468	2811.256	23.84819			
110680	2817.256	35.10447			
112106	2854.363	34.86364			
114230	2912.168	25.55697			
114795	2925.314	35.67595			
115312	2939.149	33.76711			
117658	2999.447	36.05018			
118163	3011.387	29.74951	LTGSpGSpGpDGKTGPPGPAGQDGRPGPpGppG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
118224	3013.292	22.2946	ESGREGApGAEGSpGRDGSpGAKGDRGETGPA	Collagen alpha-1 (I) chain	CO1A1_HUMAN
118694	3023.356	24.55931			

119292	3035.191	42.02093			
119385	3037.409	36.00759			
119538	3041.375	29.98314			
120367	3063.444	30.09824	RTGEVGAVGpPGFAGEKGPSGEAGTAGpPGTpGP	Collagen alpha-2 (I) chain	CO1A2_HUMAN
120693	3069.376	36.00438			
121337	3079.413	30.07032	RTGEVGAVGpPGFAGEkGPSGEAGTAGpPGTpGP	Collagen alpha-2 (I) chain	CO1A2_HUMAN
121772	3092.439	36.29566	TGEVGAVGPpGFAGEKGPSGEAGTAGPpGTpGPQG	Collagen alpha-2 (I) chain	CO1A2_HUMAN
121775	3092.464	31.24934	ADGQPGAkGEPGDAGAKGDAGPPGPAGpAGpPGPIG	Collagen alpha-1 (I) chain	CO1A1_HUMAN
121940	3098.436	30.05541	LTGNpGVQGPEGKLGPLGApGEDGRpGpPGSIG	Collagen alpha-2 (V) chain	CO5A2_HUMAN
121989	3100.42	29.96433			
122520	3112.365	22.62618			
123565	3145.459	38.89295			
123643	3148.458	31.26646			
123671	3149.46	31.24549	GADGQPGAKGEpG	Collagen alpha-1 (I) chain	CO1A1_HUMAN

			DAGAKGDAGPpGP AGpAGPPGPIG		
123969	3158.439	29.71371	GERGSpGGpGAAG FpGARGLpGpPGSN GNPGPpGp	Collagen alpha-1 (III) chain	CO3A1_HUMAN
124172	3165.462	31.32057			
124268	3168.357	24.70453	GEpGRDGVpGGpG MRGmpGSpGGpGS DGKpGPpG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
124688	3185.465	25.4677			
125529	3215.396	19.99446			
126699	3248.529	30.66041	RTGEVGAVGpPGF AGEKGPSGEAGTA GPPGTpGpQG	Collagen alpha-2 (I) chain	CO1A2_HUMAN
127351	3264.556	25.75167	AAGEPGkAGERGV pGPpGAVGPAGKD GEAGAQGPPGP	Collagen alpha-1 (I) chain	CO1A1_HUMAN
127402	3265.431	36.08731			
127852	3280.556	25.81551			
127899	3281.434	36.0914			
128086	3286.555	30.92069			
128140	3287.479	30.97061			
128689	3303.412	36.0889			
128908	3310.493	36.57161			
129019	3314.431	20.14047			
129131	3318.546	30.99198			
129150	3319.382	36.08194			
130482	3350.549	31.01736			

130504	3351.545	36.38531			
130747	3359.578	31.89787			
131294	3375.574	31.91691			
131589	3385.547	25.48872			
131879	3397.56	31.84766			
132057	3401.66	23.49281			
132411	3406.568	31.24378			
132504	3409.617	31.93033	NTGApGSPGVSGPKGDAGQPGEkGSpGAQGPPGAPGPLG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
132834	3421.555	25.99412			
132950	3425.605	31.27027	ASHTSDSDVPSGVTEVVVKLFDSDPITVTVPVE	Clusterin	CLUS_HUMAN
133430	3441.609	31.38498	NTGAPGSpGVSGpKGDAGQpGEKGSpGAQGpPGAPGPLG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
134470	3473.596	31.47644	NTGApGSpGVSGpKGDAGQpGEKGSpGAQGpPGAPGpLG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
135047	3495.56	31.9667			
141672	3718.721	32.4816			
142080	3734.721	32.49717			
143372	3775.616	37.25807			
144635	3831.81	28.48446			
145456	3870.814	33.49116			
145889	3891.752	24.52856			
146624	3927.821	33.59714			

146936	3943.83	33.62845			
148557	4002.618	20.65664			
148640	4005.81	33.05918			
149717	4043.806	33.12202			
156445	4305.937	28.82961	ARGNDGARGSDGQpGpPGPpGTAGFpGSpGAKGEVGpAGSpGSNGApG	Collagen alpha-1 (III) chain	CO3A1_HUMAN
162922	4549.146	26.55078			
179138	6688.901	20.81745			
190524	14109.54	21.93083			

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