Pre-eclampsia: predicting onset and poor outcome

By

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In memory of my inspiring grandmother

Eibhlín Uí Bhreisleaín

1914 - 2003

Declaration

I declare that all the experimental work presented in this thesis are of my own work, except the running of the plasma samples which were loaded onto the Synapt mass spectrometer by Dr. Susan Slade. In addition, Dr. Slade performed the initial database interrogation, transferring the findings onto Microsoft Excel datasheets. Further database interrogation, statistical analysis and processing were carried out by myself.

Work presented in this thesis has been published (or submitted for publication) in peer-reviewed journals to include;

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- World Congress in Fetal Medicine, Rhodes 2010
- Blair Bell Society Meeting, London 2010

- American Society of Mass Spectrometry, Salt Lake City, 2010
- British Maternal and Fetal Medicine Society / Perinatal Medicine Meeting, Harrogate 2011
- Society for Maternal and Fetal Medicine, San Francisco, 2011
- Birmingham and Midlands Obstetrics and Gynaecology annual meeting, Birmingham 2011
- Royal Society of Medicine, London 2011

I confirm that this thesis has not been submitted to another university.

Summary

Pre-eclampsia is a pregnancy-specific, multi-system disorder of placental origin. Affecting between 2-8% of women, it is one of the leading causes of both maternal and fetal morbidity and mortality in the United Kingdom. Although the disorder presents after 20 weeks gestation with the classic symptoms of hypertension and proteinuria, the pathological process(es) leading to this syndrome initiate early in the first trimester. In current clinical practice, prediction of those who will get the disease and those who will have a poor outcome once it develops is poor.

This thesis focuses on novel ways to enhance the prediction of the development of pre-eclampsia and poor outcome once the syndrome manifests.

Firstly, the current risk factors for pre-eclampsia were challenged. Racial variation in these risk factors has demonstrated the importance of considering maternal ethnicity when assessing the likelihood of developing the disease. Obese Black women were more likely to develop pre-eclampsia than obese white women (aOR 2.06 (95%CI 1.34-3.23) p=0.002). In the normal BMI group Black women were less likely to develop pre-eclampsia than White women (aOR 0.421 (95%CI 0.24-0.73) p<0.001). Younger (<20 years of age) Black women were less likely to develop pre-eclampsia than younger white women (aOR 0.628 (95%CI 0.49-0.83) p<0.001). Conversely, older (>35 years of age) Black women were more likely to develop pre-eclampsia than older White women (aOR 1.67 (95%CI 1.39-1.99) p<0.001).

Secondly, first trimester maternal plasma studies have identified a cohort of potential disease (and pathophysiological) markers that may allow for the development of an early screening test for pre-eclampsia. The ratio of angiotensinogen to Kallikrein is raised in the first trimester of pregnancies that later develop pre-eclampsia (p<0.001). The receiver operator curve (ROC) for the ratio of angiotensinogen to kallikrein had an area under the curve (AUC) of 0.81 (SE=0.05). A cut-off value of >0.27 has a sensitivity of 0.9 (95% CI = 0.74 – 0.97), a specificity of 0.5 (95% CI = 0.24-0.65), positive predictive value (PPV) 0.63 (95%CI = 0.47-0.75) and negative predictive value (NPV) of 0.87 (95% CI = 0.53-0.96) in predicting the onset of pre-eclampsia.

Common haematological and biochemical tests are presented as markers for both the development of the disease and poor outcome when it occurs. The ratio of Neutrophil to Lymphocyte (NLR) is raised in the first trimester of pregnancies that develop pre-eclampsia (p<0.001). The ROC of the ratio to Neutrophil to Lymphocyte has an AUC of 0.84 (95% CI = 0.85-0.95). A cut off value of 2.53 has a sensitivity of 0.92 (95%CI = 0.85-0.95), specificity of 0.6 (95%CI = 0.51-0.67), PPV 0.68 (95%CI = 0.6-0.74) NPV 0.87 (95%CI = 0.8-0.93) for predicting the onset of pre-eclampsia. At the time of diagnosis of pre-eclampsia, a raised NLR predicts poor maternal outcome and the need for a caesarean section due to fetal distress (p<0.05). In addition, a reduced level of bilirubin predicts both poor fetal and maternal outcome and the need for a caesarean section (p<0.05).

Abbreviations

2D	Two-dimensional
ACOG	American College of Obstetrics and Gynaecology
ADAM12	A disintegrin and metalloprotease-12
AMBP	Alpha-1-microglobulin/bikunin precursor
aRR	Adjusted relative risk
ART	Artifical reproductive technology
AT1-AA	Angiotensin II type I receptor agonistic autoantibodies
AUC	Area under the curve
BMI	Body mass index
CI	Confidence interval
CMACE	Centre for maternal and child enquires
COMT	Catechol-O-methyltransferase
CRL	Crown-rump length
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
EDF	End diastolic flow
EVT	Extravillous trophoblast
FLT-1	Fms-related tyrosine kinase -1
g	Grams
HELLP	Haemolysis, elevated liver enzymes and low platelets
HIV	Human immunodeficiency virus

HLA	Human leukocyte antigen
HzR	Hazard Ratio
ICU	Intensive Care Unit
IL	Interleukin
ISSHP	International society for the study of hypertension in pregnancy
ml	Millilitres
mm	Millimetres
mmHg	Millimetres of mercury
MoM	multiples of median
NICE	National Institute for Health and Clinical Excellence
NK	Natural Killer (cells)
nl	Nanolitres
NPV	Negative predictive value
NT	Nuchal Translucency
p	probability
PEER	Perinatal Episode Electronic Record
PI	Perinatal Institute
PI	Pulsatility Index
PlGF	Placenta growth factor
PP13	Placenta protein 13
PPV	Positive predictive value
PTB	Preterm birth

RI	Resistance Index
RNS	Reactive Nitrogen species
ROC	receiver operator curve
ROS	Reactive oxygen species
RR	Relative risk
SA	South Asian
SCOPE	Screening for pregnancy endpoints
sENG	Soluble endoglin
SGA	Small for gestational age
SLE	Systemic lupus erythematosus
T2DM	Type 2 diabetes mellitus
TGF-β	Transforming growth factor beta
TNF	Tumour Necrosis Factor
UK	United Kingdom
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2
VIP	vitamins in pregnancy (study)
WHO	World health organisation
WM	West Midlands
µl	Microliters

Chapter 1

Introduction

1. Introduction

Some form of hypertension occurs in 15-20% of pregnancies (James et al, 2003). These hypertensive disorders of pregnancy are a leading cause of maternal and fetal/neonatal morbidity, with the main cause being pre-eclampsia.

Pre-eclampsia is a multi-organ, pregnancy specific condition characterised by hypertension, proteinuria ± oedema (Walker et al, 2000) and in the western world affects 2-8% of all pregnancies (North et al, 2011). It is a spectrum disorder. Some women develop mildly raised blood pressure with proteinuria at term, requiring no further action other than enhanced surveillance. Others need to be delivered early in the third trimester due to a fulminating form of the disease potentially leading to multi organ failure and seizures. The classic triad of symptoms of headache, visual disturbance and epigastric pain represent abnormal cerebral perfusion / retinal arteriolar spasm or oedema / hepatic capsular distension respectively and are the commonest symptoms that precede an eclamptic fit (James et al, 2003). However, even at it's worst, the disease can be surprisingly symptomless. Given how severe the disease can be, much of the standard United Kingdom (UK) antenatal practice is geared towards detecting it, with every woman having her blood pressure checked and a urinalysis performed at each clinical visit. With appointments only occurring every 3 weeks until 34 weeks gestation, then 2 weeks until 38 weeks

(www.choices.nhs.uk), detection of the disease can be troublesome. Although blood pressure can be managed by a variety of agents, the only "cure" for pre-eclampsia is delivery of the placenta and fetus. In view of this, there has been a move to improve prediction rates of women who will develop pre-eclampsia and if deemed high risk, offer some form of prophylaxis or intervention such as enhanced care (NICE, 2010).

In this chapter I will discuss current ideas regarding the classification of the disease, its pathophysiology, the current standard screening methods and those novel screening methods that have been suggested in the literature.

1.1 Definition and diagnostic criteria

1.1.1 Defining preeclampsia

The standard definition of pre-eclampsia being hypertension, proteinuria ± oedema occurring after 20 weeks gestation is universally accepted. However, the differing cut-offs for defining both hypertension and proteinuria have not been so standard. The international society for the study of hypertension in pregnancy (ISSHP) therefore defined, via a consensus working group in 2000, pre-eclampsia as being:

- Hypertension = Systolic Blood Pressure (SBP) >140mmHg and / or a Diastolic Blood Pressure (DBP) of > 90mmHg on two consecutive readings 6 hours apart.

- Proteinuria = ≥300mg/l/24 hrs or a protein: creatinine ratio of >30mg/mmol, and where this test is not available, a 1+ proteinuria on a urine dipstix, with both occurring after 20 weeks and returning to normal postnatally (Brown et al, 2001).

Whilst this has been taken to be the standard definition of pre-eclampsia, definitions for characterising severe pre-eclampsia and early onset pre-eclampsia remain somewhat arbitrary (Tranquilli et al, 2013).

1.1.2 Defining severe pre-eclampsia

The National Institute for Health and Care Excellence (NICE) define severe pre-eclampsia as being pre-eclampsia with severe hypertension and/or with symptoms, and/or biochemical and/or haematological impairment with a sub definition for severe hypertension as being a systolic BP ≥160mmHg and/or a diastolic blood pressure ≥ 110mmHg (NICE, 2010).

The American College of Obstetrics and Gynaecology (ACOG) have also issued guidance on the definition of severe pre-eclampsia. They define pre-eclampsia as "Blood pressure of 140 millimeters of mercury (mmHg) systolic or higher or 90 mmHg diastolic or higher that occurs after 20 weeks of gestation in a woman with previously normal blood pressure and proteinuria defined as urinary excretion of 0.3 grams (g) protein or higher in a 24-hour urine specimen". To be classified as severe they suggest one or more of the following features:

- Blood pressure of 160 mm Hg systolic or higher or 110 mm Hg diastolic or higher on two occasions at least 6 hours apart while the patient is on bed rest
- Proteinuria of 5 g or higher in a 24-hour urine specimen or 3+ or greater on two random urine samples collected at least 4 hours apart
- Oliguria of less than 500 millilitres (mL) in 24 hours
- Cerebral or visual disturbances
- Pulmonary edema or cyanosis
- Epigastric or right upper-quadrant pain
- Impaired liver function
- Thrombocytopenia
- Fetal growth restriction

The ACOG definition has been adopted by many clinicians and researchers. Whilst it is useful to have guidance for the definition of both pre-eclampsia and severe pre-eclampsia, the ACOG guidance does not address the problem of pre-eclampsia as a result of pre-existing medical disorders, including pre-existing hypertension, the so-called 'superimposed pre-eclampsia'. Both the ISSHP and NICE have addressed this and split hypertensive disorders of pregnancy into different categories (NICE, 2010, Tranquilli et al, 2013). These are:

- Chronic hypertension (hypertension detected at booking or before 20 weeks gestation, or women taking anti-hypertensive medication pre-pregnancy)

- Gestational hypertension (hypertension occurring after 20 weeks gestation without significant proteinuria)
- Pre-eclampsia (hypertension occurring after 20 weeks gestation with significant proteinuria).

1.1.3 Defining early and late onset pre-eclampsia

The timing of the development of pre-eclampsia is also categorised. It is thought that early and late onset pre-eclampsia are heterogeneous pathophysiological processes that lead to the clinical syndrome of pre-eclampsia (Raymond et al, 2011). Outcomes vary according to the timing of onset of the disease, with those women affected by early onset pre-eclampsia suffering from a more aggressive form of the syndrome. They are more likely to have lower birth weight babies and suffer more morbidity themselves (Vatten et al, 2004). In view of this, most researchers define pre-eclampsia into either the early onset or late onset disease categories. Whilst definitions alter, the ISSHP have defined early onset pre-eclampsia as pre-eclampsia occurring less than 34 weeks of gestation, and late onset occurring after 34 weeks of gestation.

For the purpose of this thesis, I have defined pre-eclampsia, severe pre-eclampsia and early onset pre-eclampsia in line with the above ISSHP definition.

1.2 Epidemiology

Pre-eclampsia is a common disorder in pregnancy, occurring in 2-8% of pregnancies (North et al, 2011) and it is estimated that a woman in her first pregnancy has a 1:250 chance of delivering preterm due to pre-eclampsia (Hernandez-Diaz et al, 2009). The majority of these cases occur at late gestations of pregnancy and have only a negligible increased risk for adverse pregnancy outcome (Raymond et al, 2011).

1.2.1 Maternal mortality and morbidity

Despite this, pre-eclampsia represents one of the largest killers of mothers, both in the UK and worldwide. Consistently, hypertensive disorders are in the top three causes of maternal death in the UK and the last Centre for Maternal and Child Enquires (CMACE) triennial report from 2006-2008 revealed that it was the second leading cause of direct maternal deaths (that is, a death due to a complication of pregnancy, rather than a pre-existing condition leading to death (indirect maternal death)) after sepsis. The incidence of pre-eclampsia is almost 7 times higher in the developing world than in the developed with 63000 women dying from the disease in 2000 (representing 12% of maternal deaths) (CMACE, 2011). Pre-eclampsia also represents a leading cause of morbidity in mothers. It has been estimated that one-third of significant maternal morbidity is due to pre-

eclampsia (Waterstone et al, 2001) with 5% of women with pre-eclampsia requiring intensive care unit (ICU) admission (Tuffnell et al, 2005).

1.2.2 Fetal morbidity and mortality

The effect on the fetus is also significant. In stillbirths that occur to non-anomalous fetuses, 1 in 20 are due to pre-eclampsia (CMACE, 2011). Babies born to mothers with pre-eclampsia are also at greater risk of being born growth restricted. 20-25% of babies born preterm (and 14-19% of those born at term) to pre-eclamptic mothers have birth weights on the 10th centile or less (Rasmussen et al, 2006). The preterm birth rate is also proportionally raised in women with pre-eclampsia, with 10% of pre-term births being attributable to pre-eclampsia (Sibai et al, 2011).

1.3 Risk factors

Several risk factors have been identified for developing pre-eclampsia:

1.3.1 Nulliparity and primipaternity

Notably pre-eclampsia is often referred to as a disease of the first pregnancy. The incidence increases in those with a limited exposure to the paternal sperm before conception. After a previous early pregnancy loss

(iatrogenic or spontaneous) or a healthy term delivery, the risk decreases, but this protection is lost with a change of partner (Broughton-Pipkin et al, 1994). Whole population studies have shown that fathers who have fathered pre-eclamptic pregnancies are almost twice as likely to father a pre-eclamptic pregnancy in other women, irrelevant of their parity (Lie et al, 1998). It is estimated that nulliparity triples the risk of developing pre-eclampsia with a large systematic review from Duckitt et al suggesting an odds ratio of 2.91 (with confidence interval (C.I.) = 1.28-6.61) for nulliparous women developing the disease (Duckett et al, 2005).

1.3.2 Age

Whilst extremes of age are commonly given as a risk factor for developing pre-eclampsia the above mentioned systematic review by Duckitt suggested that mothers less than the age of 17 years were not significantly at risk of developing pre-eclampsia (odds ratio 2.98, CI 0.39:22.76). Despite this, it has been suggested by other authors that younger women are more likely to develop the condition, possibly due to their limited exposure to sperm before conception. It is well established that women over the age of 40 are at higher risk of developing the disease, furthermore this has been shown to be irrelevant of her parity (NICE, 2010, Broughton-Pipkin et al, 1994).

1.3.3 Obesity

It is abundantly clear that there is a causal link between obesity and pre-eclampsia (RCOG, 2010). The risk of developing pre-eclampsia increases as body mass index (BMI) increases, and for every 5-7kg/m^2 increase in BMI there is doubling of the incidence of the disease. This has been seen in studies that have controlled for both blood pressure and other risk factors (O'Brien et al, 2003). It could be suggested that this link is due to insulin resistance which is associated with obesity. Possible explanations for the link between obesity and pre-eclampsia include: increased stress due to the hyperdynamic circulation associated with obesity; dyslipidaemia or increased cytokine-mediated oxidative stress; and direct haemodynamic effects of hyperinsulinaemia (increased sympathetic activity and increased tubular sodium desorption) (Dekker et al, 2001).

1.3.4 Ethnicity

Different ethnic groups have different risks of developing pre-eclampsia and this is discussed in later chapters.

1.3.5 Fertility treatment

Women who conceive via artificial reproductive techniques (ART) are more likely to develop pre-eclampsia. Generally these women are older, more

obese (+/- polycystic ovarian syndrome (PCOS)) and therefore the cohort themselves are at greater risk of developing pre-eclampsia, irrespective of conception method. In addition, ART themselves can increase the likelihood of developing pre-eclampsia. Methods that introduce gametes that the woman has previously not been exposed to before (such as ovum, semen or embryo donation) may have an effect on the fetal-maternal immune interaction, and as such increase the risk of developing pre-eclampsia. ART increases the possibility of having multi-fetal pregnancies, which itself predispose to developing the disease (Sibai et al, 2005).

1.3.6 Multi-fetal gestation

Multi-fetal gestations increase the risk of developing pre-eclampsia. This is thought to be due to the larger placental mass inducing a greater degree of first trimester inflammation (Redman et al, 2000). A primigravidae who is pregnant with twins is 5-times more likely than a primigravidae with a single fetus to develop pre-eclampsia and for multiparous women, this is as high as 10-times greater (MacGillivray et al, 1998).

1.3.7 Pre-existing maternal medical conditions

There are several medical conditions that predispose women to developing pre-eclampsia. Whilst these disorders may affect several different organ systems, those that have pathological effects on endothelium and/or

increase systemic inflammation appear to be the main causes. These conditions are summarised in table 1.

Disease	Increased incidence of PET
Diabetes Mellitus (DM)	15-18% of type 1 DM[a]
Thrombophilia	5 fold increase[b]
Systemic Lupus Erythamatous (SLE)	2 fold increase[c]
Renal disease	3 fold increase[d]

Table 1: Summary of medical conditions associated with pre-eclampsia. (a=Middleton, 2010; b=Kingdom, 2011; c=Paez, 2013; d=Vellanki, 2013)

1.4 Pathophysiology

The true pathophysiological mechanism which causes pre-eclampsia remains elusive. Although pre-eclampsia is a systemic, multi-organ disorder, it is widely accepted that it originates from the placenta (as pre-eclampsia can occur in molar pregnancies where there is no fetus and the condition resolves when the placenta is delivered). As previously mentioned, although the clinical manifestation of the disease is the same, that is, hypertension and proteinuria after 20 weeks gestation, there

appears to be distinct pathological mechanisms in disease development (Huppertz et al, 2008). In late onset pre-eclampsia there is

- A normally grown baby with no signs of growth restriction
- Normal or only slightly altered behaviour of the uterine arteries
- No changes in blood flow of the umbilical arteries
- An increased risk for pregnant women displaying an enlarged placental mass or surface (diabetes, multiple pregnancies etc.)

This is in contrast to early onset pre-eclampsia, which comprises of:

- An inadequate and incomplete trophoblast invasion of maternal spiral arteries
- Change of blood flow within the uterine artery, suggesting impedance in the uterine spiral arteries
- An increased peripheral resistance of placental vessels that leads to abnormal waveform in umbilical artery Dopplers
- Clear signs of fetal growth restriction.

Generally, early onset pre-eclampsia can be seen as a two-stage disorder. The first (sub-clinical) stage is characterised by poor placental perfusion, which results in maternal endothelial dysfunction characterising the second (clinical) stage (Huppertz et al, 2008).

1.4.1 Normal placental development

The trophoblast is the first cell linage to differentiate at the stage of the blastocyst, occurring at about 6 days post conception (Huppertz et al, 2005). This then further differentiates into 2 different types, the villous and the extravillous trophoblasts. The human placenta is haemochorial; implying that maternal vascular integrity is disrupted by the invasive extravillous cytotrophoblast (EVT) cells, bringing maternal blood into direct contact with the placenta (Kingdom et al, 2011). This process is takes place under low oxygen tension (to avoid oxygen toxicity during embryogenesis) and is achieved by to effective (but controlled) haemostasis.

The EVT is a differentiated cell lineage possessing a unique phenotype and is specialized for adhesion, degradation, and migration through uterine stromal extracellular matrix and restructuring uterine spiral arteries. The EVT cells move within the spiral arteries, replacing the endothelium and acquiring a pseudoendothelial phenotype as they do so. Uteroplacental blood flow increases exponentially in the second trimester of pregnancy, far exceeding the required rate needed for adequate oxygenation and nutrient deliverance, and the replaced endothelium allows this process to occur at relatively low pressure (Hubbertz et a, 2008) (Figure 1).

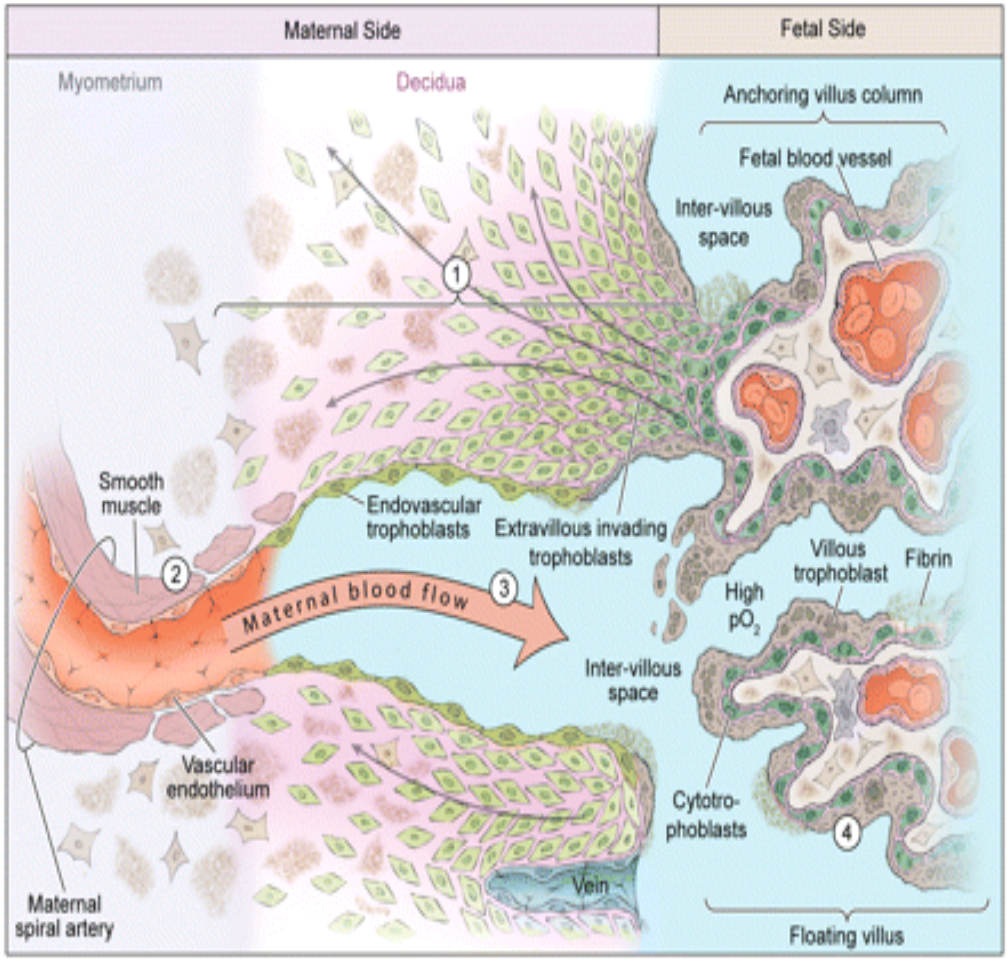


Figure 1

Normal placental development. Extravillous cytotrophoblasts proliferate in anchoring columns to successfully invade through the decidua (1) and transform the distal spiral arteries (2). These changes mediate high volume flow at low pressure into the intervillous space (3). The placental villi are covered by the villous trophoblast compartment (4), comprising cytotrophoblasts that proliferate to generate the outer syncytiotrophoblast in direct contact with maternal blood (reproduced from Kingdom et al, 2011).

1.4.2 Placental development in pre-eclampsia

The pathogenesis of pre-eclampsia is largely thought to be poor invasion of the uterine spiral arteries by the trophoblast, leading to hypoperfusion, necrosis and a high resistance circulation (figure 2).

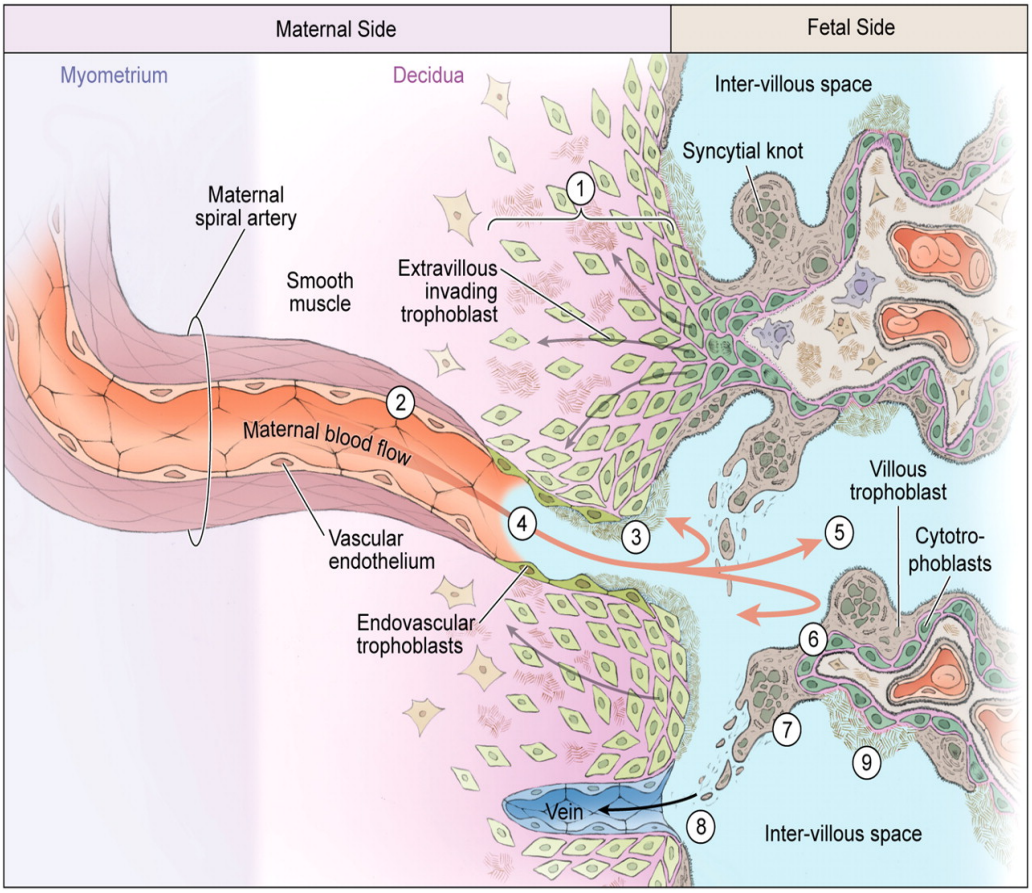


Figure 2 *Uteroplacental vascular insufficiency. Extravillous cytotrophoblasts are less successful in invading the maternal decidua and may be removed by the maternal immune system (1). Consequently the distal spiral arteries are narrower (2) and diseased, accompanied by atherosis or local fibrin deposition (3) and reduced endovascular invasion (4). Hypoxia or hypoxia-reoxygenation injury (5) has direct effects on the villous trophoblast compartment, reducing syncytial fusion (6) that may trigger the formation of syncytial knots (7). These accumulate but may fragment and shed into maternal blood (8), whereas areas deficient in syncytial fusion may exhibit focal necrosis (9). (Adapted from Kingdom et al, 2011).*

The method by which this occurs remains unclear, although there does appear to be genetic factors, immune abnormalities and other factors (such as oxidative stress) involved.

1.4.3 Genetic factors

The majority of cases of pre-eclampsia occur in nulliparous women with no family history of the disease. However, as previously mentioned there does appear to be some genetic link, with the incidence of pre-eclampsia 3-4 times higher in women with a first degree relative affected (Duckett et al, 2005). This, combined with “the paternal factor” in the pathogenesis of pre-eclampsia, suggests a potential genetic link.

Dekker et al suggest that genomic imprinting results in involvement of paternal genes in the control of invasion and placental growth, whereas maternal genes inhibit it and are responsible for the adaptive immune response of pregnancy (Dekker et al, 2011). These epidemiological-genetic links have led researchers to try to discover “the pre-eclampsia gene”. Goddard et al (2007) studied 775 single-nucleotide polymorphisms (SNP) in 190 genes, in 350 women and offspring pairs with pre-eclampsia and 600 control pairs. They detected six genes with a significant maternal-fetal genotype interaction related to pre-eclampsia in IGF1, IL4R, IGF2R, GNB3, CSF1, and THBS4 (table 2).

Gene	Function	SNP
IGF1	Codes for insulin like growth factor 1	rs5742620
IL4R	Interleukin 4 receptor	rs3024678
IGF2R	insulin-like growth factor 2 receptor	rs2274849
GNB3	Guanine nucleotide-binding protein	43188143
CSF1	Macrophage colony-stimulating factor	rs1058885
THBS4	Thrombospondin 4	rs256439

Table 2: Genes associated with increased incidence of pre-eclampsia

These and others findings suggest a multifactorial polygenic inheritance with a genetic component in the development of this disease (Valenzuela et al, 2012).

1.4.4 Immune factors

As previously discussed, women who have less exposure to paternal semen are more at risk of developing the condition, suggesting pre-eclampsia may be due to an impaired maternal response to fetal antigens.

The immune environment of the uterus is such that, in pregnancy it allows a semi-allogenic entity to survive and thrive. In order for this to happen, there must obviously be a change within immune pathways. From studying women who have human immunodeficiency virus (HIV), it appears that an intact innate immune system is required. Women who have untreated HIV are less likely to develop pre-eclampsia, but when treated with anti-retroviral medications, their risk returns to the level of the non-HIV population (Hall et al, 2007).

Natural Killer (NK) cells, macrophages and dendritic cells are mediators of innate immunity, with macrophages and dendritic cells being the major antigen presenting cells within the uterus. The presence of these cells allows adaption of the maternal immune system and prevents pregnancy loss. Studies of placental macrophages have shown that in pregnancies affected by pre-eclampsia there are significantly more macrophages in this cohort's placentae, and it has been suggested that macrophage infiltration is implicit in impaired trophoblast invasion (Zhang et al, 2013).

NK cells have been shown to initiate smooth muscle remodeling of the maternal spiral arteries (Robson et al, 2011) and genetic studies of polymorphisms in the killer immunoglobulin receptors (KIRs) on maternal NK cells and the fetal human leukocyte antigen (HLA)-C haplotype suggest patients with the KIR-AA genotype and the fetal HLA-C2 genotype are at a significant risk of developing pre-eclampsia (Hubbertz et al, 2008).

1.4.5 Inflammation and oxidative stress

Redman et al have shown that normal pregnancies are associated with a maternal systemic inflammatory response, and suggest that this response is exaggerated in pre-eclamptic pregnancies (Redman et al, 2003). This inflammatory state activates several associated pathways, including those involved in oxidative stress. Reactive Oxygen Species (ROS) are widely used as second messengers to propagate pro-inflammatory or growth stimulatory signals. In consequence, oxidative stress and exaggerated inflammation are related, and perhaps are an inseparable phenomena (Redman et al, 2000). Oxidative stress has been shown to be involved in vasculopathy and has therefore been postulated to be involved in pre-eclampsia development. Some work has looked at the link with prevention of pre-eclampsia with anti-oxidants such as vitamins C and E. This work is described later.

Although several mechanisms have been postulated as being a cause of pre-eclampsia, the placental under perfusion leading to ischaemia and necrosis/infarction is agreed to be the end point that results in the systemic syndrome. The injured placental villi release splice variant decoy receptor proteins such as soluble fms-like tyrosine kinase (sFLT1), which antagonises the actions of proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and placenta-like growth factor (Hubbertz et al, 2008). By binding with these growth factors, sFLT1 prevents their pro-angiogenic effect. Other vascular angiogenic proteins are also deranged in

pre-eclampsia. Soluble endoglin (sEng) is a truncated form of endoglin, a cell surface receptor for Transforming Growth Factor Beta (TGF-β). sEng amplifies vascular damage mediated by sFLT1 and studies have shown, that when administered to rodents it induces a severe pre-eclampsia like disorder (Maynard et al, 2011).

It is thought that these deranged angiogenic proteins, cause systemic vascular dysfunction, capillary leaking and vasospasm, which in turn leads to the clinical spectrum of pre-eclampsia (figure 3).

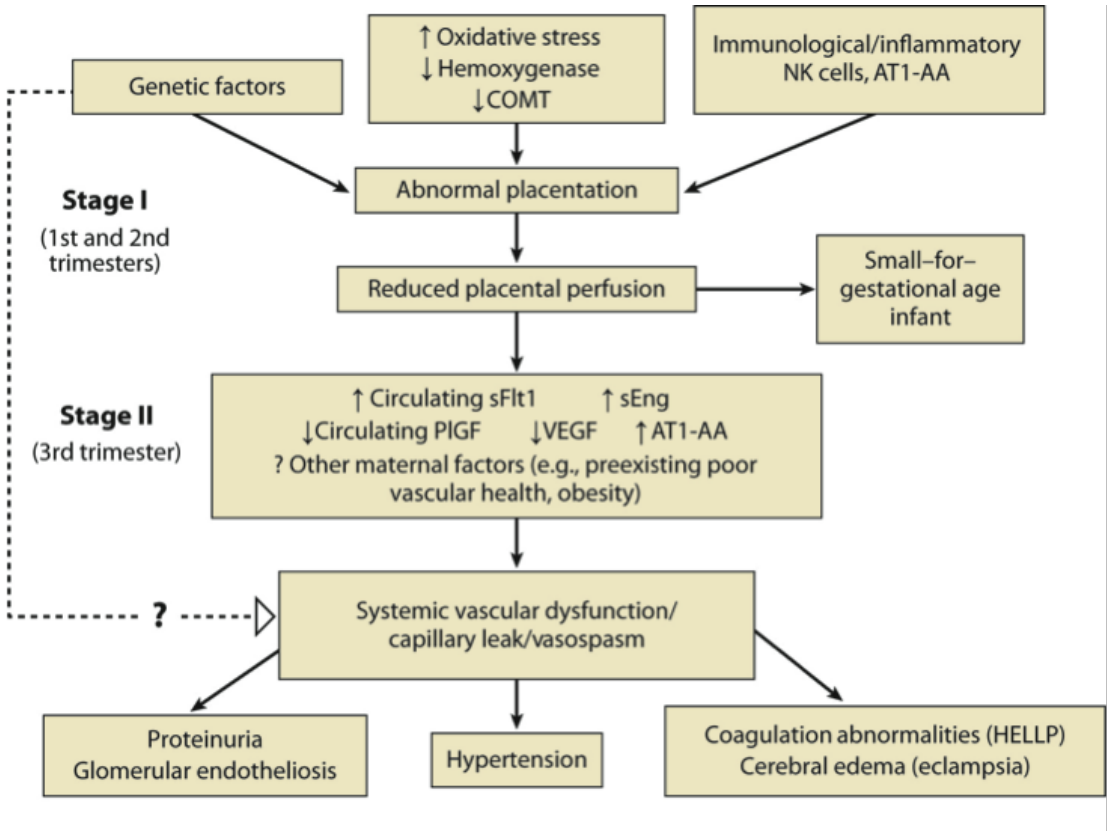


Figure 3 *Summary of the pathogenesis of preeclampsia. Genetic factors, immune abnormalities [natural killer (NK) cell/human leukocyte antigen (HLA)-C axis], and other factors such as oxidative stress may cause placental dysfunction, which in turn leads to the release of antiangiogenic factors [such as soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng)] and other inflammatory mediators to induce hypertension, proteinuria, and other complications of preeclampsia. Abbreviations: AT1-AA, angiotensin type II receptor; COMT, catechol-O-methyltransferase; HELLP, hemolysis, elevated liver enzymes, and low platelet syndrome; PlGF, placental growth factor; VEGF, vascular endothelial growth factor (adapted from Young et al, 2010)*

1.5 Current screening methods for pre-eclampsia

Several attempts have been made to predict those women who are at risk of developing pre-eclampsia. If we could accurately identify these women, then interventions could be implemented with the aim of preventing disease progression.

1.5.1 Maternal History

Although a poor predictor of pre-eclampsia, NICE recommend screening for pre-eclampsia according to clinical history. However, this guideline has only moderate ability to predict pre-eclampsia, as it identifies roughly 60% of pregnant women as high-risk with only 30% of those deemed high risk developing the disease (NICE, 2010). A large prospective international study looked at phenotypic characters aimed at predicting those at risk of developing the disease. The Screening for Pregnancy Endpoints (SCOPE) study reviewed healthy nulliparous women with the aim of stratifying them into high and low risk of developing pre-eclampsia. They developed a referral framework (figure 4) based on assessment of several clinical risk factors, but warned that it would only moderately predict pre-eclampsia and further validation models are required. The clinical risk factors included; decrease of >5 years of age*, increase of 5mmHg in mean arterial blood pressure*, increase of 5 of BMI*, family history of pre-eclampsia, family

history of coronary heart disease, decrease of 500g in maternal birth weight, > 5days of vaginal bleeding, bilateral notching of the uterine artery Doppler and an increase of 0.1 of the mean uterine artery resistance index (RI) (* from the mean of the group) (North et al, 2011).

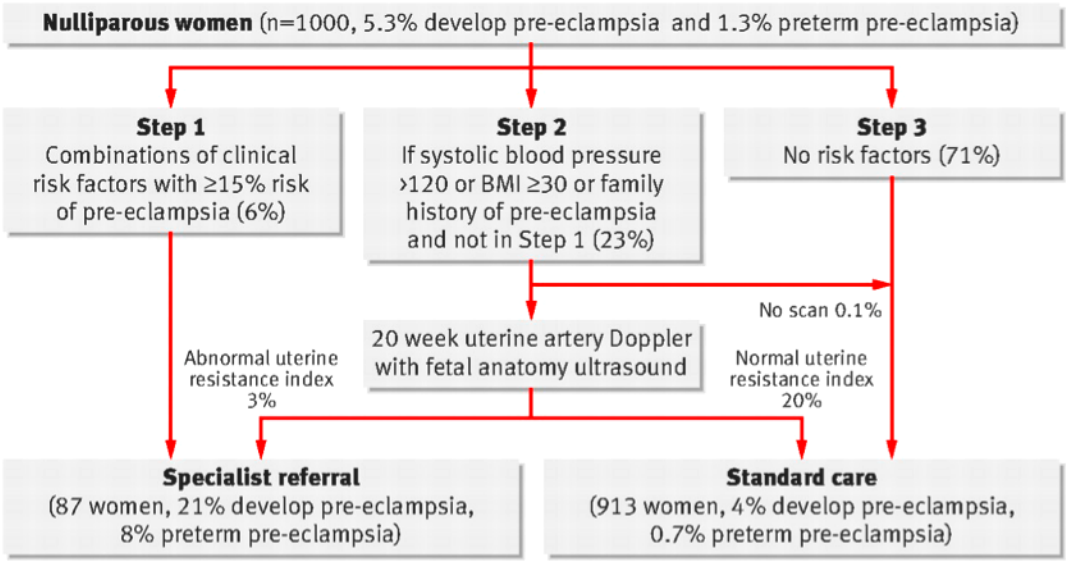


Figure 4: Framework for specialist referral when estimated risk of pre-eclampsia is ≥15% in model or presence of clinical risk factor with abnormal result on uterine artery Doppler scan.

1.5.2 Blood pressure

Subtle changes in blood pressure in the first and second trimester have been suggested to predict the onset of pre-eclampsia. A systematic review by Cnossen suggested that mean arterial pressure (that is, twice the diastolic plus the systolic blood pressure, divided by three) of greater than 90 mmHg obtained in the second trimester predicts the onset of pre-

eclampsia in low risk women, with a positive likelihood ratio of 3.5 and a negative likelihood ratio of 0.39. In high risk populations a diastolic blood pressure of 75 mm Hg or more at 13 to 20 weeks' gestation best predicted pre-eclampsia, although the accuracy of prediction was modest (positive likelihood ratio 2.8; negative likelihood ratio 0.39). Systolic blood pressure; an increase of systolic blood pressure or increase of diastolic blood pressure, or both, predicted pre-eclampsia poorly (area under the curve <0.70) (Cnossen et al, 2008).

1.5.3 Uterine Artery Doppler

In the non-pregnant state, uterine artery Doppler waveforms are characterised by a rapid rise and fall of uterine artery velocity in systole, and the high resistance spiral arteries cause notching of the velocity in early diastole. During normal pregnancy, the high resistance system is replaced by a low resistance system, which leads to loss of this diastolic notching (figure 5).

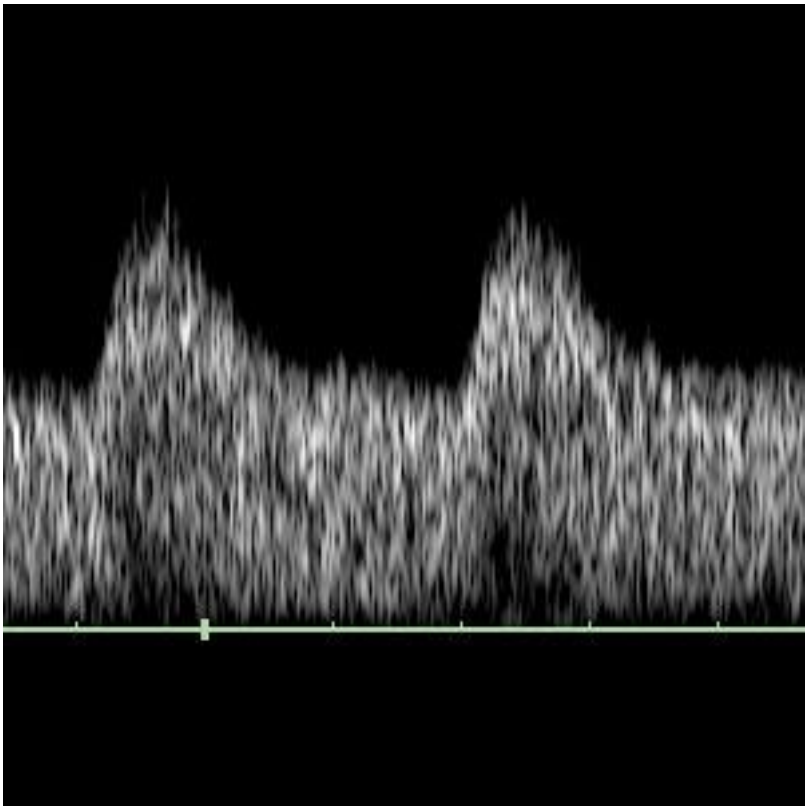


Figure 5*: Uterine artery Doppler at 24 weeks gestation (adapted from Nicolaides, 2000)*

In pre-eclamptic pregnancies, the impaired trophoblastic invasion of the spiral arteries leads to a persistence of the high resistance state. This placental dysfunction is characterised by the presence of notching of the uterine artery velocity during early diastole, and an increase in resistance index (RI) and pulsatility index (PI) (where the RI = maximum-minimum velocity divided by the maximum velocity, representing the resistance to blood flow in a vascular bed distal to the site of measurement and PI = maximum-minimum velocity / mean velocity, representing the average flow of blood) (Nicolaides et al, 2000).

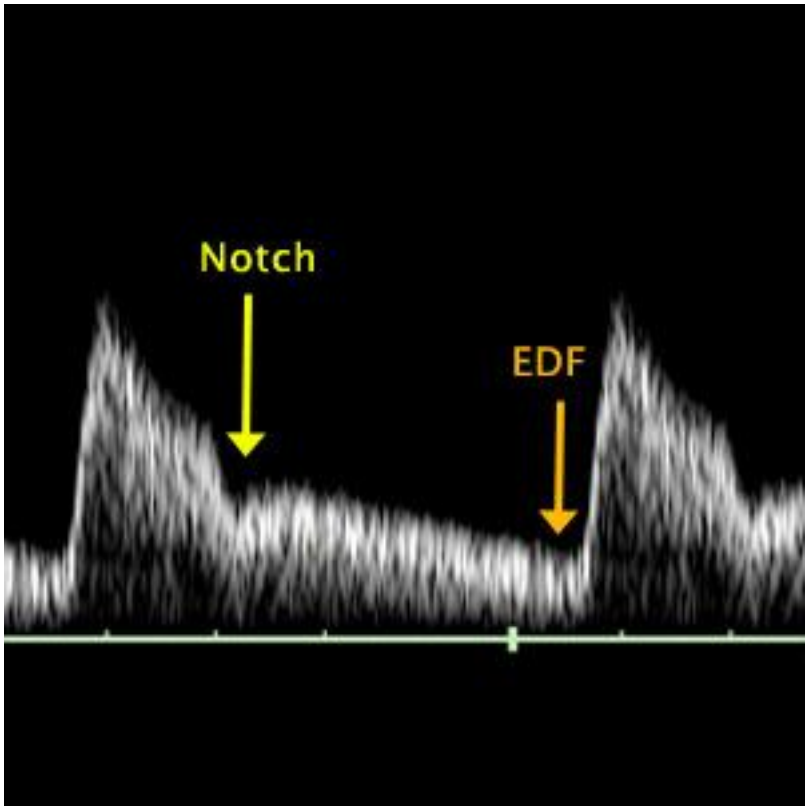


Figure 6: *Flow velocity waveform from the uterine artery at 24 weeks of gestation in a pregnancy with impaired placentation; in early diastole there is a notch (yellow arrow) and in late diastole there is decreased flow (orange arrow) (adapted from Nicolaides et al, 2000)*

Cnossen showed that Severe pre-eclampsia in low-risk patients was best predicted in the second trimester by an increased pulsatility index (positive likelihood ratio 15.6, 95% CI 13.3–17.3; negative likelihood ratio 0.23, 95% CI 0.15–0.35) and bilateral notching (positive likelihood ratio 13.4, 95% CI 8.5–17.4); negative likelihood ratio 0.4 (95% CI 0.2–0.6). Among high-risk patients, pre-eclampsia was best predicted in the second trimester by unilateral notching (positive likelihood ratio 20.2, 95% CI 7.5–29.5; negative

likelihood ratio 0.17, 95% CI 0.03–0.56) and an increased pulsatility index with notching (positive likelihood ratio 21.0, 95% CI 5.5–80.5; negative likelihood ratio 0.82, 95% CI 0.72–0.93). These results from 2008, studying 79517 women, challenged previously held ideas about the lack of sensitivity of uterine artery Doppler to predict pre-eclampsia. However, due to the high false positive rate, the authors do offer caution in using uterine artery Doppler to predict pre-eclampsia in high-risk women (Cnossen et al, 2008).

1.6 Biomarkers

Given the significant morbidity that pre-eclampsia can bring, many people have sought to find the elusive perfect screening test(s) which can predict the development of pre-eclampsia, much in the same way that screening for trisomy 21 is undertaken.

1.6.1 Angiogenic factors

As previously described, one of the hallmarks of the pathogenesis of pre-eclampsia is alteration in angiogenesis in the ischaemic placenta. The hypoxic placenta releases a host of angiogenic factors, with alteration in the normal equilibrium of pro-angiogenic versus anti-angiogenic factors detectable in maternal blood from as early as the first trimester of pregnancy (Myatt et al, 2012).

Vascular Endothelial Growth Factor (VEGF) can promote endothelial cell proliferation, migration and survival, and exerts its biologic effects through two high-affinity tyrosine kinase receptors: VEGFR-1 (VEGF receptor-1 also known as fms-like tyrosine kinase-1 (FLT-1)) and VEGFR-2 (VEGF receptor-2 or KDR/Flk-1 also known as kinase domain receptor). VEGFR-1 has two isoforms: a transmembranous form and a soluble form (sVEGFR-1). The latter is generated by a splice variant of the VEGFR-1 gene and contains the extracellular ligand-binding domain, while lacking the signaling tyrosine kinase domain. Thus, this isoform binds VEGF and inhibits its biological activities (Shibuya et al, 2013). A similar pro-angiogenic protein secreted in pregnancy is placental growth factor (PlGF). PlGF is a member of the VEGF family and shares 42% of the amino acid sequence identity with VEGF and they also share significant structural similarity (Sibiude et al, 2012). Both VEGF and PlGF rise gradually in pre-eclampsia and reach a peak concentration at 29-32 weeks gestation (Chen, 2009). Placental cells also secrete a soluble isoform of FLT-1 (sFLT-1), which is generated through alternative splicing of the messenger RNA and acts as an anti-angiogenic factor by interacting with, and thereby neutralising, PlGF and VEGF (Monte et al, 2011). There is strong evidence for the occurrence of higher placental expression of sFLT-1 in pre-eclampsia. There are also elevated circulating levels of sFLT-1 and reduced free bioactive PlGF and VEGF and it has therefore been suggested that a part of this excess of circulatory sFLT-1 may in fact stem from the placenta (Chen et al, 2009). In women who develop pre-eclampsia, sFLT-1 is elevated from the second

trimester onwards (Monte et al, 2011), with lower levels of VEGF and PlGF detected even as early as the first trimester of pregnancy (Romano et al, 2008). Soluble endoglin (sEng), a circulating antagonist to TGF-β, has been found to be increased in both the placenta and serum of women with preeclampsia. Exogenous sEng exacerbated proteinuria, hypertension, and resulted in a HELLP-like syndrome in a rat model of sFLT1-induced preeclampsia, suggesting sEng may contribute to the pathogenesis of the disease (Hladunewich et al, 2007). Subsequent studies have verified that sEng, in addition to sFLT1 and PlGF, are altered several weeks prior to the clinical manifestations of pre-eclampsia in healthy, nulliparous women (Chen et al, 2009). A further systematic review by Kleinrouweler reviewed 32 studies that identified angiogenic factors as potential biomarkers for predicting pre-eclampsia. Although PlGF, sFLT1 and sENG showed modest but significantly different concentrations before 30 weeks of gestation in women who developed pre-eclampsia, test accuracies of all four markers remain too poor for an accurate prediction of women who will ultimately develop pre-eclampsia in clinical practice (Kleinrouweler et al, 2012).

1.6.2 Uric acid

Siemons et al were the first group to show the link between pre-eclampsia and raised plasma uric acid levels (Siemons, 1917). Uric acid is a product of purine breakdown and with an increasing cell turn over in the placental

bed with advancing gestation, plasma concentration rises naturally as a pregnancy advances. However, several studies have shown that plasma uric acid concentration is raised further during pre-eclampsia. This has been suggested to be the case for two reasons. Firstly, the injured placenta has an increase in cell breakdown with an associated increase in purine breakdown. Secondly, absorption of uric acid from the loop of Henle is impaired in pre-eclampsia. Thangaratinam undertook a systematic review of 18 articles that analysed the ability of uric acid to predict outcomes in women with pre-eclampsia. They concluded that uric acid is a poor predictor of maternal and fetal complications in women with pre-eclampsia (Thangaratinam et al, 2008). Work has also been undertaken to review whether uric acid can be used as a predictor of disease development. Cnossen undertook a systematic review and meta-analysis of 5 articles consisting of 572 women, 44 of whom had pre-eclampsia. Their review was unable to identify uric acid having the ability to predict those who will develop the condition (Cnossen et al, 2005).

1.6.3 Plasma protein 13 (PP13)

PP13 is produced predominantly by the syncytiotrophoblast and is thought to play a major role in the implantation of the blastocyst. From the first-trimester onward, levels of PP13 slowly increase in healthy pregnancies. First-trimester concentrations of PP13 have been shown to be significantly lower in the first trimester, but higher in the second and third trimesters in

association with pre-eclampsia (Sammar et al, 2011). More specifically, several studies reported significant differences between the median PP13 Multiples of Median (MoM) of early onset pre-eclampsia and control pregnancies in the first trimester of pregnancy (Chafetz et al, 2007). Schneuer et al performed analysis of 2678 women, 71 who had pre-eclampsia. They showed that PP13 had a moderate ability to predict the onset of pre-eclampsia (area under the curve (AUC) = 0.72). However, in the same study they undertook a systematic review of PP13 and whether it could predict the onset of pre-eclampsia. Despite their own relatively significant results, they were unable to reproduce this from a meta-analysis of 8 papers. They therefore concluded that PP13 is unable to predict the onset of pre-eclampsia (Schneuer et al, 2012).

1.6.4 ADAM 12

ADAM 12 is a member of the A Disintegrin And Metalloproteinase (ADAM) family. It has been identified in pregnancy, but not in non-pregnant women, and is thought to be involved in trophoblast development. Studies have shown that ADAM 12 is significantly reduced in first trimester plasma samples of women who go on to later develop pre-eclampsia (Laigaard et al, 2005). However, other studies have contradicted these findings (Poon et al, 2008), and therefore further work (or a systematic review and meta analysis) is required to confirm its ability to predict pre-eclampsia.

1.6.5 Human Chorionic Gonadotropin and Pregnancy Associated Plasma Protein-A

These two proteins are currently tested in the first trimester of pregnancy as part of the national Down syndrome screening programme. Human chorionic gonadotropin (hCG) is released by the syncytiotrophoblast and promotes:

- Progesterone production by corpus luteum cells;
- Angiogenesis in uterine vasculature;
- The fusion of cytotrophoblast cell and differentiation to make syncytiotrophoblast cells;
- The blockage of any immune or macrophage action by mother on foreign invading placental cells
- Uterine growth parallel to fetal growth;
- Suppression of any myometrial contractions during the course of pregnancy;
- Growth and differentiation of the umbilical cord;
- Signals to the endometrium about forthcoming implantation;
- Receptor stimulation in mother's brain causing hyperemesis gravidarum,

And also promotes growth of fetal organs during pregnancy (Cole et al, 2010). Given its role as a marker of pregnancy and it's altered concentration in trisomic pregnancies, hCG has been studied as to whether

it is altered in pre-eclampsia (both during the clinical syndrome and prior to the onset of the clinical disease). Studies from early in pregnancy (during the first or second trimester) have shown that before the disease state, women with high levels of hCG are more at risk of developing pre-eclampsia, than those who do not (Basirat et al, 2006, Olsen et al, 2012). This is thought to be due to placental injury releasing more of the syncytiotrophoblast derived hCG into the maternal circulation.

PAPP-A is a metalloproteinase that is involved in the function of insulin like growth factors and local proliferative processes. It has been shown to be reduced in pregnancies that go on to develop pre-eclampsia, and this reduction is consistent across all trimesters (Yaron et al, 2002).

Morris et al undertook a systematic review and meta analysis to see whether markers currently used to predict Down's syndrome can be used to predict pre-eclampsia. 21 studies identified hCG as a possible candidate, with all but 3 of the studies using second trimester samples. hCG > 2.0 MoM in the second trimester was the best marker to predict the risk of developing pre-eclampsia (LR+ 2.45 (1.57,3.84), LR- 0.89 (0.83,0.96)). 16 studies, all from first trimester samples, studied whether PAPP-A has the ability to predict the onset of pre-eclampsia. The most accurate predictor was PAPP-A<5th centile (LR+ 2.10 (1.57,2.81), LR- 0.95 (0.93,0.98)). However, they concluded that Down serum screening analytes had a low predictive accuracy for pre-eclampsia. They advised that they maybe a useful means of risk assessment or be of use in prediction when combined

with other tests, and advised that further studies were required (Morris et al, 2008).

1.7 Factors that reduce the risk of developing pre-eclampsia

Currently there is no cure for pre-eclampsia. When the clinical syndrome develops, a risk-benefit decision is made regarding the benefit of prolonging the pregnancy to improve fetal maturity, which is done mainly by managing hypertension and the risk of continuing the pregnancy and worsening of the maternal disease and fetal consequences. There has been a move to prevent the disease in women who are at high risk of developing the condition, and this prevention occurs before the clinical syndrome has developed.

1.7.1 Aspirin

Pre-eclampsia is associated with reduced production of prostacyclin, a powerful vasodilator, and an increase in thromboxane, a vasoconstrictor and a stimulant for platelet aggregation (Briceno-Perez et al, 2009). Given this, there has been much work on the effects of anti-platelet agents on pre-eclampsia and their use in preventing disease development. Aspirin is a cyclooxygenase (COX) inhibitor. By preventing COX from irreversibly catalysing the transformation of arachidonic acid into thromboxane, aspirin

prevents platelet aggregation in the placental bed. A Cochrane review of 59 trials, consisting of 37560 women, suggests an overall 17% reduction in pre-eclampsia (relative risk 0.83, 95% confidence interval (CI) 0.77 to 0.89). Women at high risk of developing pre-eclampsia had a significant increase in the absolute risk reduction of pre-eclampsia for high risk (risk difference (RD) -5.2% (-7.5, -2.9), NNT 19 (13, 34)) compared with moderate risk women (RD -0.84 (-1.37, -0.3), NNT 119 (73, 333)) (Duley et al, 2007). Since this cochrane review, others have tried to emulate their findings. Villa et al (2013) performed a randomised placebo control trial assessing whether aspirin could prevent pre-eclampsia in high risk women, and in addition undertook a further systematic review and meta-analysis. Whilst their trial suggested no significant difference in pre-eclampsia rates between the placebo and the treatment group, their meta-analysis suggested aspirin could reduce the incidence of pre-eclampsia in high risk women (Villa, 2013). NICE have adopted this policy for advising the prescribing of aspirin to some groups of women in pregnancy (NICE, 2010). They recommend that women who are at high risk of developing pre-eclampsia should start aspirin at 12 weeks of gestation until birth of their baby. They define high risk women as a woman with any of the following:

- Hypertensive disease during a previous pregnancy
- Chronic kidney disease
- Autoimmune disease such as systemic lupus erythematosus or antiphospholipid syndrome

In addition, they suggest that women who are at moderate risk of developing pre-eclampsia should also take aspirin from 12 weeks of gestation. Women with more than one moderate risk factor for pre-eclampsia should take 75 mg of aspirin daily from 12 weeks until the birth of the baby. Factors indicating moderate risk are:

- first pregnancy
- age 40 years or older
- pregnancy interval of more than 10 years
- body mass index (BMI) of 35 kg/m2 or more at first visit
- family history of pre-eclampsia
- Multiple pregnancies.

1.7.2 Calcium supplementation

An inverse relationship between calcium intake and hypertensive disorders of pregnancy was first described in 1980 but the exact mechanism by which calcium may reduce pre-eclampsia remains uncertain (Belizan et al, 1980). Calcium may increase blood pressure by stimulating either parathyroid hormone or renin release, thereby increasing intracellular calcium in vascular smooth muscle and leading to vasoconstriction. A possible mode of action for calcium supplementation is that it reduces parathyroid release and intracellular calcium and so reduces smooth muscle contractility (Hofmeyr et al, 2004).

A Cochrane review in 2010 concluded that calcium supplementation could reduce the incidence of pre-eclampsia by almost half (Hofmeyr et al, 2006). Thirteen studies of good quality (involving 15,730 women) were included in the review. The average risk of high blood pressure was reduced with calcium supplementation rather than placebo (twelve trials, 15,470 women: risk ratio (RR) 0.65, 95% confidence interval (CI) 0.53 to 0.81). There was also a reduction in the average risk of pre-eclampsia associated with calcium supplementation (thirteen trials, 15,730 women: RR 0.45, 95% CI 0.31 to 0.65). The effect was greatest for women with low baseline calcium intake (eight trials, 10,678 women: RR 0.36, 95% CI 0.20 to 0.65) and those selected as being at high risk (five trials, 587 women: RR 0.22, 95% CI 0.12 to 0.42).

1.7.3 Heparin

As discussed previously, the placenta in pre-eclamptic pregnancies is associated with thrombosis, necrosis and infarction. In view of these findings it has ben suggested that heparin might be used to prevent these pathological changes and potentially prevent development of the disease. Heparin is a large, complex macromolecule that has been used in pregnancy to improve perinatal outcomes. This was initially thought to be via its broad anticoagulation properties. However, heparin has several non-anticoagulant effects on the placenta (Kingdom et al, 2011):

- Heparin promotes cytotrophoblast proliferation: Heparin facilitates dimerisation of FGFs to enhance mitotic signalling. This action of heparin may reduce the risk of severe pre-eclampsia by promoting the production of cytotrophoblasts for syncytial fusion and maintenance of a healthy outer syncytiotrophoblast in contact with maternal blood.
- Suppression of complement pathway activation. Women destined to develop pre-eclampsia have elevated circulating levels of complement-activation factor Bb, but not C3a or sC5b-9. Because Bb levels are associated with maternal obesity, complement activation may be a dominant pathway in the maternal phenotype of pre-eclampsia. By suppressing factor Bb this may reduce systemic and endometrial inflammation.

  Unfortunately the findings from randomised trials have failed to show significant reduction in pre-eclampsia when treated with heparin (Kingdom et al, 2011).

1.7.4 Vitamin D

Vitamin D is a fat-soluble protein that has anti-inflammatory and immune modulation effects. Several large studies have showed the link between vitamin D deficiency and the increased incidence of pre-eclampsia (Tebesh et al, 2013). Given the theory that pre-eclampsia involves an exaggerated

inflammatory process and the importance of immune factors in the disease pathogenesis, one could suggest the link between the two factors.

A Cochrane review from 2012 studied the effect of vitamin D supplementation on pregnancy outcomes. Although they studied 6 trials with a total of 1023 women, only 1 trial (n=400 women) specifically had the primary outcome for reducing the incidence of pre-eclampsia. Women who received 1200 IU vitamin D along with 375 mg of elemental calcium per day were as likely to develop pre-eclampsia as women who received no supplementation (average risk ratio (RR) 0.67; 95% confidence interval (CI) 0.33 to 1.35) (De-Regil et al, 2012). Further work is needed to assess the effect vitamin D supplementation has on reducing the incidence of pre-eclampsia, the correct dose of supplementation and the safety of supplementation.

1.7.5 Vitamins C and E

Pre-eclampsia is associated with marked oxidative stress. Given that Vitamins C and E are powerful antioxidants, several studies were undertaken to identify whether supplementation with these vitamins can reduce the incidence of pre-eclampsia. Chappell et al published a promising trial that randomised 283 women to receive vitamin C and E supplementation or placebo. In the cohort who completed the study (81 placebo group, 79 vitamin group), the odds ratio for pre-eclampsia was 0·24 (0·08–0·70, p=0·002) (Chappell et al, 1999). These exciting findings were

then studied further in a large multicentre trial that was appropriately powered. The Vitamins in Pregnancy (ViP) trial was published in 2006. This randomised placebo-controlled trial randomised 2410 women equally into placebo or treatment group. They were unable to reproduce the reduction in the incidence of pre-eclampsia in the treatment group. However, in the treatment group, more babies were born with low birth weight (adjusted odds ratio 1·15 95%) confidence interval [1·02–1·30]) (Poston et al, 2006). Given these findings, vitamin C and E supplementation cannot be advocated as a treatment to prevent the development pre-eclampsia.

1.8 Aims of this thesis

Pre-eclampsia remains one of the most serious morbidities of pregnancy. It has an impact on maternal and fetal/neonatal health with a growing body of evidence suggesting its link to long-term cardiovascular and renal disease. Although there are current mechanisms in clinical practice aimed at predicting the onset of pre-eclampsia, none of these have developed a tool which will do so accurately in all cohorts of women. Secondly, in those women who develop pre-eclampsia, there are few methods to predict those who will suffer worse outcomes. The overall aim of this work is to contribute

to our ability to accurately predict women who will ultimately develop pre-eclampsia.

1) The first hypothesis that this thesis will test is that known risk factors for developing pre-eclampsia can be used together to identify previously unidentified links. Identifying a subset of women who can be more accurately predicted to develop pre-eclampsia would allow enhanced surveillance of this group, and offer appropriate prevention strategies. To test this hypothesis I shall undertake one of the largest epidemiological studies in England and analyse a database covering a large ethnically diverse group.

2) The second hypothesis that this thesis will test is that proteomics can be used to identify markers that are altered in the first trimester of pregnancy between women who develop pre-eclampsia and those who do not. These alterations can be used to further the development of a screening test for pre-eclampsia. To test this hypothesis I shall use plasma proteomics of individual plasma samples.

3) The third hypothesis that this thesis will test is that standard clinical tests can be used to detect subtle differences in the inflammatory state during early pregnancy in women who go on to develop pre-eclampsia. In doing so, this may allow us to further understand the pathogenesis of the disease and could potentially offer a novel disease marker. To test this hypothesis I shall undertake a large retrospective case-control study from a large ethnically diverse cohort.

4) The fourth hypothesis that this thesis will test is that we can predict poor outcome in women who develop pre-eclampsia using biochemical parameters that already form part of clinical investigation during the diagnosis of pre-eclampsia. To test this hypothesis I shall undertake a retrospective cohort study.

Chapter 2

Racial variation in risk factors for developing pre-eclampsia

2.1 Introduction

Ethnicity can have a huge impact on health and health outcomes. According to genetic studies, human DNA can be clustered into groups that correspond to their ancestral geographic origins and this variation may account for the health disparities between populations (Balchin, 2011). For example, People from South Asia (SA) are more susceptible to type 2 Diabetes Mellitus (T2DM) than their European counterparts. Given this variation, it is important for different prevalence and risk factors for diseases to be identified amongst different ethnic groups, and tailor management accordingly.

2.1.1 Ethnicity

2.1.2 Definition

Ethnicity refers to “the cultural practices and outlooks of a given community of people that set them apart from others” and is derived from the ancient Greek word, Ethos, meaning custom (Giddens, 1997). The subjective nature of this definition lends itself to controversy, as it represents a person’s self-identity and can therefore be liable to change.

2.1.3 Measuring ethnicity

Measuring ethnicity can be difficult but it provides vital information for government, policy makers and researchers and therefore it is vital it is done as robustly as possible. The current mechanism of measurement is

based on the classification of ethnicity from the 2011 UK Census (shown in figure 7).

Figure 7: classification of ethnicity from the 2011 UK Census.

2.1.4 Ethnicity and pregnancy outcomes

Women from an ethnic minority (that is; a group within a community which has different national or cultural traditions from the main population) who live in the UK are more likely to have an adverse

pregnancy outcome than their European counterparts (Balchin, 2007). In the last triennial report into maternal death in the UK, Black women were three times more likely to die than white or SA women. There is also a higher rate of perinatal mortality amongst ethnic groups with black women being twice as likely and SA women being 1.6 times more likely to have a stillbirth or neonatal death than mothers of white ethnic origin (CMACE, 2009). Medical complications are also higher amongst mothers from an ethnic minority background. Gestational diabetes is one of the commonest medical conditions affecting pregnancies in the UK and there is a huge racial disparity in the prevalence across ethnic groups with an 11-fold increase amongst SA women, and 3-fold increase in Black women compared to their White counterparts (Dornhorst, 1992).

2.1.5 Ethnicity and pre-eclampsia

There is an obvious link between ethnicity and pre-eclampsia and it is well established that black women are more at risk of developing the disease. In a large American cohort study, black women were more at risk (odds ratio 1.41, 95% CI 1.25-1.62) (Caughey, 2005) and Asian women were less likely to develop pre-eclampsia than white women. A similar UK study showed black women at higher risk of the disease (Wright, 2012).

Being black also increases the likelihood of a recurrence of pre-eclampsia. This increase may be due to the direct effect of ethnicity or factors associated with ethnicity, such as deprivation (Sibai, 2005).

2.1.6 Obesity

Obesity is a worldwide epidemic that is worsening year on year. It has an impact on almost all aspects of clinical medicine from cardiovascular disease to cancer and it results from a complex interaction between the environment, genetic predisposition and human behaviour (Nguyan, 2010).

2.1.7 Definition of obesity

The World Health Organisation (WHO) defines obesity as “an abnormal or excessive fat accumulation that may impair health” however, to define obesity clinically, the Body-Mass Index (BMI) scale is used (table 3).

BMI	Weight Status
Below 18.5	Underweight
18.5 – 24.9	Normal
25.0 – 29.9	Overweight
30 – 34.9	Obese (mild, Class I)
35-39.9	Obese (moderate, Class II)
40+	Obese (severe, Class III)

Table 3: Body-Mass Index

2.1.8 Obesity in pregnancy

Obesity in pregnancy is defined as a Body Mass Index (BMI) of 30 kg/m^2 or more at the first antenatal consultation.

The prevalence of obesity in pregnancy has increased markedly from the early 1990s where it was 9-10% to the late 2000s where it is 16-19% (CMACE/RCOG, 2010) (Figure 8).

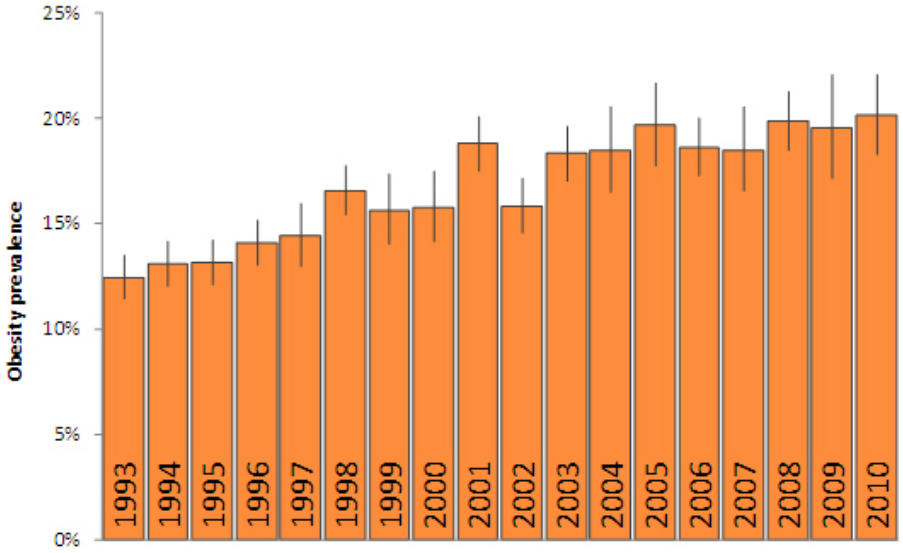


Figure 8 Prevalence of obesity in the UK amongst pregnant women since 1993 (Public Health England, 2013)

Obesity is now one of the most commonly occurring risk factors to affect obstetric practice and is associated with an increase in a number of serious adverse outcomes including miscarriage, fetal anomaly, thromboembolism, gestational diabetes, labour dystocia, postpartum haemorrhage, wound infections, stillbirth and pre-eclampsia (CMACE/RCOG, 2010).

2.1.9 Obesity and pre-eclampsia

The link between obesity and pre-eclampsia is well established. A systematic review suggested that, when compared to a normal BMI, an increased BMI at booking doubled the risk of pre-eclampsia and a BMI of > 40 increased the risk of developing pre-eclampsia 4-fold (Duckitt, 2005).

2.1.10 The West-Midlands

The West Midlands (WM) is an area of 13000 square kilometres and shares its borders with the Southeast, the South West, the East Midlands and North West regions of England and with Wales (Medland, 2011). It contains some of the largest urban areas outside of London and is home to some of the countries most deprived areas (based around Birmingham, Coventry and Stoke-on-Trent) and some of the most affluent (Such as Solihull and Warwickshire) (See figure 9).

Figure 9: Map of the West Midlands region (adapted from picturesofengland.com)

2.1.11 The West-Midlands' population

The WM had a population of just over 5.4 million in 2009, with the highest proportion of children under the age of 16 in the UK. It also has the largest non-white population outside of London, with 14 per cent of the population being classified as a non-white ethnicity. Asian or British Asian people make up 8.5% and Black or British Black make up 2.7 per cent of the

population. In addition, the WM consist of some of the most and least deprived areas in the country with Birmingham and Coventry accounting for those areas where the largest deprivation is seen (Medland, 2011).

2.1.12 Measuring Deprivation

The indices of deprivation are a method of measuring deprivation based on a concept that deprivation refers to more than just poverty; Poverty is not having enough money to get by on where as deprivation refers to a general lack of resources and opportunities (UK government, 2010). It is based on 38 separate indicators covering areas such as income, employment, health, education, crime access to services and living environment with each UK postcode generating its own index of multiple deprivation (IMD) on a scale of 1-5 (1 being the least deprived and 5 being the most deprived).

2.1.13 The Perinatal Institute

The Perinatal Institute (PI) is a not-for-profit organisation set up to improve the quality and safety of maternity care. This achieved via numerous avenues

- Provision and training of standardised maternity note and note-keeping
- Perinatal audit
- Fetal growth assessment

2.1.14 Standardised Maternity notes

The PI maternity notes provide complete coverage of events in a woman's pregnancy, including the antenatal, intrapartum and postpartum periods. The antenatal notes form part of the mother's hand held documentation of her pregnancy care and whilst there is currently no consensus on a layout or style of how these notes should structured (with each maternity unit able to use their own version of maternity notes), over half of maternity units in the UK use the PI notes, accounting for two thirds of all pregnant women.

2.1.15 Perinatal audit

Perinatal mortality and morbidity in the WM is amongst the highest in the UK. To try and reduce this, the PI has been funded to a) audit the care of women who have the babies in the WM and b) introduce a robust fetal growth surveillance programme. In order to undertake the perinatal audit, the pregnancy details of the women where uploaded to the NHSnet based perinatal episode electronic record (PEER) database.

2.1.16 Perinatal episode electronic record (PEER) database.

Collection for the PEER database was undertaken prospectively from the handheld maternity notes, used by the 19 maternity units in the WM, from April 2009. Trained data-entry clerks transferred the information from the notes onto the database at the end of the pregnancy, with a robust quality assurance programme through the use of training workshops and regular on-site data quality audits. 87 data items were collected including age, parity, ethnic origin, and maternal height and weight (expressed as body mass index); social factors, including employment status of the mother and her partner, consanguinity with the partner, and index of multiple deprivation; history of mental health problems, pre-existing diabetes or hypertensive disease, or previous stillbirth; smoking status, alcohol consumption, non-prescription drugs, folic acid intake, and time of first visit in pregnancy (the information for all these previous variables was usually recorded at the early pregnancy booking visit); complications in pregnancy, including gestational diabetes, antepartum haemorrhage, pregnancy induced hypertension, and pre-eclampsia and fetal or neonatal characteristics, including sex, gestational age and weight at birth, and estimated weight during pregnancy (Gardosi, 2013).

2.2 Aim

The aim of this work is to identify ethnic variations of risk factors for developing pre-eclampsia and whether there are variations in poor outcome once the clinical syndrome has established.

2.3 Methods

2.3.1 Study design

This work was part of a much larger prospective observational study. An observational study is one "in which no intervention is made (in contrast to a experimental study) and such studies provide estimates and examine associations of events in their natural settings without recourse to experimental intervention" (Mann, 2003). There are many different types of observational studies such as cross-sectional (where the prevalence is calculated and any determining risk factor identified at one specific point in time), case-control (comparing those with a disease; the case, with those who do not; the control) and cohort studies. A cohort study follows a defined group of people and they are generally seen as the best way of determining incidence and natural history of a condition (Mann, 2003). This

work studies a group of women, all of who have pre-eclampsia, and is undertaken retrospectively by interrogating a pre-existing database. It is therefore a retrospective cohort study.

Retrospective cohort studies can provide powerful results. They are advantageous when assessing rare disorders or groups and have the benefit of being able to examine multiple outcomes. However, they are only as good as the data collected. Poorly collected or incomplete data can limit the study findings and this can lead to selection bias. The PEER database prospectively collects data regarding nearly all of the clinical and social aspects of a woman's pregnancy. Interrogating such a robust database allows for all potential cofounders and exposures to be assessed and reduces any potential recall bias as data is collected prior to the follow up and without any knowledge of any hypotheses being tested (Baron, 2000).

Ethical approval was not required; data were collected with patient consent and were pseudonymised before analysis (Gardosi, 2013).

2.3.2 Cohort Identification

Data was obtained on women from April 2009 to August 2011 who were recorded as having pre-eclampsia during their first pregnancy. This prevented women who had pre-eclampsia in several pregnancies being included multiple times. For the purpose of this study the ISHHP definition

of pre-eclampsia was used (chapter 1). The diagnosis of pre-eclampsia was made by clinical staff, and recorded in the patient's records. A trained data clerk extrapolated data and the inclusion and exclusion criteria of the search are listed in table 4.

Inclusion Criteria	Exclusion Criteria
Diagnosis of pre-eclampsia	Multiparity
Primigravidae	Multiple pregnancies
Singleton pregnancies	

Table 4: Entry criteria during patient selection

After a woman self-identified her ethnicity, this was then grouped into White, Black, South Asian Middle-Eastern or Other. In order to assess the racial variation of certain risk factors for developing pre-eclampsia, a list of other known risk factors was developed and obtained from the database. These are listed in table 5.

Risk factor for developing pre-eclampsia
Age
Pre-existing medical conditions (diabetes / hypertension)
Smoking history

Table 5: Risk factors for developing pre-eclampsia

2.3.3 Statistical analysis

Logistic regression models with the outcome pre-eclampsia and the null hypothesis that model coefficients are not different between racial groups were used to generate odds ratios to test the difference in rates of pre-eclampsia between racial groups. The predictor variables were racial groups, using white women as a reference, and body mass index. Odds ratios were corrected for confounding factors of known clinical relevance and from previous publications (Duckitt, 2005) and included age, previous medical history and smoking history.

Frequency and percentages were calculated to show the distribution of the study variables. Cross tabulation was used to show the comparisons between key study variables. Variables were treated as categorical to account for potentially non-linear relationships. X^2 was used to analyse categorical variables and Mann-Whitney U was used to analyse continuous variables. Variables were entered using the manual stepwise (forward-

backward) method, and those reaching a 0.05 significance level were retained in the model. There was no correction for multiple testing because these variables has strong association with pre-eclampsia (p=<0.001) and therefore would be significant even if the Bonferroni rule were applied (Perneger, 1998).

In addition, the outcomes in the women diagnosed with pre-eclampsia were studied. The predictor variables were racial groups (again, using white women as a reference) and fetal and maternal outcomes. The presence of intrauterine growth restriction was established on the basis of a birth weight below the 10th weight for gestational age centile, using the gestation related optimal weight standard (GROW)(Gardosi,1992), with coefficients derived from the West Midlands population. This method defines the fetal growth potential by excluding pathological factors such as smoking and diabetes, and individual adjustment or “customisation” for the baby’s sex and the mother’s height, weight, ethnic origin, and parity (Gardosi, 1995. Gardosi, 2012). A weight that is small for gestational age after such adjustment by growth potential has been shown to represent pathological smallness and is referred to as fetal growth restriction. We applied the 10th centile as the cut-off, as it is in standard clinical use and has been validated through receiver operator curves as being close to optimal for predicting pathology by customised centiles.

The SpSS computer programme (SpSS v19, Chicago, USA) was used for the statistical analysis and statistical significance was defined as p<0.05.

2.4 Results

2.4.1 Maternal demographics

A total of 109576 women were initially identified for the selection period between April 2009 and August 2011. There were 1756 multiple pregnancies and therefore excluded, leaving a population of 107820 women. Of these there were 83237 (77.2%) white women, 18005 (1.7%) SA women and 6578 (6.1%) Black women. The maternal demographics of the group are featured in table 6.

Demographic feature	Number (%)
Body Mass Index (kg/m^2)	
<18.5	3493 (3.3)
18.5-24.9	51550 (48.7)
25-29.9	29533 (27.9)
30-34.9	13443 (12.7)
>35	7833 (7.4)
Missing	*1968 (1.8)*
Maternal age (years)	
<20	7548 (7.1)
20-24	23950 (22.4)
25-29	31504 (29.5)
30-34	26851 (25.1)
≥35	17035 (15.9)
Missing	*932 (0.9)*
Parity	
0	45191 (42.4)
1	34853 (32.7)
2	15668 (14.7)
≥3	10871(10.2)
Missing	*1237 (1.1)*

Table 6: Demographic features of the women in the perinatal institute's database

Demographic feature	Number (%)
Index of Multiple Deprivation (fifths)	
1-3 (least deprived)	40109 (37.2)
4	22750 (21.1)
5 (most deprived)	44961 (41.7)
Pre-existing DM	
No	105627 (99.2)
Yes	852 (0.8)
Missing	*1791 (1.6)*
Pre-existing Hypertension	
No	103484 (97.3)
Yes	2871 (2.7)
Missing	*1465 (1.4)*
Smoking	
No	61362 (61.5)
Yes	20853 (20.9)
Passive Smoking	17461 (17.6)
Missing	*8144 (7.5)*
Developed Gestational DM	
No	103002 (96.8)
Yes	3405 (3.2)
Missing	*1413 (1.3)*

Table 6 (continued) Demographic features of the women in the perinatal institute's database

2.4.2 Fetal and neonatal characteristics

The fetal and/or neonatal characteristics from the cohort that are associated with pre-eclampsia are summarised in table 7. Overall the mean birth weight was 3343.1grammes (Standard deviation ±569.1). The median gestational age at delivery was 280 days (Interquartile range 272-286), with 8455 babies being born before 37 weeks gestation (representing a pre-term birth rate of 7.83%).

Fetal / Neonatal characteristic	Number (%)
Sex	
Boy	55262 (51.7)
Girl	52462 (48.3)
Missing	*96 (0.09)*
Gestation Related Optimised Weight (GROW) Centile	
Median (IQR)	41.5 (19.5-67.5)
≤10	13075 (13.3)
10-90	77934 (79.2)
>90	7310 (7.5)
Missing	9501 (8.8)

Table 7: Fetal and neonatal characteristics of the babies born to the women from the perinatal institute's database

2.4.3 The pre-eclampsia population

2.4.4 Maternal characteristics

Women who had been diagnosed with pre-eclampsia were identified (n=7655) and of these, 6813 were primigravid. These were then separated according to ethnicity and BMI group (see figure 10). Within this group, 6109 were identified as White, Black or South-East Asian. The remaining 704 of these women were either Middle-Eastern (n=105), mixed-unclassified (n=112) or their ethnicity was not recorded (n=487). Given the low number of Middle-Eastern women, they were not included in the study. The maternal, fetal and neonatal characteristics are featured in tables 8 and 9.

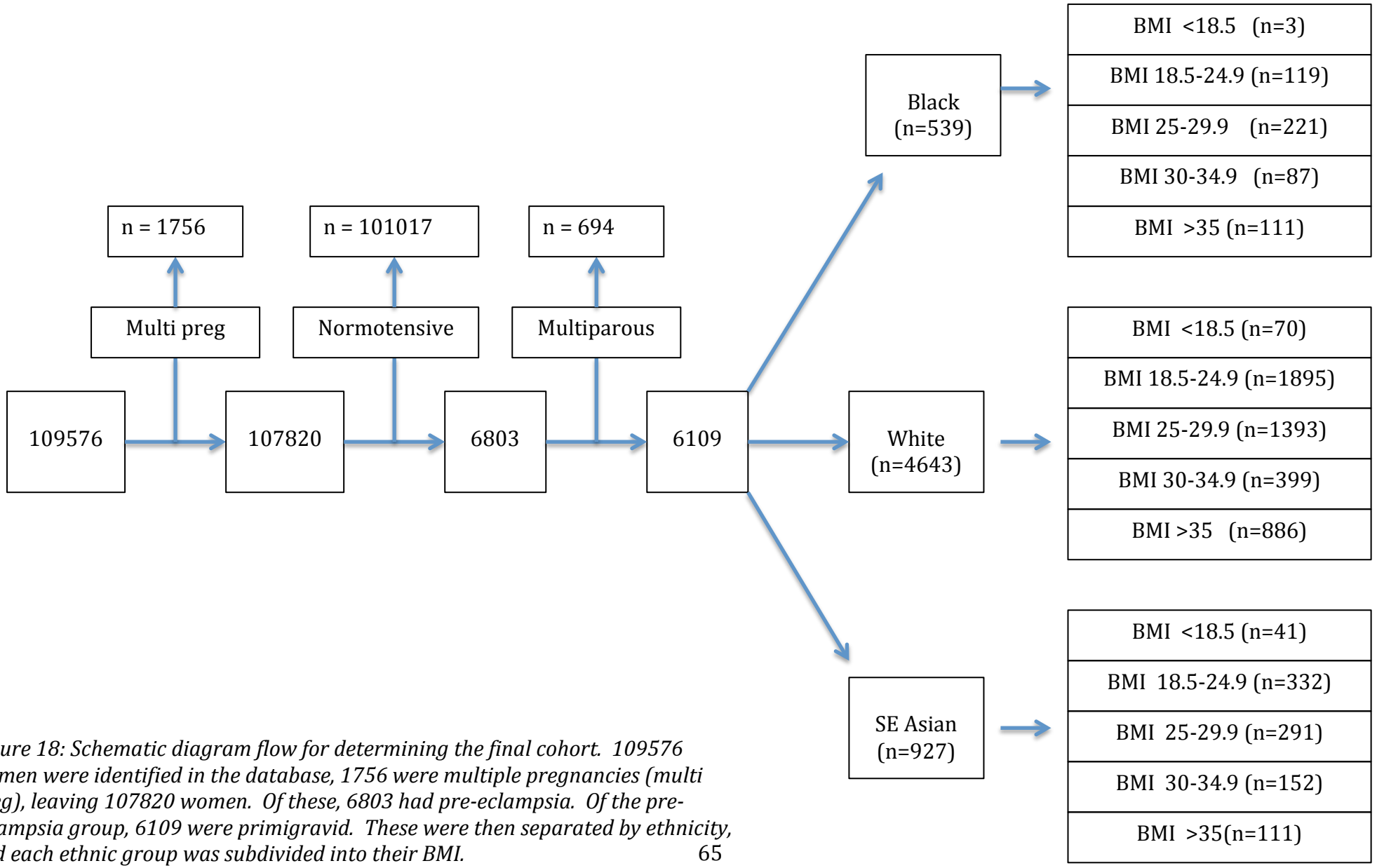


Figure 18: Schematic diagram flow for determining the final cohort. 109576 women were identified in the database, 1756 were multiple pregnancies (multi preg), leaving 107820 women. Of these, 6803 had pre-eclampsia. Of the pre-eclampsia group, 6109 were primigravid. These were then separated by ethnicity, and each ethnic group was subdivided into their BMI.

Demographic feature	Number (%)	Odds Ratio (95% C.I.)*
Body Mass Index (kg/m^2)		
<18.5	114 (1.8)	0.576 (0.477-0.696)
18.5-24.9	2402 (39.3)	Reference
25-29.9	1655 (27.1)	1.833 (1.729-1.943)
30-34.9	982 (16.1)	3.49 (3.205 – 3.806)
>35	956 (15.7)	3.383 (3.106 – 3.685)
Maternal age (years)		
<20	968 (15.8)	1.262 (1.149 –1.386)
20-24	1173 (19.2)	0.99 (0.9 – 1.08)
25-29	819 (13.5)	Reference
30-34	1411 (23.1)	1.264 (1.158 – 1.379)
≥35	1738 (28.4)	2.195 (1.876 – 4.093)
Index of Multiple Deprivation (fifths)		
1-3 (least deprived)	1808 (29.6)	Reference
4	1057 (17.3)	1.108 (0.972 – 1.581)
5 (most deprived)	3244 (53.1)	2.694 (2.501 – 2.942)

Table 8: Maternal characteristics of the women who developed pre-eclampsia

Demographic feature	Number (%)	Odds Ratio (95% C.I.)*
Pre-existing Diabetes		
No	5979 (97.9)	Reference
Yes	110 (1.8)	2.324 (1.902 – 2.839)
Missing	*20 (0.3)*	
Pre-existing Hypertension		
No	5582 (91.5)	Reference
Yes	516 (8.5)	3.332 (3.022 – 3.673)
Missing	*11 (0.18)*	
Smoking		
No	4172 (68.3)	Reference
Yes	1006 (16.5)	0.745 (0.695-0.799)
Passive	931 (15.2)	0.847 (0.788 – 0.909)
Developed GDM		
No	5817 (96.3)	Reference
Yes	225 (3.7)	1.17 (1.020-1.343)
Missing	*67 (1.1)*	

Table 8 cont'd: Maternal characteristics of the women who developed pre-eclampsia

Demographic feature	Number (%)	Odds ratio (95% C.I.)*
Sex		
Male	3138 (51.4)	Reference
Female	2971 (48.6)	0.99 (0.943-1.045)
GROW Centile		
Median (IQR)	38 (12.5-54.5)	
≤10	1879 (31.3)	2.974 (2.808-3.150)
10-90	3812 (63.6)	Reference
>90	307 (5.1)	1.119 (0.703-1.895)
Missing	111(1.8)	

Table 9: Fetal/neonatal characteristics of the women who developed pre-eclampsia

2.4.5 Body Mass index and the risk of pre-eclampsia

On the whole, Black women were significantly more likely to develop pre-eclampsia than white women (p=0.002, aOR 1.613 (CI 95% = 1.42 - 1.756)). There was no significant difference in pre-eclampsia rates between White and SA women (p=0.48 OR=1.113 (95%CI=0.849 - 1.457)). Black women who had a normal BMI (18.5-24.9) were significantly less likely to develop pre-eclampsia, than white women with a normal BMI (p=0.001, aOR 0.267[95% C.I. = 0.21-0.]). When overweight (BMI= 25-29.9), Black women were more likely to develop pre-eclampsia, than white women (p=0.001 aOR 2.06 [1.34-3.23]). Once in the obese group, pre-eclampsia rates were not significantly different across the racial groups (table 10).

Body Mass Index	Ethnicity								
	White (Reference) N= 4623	Black N= 539	OR (C.I.)	aOR (C.I.)	p value	S Asian N= 947	OR (C.I.)	aOR (C.I.)	p value
<18.5	70	3				41			
18.5-24.9	1895	115	0.39 (0.32-0.48)	0.421 (0.24-0.73)	0.002	392	1.1 (0.91-1.22)	0.99 (0.7-1.41)	0.94
24-29.9	1193	211	2.1 (1.76-2.55)	2.06 (1.34-3.23)	<0.001	251	0.96 (0.83-1.13)	1.27 (0.9-1.84)	0.23
30-34.9	729	101	1.24 (0.98-1.56)	0.84 (0.47-1.50)	0.66	152	1.02 (0.84-1.24)	0.89 (0.38-1.93)	0.12
>35	736	109	1.08 (0.86-1.34)	1.33 (0.78-2.23)	0.37	111	0.83 (0.67-1.03)	0.67 (0.40-1.12	0.07

Table 10: Effect of ethnicity on the association of body mass index and pre-eclampsia

2.4.6 Maternal age and the risk of pre-eclampsia

Advanced maternal age (>35 years of age) is a risk factor for developing pre-eclampsia (relative risk = 2.195 (1.876 – 4.093)). However, there is racial variation of rates of pre-eclampsia across different ages groups. Young (<20 years of age) Black women are less likely to develop pre-eclampsia than their White counterparts. This relationship is reversed when maternal age is >35, with Black women at more risk of developing the disease than white women. When aged between 30 and 35, SA women are more at risk of developing pre-eclampsia than White women (See table 11). This trend however is not continued when SA women are aged over 35.

Age	Ethnicity	Number	OR (95% CI)	Adjusted OR (95% CI)	p value
<20	White	735	Reference	Reference	
	S Asian	147	0.873 (0.686-1.112)	1.903 (0.905-1.320)	0.38
	Black	86	0.240 (0.176 - 0.329)	0.628 (0.490 – 0.803)	<0.001
20-24	White	876	Reference	Reference	
	S Asian	197	0.549 (0.447 – 0.674)	0.965 (0.823-1.113)	0.64
	Black	113	0.314 (0.249 – 0.397)	0.635 (0.518 – 0.777)	<0.001
25-29	White	622	Reference	Reference	
	S Asian	186	0.980 (0.782 – 1.276)	0.884 (0.725 – 1.077)	0.24
	Black	59	0.856 (0.680 – 1.801)	1.044 (0.797 – 1.369)	0.81
30-34	White	1334	Reference	Reference	
	S Asian	202	0.965 (0.830 – 1.122)	1.428 (1.227 – 1.661)	<0.001
	Black	125	1.020 (0.738-1.255)	0.845 (0.699 – 1.022)	0.091
>35	White	1076	Reference	Reference	
	S Asian	195	0.958 (0.851 – 1.079)	1.15 (0.98 – 1.347)	0.089
	Black	156	1.77 (1.590-1.973)	1.67 – 1.397-1.996	<0.001

Table 11: Effect of ethnicity on the association of age and pre-eclampsia

2.4.7 Maternal and Fetal outcomes

Whilst demonstrating a clear racial variation in two of the strongest risk factors for developing pre-eclampsia (BMI and maternal age), I studied whether there was also racial variation in the maternal and fetal outcomes (tables 13 and 14).

2.4.8 Preterm birth

Preterm birth (PTB) was defined as delivery less than 37 completed weeks. The PTB rate for the whole cohort was 7.8% (8409 births). In the pre-eclampsia group the overall PTB rate was 19.65% (1201 births) of which the majority (94.7%) were iatrogenic deliveries (that is, deliveries based on medical grounds)(1140 births). The racial variation of the iatrogenic births is shown in table 12 (overleaf).

Ethnicity	Number	RR	Adjusted RR	p value
White	866	Reference		
Black	101	1.670 (1.397-1.996)	1.587 (1.268 – 1.987)	<0.001
S Asian	173	0.792 (0.668-0.938)	0.836 (0.706-0.989)	0.04

Table 12: Racial variation in the relative risk (RR) for preterm birth. Adjusted relative risk adjusted for BMI, smoking, maternal age and Gestation Related Optimised Weight (GROW) centile.

2.4.9 Maternal complications of pre-eclampsia

Complication	Ethnicity	N	RR (95% CI)	Adjusted RR (95% CI)	p value
Placental abruption	White	221	Reference		
	Black	48	2.92 (2.152-3.976)	1.73 (1.527-1.963)	0.001
	S Asian	43	1.163 (0.790-1.691)	1.144 (0.996-1.313)	0.062
HELLP syndrome	White	285	Reference		
	Black	32	0.556 (0.468-0.661)	0.75 (0.587-0.956)	0.025
	S Asian	44	0.420 (0.423-0.608)	0.635 (0.491-0.820)	0.001

Table13: Racial variation in the relative risk (RR) for maternal complication in women with pre-eclampsia. Adjusted relative risk adjusted for BMI, smoking, maternal age and Gestation Related Optimised Weight (GROW) centile (N=number).

2.4.10 Fetal complications of pre-eclampsia

Complication	Ethnicity	N	RR	Adjusted RR	p value
Fetal growth restriction	White	735	Reference		
	Black	75	1.10 (0.975-1.26)	1.087 (0.954-1.239)	0.7
	S Asian	183	1.323 (1.165-1.500)	1.278 (1.126-1.450)	0.002
Admission to NNU	White	740	Reference		
	Black	78	0.892 (0.769-1.036)	0.871 (0.751-1.118)	0.073
	S Asian	176	1.364 (1.137-1.636)	1.25 (1.042-1.520)	0.017

Table 14: Racial variation in the relative risk (RR) for fetal/neonatal complications in women with pre-eclampsia. Adjusted relative risk adjusted for BMI, smoking, maternal age and Gestation Related Optimised Weight (GROW) centile (N=number).

2.5 Discussion

In this cohort study consisting of 109576 women, I studied previously identified risk factors for developing pre-eclampsia and assessed whether there is variation in these risk factors across different ethnicities. In addition to studying the risk factors for developing pre-eclampsia, I also investigated whether there was a difference in maternal and fetal/neonatal outcomes once the disease was diagnosed.

2.5.1 Summary and discussion of key findings

The overall rate of pre-eclampsia was 6.98%. This is in keeping with standard worldwide range of between 2-7% that is widely quoted (Sibai et al, 2005). Whilst the rate of 6.98% is higher than other Northern European groups have identified (In a Swedish study the overall risk of developing pre-eclampsia was 4.1% Hernandez-Diaz et al (2009)), this most likely represents the difference in population. The West-Midlands have some of the most deprived and obese mothers in Europe with a much greater variation in ethnicity across the population (Public Health England, 2013). The risk of developing pre-eclampsia in my cohort is similar to other UK based cohort studies (Bramham et al, 2011).

This cohort study corroborated previous work that showed that Black women were significantly more likely to develop pre-eclampsia. Caughey et

al (2005) undertook a similar sized retrospective study based on a Californian cohort that showed that Black women had an odds ratio of 1.41 (95% CI 1.25-1.62) when compared to white women, (which is synonymous with this study, RR 1.613 (CI 95% = 1.42 - 1.756)). However, this study did not study South Asian women. Work undertaken by the Fetal Medicine Foundation (London), studied a population in South London (n=76158). Again, they confirmed the link between Black ethnic origin and pre-eclampsia. However, unlike this study, they found SA women were at higher risk of developing pre-eclampsia also (RR 1.73 95% CI (1.39 – 2.15)) (Khalil et al, 2013). One possible reason for this is the smaller number of SA women included in their study (n=6314) compared to this study (n=18005).

As mentioned in the introduction, it is widely acknowledged that the risk of pre-eclampsia increases with increasing maternal BMI. This link has been confirmed in this cohort study. Once in the obese group, the risk of developing pre-eclampsia is three fold. This reiterated the findings of a large study from the east coast of the USA (Roberts et al, 2011). One possible reason why obesity predisposes to development of pre-eclampsia is the increase in insulin resistance that is found in obese people. Insulin resistance is thought to be involved in pathogenesis of pre-eclampsia (Kaaja, 1998) and appears to remain, even many months after the delivery of the fetus and placenta (Lampinin, 2008). Given the link between insulin resistance and cardiovascular disease, this residual insulin resistance may

be responsible for the long-term cardiovascular morbidity seen in women with previous pre-eclampsia (Carty et al, 2010).

Pre-eclampsia is commonly thought to affect the “extremes of reproductive age” (Dekker et al, 2001). The results of this cohort study would support this idea. My results showed a “U” shaped distribution, with teenagers being at an increased risk of developing pre-eclampsia (RR - 1.262 (95% 1.149-1.386) p=0.002), when compared to women aged 25-29. This risk becomes non significant when a woman is in her early twenties. However, this risk increases again when a woman is in her thirties, and when over 35 the RR of developing pre-eclampsia doubles (2.195 (1.876-4.093, p=<0.001). Although this distribution is widely reported in literature (Sibai et al, 1995, Conde-Agudelo et al, 2000) the mechanisms by which these two age groups develop pre-eclampsia are most likely to be different. Teenagers are thought to get pre-eclampsia through unfamiliar sperm exposure, which causes the heightened maternal inflammatory response to paternal antigens in the placenta seen in pre-eclampsia (Robillard et al, 1999). Whilst older women may develop pre-eclampsia due to their vascular endothelium adapting poorly to the inflammatory response of normal pregnancy (Redman et al, 1999) due to subclinical chronic vascular disease.

2.5.2 Racial variation in the association of risk factors and developing pre-eclampsia

2.5.3 Body Mass Index and Pre-eclampsia

This study showed that, once obese, there is no additional racial variation in risk of PE accorded to ethnicity. However, in those women whose BMI lies below the obese range (those overweight and of normal weight) there are clear ethnic variations. Table 10 within the results section demonstrates these variations and is demonstrated graphically in figure 11.

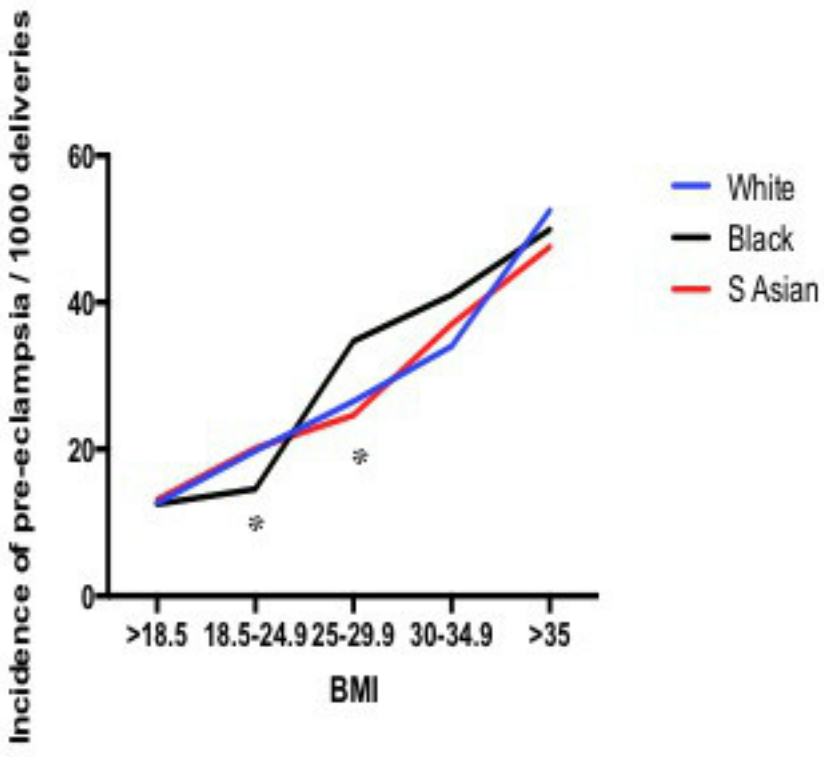


Figure 11: Racial variation of the link between body mass index and ethnicity (= statistically significant).*

It appears that when their BMI is within the normal range, Black women are less likely to develop pre-eclampsia, when compared to White women. This "protection" against developing pre-eclampsia is lost when overweight and when in this range, Black women appear more susceptible to developing the disease than their white counterparts. There is no significant variation in the risk of developing pre-eclampsia amongst obese women, regardless of their ethnicity.

Whilst initially puzzling, these findings maybe explained by the racial variation in fat distribution. It has been well described that different ethnicities carry body fat differently, with Black women carrying more fat around their trunk and hips than White or S Asian women (Stults-Kolehmainen et al, 2012). In addition, Lear et al (2007) found that BMI underestimated visceral adipose tissue in Black women. Visceral adipose tissue is functionally different from cutaneous fat; it produces more CRP (c-reactive protein) and other inflammatory cytokines and less leptin, contributing to more oxidative stress (Roberts et al, 2011). Black women, who are overweight but not obese, may have a heightened subclinical systemic inflammation than other ethnicities at a comparable BMI. It could therefore be argued that this leads to a more aggressive inflammatory response during early pregnancy, which has been proposed as a precursor for the development of pre-eclampsia (Redman et al, 1999).

2.5.4 Age and pre-eclampsia

The link between age and pre-eclampsia is still apparent when separated across ethnicities. However, despite the "U" shaped distribution appearing across the three ethnicities, there were significant differences between the groups. These are described in table 11 and represented graphically below in figure 12.

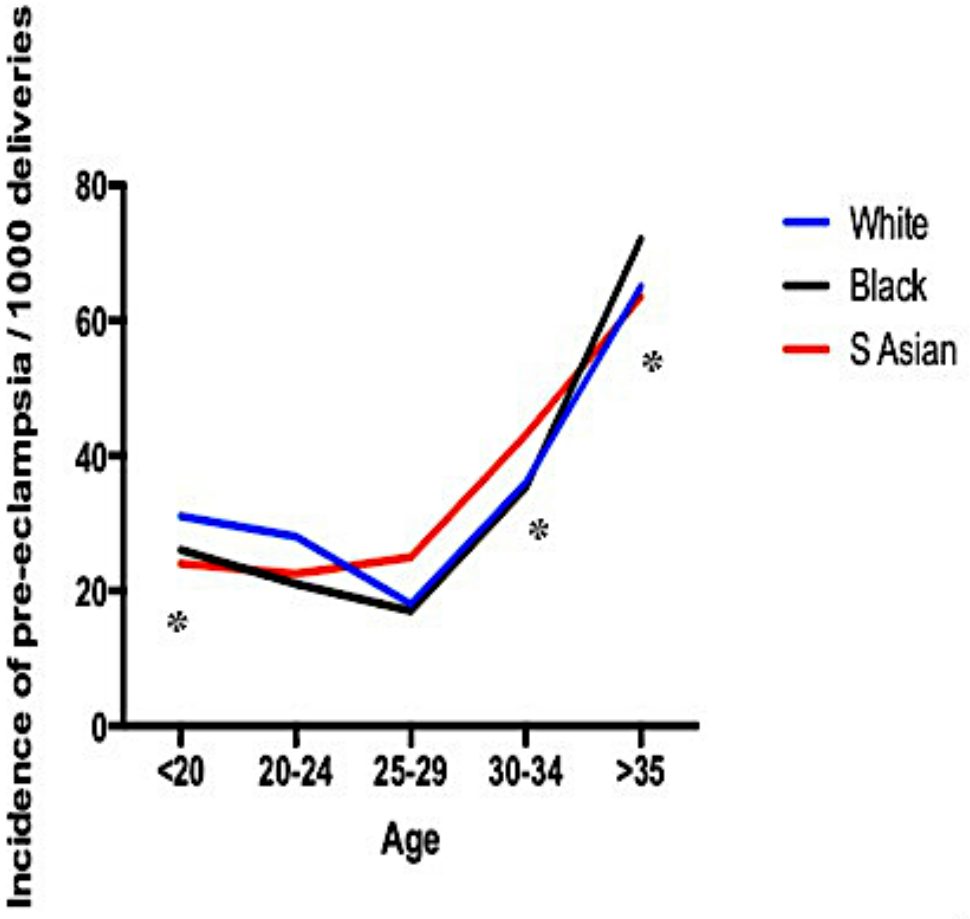


Figure 12: Ethnic variation of the link between maternal age and pre-eclampsia (=statistically significant)*

As mentioned previously, it is thought that teenage women are at greater risk of developing pre-eclampsia than older women due to their lack of exposure to their partner's semen. The reasons why girls become sexually active during their teenage years are complex and involve many psychological and social issues that go beyond the measurable parameters within the perinatal institute's database. There is however, clear evidence of racial variation in the sexual activity of teenagers. Black teenage girls are more likely to have coitus earlier than White teenage girls (Smith et al, 1985) and in those girls who are sexually active, whilst contraception use is lacking across all ethnic groups, White teenagers are more likely to use barrier contraception than Black teenagers (Coles et al, 2011). It could be suggested that sexually active Black teenage girls have more exposure to semen and this may account for their reduced incidence of pre-eclampsia.

The increased risk of developing pre-eclampsia with advancing maternal age spanned all ethnicities. However, South Asian women were more likely to develop pre-eclampsia than White women in the 30-34 age group and Black women were at greater risk of developing pre-eclampsia than White women in the >35 age group. This suggests a difference in vascular aging in different ethnicities. Pre-eclampsia has been described as a disorder of the maternal endothelium (Powe et al, 2011), so to investigate possible explanations for this racial variation, we need to look at racial variation of endothelial dysfunction in the general population. Healthy older Black adults are more likely to have overall greater endothelial dysfunction (with

significant impairment to microvascular vasodilatatory function and greater arterial stiffness) than White adults (Morris et al, 2013) which has been attributed to the higher incidence of cardiovascular disease in this population (Mulukutla et al, 2010). On a whole the South Asian populations in their late 30's and early 40's have a greater insulin resistance when compared to White populations (Mente et al, 2010) and this insulin resistance can lead to impairment of circulating angiogenic progenitor cell function and a delay in endothelial repair (Kahn et al, 2011). When pre-existing vascular endothelium is damaged or impaired, the mild inflammatory state of "normal" pregnancy is enhanced leading to a) a more aggressive systemic inflammation (Redman et al, 1999) and b) greater circulating concentrations of anti-angiogenic proteins (Powe et al, 2011) both of which have been identified as possible mechanisms in the development of pre-eclampsia.

2.5.5 Racial variation in the Maternal and Fetal outcomes when diagnosed with pre-eclampsia

2.5.6 Preterm birth in pre-eclamptic pregnancies

In the pre-eclampsia group, Black women were more likely to be delivered earlier than White women (aRR 1.587 (95% CI 1.268-1.987)). Iatrogenic delivery in women with pre-eclampsia occurs either because there is

significant maternal morbidity or there is evidence of fetal compromise. S. Asian women were more likely to deliver due to concerns over fetal health ($p<0.001$) than white women (with no difference in the deliveries due to fetal compromise in the Black cohort ($p=0.07$)). However, Black women were more likely to deliver early due to concerns about maternal morbidity ($p=0.002$) than White women (there was no significant difference in the risk of delivering early due to maternal morbidity between Asian women and White women ($p=0.81$)).

To my knowledge, this is the first study to assess the racial variation of iatrogenic PTB in pre-eclamptic pregnancies. Why this variation exists, is not completely clear. As discussed in this thesis's introduction, whilst the diagnosis of pre-eclampsia is based on clinical criteria, the pathophysiology leading to the clinical syndrome is most likely via different mechanisms. It is therefore not unreasonable to suggest that some ethnicities may be susceptible to different pathophysiological processes than others causing a fetal compromise dominant picture in one group and a maternal syndrome dominance in another (Ness et al, 2006).

2.5.7 Maternal Outcomes and pre-eclampsia

2.5.8 Placental abruption

Placental abruption (premature separation of the placenta from the uterine wall whilst the fetus is still in utero) was more common in Black women who

had pre-eclampsia, when compared to White women (aOR 1.73 (95% CI 1.527 – 1.963, p=0.001). This was after controlling for confounding factors such as smoking, BMI and maternal age. The link between ethnicity and placental abruption has been reported before with both Shen et al (2008) and Balchin et al (2009) reporting that placental abruption was higher in Black women, although this is the first study to confirm the link in pre-eclamptic pregnancies.

The pathogenesis of placental abruption is complicated but there does seem to be at least two distinct clinical pathways 1) acute inflammation, and 2) chronic vasculopathic processes, such as chronic hypertension and smoking (Shen et al, 2008). As mentioned earlier, Black women are more likely to have endothelial dysfunction than White women. It could therefore be suggested that the acute inflammation associated with the clinical syndrome of pre-eclampsia combined with pre-existing endothelial damage puts Black women at greater risk of preeclampsia than White women.

2.5.9 HELLP syndrome

White women were more likely to develop HELLP syndrome than both Black and SA women. The clinical presentation of pre-eclampsia lies on a spectra of signs and symptoms with HELLP syndrome lying at the severe end of that spectrum, being associated with significant maternal morbidity and mortality. These findings concur with the findings from a small

American study, which assessed the variation between Black and White women (but not S Asian women) (Goodwin, 2005). An understanding of the pathophysiology behind HELLP syndrome remains elusive, with several theories about the diseases origin. One of the strongest theories is that placenta-mediated anti-angiogenic factors are released which cause vasoconstriction and endothelial damage to the hepatic microcirculation. Fibrin deposition within these small damaged vessels, leads to reduction in hepatic blood flow. This causes hepatic necrosis with the endothelial damage leading to consumption of circulating platelets (Eastabrook et al, 2011). The reason why this may occur more frequently in White women than Black women remains unclear.

2.5.10 Fetal outcome in pre-eclamptic pregnancies

2.5.11 Fetal growth restriction

South Asian women were more likely to have a growth-restricted infant (that is, a baby that is less than the 10th GROW centile) than White women ($p=0.002$) with no significant difference between infants born to Black and White women. This is in contrast to studies of fetal growth restriction without pre-eclampsia that show both Black and S Asian women were at higher risk of FGR than White women (Balchin et al, 2007; Reagan et al, 2007). However this is the first study to assess the racial variation of fetal

growth restriction in women from the UK with pre-eclampsia. Whilst both fetal growth restriction and pre-eclampsia are disorders of poor placentation, it is thought that they arise from different mechanisms (Ness et al, 2006, Egbor et al, 2006). It could be argued that (as previously discussed) different ethnicities, with their differences in insulin sensitivity, pre-existing endothelial dysfunction and genetic variations, have different "types" of pre-eclampsia, with SA women tending towards the fetal growth restriction ("fetal") type of placentation and Black women tending towards the severe hypertension and systemic endothelial damage ("maternal") end.

2.5.12 Strengths of this study

The greatest strength of this study is the large dataset that included a large non-White population giving the study high statistical power. In addition the database was well constructed, with paid data entry clerks meaning that there was minimal missing data.

The robust dataset allowed many potentially confounding variables to be studied together, and on reviewing the literature, few studies are able to control for age, BMI, ethnicity, social class, smoking history and socio-economic status.

Another significant strength of this study is in the fact that the database spans all hospitals in the West-Midlands ranging from inner city highly deprived areas, such as Coventry to rural high-income areas such as

Warwick. This span of ethnicity and socio-economic status allows us to use this data as a snapshot of modern day Britain.

When self-reporting weight and height pregnant women are likely to underestimate, leading to potential study bias (Gorber et al, 2007). In view of this, there is a region wide policy of measuring height and weight at booking, with weighing scales being calibrated regularly.

2.5.13 Weaknesses of this study

The demographic variables (BMI, smoking history, etc.) used in this study were recorded at the booking appointment. They did not account for changes, for example, of excessive weight gain in pregnancy or smoking cessation. Excessive weight gain in pregnancy is reported to increase maternal morbidity, even in normal and overweight BMI ranges (Savitz et al, 2011) and a large cohort study reported that women who stopped smoking by 15 weeks gestation had the same perinatal outcomes as non-smokers (McCowan et al, 2009). Additionally, smoking status is self reported and not tested objectively, which may have lead to confounding results. However, the findings in the study (that is, the reduced rate of pre-eclampsia in smokers) are supported by the literature, suggesting that any underreporting, if any, is small.

Given how large this dataset is there was a huge choice of possible predictors for pre-eclampsia and this could have led to variable selection

bias. However, the clinical risk factors and their strength of association with pre-eclampsia found in this study are consistent with other international studies.

2.6 Conclusion

This chapter reviewed the racial variation of the known risk factors for developing, and the outcomes associated with, pre-eclampsia. Using a large database, I have been able to identify previously unreported associations;

- BMI had a complex interaction with ethnicity in terms of developing pre-eclampsia
- There were different indications for iatrogenic preterm delivery in PE for women with different ethnicities.

The clinical manifestations of pre-eclampsia represent a variety of pathophysiological processes. Biochemical markers that allow prediction of the disease would be useful but may be specific to different populations, such as Black, Asian, obese or older women.

Chapter Three

A study of plasma proteomics to predict pre-eclampsia

3.1 Introduction

3.1.1 Proteomics

Proteomics is the study of the proteins, their structure and function, within a cell/system/organism (Gramolini et al 2008) and has emerged as an important tool in several different fields of medical research for early disease detection, for assessment of response to treatment and for unraveling underlying pathophysiological processes (Wheelock et al 2013).

After Richard Levine's work showed how circulating angiogenic factors are altered in pre-eclamptic pregnancies up to five weeks before the onset of the clinical syndrome, many groups have aimed to identify "biomarkers" (biological markers) for predicting the onset of pre-eclampsia (Levine et al 2004). It is hoped that by discovering these biomarkers we can accurately predict the onset of the disease and study potential interventions that may reduce the severity of the disease, or prevent its development altogether.

The identification of one unique protein that could accurately diagnose pre-eclampsia is, perhaps, unrealistic. Given that trisomy 21 is screened by combination of several biochemical factors and an ultrasound parameter, the same could be hoped of for pre-eclampsia.

The quest for a clinically utilizable biomarker is split into two processes (see figure 13). The first aims to discover the potential marker. This involves choosing the appropriate biological sample, reducing the complexity of the

sample (this can involve removing the abundant proteins and/or separating the proteins according to their molecular weight), analyzing the sample using mass spectrometry, interpreting the results using a defined database and selecting the candidate protein/peptide(s) for further study. The next stage involves prospective confirmation of these candidate proteins/peptides as biomarkers, using a larger and more diverse study group (Gramolini et al 2008). Only when these have been shown to: a) be reproducible across a wide selection of patients b) have the appropriate sensitivity and specificity c) be clinically useful at either predicting diagnosis or prognosis of a disease state can a candidate protein/peptide be called a biomarker (Figure 13).

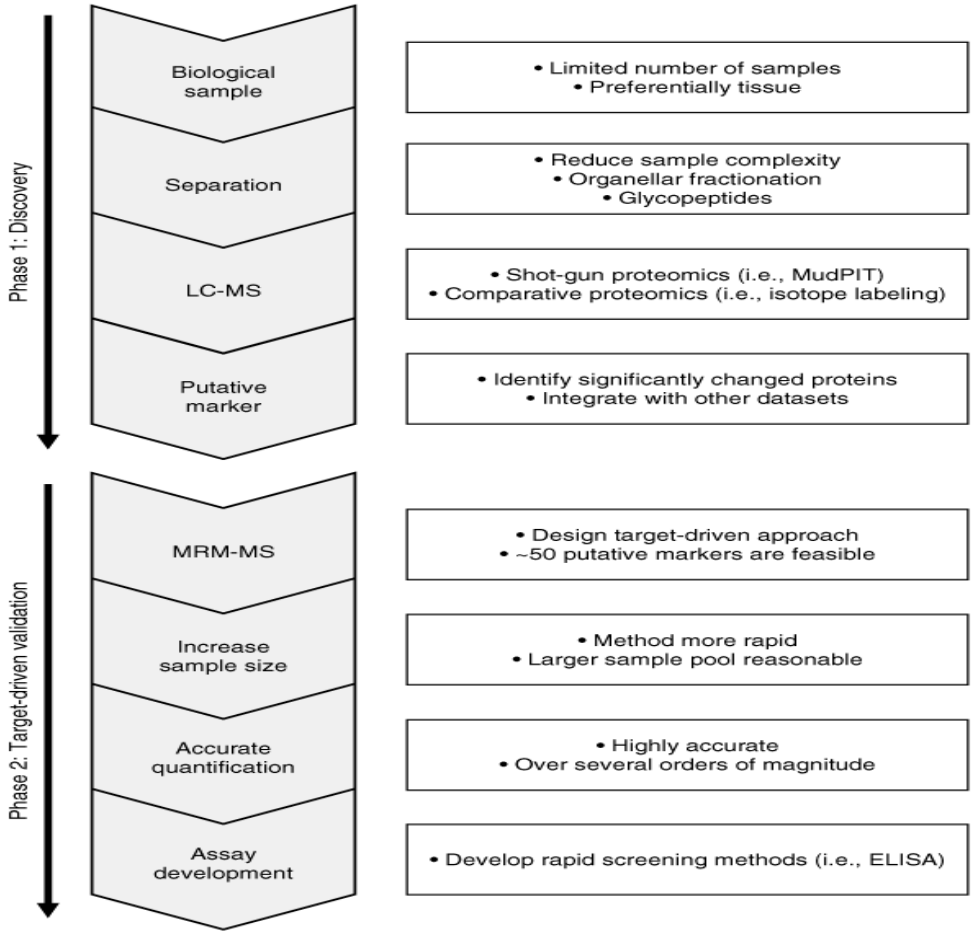


Figure 13

A proteomics-based biomarker discovery pipeline. A potential biomarker discovery platform could combine global proteomic profiling in tissue (discovery phase) with sensitive quantitation by target-driven mass spectrometry (MS) (target-driven validation) of putative biomarkers directly in plasma. ELISA, enzyme-linked immunosorbent assay; LC-MS, liquid chromatography–MS; MRM-MS, multiple reaction monitoring mass spectrometry; MudPIT, multidimensional protein identification technology (from Gramolini et al 2008)

3.1.2 Sample choice

Most human tissue types can provide a platform for proteomic analysis. In pre-eclampsia research plasma, urine and placental tissue have all been studied with a significant focus on plasma studies in the quest for an early pregnancy pre-eclampsia biomarker (Horgan et al 2011). Whilst Plasma is an ideal medium for detecting systemic markers of a disease process, its use in proteomics is fraught with challenges. 95% of the plasma proteome is made up of just 14 proteins and this highly abundant group prevents adequate study of the remaining 5% where the potential biomarker(s) probably lie (Millioni et al 2011). In addition, the complexity of the plasma proteome and the role genotype and environmental influences play on the individual plasma proteomes means that researchers need to overcome significant inter-person variation (Molloy et al 2003). Plasma proteomics can generate huge amounts of data. Without the correct analysis platforms and the ability to link (patho)physiological processes and the peptides/proteins can mean researchers are searching for the elusive needle in the proteomic haystack.

3.1.3 Protein identification by mass spectrometry

Mass spectrometry is an analytical process by which a the content of a sample is separated according to the mass-to-charge ratio of the ionized molecules contained within it. The resulting spectra of these mass-to-charge ratios are then used to identify and quantify the molecules. These molecules can then be interrogated in large databases, allowing the content of the original sample to be discovered (El-Aneed et al 2006).

Before a biological sample undergoes mass spectrometry, it needs to undergo preparation to allow a more efficient analysis and production of relevant data that can be studied further. This preparation can consist of separating the proteins according to their mass and digesting the proteins into peptides that allow for easier ionization. Some of the commonest preparation methods are discussed in the following paragraphs.

3.1.4 2 Dimensional gel electrophoresis

2 dimensional (2D) gel electrophoresis separates proteins according to their mass and their isoelectric point. The sample to be studied is placed in a gel-based matrix. The matrix contains a proteolytic agent, digesting the protein into ionised peptides. Across the matrix there are different pH gradients, with one end of the gel being more positive than the other. When a charge is applied, the peptide moves towards the different the end with

the charge opposite to that of its own. It eventually stops at the pH where it has no net charge, this is known as the isoelectric point of the protein. As some peptides are heavier than others, the lighter ones are able to travel further through the gel matrix. This occurs at a 90° angle from the isoelectric filter. The resulting "spots" which represents a specific peptide are then extracted and undergo mass spectrometry analysis. Drawbacks of 2D gel electrophoresis include the inability to analysis large complex solutions and it's poor reproducibility (Issaq et al, 2008).

3.1.5 Liquid Chromatography

Liquid chromatography (LC) is "the separation of components of a mixture based upon the rates at which they elute from a stationary phase over a mobile phase gradient". The sample is introduced to a solvent (the mobile phase, as the solvent moves through the equipment) and crosses fixed columns containing the chromatographic medium. Separation occurs according to how easily the proteins pass along the liquid phase, through the "pores" of the fixed columns, with the lighter proteins passing through easier than heavier proteins (Bansal et al, 2009) (figure 14).

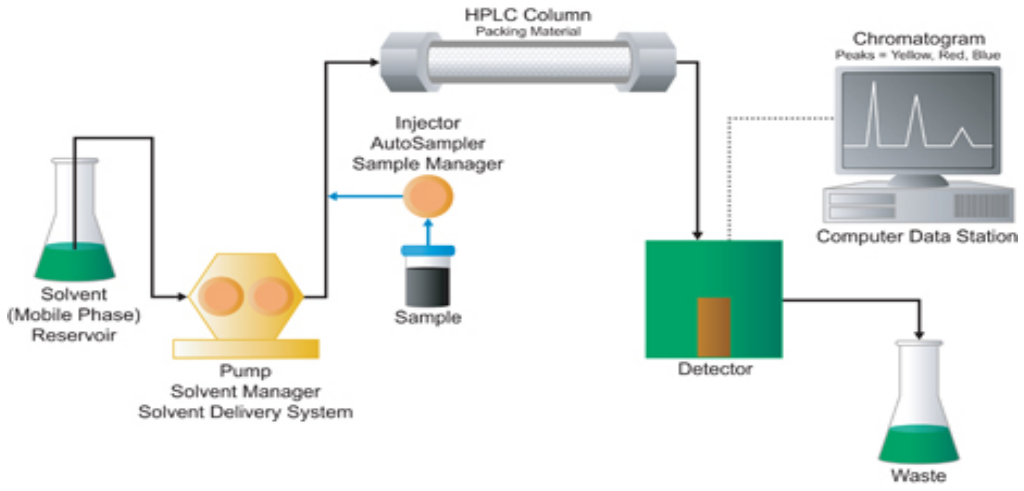


Figure 14

A schematic depiction of liquid chromatography (in this case high performance liquid chromatography (HPLC)). The Solvent passes through the system under high pressure generated by the pump. Sample is introduced into the solvent and passes through the HPLC column where the proteins are separated and are detected digitally to produce a chromatogram. Adapted from waters.com

3.1.6 Gas phase chromatography

Gas phase chromatography (or, more accurately, gas-liquid phase chromatography) is a separation process where the mobile phase is gas. The sample is vapourised in an inert gas, and passed over a liquid-covered solid chromagraphic platform. The ease at which the vaporized sample passes through the liquid allows it to be separated according to its mass.

The high temperatures required for gas phase chromatography means it is generally not suited to proteomic studies (Mondello et al, 2008).

3.1.7 Mass spectrometry

All mass spectrometers contain three common components; an ion source, a mass analyser and an ion detection system (figure 15).

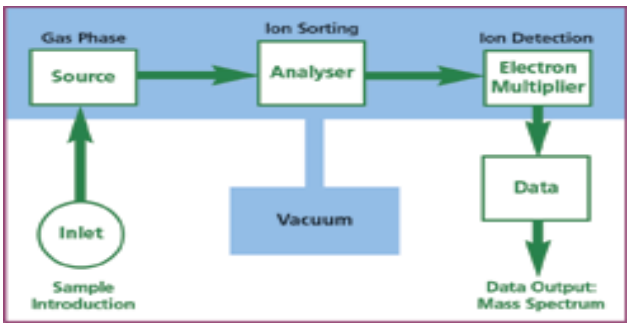


Figure 15:

A schematic interpretation of the basic steps involved in mass spectrometry. Reproduced from SGE analytical Science.

- Ionisation

  During ionization the compound to be studied is bombarded with electrons. These electrons cause the compound's component molecules to lose an ion, making them positively charged.

- Mass analyser

  When ionized, the molecules then pass through an accelerator, where they form a fine beam. This beam then passes through a field of

electromagnetic energy. This deflects the fine beam of analyte. Two main factors contribute to how much the beam deflects; the mass of the molecule and the charge of the molecule (heavier molecules will deflect less than lighter molecules and molecules with +1 charge will deflect less than those of higher charge +2). These two factors give each molecule its own mass to charge ratio (m/z) (de Hoffmann 2007).

- Ion detection system

  The m/z ratios of the analytes molecules are recorded electronically through complex informatics technology. The results are compared to huge databases of proteins, allowing for the content of the protein compound to be identified (de Hoffmann 2007).

3.1.8 The Synapt HDMS system

Although the basic principles of mass spectrometry are relatively simple (produce an ion, accelerate it, deflect the components of the beam according to the m/z and record the results) the technicalities of the procedures are much more complex. There are numerous methods of ionizing the samples and hundreds of different machines that deflect the mass of the compound, all with slightly varying methods. For the purpose of this study, the samples were performed on an Electrospray Quadrupole Time-of-Flight (ESI Q-ToF) Synapt high definition mass spectrometer (HDMS) (“the Synapt”).

3.1.9 Electrospray Ionization

Ionization of the protein/peptide analyte is achieved by electrospray ionization (ESI) (figure 16).

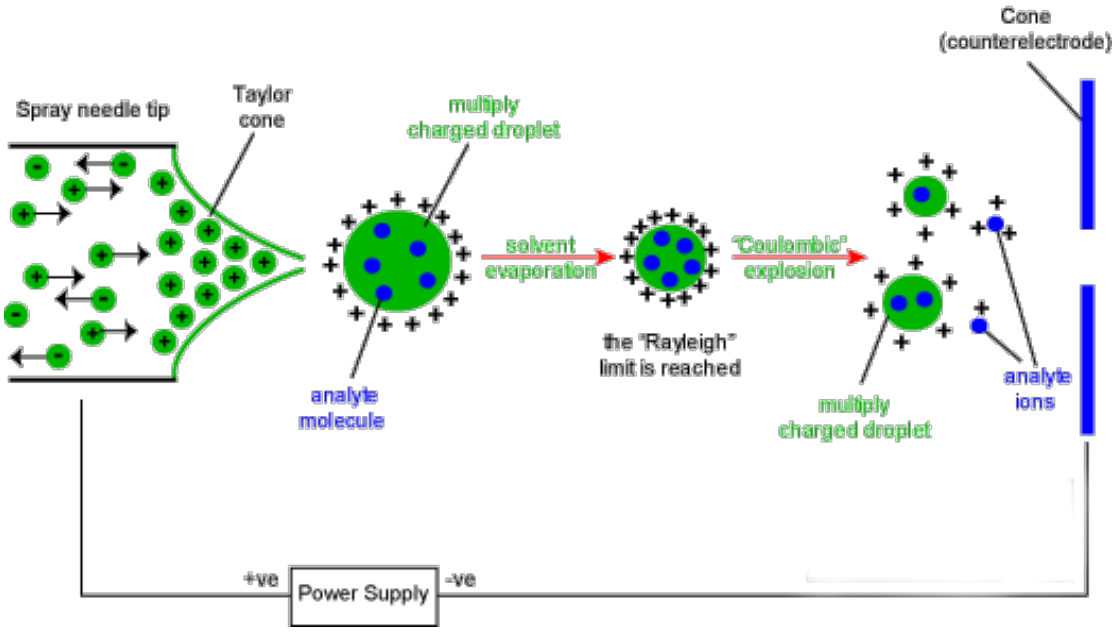


Figure 16: Schematic interpretation of electrospray ionization, adapted from the University of Bristol's Chemistry department (http://www.chm.bris.ac.uk)

The analyte is dissolved in a volatile solvent and passed through a spray needle, which has a large potential difference (in reference to the cone/counter electrode). The needle repels those ions with similar charge to it, towards the oppositely charged counter electrode. Whilst travelling through the space between the needle and the cone, the solvent evaporates. When the droplet of solvent/analyte shrinks until the surface tension can no longer sustain the charge ("the Rayleigh limit") the droplet is

torn apart via the Coulombic explosion, producing multiple smaller solvent/analyte droplets and charged analytes only. These solvent/analyte compounds undergo the Coulombic explosion, until only ionic analytes travel through the counter electrode (Ho et al, 2003).

3.1.10 The Quadrupole mass analyzer

A quadrupole mass analyzer consists of four cylindrical electromagnetic rods in parallel to each other (see figure 17).

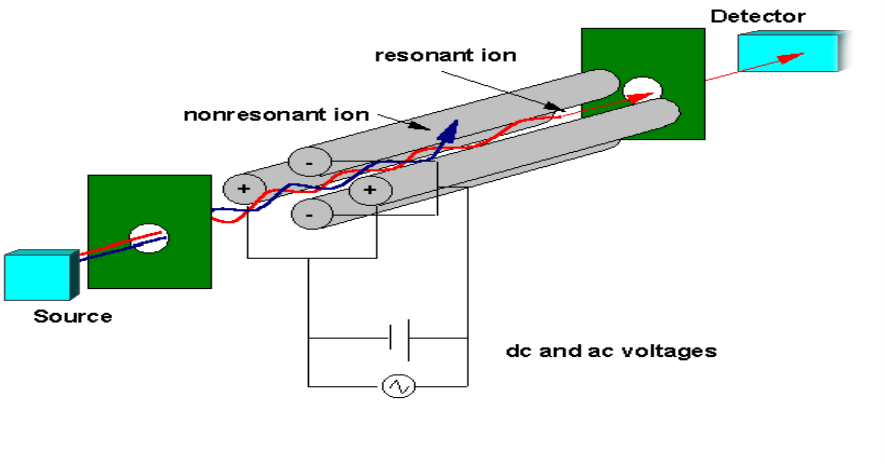


Figure 17: A quadrupole mass analyser. The ion sample is passed through the middle to the four charged rods. When the voltage applied across the rods varies, only ions with specific m/z will reach the detector. Adapted from the University of Bristol's Chemistry department (http://www.chm.bris.ac.uk)

The rods have a fixed current applied to them and an alternating radiofrequency voltage applied across them. The ionized analyte is passed through the middle of the rods with their trajectory through these rods dependent on the radio frequency voltage applied across them. By alternating this voltage, ions of varying m/z can be focused onto the detecting equipment allowing different ions to be studied (Dawson et al, 1986, de *Hoffmann 2007*).

3.1.11 Time-of-Flight mass spectroscopy

Time-of-Flight (TOF) mass spectrometry is a method of m/z detection of an ion by measuring the time it takes for it to pass between a source and a detector. The ion passes through the source via pulsatile electrical field of known quantity. This means that every ion passes into the TOF-MS with the same kinetic energy. It passes along a fixed distance to the detector and the time it takes to reach the detector is recorded. Ions with low mass reach are detected first. With the distance travelled and the time taken to cross the distance known, the mass of the molecule can therefore be calculated (Guilhaus, et al 1995).

3.1.12 Protein quantification

Whilst standard proteomic mass spectrometry readily identifies proteins, quantifying these proteins is more challenging. In order for studies to be

useful in identifying alteration in the proteomes between disease and control states, it is imperative that we can quantify these alterations. There are two main methods of protein quantification using MS technology, relative and absolute. Relative quantification identifies those proteins that are up or down regulated and this allows for a trend to be determined. Absolute quantification allows for proteins to be quantified by their concentration in a sample (such as femtomoles or picograms per amount of cell material). Absolute quantification can be achieved using label or label free techniques (Wasinger et al, 2013).

3.1.13 Label dependent absolute protein quantitation

An isotope-labeled peptide behaves chemically identical to its native counterpart and therefore the two peptides behave identically through the chromatographic and/or mass spectrometric analysis (Bantscheff 2007). Given that a mass spectrometer can recognize the mass difference between the labeled and unlabeled forms of a peptide, quantification is achieved by comparing their respective signal intensities. Isotope labels can be introduced as an internal standard into amino acids either (i) metabolically (during cell growth and division) or (ii) chemically/enzymatically (where an isotope “label” is added to a protein’s cysteine residue).

3.1.14 Label-free protein quantification

There are currently two methods of label-free protein quantification, spectral counting and LCMS/LCMS^{E} area under the curve.

As the abundance of a protein and the precursor peptides increase, so to do the MS spectra collected from their analysis. These spectra are then counted, compared to the spectra obtained from other samples and the differences quantified. This allows for a change in two samples to be identified, but not for the magnitude of change to be known (Old et al, 2005). During HPLC, the peak which the sample enters the mass spectrometer is measured. The area under this peak is then calculated and together with an internal standard of known quantity, comparison of the two areas under the curve can be compared and mass of the peptide determined.

3.2 Aim

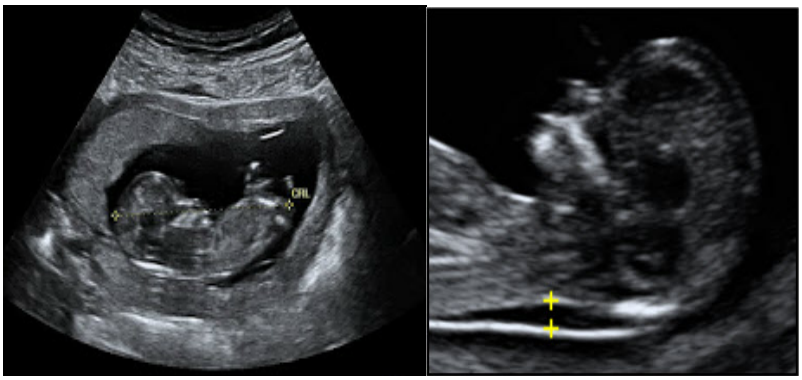
The aims of this work are:

- To study the proteome of women who did and did not develop early onset pre-eclampsia from samples obtained in the first trimester of pregnancy
- Comparing the two groups, identify markers that may a) identify putative markers that may allow early detection of disease onset and b) suggest pathophysiological process involved in disease development.

3.3 Materials and methods

3.3.1 Study population

This was a case–control study drawn from a large prospective observational study for hypertensive complications of pregnancy in women attending for their routine first hospital visit in pregnancy at King's College Hospital, London. At this visit, women where given a patient information leaflet to read, prior to their ultrasound scan, and if they agreed to participate they signed a patient consent form. Women who could not speak English were excluded from the study. All women had a first trimester combined anomaly screen performed. This consisted of a blood test for PAPP-A and βhCG and an ultrasound scan to measure the crown-rump length (CRL) of the fetus the width of nuchal translucency (NT) (figure 10).



(a) (b)

Figure 18

Figure 18(a) shows measurement of the crown-rump length (CRL) of a fetus with a gestational age of 12 weeks. Figure 18(b) shows the same fetus with the normal Nuchal Translucency (NT) measurement of 1.6mm.

Nuchal Translucency is measured between 11+0 and 13+6 weeks gestation, therefore women booked outside of this gestational age range were excluded. Women whose screening test deemed them at high-risk for having a fetus with aneuploidy were also excluded. Patients were asked to complete a questionnaire on maternal age, racial origin (white, black, Indian or Pakistani, Chinese or Japanese and Mixed), cigarette smoking during pregnancy (yes or no), method of conception (spontaneous or assisted), medical history (including chronic hypertension, diabetes mellitus, antiphospholipid syndrome, thrombophilia, and sickle cell disease), medication (including antihypertensive, antidepressant, antiepileptic, aspirin, steroids, betamimetic, insulin, and thyroxin), parity (parous or nulliparous if no delivery beyond 23 weeks), obstetric history (including previous pregnancy with pre-eclampsia), and family history of pre-eclampsia (mother). Clinical examination consisted of measuring the woman's height and weight, allowing for body-mass index to be calculated. Blood pressure was recorded and urinalysis was also performed. Abdominal Doppler ultrasound was performed to visualize the left and right uterine artery, the pulsatility index was recorded and any notching of the Doppler flow noted.

Full ethical approval was granted by the King's College ethics committee (reference: 02-03-033).

3.3.2 Specimen collection

Following study interview and clinical examination, blood was obtained from the antecubital fossa into an EDTA tube via a vacutainer giving set. It was then taken to the research laboratory, where it was centrifuged twice at 3000rpm for 10 minutes at 20°C. The resulting plasma was then stored at -80°C until immediately prior to being studied. From the over all cohort, 30 "pre-eclampsia" and 30 "normal" samples were analysed.

3.3.3 Materials (and suppliers)

- Seppro IgY14 LC2 column kit (Sigma Aldrich, Gillingham, UK). This included an LC column and dilution, stripping and neutralizing buffers.
- Rapigest surfactant (Waters Corporation, Milford, MA. USA)
- Dithiothreitol (Melford laboratories, Ipswich UK)
- Glu^{1}-Fibrinopeptide B peptide (human) (Sigma Aldrich, Gillingham, UK)
- Sodium azide (Sigma Aldrich, Gillingham, UK)
- Iodoacetamide (Sigma Aldrich, Gillingham, UK)
- Ammonium bicarbonate (Sigma Aldrich, Gillingham, UK)
- Acetonitrile (Sigma Aldrich, Gillingham, UK)
- Trypsin (Promega, Madison, WI, USA)
- Mass spectrometry solvents (MallinckrodtBaker Inc, Phillipsberg, NJ, USA)

- Spin X cellulose acetate centrifuge tube filters (Cosar, Tewksbury MA, USA)
- 2ml square 96-well trays (Beckman Coulter. Fullerton, USA)
- 5 kDa nominal molecular weight cut off (NMWCO) spin columns (Millipore, Billerica, MA, USA)
- 0.45 µm nitrocellulose filter discs (Millipore, Billerica, MA, USA).

3.3.4 Sample preparation

Each sample was thawed to room temperature and 200µL removed. This was then diluted to 1mL with the addition of dilution buffer and centrifuged using a 0.22µm spin-X cellulose acetate centrifuge tube filter.

3.3.5 Sample fractionation

The purpose of the fractionation step was to deplete the maternal plasma of the 14 most abundant proteins (which, as previously mentioned, make up 95% of the total plasma proteome) and these proteins are listed in table 15.

Proteins removed by Seppro IgY-14 fractionation

Alpha2-macroglobulin	Alpha1-Acid glycoprotein
IgG	Alpha1-antitrypsin
Fibrinogen	Transferrin
Apo-A-I HDL	IgA
Complement C3	IgM
Haptoglobin	Albumin

Table 15: Proteins removed by Seppro IgY-14

Fractionation utilised the ProteomeLab PF2D system (Becker Coulter, Fullerton, USA) via an IgY-14 LC2 chromatography column. The ProteomeLab system comprised a single quaternary pump, sample loop injector with UV detector and fraction collection using a 2mL capacity square 96-well plate.

The system was flushed with water to remove any residual organic solvent in the flow path (with a connector used instead of the Seppro column). A method was developed to load the diluted plasma onto the IgY-14 column whist sequentially using dilution, stripping, neutralization and dilution buffer over a 50-minute cycle. To allow equilibration of the column, during the final cycle of the day the last step with dilution buffer included sodium azide. Fractions were collected from the column during the chromatographic steps

and detection of protein elution was achieved using absorbance at 280nm with a 5Hz sampling rate.

Once the Seppro column was attached, a full cycle of fractionation was undertaken, using 250µL of dilution buffer before the plasma sample was run. Each plasma sample then underwent two depletion steps, ensuring there was enough sample for the mass spectrometry. Finally between different plasma samples, a blank run, using the dilution buffer, was undertaken to ensure no cross contamination.

3.3.6 Tryptic digestion of IgY-14 fractionated plasma

The two IgY-14 depleted plasma samples were combined, making a total of approximately 1mL. The sample was then transferred to vial containing lyophilised 0.1% w/v Rapigest surfactant (to help the protein to solubilise, allowing them to be more amenable to enzyme cleavage) and the solution agitated gently to allow the plasma to fully dissolve. The contents were then transferred to an 5kDa NMWCO spin column and centrifuged at 14,000rrm, 4°C, until the volume halved to approximately 50µL. The contents were transferred to a 0.5mL microfuge tube (Fisher Scientific Ltd, Loughborough, UK) and incubated in a water bath for 15 minutes at 80°C. 5µL of 100mM dithiothreitol in 100mM ammonium bicarbonate was added

to plasma (to reduce any disuphide bonds), the solution agitated and incubated at 60°C for 15 min, followed by the addition of 5µL of 200mM iodoacetamide in 100mM ammonium bicarbonate and incubated at room tempature, in the dark, for 30 minutes (the iodacetamide binds with the cysteine residue of peptides, preventing (re)formation of disulphide bonds).

A 20µg of trypsin was resolubilised in 20µL of 100mM ammonium Bicarbonate, and 2µL transferred to the plasma sample and thoroughly agitated. The sample was incubated overnight at 37°C. The next morning, 2µL of formic acid was added to the sample, incubated at 37°C for 15 minutes and filtrated through a 0.22µm Spin-X cellulose acetate centrifuge tube filter. Typically 45-50µL was obtained from each depleted sample.

The tryptically-digested sample is diluted 8 fold and combined with 200fmol μL^{-1} MassPREP glycogen phosphorylase (PhosB) tryptic digestion standard in 0.1% v/v aqueous formic acid. This produced a sample containing approximately 300ng μL^{-1} depleted plasma and 100fmol μL^{-1} PhosB. The PhosB was used as an internal standard for the estimation of protein concentrations.

3.3.7 Liquid Chromatography-Mass Spectrometry (LC-MS ^{E})

Liquid chromatographic separation was performed using a NanoAcuity UPLC system (Waters Corperation, Milford, MA, USA). This was comprised of a binary solvent, an auxiliary solvent and sample manager fitted with a heating and trapping module.

LC separation was performed using a Symmetry C18 trapping column (180µm x 20mm x 5 µm) and a C18 analytical column (75µm x 250 mm x 1.7µm). Solvent A was 0.1% v/v aqueous formic acid and solvent B was 0.1% v/v formic acid in acetonitrile.

The digested plasma sample/internal standard mixture was loaded to the trapping column and flushed with solvent B for 2 min at a flow rate of 15 µL min^{-1}. Sample elution was at a rate of 250 nL min^{-1} by increasing the organic solvent B concentration from 3% to 40% over 90 minutes.

Each sample was conducted in three technical replicates.

Before and after each of the technical replicates, a quality control process was undertaken with 50fmol of PhosB being analysed by LC-MS^E. Where peptide sequence coverage fell below 35% for PhosB no further samples were run, and the cause of the fall in peptide identification investigated and resolved. Four quality control processes were carried out every 24 hours during sample processing.

MS^E data acquisition was performed on a Synapt HDMS instrument (Waters Corporation) with MS^E configuration achieved through the MS method editor controlled by MassLynx software (v4.1). TOF analyser of the mass spectrometer was calibrated using the MS/MS spectrum obtained from the doubly charged precursor of the GFP peptide over the range m/z 50 to 1300. This was validated with an average ppm error across the mass range of <10ppm.

In low energy MS, data was collected at a constant trap collision energy of 6eV and in high energy MS, data was collected at alternating energies from 15eV to 30eV. All data was post-acquisition lockmass corrected using monoisotopic ion of the doubly charged precursor of GFP (m/z 785.8426).

3.3.8 Data processing and database interrogation

The MS^E data were processed by and proteins quantified using the Identity and Expression algorithms in PLGS v2.4. The data was collected in separate data functions. The first contained data from low energy MS and the second contained data from elevated energy MS and the data from the Phos B runs for accurate mass correlation. The lockmass-corrected spectra are deisotoped and charge-state reduced to produce a single accurately mass-measured monisotopic mass for each peptide and its related fragment ion.

The database search parameters used the following variable modifications; N-terminal acetylation, deamidation of N/Q and oxidation of M residues. The IPI human database rel. 3.69 was appended to include the sequences for the internal standards. A database was then generated which included one random entry for each original sequence in the file and was used for all subsequent interrogations.

The protein tables from PLGS IdentityE were compiled in Excel and pivot tables were used to identify proteins observed in a minimum of 2 replicate analyses from each individual patient. The protein abundance (as a % of the total loading) was then calculated for the confident identifications. The number of random entries in the confident protein table was used to determine the false positive rate for the analyses.

3.3.9 Patient outcome and diagnosis of early onset pre-eclampsia

For the purpose of this study the definition of early onset pre-eclampsia was Hypertension of SBP>140 and / or DBP >90 over two consecutive readings, 6 hours apart, with proteinuria of 300mg/24 hrs or a protein: creatinine ratio of >30mg/mmol, and where not available, a + proteinuria on a urine dipstix, occurring after 20 weeks gestation, but before 34 weeks gestation and resolving post delivery.

In addition to this definition, all patients who developed pre-eclampsia had abnormal uterine artery Doppler studies (either notching of the uterine artery Doppler (bilateral or unilateral) or an raised uterine artery Doppler PI) at 24 weeks gestation.

3.3.10 Delivery information

Delivery information was obtained from hospital informatics and cross-referenced with delivery notes. This information included gestation of

delivery, mode of delivery, birth weight and blood pressure reading pre-delivery and post-delivery.

3.3.11 Statistical analysis

All statistical analysis was performed on the SPSS software package (SPSS inc., Chicago,USA). Normally distributed data were expressed as mean +/- standard deviation, whereas non-normally distributed data were reported as median +/-interquartile range. Data distribution was assessed for normality via the Kolmogorov-Smirnov test. Comparison of categorical data between the two groups was through Fisher's exact test and continuous data were compared by the Mann Whitney U test if the data was skewed, or by the student's t test if the data was normally distributed. Statistical significance was taken as a p value of <0.05.

3.4 Results

3.4.1 Patient demographics

All women who developed pre-eclampsia had bilateral notching of the uterine arteries at 20 weeks gestation, whilst those in the “normal” group did not. The pre-eclampsia group developed significantly higher blood pressure, both at booking and (by definition) at the time of delivery, than those in the normal group (table 16). The pre-eclampsia group required delivery before 34 weeks gestation, the normal group were delivered at term (>37 completed weeks of gestation). Birth weight, plotted on customised growth charts, were lower in the pre-eclampsia group than the normal group at delivery.

	Controls	Pre-eclampsia
Maternal age Median (IQR)	31.2 (27.5-35.8)	31.6 (25.2-37.1)
Maternal BMI in kgm^{-2}, Median (IQR)	24.2 (21.9-27.3)	28.3 (23.5-32.7)
Crown-rump length in mm, median (IQR)	63.3 (58.0-68.5)	62.9 (57.0-68.1)
Gestation at sampling (weeks), median (IQR)	12.4 (12.1-12.9)	12.4 (12.0-12.8)
Racial origin		
Caucasian, (%)	58	36
African, (%)	32.5	46
Asian, (%)	9.5	18
Parity		
Nulliparous, n (%)	46	56.7
Cigarette smoker, n (%)	7	3

Table 16

Demographic details of women who did and did not (Control) develop pre-eclampsia

3.4.2 Quantification of plasma proteins

For each patient, the three technical replicates were compared and protein abundances were calculated from the Identity^{E} tables (as a % of the total loading) for each protein that observed in two or more replicates. In total, 1625 proteins were identified. However only 371 of these were identified in two of the three technical replicates of the individual samples. 894 were identified in only one of these technical replicates. These were used to calculate the false positive protein detection rate, which was <0.3%. Of these 371 proteins, 35 were statistically significant between the normal and pre-eclampsia samples ($p<0.05$ Mann Whitney U test) and are listed in table 17.

Over represented in pre-eclampsia	Under represented in pre-eclampsia
Angiotensinogen	Tissue Kallikrein-1
Pregnancy zone protein	Complement C1 inhibitor
Complement component C4a	Thyroxine binding globulin
Complement component C3Bb	Vitamin D binding globulin
Complement component C1q	C4b binding protein
Complement C2	Complement factor H
Complement C1r	Extracellular matrix protein 1
Complement factor B	AMBP
hCG	Paraoxonase-1
Pigment epithelium derived factor	
Alpha 1 anti protease	
Fetuin B	
Hyaluronan	
Vitronectin	
Pigment Epithelium Derived Factor	
Plasma Kallikrein	
Prothrombin	
Sex Hormone Binding Globulin	
Extracellular matrix protein 1	
Clusterin	
Coagulation factor X	
Coagulation factor V	
Pregnancy specific glycoprotein 9	
Pregnancy specific B1 glycoprotein 11	
Ceruloplasmin	
Inter alpha trypsin inhibitor	

Table 17: A list of peptides that are statistically significantly (P=<0.05) altered in first trimester individual samples from women who did and did not subsequently develop pre-eclampsia.

Angiotensinogen was significantly raised in pregnancies that went onto develop pre-eclampsia ($p<0.0001$) (figure 19).

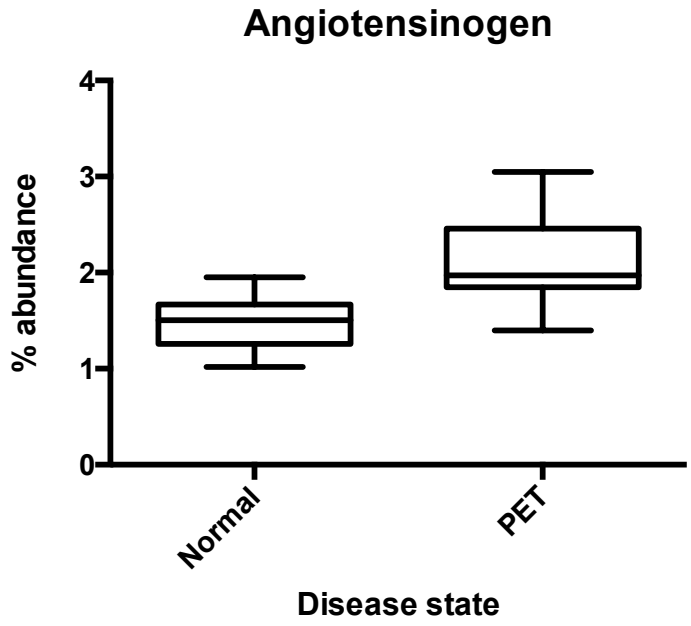


Figure 19: Box plot of the median value for the % abundance of Angiotensinogen in the first trimester individual samples from women who did and did not go on to develop pre-eclampsia. Angiotensinogen is significantly increased in the pre-eclampsia samples (p=<0.001). The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points

Tissue Kallikrein 1 was significantly lower in the pre-eclampsia group compared to the normal group (figure 20).

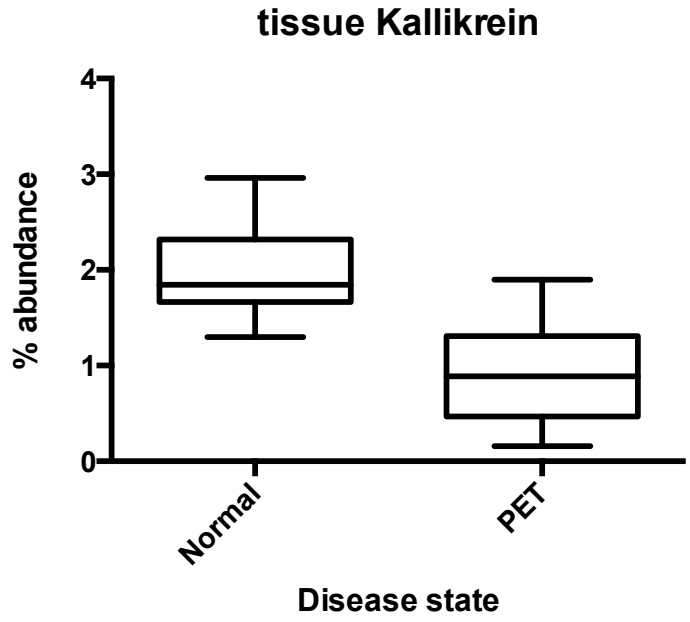


Figure 20: Box plot of the median value for the % abundance of tissue in the first trimester individual samples from women who did and did not go on to develop pre-eclampsia. Tissue kallikrein is significantly decreased in the pre-eclampsia samples (p=<0.001). The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points.

The ratio of angiotensinogen to tissue kallikrein is significantly up-regulated in women who go on to develop pre-eclampsia than those who do not ($p<0.0001$). In addition when plotted on a Receiver-Operator Curve (ROC) the area under the curve (AUC) is 0.81 (figure 21).

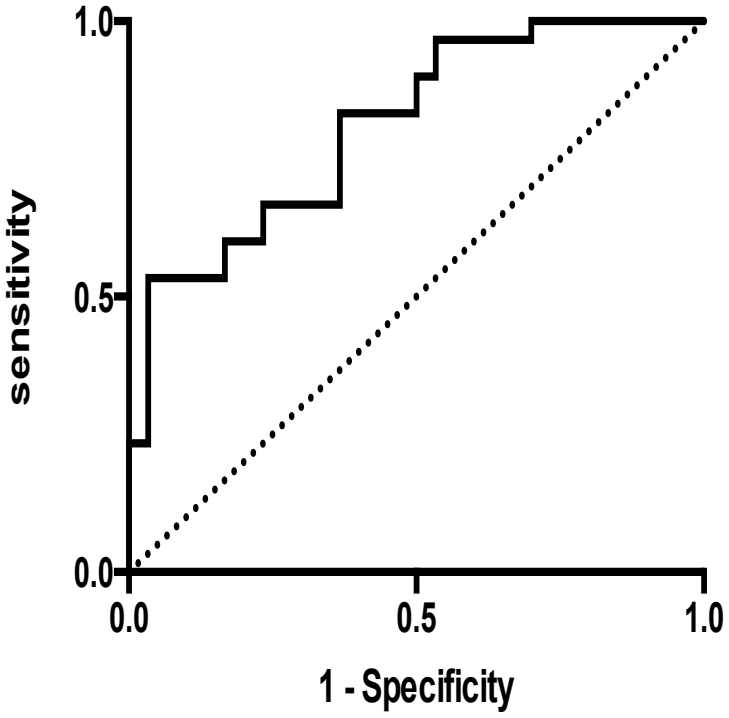


Figure 21: ROC curve for angiotensinogen: tissue kallikrein. AUC=0.81 (Standard Error (SE) = 0.05). A cut-off value of >0.27 gives a sensitivity of 0.9 (95% CI = 0.74 – 0.97), a specificity of 0.5 (0.24-0.65), positive predictive value (PPV) = 0.63 (0.47-0.75) and a negative predictive value of 0.87 (0.53-0.96).

When the relationship between angiotensinogen and tissue kallikrein levels was defined there was a significant negative correlation between the two with a correlation coefficient r = -0.5513 (95% CI = -0.71-0.34) (Figure 22).

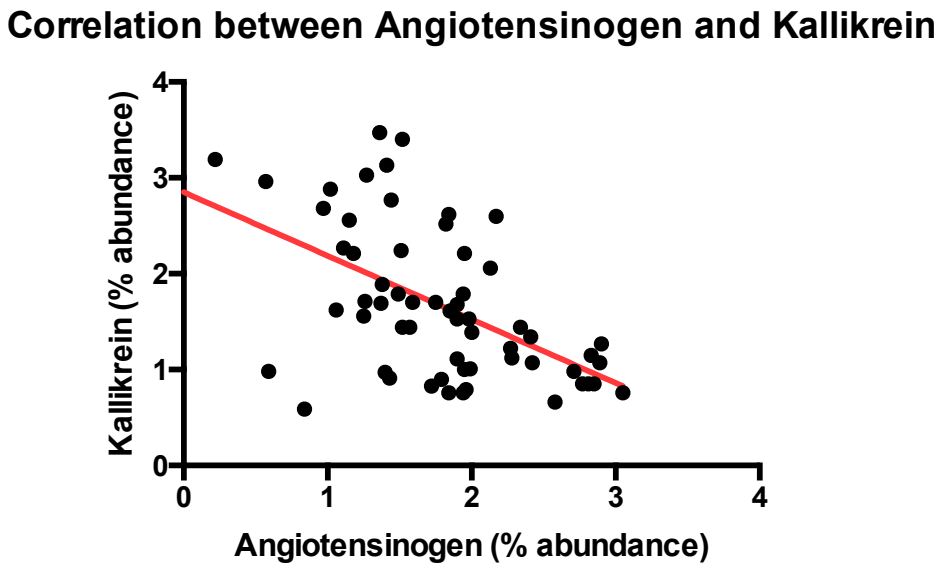


Figure 22: Correlation between Tissue kallikrein and Angiotensinogen.

Complement C3Bb and Complement factor B were significantly raised in first trimester samples that later develop early onset pre-eclampsia (figure 23)

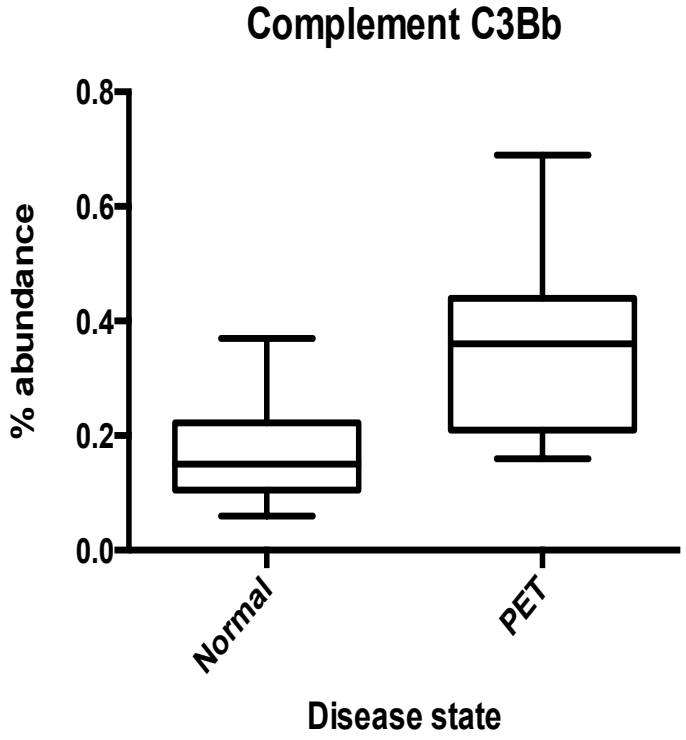


Figure 23 (continued overleaf)

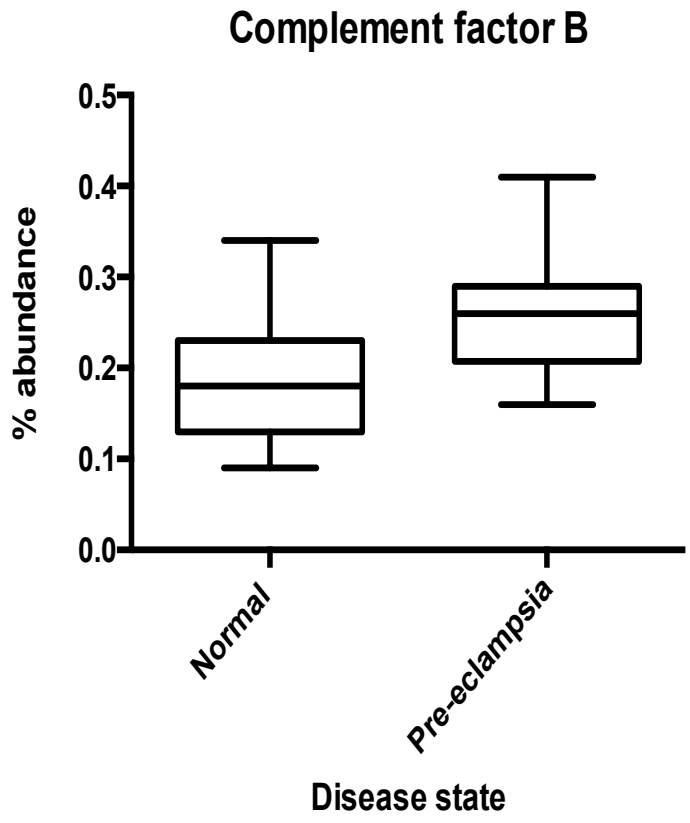


Figure 23: Box plot of the median value for the % abundance of complement factors involved in the alternative pathway of complement (Complement C3Bb and Complement factor B) in the first trimester individual samples from women who did and did not go on to develop pre-eclampsia. All proteins are significantly increased in the pre-eclampsia samples (p=<0.001). The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points

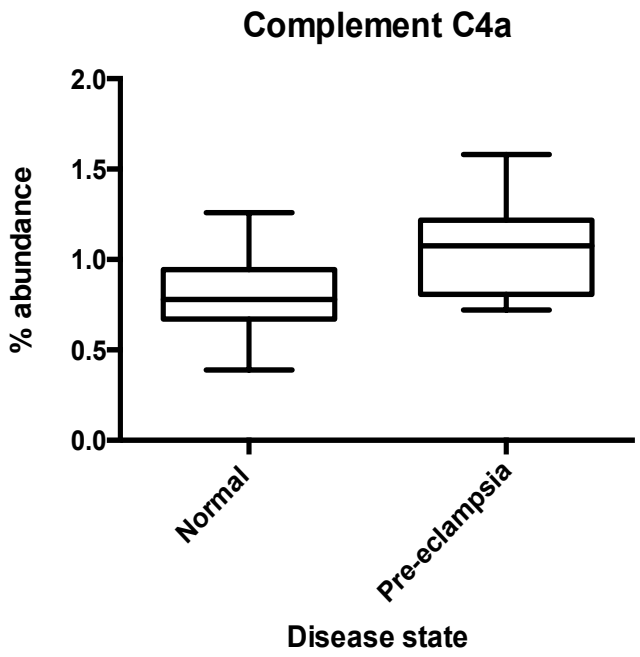


Figure 24: Box plot of the median value for the % abundance of complement factors involved in the classical pathway of complement (complement C4a) in the first trimester individual samples from women who did and did not go on to develop pre-eclampsia. All proteins are significantly increased in the pre-eclampsia samples (p=<0.001). The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points.

Complement factor H and Complement C4b are significantly down regulated in individual first trimester plasma samples of women who go on to develop pre-eclampsia ($p<0.0001$ and $p<0.0003$ respectively) (figure 25).

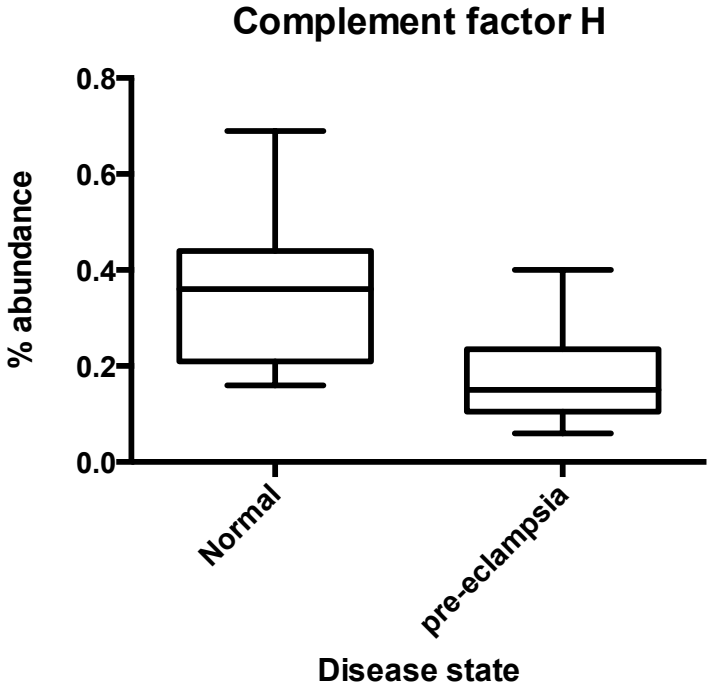


Figure 25 (continued overleaf)

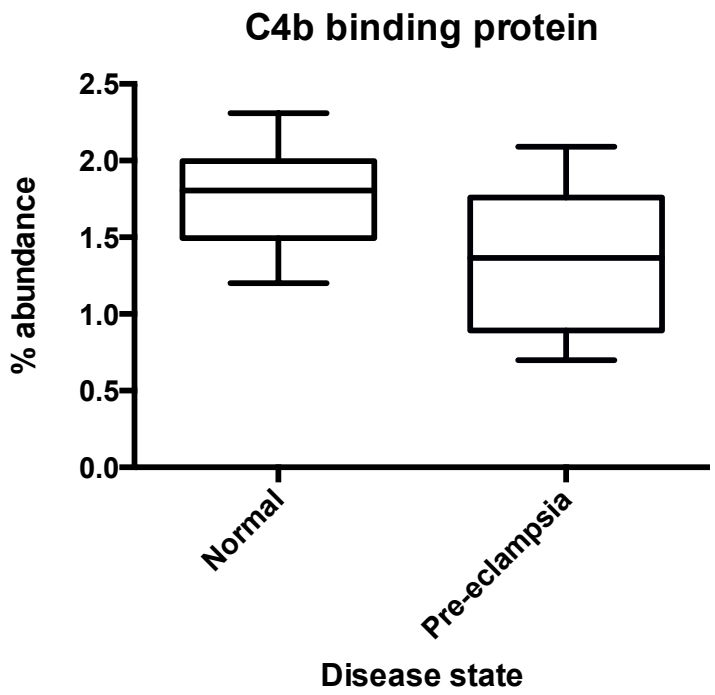


Figure 25: Box plot of the median value for the % abundance of proteins involved regulation of complement pathways in first trimester individual plasma samples from women who did and did not go on to develop pre-eclampsia. Both proteins are significantly decreased in the pre-eclampsia samples (Complement factor H p<0.0001, C4b binding protein p<0.0003). The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points.

Paraoxonase-1, a powerful anti-oxidant is significantly lowered in the first trimester of pregnancies that go on to develop pre-eclampsia (p=0.0034) (figure 26).

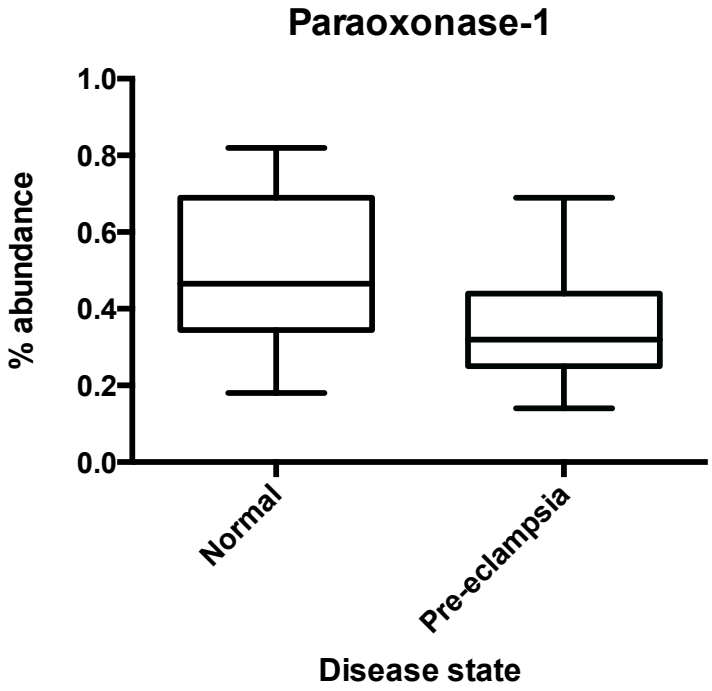


Figure 26: Box plot of the median value for the % abundance of paraoxonase-1 in first trimester individual plasma samples from women who did and did not go on to develop pre-eclampsia. Levels are significantly lower in the pre-eclampsia samples (p=0.0034). The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points

3.5 Discussion

The results of this study show that even in the first trimester of pregnancy, there are significant alterations in the plasma proteome of women who later develop severe early onset pre-eclampsia and those who do not.

Although this pilot study only had a relatively small number of samples (n=30 in each group), the methodology used is such that it overcomes many of the obstacles faced by other proteomic groups. In view of the enormous cost attributed to undertaking proteomics and mass spectrometry, several groups limit the amount of mass spectrometry undertaken by pooling the individual samples to form (usually) two specimens to be studied, “disease” versus “normal”. The problems associated with this are quite obvious, if only one person in a cohort has an abnormally raised protein concentration, then the results of the whole group are altered. This is even the case for large multi-centred prospective studies, such as the SCreening fOr Pregnancy Endpoints (SCOPE) study (Blankley, et al, 2013).

As previously mentioned, in order to study the 5% of the proteome that is of interest to researchers, the remaining proteome needs to be removed. This, and the latest equipment, will allow a detection limit to study small changes between samples. This study removed the 14 most abundant proteins (whereas a lot of other studies remove only the 10 or 12 most abundant proteins) allowing us to push our detection limit further.

Not only has this study shown significantly altered proteins between the two groups of individual plasma samples, these proteins, may allow us to postulate potential pathophysiological pathways involved in disease development and/or allow us to suggest candidate proteins which could allow a screening tool for the disease.

To validate any potential marker, further work on the individual samples must be done.

3.5.1 Angiotensinogen and Tissue kallikrein are altered in pre-eclamptic samples

Two proteins identifiable in the results that are associated with the development of hypertension are altered between the pre-eclamptic and normal groups. Angiotensinogen is converted to angiotensin I by the action of renin, which is produced in the juxtaglomerular apparatus of the kidney. The angiotensinogen I is converted to angiotensin II by angiotensin converting enzyme. Angiotensin II is a powerful vasoconstrictor that leads to systemic hypertension (Prieto et al, 2013). Whilst renin, though commonly thought to be the rate-limiting step of this pathway, some groups have suggested that the plasma concentration of angiotensinogen is and have shown that Increased plasma levels of angiotensinogen have been associated with development of hypertension in the non-pregnant subject (Klett et al, 2001).

Kallikrein belongs to the kallikrein-kinin system, which plays a role in inflammation, apoptosis and blood pressure control (Marcondes et al, 2005). Bradykinin is formed from its inert precursor, high molecular weight kininogen, by kallikrein. The Bradykinin formed causes vasodilatation and smooth muscle relaxation that results in an overall reduction in blood pressure. Kallikrein exists in several forms, mainly plasma kallikrein and tissue kallikrein. Plasma kallikrein, originally thought to be part of the clotting cascade, is involved in inflammation and fibrinolytic activation whilst tissue kallikrein is found in a variety of tissues including kidney and vascular endothelium. Of the many tissue kallikrein proteins, only one, tissue Kallikrein 1 (tK1) is a kininogenase, that is, able to convert kininogen to bradykinin (Madeddu et al, 2007).

As angiotensinogen is raised and tissue kallikrein 1 is reduced in the first trimester of pregnancies that go on to develop pre-eclampsia, it could be suggested that these changes contribute to the later hypertensive syndrome. It has also been shown that the Renin-Angiotensin-System (RAS) plays some role in spiral artery remodeling (Morgan et al, 1998). With alteration of the RAS (as evidenced by the significant increase in angiotensinogen) in the first trimester it can be suggested that this may represent a poorly understood component of the multifactorial pathogenesis of pre-eclampsia, with endothelial damage, oxidative stress and autoimmunity all being suggested as possible mechanisms (Yang et al 2013).

Tissue kallikrein has previously been shown to be altered in pre-eclamptic pregnancies. Khedun et al studied tissue kallikrein levels in women who were hypertensive in pregnancy, and demonstrated a reduction in plasma concentration of the protein (Khedun et al 2000). Other groups have demonstrated how urinary tissue kallikrein: creatinine ratios were altered in pre-eclamptic pregnancies at 28 weeks, but the results were not strong enough to support a possible predictive role. (Kyle et al1996).

Not only are both significantly altered between the two groups, but also the relationship between them suggests a strong correlation ($r = -0.55$, $p<0.001$). Given that both proteins can be up-regulated in inflammation, one could argue that it is due to the presumed heightened inflammatory state of pre-eclampsia that these proteins are altered. However, as the relationship shown is negative and tissue kallikrein is reduced in the pre-eclampsia group this would support the theory that this may represent a pathophysiological pathway and not just a non-specific inflammatory response.

When the ratios of angiotensinogen to tissue kallikrein are plotted on a ROC curve, the area under the curve = 0.81 (SE=0.05) which suggests that this ratio may be able to predict the development of early onset pre-eclampsia. Having a cut-off value greater than 0.27 gives a sensitivity of 0.9 (95%CI = 0.74-0.97), a specificity of 0.5 (0.24-0.65), PPV= 0.63 (95%CI = 0.47-0.75) and a NPV of 0.87 (95%CI = 0.53-0.96). In high-risk women (defined as those having had severe early onset pre-eclampsia

previously) PP13 had a greater ability to predict pre-eclampsia in the first trimester (AUC=0.87), but this has not been reproducible in low-risk women (Khalil et al, 2010). Several other groups have demonstrated an enhanced ability to predict pre-eclampsia, but to obtain this higher overall accuracy the biochemical results were combined with ultrasound indices. Even then, the AUC was marginally greater than the one presented in this chapter at 0.84 (Thaliganathan et al, 2010). Another study showed how early third trimester (20-24weeks) placental Growth Factor (PlGF) levels combined with abnormal uterine artery Doppler indices had similar findings to the results presented in this thesis (AUC=0.8) for predicting severe early-onset pre-eclampsia (Espinoza et al, 2007). However, the clinical usefulness of a test for prediction of severe early-onset pre-eclampsia should be questioned. This would offer limited opportunity for interventions to prevent disease occurrence / reduce disease severity (but could serve as a marker for those women where earlier, more intense surveillance is necessary).

3.5.2 Inflammatory markers are altered in pre-eclampsia samples

Several markers of inflammation are upregulated in the first trimester samples that go on to develop pre-eclampsia. These inflammatory proteins are generic, non-specific proteins that include the members of the complement system. With up-regulation of C4a indicating potential activation of the classic pathway of complement and complement factor B

and C3bB indicating potential activation of the alternative pathway of complement, this suggests that a strong inflammatory proteome is present in the first trimester of pregnancies later complicated by pre-eclampsia. This idea is supported further by the down-regulation of proteins involved in controlling the above pathways (Complement factor H and C4b binding protein).

Whilst pregnancy itself is an inflammatory state, pre-eclampsia appears to be associated with an aggravated form of this state with both systemic and placental inflammation noted during the clinical syndrome (Redman et al, 1999). This may explain the crossing of the interquartile ranges between the normal and pre-eclampsia groups.

Inflammatory (innate) response to stimuli is quick and (generally) non-specific and when activated releases cytokines or chemokines that attract and instruct adaptive immune cells to generate antigen-specific responses in the form of antibodies or cytotoxic cells. First trimester placentation induces a localised innate response (including uNK activation) crucial for normal spiral artery remodeling, which results in the release of inflammatory markers into the systemic circulation that are responsible for the normal systemic inflammatory state of pregnancy (Borzychowski et al, 2006). It could be postulated that the poor placentation of pre-eclamptic pregnancies leads to a more aggressive form of this localized inflammatory response (due to tissue necrosis and increased oxidative stress) and this in turn leads to a more aggressive systemic inflammatory response (figure 27).

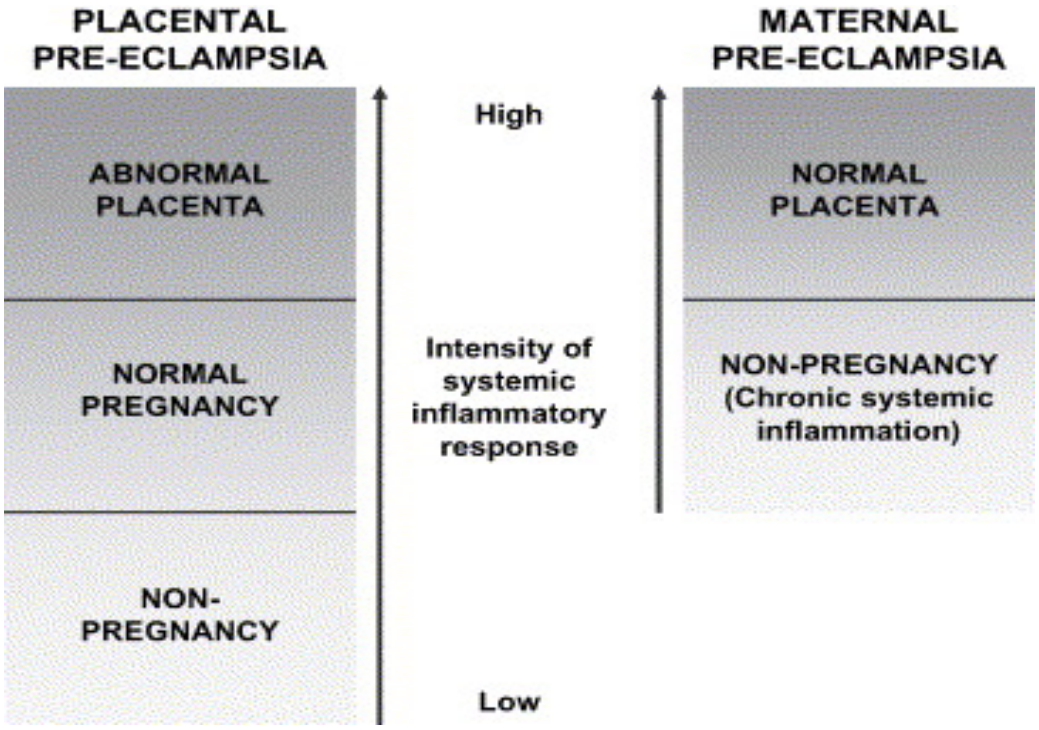


Figure 27: A hypothetical grey scale of increasing systemic inflammation. In a normal woman, although pregnancy stimulates a systemic inflammatory response, it is not intense enough to generate the signs of pre-eclampsia. To do that requires the abnormal stimulus from an oxidatively stressed placenta (left column: Placental Pre-eclampsia). In a woman with chronic systemic inflammation associated with conditions such as chronic hypertension, diabetes or obesity, which predispose to pre-eclampsia, the starting point is abnormal enough such that even a normal placenta can stimulate a systemic response of a sufficient intensity to produce the signs of pre-eclampsia (right column: Maternal Pre-eclampsia). In clinical practice there are many mixed presentations with both maternal constitution and placental ischaemia contributing to the presentation (Adapted from Borzychowshi et al., 2006).

We have shown an increase in non-specific inflammatory proteins in the first trimester of pregnancies later affected by severe pre-eclampsia, which would support the idea of a heightened innate (that is, a non-specific inflammatory) immune response.

3.5.3 Paraoxonase-1 is lower in pregnancies that go on to develop pre-eclampsia

The powerful antioxidant Paraoxonase-1 is significantly reduced in women who go on to develop pre-eclampsia ($p=0.0032$). Antioxidants stop free radicals from causing tissue damage and in-vivo levels have been studied as markers of cardiovascular health. Lower levels of anti-oxidants are associated with a greater degree of cardiovascular disease (Madamanchi et al, 2005). Higher levels of free radicals and oxidative stress have been found in pre-eclampsia and it could be argued that reduced paraoxonase-1 represents either an intrinsic antioxidant deficiency that leads to pre-eclampsia or greater consumption during oxidative homeostasis. This is explored further in chapter 5.

3.5.4 Potential biomarkers?

Whilst this study has shown significant alteration in the first trimester

plasma proteome of women who go on to develop pre-eclampsia, Are any of these markers capable of predicting the disease? The simple answer is, no. In order for a candidate protein to be introduced into a clinically useful test it must go through several stages (figure 28).

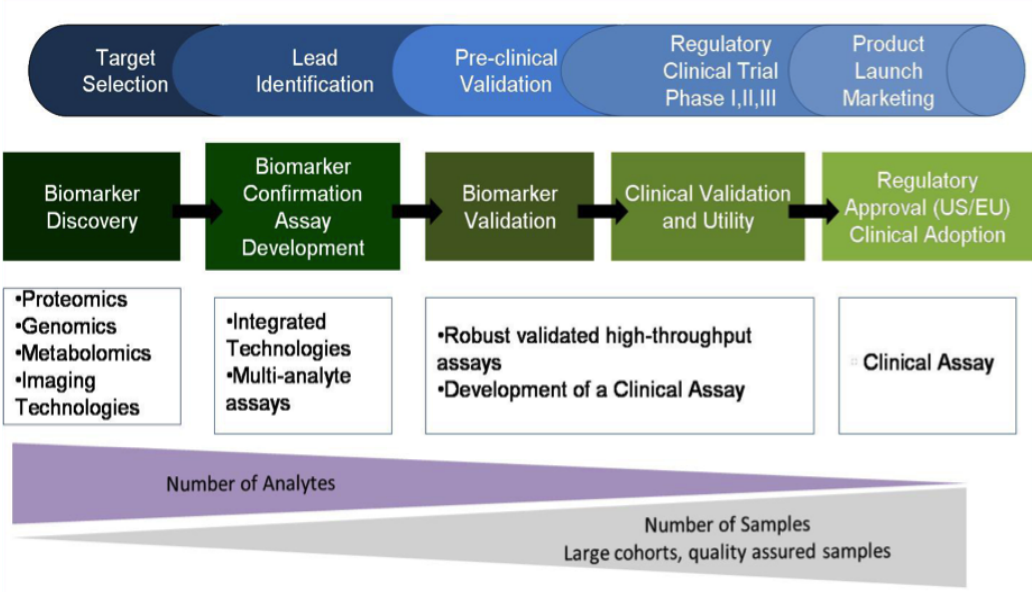


Figure 28: Schematic representation of biomarker development. (adapted from molecularmedicineireland.ie)

What this study does provide is a selection of novel candidate proteins to undergo further prospective validation studies.

3.6 Conclusion

This study represents the first part of this journey; biomarker discovery. One of the crucial stages of biomarker discovery is ensuring that robust study

design. This was achieved in this study by:

- Phenotypically identical specimens
- Similar gestational age
- Alternation of samples for mass spectrometry measurement

In order for any of these candidate proteins to be introduced as a clinically viable biomarker, then large validation steps would need to be undertaken.

What this work has identified are “themes” of potential pathophysiological pathways and disease markers. In the next chapters, I shall explore whether these themes are reproducible – that is, do we already have the ability to accurately predict the onset of the disease or predict poor outcomes after the development of the disease by utilising the standard investigations undertaken during routine clinical care?

Chapter 4

Ability of common inflammatory markers to predict pre-eclampsia

4.1 Introduction

As discussed in previous chapters, the clinical syndrome of pre-eclampsia occurs many months after the initial insult that lead to the systemic endothelial dysfunction. These two distinct stages have led to pre-eclampsia being seen as “a two stage disorder” (Borzychowski et al, 2006). Normal pregnancy is associated with a raised inflammatory condition and the two stages of pre-eclampsia are thought to either result from, or cause, this inflammatory state to become heightened in some women (Walker et al, 2011).

This inflammatory state is thought to be innate immune mediated. This leads to a quick, non-specific response to stimuli and does not rely on the presence of a foreign antigen to initiate an immunological “memory”. The innate system can interact with the adaptive immune system but does not require this interaction to function, whereas, the adaptive system cannot function without signals from the innate system. A systemic inflammatory response is not necessarily generated by antigenic stimulation. Indeed, in pregnancy, it almost certainly does not result from antigen stimulation by a genetically foreign fetus (Borzychowshi et al, 2006).

4.1.1 Pre-eclampsia; a two-stage disorder

One of the initial insults thought to play a significant role in developing pre-eclampsia is the poor invasion of embryonic trophoblast into the maternal

spiral artery vasculature. In a nutshell, this causes hypoxia, placental necrosis and predisposes to fibrin and thrombus formation (Walker et al, 2011). This now "damaged" placenta results in alteration of the finely balanced angiogenic-antiangiogenic partnership with an increase in circulating anti-angiogenic markers. These then lead to a series of pathological changes that result in the clinical syndrome of pre-eclampsia (explained further in figure 29).

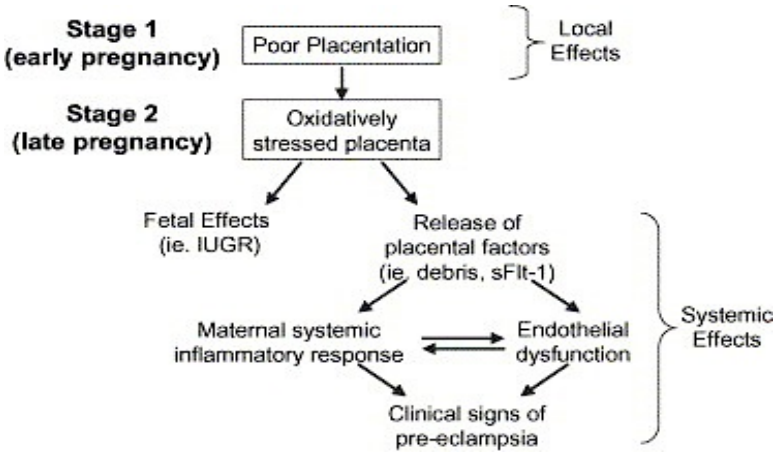


Figure 29: The two stages in the development of pre-eclampsia; Pre-clinical stage 1 pre-eclampsia occurs in early pregnancy when insufficient trophoblast invasion leads to poor placentation resulting in placental hypoxia. Stage 2 occurs systemically when an oxidatively stressed placenta releases factors into the maternal circulation, which cause the maternal systemic inflammatory response and endothelial dysfunction that lead to the clinical signs of pre-eclampsia (Adapted from (Borzychowshi et al, 2006).

4.1.2 Inflammation

Inflammation is “an essential response provided by the immune system that ensures survival during infection and tissue damage” (Ramma et al, 2011). It allows the host tissue to repair and removes harmful stimuli. As inflammation is usually initiated by the innate immune system, cells involved in the innate response are pivotal in the inflammatory processes (Akira et al, 2006) (these cells are depicted in figure 30)

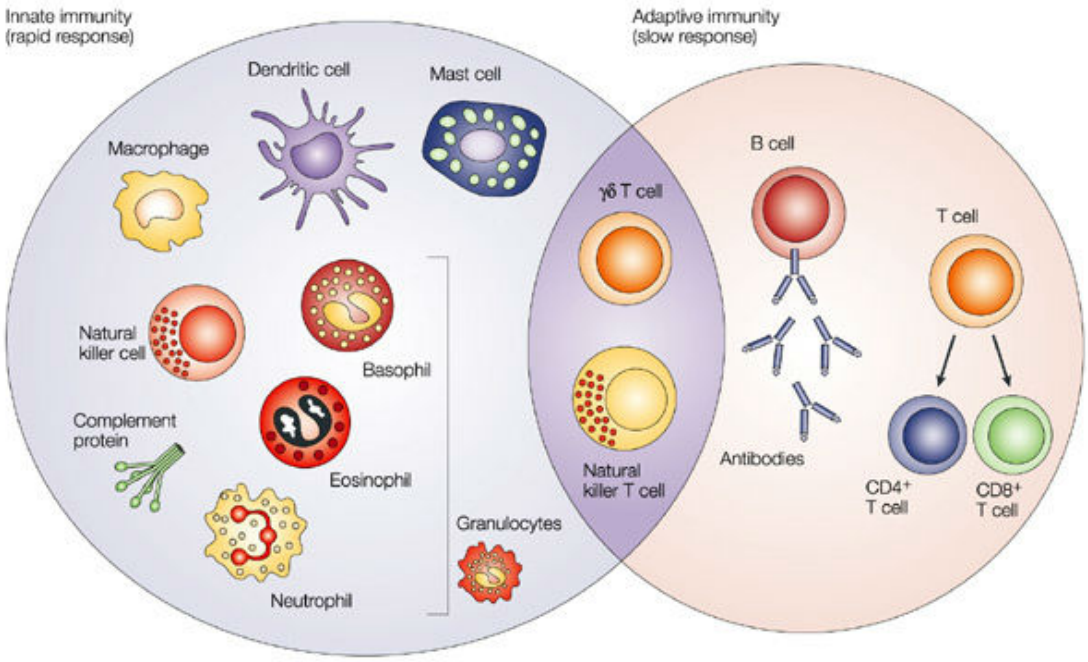


Figure 30: The innate immune response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells (adapted from Dranoff et al, 2004)

Even though deemed non-specific, different tissue damage results in different aspects of the inflammation pathways to be activated. These tissue/pathogen specific receptors induce the production of inflammation mediators, which include cytokines, (such as tumour necrosis factor (TNF) and members of the interleukin (IL) family) and chemokines. These accelerate the progression of inflammation through modifying vascular endothelial permeability as well as recruiting neutrophils and excess plasma (containing antibodies and complement factors) (Ahmed et al, 2011).

Chemokines recruit T lymphocytes that, in turn, secrete large amounts of cytokines and a subgroup of T lymphocytes that are the most prolific producer of cytokines are known as T-helper cells, or CD4 cells. Whilst vast types of cytokines are produced, they are generally split into two functions. The first group are pro-inflammatory, consisting of interferon-gamma, IL-2 and TNF-beta, and activate macrophages (and are involved in phagocyte dependent protective responses). The T helper cells that produce these are known as Th1, and form the basis of cell-mediated immunity. In order to halt the cascade of inflammation, a second group of T helper cells produce anti-inflammatory cytokines (mainly members of the interleukin family) and are referred to as Th2 cells. They also produce antibodies, activate eosinophils and inhibit several macrophage functions (and are therefore involved in phagocyte independent protective responses) and form the basis of humoral immunity (Romagnani et al, 1999). Th2

cytokine production also occurs in non-lymphoid tissue (including the trophoblastic tissue). In the non diseased, non pregnant state there is a perfect equilibrium between the Th1 and Th2 states.

The duration of inflammation depends on the extent of tissue damage and in most cases the excessive production of cytokines mediate release of acute phase proteins (including c-reactive protein (CRP) and coagulation factors) leading to systemic markers of local inflammation.

4.1.3 Inflammation and pregnancy

Pregnancy was initially thought to be anti-inflammatory, with a predilection for Th2 or humoral immunity. Pregnancies with a prominence of Th1 type immunity were thought to be at greater risk of developing poor outcomes such as miscarriage, preterm birth and pre-eclampsia (Challis et al, 2009). However, evidence supporting this was contradictory. Mor et al (2011) propose that these inconsistencies were due to researchers seeing pregnancy as a single “immune event”. They argue that pregnancy is split into three distinct immunological states, a pro-inflammatory first trimester (Th1 mediated), an anti-inflammatory second trimester (Th2 mediated) and a return to a heightened inflammatory state in the third trimester.

4.1.4 Measuring markers of inflammation in clinical practice

It is common practice to investigate the aetiology of symptoms or monitor disease activity by monitoring markers of inflammation. These markers include acute phase proteins and the cells involved in innate immunity (leucocytes). Leucocytes are measured clinically as part of a full blood count.

4.1.5 The Full Blood Count

Haemoglobin, White blood cells and platelet count are measured in the UK via the Full Blood Count (FBC). Being one of the most commonly requested haematological investigations, it provides screening for anaemia, infections, inflammation, some clotting disorders and can be used to diagnose malignancy. The elements of a FBC sample (with normal non-pregnant and pregnant values) are listed in table 18 overleaf.

Parameter (units)	Non[$] pregnant	Pregnant* 1st Trimester	Pregnant* 3RD Trimester
Haemoglobin (g/L)	115 - 160	110-140	105-140
Platelets ($x10^9$/L)	150 - 400	150-400	150-400
Mean Cell Volume (fL)	78 - 100	78-100	78-100
RBC ($x10^{12}$/L)	3.8 - 5.8	3.4-4.55	2.7-4.4
Mean Corpuscular Haemoglobin (pg)	27 - 32	27-32	27-32
Red Cell Distribution Width (%)	11.5 - 15.0	11.5-15	11.5 - 15
White Blood Cell ($x10^9$/L)	4 - 11	6-14	5.9-16.9
Neutrophils ($x10^9$/L)	2 - 7.5	3-10	6 - 17
Lymphocytes ($x10^9$/L)	1.0 - 4.5	1-4.5	1 - 4.5
Monocytes ($x10^9$/L)	0.2 - 0.8	1-1.1	0.1 - 1.4
Eosiniphils ($x10^9$/L)	0.04 - 0.40	0-0.6	0 - 0.6
Basophils ($x10^9$/L)	<0.1	<0.1	<0.1

Table 18 Full Blood Count, its components and normal values in non pregnant women and women in the 1st and 3rd* trimester of pregnancy ([$]adapted from Nelson-Piercy, 2006, * Adapted from Abbassi-Ghanavati et al, 2009)*

4.1.6 Leucocytes in the non-pregnant state

The main function of leucocytes in both the pregnant and non-pregnant population is to fight infection, with each leucocyte subtype having their own function (table 19, overleaf).

In addition there has been a lot of work assessing the potential of leucocytes as markers of the inflammation. In 1974, Freidman et al were the first to link leucocyte count as a predictor for myocardial infarction and since then studies have cemented the association of raised leucocyte count with atherosclerosis and cardiovascular events (Hoffman et al, 2003). Raised neutrophil and low lymphocyte counts were both shown to be a better predictor of cardiovascular disease (hazard ratio (HzR) for raised neutrophil count – 2.06, HzR for lower lymphocyte count – 0.41). However, when assessed together, lymphocyte to neutrophil ratio was the best predictive marker with an HzR of 2.73 ($p<0.001$) (Horne et al, 2005)

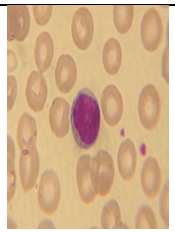
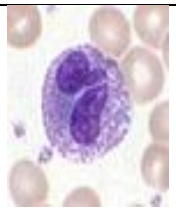
Leucocyte component	Appearance	% in adults	Function/ Target	Lifetime
Neutrophil		62%	Bacteria /Fungi	6hr - several days
Lymphocyte		30%	B cells release antibodies and activate T cells. T cells have many components antibacterial and immune functions.	Years for memory cells. Weeks for all others
Monocyte		5.3%	Migrate to other tissues and differentiate into tissue resident macrophages	Hours to days
Eosinophil		2.3%	Larger parasites Modulate allergic inflammatory responses	8-12 days
Basophil		0.4%	Release histamine for inflammatory response	Hours to days

Table 19: Leucocyte overview (adapted from Wikipedia)

4.1.7 Leucocytes in pregnancy

Pregnancy is associated with a physiological leucocytosis that is thought to represent both the generalized systemic inflammatory state associated with normal implantation and placentation (Canzoneri et al, 2011) and a means of protecting the fetus from ascending infection (Korgun et al, 2002) with the main reason for this leucocytosis being due to a rise in neutrophil count (Von Dadelszen et al, 1999).

4.1.8 Leucocytes in pre-eclampsia

Pre-eclampsia is associated with endothelial activation and dysfunction which in turn leads to a heightened inflammatory state. This inflammatory state is thought to cause a leucocytosis, greater than that seen in normal pregnancy, and again, neutrophilia plays the largest part in creating the leucocytosis (Canzoneri et al, 2009).

Despite the evidence that there is a significant rise in neutrophil count during pre-eclampsia, there is limited work on leucocyte count before pre-eclampsia develops. The PIERS group (2011) included total leucocyte count in their model to predict pre-eclampsia, but found it was unable to do so.

4.2 Aim

The aim of this work is to identify parameters from a full blood count, obtained in the first trimester of pregnancy, that are altered between women who go on to develop pre-eclampsia and those who do not.

4.3 Methods

A retrospective case-control study of women who delivered at University Hospitals of Coventry and Warwickshire NHS Trust (UHCW) between 1999-2010 (n=50,712) was performed. UHCW is a large tertiary referral teaching hospital in the West Midlands serving a multi ethnic low socio-economic population. Women were included if they were diagnosed with pre-eclampsia between 1999 and 2010 with pre-eclampsia being defined as a blood pressure ≥ 140/90mmHg (with Korsokoff V used to determine diastole) with proteinuria of either 0.3g/L/24hrs or (+) proteinuria on urinary dipstix analysis (ISSHP, 2001). For each woman diagnosed with pre-eclampsia, a control was selected (from women who had the same estimated delivery date) who did not develop pre-eclampsia. Both sets of women were identified from this hospital database and their mode of delivery and neonatal outcomes were retrieved. Severe pre-eclampsia was diagnosed in line with the ACOG guidelines (one or more of the following

features: Blood pressure of 160 mm Hg systolic or higher or 110 mm Hg diastolic or higher on two occasions at least 6 hours apart while the patient is on bed rest / Proteinuria of 5 g or higher in a 24-hour urine specimen or 3+ or greater on two random urine samples collected at least 4 hours apart / Oliguria of less than 500 mL in 24 hours / Cerebral or visual disturbances / Pulmonary edema or cyanosis / Epigastric or right upper-quadrant pain / Impaired liver function / Thrombocytopenia / Fetal growth restriction). Following delivery, midwives entered the antenatal history and delivery details into the hospital database (Evolution, CSC Healthcare IT).

Women were excluded if they first visited the midwife (the booking visit) after 14 weeks gestation, if she had any past history of liver or haematological disease, had multiple pregnancies or was less than 20 years old. Smoking can cause neutrophilia, so women who self reported that they were smokers were also excluded.

When the woman first presented to her midwife the midwife performed a series of investigations known as the 'booking bloods'. These consisted of blood group, antibody screen, infection screen and a FBC. For the FBC, 3-4ml of blood was collected into an EDTA (ideal concentration 1.5-2.2mg/ml) anti-coagulate sample bottle from the patient's antecubital fossa and the sample was mixed well. The blood was then placed in an automated cell analyser (SIEMENS Advia 2120, USA), which counts the components of the FBC, flagging any morphologically abnormal cells in the process.

The first trimester blood results were obtained from the Clinical Results Recording System (CRRS). Neutrophil to Lymphocyte ratio was calculated by dividing the neutrophil count by the lymphocyte count. Those who had abnormal parameters on FBC or liver function test were excluded.

The SPSS computer programme (SPSS v19, Chicago, USA) was used for the statistical analysis and statistical significance was defined as p=<0.05. We compared categorical variables using the χ^2 test and continuous variables using t-tests. Non-parametric data were analysed by the Mann-Whitney-U test.

4. Results

4.4.1 The whole pre-eclampsia group

4.4.2 Maternal demographics

992 consecutive women met the inclusion criteria (had pre-eclampsia, singleton pregnancy, older than 20 years and had FBC and LFT within the normal range) 67 self-classified themselves as smokers and were excluded, leaving 925 women. These were matched against 925 women who did not develop pre-eclampsia. The demographics of these participants are listed in table 20.

As expected, women in the pre-eclampsia group were significantly more likely to have an increased BMI ($p=0.03$), have higher booking blood pressure ($p=0.01$), deliver before 37 weeks gestation and deliver a baby that was less than the 10^{th} GROW centile (both $p<0.001$).

	Patient group Mean (Standard deviation)		P value
	Pre-eclampsia	Control	
Age (years)	25.1(3)	24.7(2.8)	0.7
Height (cm)	162 (7)	160(8)	-
Weight (kg)	70.8 (20)	64.5(15.5)	-
BMI (Kg/m^2)	26.9 (8)	25.1(5.3)	0.03
Gestation at booking	13.9 (1.7)	13.8 (1.5)	0.64
SBP at booking	121 (17)	112(14.5)	0.01
DBP at booking	75 (11.3)	67 (10.9)	0.01
DBP at diagnosis of PET*	160 (17.8)	119 (14.5)	-
DBP at diagnosis of PET*	106 (14)	70 (8.7)	-
Delivery before 37 weeks (n and %)	79 (8.6%)	47 (5.1%)	<0.001
SGA $<10^{th}$ GROW centile (n and %)	152 (16.5%)	53 (6%)	<0.001

*Table 20: Demographic features for the "pre-eclampsia" and the "normal" group (BMI = Body Mass Index, PET = pre-eclampsia, n=number, GROW =gestation related optimised weight) *In the control group this reading represents the highest third trimester blood pressure.*

4.4.3 Full Blood Count comparisons

There were no differences in the standard measured parameters of first trimester FBC samples between the two groups, Although neutrophil count appeared higher in the pre-eclampsia group, this did not reach significance (p=0.063). Table 21 demonstrates the difference between the normal and pre-eclampsia groups.

Parameter	Normal	Pre-eclampsia	p value*
	(median with IQR)		
Haemoglobin (g/L)	122 (18)	121 (18)	0.76
Platelets (x 10^9/L)	217 (29)	223 (32)	0.11
WBC (x 10^9/L)	7.6 (1.7)	8.2 (2.1)	0.54
Neutrophil (x 10^9/L)	4.5 (1.5)	5.1 (1.6)	0.06
Lymphocyte (x 10^9/L)	2.4 (6.1)	2.03 (7.4)	0.09
Monocyte (x 10^9/L)	0.5 (0.3)	0.6(0.3)	0.82
Eosinophil (x 10^9/L)	0.15 (0.01)	0.17 (0.02)	0.60
Basophil (x 10^9/L)	<0.01	<0.01	-
N:L ratio	2.04	2.47	<0.001

Table 21: First trimester Full Blood Count parameters between women who went on to develop pre-eclampsia in the third trimester (the Pre-eclampsia group) and those who did not (the normal group). WBC=White Blood Cell, N: L ratio= Neutrophil to lymphocyte ratio. Significance taken as p<0.05.

4.4.4 Neutrophil to Lymphocyte ratio

Although not a commonly reported parameter of the FBC, the Neutrophil to lymphocyte ratio was raised in women who went on to develop pre-eclampsia ($p<0.001$), with the difference between the two groups represented in figure 31:

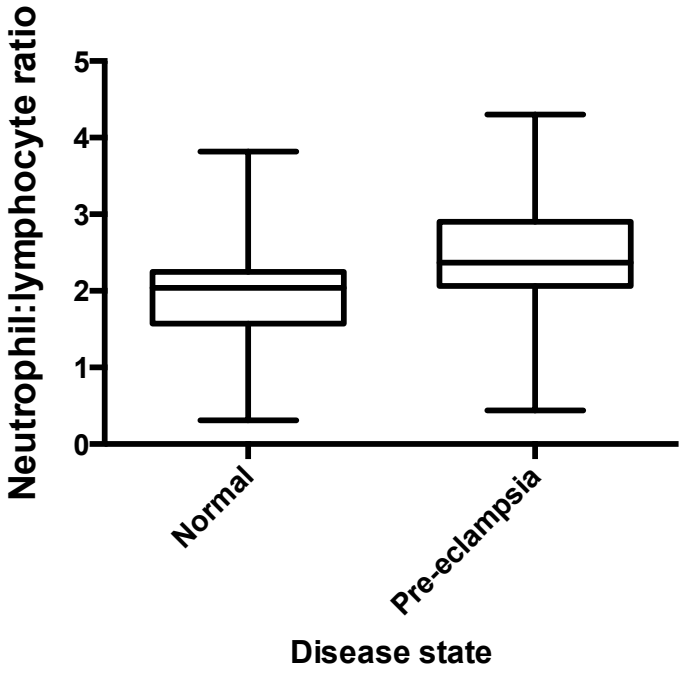


Figure 31: Box plot of the median value of neutrophil to lymphocyte ratios in first trimester FBC samples from women who did and did not go on to develop pre-eclampsia. The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points

To assess the incremental predictive ability of the neutrophil to lymphocyte ratio to predict the onset of pre-eclampsia, a Receiver-Operator Characteristic (ROC) curve was produced (figure 32, below), with the area under the curve (AUC) calculated (AUC = 0.7237).

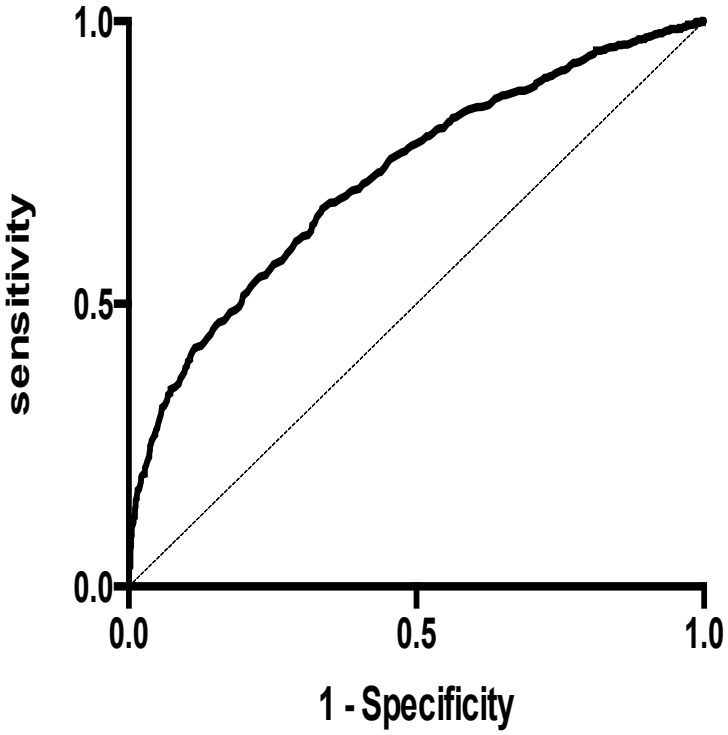


Figure 32: Receiver Operator Characteristic curve of neutrophil to lymphocyte ratio values in predicting the development of pre-eclampsia (AUC = 0.7237 (95% CI 0.7009 – 0.7466); at a cut off of 2.47 Sensitivity =0.81 (95%CI 0.73-0.87), specificity =0.56 (95%CI 0.48-0.65), PPV=0.63 (95% CI 0.56-0.70), NPV=0.77 (0.67-0.84).

4.4.5 The Severe pre-eclampsia group

4.4.6 Maternal demographics

144 women met the inclusion criteria (had severe pre-eclampsia as defined by the ACOG), singleton pregnancy, older than 20 years and had FBC and LFT (within the normal range) 8 self-classified themselves as smokers and were excluded, leaving 136 women. These were matched against 136 women with the same estimated delivery date who did not develop pre-eclampsia. The demographics of these participants are listed in table 22.

Women in the severe pre-eclampsia group were significantly more likely to have an increased BMI ($p=0.01$), deliver before 37 weeks gestation and deliver a baby that was less than the 10^{th} GROW centile (both $p<0.001$). There was, however, no difference between booking blood pressures between the two groups.

	Patient group (Mean (standard deviation))		P value
	Pre-eclampsia	Control	
Age (years)	25.7(3.1)	24.4(2.9)	0.09
Height (cm)	162 (7)	160(8)	-
Weight (kg)	70.8 (20)	64.5(15.5)	-
BMI (kg/m^2)	27.1(8.2)	25.0(5.6)	0.01
Gestation at booking	13.2 (1.6)	13.6 (0.9)	0.64
SBP at booking (mmHg)	124 (17)	113(14.5)	0.01
DBP at booking (mmHg)	79 (11.3)	67 (10.9)	0.01
SBP at diagnosis of PET* (mmHg)	163 (17.8)	117 (13.9)	-
DBP at diagnosis of PET* (mmHg)	106 (14)	71 (8.3)	-
Delivery before 37 weeks (n and %)	54 (39.7%)	7 (5.2%)	<0.001
SGA <10th GROW centile (n and %)	152 (16.5%)	53 (6%)	<0.001

*Table 22 Demographic features for the "Severe pre-eclampsia (ACOG)" and the "normal" group (BMI = Body Mass Index, PET = pre-eclampsia, n=number, GROW =gestation related optimised weight) *In the control group this reading represents the highest third trimester blood pressure.*

Full Blood Count comparisons

In women who went on to develop severe pre-eclampsia, there was a significant difference in neutrophil count (p=0.03). Other haematological findings between the two groups were not significant. Table 23 demonstrates the difference between the normal and pre-eclampsia groups.

Parameter	Normal	Pre-eclampsia	p value*
	(median with IQR)		
Haemoglobin (g/L)	128 (21)	126 (15)	0.79
Platelets (x 10^9/L)	225 (31)	236 (39)	0.15
WBC (x 10^9/L)	7.8 (1.6)	8.5 (2.0)	0.58
Neutrophil (x 10^9/L)	4.6 (1.6)	6.7 (2.1)	0.03
Lymphocyte (x 10^9/L)	2.5 (6.3)	2.09 (7.4)	0.08
Monocyte (x 10^9/L)	0.6 (0.3)	0.6(0.3)	0.83
Eosinophil (x 10^9/L)	0.18 (0.01)	0.2 (0.01)	0.58
Basophil (x 10^9/L)	<0.01	<0.01	-
N:L ratio	1.99	2.53	<0.001

Table 23: First trimester Full Blood Count parameters between women who went on to develop severe pre-eclampsia in the third trimester (the Pre-eclampsia group) and those who did not (the normal group). WBC=White Blood Cell, N: L ratio= Neutrophil to lymphocyte ratio. Significance taken as p<0.05.

Neutrophil to Lymphocyte ratio

The Neutrophil to lymphocyte ratio was significantly raised in women who went on to develop severe pre-eclampsia ($p<0.001$), with the difference between the two groups represented in figure 33 (below):

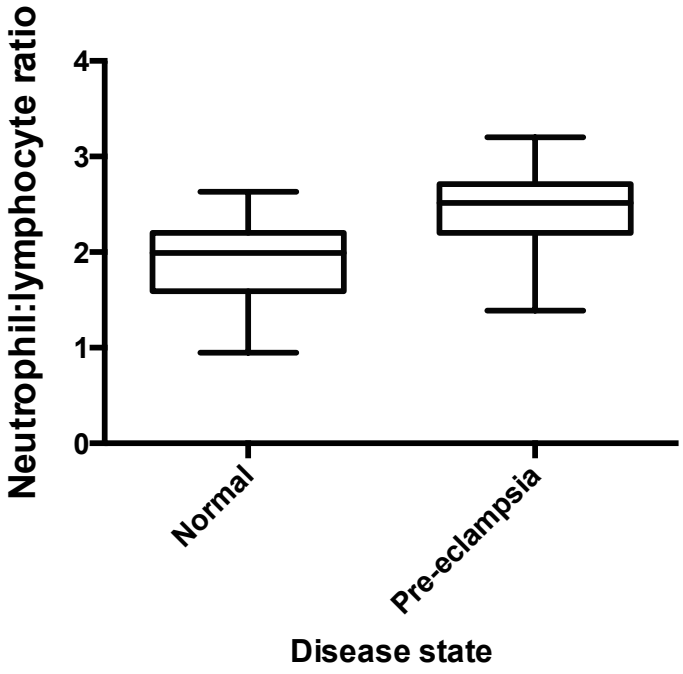


Figure 33: Box plot of the median value of neutrophil to lymphocyte ratios in first trimester FBC samples from women who did and did not go on to develop severe pre-eclampsia. The bottom and top of each box represents the 25th and the 75th percentile respectively; the line represents the median value. Whiskers extend to the most extreme data points

To assess the incremental predictive ability of the neutrophil to lymphocyte ratio to predict the onset of severe pre-eclampsia, I again produced a ROC Curve (figure 34, below) and from this, the AUC (AUC = 0.834) was calculated.

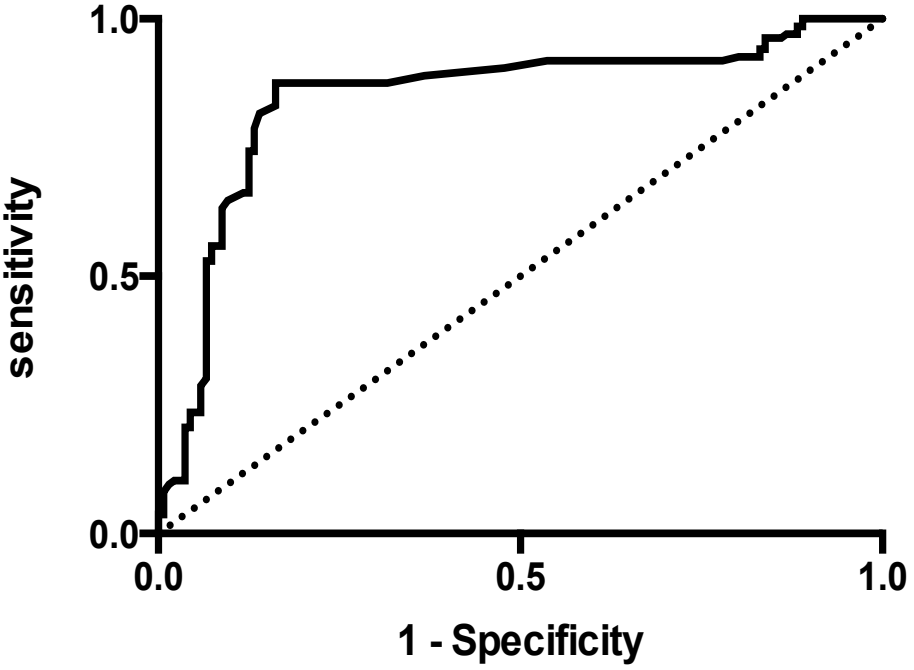


Figure 34: Receiver Operator Characteristic curve of neutrophil to lymphocyte ratio values in predicting the development of severe pre-eclampsia (AUC = 0.84 (95% CI 0.79 – 0.91); at a cut off of 2.53, sensitivity 0.914 (95% CI=0.85-0.95), specificity = 0.6 (95% CI = 0.51-0.67), PPV=0.68 (95% CI = 0.6-0.74), NPV=0.87 (95% CI 0.8 – 0.93).

4.5 Discussion

This study assesses the difference in commonly tested haematological parameters undertaken in the first trimester of pregnancy from women who do and do not go on to develop pre-eclampsia. It evolves from work presented in this thesis, which demonstrates that women who later develop pre-eclampsia have a distorted plasma proteome in the first trimester of pregnancy and part of this altered state involves changes in markers of inflammation.

4.5.1 Summary and discussion of key findings

The maternal demographics between the women who developed pre-eclampsia and those that did not were on a whole expected and did not differ greatly from those discussed in chapter 2. The pre-eclampsia group was statistically more likely to have an increased BMI, have a raised booking blood pressure and have a growth restricted baby (less than 10th GROW centile).

Despite the fact that pre-eclampsia has been shown to be an excessive inflammation state (Borzychowshi et al, 2006), this excessive inflammation is not widely evident in the first trimester, and although various studies have shown up-regulation in certain IL and TNF concentrations (Freeman et al, 2004) there is no difference in the common leucocytes between the normal

and pre-eclampsia groups. However, this is to be expected. Before the systemic clinical syndrome of pre-eclampsia develops, the main site for pre-clinical pathological changes is placental tissue (Sibai et al, 2005). This area of localized inflammation is unlikely to produce an overt inflammatory state that would significantly alter the leucocyte population.

In cardiovascular literature, there is evidence that long before a major pathological event, such as myocardial infarction, or diagnosis with angina (both of which are associated with alteration in leucocytes, thought to represent the inflammatory state of the disease), there are subtle changes suggesting a low-grade inflammation and this low-grade inflammation can be assessed by measuring the neutrophil to lymphocyte ratio (Bhat et al, 2013).

In this study we studied two populations of women with pre-eclampsia. The first were women who developed pre-eclampsia according to the ISSHP guidelines (the ISSHP group), which does not account for the severity of the disease. The second group of women were those who developed severe pre-eclampsia, as defined by the ACOG (the ACOG group). Both of these groups had raised neutrophil to lymphocyte ratios compared to women who did not develop pre-eclampsia, suggesting a marked low-grade inflammation. The neutrophil to lymphocyte ratio was able to predict pre-eclampsia more accurately in the ACOG group than the ISSHP group (AUC = 0.834 versus AUC= 0.7237). These are both greater than other studies involving first trimester markers for pre-eclampsia. Baumann et al (2008)

reviewed several markers to predict late onset pre-eclampsia, showing that when soluble Eng, Inhibin-A and sFlt1 were combined the AUC was 0.656. This could be due to their definition of late pre-eclampsia, and whether late pre-eclampsia is associated with greatly exaggerated endothelial damage. Conversely, the AUC from my study is less than that reported by Bosio et al(2001). They showed that using p-Selectin in the first trimester had the ability to predict pre-eclampsia (AUC=0.93). P-Selectin was expressed by platelets and endothelial cells upon activation and involved in inflammation, and is thought to reflect disturbance of the vascular system. However, in subsequent work P-Selectin has only limited ability to predict pre-eclampsia, with a detection rate of 59% and a false positive rate of 5%, making it inappropriate as a screening tool (Monte et al, 2011).

Imtiaz et al (2012) studied leucocyte concentration and neutrophil to lymphocyte ratios in a general population as a systemic inflammation in chronic diseases. They found that the neutrophil to lymphocyte ratio was increased in people with previously undiagnosed diseases that commonly affect the cardiovascular system, such as diabetes and hypertension. These findings were not present in non-vascular diseases such as asthma and arthritis. This may suggest that the neutrophil to lymphocyte ratio is a marker of inflammation due to endothelial injury. This is further supported by the numerous works involving the neutrophil to lymphocyte ratio as a marker for severity of cardiovascular disease (Bhat et al, 2013).

Neutrophilia accounts for the leucocytosis seen in pre-eclampsia (Canzoneri et al, 2009) and with a lack of first trimester studies that review neutrophils in women who develop pre-eclampsia, this could represent a (unidentified) long-standing up-regulation existing from early pregnancy (or before). As neutrophilia is a marker of inflammation, an increased neutrophil count may suggest an increased level of inflammation (Butterfield et al, 2006).

4.5.2 Strenghts of this study

One of the main strengths of this case-control study is that the data were retrospectively analysed from robust prospectively collected information entered into the maternity information system. Midwives were trained on data entry and, in addition, at the end of the pregnancy the data was checked for completeness by administration teams, cross referencing patient records for procedure/diagnosis coding and payment reasons.

The gestation cut-off of 14 weeks meant that physiological variation in leucocyte concentration was minimised and to reduce selection bias further, the study consisted of 925 *consecutive* women, rather than randomly generating the pre-eclampsia group.

4.5.3 Weakness of the study

The definition of pre-eclampsia is specific, but there is a possibility of falsely diagnosing pre-eclampsia (for example, hypertension in the presence of a urinary tract infection, or urine contamination from amniotic fluid). Including women in the pre-eclampsia group who did not have the disease, may account for the difference in results between the ISSHP and the ACOG group.

In addition, women who truly had pre-eclampsia may have been categorised as having similar disorders such as pregnancy-induced hypertension that could have classified them within the “normal” cohort.

The control group consisted of women who were matched to women developing pre-eclampsia by their estimated due date. In hindsight, it may have been more advantageous to match women according to their age (although in both the ISSHP and ACOG group the difference in ages between the case and control cohorts was not significant).

This case-control study consisted of women from the West-Midlands, which has one of the highest rates of cardiovascular disease in England. Therefore the difference in booking blood pressures between the normal and pre-eclamptic groups may be due to pre-existing hypertension, which may not be reflective of the pregnancy population as a whole.

4.6 Conclusion

Full Blood Counts are routinely performed in pregnant women at the beginning of their pregnancy. Not only do they usually take place before 16 weeks gestation (that is, before placentation is completed) but also, the test is relatively cheap. Given that this study has shown the neutrophil to lymphocyte ratio is increased in women who go one to develop pre-eclampsia, this may serve as a predictor for the disease.

What this study does not assess is whether we are able to predict those who will have poor outcome in pre-eclampsia. In order to do this, I have continued with the theme of assessing commonly ordered investigations in women diagnosed with pre-eclampsia and whether they are able to predict those, that when diagnosed with the disease, will have a poorer outcome. This is presented in the following chapter.

Chapter 5

Inflammatory and antioxidant markers to predict poor outcome in women diagnosed with pre-eclampsia

5.1 Introduction

Failure of the implanting trophoblast to properly invade the maternal spiral arteries has been widely accepted to play a key role in the pathogenesis of pre-eclampsia but the mechanism by which this occurs and the consequence of this event, remain unclear. However, what is becoming clear, is the role placental oxidative and endoplasmic reticulum stress plays towards the development of the clinical disease (Steegers et al, 2010). The placenta appears to be the principal source of free radical synthesis but maternal leucocytes and the maternal endothelium are also likely contributors. In addition studies have suggested an important role for placental trophoblast NAD(P)H oxidase in free radical generation in preeclampsia (Raijmakers et al, 2004).

5.1.1 Oxidative Stress

Oxidative stress occurs when the generation of free radicals (that is, substances with one or more unpaired electrons) exceeds the capacity of antioxidant defense mechanisms (that is, pathways that provide protection against harmful effects of free radicals) (Poston et al, 2004). Free radicals primarily consist of oxygen (collectively known as oxygen free radicals or reactive oxygen species (ROS)) or nitrogen (known as reactive nitrogen species (RNS)) metabolites and many are by-products of common aerobic cellular processes. They are primarily

formed in mitochondria, where the superoxide anion is generated from complex I and III of the electron transfer chain. The superoxide anion ($O^{\bullet}_{2}^{-}$) is formed by the univalent reduction of triplet-state molecular oxygen ($^{3}O_2$). This process is regulated enzymatically by NAD(P)H oxidases and xanthine oxidase and non-enzymatically by redox-reactive compounds such as semi-ubiquinone compound). $O^{\bullet}_{2}^{-}$ is detoxified by manganese superoxide dismutase enzyme (MnSOD) in mitochondria and by copper/zinc superoxide dismutase enzyme (Cu/ZnSOD) in cytoplasm. The SOD enzymes convert $O^{\bullet}_{2}^{-}$ into hydrogen peroxide (H_2O_2) that in turn can be converted into water. Alternatively in the presence of transition metals, $O^{\bullet}_{2}^{-}$ can be converted into the highly reactive hydroxyl radical $^{\bullet}OH$, the most potent of all ROS (see figure 35) (Cindrova-Davies et al, 2009). In physiological conditions, moderate levels of ROS and RNS play a role in cell proliferation and survival. However, when this redox homeostasis is disturbed, there is oxidative stress that can lead to cell death and contribute to disease development (Trachootham et al, 2008).

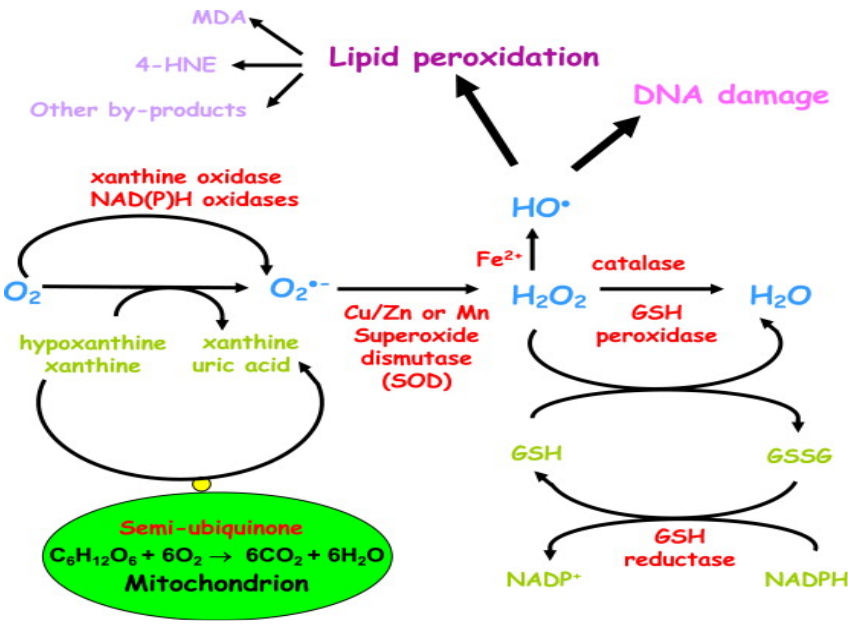


Figure 35: An overview of redox generation and clearance. The superoxide anion (O2−) is formed by the univalent reduction of triplet-state molecular oxygen (^{3}O2). This process is regulated enzymatically by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds (e.g. semi-ubiquinone compound). O2− is detoxified by manganese (if in mitochondria) or copper/zinc (if in cytosol) superoxide dismutase enzyme (MnSOD or Cu/ZnSOD). SOD converts superoxide into hydrogen peroxide (H2O2), which can subsequently be converted into water by the enzymes catalase or glutathione peroxidase. Alternatively, H_2O_2 can be converted into the highly reactive hydroxyl radical (OH) in the presence of reduced transition metals via the Fe^{2+}-dependent Fenton reaction. GSH – glutathione; GSSG – glutathione disulphide. (Adapted from Cindrova-Davies, 2009).

5.1.2 Anti-oxidants

As mentioned, antioxidants play a crucial role in redox homeostasis and these exist in enzymatic and non-enzymatic forms. In general anti-oxidants work by stopping free radicals by removing the unpaired electron(s), reducing the substrate and being oxidised themselves in the process. Non-enzymatic anti-oxidants include vitamin C, vitamin E, quinones, carotenoids and bilirubin (Rock et al, 1996).

Given the effect free radicals and oxidative stress may play in the pathophysiology of many diseases, a significant number of trials have been undertaken with a view to assessing the effect anti-oxidant supplementation has in preventing disease development and progression. However, when these trials have been assessed in robust systematic reviews the results were not as expected. Supplementation with some antioxidants (Beta carotene, vitamin A and vitamin E) increased mortality (Bjelakovic et al, 2007).

Despite this negative association with exogenous antioxidants, endogenous antioxidants have been used as a marker of health. In particular bilirubin has been shown to be protective against cardiovascular morbidity.

5.1.3 Bilirubin

Bilirubin is a powerful antioxidant with the ability to prevent lipid (amongst others) oxidation with greater efficacy than vitamin E (Otero Regino et al, 2009). It is formed via the breakdown of haem, the oxygen-carrying component of red blood cells. This is described further in figure 36:

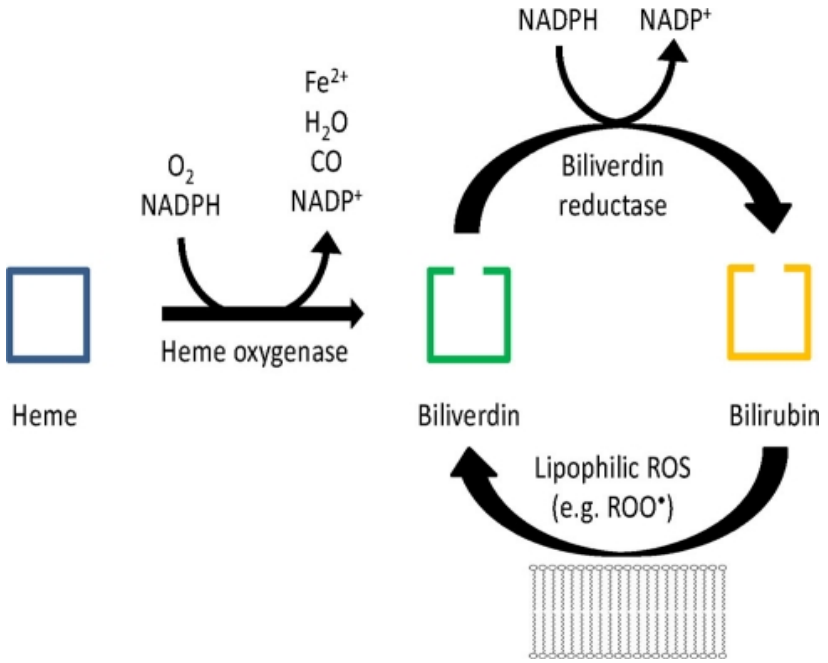


Figure 36: The antioxidant-redox cycle of the Haemoxygenase /Bilirubin system.

Haemoxygenase (HO) itself is a powerful antioxidant and is protective in various pathophysiological states such as cardiovascular and neurodengerative diseases, whilst the HO-derived bilirubin is an efficient scavenger for ROS and RNS (Jansen et al, 2010). Physiological concentrations of bilirubin are therefore beneficial, but when elevated

bilirubin is neurotoxic.

In 1994, Schwertner et al showed an inverse association between bilirubin and coronary artery disease and since then there have been many studies that showed the similar association between low bilirubin levels and worse clinical outcomes in other vascular and respiratory disorders. This included secondary analysis of the seminal Framingham study that showed how elevated (but still physiological) levels of bilirubin had a reduced incidence of cardiovascular morbidity (Lin et al, 2006; Horsfall et al, 2011; Vitek et al, 2012).

5.1.4 Anti-oxidants and pre-eclampsia

Pregnancy is a state of heightened oxidative stress, with both an increase in circulating levels of oxidised low-density lipoproteins and a reduction in the total antioxidant capacity compared to non-pregnant individuals. Pregnancy is also associated with a systemic inflammation characterised by a greater activation of circulating lymphocytes and granulocytes, both of which produce a large number of ROS, testimony that these two pathways are interlinked (Burton et al, 2011).

Both oxidative stress and systemic inflammation are more pronounced in pre-eclampsia. However, as mentioned in previous chapters, the nature in which the placental injury leads to these heightened pathways, and how they

lead to the clinical syndrome, remain unclear. Redman and Sargent (2009) describe two scenarios. The first, is due to reduced uteroplacental arterial perfusion from inadequate remodeling of the spiral arteries, which causes a state in which the placenta is chronically hypoxic. The second scenario is due to intermittent blood flow through the spiral arteries that causes ischaemic reperfusion of the placenta. This hypoxia/reoxygenation is a potent stimulus to the activation of xanthine oxidase, an important source of superoxide generation, which is abundantly expressed in cytotrophoblast, syncytiotrophoblast, and villous stromal cells (Raijmakers et al, 2004). This leads to an environment of increased oxidative stress and increased ROS, provoking release of sFLT1, causing the heightened inflammatory state and the resulting maternal systemic syndrome (Redman et al, 2009).

The role oxidative stress has on the development of the pre-eclamptic placenta is explored further in figure 37.

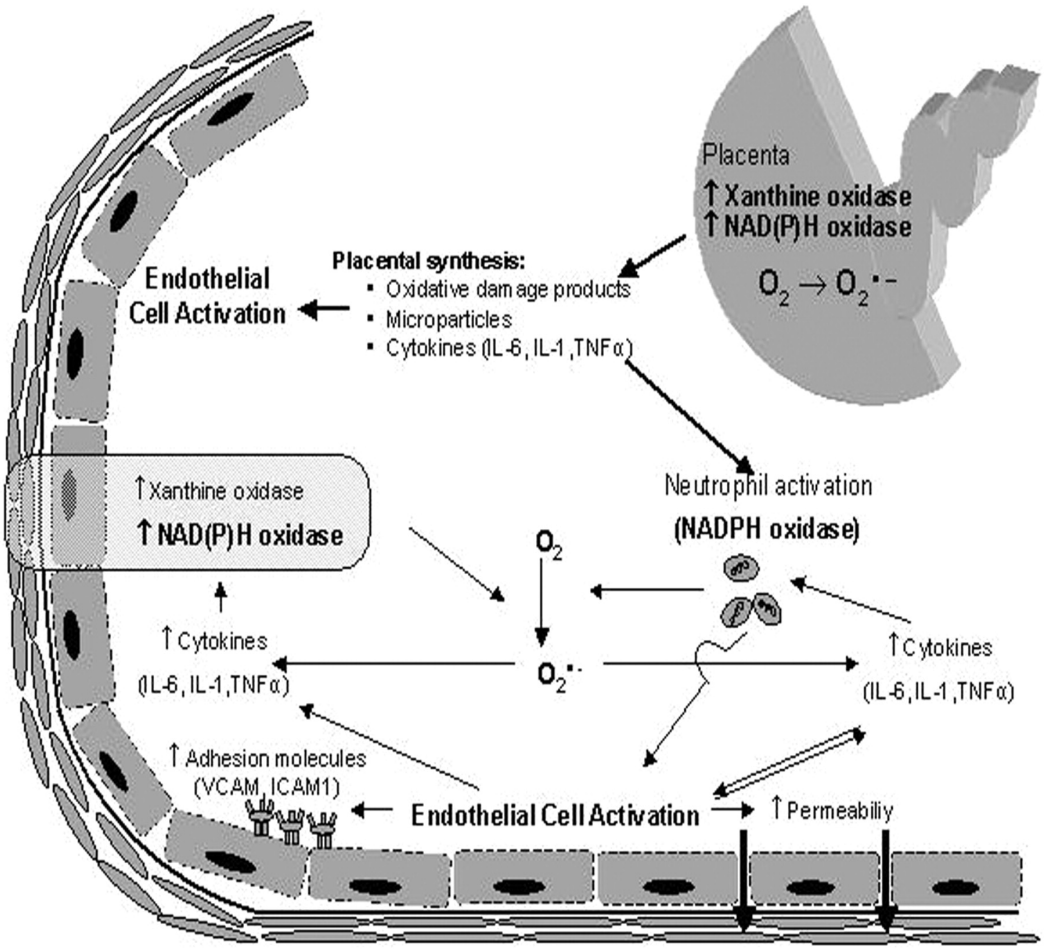


Figure 37: Proposed association between placental oxidative stress and maternal vascular dysfunction in preeclampsia. It is hypothesized that free radical generation through xanthine oxidase or NAD(P)H oxidase in the placenta leads indirectly to maternal neutrophil activation. In the maternal circulation, a vicious cycle of maternal endothelial and neutrophil activation may result in sustained NAD(P)H oxidase activity and release of superoxide (Adapted from Raijmakers et al, 2004)

5.2 Aim

The first aim of this work was to assess the ability of bilirubin concentration to predict poor outcome in women who develop the disease. Secondly, this work also evaluated the ability of the neutrophil to lymphocyte ratio (discussed in the previous chapter) to predict poor maternal and fetal outcome at the time of diagnosis with pre-eclampsia.

5.3 Method

A retrospective observational study of women who delivered at University Hospitals of Coventry and Warwickshire NHS Trust (UHCW) between 1999-2010 (n=50,712) was performed. Women were included if they were diagnosed with pre-eclampsia between 1999 and 2010. Pre-eclampsia was defined as a blood pressure ≥ 140mmHg/90mmHg (with Korsokoff V used to determine diastole) with proteinuria of either 0.3g/L/24hrs or (+) proteinuria on urinary dipstix analyisis. On diagnosis of pre-eclampsia "pre-eclampsia bloods" were taken. These haematological and biochemical tests consisted of FBC, urea & electrolytes, uric acid level and liver function test (a component of the liver function test is bilirubin). Following delivery, midwives entered the antenatal history and delivery details into the hospital database (Evolution, CSC Healthcare IT, USA). Women diagnosed with pre-eclampsia in a singleton pregnancy were identified from this hospital database and their mode of delivery and neonatal outcomes were retrieved. Poor maternal

outcomes were defined as placental abruption, HELLP syndrome, eclampsia and maternal death. Poor fetal outcomes were defined as Apgar's less than 7 at 5 minutes post delivery, low birth weight (<10th centile at birth), unexpected admission to neonatal unit and late fetal demise.

Women were excluded if they had any past history of liver disease; the liver function test showed any abnormal liver function parameters, had multiple pregnancies or were less than 20 years old (Bilirubin levels stabilise after 20 years of age (Wilding et al, 1972)). As smoking can cause change in plasma bilirubin levels smokers were excluded from the study (Van Hoydonck, 2001). Haematological parameters and bilirubin levels were obtained from the Clinical Results Recording System (CRRS).

The SPSS computer programme (SPSS v19, Chicago, USA) was used for the statistical analysis and statistical significance was defined as $p<0.05$. Categorical variables were compared using the χ^2 test and continuous variables using *t* tests. Non-parametric data were analysed by the Mann-Whitney-U test. Comparisons of bilirubin quintile levels (with the median range acting as reference) are presented as two tailed p values.

5.4 Results

992 consecutive women met the inclusion criteria (pre-eclampsia, singleton pregnancy, older than 20 years and had liver function tests within the normal range) with 67 self-classified themselves as smokers and were excluded, leaving 925 women. The demographic data of the women has already been discussed and can be found in chapter 4.

The median time between the blood tests being performed and the poor outcome was 5.4 days (interquartile range = 1-13 days).

5.4.1 Bilirubin

The lowest quintile of bilirubin levels were associated with a greater risk of requiring a caesarean section for fetal distress (p=0.001 aOR=2.25 (95% CI=1.27-5.61) (Table 24).

	CS for fetal distress		Poor maternal outcome		Poor infant outcome	
Bilirubin Level (mg/L)[$]	Crude OR (95% C.I.)	Adjusted OR^ (95% C.I.)	Crude OR (95%C.I.)	Adjusted OR^ (95% C.I.)	Crude OR (95%C.I.)	Adjusted OR^ (95% C.I.)
1st quintile 0-0.21 (n= 157)	2.96 (1.52-5.74)*	2.25 (1.27 – 5.61)*	3.05 (1.63-5.72)*	3.12 (1.2-6.87)*	3.9 (2.5-5.6)*	2.5 (1.25-5.19)*
2nd quintile 0.22-0.43 (n=204)	1.49 (0.86-2.61)	0.94 (0.51-2.0)	1.91 (0.98-3.67)	1.85 (0.76-2.64)	1.3 (0.86-1.71)	1.24 (0.97-1.88)
3rd quintile 0.44-0.65 (n=270)	REFERENCE	REFERENCE	REFERENCE	REFERENCE	REFERENCE	REFERENCE
4th quintile 0.66-0.87 (n=190)	1.69 (0.99-2.86)	1.63 (0.12-3.95)	1.66 (0.85-3.3)	2.77 (0.59-4.54)	1.55 (1.2-2.6)	0.97 (0.2-1.8)
5th quintile 0.88-1.05 (n=104)	0.59 (0.38-0.97)*	0.65 (0.15-1.31)	1.14 (0.56-2.30)	1.31 0.66-2.84)	0.86 (0.41-1.30)	0.91 (0.34-1.79)

^ Adjusted for age, BMI, ethnicity, gestation at delivery, Diastolic and Systolic blood pressure at diagnosis and amount of proteinuria.
*p=<0.05
$ To convert mg/L of bilirubin to µmol/L multiply by 17.1

Table 24: Ability of Bilirubin to predict poor outcome in women with pre-eclampsia

The area under the receiver operator characteristic curve was 0.78 (95% confidence limit 0.65 - 0.89). Sensitivity was 0.87, specificity was 0.55, positive predictive value was 0.7 and negative predictive value was 0.86 (Figure 38).

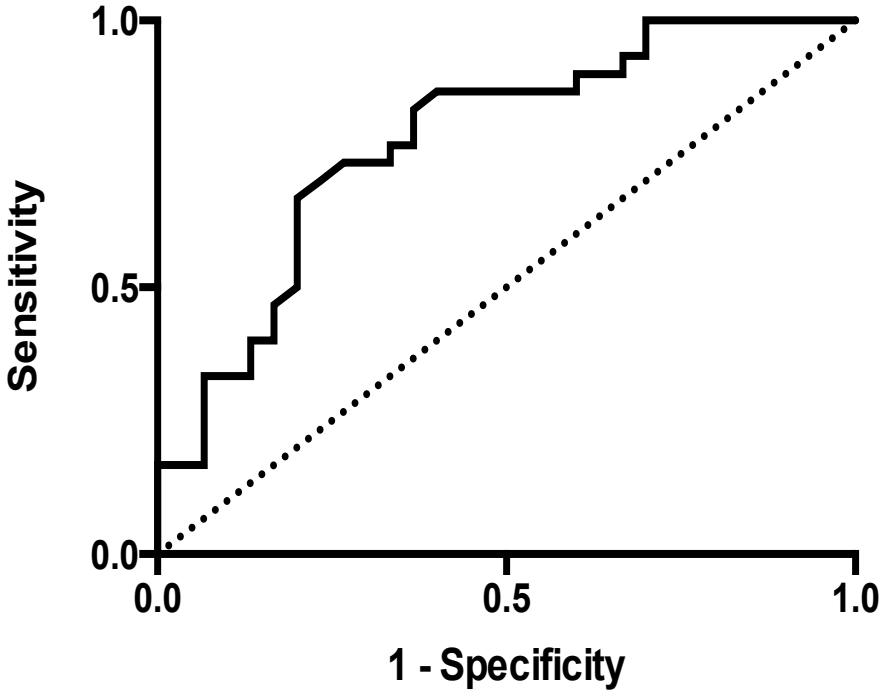


Figure 38: Receiver Operator Characteristic curve of bilirubin concentration to predict delivery by caesarean section for fetal distress (AUC = 0.78 (95% CI 0.65 – 0.89). At a cut off of 0.17mg/L; Sensitivity =0.87 (95% CI 0.72-0.96), specificity =0.55 (95% CI 0.43-0.68).

Maternal poor outcomes; placental abruption (n=31), HELLP syndrome (n=78), eclampsia (n=2) and maternal death (n=1) were grouped together for the logistic regression analysis due to too the small number of individual incidences. The first quintile of bilirubin levels were associated with a poor maternal outcome after adjusting for age, BMI, ethnicity and gestation of delivery (p=0.002 aOR3.12 (95% CI=1.2-6.87) (table 16). The AUC=0.73, with a cut off of 0.17 mg/L; sensitivity = 0.93 (95% CI 0.85-0.99), specificity = 0.42 (95% CI 0.3-0.57). Poor infant outcomes were also relatively few (Apgar's less than 7 at 5 minutes of life n=53, low birth weight (<10th centile at birth) n= 30, unexpected admission to neonatal unit n=37 and late fetal demise n= 11). Hence a combined measure of all these poor infants outcomes born to pre-eclamptic mothers was used for the multivariate analysis. When bilirubin concentrations were in the lowest quintile, there were more poor infant outcomes after adjusting for age, BMI, ethnicity and gestation of delivery, Blood pressure and proteinuria (p=0.015, aOR 2.5 (95%CI 1.25-5.19)). However, the sensitivity of the lowest quintile for predicting poor fetal outcome was poor (0.61 (95% CI 0.48 – 0.73).

5.4.2 Neutrophil to Lymphocyte Ratio

The lowest quintile of NLR was associated with a lower incidence in fetal distress necessitating delivery by CS and maternal complications (including HELLP and placental abruption) while conversely, the highest quintile of NLR was associated with a greater number of complications when compared to the median quintile. There was no difference in fetal / neonatal complications between the reference groups and the other NLR ratios (See table 25).

In the poor maternal outcome group the AUC was 0.7239 (95% CI 0.59 - 0.86, p=<0.002). At a cut-off of 5, the sensitivity was 0.9, with a specificity of 0.36.

	CS for fetal distress		Poor maternal outcome		Poor infant outcome	
NLR	Crude OR (95% C.I.)	Adjusted OR^ (95% C.I.)	Crude OR (95%C.I.)	Adjusted OR^ (95% C.I.)	Crude OR (95%C.I.)	Adjusted OR^ (95% C.I.)
1st quintile 0-1.24 (n= 113)	1.14 (0.72-2.0)	1.25 (0.83 – 1.78)	0.72 (0.60-0.85)*	0.78 (0.67-0.9)*	0.89 (0.6-1.13)	1.02 (0.74-1.5)
2nd quintile 1.24-2.48 (n=204)	1.2 (0.86-2.61)	1.17 (0.7-1.98)	1.38 (0.86-2.18)	1.44 (0.87-2.27)	1.42 (0.94-1.60)	1.13 (0.77-1.58)
3rd quintile 2.48-3.71 (n=252)	REFERENCE	REFERENCE	REFERENCE	REFERENCE	REFERENCE	REFERENCE
4th quintile 3.71-4.95 (n=239)	1.42 (0.98-1.98)	1.60 (1.0-2.54)	1.34 (0.84-2.1)	1.24 (0.91-1.62)	1.32 (0.84-2.81)	1.19 (0.82-1.86)
5th quintile 4.95-6.20 (n=117)	1.72 (1.08-2.85)*	1.53 (1.09-1.91)*	1.69 (1.13-1.98)*	1.4 (1.12-1.67)*	1.36 (0.82-2.3)	1.1 (0.82-1.97)

^ Adjusted for age, BMI, ethnicity, gestation at delivery, Diastolic and Systolic blood pressure at diagnosis and amount of proteinuria.
*p=<0.05

Table 25 Ability of Bilirubin to predict poor outcome in women with pre-eclampsia

5.4.3 Correlation between bilirubin and neutrophil to lymphocyte ratio

The relationship between bilirubin and NLR concentrations suggests that there is a correlation between the bilirubin and Neutrophil to lymphocyte ratio values in women with pre-eclampsia of (correlation coefficient *r*=-0.4 (95% -0.4525 to -0.3444) r^2=0.1599, p=<0.001) (see figure 39).

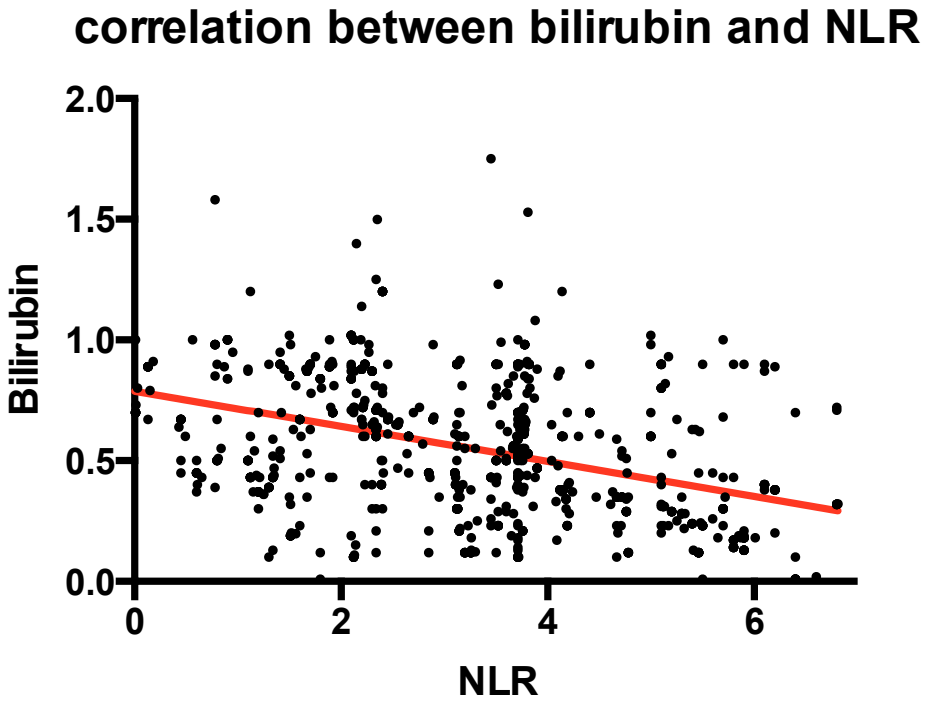


Figure 39: Correlation between bilirubin (in mg/L) and neutrophil to lymphocyte ratio in women diagnosed with pre-eclampsia.

5. Discussion

This is novel data because this is the first study to have found an association between bilirubin levels in the lowest quintile (of the normal range) along side NLR in the highest quintile and poor outcomes in mothers and infants in women with pre-eclampsia.

5.5.1 Summary and discussion of key findings

Previously Kozic et al (2011) investigated the ability of abnormal liver function tests to predictor poor clinical outcomes in pre-eclampsia. These authors found a strong association between abnormally elevated bilirubin levels and poor clinical outcomes in women with pre-eclampsia. However, bilirubin levels within the normal range were not stratified and it is thus likely that Kozic compared two different grades of pre-eclampsia from the known spectrum of the clinical presentation of the disorder (e.g., the abnormal liver function representing a form of HELLP).

Given the potent anti-oxidant properties of bilirubin, our data suggests that low levels of bilirubin leave the mother and fetus susceptible to the syndrome and clinical presentation of pre-eclampsia. The higher concentrations of

bilirubin did not, as initially hypothesised, confer protection from developing poorer outcomes than the median group.

This may be due to the more severe form of pre-eclampsia having a higher oxidative stress response and therefore consuming more anti-oxidants e.g. bilirubin. Or it could be that there is just not a direct causal relationship.

The neutrophil to lymphocyte ratio, used as a marker of systemic inflammation, was raised in women who had greater number of poor maternal outcomes or required delivery by caesarean section for fetal distress. From this it could be reasoned that in pre-eclamptic pregnancies, greater systemic inflammation is an independent marker of poor pregnancy outcomes. Conversely, NLR at the lower quintile (which could be taken as a marker of reduced systemic inflammation) was associated with fewer poor maternal outcomes. There was no significant difference in the number of caesarean sections for fetal distress between the lowest and median quintile groups.

Interestingly, NLR could not identify those pregnancies that would have poorer infant outcome. Whether this is due to systemic inflammation not having a direct link on the fetus is unlikely. A more plausible reason could be that the study was insufficiently powered to assess the link between NLR and infant outcome.

Given the link between inflammation and oxidative stress, it would be suggestive that there is a relationship between bilirubin and NLR. Given a correlation coefficient of -0.4 it would suggest that, whilst there is a link

between the two, it is not a strong one. The clinical syndrome of pre-eclampsia is complex and finding a strong correlation between the two is unlikely from clinical studies. However, given that the link is negative, it is more in keeping that this is a true relationship. If the link were positive, it would more likely be due to an up-regulation of many disease processes rather than a true association.

5.5.2 Strenghts of this study

The strength of this study lies in the thoroughness of the database used to collect the information. Using standardised hospital notes ("the green notes (patient hand-held) notes"), standardised definitions and robust protocols for managing pre-eclampsia meant that this study overcame the largest hurdle that faces retrospective data, missing data.

5.5.3 Weaknesses of this study

The results suggest that lower levels of bilirubin can aid the prediction of those fetuses that do not cope well with labour (and therefore require delivery by emergency caesarean section). However, possibly due to relatively low numbers of poor outcomes, further work would be needed before bilirubin levels could be used to accurately detect those women at risk of poor maternal and neonatal outcomes.

Fetal distress necessitating delivery by caesarean section is, unfortunately, quite often a subjective decision. Despite training on cardiotocograph (CTG) interpretation being robust and the interpretation of a CTG together with a more objective fetal blood sample (FBS) being advocated, quite often the indication for CS is intuitively made my the senior member of the delivery suite team. It could be that some of these CS were carried out unnecessarily.

The question whether the study was appropriately powered needs to be addressed. Retrospective studies are limited by number of patients available and therefore at risk of type II error. Given that for both CS for fetal distress and poor maternal outcome with attained levels of statistical significance ($P<0.05$) we can assume that for these the study was appropriately powered. The study number was limited by the number of women who had pre-eclampsia, so whilst this number was appropriately powered for the maternal complications and risk of caesarean section, it may be that it was insufficiently powered to study infant outcomes.

5.6 Conclusion

These findings suggest that the commonly ordered haematological and biochemical tests in women diagnosed with pre-eclampsia can, in addition to establishing those with HELLP or renal injury, predict those women who are at risk of developing further complications of the disease. Further prospective work is required to validate these as a potential screening tool.

Chapter 6

General discussion and conclusion

6. Discussion

6.1 Introduction

The vast spectrum that is pre-eclampsia encompasses many different clinical presentations, with varying fetal and maternal considerations. Whilst the extent to which these various manifestations occur may alter, systemic inflammation and endothelial dysfunction are universally present. Once diagnosed, the only cure for the disease is delivery of the placenta and this can lead to dilemmas for clinicians as to the best time to deliver a (potentially) preterm baby, risking complications of prematurity or to continue with the pregnancy and risk complications of pre-eclampsia. National guidelines exist to aid in the management of the disease but no current intervention can reverse effect that the disease. Instead most treatments aim to prevent further worsening of the condition (Von Dadelszen et al, 2011).

The overall aim of this thesis was to develop markers that could potentially work towards predicting pre-eclampsia. Prediction of pre-eclampsia would allow optimal allocation of the resources needed to deliver high-risk obstetric care. The thesis comprised epidemiological, laboratory (basic science) and clinical studies. Firstly, I undertook one of the largest cohort studies of risk factors for developing pre-eclampsia in the UK. This study contained a significant number of women from ethnic minorities and the large number of women within the cohort allowed the currently accepted risk factors to be challenged. To identify novel plasma markers that are

altered in women who go on to develop pre-eclampsia I performed a quantitative proteomic analysis of individual first trimester plasma. The proteomic work identified several novel results not previously been identified. Although encouraging, before a biomarker can be identified as a possible predictor of pre-eclampsia it must undergo several robust prospective studies. This necessary process can take years, so a retrospective study was then undertaken that assessed the ability of commonly ordered clinical tests to predict the onset of pre-eclampsia. However, simply predicting pre-eclampsia whilst useful may have limited clinical application. So the same cohort was used to identify clinical parameters that could predict those women who are at risk of poorer outcomes when diagnosed with the condition.

6.2 Summary of key findings

6.21 The epidemiological study

Identification of risk factors for developing pre-eclampsia is important as it is currently the only established way to predict who is at risk of developing the disorder in clinical settings. However, several known risk factors (such as ethnicity) are not featured in protocols for preventing and managing hypertensive disorders of pregnancy (NICE, 2010). Using a database of 109576 women, the first part of the study was to assess the impact conventional risk factors have on developing pre-eclampsia for this multi-ethnic cohort. As expected; Black ethnicity, Age, BMI and previously

diagnosed diabetes and hypertension all increase the likelihood of developing the condition. Having such a large cohort, these risk factors were then able to undergo further analysis. This is the first study to show that there is significant racial variation in both the standard risk factors for developing pre-eclampsia and the risk of having a significant morbidity amongst the three largest ethnic minorities in the UK. To consider why these disparities may be the case we looked at the variation in cardiovascular and metabolic diseases amongst these groups. Miller (2007) suggests that the difference in the incidence and presentation of many cardiovascular diseases lies in the difference in adhesion molecules and cytokines, that lead to altered inflammatory states. If this theory of altered inflammatory states applies to pre-eclampsia, this may account for the variation in clinical presentation.

6.22 Proteomic studies

Another leading theme of this thesis was to identify markers of pre-eclampsia that could potentially be used to predict the onset of the disease from as early as the first trimester of pregnancy. Since Levine et al's (2004) seminal piece of work illustrating alteration in plasma concentrations of angiogenic proteins at 20 weeks gestation in women who go on to develop pre-eclampsia, there has been much research aimed at predicting the onset of the disease. Whilst many proteins have been identified as potential markers, none are used in clinical practice. There are several reasons for

this lack of usefulness of the current biomarkers. The first is usually as a result of poor study design. The cost of running proteomic studies can be immense and to reduce overheads many groups use pooled plasma samples in their biomarker discovery stage (usually running just two samples, disease versus normal states). When individual samples are then interrogated they fail to reproduce the initial exciting results (Barker et al, 2005). In the proteomic chapter of this thesis, not only were individual plasma samples ultilised, these samples were also run in triplicate, to reduce the incidence of false positive discoveries. Another strength of my study is that the samples used were matched for age, BMI, ethnicity and gestational age. This allowed biological variation to be as minimised as much as possible. In my study markers of inflammation, hypertension and oxidative stress were all altered in the pre-eclampsia group. This is the first study to show how angiotensinogen, paraoxonase-1, Kallikrein, vitamin D binding protein and certain complement proteins are altered in the first trimester of pregnancies that subsequently developed early onset severe pre-eclampsia.

There are currently no clinically useful biochemical tests to predict the onset of pre-eclampsia in low-risk women. Chappell et al (2013) have recently studied the diagnostic accuracy of PIGF in its ability to predict delivery within 14 days due to pre-eclampsia in women suspected of the disease. PIGF $<5^{th}$ Centile was able to predict the onset of the disease with Sensitivity of 0.96 (95% C.I. 0.85-0.99) and an AUC of 0.87. Only 14 days

warning of pre-eclampsia gives clinicians insufficient time to alter obstetric management. In contrast the results presented in chapters 3 and 4 were on samples taken more than 16 weeks prior to disease onset. I demonstrated an angiotensinogen to kallikrein ratio greater than 0.267 had a sensitivity of 0.9 (95% C.I. 0.8-0.96) and an AUC of 0.831 (SE=0.042, 95% CI 0.74-0.91) for predicting early onset severe pre-eclampsia. Thus the angiotensinogen to kallikrein ratio may allow for the development of an accurate first trimester predictor of the disease. Further, larger prospective studies are needed to validate these novel findings.

6.23 Clinical inflammatory markers studies

A common pattern within the literature (and this thesis) is the role inflammation plays in pre-eclampsia; although whether this causes the disease or results from the disease is in dispute. Either way, the finding of an inflammatory state in pre-eclampsia has been repeatedly demonstrated (Redman et al, 1999). The findings in chapter 3 support these studies by demonstrating a raised inflammatory proteome in first trimester pregnancies that later develop pre-eclampsia.

The nature of antenatal care in the UK is such that on a woman's first visit to see her midwife (the booking visit) she undergoes several haematological investigations to screen for conditions such as anaemia. As the majority of

women book during the first trimester, it would be extremely useful to identify markers from these booking tests that may predict those women who develop pre-eclampsia.

In chapter 4 I demonstrated that there is an increased neutrophil: lymphocyte ratio (a marker of a heightened inflammatory state) in women who later develop pre-eclampsia. The results suggest that this ratio may be able to predict the onset of severe pre-eclampsia.

6.24 Predicting poor outcome

Although 2-8% of women get pre-eclampsia, not all of them will have a poor outcome. In fact, the majority of women with pre-eclampsia will have a mild form with no complications. As Chappell (2013) discussed, much resources are spent on monitoring these women suggesting that being able to predict those at risk of a poor outcome would be very clinically useful in terms of better recourse allocation. In chapter 5 I have found that, commonly ordered haematological and biochemical investigations may help towards pre-eclampsia prediction. Bilirubin and the neutrophil to lymphocyte ratio both have the ability to predict poor outcomes after the disease process is detected clinically.

However their were relatively low numbers of adverse outcomes in my data suggests that much larger studies would be required to corroborate these findings.

6.25 A potential screening test?

An ideal screening tool would be 100% sensitive and 100% specific; that is, it would be positive for all those with the disease and negative for all those who did not. Unfortunately no screening tool is ideal. The predictive ability of the findings presented in this thesis all have AUC > 0.7 indicating that they have a relatively good overall predictive value. However, AUC on its own offers relatively limited clinical information. To assess a screening tool's predictive ability, sensitivity, specificity, positive predictive value and negative predictive value should be evaluated (Deeks et al, 2004). Whilst the results shown have relatively high sensitivity (>0.85) their corresponding specificity are not so encouraging. Despite this, these results still may have the potential to predict the onset of the disease in medical practice. With a high sensitivity, most women who are going to develop the disease will screen positive. With a lower specificity, it means that some women who will not develop the disease will also screen positive. In clinical terms this means that few women who will develop pre-eclampsia will not have enhanced surveillance and/or intervention treatment (currently aspirin, with its relatively low side effects) and some women who will not develop the disease will have unnecessary monitoring. These findings are in keeping with the conclusion from a systematic review that suggests that any

screening tool for pre-eclampsia should have high sensitivities (Cnossen et al, 2004).

6.3 Disease pathogenesis

The aim of this study was not to try and understand the initial insult that causes pre-eclampsia. However, with any disease where there is a lack of understanding regarding its development, it is tempting to suggest markers of pathophysiological pathways that answer some of the many unanswered questions.

- Different ethnicities appear to be at risk of different clinical presentations of pre-eclampsia. Some of these differences may be a result of either different fat distribution and alteration in the resulting inflammatory states or different ethnicities predilection towards insulin resistance and heightened oxidative stress.
- The first trimester proteome of women who develop pre-eclampsia have increased markers of inflammation and oxidative stress, suggesting that these may play a role in the early pathophysiology of the disease.
- Markers of both inflammation and oxidative stress are again present at the onset of the disease and during the clinical presentation of the disease and there appears to be a correlation between the two.

These findings may help to answer some of the questions regarding the pathophysiology of the disease, but they also throw open several other questions; does a heightened inflammatory state (representing existing endothelial damage) pre-exist before the onset of the disease? If oxidative stress plays such an important role in the development of the disease, why does treatment with anti-oxidants not prevent disease development (Poston et al, 2004)?

Conclusion

This thesis evolved during a time of great excitement and trepidation at the possibility of finding one of the holy grails of obstetrics – predicting the onset of pre-eclampsia. With its associated morbidities, healthcare costs and impact on future cardiovascular health much energy and resources have been spent trying to develop a risk score, similar to that found in screening for fetal aneuploidy, to predict the onset of pre-eclampsia. Unfortunately this test has of yet to be discovered. The excitement has turned to cynicism, especially towards the field of proteomics; now often compared to “finding a needle in a haystack”. Hopefully the results presented in this thesis offer some renewed interest in this quest. The epidemiology chapter reveals the importance of including ethnicity when assessing risk of developing pre-eclampsia and the proteomics chapter illustrates how the different markers altered in the first trimester that may eventually lead to the development of screening tool for the disease. The

next two chapters explored how markers of inflammation and oxidative stress currently available in obstetric practice that are altered in pre-eclamptic pregnancies.

Specifically, the data presented in this thesis add to this existing knowledge by providing:

- One of the largest cohort studies of risk factors for pre-eclampsia involving a multi-ethnic UK population.
- For the first time, differences in the rates of pre-eclampsia across different BMI and revealing how Black women are at risk of developing pre-eclampsia at lower BMI than White women.
- Differences occur between age related risk of developing pre-eclampsia across ethnicities.
- The finding that White women are more at risk of developing poor maternal outcomes that Black or S Asian women, whereas S Asian women with pre-eclampsia are more likely to have fetal complications.
- Evidence that Angiotensinogen is raised and Kallikrein is lower in the first trimester of pregnancies that subsequently develop pre-eclampsia. These findings may suggest how these proteins play a role in the development of hypertension later in the pregnancy.
- A ratio of angiotensinogen to kallikrein > 0.27 predicts the onset of pre-eclampsia with a sensitivity of 0.9 (95% CI 0.74-0.97), specificity

of 0.5 (95% CI 0.24-0.65), PPV = 0.63 (95%CI 0.47-0.75) NPV =0.87 (95%CI 0.53-0.96), with an AUC of >0.81 (SE=0.05).

- Confirmatory evidence of raised inflammatory markers and down regulation of inhibitors of inflammation in the first trimester.
- Evidence of down regulation of paraoxonase-1 in the samples that develop pre-eclampsia.
- Findings that show how the neutrophil to lymphocyte ratio is raised in the booking bloods of women who go on to develop pre-eclampsia.
- Confirmation that bilirubin and neutrophil to lymphocyte ratio can predict poor outcomes in women diagnosed with pre-eclampsia and how these two parameters have mild correlation.

Like most research projects, this thesis has led to many further questions that need to be answered. Further research areas I intend to pursue include:

- Undertaking a prospective large cohort study starting pre-pregnancy. This would hopefully answer once and for all the question of which came first, the pre-eclampsia or the inflammation.
- Develop a large statistical model of demographics (including ethnicity), first trimester proteomics together with ultrasound parameters to form a robust screening tool to predict the onset of pre-eclampsia

- Hone current chemoprophylactic agents towards those deemed at risk from the model, therefore reducing the number of women receiving medication unnecessarily.

From the findings presented in this thesis (and the further research planned) there is not only the possibility of being able to predict the onset of pre-eclampsia, but this work may also further the understanding of the diseases origin.

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