BIOCHEMICAL AND CLINICAL FACTORS WHICH ARE ASSOCIATED WITH OR PREDICTIVE OF PRE-ECLAMPSIA IN HEALTHY WOMEN AND THOSE WITH CHRONIC KIDNEY DISEASE

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ABSTRACT

Pre-eclampsia (PE) complicates around 5% of all pregnancies and over 20% of pregnancies in women with chronic kidney disease (CKD). The cause of PE is unknown, but dysregulation of the maternal immune system and an imbalance in angiogenic factors, characterised by a high ratio of soluble fms-like tyrosine kinase-1 (sFLT-1) to placental growth factor (PIGF), are implicated as important aetiological factors. There are limited data published on disturbances in humoral-mediated immunity in PE and it is not well established how important immunological factors are in the development of superimposed PE in CKD. The work of this thesis comprises of three studies and I report:

- An increase in polyclonal free immunoglobulin light chains and a reduction in IgG whole immunoglobulin, specifically IgG1 and IgG3, concentrations at the time of PE in previously healthy women compared to uncomplicated pregnancies at term. Beta-2-microgobulin levels had comparable diagnostic association for PE with sFLT-1/PIGF.
- Healthy pregnant women who subsequently develop PE had lower levels of circulating IgM compared to matched healthy pregnant women who had uncomplicated pregnancies. Higher IgG levels were independently predictive of adverse pregnancy outcomes in the PE but not in the non-PE group.
- In CKD pregnancies, immune system and angiogenic markers were not associated with, or strongly predictive of, superimposed PE. This is in contrast to findings in the healthy pregnant cohort and suggests that the pathophysiological mechanisms involved in the development of PE differ between the two groups.

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STATEMENT OF OWN WORK

I confirm that the work of this thesis is mine. I planned the study methodology, sought ethical approval, recruited patients, collected clinical samples and data. I prepared the samples for laboratory analysis. The analysers used for the purpose of sample analysis were operated by trained laboratory staff, whilst I was fully acquainted with the methodology, validation and quality assurance of the assays and present and assisting. The data analysis was performed by me and supported by statisticians.

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LIST OF ABBREVIATIONS

ACR	Albumin to Creatinine Ratio
ADPKD	Autosomal Dominant Polycystic Kidney Disease
AKI	Acute Kidney Injury
AN	Antenatal
AUROC	Area Under the Curve of the Receiver Operating Characteristic
B2-M	Beta 2 microglobulin
BMI	Body Mass Index
BNP	B-type natriuretic peptide
BP	Blood Pressure
BWH	Birmingham Women's Hospital
cFLC	Combined Free Light Chains
C3/4	Complement Proteins 3/4
CI	Confidence Interval
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CRP	C-reactive Protein
CTD	Connective Tissue Disease
CV	Coefficient of Variation
ELISA	Enzyme-linked immunosorbent assay
ESRD	End Stage Renal Disease
EVT	Extravillous Trophoblast
FGR	Fetal Growth Restriction
FLT-1	fms like Tyrosine Kinase 1
FLC	Free Light Chains
GFR	Glomerular Filtration Rate
HBRC	Human Biomaterials Resource Centre
HC	Head Circumference
HDU	High Dependency Unit
HELLP	Haemolysis Elevated Liver Enzymes and Low Platelets
HLA	Human Leukocyte Antigen
hs-CRP	High-sensitivity C-reactive protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	interquartile range
ISSPH	International Society for the Study of Hypertension in Pregnancy
IUD	Intrauterine Death
κ	Kappa
KDOQI	Kidney Disease Outcomes Quality Initiative
KIM-1	Kidney Injury Molecule - 1
λ	Lambda
LSCS	Lower Segment Caesarean Section
LMP	Last Menstrual Period

MDRD	Modification of Diet in Renal Disease Study
MGUS	Monoclonal Gammopathy of Undetermined Significance
NGAL	Neutrophil gelatinase-associated lipocalin
NICE	National Institute of Clinical Excellence
NICU	Neonatal Intensive Care Unit
NK	Natural Killer
NNU	Neonatal Unit
NND	Neonatal Death
OR	Odds Ratio
PCR	Protein to Creatinine Ratio
PE	Pre-eclampsia
PIGF	Placenta Growth Factor
PN	Postnatal
PROM	Premature Rupture of Membranes
ROC	Receiver Operating Characteristic
SCBU	Special Care Baby Unit
SD	Standard deviation
SE	Standard Error
sFLC	Serum Free Light Chains
sFLT-1	Soluble fms like Tyrosine Kinase 1
SGA	Small for Gestational Age
SLE	Systemic Lupus Erythematosus
SPE	Superimposed Pre-eclampsia
TGF	Transforming Growth Factor
Th	T-helper
TNF	Tumour Necrosis Factor
UA	Uric Acid
uACR	Urine Albumin to Creatinine Ratio
UHB	University Hospitals Birmingham
UK	United Kingdom
uPCR	Urine Protein to Creatinine ratio
USS	Ultrasound Scan
UTI	Urinary Tract Infection
VEGF	Vascular Endothelial Growth Factor

1 INTRODUCTION

1.1 Thesis Overview

Pre-eclampsia (PE) is a leading cause of maternal and perinatal morbidity and mortality, and often manifest by the onset of de novo hypertension and proteinuria in the second half of pregnancy. There is no recognised cure for PE other than delivery and, despite extensive research, the precise cause remains unknown. Poor placentation is viewed as a critical trigger for the development of PE and is thought to lead to uteroplacental hypo-perfusion triggering widespread endothelial dysfunction in later, clinical stages of disease.

As the diagnosis of PE is established on clinical criteria without an available diagnostic gold standard, misdiagnosis may occur leading to unnecessary intervention including pre-term delivery. In pregnant women with chronic kidney disease (CKD), the risk of developing PE, also known as superimposed pre-eclampsia (SPE), is increased and diagnosis is often more difficult to establish, due to pre-existing hypertension and abnormal kidney function, with limited formal diagnostic criteria available to aid diagnosis. Identifying biochemical markers which are diagnostic and/or predictive of PE would help establish diagnosis in challenging cases and help identify women at high risk of developing disease, allowing closer clinical monitoring. Also, it would provide useful insight in the pathophysiological mechanisms involved in PE, as well as identifying potential targets for therapeutic intervention.

The circulating pro-angiogenic placenta growth factor (PIGF) and antiangiogenic soluble fms-like tyrosine kinase-1 (sFLT-1) proteins have been demonstrated in an increasing number of studies to assist diagnosis and prediction of PE in the general population. Moreover, pre-

eclamptic pregnancies are characterised by increased activation of both the innate and adaptive immune system resulting in a pro-inflammatory response compared to healthy pregnancies. Thus, dysregulation of the maternal immune and angiogenic system response are viewed as important features of PE, though there are limited data exploring changes in the humoral system in pregnancies complicated by PE. Furthermore, there is little data on whether these factors play an important role in the development of SPE in women with CKD or whether, in these individuals, other pathological mechanisms may be more important.

This thesis involved three cohorts of patients, healthy pregnant women at term (chapter three), healthy pregnant women in early pregnancy (chapter four) and pregnant women with CKD (chapter five). Each cohort was then split into women who developed or subsequently developed PE and compared to those with a healthy pregnancy. The aims of this thesis were 1) to assess markers of the humoral system at the time of PE in healthy women along with other clinical immune system markers and compare their diagnostic performance to that of sFLT-1/PIGF (chapter three); 2) assess the same immune system markers in the first trimester of pregnancy in healthy women, to determine whether early differences in these may pre-dispose to the subsequent development of PE (chapter four); 3) assess whether the immune and angiogenic system biomarkers associated with PE in healthy women (chapters three and four) behaved similarly in SPE occurring in pregnant women with CKD by taking serial measurements of the same markers and sFLT-1/PIGF in a cohort of pregnant women with CKD (chapter five).

1.2 Pre-eclampsia – Overview

1.2.1 Definition and Epidemiology

Pre-eclampsia is a multisystem, pregnancy specific condition and estimated to affect 3% - 5% of pregnancies [1-7]. It is a leading cause of both maternal and fetal morbidity and mortality worldwide contributing to 15-20% of maternal deaths and 15% of preterm deliveries. The development of PE can lead to several serious maternal complications such as liver dysfunction, cerebrovascular accidents, acute respiratory distress syndrome, acute kidney injury, Haemolysis Elevated Liver Enzymes and Low Platelets (HELLP) syndrome, cerebral haemorrhage, disseminated intravascular coagulation and progression to eclampsia (seizures). In one study the rate of major complications in a pregnancy associated with PE was increased 3-25 fold [8]. The most serious complications for the fetus arise from fetal growth restriction (FGR), premature delivery, placental abruption and death (still birth or neonatal death). It is estimated that 50,000-76,000 women and 500,000 infants die from PE each year worldwide [4]. Whilst the disease can involve a number of or multiple organ systems, it most commonly causes hypertension and renal glomerular injury manifest as proteinuria [9]. Poor maternal outcome can be mitigated by timely delivery whilst much of the adverse fetal outcome arises from requirement for pre-term birth [10].

Pre-eclampsia is the most common cause for a woman developing a pregnancy-related renal complication [11-13]. The histological renal lesion specific to PE is glomerular endotheliosis which is characterised by endothelial swelling with loss of endothelial fenestrae and microvascular occlusion of the capillaries [14, 15]. The maternal complications tend to resolve fairly rapidly following delivery; however long term sequelae

are increasingly recognised as women with a history of PE have an increased risk of developing future Cardiovascular Disease (CVD), Cerebrovascular Disease and Chronic Kidney Disease (CKD) [1, 11, 16-19].

Whilst many risk factors for developing PE are recognised, most cases occur in healthy women experiencing their first pregnancy (nulliparous pregnancy) [25]. Recognised risk factors include first pregnancy, previous history of PE, autoimmune illness, diabetes mellitus, obesity, age over 40 years, a positive smoking history and black ethnicity. A genetic component in both parents is also recognised [18, 20-22]. The presence of chronic hypertension and/or CKD, significantly increase the chance of superimposed PE (SPE) developing [20, 21]. In those with a previous history of PE, the risk of recurrence increases progressively the earlier in gestation that PE occurred. In women who delivered at term with the affected pregnancy the risk of recurrence is 16%, increasing to 25% if the mother delivered before 34 weeks' gestation and 55% if she delivered before 28 weeks' gestation [11, 29]. Low dose aspirin started at 12 weeks' gestation has been shown to reduce the risk of PE developing by 17% in high risk pregnancies [23, 24]. Therefore, in the absence of contraindications, all pregnant women with significant risk factors for developing PE, including those with a history of CKD or chronic hypertension, should be offered aspirin [25].

Traditionally PE used to defined on the basis of new onset hypertension (blood pressure $\geq$140/90 mmHg) and proteinuria (spot urine protein creatinine ratio (uPCR) > 30 mg/mmol or 24h urine protein excretion > 300mg) developing after 20 weeks' gestation [25]. However national and international diagnostic criteria have been revised and proteinuria is no longer a necessary criterion for a diagnosis of PE, as long as there is evidence of other maternal

organ dysfunction and/or uteroplacental dysfunction [26-28]. Pre-eclampsia according to the international society for the study of hypertension (ISSHP) criteria and National Institute for Health and Care Excellence (NICE) guidelines is new onset hypertension (≥140/90 mmHg) developing after 20 weeks' gestation accompanied by one of the following new onset conditions [26, 27]:

- Proteinuria (uPCR≥30 mg/mmol or uACR≥8 mg/mmol or at least 1g/lite (2+) on dipstick testing**) or**
- Other maternal organ dysfunction:
    - *renal insufficiency* (creatinine >90 umol/L)
    - *liver involvement* (elevated transaminases and/or severe right upper quadrant or epigastric pain)
    - *neurological complications* such as eclampsia, altered mental status, blindness, stroke, clonus, severe headaches or persistent visual scotomata
    - *haematological complications* such as thrombocytopaenia (platelet count below 150,000/microlitre), disseminated intravascular coagulation or haemolysis
- Uteroplacental dysfunction such as fetal growth restriction, abnormal umbilical artery Doppler waveform analysis, or still birth.

The ISSHP acknowledges that PE can occur in the absence of overt hypertension; however, they have elected to keep it as an essential diagnostic criterion for now. The revised definition of PE reflects the heterogeneous presentation of the disease and the gestation

time of onset also impacts on the clinical presentation. Early onset PE is defined as occurring at <34 weeks' gestation and late onset disease as after > 34 weeks [1]. Early onset PE is associated with more severe disease at presentation, increased risk of maternal and fetal morbidity including FGR, stillbirth and neonatal death compared to late onset PE [11, 29, 30]. The diagnosis of PE remains notably based on clinical criteria, despite substantial interest over the past decade in identifying biomarkers which can help predict and/or diagnose the condition [31].

1.2.2 Treatment and Delivery

There is no recognised treatment for PE other than delivery of the placenta and baby and supportive care [1, 6, 32]. However the decision to deliver is also heavily influenced by the gestation at which the woman presents with PE and balancing the fetal benefits by prolonging pregnancy, with the risks posed by progressive disease to the mother and fetus [6, 18, 33]. Premature delivery is a leading cause of neonatal morbidity and mortality, but prolonging pregnancy may lead to severe hypertension, organ failure and/or eclampsia in the mother. For presentations at or near term (37 weeks onwards) delivery rather than expectant management is usually indicated, irrespective of severity of disease. Neonatal outcomes are considerably improved from 34 weeks onwards and if PE develops before then and the maternal condition allows, the pregnancy is managed conservatively with close maternal/fetal monitoring in the hope of enabling further fetal development in utero before delivery. After 34 weeks' gestation the benefit of prolonging pregnancy may be outweighed by maternal risk and so a plan for delivery is usually indicated. Nevertheless, every case and its associated risk profile are specific to the individual mother and baby and warrant tailored

management plans for each pregnancy. Stabilisation of the mother's condition and delivery are always indicated for unstable maternal or fetal condition, regardless of gestation.

1.3 Introduction to the Pathology of Pre-eclampsia

The aetiology of PE is poorly understood and has been described as a 'disease of theories' with multiple factors including genetic, environmental and immune mechanisms likely to play a role [11, 29, 32, 34-36]. The presence of the placenta is essential for the development of PE; the disease can occur in the presence of placental tissue without a fetus, as is demonstrated in molar pregnancies (hydatiform mole) and delivery of placenta is the only recognised cure for PE [1, 37]. The initial trigger for PE is hypothesised to be a failure of normal placentation with subsequent poor angiogenesis, dysregulation of the immune system and excessive maternal inflammation leading to generalised endothelial dysfunction viewed as important hallmarks of the condition [1, 4]. These components will be discussed in further detail and the work of this thesis concentrated on biomarkers which represent these disease pathways and examined whether they differ in PE occurring in healthy women compared to those with CKD.

1.3.1 Placental versus Maternal Pre-eclampsia

The clinical presentation of PE can be markedly heterogeneous and the precise underlying aetiology may vary depending on pre-disposing factors and maternal co-morbidities [29, 38, 39]. The disease has been described as either placental or maternal in origin and proposed to converge at the point of endothelial injury [40]. Whilst the precise aetiology of PE is incompletely understood early-onset disease is thought to be related to abnormally shallow placental invasion which leads to placental hypoxia and increased oxidative stress [41]. In

contrast, late onset PE is believed to be linked with pre-existing maternal endothelial dysfunction and vascular disease.

In the first few weeks of gestation during placentation extravillous trophoblasts (EVT) invade the decidua, which is the mucosal lining of the uterus, and remodel maternal spiral arteries [1, 42-45]. The arteries are transformed into a wide-calibre low resistance arteriolar system which allows adequate blood flow and oxygen delivery to the fetus during pregnancy. The spiral arteries should be lined entirely by trophoblasts by the end of the second trimester. In PE, this physiological process is thought to fail and the EVT do not invade as deeply as they should be, resulting in transformation of arteries only in the superficial layer of the decidua (as illustrated in Figure 1-1). This leads to narrower spiral arteries causing placental hypoperfusion, oxidative stress, shallow placentation and increased predisposition to thrombosis. This phenomenon is not unique to PE and has also been demonstrated to occur in other adverse pregnancy conditions such as miscarriage, FGR, fetal death, premature rupture of membranes (PROM), placental abruption and pre-term labour [1, 36].

Maternal disease in contrast often presents at later gestation and is thought to be triggered by maternal co-morbidities associated with increased predisposition to inflammation and/or vascular problems [29]. Placentation may be 'normal' and maternal rather than placental-derived factors are implicated in the predisposition to endothelial dysfunction. There are fewer adverse fetal and perinatal outcomes, including fetal growth issues, in maternal compared to placental disease. However, the distinction between the two categories is not clear cut and many patients with PE have overlapping features of both maternal and placental disease.

Figure 1-1: The abnormal re-modelling of the spiral arteries in Pre-eclampsia

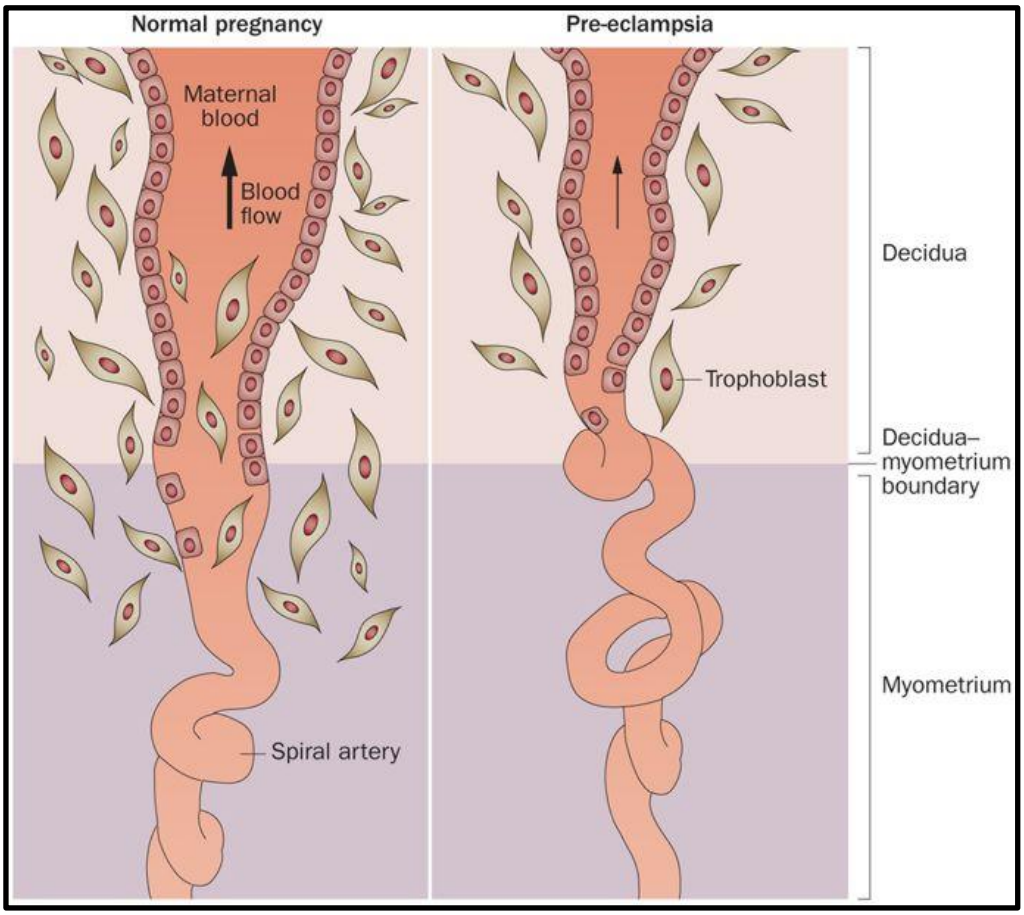


Figure reproduced from reference [1] with permission obtained from Nature Publishing Group (license number 4496730700387). In healthy pregnancy (left) physiological transformation of the maternal spiral arteries occurs with invasion of the trophoblasts extending down to the myometrium segment of the uterus. The arteries are converted into widened diameter blood vessels with increased capacitance increasing blood flow to the placenta. In Pre-eclampsia (right) the trophoblasts do not invade as deeply as in healthy pregnancy and this results in narrower spiral arteries causing uteroplacental hypo-perfusion.

1.3.2 Endothelial Dysfunction

The clinical manifestations of PE are thought to arise from widespread endothelial dysfunction [15, 18]. The endothelium forms a single layer of cells lining the vascular system and has a number of important regulatory functions including fluid filtration in the glomeruli, controlling the fine balance of thrombosis and thrombolysis, neutrophil recruitment, platelet adherence and maintenance of appropriate vascular tone [40, 46, 47]. Endothelial cells

interact closely with the immune and angiogenic system which are both implicated in the pathogenesis of PE. The endothelial system can be activated in several ways and responds to a number of mediators such as cytokines, chemokines and cell adhesion molecules. During PE, endothelial cells lose function and develop a typical flat morphological appearance resulting in them becoming 'leaky'. This is best characterised in the glomerular endothelium and histologically defined as endotheliosis [15, 48]. Whilst the endothelial dysfunction can affect any organ system, most commonly it causes systemic hypertension and renal glomerular dysfunction [9].

It is unclear what activates endothelial cells in PE and various factors have been implicated. For instance, over activity of leucocytes in the maternal circulation, angiogenic factors and the release of syncytiotrophoblast microvesicles from the placenta into the maternal circulation [40, 49]. Others have suggested that endothelial cell activation is a normal physiological response in pregnancy, occurring as a result of a pro-inflammatory state, and that this response becomes exaggerated or dysfunctional in PE [40]. Outside pregnancy, endothelial cell dysfunction is associated with conditions predisposing to the development of PE, such as CVD, hypertension, diabetes, CKD and is characterised by other features of PE including a pro-inflammatory state, pro-thrombotic tendency and vasoconstriction [47]. Furthermore, CVD and PE are both associated with endothelial dysfunction, metabolic syndrome, oxidative stress and share mutual risk factors such as positive smoking history, obesity, CKD and diabetes [50]. It is unclear whether the increased risk of developing subsequent CVD, and possibly even CKD, in women with a history of PE is due to progressive endothelial damage triggered by PE or alternatively due to mutual risk factors which

pre-dispose the mother to develop PE, as well as these other long-term health conditions.

1.3.3 The Maternal Immune Response in Pregnancy and in Pre-eclampsia

Dysregulation of the maternal immune system is proposed to play an important role in a number of pregnancy-related complications including PE [51-53]. This is supported by underlying autoimmune illness pre-disposing to the development of PE [54]. Whether maladaptation of the immune system plays a similar role in PE occurring in CKD pregnancies has not been extensively investigated previously.

During pregnancy, the maternal immune system must maintain tolerance to the fetal allograft yet remain able to protect the mother against infection [55, 56]. This balance is crucial to facilitate a successful pregnancy. Dysregulation of these mechanisms, caused by abnormal maternal adaptive and innate immune responses, are implicated in the pathogenesis of PE [1, 56-59]. It is hypothesised that an altered immune response first occurs locally following recognition of paternal antigens present on trophoblast cells by the maternal immune system, resulting in a systemic inflammatory response in the later stages of pregnancy [54].

1.3.3.1 The Innate and Adaptive Immune Response

The immune response can be divided into innate and adaptive branches [56, 60]. The innate response can proceed more rapidly to stimuli in a non-specific, antigen-independent manner, recognising molecular patterns which are usually found on non-self pathogens. Cells involved in the innate immune system include phagocytes, granulocytes, mast cells and Natural Killer (NK) cells. They recognise pathogens via toll like receptors which activate the

innate immune system to produce chemokines which are a chemoattractant for immune cells. In contrast, the adaptive immune system generates a slower response but is targeted against specific antigens and leads to the formation of long-term immunological memory against these antigens. Specific antigens are processed by dendritic cells and presented to B or T lymphocytes leading to antigen-specific humoral and cell mediated immune responses through formation of antibodies and cytotoxic cells respectively.

T cells can be categorised as T helper 1 (Th1) and Th2 cells [61]. T helper 1 cells produce type 1 cytokines, such as Interleukin (IL)-2, Interferon (IFN)- γ and Tumour Necrosis Factor (TNF)-β, and induce cell-mediated immunity which is responsible for removal of intracellular pathogens, tumour cells and graft rejection. In comparison, Th2 cells produce type 2 cytokines, such as IL-4-6 and IL-13, and induce humoral immunity with antibody production. Furthermore, Th1 cells promote inflammation which is regulated by type 2 cytokines and immunoregulatory T cells. In pregnancy, there is a predominance of cytokines produced by Th2 cells which facilitates the maintenance of pregnancy and is discussed in further detail later on in this section.

1.3.3.2 Immune Intolerance

It has been proposed that maternal immune intolerance may contribute to abnormal trophoblast invasion of maternal arteries which is viewed as central to the development of PE [45]. The mother's innate immune system is involved in the process of placentation, with uterine decidual NK cells secreting cytokines which allow trophoblasts to invade the spiral arteries [60]. Therefore, during its early stages, pregnancy requires a localised inflammatory response to facilitate placentation [62]. In PE, it has been suggested that a systemic

dysregulation of the maternal immune response arises from early immune intolerance to paternal alloantigens present on trophoblast cells [45, 63]. In particular, it has been proposed that poor expression of Human Leukocyte Antigen (HLA)-G by trophoblasts impairs their ability to invade blood vessels, with reduced stimulation of local NK cells, resulting in a change in release of cytokine and angiogenic factors [52]. It is thought that HLA-G expressing trophoblasts play an important role in embryo implantation and establishing maternal–fetal immune tolerance [52, 64]. Antiphospholipid antibodies have also been demonstrated to inhibit uterine invasion of trophoblasts suggesting multiple components of the immune system could perhaps influence this process [56]. This concept, that immune intolerance to paternal antigens is a trigger for PE is supported by the epidemiology of PE. First pregnancy or a first pregnancy with a new partner, pregnancy from gamete donation, long inter-pregnancy intervals, use of barrier contraception and reduced duration of sexual cohabitation pre-pregnancy are all risk factors for developing PE [54, 56]. In contrast, a second pregnancy with the same partner after a short interval is protective against PE [63]. These all suggest that inadequate exposure to paternal antigens before pregnancy may predispose to maternal intolerance to paternal antigens, increasing the risk of PE. A transient state of tolerance towards paternal antigens has been demonstrated in maternal T cells in pregnant mice and this tolerance is thought to involve memory T cells [56]. Also, the number of regulatory T cells in normal pregnancy are reported to be increased, with depression of NK and T cell activity, which may facilitate maternal immune tolerance to the growing fetus [63].

Nevertheless, normal pregnancy is characterized by a state of mild controlled inflammation with increased activation of granulocytes and monocytes, which is thought to a physiological response to pregnancy and beneficial to the mother [4, 40, 60, 65]. Therefore, there is a balance between activation and suppression of maternal immune response during healthy pregnancy and it is reported that this balance is lost in PE. It is hypothesised that the normal beneficial inflammatory response of pregnancy becomes exaggerated and dysregulated in PE resulting in an uncontrolled, pathological inflammatory state, which is discussed further below [6, 40, 52, 57, 60, 66-71].

1.3.3.3 Systemic Immune Response in Pre-eclampsia

Pregnancies complicated by PE when compared to healthy pregnancies are associated with a heightened systemic inflammatory response involving a number of circulatory immune cells and protein systems [60]. Affected pregnancies have been demonstrated to manifest an increase in number and/or activation of pro-inflammatory cells such as neutrophils, phagocytes, NK cells and pro-inflammatory cytokines, enhanced cell mediated immunity, reduction in regulatory T cells, increased activation of the complement system and clotting cascade in addition to enhanced activation of numerous other immune pathways and cell types [1, 6, 40, 45, 51, 52, 56-58, 66-69, 72, 73]. Increased levels of complement breakdown products and IgG are observed in placentae in women suffering PE compared to normal pregnancies [4]. These findings suggest that multiple immunological mechanisms are likely to involved in the pathogenesis of PE.

It has been proposed that following poor placentation, placental hypoxia develops leading to a generalised immune response which is much more exaggerated compared to normal

pregnancy [51, 59, 68]. The abnormal inflammatory response is hypothesized by some to cause necrotic shedding of syncytiotrophoblast fragments into maternal blood [40, 56], followed by activation of the endothelial system with increased expression of surface markers which allow leucocyte adhesion [60]. Obstetric complications other than PE, such as pre-term labour, FGR and fetal loss known collectively as the 'great obstetrical syndromes', have also been linked to defective placentation and abnormal maternal inflammatory response [69, 74].

The clinical features of PE can be reproduced in pregnant rates using low dose infusion of both endotoxin and the pro-inflammatory cytokine TNF-α [35, 75-77]. In vitro studies demonstrate that TNF-α can also inhibit trophoblast-like cells integration into maternal endothelial cellular networks and have harmful effects on these cells. Furthermore, an association between PE and the pro-inflammatory response gene SEPS1 has previously been demonstrated [78]. These data support the concept that the increased inflammatory response associated with PE may play a role in the pathophysiology of disease rather than being secondary consequence of the disease process. Autoimmune disease and obesity are well recognised risk factors for PE and both associated with chronic inflammation which may thus act as a trigger for an exaggerated inflammatory response in maternal PE [70]. Moreover, there is a strong interplay between inflammation and other disease processes which have been implicated in PE, such as hypoxia, endothelial dysfunction, angiogenic disturbance and oxidative stress [67-71, 77, 79-81]. Pro-inflammatory cytokines exert a powerful effect on endothelial cells and have been shown to interfere with cell permeability and function [4, 67]. In-vitro, trophoblast cell exposure to hypoxia induces a greater

inflammatory response [82] and the relationship between hypoxia and inflammation has been described as interdependent [77]. Reduction of uterine perfusion in pregnant rats increases circulating levels of TNF-α and soluble fms-like tyrosine kinase-1 (sFLT-1) demonstrating a link between hypoxia, inflammation and angiogenic factors in rodent pregnancy [83]. It has also been proposed that the maternal endothelium is sensitised by inflammatory markers in PE, allowing placental derived factors to contribute further to the disease process [42].

1.3.3.4 The Humoral response

In pregnancy, B-cell counts, B-cell function and antibody production following vaccinations are reported to be normal which is in contrast to T cell activity which is supressed during pregnancy [63, 65]. This would suggest that pregnant women are more likely to respond to infection with antibody rather than T cell mediated responses. In keeping with these findings, normal pregnancy is associated with a shift towards a Th2 dominated response, in contrast to a balance between Th1 and Th2 responses in healthy non-pregnant women [45, 56, 61, 72, 84, 85]. There is increased production of progesterone and cytokines (IL-4-6 and 13) in normal pregnancy, which promote a Th2 environment and suppression of Th1 cytokines (IL-12, IFN-γ and TGF-β) [45, 52, 84, 86]. As discussed earlier Th1 cytokines help induce an inflammatory response whereas Th2 cytokines regulate the inflammatory response [56]. It is proposed that a maternal Th2 response during pregnancy favours an immune tolerant environment, thereby protecting against cell-mediated rejection of the fetus [86, 87].

In contrast to normal pregnancy, PE is associated with an increase in Th1 cytokine production and suppression of Th2 cytokines and thus a switch towards a Th1 phenotype [61, 84, 86]. The switch to Th1 cytokine phenotype in PE has been demonstrated to be due to a higher number of circulating Th1 over Th2 cells [61]. In contrast, Th2 subpopulations predominate in second and third trimester of healthy pregnancy. Additionally, the transfer of Th1 cells into pregnant mice results in the mice developing features of PE such as high blood pressure and proteinuria [84]. However, not all studies have demonstrated the switch to Th1 cytokine profile in PE and the concept of Th1 versus Th2 phenotype in affected pregnancies has been suggested as being 'too simplistic' [52, 86, 87]. Furthermore, some women with PE have detectable circulating antibodies against type-1 angiotensin II receptor and these have been implicated in the pathogenesis of PE in rats [1].

A Th1 phenotype during pregnancy has also been associated with other adverse obstetric outcomes such as pre-term birth, miscarriages, embryonic rejection and growth restriction [4, 88]. In one study, immunosuppression with tacrolimus was shown to improve pregnancy rate and live birth rate in women with a history of recurrent implantation failures and elevated Th1/Th2 ratios [88]. Also, In pregnant rats and mice, injection of T-helper 1 cytokines results in fetal demise [89]. An inability to sufficiently switch to a Th2 response in pregnancy may therefore play a part in adverse pregnancy outcome including PE. There are limited published studies which have reported changes in antibody concentrations in PE compared to healthy pregnancy and this is discussed later, as the work of this thesis has attempted to focus on markers of the humoral system at the time of and/or before PE developing.

1.3.4 Angiogenic Factor Imbalance

Placental PE is often described as developing in two stages [60, 68]. A pre-clinical phase lasting for weeks is hypothesised to occur before onset of the clinical manifestations of the disease, during which time the ischaemic placenta secretes factors into the circulation which in turn drive the widespread endothelial dysfunction and vasomotor imbalance giving rise to the clinical manifestations of PE [1, 90]. There has been significant interest over the past decade trying to identify the biochemical triggers secreted by the placenta and whilst this has not been definitively identified there has been recently increasing focus on soluble angiogenic factors. It has been proposed that in both maternal and placental PE, the clinical features of disease result from the effects of circulating anti-angiogenic factors released from the placenta [41].

Pre-eclampsia is associated with an imbalance between pro and anti-angiogenic factors, resulting in a shift towards an antiangiogenic state [1, 5, 30]. In particular, changes in the concentrations of the anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFLT-1) and pro-angiogenic factor placental growth factor (PIGF) which are both secreted by the placenta have emerged as strong biomarkers for diagnosis and prediction of PE [91]. These angiogenic factors are thought to play an important role in the pathogenesis of PE and in particular induction of endothelial dysfunction [92, 93].

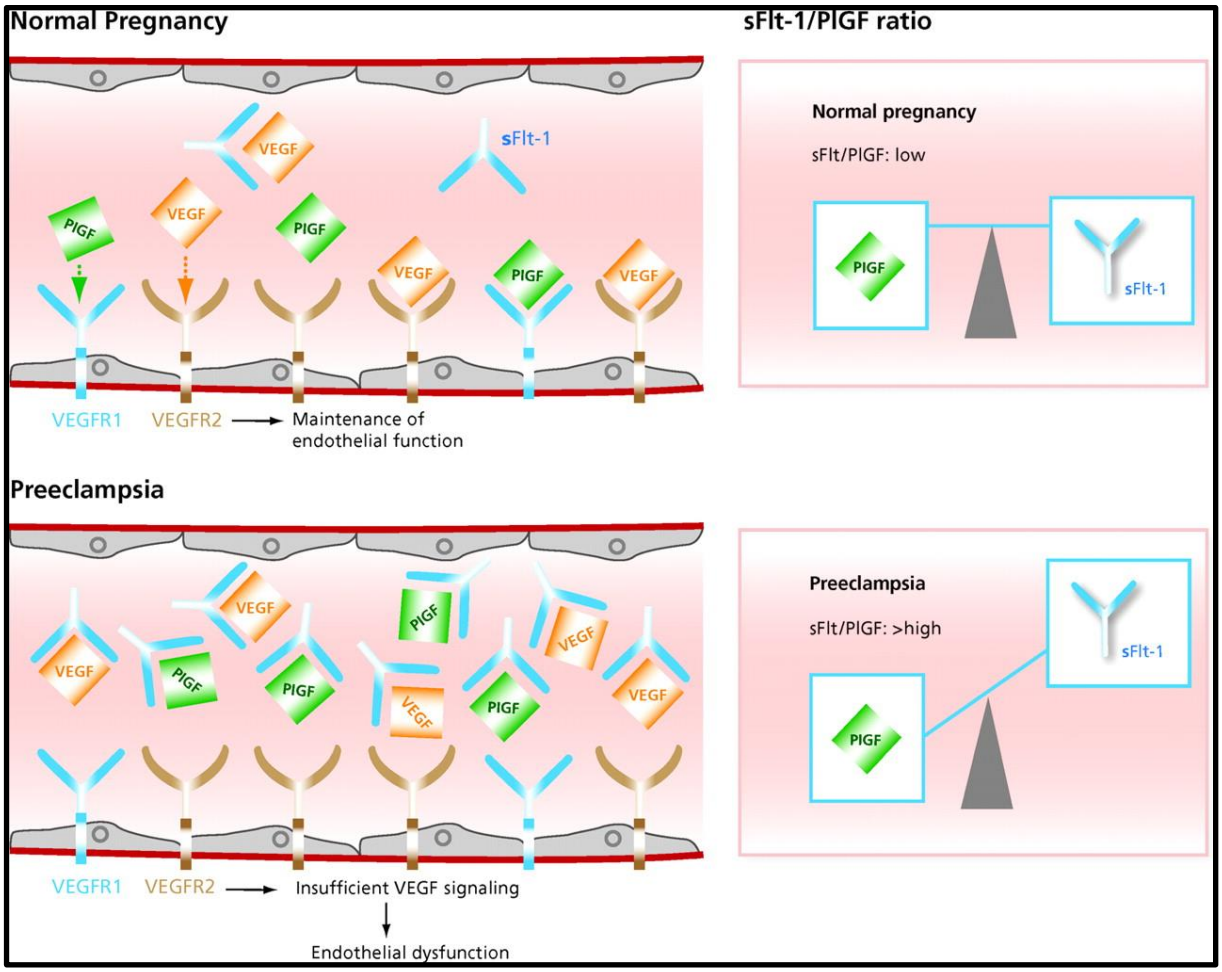
Vascular endothelial growth factor (VEGF) plays a key role in triggering angiogenesis and maintaining normal endothelial function [15, 94-97]. It is one of the most important angiogeic mediators, acting through two endothelial cell receptors. These are VEGF-receptor-1 which is also known as fms-like tyrosine kinase-1 (FLT-1) of which the soluble

isoform present in the circulation is known as sFLT-1, and the second receptor is VEGF-receptor-2. Placental growth factor is a pro-angiogenic molecule derived from the VEGF family and is expressed in large amounts by syncytiotrophoblasts in the placenta. Soluble FLT-1 is also mostly produced by the placenta and behaves as a circulating anti-angiogenic protein by binding to and thereby antagonising the actions of both VEGF and placental growth factor (PIGF). As Figure 1-2 illustrates, excessive concentrations of circulating sFLT-1 results in altered VEGF signalling leading to endothelial cell dysfunction. The circulating concentrations of VEGF are below limit of detection using most commercial enzyme-linked immunosorbent assay (ELISA) kits and are not measured in clinical practice [15].

In normal pregnancy VEGF and PIGF play an important role in placental vascular development, in particular regulation of trophoblast growth, villous angiogenesis and spiral artery remodelling [43]. During pregnancy, the interaction between the receptor FLT-1 (VEGFR-1) with VEGF and PIGF promotes placental angiogenesis. However, interaction between sFLT-1 with VEGF and PIGF prevents their binding to transmembrane receptors and thus induces an anti-angiogenic state. Pre-eclampsia is thought to develop when the functional activity sFLT-1 exceeds that of VEGF causing impaired angiogenesis and placental development. The renal capillary endothelium is very sensitive to the effects of VEGF as this is required to maintain normal fenestration of glomerular endothelial cells and maintenance of the glomerular filtration barrier [50]. This would explain why proteinuria, arising from breakdown of the glomerular filtration barrier is an important characteristic feature in PE. It has been suggested, that in women with underlying co-morbidities such as hypertension, diabetes and obesity, there is already an element of endothelial dysfunction and less sFLT-

1/PlGF imbalance is required to produce the symptoms of PE [41]. It is therefore possible that pre-existing endothelial dysfunction arising from CKD may also have a similar effect.

Figure 1-2: The effect of sFLT-1 on PIGF and VEGF mediated endothelial cell signalling pathways



High sFLT-1 levels inactivate the pro-angiogenic proteins by blocking their binding to their transmembrane receptors which normally helps maintains endothelial function. Figure reproduced from reference [15] with permission obtained from copyright.com (confirmation number 11812224).

A hypoxic state arising from failed spiral artery modelling and resulting oxidative stress is postulated to promote the angiogenic factor imbalance in PE [43, 98]. Uteroplacental hypo-perfusion resulting in ischaemia has been shown to lead to increased circulating levels of sFLT-1 and a reduction in PIGF in animal models. Furthermore, in-vitro studies on human

trophoblast cells have demonstrated hypoxia promotes increased production of sFLT-1 [99]. It has been suggested that placental hypoxia leads to over activation of the hypoxia-inducible factor 1α which targets VEGF and sFLT-1 genes resulting in increased placental production and circulating levels of both factors [43].

Administration of sFLT-1 in pregnant rats results in hypertension, proteinuria and glomerular endotheliosis; the clinical manifestations of PE [100]. Furthermore, administration of anti-VEGF therapy can induce hypertension and proteinuria when used therapeutically in patients with malignancy [70]. Markers of inflammation and the complement system have also been shown to be correlated with angiogenic markers in the setting of PE and other adverse human pregnancy outcome [70, 71, 79, 81, 101-103]. Experimental and clinical research is ongoing into the effects of directly targeting anti-angiogneic proteins in PE [18]. The predictive capability of soluble angiogenic markers in PE is discussed later in the chapter.

Figure 1-3 depicts the various factors that are hypothesised to be involved in the pathophysiology of PE, these include abnormal trophoblast invasion, increased oxidative stress, altered immune system response and an anti-angiogenic imbalance [6]. It is likely the factors implicated in the pathophysiology of PE are interlinked rather than act by independent pathways and there are probably other important factors that are also involved. It is far from established how these various disease pathways interact and their respective importance in the development of PE in CKD pregnancy.

Figure 1-3: Factors involved in the pathophysiology of Pre-eclampsia

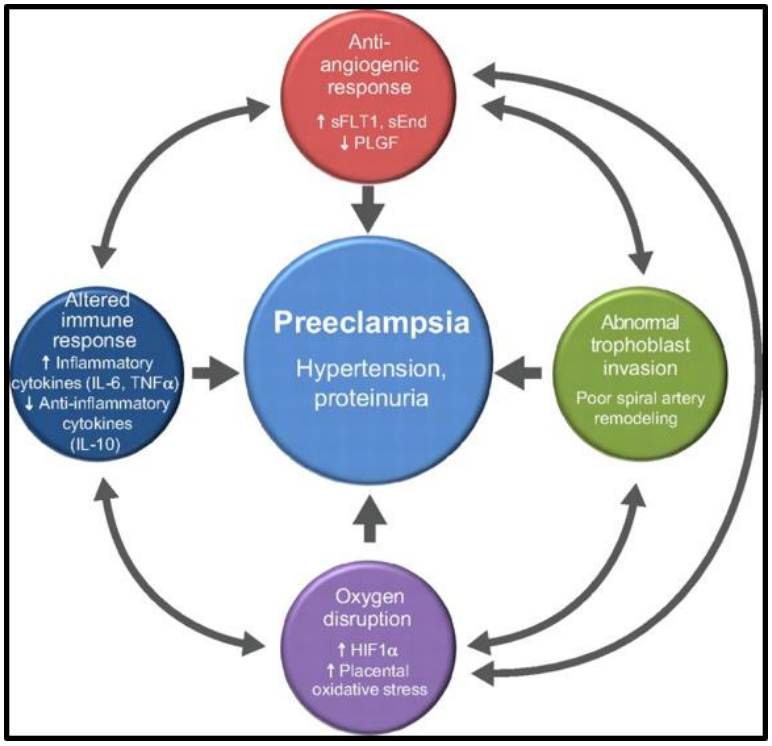


Figure reproduced from reference [6] with permission granted from publisher.

1.4 Chronic Kidney Disease - Overview

Chronic Kidney Disease is defined as a chronic reduction in kidney function with or without structural kidney damage [104-107]. It is increasingly recognised as a global health problem as a consequence of its high prevalence and significant health implications. Current estimates report the prevalence of CKD in the general population to be around 11% to 15%. However, the true prevalence of CKD is unknown as earlier stages of CKD are usually asymptomatic and symptoms are unlikely to occur until glomerular filtration rate (GFR) falls to less than 25% of normal [108]. The cause of CKD can be due to primary renal pathology or secondary to other diseases. Common secondary causes of CKD include diabetes mellitus, chronic hypertension and CVD [107, 109]. The prevalence of CKD worldwide is expected to continue to rise given the predicted increase in these conditions. In the United states for

instance, the prevalence of CKD stages 1 to 4 has increased from 10% in 1988-1994 to 13.1% in 1999-2004 [106].

Figure 1-4: KDOQI classification of CKD stages

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30–300 mg/g 3–30 mg/mmol	>300 mg/g >30 mg/mmol
GFR categories (ml/min per 1.73 m^2) Description and range	G1	Normal or high	⩾90			
	G2	Mildly decreased	60–89			
	G3a	Mildly to moderately decreased	45–59			
	G3b	Moderately to severely decreased	30–44			
	G4	Severely decreased	15–29			
	G5	Kidney failure	<15			

Adapted from guideline reference [110]. The coloured cells denote rate of CKD progression as green: low risk, yellow: moderately increased risk, orange: high risk and red: very high risk. The staging system is also the same for renal transplant patients with a T designating transplant status for instance CKD stage 2T represents a transplant recipient with a GFR of 60-89 mL/min/1.73m^2.

The National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI) in 2002 proposed a five-stage classification system of CKD. This has been subsequently updated and the current staging classification is summarised in Figure 1:4 [110, 111]. This classification system uses the estimated GFR (eGFR), as a measure of kidney function, and quantification of proteinuria (albuminuria), as a marker of kidney damage, to define the

stages of CKD. The GFR is a measure of the total amount of fluid filtered through all of the functioning nephrons per unit of time [112]. This can be estimated from measuring serum creatinine concentration and using equations, such as the Modification of Diet in Renal Disease (MDRD) or the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations, with the latter deemed to be a more accurate marker of renal function and predictor of clinical risk [104, 110, 113].

Normal urine contains several low molecular weight proteins; however due to glomerular capillary wall impermeability albumin is not usually present in significant concentrations in urine [112]. Traditionally, a 24-hour urine collection has been used as the most accurate method to measure urine protein excretion. However, this method can be cumbersome and inconvenient for patients and can lead to significant errors in collection. Currently, 'spot' urine sample measurements offer comparable results, are more convenient and preferentially used in clinical practice to quantify proteinuria. Using a spot urine sample, an urine albumin to creatinine ratio (uACR) of over 30 mg/mmol supports the presence of glomerular pathology; however even values above 3mg/mmol are considered abnormal [113].

Stages 3–5 CKD is defined as a GFR of less than 60 ml/min/1.73 m^2 with or without other evidence of kidney damage for at least 3 months. Stages 1 and 2 CKD are associated with normal or mildly impaired kidney function but with evidence of kidney damage which includes presence of albuminuria, urine sediment abnormalities, electrolyte abnormalities due to tubular disorders, histological changes present on kidney biopsy, structural abnormalities detected by imaging or a history of renal transplantation [111]. The risk of

progression of renal disease increases as GFR drops and as the degree of albuminuria increases and this is illustrated in Figure 1-4 [110, 114]. End stage renal disease (ESRD) usually develops once GFR falls to 5-10 ml/min/1.73m^2 and it is at this point that either dialysis (haemodialysis or peritoneal dialysis) or renal transplantation is necessary for patient survival. The majority of patients with CKD have stage 3 disease [104]. The reported global mean CKD prevalence is Stage 1: 3·5%; Stage 2: 3·9%; Stage 3: 7·6%; Stage 4: 0·4% and Stage 5: 0·1%.

Chronic Kidney Disease is independently associated with an increased risk of all-cause mortality, CVD related morbidity and mortality [110, 114]. Cardiovascular disease is the primary cause of death and morbidity in CKD, the risk of which increases as GFR drops and is independent of other traditional CVD risk factors such age and sex [115]. Increasing proteinuria is also independently associated with an increased CVD risk even at early stages of albuminuria [115-118]. Furthermore, increased proteinuria is associated with worse prognosis compared to reduced eGFR by itself [119, 120]. For example, individuals with stage 1–2 CKD and microalbuminuria (uACR>30mg/mmol) have worse cardiovascular disease outcomes compared to patients with stage 3 CKD without micro-albuminuria (uACR <30 mg/mmol).

1.5 Chronic Kidney Disease in Pregnancy

In developed countries CKD is estimated to affect up to 6% of women of child bearing age and 3% of pregnant women [108, 121, 122]. Pregnancy in women with advanced CKD is not very common due to reduced fertility but also, perhaps, because of a reported increased rate of miscarriage [108, 123-127]. However, due to increasing prevalence of CKD in the general population, this figure is likely to increase [106, 128]. Most pregnant women with CKD have mild renal impairment and 18-29% are newly diagnosed with CKD during pregnancy [129]. Renal function is not routinely measured during pregnancy in the United Kingdom (UK); though, urinalysis and blood pressure are checked during routine antenatal visits. The discovery of urinalysis abnormalities (haematuria or proteinuria) with or without hypertension allows the identification of new CKD cases.

1.5.1 Renal physiology in pregnancy

The kidney undergoes a number of significant anatomical, physiological and functional changes during pregnancy, which are summarised in Figure 1-5 and Table 1-1 [108]. The bipolar length of the kidney increases by approximately 1cm with associated pelvicalyceal dilatation; which is more pronounced on the right side. Physiological hydronephrosis occurs in 43 to 100% of pregnancies and is thought to be due to progesterone-induced ureteric smooth muscle relaxation, as well as some mechanical compression due to the gravid uterus [85, 130]. This leads to urinary stasis and increased risk of developing Urinary Tract Infections (UTI) in pregnancy including pyelonephritis.

During pregnancy, there is an increase in circulating blood volume, increase in cardiac output and systemic vasodilatation resulting in decreased systemic vascular resistance and first

trimester blood pressure [108, 130]. These changes result in increase in renal blood flow by 50-85% and increase in GFR by over 50% compared to the non-pregnant state. Blood pressure decreases at the start of pregnancy reaching a nadir at around 20 weeks' gestation. This gradually returns to baseline towards the third trimester of pregnancy. These adaptations are important for optimal pregnancy outcome but may not be achieved in women with CKD, in whom the increased physiological demands may lead to deterioration in maternal kidney function [92]. For instance, gestational hyperfiltration is absent in advanced CKD [108].

In women with preserved kidney function or mild CKD, serum creatinine values will fall during pregnancy as a consequence of glomerular hyperfiltration (Table 1-1). Therefore, a serum creatinine over 77 umol/l during pregnancy may be considered abnormal compared to outside pregnancy where the normal range for females is 45-90 umol/l [131]. Uric acid (urate) levels also decrease in early pregnancy due to increased renal excretion [126]. Metabolic acidosis may result from increased renal excretion of bicarbonate, though hyperventilation occurring in later pregnancy may compensate for this [132]. Other changes include glycosuria (increased glucose in the urine), due to reduced renal reabsorption threshold, and a lowering of serum sodium levels [108].

Total urinary protein excretion is higher during healthy pregnancy, albeit below 300 mg a day, when compared to a normal range outside pregnancy of 60-90 mg a day [3, 85]. Albuminuria accounts for approximately 10% of the excreted urine protein during pregnancy [133]. The increase in proteinuria is believed to be due to gestational glomerular hyperfiltration and possibly increased glomerular permeability arising from an increase in

glomerular pore size [3, 85]. In a study of 202 pregnant women, where only one patient had CKD, over 10% developed significant proteinuria in the second and third trimester with ≥ 1+ protein on their urine dip [134]. There was no associated decline in kidney function and proteinuria resolved post-partum. If significant proteinuria is first detected in pregNancy after 20 weeks' gestation, PE needs to be excluded; whereas if evident in early pregnancy, proteinuria is more likely due to pre-existing CKD.

Figure 1-5: Summary of the physiological changes that occur in the kidney during pregnancy

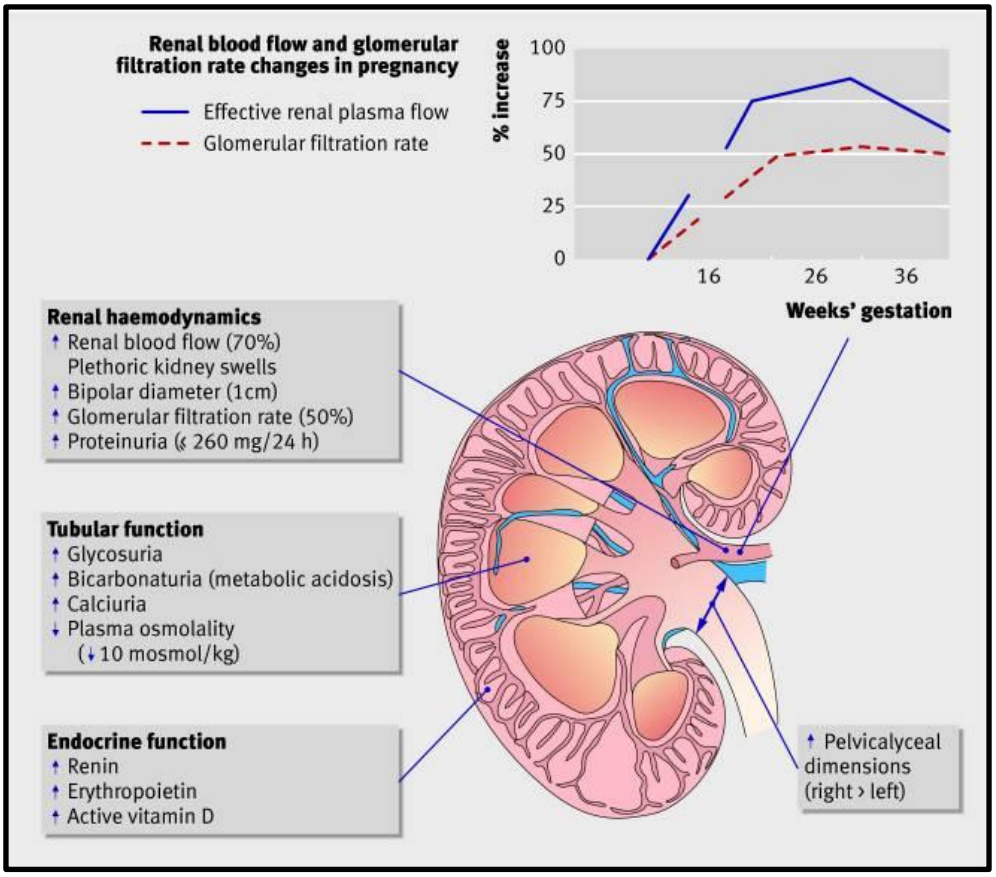


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Table 1-1: Changes in renal function and other clinical markers during pregnancy in healthy women

Measure	Stage of pregnancy			
	Before Pregnancy	First trimester	Second trimester	Third trimester
Effective renal plasma flow (ml/min)	480 (72)*	841 (144)	891 (279)	771 (175)
Measured GFR (ml/min) by inulin clearance	105 (24)	162 (19)	174 (24)	165 (22)
Measured GFR (ml/min) by 24h creatinine clearance	98 (8)	151 (11)	154 (15)	129 (10)
Serum creatinine (umol/l)	73 (10)	60 (8)	54 (10)	64 (9)
Plasma urea (mmol/l)	4.3 (0.8)	3.5 (0.7)	3.3 (0.8)	3.1 (0.7)
Plasma uric acid (umol/l)	246 (59)	189 (48)	214 (71)	269 (58)
Plasma osmolality (mosmol/kg)	290 (2)	280 (3)	279 (3)	279 (5)

*Adapted from reference [108] with permission obtained from BMJ Publishing group Ltd (license number 4492640392293). *Values provided are in mean with standard deviation (SD) in brackets.*

1.5.2 Pregnancy Outcomes in Chronic Kidney Disease

Historically, clinicians often advised women with CKD against pregnancy due to the perceived high risk to maternal health and poor fetal outcomes [135]. However, current advice is substantially different with recognition that most pregnancies in women with CKD do not result in significant long-term harm to the health of the mother or baby. Nevertheless numerous studies, mostly observational, and reviews on the subject have consistently demonstrated high rates of maternal, fetal and obstetric complications in women with CKD compared to healthy women [85, 108, 127, 128, 136-144]. Figure 1-6 summarises the increased rate of adverse pregnancy outcome associated with worsening kidney function; defined according to pre-pregnancy serum creatinine (a-b) or CKD stage (c-d). These include a higher risk of developing superimposed PE, FGR, premature delivery (delivery < 37 weeks), delivering low birth weight (LBW) babies, admission to neonatal unit (NNU) and a temporary

or permanent decline in kidney function following pregnancy. The risks of pregnancy related complications are recognised to be stepwise according to pre-pregnancy renal function and demonstrated in Figure 1-6 [108, 142]. Other factors which have been shown to be associated with adverse pregnancy outcome independent of renal function are pre-existing and uncontrolled hypertension, increased baseline proteinuria, renal transplant status, lupus nephritis, diabetic nephropathy and PE in a previous pregnancy [108, 126, 136, 142].

Whilst it is accepted that pregnancies in women with advanced CKD are at the highest risk of pregnancy complications, the area of contention is the stage at which this increased risk occurs or whether all CKD pregnancies should be treated as high risk [121, 123, 126, 142]. The rates of reported pregnancy complications have also varied in studies. Heterogeneity in the degree of renal impairment, whether this was defined pre or during pregnancy, and primary renal pathology in the study populations may explain why reported pregnancy outcomes in the literature have not been consistent. Furthermore, studies have often been small and retrospective. Methods used to measure renal function and define stage of CKD have also differed as have outcome measures [122].

Piccoli et al. in an Italian cohort study comparing 504 CKD and 836 non-CKD pregnancies demonstrated a stepwise increase in adverse pregnancy outcome (caesarean section delivery, pre-term birth, small for gestational age baby (SGA), admission to neonatal intensive care unit (NICU), worsening hypertension and/or proteinuria) with increasing CKD severity (Figure 1-6) [142]. The study also demonstrated that CKD stage 2 was associated with increased risk compared to CKD stage 1 pregnancies. Furthermore, women with CKD stage 1 without co-existing hypertension, systemic disease or proteinuria compared to

pregnant women without CKD still had an increased risk of adverse pregnancy outcome with an odds ratio (OR) and 95% confidence interval (CI) of 1.88 and 1.27-2.79 respectively, raising possibility that CKD itself, even with preserved kidney function, is a risk factor for adverse pregnancy outcomes. However, in their study, where pre-pregnancy renal function was not available CKD stage was defined by MDRD and CKD-EPI equations calculated during pregnancy, which as discussed later in the chapter are not deemed accurate in pregnancy. This may have led to an overestimate of renal function and misclassification of CKD stage [126]. Female live kidney donors, who in general are likely to have preserved renal function, have been demonstrated to have a small, but higher, rate of PE following donation compared to appropriately matched controls (OR 2.4, 95% CI 1.2-5.0) [145].

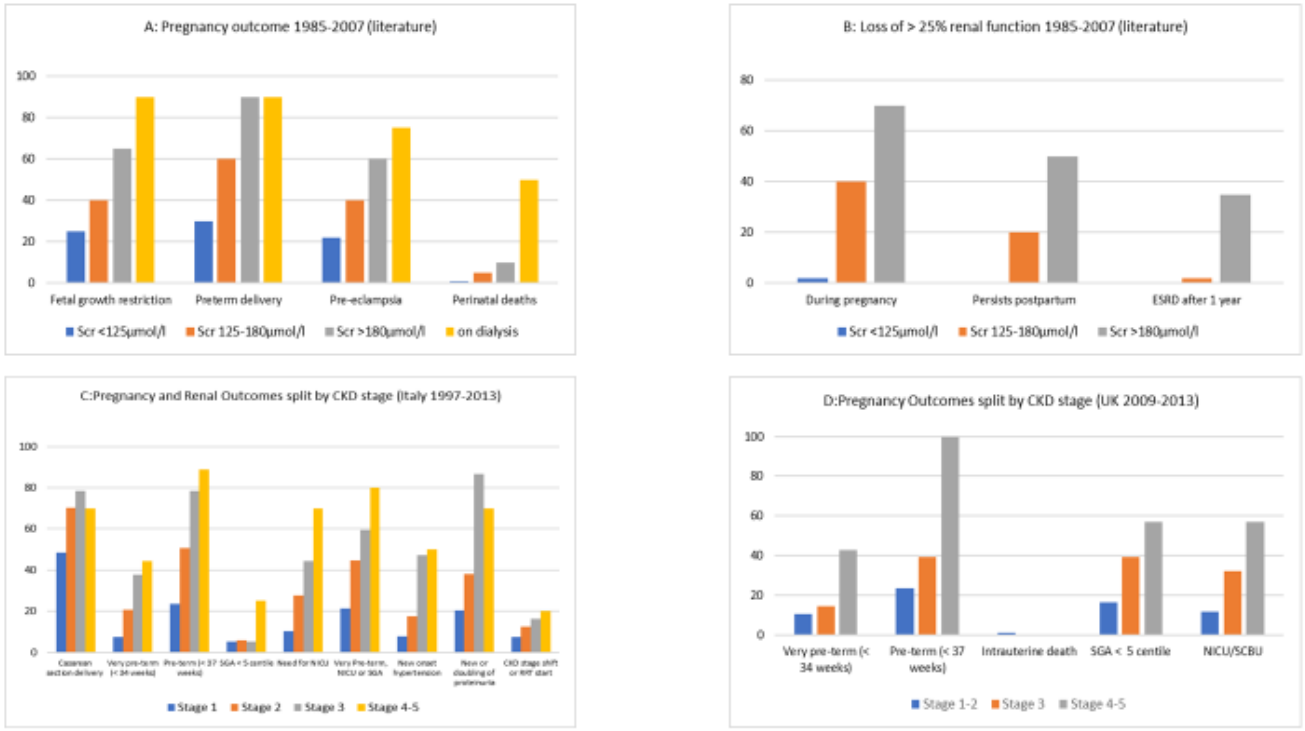
In a further retrospective study comparing CKD and non-CKD pregnancies, women were matched for age, race, history of diabetes, chronic hypertension, liver disease and connective tissue disease [146]. Compared to controls women with CKD, despite matching, still had an increased risk of preterm delivery (OR 1.52, 95% CI 1.16-1.99), delivery via cesarean section (OR 1.33, 95% CI 1.06-1.66), admission to NICU (OR 1.71, 95% CI 1.17-2.51), delivery of LBW baby (OR 2.38, 95% CI 1.64-3.44). The risk of maternal death was not increased in the CKD cohort.

It is unclear why CKD stages 1 or 2, without co-existing hypertension or proteinuria, may be associated with a higher risk of adverse pregnancy outcome. It has been suggested that the renal adaptations specific to pregnancy, may not be fully achievable in women with CKD, possibly due to lack of renal reserve resulting from glomerular scarring (sclerosis), pre-existing arteriolar vasodilatation, arterial disease (arteriosclerosis) and over-activation of the

renin-angiotensin system [137]. It has been demonstrated that there is no difference in pregnancy outcome (caesarean section delivery, premature delivery, need for NICU) between women with CKD stage 1 who show evidence of glomerular hyperfiltration (defined as estimated GFR >120 mL/min/1.73m^2) compared to those who do not, which may indicate that factors other than failure of appropriate renal physiological adaptation are responsible for the increased risk in pregnancy in these women [147]. Not all studies have demonstrated an increased pregnancy risk in earlier stages of CKD. A population based Norwegian study of 5655 singleton CKD pregnancies with an eleven year follow up period reported that women with CKD stages 1 and 2 were not at an increased risk of PE, SGA or preterm delivery unless they also had underlying hypertension [148].

A literature based review spanning over 25 years, including the author's own records, summarised pregnancy outcomes splitting patients into three groups based on their pre-pregnancy serum creatinine; these were mild (< 125umol/l), moderate (125-180umol/l) and severe (>180 umol/l) renal impairment [108]. Women with preserved or mildly decreased kidney function had over 95% live births and three quarters of these babies were born at an appropriate size. Women with a pre-pregnancy serum creatinine of >180 umol/l had much higher complications rates; specifically, FGR (over 65%), preterm delivery (over 90%), PE (60%) and perinatal deaths (10%). In comparison, the rates of complications were lower in the moderate group; however, the lowest rates of these complications were observed in the mild group; occurring in 25%, 30%, 22% and 1% of pregnancies respectively. Pregnancies in women on dialysis pre-pregnancy were associated with substantial risk of serious adverse outcome. FGR and pre-term delivery were identified in over 90% of pregnancies and there

Figure 1-6: Pregnancy outcomes according to pre-pregnancy kidney function (various studies)



Values are displayed as percentages on the y-axis. Figures A and B are taken from reference [108] and is based on a literature review, groups are split according to pre-pregnancy serum creatinine (Scr). Figures C and D are taken from reference [142] and [139] with findings based on prospective Italian and UK cohort studies respectively. ESRD = end stage renal disease, SGA= small for gestation age, NICU= neonatal intensive care unit, SCBU = special care baby unit. The graphs illustrate worsening of pregnancy and renal outcomes as severity of CKD increases.

was a one in two risk of perinatal death. Over the past few years, women with advanced CKD commenced on an intensive haemodialysis regimen during pregnancy have yielded much improved pregnancy outcomes [126, 137]. Intensive haemodialysis (defined as an average of 43 hours per week) resulted in an 86% live birth rate at a mean gestational age of 36 weeks compared to respective figures of 61% and 27 weeks in women receiving a standard haemodialysis regimen of an average of 17 hours per week in one study [149].

More recently, a meta-analysis, including 23 observational studies comprising of 1514 CKD pregnancies, summarised pregnancy outcomes in women with CKD [136]. Compared to pregnancies in women without CKD, those with CKD had a higher risk of PE (OR 10.36, 95% CI 6.28- 17.90), premature delivery (OR 5.72, 95% CI 3.26 -10.03), delivery of SGA or LBW baby (OR 4.85, 95% CI 3.03 - 7.76) and delivery by caesarean section (OR 2.67, 95% CI 2.01 - 3.54). Failure of pregnancy, defined as a combined outcome of stillbirth, neonatal or fetal death, was also increased (OR 1.80, 95% CI 1.03 - 3.13). Pre-existing macroproteinuria (defined as per 2012 KDIGO guidelines) was associated with a higher risk of PE and/or pre-term birth. Therefore, pre-existing proteinuria as well as hypertension and pre-pregnancy renal excretory function, is linked to an increased risk of adverse pregnancy outcome in CKD pregnancy.

Transplantation usually restores fertility in women with advanced CKD and pregnancy is estimated to occur in 12% of transplant recipients of child bearing age [150]. Renal transplant recipients have been shown to have an increased risk of adverse pregnancy outcome in the context of pre-existing transplant dysfunction, hypertension and/or proteinuria [121, 151]. In a meta-analysis of 50 studies, reviewing pregnancy outcomes of

4706 pregnancies in 3570 kidney transplant recipients, the live birth rate was similar to the general population (73.5% vs. 66.7% respectively). However rates of PE (27% vs. 3.8%), gestational diabetes (8% vs. 3.9%) and premature delivery (45.6% vs. 12.5%) were significantly higher in the transplant compared to non-transplant group respectively [124]. Urinary tract infections are more common in pregnancy and this risk is further increased in some women with CKD, for instance those with a history of reflux nephropathy or structural kidney abnormalities [137]. Asymptomatic bacteriuria should be screened for and treated during pregnancy, as there is an association between UTIs and adverse pregnancy outcome (spontaneous preterm labour and infant mortality) [152].

Williams et al. reported an increase in accelerated decline in kidney function following pregnancy in women whose pre-pregnancy creatinine values were above 125umol/l [108]. As illustrated in Figure 1-6, renal outcomes, in addition to pregnancy outcomes, worsen as CKD severity increases. Loss of over 25% renal function occurred in pregnancy in 2% of women with a pre-pregnancy creatinine <125umol/l, increasing to over 40% and 70% respectively in women with serum creatinine of 125-180umol/l and >180umol/l. This decline was not reported to persist postpartum in the mild group but persisted in 20% and 50% in the moderate and severe group respectively. Pre-existing hypertension and proteinuria, as is the case with pregnancy complications, both increased the risk of decline in renal function following pregnancy.

In the meta-analysis reviewing CKD pregnancy outcomes, at a median follow up period of 5 years (interquartile range [IQR] 5-15 years), no increased risk of adverse renal outcomes (OR 0.96, 95% CI 0.69 - 1.35) was noted in the CKD versus non-CKD group [136]. The adverse

renal outcomes assessed included doubling of pre-pregnancy serum creatinine, a 50% drop in estimated GFR/creatinine clearance or development of ESRD. However, in these analysis women with CKD stage 4 were excluded due to paucity of relevant literature. A case-control study examining pregnancy outcomes in women with mild CKD (defined as pre-pregnancy serum creatinine < 100 umol/l) due to primary glomerulonephritis, with controlled blood pressure, identified little adverse renal outcome on long term follow up of up to 25 years [153]. The authors concluded that pregnancy does not impact on the course of renal disease in women who have normal renal function at the time of conception. In contrast, Piccoli et al. reported adverse kidney function outcome in CKD stage 1 pregnancies, with an increased risk of decline in long-term kidney function [147]. They reported 7.6% of women with stage 1 CKD had a post-partum decline in kidney function. However as described earlier, women in this study may have been misclassified to their CKD stage.

In a retrospective study of 70 pregnancies, 43% of women with an early pregnancy serum creatinine >168umol/l had a 25% reduction in kidney function at 6 weeks post-partum and 11% had progressed to ESRD [154]. In another study reporting pregnancy outcome in women with CKD stage 3-5, the presence of a pre-pregnancy estimated GFR <40 ml/min/ $1.73m^2$ and proteinuria >1g per day was associated with accelerated decline in kidney function at 6 months following delivery compared to either factor alone (from 0.55± 0.39 to 1.17±1.23 mls/min/1.73m2/month) and was associated with poorer fetal outcomes [140]. Pregnancy was not shown to accelerate decline in kidney function in stage 3-4 compared to stage 1 CKD pregnancies in a recently published retrospective study of a Chinese cohort with a median post-partum follow up period of 49 months [128]. However, there were a smaller

number of pregnancies in the CKD stage 3-4 group (30 pregnancies) compared to the other groups and CKD stage in this study was defined in some patients based on estimated GFR equations calculated during pregnancy. It has been suggested that a third of women with CKD stage 4-5 during pregnancy will require dialysis within 12 months of delivery [137]. Transplant loss following pregnancy is rare if pre-pregnancy serum creatinine is under 150umol/l [155] and, in the meta-analysis on renal transplant pregnancy outcomes, it was reported as 5.8% at 1 year and 6.9% at 5 years [124].

1.6 Superimposed Pre-eclampsia

PE developing in the context of pre-existing hypertension or CKD is termed superimposed PE (SPE). The precise incidence of SPE is unknown [156]. Figure 1-7 is a forest plot published in a meta-analysis reporting a greater than tenfold increase in rates of PE in CKD versus non-CKD pregnancies (OR 10.36) [136]. The risk is proportional to the severity of CKD, with a range in risk of developing SPE of 20% in those with mild kidney disease to over 50% in those with more advanced CKD, including those who become pregnant whilst receiving dialysis [157, 158]. There is also evidence to suggest that PE is more severe when superimposed on CKD, as it is more likely to occur earlier, with one paper reporting a third of cases occur before 34 weeks' gestation [127]. It is estimated that about 20% of women who develop severe early onset PE have underlying CKD [159]. Pregnant women with chronic hypertension are already at increased risk of maternal and fetal complications, which is further increased if they develop SPE [156].

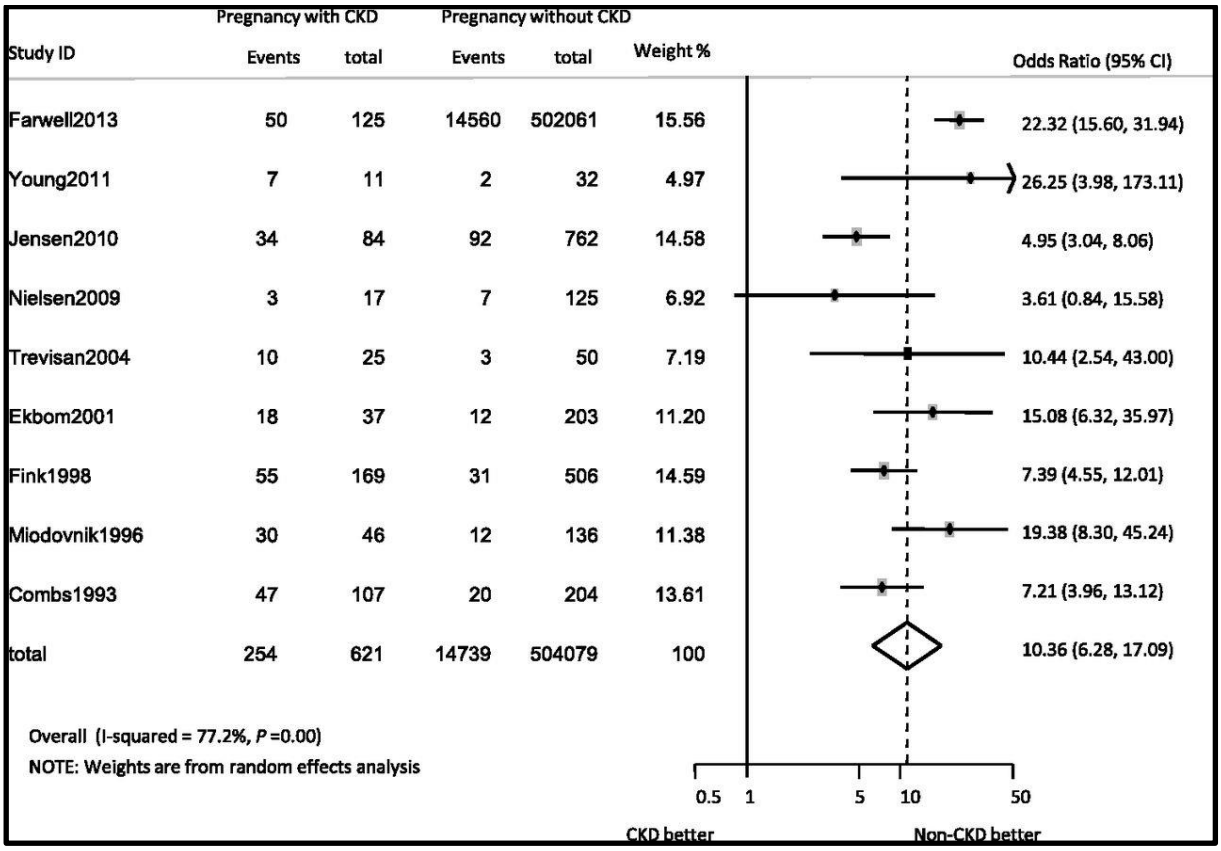
1.6.1 Diagnosis of Superimposed Pre-eclampsia in CKD

Whilst the incidence is considerably increased, the diagnosis of SPE in CKD pregnancy can be challenging and sometimes impossible [126, 160]. There is a lack of universal definition or consensus guidelines to aid diagnosis of superimposed PE in CKD. In clinical practice, diagnosis is usually made on the basis of rapidly worsening hypertension, proteinuria or renal function occurring after 20 weeks' gestation. However, hypertension, proteinuria and renal dysfunction may already be present pre-pregnancy in women with CKD and worsen due to increased physiological demands on the kidney as pregnancy progresses [126, 137]. The distinction is important as PE will only resolve following delivery, whereas worsening of underlying renal disease may stabilise with supportive care, without need for immediate delivery, allowing pregnancy and further fetal maturation in utero to continue for longer. Furthermore, as noted earlier, PE may be associated with more frequent adverse outcome in CKD which would favour earlier delivery [43, 137].

Maternal clinical risk factors are estimated to predict just 30% of women in the general population who go on to develop PE [161]. Data as to whether the same clinical factors are as, or more or less, predictive for SPE developing in CKD are lacking. In recent years, there has been increasing interest in identifying diagnostic and/or predictive biomarkers for PE [31]. Similar data for the use of biomarkers to aid diagnosis of SPE in CKD pregnancies is scarce. Predictive and/or diagnostic biomarkers could potentially allow recognition of high-risk pregnancies, timely diagnosis and early intervention to minimise the risk of adverse maternal and fetal outcomes. Being able to reliably exclude PE is also important and identifying 'low risk' pregnancies, unlikely to develop PE, would provide reassurance and

potentially allow less intensive antenatal monitoring. Additionally, studying disease biomarkers facilitates a greater understanding of the pathophysiological mechanisms of PE, including insights into why the risk is increased in CKD and may indicate potential therapeutic targets for further study.

Figure 1-7: Forest plot showing risk of developing Pre-eclampsia in CKD versus non-CKD pregnancy



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1.7 Background and Rationale for Serum Markers Analysed in Thesis

1.7.1 Immunoglobulins in General Health

Serum antibody levels in PE have received little attention yet could potentially provide important insight into underlying disturbances in adaptive humoral immunity. Antibody is secreted from millions of different clonal expansions of plasma cells, with each clone directed against a specific and different molecular target (antigen), that is ideally a microbial pathogen or, adversely, a self-antigen (autoimmunity) or a fetal antigen such as rhesus [162]. Whole antibody is made of two identical immunoglobulin (Ig) heavy chains and two identical immunoglobulin light chains. The heavy chains (HC) are usually of IgM, IgG or IgA isotype. IgM and IgA have serum half-lives of 5 and 6 days respectively. IgG's half-life is prolonged to 23 days by recycling via the neonatal Fc receptor (FcRn) that also transports maternal IgG across the placenta in the last trimester [162-169].

In a new antibody response, low affinity IgM initially dominates followed by higher affinity IgG, or IgA if the response is against antigens from the mucosal surfaces. IgG is the most abundant of the immunoglobulins and the only isotype to cross the placenta in pregnancy [170]. Circulating IgD and IgE are found in very low concentrations with short half-lives. The function of IgD is not very clear and IgE is involved in defence against parasites and is involved in type 1 hypersensitivity (allergic reactions). IgG can be further divided into 4 subclasses IgG1-IgG4. The functional capabilities of the different isotypes and subclasses vary as illustrated in Table 1-2, for instance their ability to trigger antibody-dependent cell mediated cytotoxicity or activate complement [162, 171]. IgG1 and IgG3 are the strongest

complement activators and both respond to protein antigens rather than polysaccharide antigens which are recognised by IgG2 and IgG4.

Table 1-2: Immunoglobulin structure, function and relative proportion, adapted from reference [162]

	Proportion in Sera	Structure	Complement Fixation	Opsonizing	Cross Placenta	Other Functions
IgG	75 %	Monomer	+	+++	+	Secondary response, neutralise toxins and viruses
IgG1	67% (IgG)	Monomer	Yes	Yes	+	
IgG2	22% (IgG)	Monomer	Yes	Yes	+	
IgG3	7% (IgG)	Monomer	Yes	Yes	+	
IgG4	4% (IgG)	Monomer	No	No	+	
IgM	10%	Pentamer	+++	+++	-	Primary response
IgA	15%	Monomer and Dimer	-	-	-	Mucosal response
IgD	<0.5%	Monomer	-	-	-	Homeostasis
IgE	<0.01%	Monomer	-	-	-	Allergy

1.7.2 Immunoglobulins in Normal Pregnancy and in Pre-eclampsia

Intact Immunoglobulin levels change in healthy pregnancy, with reduction in IgG concentrations as pregnancy progresses; whereas conflicting trends for IgA and IgM levels have been reported [172-174]. Pregnancy has also been shown in one study to be characterised by B-cell lymphopenia followed by a recovery of B cells in the post-partum period [175, 176]. Limited and now mostly historic studies which examined serum antibody levels in PE compared to healthy pregnancies, have also reported a difference (reduction) in IgG concentrations in PE, whereas findings for IgA and IgM levels have again been more variable [177-183]. These studies have often included women with gestational hypertension in the PE group, comprised small patient numbers and relationship of circulating Ig

concentration to proteinuria, which can lead to renal loss of immunoglobulins, was not adequately described.

1.7.3 Serum Free Light Chains in Health and Disease

The light chain component of the immunoglobulin molecule also circulates, unbound from the intact Ig molecule, as serum free light chains (sFLC). There are two light chain isotypes present, kappa (κ) and lambda (λ), with each B cell and its progeny producing just one type. The measurement of sFLC using an automated assay is now well established in clinical practice to help diagnose and monitor multiple myeloma and other monoclonal gammopathies [184-187].

Serum free light chains have a half-life of just a few hours as they are filtered at the renal glomerulus and catabolised by the renal tubules [186]. Serum concentration of FLCs is milligrams/l, compared to grams/l for whole antibody, and increase several fold if glomerular filtration is severely reduced [188]. Because their half-life is just a few hours, compared to days to weeks for whole antibody, sFLC levels may be used to identify acute changes in antibody secretion if glomerular filtration rate is stable. A polyclonal increase in combined FLC (cFLC; sum of κ and λ isotypes) occurs when there is increased production of multiple immunoglobulins or reduced renal clearance, which should not be associated with a major change in the κ/λ ratio as the increase in sFLCs is non-clonal [186, 188-190]. Stimulation of the immune system and B-cell activation, for instance as a result of infection, inflammation or autoimmune disease, is characterised with polyclonal increase in sFLC levels.

Previous studies have reported the use of raised sFLC levels as a marker of disease activity in various inflammatory conditions such as such as rheumatoid arthritis, Systemic Lupus Erythematous (SLE) and Chronic Obstructive Pulmonary Disease (COPD) [190-193]. Furthermore, FLCs have been associated with a number of biological mechanisms which, as discussed in this chapter, have been implicated in the pathology of PE, for instance anti-angiogenic activity and activation of the complement system [194-196]. Increased sFLC levels have also been linked to future risk of CVD and development of CKD, as well as its progression to renal replacement therapy; all of which are recognised as long-term complications following PE [17, 18, 36, 197, 198]. These overlapping features with PE may thus allow sFLC to act as a biomarker for the diagnosis and/or prediction of PE. In addition, measurement of IgG subclasses, with their known individual functional effects, has not been previously reported in PE. It may aid a more detailed assessment of humoral immunity in PE and may assist in unraveling its pathophysiology.

1.7.4 Routine Clinical Laboratory Biomarkers Associated with PE

Pro-inflammatory markers, as described earlier in this chapter, have been hypothesised to play a role in the pathophysiology of PE [199]. **C-reactive protein (CRP)** forms an important component of the innate immune system and works with complement components to assist in the removal of foreign pathogens by binding to phosphocholine on dying cells and pathogens [60, 199-201]. It is a classic acute phase reactant and is produced mainly in the liver in response to inflammatory stimuli. Concentrations of CRP increase with inflammatory states [201] and CRP blood levels are used in clinical practice to monitor infectious and inflammatory diseases [200]. It acts as a scavenger protein in response to a range of

molecules or inflammatory stimuli and binds to a number of intrinsic and extrinsic ligands present on damaged cell membranes and micro-organisms respectively [60]. Ten plus fold acute elevations in CRP are associated with extensive tissue damage or bacterial sepsis and this transient response is beneficial. However, lower but sustained elevations in CRP, measured using high-sensitivity CRP assays (hs-CRP), are deemed to be harmful and associated with tissue damage. For instance, a chronic mildly elevated CRP is associated with increased long term risk of CVD and the metabolic syndrome, which are also potential sequelae of PE [36, 202, 203]..

Inflammatory makers, including CRP, are also elevated in pregnancy compared with the non-pregnant state [49]. Amniotic fluid and placental production of CRP in pregnancy has also been described [204, 205]. A number of studies have demonstrated that CRP is increased further in PE compared to women with healthy pregnancies [49, 200, 206-208] and is correlated with PE severity as well as other adverse pregnancy outcomes [49, 71, 200]. CRP injected into mice has been shown to result in hypertension and glomerular damage and promote release of sFLT-1 [49, 199]. However, confounding factors, such as body mass index (BMI), have been shown by many to impact on the association between CRP levels and PE [200]. Also, the value of CRP as a predictive marker in early pregnancy has not been well studied, especially in the context of SPE.

A reduction in **serum albumin** is another marker of the acute phase response and may also be an indicator of protein loss resulting from PE induced glomerular endotheliosis. However, a reduction in serum albumin levels is reported to occur in the second and third trimester of healthy pregnancy, which is hypothesised to be related to increased plasma and

extravascular volume associated with pregnancy, rather than a change in capillary permeability [209-211].

The complement system is an essential constituent of both the innate and adaptive immune system and comprises over 30 proteins [212]. There are three different pathways that can trigger the complement system; these are the classical, lectin and alternative pathways. Moreover, both **C3 and C4** complement components are, like CRP, acute phase proteins [201]. C3 activation is the critical first step in producing functionally active complement components for all three complement pathways. C4 is key in initiating the lectin pathway (includes CRP) and classical pathway (antibody mediated), and C4 levels fall as it is consumed when these pathways are active [213]. At the time of PE , decreased serum C3 and C4 concentrations have been reported in the maternal circulation, together with an increase in the serum C3a/C3 ratio, C5a and sC5b-9 levels, supporting activation of the complement cascade at the time of disease [81, 102, 183, 214-226]. Heterozygous mutations in complement regulatory genes were, in one prospective study, reported to be present in 18% of women who subsequently developed PE [227].

Beta 2 microglobulin (B2-M) forms part of the FcRn receptor which is necessary for IgG recycling and placental transfer [164, 228]. It also forms part of the MHC I molecule which inhibits NK cell activation and presents cellular and viral peptides to CD8 cytotoxic T cells. Serum levels of B2-M are increased in inflammatory states connected with activation of the lymphoid system, autoimmune disease, infection, malignancy, proliferative disorders and have also been shown to be increased in women at the time of PE [229-235]. However, there are discordant findings in studies as to whether it can act as a predictive marker of PE

[9, 234-236] and limited data for SPE specifically. It's ability to act as a marker of renal function is discussed in the next section, and therefore concentrations of B2-M may reflect multiple disease pathways associated with PE.

1.7.5 Comparison with Ethnicity and BMI

As has been described earlier in this chapter, both ethnicity and obesity are known risk factors for the development of PE. They are also recognised to be associated with variations in immune responses. Obesity is associated with chronic systemic inflammation and obese individuals have an increased susceptibility to bacterial and viral infections [60, 237]. The precise mechanisms which cause impaired immune responses in obesity are not known. A number of studies have demonstrated that obesity is characterised by dysfunction of macrophage, NK and dendritic cells, in addition to weakened antigen and cytokine production. Variations in immune responses amongst different ethnic groups have also been demonstrated. For instance, black individuals have higher circulating B cells, activated T cells and IgG levels compared to White individuals, in keeping with increased B cell activity in these individuals [238-240]. Furthermore, there is racial and ethnic variation in pre-disposition to certain autoimmune conditions for example Multiple Sclerosis occurs most commonly in individuals from a White European background compared to other ethnic groups [241]. I have therefore considered in the results chapters the impact of ethnicity and BMI of the patient when exploring the relationship of the immune system markers to, or to the subsequent development of, PE.

1.7.6 Immune Dysfunction in Chronic Kidney Disease

It is possible that pathophysiological mechanisms differ in SPE occurring in CKD pregnancy compared to PE in healthy pregnant women. For instance it has been suggested that the inability of the kidneys to adapt physiologically in women with CKD may compromise placental blood flow which may predispose to the development of PE [92, 137]. It is nevertheless increasingly recognised that the immune response is impaired in CKD, with an underlying pro-inflammatory state. Thus, if maternal immune intolerance plays a role in the development of PE, it may be possible that the relationship between CKD and PE is related to this [242-245]. Some of the described immune features in individuals with CKD include reduced B and CD4 T cell populations, reduced T-cell response to antigen stimulation, reduced neutrophil function and reduced phagocytosis in individuals with ESRD. There is conflicting data as to whether there is a shift towards a Th1 or Th2 phenotype in CKD. A comparison of the immune response in CKD and healthy pregnancy in the context of development of PE will therefore to provide insight into this understudied area.

1.7.7 Markers of Renal Function in Pregnancy and Pre-eclampsia

As previously discussed, the kidney plays an important role in the normal physiological adaptation of pregnancy and renal impairment is a common and characteristic feature of PE. Thus, early detection in changes in renal function, including in women with CKD, could allow for timely and accurate diagnosis or prediction of PE. However, it is not well established which are the best biomarkers to assess renal function during pregnancy, and even less data in CKD pregnancy. The MDRD equation and CKD-EPI formulae are not reliable for estimating GFR in pregnancy as these underestimate GFR as measured by inulin clearance the gold

standard method of measuring GFR [137, 246, 247]. Similarly, the Cockcroft and Gault equation which is based upon weight and estimates creatinine clearance is also unreliable to in pregnancy as it tends to overestimate true GFR. Creatinine clearance does correlate with inulin clearance; however, this requires 24-hour urine collections and timed blood samples. Thus, it is not practical to undertake in routine clinical practice. At present serum creatinine is used by most clinicians in clinical practice as a measure of renal function during pregnancy. The limitations of serum creatinine as a marker of kidney function are recognised both outside and during pregnancy. Glomerular filtration rate can be reduced by 50% in non-pregnant individuals, before a rise in serum creatinine is observed. As discussed earlier, physiological adaptations in pregnancy, including glomerular hyperfiltration, result in a reduction in concentration of creatinine in women with normal or mildly impaired kidney function. Therefore, changes in serum creatinine concentrations during pregnancy may be related to physiological changes and not be easily interpretable.

Uric acid (UA) is an end product of purine metabolism and circulating concentrations depend on dietary intake, endogenous production and elimination, two-thirds of which is by the kidney [248]. It is freely filtered through the glomerular membrane and both reabsorbed and secreted by tubular cells [209]. Serum levels of UA decrease in the first half of pregnancy and then stabilise, increasing towards pre-pregnancy levels by the end of pregnancy. The early reduction in UA levels is due to plasma volume expansion, as well as increased renal blood flow and GFR leading to increased uricosuria [249, 250]. Women with impaired kidney function will be expected to have elevated UA levels during pregnancy due to reduced renal clearance [209].

Levels of UA are increased at the time of PE and correlate with disease severity [248-251]. It has nevertheless failed to perform as sensitive predictive marker for development of disease. It has been historically viewed that the increase of UA levels seen in PE is due to reduced renal clearance. However, more recent studies suggest that levels maybe increased due to other factors such as trophoblast breakdown, cytokine release and ischaemia [249, 252]. Moreover, it has also been proposed that UA may play a pathogenic role in PE through its ability to promote inflammation, oxidative stress and endothelial dysfunction [250, 252]. In the non-pregnant population elevated UA levels are associated with hypertension, metabolic syndrome, CKD and CVD which are all associated with PE, either as risk factors and/or long-term sequelae [17-19, 36, 251].

Both cystatin-C and B2-M are endogenous markers which are freely filtered by the glomerulus, then reabsorbed and catabolised by the proximal tubular cells, so both are markers of kidney function [235]. They are middle sized molecules (10-30 kDa) compared to creatinine (113 Da) which is a small molecule. Cystatin-C acts as an inhibitor of cysteine protease preventing the breakdown of extracellular proteins. Its concentration is independent of body weight, age, muscle mass, exogenous products and other pregnancy related changes and it has been suggested that cystatin-C reflects the GFR more accurately (outside pregnancy) and is a better marker of kidney function than serum creatinine, especially in individuals with small to moderate decreases in GFR [209, 235, 253]. Nevertheless, cystatin-C based equations have been shown not to correlate with inulin clearance in pregnancy [130].

In contrast to serum creatinine levels are which are lower throughout pregnancy compared to pre-pregnancy levels, concentrations of cystatin-C and B2-M levels do not decrease in early pregnancy and are reported to increase in the third trimester of healthy pregnancy [209]. As these former molecules are middle-sized GFR markers compared to the smaller weight molecule creatinine, it has been suggested that there is a reduction in middle molecule clearance in the third trimester, whereas increased filtration of low molecular weight molecules occurs throughout pregnancy. The decrease in filtration of these medium-sized molecules in the third trimester has been proposed to be related to a decrease in the glomerular pore size or the number of pores, or structural changes in the glomerular barrier arising from the number of anionic sites in the glomerular barrier.

The increase in cystatin-C levels in late pregnancy has been reported to be more pronounced in pregnancies complicated by PE. It has been suggested that this is related to increased proximal tubular reabsorption [9, 233]. Both cystatin-C and B-2M levels have been proposed as better markers of renal impairment compared to UA and serum creatinine levels. However the ability of cystatin-C to act as a predictive marker in PE has yielded conflicting results, which, as discussed earlier, is also the case with B2-M [9, 209, 234-236, 253]. Data on how B2-M and cystatin-C compare with creatinine levels for monitoring renal function in pregnancy in CKD is lacking. In this thesis, creatinine, B2-M, cystatin-C and UA levels were measured in all three results chapters of this thesis.

1.7.8 The sFLT-1/PIGF Ratio in Pre-eclampsia

1.7.8.1 Prediction of Pre-eclampsia in Healthy Women

In normal pregnancy, maternal levels of sFLT-1 increase with gestation. Whilst circulating levels of PIGF also increase, levels subsequently fall from the middle of the third trimester onwards [254]. An increasing number of studies have demonstrated that from 30-40 weeks' gestation, PE is characterised by an increase in circulating levels of the anti-angiogenic factor sFLT-1 and decreased levels of the pro-angiogenic PIGF; resulting in an increased sFLT-1/PIGF ratio [15, 30, 33, 91, 100, 255-264] . These changes have been detected 6-10 weeks prior to the clinical onset of PE and even earlier in those that develop early onset disease. The sFLT-1/PIGF ratio is used as a measure of antiangiogenic activity and is a better predictor of disease than either biomarker individually [15, 43]. Figure 1-8 illustrates that the sFLT-1/PIGF ratio increases weeks before clinical manifestation of PE [100]. The levels of PIGF in women who go onto develop PE start to decrease from as early as 11 weeks in those that develop early onset PE [265, 266], whereas levels of sFLT-1 increase from 15 weeks' gestation [260]. The difference in sFLT-1/PIGF ratio is more marked in those with early onset PE or if disease is complicated by FGR, with sensitivities and specificities of over 90% reported for early onset disease [5, 15, 258, 267]. In a meta-analysis assessing the predictive accuracy of PIGF and sFLT-1, measurements of these markers before 30 weeks' gestation were too poor to be deemed useful in clinical practice [94]. Nevertheless, in 2016 NICE issued clinical guidelines supporting the use of the PIGF based testing (including Elecsys immunoassay sFLT-1/PIGF ratio or Triage PIGF test) alongside clinical assessment to help exclude PE in women presenting with suspected PE between 20 and 34+ 6 weeks' gestation [27, 268]. Furthermore, a pilot study has demonstrated that removal of sFLT-1 in women with PE,

through extracorporeal aphersis, results in reduced proteinuria and improvement in blood pressure control, potentially allowing for prolongation of pregnancy [48].

The NICE guidelines followed the publication of a large multicentre prospective study, which demonstrated that a sFLT-1/PIGF ratio (measured by Elecsys immunoassay) of 38 or lower can be used to predict the short-term absence of PE where clinically suspected [91]. This study included 1050 women with singleton pregnancies presenting with suspected PE at 24-37 (median 32) weeks' gestation. A sFLT-1/PIGF ratio of ≤38 was deemed most accurate to predict absence of PE within 1 week and a ratio of > 38 was best for predicting PE developing within four weeks from assessment. In a separate validation cohort, a ratio of ≤38 provided a high negative predictive value (99.3%, 95% CI 97.9 – 99.9%) in identifying women who are unlikely to progress to PE within the following week. A low ratio was also associated with a low likelihood of adverse fetal outcome. The positive predictive value for those with a 'high' ratio for development of PE in the next 4 weeks, was much lower at 36.7% (95% CI 28.4-45.7%). On post-hoc analysis the sFLT-1/PIGF ratio still provided a higher positive predictive value than clinical factors alone.

An elevated sFLT-1-1/PIGF ratio has been shown in other studies to predict the occurrence of adverse outcome and imminent delivery in PE, as well as being associated with other placental disorders such as FGR and stillbirth [5, 18, 33, 258, 269-274]. However, as noted earlier, neither angiogenic marker alone has sufficient predictive accuracy to be used in clinical practice. It has been suggested they may be better at predicting early onset, overt disease [160, 262]. Moreover, there is an overlap of the sFLT-1/PIGF values between women who develop PE with those who do not, leading to uncertainty in clinical practice regarding

its ability to distinguish between the two groups [275]. The predictive accuracy of the sFLT-1/PIGF ratio declines after 34 weeks' gestation, related to physiological changes in concentrations of both markers, resulting in an increase in the ratio towards the end of normal pregnancy [5]. This has been demonstrated in a study showing a five-fold increase in false positive rates when comparing the predictive accuracy at 31-44 versus 35-37 weeks' gestation. Normal gestation-specific ranges, using the automated Elecsys sFLT-1 and PIGF assay, have been determined [259, 276, 277].

Figure 1-8: The sFLT-1/PIGF Ratio in the weeks prior to the onset of clinical signs and symptoms of Pre-eclampsia

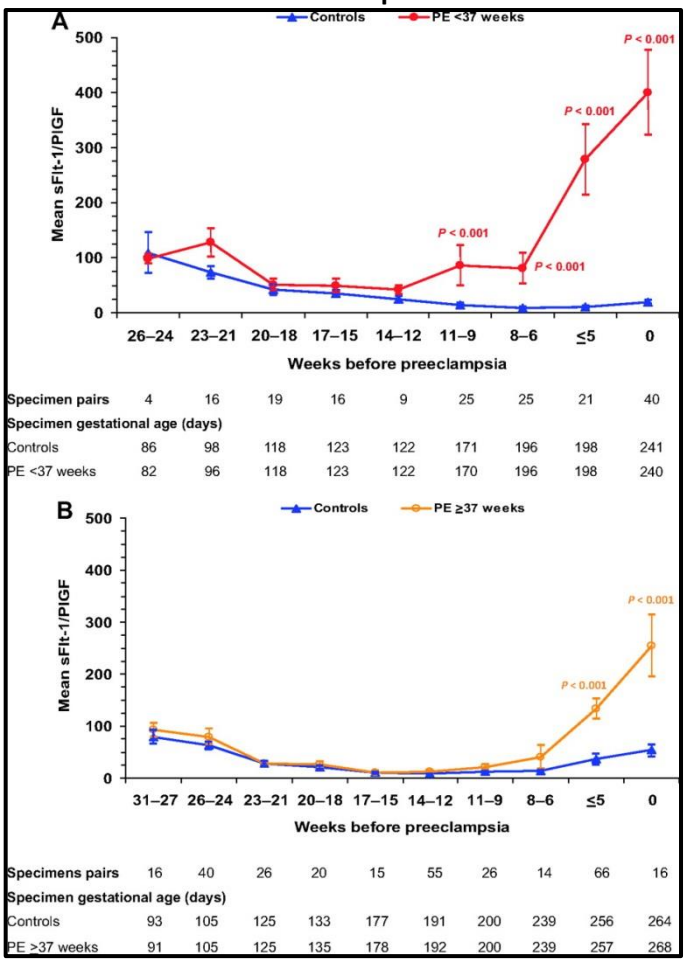


Figure taken from reference [15] with permission to reproduce obtained from copyright.com (confirmation number 11812318).

Most studies on changes in sFLT-1 and PIGF in PE have been cross-sectional in design and have not examined longitudinal changes in angiogenic makers in women who go on to develop PE [260]. Since the levels of angiogenic factors change with gestation in normal pregnancy, it has been suggested that longitudinal changes in concentrations may be better at predicting PE compared to single measurements, especially in early onset PE [15, 98, 260, 278]. Vatten et al. have demonstrated that a low PIGF and high sFLT-1, with an increase in ratio, between the first to second trimester is a strong predictor for subsequent development of PE [279]. These findings have been supported by other studies showing that the trend or sequential change in angiogenic marker levels, rather than isolated measurements, maybe more useful to predict disease and differentiate PE from non-PE pregnancies [260, 280-282].

1.7.8.2 High Risk Pregnancies

Most of the earlier mentioned studies have investigated the predictive capability of sFLT-1 and PIGF for PE in the healthy pregnant women. There are limited studies which have examined pregnant women with pre-disposing risk factors, such as chronic hypertension, CKD, diabetes or obesity and data are also lacking for SPE [281, 283]. A longitudinal study which screened women with at least 1 major risk factor for PE, found that the sFLT-1/PIGF ratio was highly predictive for early onset PE from 22-26 weeks onwards, with an area under the ROC curve (AUROC) of 0.97 [281]. For women developing either early or late onset PE, the levels of sFLT-1 were found to increase from 31 weeks onwards and, a rapid rise of the sFLT-1/PIGF ratio during an individual pregnancy was demonstrated to be predictive of PE. However, this was a small study with PE developing in only 12 patients and only 7 out of 94

participants had CKD. Nevertheless, these results suggested that high predictive values maybe achieved by targeting antenatal screening at high risk populations.

Verlohren et al. has previously demonstrated that the sFLT-1/PIGF ratio is able to discriminate between PE and other hypertensive disease of pregnancy (chronic or gestational hypertension) [258]. However, there is published literature to suggest that the switch to an anti-angiogenic profile is less marked in SPE, which usually develops on a background of chronic hypertension, compared to PE developing in a previously healthy population [283, 284]. In a study carried out in Brazil by Costa et al. SPE developing in women with chronic hypertension was characterised by a higher sFLT-1/PIGF ratio only at 32 weeks' gestation, when compared to hypertensive women who did not develop PE (9.98 vs. 2.51, p = 0.039)[283]. In contrast, in women without chronic hypertension, development of PE was associated with an increase in sFLT-1/PIGF at 26, 32 and 36 gestational weeks. Perni et al. in another longitudinal study followed 109 pregnant women with chronic hypertension [285]. They reported that, whilst changes in angiogenic factors were seen before and at the time of disease for early onset SPE, changes in the sFLT-1/PIGF ratio only occurred at the time of diagnosis in women with late onset SPE, compared to women who did not develop PE.

1.7.8.3 Superimposed Pre-eclampsia in CKD

There are only a handful of studies which have studied the sFLT-1/PIGF ratio specifically in CKD pregnancies and these have mostly been comprised of small patient numbers [43, 92, 139, 160, 286]. Masuyama et al. in 2006 in a Japanese study of 30 pregnant women with CKD secondary to chronic glomerulonephritis, demonstrated that those with SPE had higher sFLT-

1 and lower PIGF levels with a higher sFLT-1/PIGF ratio compared to women in the CKD group that did not develop SPE [286]. Samples were taken at the time of diagnosis or towards the end of pregnancy respectively. Similar results were reported by the same authors in a follow up study in 2012, with the CKD cohort this time comprising 90 women [92]. Diagnostic criteria in these studies for SPE was, the same as defined in the non-CKD population, new onset hypertension and proteinuria [25].

Rolfo et al. in their Italian cross-sectional study published in 2013, compared 23 patients with CKD during healthy pregnancy, 34 women with PE (without CKD) and 38 healthy pregnant women [43]. Similar to the previously described Japanese study [92], they found no difference in the sFLT-1/PIGF ratio in pregnant women with CKD and without PE compared to healthy pregnant controls without CKD. The median ratio observed in the PE group, controls and CKD group without PE was 436, 9.4 and 4.0 respectively. Samples were taken in the third trimester and, whilst women in the CKD group were deemed not to have PE, only those with hypertension and proteinuria at presentation were recruited. A follow up prospective study by the same researchers, reported a higher sFLT-1/PIGF ratio in 24 women with CKD and PE, without pre-existing proteinuria and hypertension, compared to 35 pregnant women with CKD, who did not develop proteinuria or hypertension during pregnancy [160]. Single measurements were taken in the second or third trimester. A sFLT-1/PIGF ratio of $\geq$ 32.8 provided a sensitivity of 82.9% and specificity of 91.4 % for diagnosing PE in this study .

Most recently Bramham et al. in a longitudinal study (2016) of a UK cohort, comprising 121 pregnant women with CKD and 44 with chronic hypertension, reported lower PIGF

concentrations from 24 weeks until 42 weeks' gestation in women destined to develop SPE (24% of cohort) [139]. The AUROC for PIGF concentration and PE prediction was 0.85, and 0.82 when validated on a separate cohort. The Triage test was used to measure PIGF concentrations in this study, whereas the Elecsys assay was used in the CKD studies mentioned previously. The definition of SPE in this paper was broader compared to other studies, with evidence of other organ dysfunction (abdominal pain, abnormal liver function tests, thrombocytopenia, pulmonary oedema or neurological/visual symptoms) used in place of hypertension or proteinuria, if one of these features pre-existed. If both hypertension and proteinuria were already present, the definition of SPE required severe hypertension (systolic $\geq$ 160 or diastolic $\geq$ 110 mmHg). The overall PIGF concentration during pregnancy in the longitudinal cohort was not different in the women, with pre-existing chronic hypertension or CKD, who developed SPE compared to women, without pre-existing disease, who developed PE. Additionally, a low PIGF concentration was shown to have a high diagnostic accuracy for SPE requiring delivery within 14 days, with an AUROC of 0.85, and a value of PIGF less than the fifth centile was found to have the best diagnostic performance. The same study also looked at other biomarkers, including renal markers (neutrophil gelantinase-associated lipocalin [NGAL] and relaxin) and cardiac markers (B-type Natriuretic peptide [BNP]); none of these were predictive of SPE in the longitudinal cohort. In a separate validation cohort, plasma samples were taken at the time of suspected PE, between 20 and 37 weeks' gestation, and these were tested for PIGF, and sFLT -1 using the Triage test. This separate cohort comprised 29 women with CKD, 94 with chronic hypertension and 366 women without pre-existing illness. Women who developed SPE, requiring delivery within 14 days, had lower PIGF concentrations and higher sFLT-1 and sFLT-1/PIGF ratios compared to

women with chronic hypertension and CKD who did not develop SPE. The sFLT-1/PIGF ratio was similar in all three groups that developed PE, as was the case in the three groups that did not develop PE. The authors found that the diagnostic performance of low PIGF concentration in isolation to predict SPE, requiring delivery within 14 days, was similar to that provided by sFLT-1/PIGF ratio.

1.7.8.4 Relationship of Angiogenic Markers with Renal Function

The findings of an imbalance in circulating angiogenic factors, similar to that reported in the general pregnant population, would support the concept that abnormal placentation also occurs in SPE in CKD pregnancies. However, it is unclear whether abnormal kidney function may pre-dispose to an abnormal angiogenic profile, and, in part, explain why the risk of PE increases with increasing CKD severity. Concentrations of circulating PIGF and sFLT-1 have been demonstrated to be raised in the non-pregnant CKD population, with the latter having an impact on endothelial dysfunction and atherosclerosis. Increased PIGF is associated with an increased risk of mortality and CVD in the CKD population [287-290]. Women with CKD may, therefore, be predisposed to developing PE due to pre-existing imbalance in their angiogenic factor profile [92]. As was discussed earlier in this chapter, it's also conceivable that a lower threshold of angiogenic imbalance is required to trigger endothelial dysfunction in pregnant women with pre-existing CVD and possibly CKD.

Rolfo et al. reported lower median PIGF concentrations in pregnant women with CKD (270 pg/mL) compared to the normal reference range in pregnancy (439 pg/mL) [160]. However, the measurements covered a range of gestations and, as described earlier, levels of PIGF change as pregnancy progresses, with levels increasing during the first two trimesters and

then falling as pregnancy reaches term. Interestingly, Masuyama et al. reported a lower sFLT-1 (median 3220 versus 9122 pg/mL) and higher PIGF concentration (median 222.5 versus 98.3 pg/mL) in SPE associated with worsening kidney function compared to SPE without worsening kidney function in their CKD cohort. In the study by Bramham et al. no relationship was found between concentrations of creatinine and PIGF, and correlations between creatinine and sFLT-1 levels were not reported. Data regarding the relationship of kidney function to angiogenic factors in pregnancy and PE appear to be inconsistent and, therefore, require further investigation.

In summary, it is not established whether the sFLT-1/PIGF ratio can predict adverse obstetric outcome in CKD pregnancies as has been demonstrated in the general pregnant population [291-293]. If a relationship between baseline renal function and concentrations of circulating angiogenic factors exists, this may influence their utility as a biomarker of adverse outcome in CKD pregnancies.

1.8 Ultrasound Screening and Prediction Models in Pre-eclampsia

1.8.1 Antenatal Doppler Ultrasonography

Placental insufficiency resulting from PE can be identified at 20 weeks' gestation through identification of vascular resistance on uterine Doppler ultrasound scan [93]. This can therefore be used in pregnancy as a screening test to help identify women at high risk of developing PE [73, 93, 294, 295]. Pulsatility index is defined as peak systolic flow minus end diastolic flow divided by mean flow. Normal pregnancy is characterized by a low pulsatility index and high end-diastolic flow. Abnormalities in the uteroplacental circulation can be detected by the presence of diastolic 'notching', representing impaired perfusion characteristic of PE. Abnormal uterine artery Doppler waveforms after 20-24 weeks' gestation reflect abnormal trophoblast invasion of the spiral arteries. These findings are indicative of a high probability of developing PE. There are often associated abnormalities in flow of the umbilical arteries [93]. Changes in measured Doppler flows through the umbilical artery suggest abnormal vasculature development and are suggestive of fetal growth compromise, as well as being predictive of adverse pregnancy outcome. However, neither ultrasound test is sensitive enough to be used as a screening test to reliably predict PE [73, 296, 297]. In those women deemed to be at higher risk of developing PE, uterine and umbilical Doppler imaging may be used to identify women at particularly high risk of adverse pregnancy outcome, who require increased antenatal surveillance and monitoring. Uterine Doppler studies have been demonstrated to be better at predicting PE in placental compared to maternal aetiologies of PE [29].

There is less literature published on the use of Doppler imaging in CKD pregnancy. In one prospective study, abnormal uterine Doppler imaging in 15 CKD pregnancies provided a 100% specificity for prediction of SPE development [139]. In a further retrospective study of 61 CKD pregnancies with pre-existing hypertension and proteinuria, it was demonstrated that uterine and umbilical Doppler waveforms could distinguish between superimposed PE and absence of SPE [93]. Normal waveforms of both were associated with healthy CKD pregnancy (p=0.002) and abnormal waveforms of both were associated with SPE developing (p= 0.023).

1.8.2 Prediction Models on Pre-eclampsia

Prediction of patients at high risk of developing severe PE would enable earlier identification of disease and allow for appropriate monitoring and intervention to help minimise risk to the mother and baby. Despite much research interest in the subject over recent years, none of the biomarkers studied in PE to date have shown sufficient predictive or diagnostic accuracy values to be introduced into clinical practice [11]. Over 70 prediction models for PE have been reported, these combine a number of factors such as biomarkers, maternal demographics and clinical findings, including ultrasound Doppler data [24, 298]. Predictive models for all three trimesters have been reported. A number of these have shown that the predictive accuracy of biomarkers, such as angiogenic factors, improves when combined with other data [15, 30, 264]. For example, combining the sFLT-1/PIGF ratio with blood pressure, multiparity (previous birth) and previous history of PE has been demonstrated to predict early onset PE from 20 weeks onwards, with an AUROC of 0.86, 0.91 and 0.93 at 20, 24 and 28 weeks' gestation respectively [30]. In another large multicentre study, change in

angiogenic factors (sFLT-1 and PIGF independently) between first and second trimester combined with clinical risk factors in low risk nulliparous population predicted early onset PE, with an AUROC of 0.86 and 0.84 respectively [299]. Despite the number of predictive models reported, none appear to be used in routine clinical practice and most have not undergone validation [24].

In a recently published systematic review, 68 prediction models from 70 studies with 425,125 patients were analysed [298]. The most commonly included predictors were medical history, BMI, blood pressure, parity, uterine artery pulsatility index and maternal age. The authors reported that maternal characteristics, ultrasound markers and/or biomarkers were not clearly associated with model discrimination. Only a minority of the models were internally (4%) or externally (6%) validated. In a further review on the subject, the strongest variables in prediction models of PE were found to be BMI, first trimester uterine artery indices, PIGF and placental protein 13 [300]. The authors concluded that no single marker had an adequate test performance to incorporate into routine clinical use. They also concluded that models which combined biomarkers showed promise.

Recently updated NICE guidance has suggested considering the use of the one of two validated risk prediction models for individual patient risk assessment in the setting of PE [27]. These models have undergone internal and external validation for performance. The aim of using these models is to help guide clinicians to make decisions about appropriate place of care, for instance inpatient or outpatient setting, and thresholds at which intervention should be considered. One of these is the fullPIERS model (Preeclampsia Integrated Estimate of Risk) which is based on six predictor variables - gestation of

pregnancy, presence of chest pain or dyspnoea, oxygen saturation, platelet count, serum creatinine, and serum aspartate aminotransferase concentrations [301]. On internal validation, the fullPIERS model was able to predict within 48 hours of hospital admission an adverse maternal outcome with an AUROC of 0.88 (95% CI, 0.84–0.92).

The other model is the Prediction of Risks in Early onset Pre-eclampsia (PREP) model which can be used up to 34 weeks of pregnancy and provides individualised risk prediction of complications in early onset pre-eclampsia for overall risk and by 48 hours [302]. The PREP-S model includes maternal age, gestational age, medical history, systolic blood pressure, deep tendon reflexes, uPCR, platelet count, serum alanine amino transaminase, urea, creatinine, oxygen saturation and treatment with antihypertensives or magnesium sulphate. On internal validation, the AUROC for predicting complications by 48 hours and by discharge were 0.84 (95% CI, 0.81-0.87). Predictive models specific to development of SPE in the CKD population are lacking. It has been suggested that predictive models may provide greater predictive capability in high risk pregnancies [15].

1.9 Thesis Aims

Chapter Three: Humoral Immunity in Pre-Eclampsia and Linkage with Angiogenic and Inflammatory Markers

- To compare serum levels of intact immunoglobulin G, A and M, IgG subclasses 1-4 and free immunoglobulin light chains (sFLC) between women with Pre-eclampsia (PE) and healthy pregnant women at term.
- To correlate the above with levels of other serum proteins routinely measured in clinical laboratory practice and that have previously had some indication of association with PE. These are levels of C Reactive Protein down to the lower limits of the normal range (hs-CRP), beta2 microglobulin (B2-M), uric acid (UA), albumin and complement proteins (C3 and C4). Plus the anti-angiogenic profile by measuring levels of soluble fms-like tyrosine kinase-1 (sFLT-1) and the pro-angiogenic placental growth factor (PIGF) to derive the sFLT-1/PIGF ratio.
- Correlate differences in levels of the above proteins with renal function measured by serum creatinine and cystatin-C.
- To assess whether humoral system markers provide independent association with PE after control for angiogenic factors, inflammation and renal dysfunction.

Chapter Four: Predictive Biomarkers in Early Pregnancy for Development of Pre-eclampsia

To determine whether healthy women who developed PE in their pregnancy have any differences in the clinical markers assessed in chapter three - Igs, IgG subclasses, albumin, hs-CRP, B2-M, UA, cystatin-C and creatinine - in early pregnancy (first trimester) compared to matched healthy pregnant women who do not develop PE.

Chapter Five: Biomarkers Predictive of Pre-eclampsia in Chronic Kidney Disease

To make serial measurements of circulating markers of immunity, inflammation, renal function and angiogenic factors, studied in chapter three, in pregnant women with CKD in order to explore:

- clinical risk factors for developing SPE and other adverse pregnancy outcomes in CKD pregnancies
- whether the sFLT-1/PIGF ratio, clinical markers of the immune system, including humoral, and inflammation can predict SPE and other adverse pregnancy outcomes in CKD
- the impact of renal function on concentrations of angiogenic markers in pregnancy in the context of CKD
- which markers of renal function are most predictive of adverse pregnancy outcomes, including SPE, in CKD.

2 MATERIALS AND METHODS

This chapter covers methodology for the result chapters in the thesis (chapters three-five). Chapter three has also been published as a scientific paper in a peer reviewed journal [303] (Appendix 1). The sample analysis was similar for all three of these chapters and so this section has been combined.

2.1 Ethical Approval

Ethical approval for all research work undertaken in this thesis was provided by the University of Birmingham Human Biomaterials Resource Centre (HBRC) (reference:15/NW/0079: date of approval granted on the 21/01/2014) with the University of Birmingham (RG_14-023) acting as the sponsor (Appendix 2). All patient recruitment was undertaken at Birmingham Women's Hospital (BWH), a tertiary obstetric centre in the West Midlands, UK. Written informed consent was obtained and patients were recruited individually into the studies in chapter three and five (Appendix 3: patient information sheet and Appendix 4: patient consent form). For the chapter four study, individual patient consent was not required on the basis that the study samples were due to be discarded as they were no longer required for clinical use and that they would be anonymised at the time of analysis. Patient identifiable features associated with samples for all three studies were strictly restricted to the HBRC staff, and not recognisable to myself or others involved in analysis of sample material.

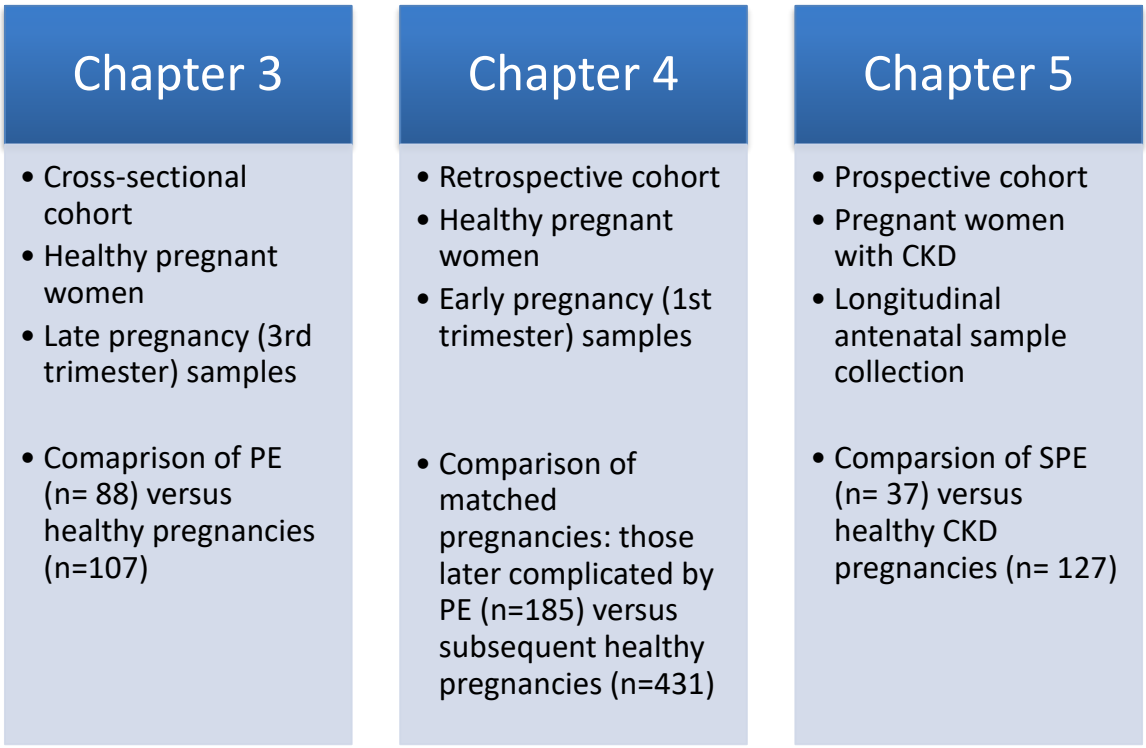
2.2 Study Design

A summary of the study designs in chapters three-five is illustrated as Figure 2-1. Each study chapter will now be discussed separately.

2.2.1 Chapter Three

This study compared previously healthy women admitted to hospital with PE to healthy pregnant women at term without PE. Cases were enrolled at the time of admission to hospital with PE and controls were recruited close to their elective caesarean section delivery date. Healthy women without prior significant co-morbid illness, including pre-existing diabetes, CKD or autoimmune illness, were enrolled as defined in the inclusion and exclusion criteria below. Women with non-singleton pregnancies and a history of chronic hypertension were enrolled into the study to facilitate achieving our target recruitment goal, which was 100 patients per group, within the study time frame. Following written, informed consent, antenatal serum samples were collected from cases and controls as later described. The diagnosis of PE was on the basis of new onset hypertension and proteinuria (BP ≥140/90 mmHg and urine protein to creatinine ratio (PCR) > 30 mg/mmol). More atypical presentations of PE outside the above criteria were excluded in order to allow analysis of a more homogenous group of patients and presentations. Controls were recruited as patients for whom an elective caesarean section was planned, this was for non-medical indications such as personal preference, breach pregnancy or previous deliveries by caesarean section. Patients were recruited over an eight-month period from February-September 2014.

Figure 2-1: Summary of study designs in chapters 3,4 and 5 of Thesis



2.2.1.1 Inclusion and Exclusion Criteria:

- Inclusion criteria:
    - Admission to BWH with a diagnosis of PE defined in text (new onset hypertension and proteinuria) - *cases*
    - Booked in for elective caesarean section for non-medical indications and attending pre-operative assessment clinic within 5 days of delivery – *controls*
- Exclusion criteria:
    - Atypical presentations of PE (without hypertension or proteinuria) – *cases*
    - Pre-existing CKD, chronic illness including autoimmune or inflammatory disease – *cases and controls*

- Previous PE or history of ≥ 3 consecutive miscarriages – *controls*
- Medical indication for caesarean section – *controls*

2.2.1.2 Data Collection

Baseline demographic and clinical details were collated from patient notes, hospital informatics data and electronic laboratory and discharge records.

The following information was recorded:

- Maternal demographics:
  - Age
  - Ethnicity
  - BMI
  - Parity
  - Co-morbidities
  - Previous obstetric history (PE/recurrent miscarriages)
- Antenatal data:
  - Urine PCR on admission (cases only)
  - Singleton or non-singleton pregnancy
  - Gestational diabetes
  - Gestation at enrolment/sample collection
  - Gestation at delivery
  - Outcome at pregnancy (live birth/IUD/still birth)
- Postnatal data:
  - Small for gestational age baby

- Low birth weight baby
- HDU admission during pregnancy or post delivery

2.2.1.3 Definition of Adverse Pregnancy Outcome

Adverse clinical outcome was defined as occurrence of one of the following: maternal admission to high dependency unit (HDU), pre-term birth (delivery < 37 weeks), SGA, delivery of low birth weight baby or admission to the NNU.

2.2.2 Chapter Four

During this study, samples taken in early pregnancy (first trimester) were compared between women who subsequently developed PE, to matched pregnant women who did not. As part of routine antenatal screening in the United Kingdom, serum samples are collected early on in pregnancy to screen for blood borne viruses. These samples are taken during the first antenatal visit with the midwife/hospital, which is usually during the first trimester. They are kept frozen at -20°C for 2 years, as per National Screening Committee recommendations, before being discarded. The samples are stored in the microbiology laboratory department at BWH and discarded on a monthly basis using electronic records, allowing identification of samples that have reached their expiry date and should be discarded.

Using a combination of BWH informatics data and these laboratory records, pregnant women, who had samples which were due to be discarded and had later developed PE in their pregnancy, were identified. These pregnancy samples were then matched to non-PE pregnancy samples which were also due to be discarded. Matching was made on the basis of age (within 2 years), ethnicity (same ethnic group), BMI (same BMI category) and

primagravidity (whether nulliparous or parous) in women who did not develop a hypertensive disorder in pregnancy. The BMI categories used for matching were <18.5 underweight, 18.5-25 normal, 25-30 overweight, 30-35 obese, 35-40 severely obese, >40 very severely obese and unknown. The matching process allowed otherwise healthy women, who developed PE in pregnancy, to be compared to women who had not and had a similar maternal demographic and clinical risk profile. Smoking history was missing in a significant proportion of pregnancies and therefore not included in the matching criteria. Exclusion criteria for both cases and controls was pre-existing diabetes mellitus, CKD, pre-existing illness including autoimmune conditions such as connective tissue disease (CTD) and hypo/hyperthyroidism. It was not possible to identify whether there was a history of chronic hypertension present in cases or controls and thus I was unable to exclude these women from the study. Once controls were identified as suitable matches for cases, the cases were split into groups with one, two or three available matches. The closest available match (where the age matched more closely) were used in pairings where more controls were available than required. For instance, if a case had three available suitable controls, where two controls were selected, these were the two closest age-matched controls.

Miscarriages and non-singleton pregnancies were also excluded in both cases and controls. Furthermore, any cases or controls found to have a serum creatinine ≥ 90 umol/l and their respective cases/controls were subsequently excluded from the study, on the basis these patients may have undiagnosed/unrecorded CKD. Samples that had been taken during April 2012-February 2013 and corresponding pregnancies were screened and collected over an eleven-month period.

2.2.2.1 Data Collection

All baseline demographic and pregnancy outcome data were provided by BWH informatics.

The data requested and subsequently used for the study was:

- Maternal demographics:
    - Maternal age at sample collection
    - Ethnicity
    - BMI
    - Parity
- Antenatal data:
    - Gestation at sample collection
    - Gestation at delivery
    - Fetal growth restriction (FGR)
    - Outcome of pregnancy (miscarriage/live birth/IUD/still birth)
- Baby data:
    - Neonatal death
    - Low birth weight or very low birth weight baby

2.2.2.2 Definition of Adverse Pregnancy Outcome

This was defined as one of the following: delivery <34 weeks' gestation, IUD, still birth, neonatal death, FGR, low birth weight or very low birth weight baby.

2.2.3 Chapter Five

For this study, longitudinal samples during pregnancy were collected from women with CKD. The University Hospitals Birmingham (UHB) and BWH have a long established joint

Renal/Obstetric clinic, providing pre-pregnancy counselling and antenatal management for women with all stages of CKD. Women attending their routine renal-antenatal appointment at this clinic were approached from 2011 - 2016 to consent for collection and storage of serial urine, plasma and serum samples. All women attending the clinic were approached. These included patients known to have CKD pre-pregnancy and women referred with a newly suspected diagnosis of CKD made during pregnancy. Women with non-singleton pregnancies were also included to increase the number of patients recruited into the study and to capture 'higher risk' pregnancies within the cohort. Attempts to collect postnatal samples were made for those who attended for a postnatal review towards the end of the study period. If recruited patients were admitted to hospital, either for delivery, an obstetric or medical complication, further serum samples were collected when possible.

2.2.3.1 Data Collection

Baseline demographic, pre-pregnancy details, serial clinical parameters and pregnancy outcomes including baby data were recorded from BWH and UHB patient notes, laboratory records, ultrasound reports and outpatient clinic letters.

- Maternal demographics:
    - Age
    - Ethnicity
    - BMI
    - Gravida and Parity
    - Co-morbidities
    - Aetiology of CKD

- Presence of structural abnormalities of renal tract
- Pre-pregnancy eGFR (ethnicity adjusted) and CKD stage (closest pre-conception) and whether renal transplant in-situ
- Pre-pregnancy creatinine and uACR (closest pre-conception)
- History of UTI (including during pregnancy)
- Smoking history
- Whether known to renal team pre-pregnancy
- Whether attended pre-pregnancy renal clinic for pre-pregnancy counselling
- Medication history

- Obstetric history:
  - Number of previous miscarriages including if ≥ 3 consecutive miscarriages
  - Previous PE
  - Previous pre-term (<37 weeks) or very pre-term (<34 weeks) delivery
  - Previous SGA or FGR
  - Previous still birth or IUD
  - Previous neonatal death
- Antenatal data
  - Assisted reproductive technique
  - Blood pressure at booking visit (first antenatal visit)
  - Urine dip at booking visit
  - Singleton or non-singleton pregnancy
  - Antenatal aspirin prescribed

- Abnormal growth scans (formal scan report) including tapering or reduction of fetal growth or FGR (growth <10th centile)
- Antenatal infections with dates (diagnosis documented in clinical notes with appropriate antimicrobial therapy prescribed)
- Development of gestational diabetes
- New onset or worsening hypertension (requiring escalation of pre-existing treatment)
- Fetal distress
- Presence of uterine artery Doppler notching (on formal scan report)
- Abnormal uterine artery velocity waveform (on formal scan report)
- Pre-delivery hospital admission (to BWH)
- Superimposed PE (defined below) with date of diagnosis
- Renal complication (worsening of proteinuria or renal function during antenatal period, considered pathological by clinicians)
- Outcome (miscarriage/live birth/IUD/still birth)
- Gestation at outcome
- Type of delivery: caesarean section (emergency or elective), vaginal delivery (instrumental or normal)
- PROM
- Spontaneous or induced labour

- Postnatal data:
  - Postpartum haemorrhage (estimated blood loss > 500 ml)

- Renal complication (worsening of proteinuria or renal function during postnatal period, considered pathological by clinicians)
- Length of maternal hospital stay post delivery
- New-onset or worsening hypertension (requiring escalation of pre-existing treatment)

- Baby data
  - Birth weight
  - Apgar score at 1 and 5 minutes
  - Length of hospital stay
  - Neonatal death
  - Admission to NNU/NICU

2.2.3.2 APGAR Score

The Apgar score is an immediate assessment of the newborn baby's clinical condition following delivery and is usually taken at 1 and 5 minutes after birth with the score ranging from 0-10 [304, 305]. A 5-minute Apgar score of 7 to 10 is considered reassuring and a score below 7 as abnormal. A low score at 5 minutes is associated with increased neonatal morbidity and increased risk of subsequent neurological conditions such as cerebral palsy and epilepsy developing.

2.2.3.3 Diagnosis of Superimposed PE

Clinical notes were reviewed following pregnancy to determine whether pregnancy was complicated by SPE. Women were classified as having developed SPE if the diagnosis was suspected on clinical grounds and there was evidence of either i) new onset (BP>140/90

mmHg) or worsening hypertension (requiring escalation of anti-hypertensive treatment) or ii) new onset proteinuria (uPCR > 30 mg/mmol) or rapid worsening of pre-existing proteinuria and at least one of the following if only one of these features was present:

- Rapidly worsening kidney function
- Abnormal liver function tests (previously normal)
- Neurological symptoms
- Hematological complications
- Evidence of uteroplacental dysfunction such as FGR

Furthermore, the indications for delivery would also have to be related to suspected diagnosis of SPE. Where uncertainty of diagnosis existed, a consensus opinion was obtained independently from a Consultant Nephrologist and Obstetrician, both with a specialist interest in Obstetric Nephrology. A diagnosis of SPE in these cases required both clinicians to independently agree. In cases where diagnosis was deemed uncertain patients were categorised as not having developed PE. Deliveries occurring at other hospitals were classified as non-PE, unless the development of PE was specified clearly in the external correspondence.

2.2.3.4 Composite adverse pregnancy outcome

This was defined on the presence of at least one of the following: miscarriage, still birth, IUD, delivery < 34 weeks, admission to NNU/NICU, FGR or very low birth weight baby (<1500g).

2.3 Laboratory Analyses

Serum samples that had already been collected as part of routine clinical care were used in chapters three and four. Sample collection for all studies was performed in accordance to local laboratory protocol, with serum samples transferred into Sarstedt clot-activator tubes.

2.3.1 Sample Preparation

2.3.1.1 Chapter Three

Routinely collected clinical samples were centrifuged within 3 hours of collection at 3000 RPM for 5 minutes (Sorvall ST16R, Thermofisher Life Sciences) and transferred to a +4°C refrigerator after routine analysis. These samples are usually transferred to the freezer (-20°C) the following day. Samples for inclusion into the study were identified on the same day of collection and transferred from the refrigerator to HBRC where they were aliquoted (250-500µl per aliquot). In cases where a routinely collected clinical sample was not available, a separate sample was taken and handled the same way as the other samples. All samples had patient identifiable labels removed and pseudo-anonymised with a unique sample number, the link to the patient being retained by the HBRC. These samples were then frozen at -80°C at the HBRC until analysis.

2.3.1.2 Chapter Four

Study samples had previously been kept frozen in aliquots (1 per sample) at -20°C in the BWH microbiology freezers after collection and analysis. Following identification of samples for inclusion in the study, samples were transferred to HBRC on dry ice, pseudo-anonymised and stored as described for chapter three.

2.3.1.3 Chapter Five

Serum research samples were collected separately, but at the same time as routine clinical care samples were taken in clinic. Samples were centrifuged at 3500 RPM for ten minutes at +4°c (Heraeus Labofuge, Thermofisher Life Sciences) within 4 hours of collection. These samples were then aliquoted (250-1000 μl per aliquot), pseudo-anonymised as described above and kept frozen at -80°C until the day of analysis.

2.3.2 Sample Analysis and Kits

Samples were thawed on the day of analysis and repeated thaw-freeze cycles were avoided. All samples were tested blindly without knowledge of who the sample belonged to or their clinical outcome. Analysis was performed in batches and separately for the clinical/immune system markers, and the angiogenic markers. Serum levels of high-sensitivity C-reactive protein (hs-CRP), albumin, creatinine and cystatin–C, complement components 3 & 4, IgM, IgA and IgG and IgG subclasses 1-4, kappa and lambda free immunoglobulin light chains and beta2 microglobulin (B2-M) were measured on the Hitachi Cobas 6000 Turbidimeter (Roche diagnostics, West Sussex, UK). The Hitachi Cobas® system is a fully automated analyser and the basis of the test relies on measurement of antigen/antibody reaction by turbidimetry. A diluted serum sample is pipetted into a cuvette, and reagent added. Measurement requires a light beam being passing through the solution with the loss in light intensity equating to a concentration when compared with a standard curve. Not all samples in chapter five were tested for IgG subclasses due to limited kit supplies and thus these were measured in random batches. All reagents were supplied by Roche diagnostics, except for those used in analysis of the IgG subclasses and the Freelite® FLC assay (The Binding Site Group Ltd,

Birmingham, UK). The coefficient of variation (CV) for the various reagents tested in this thesis are summarised in Table 2-1. The majority of the intra and inter assay CoV were under 3% and 7% respectively and were all under 10.2%, which was deemed to be within an acceptable range.

Table 2-1: Summary of the Coefficient of Variations (cvs) reported for the various assays tested in the thesis (obtained from reference [306])

Assay	Intra-assay range tested	Intra-assay CoV	Inter-assay range tested	Inter-assay CoV
IgG	8.25-21.5 g/L	0.6-1.5%	7.1-21.1 g/L	1.1-1.7%
IgA	1.55-3.23 g/L	0.7-1.0%	1.93-3.31 g/L	1.1-1.8%
IgM	0.71-1.36 g/L	0.9-1.6%	0.75-1.34 g/L	1.9-3.8%
IgG1	1.61-16.11 g/L	2.6-2.8%	1.61 -16.11 g/L	2.9-4.2%
IgG2	9.75-8.27 g/L	1.8-2.6%	9.75-8.27 g/L	0.0-2.9%
IgG3	0.13–1.10 g/L	3.1-4.9%	0.13 – 1.10 g/L	4.4-6.6%
IgG4	0.13-0.73 g/L	1.3-2.3%	0.13-0.73 g/L	2.1-4.6%
Kappa sFLC*	5.58-41.08 mg/L	1.4-2.5%	5.79-41.35 mg/L	2.9-7.2%
Lambda sFLC*	7.66-60.33 mg/L	2.8-5.5%	7.98-63.26 mg/L	2.3-10.1%
Creatinine	105-422 umol/L	1.1-2.1%	101-411 ummol/l	2.2-3.5%
UA	4.32–10.7 mg/dL	1.0-1.1%	4.35–11.1 mg/dL	1.8-1.9%
Cystatin-C	0.52-6.30 mg/L	0.6-2.7%	0.65-7.17 mg/L	2.1-4.3%
B2-M	0.79-6.07 mg/L	1.4-1.9%	1.76-4.76 mg/L	1.2-1.5%
hs-CRP	0.54-15.9 mg/L	0.4-1.6%	0.53-13.3 mg/L	1.3-8.4%
Albumin	32.1-51.3 g/L	0.7-1.2%	32.0-51.3 g/L	0.9-1.5%
C3	1.17–2.05 g/L	0.8-1.2%	1.14–2.09 g/L	1.4-2.0 %
C4	0.17–0.30 g/l	0.7-1.3%	0.170-0.404 g/L	1.4-1.8%
sFLT-1	63.1-79101 pg/mL	0.8-1.6%	63.1-79101 mg/L	0.8-1.6%
PIGF	112-9542 pg/mL	0.7-1%	112-9542 pg/mL	2.7-4.1%

**The CV for the sFLC assays were obtained from the University of Birmingham Clinical Immunology Services summary sheet for verification and validation.*

The angiogenic factors sFLT-1 and PIGF were measured separately to the above markers, as they were measured on the on the Cobas e411 analyser, using the fully automated Elecsys assays. Where sample aliquots or volumes were limited, angiogenic markers could not be

tested, as these were analysed after the immune system markers had already been. The angiogenic marker kits were also limited in number for analysis of samples in the chapter five study. All samples belonging to women with CKD stage 3-4 group (none had CKD stage 5) where available were analysed as there were less patients in these groups. For samples belonging to women with CKD stage 1-2, the two last samples, closest to and including around the time of delivery that were available, were analysed. Any postnatal samples and clinical samples (taken at the time of PE manifestation), where available, were also tested for angiogenic and immune system markers.

2.3.2.1 Serum Free Light Chain Assays

Clinical laboratory tests to measure sFLCs were first published in 2002 and developed into the commercially available Freelite® assay (measuring both kappa and lambda) [185]. The Freelite® assay is well established, and in routine clinical used to measure sFLC, with normal reference ranges established for adults [185]. The assay uses polyclonal sheep antisera directed against epitopes that are only accessible when light chains are not part of an intact immunoglobulin. When intact these epitopes are 'hidden' and are located at the interface between light and heavy chains. The sensitivity of the Freelite® assays is increased by latex enhancement, to a few mg/L and the assays can be performed on a number of automated laboratory instruments using turbidimetry or nephelometry.

2.3.2.2 Serum immunofixation

Serum immunofixation was performed on five patients in chapter three with the highest sFLC values (combined, 66.5-85.0 mg/L) to exclude presence of a mononclonal band. This was performed using Sebia Hydrays System (Sebia UK Limited) Hydrays equipment and as

per manufacturer guidelines. The serum sample was prepared to 1:6 and 1:3 dilution and the procedure was a four-step process:

- *Protein separation by electrophoresis* in which the sample is simultaneously electrophoresed in six tracts on alkaline buffered gel (10 minutes to run).
- *Immunofixation:* Antisera specific to IgG, IgA, IgM and free and bound κ and λ light chains were added to the electrophoretic migration tracks which diffuse into the gel and precipitate the corresponding proteins (left for 5 minutes). One of the tracks was overlaid with fixative and acted as a reference track.
- Soluble proteins were removed by blotting (3 minutes) and washing (5 minutes, 50°C) leaving the proteins of interest to fix onto the gel pad.
- Staining with acid violet (30 minutes) allowed the immunoprecipitates to be visualised for the presence of monoclonal bands. The immunofixed bands were compared with bands in the reference tracts.

2.3.3 Stability of Samples

Previously published data has demonstrated that levels of sFLC remain stable in serum following long-term (193-568 days) storage at -20°C [307]. Measurements of sFLC levels in these samples correlate very strongly to those obtained from freshly collected serum samples. Furthermore, the Clinical Immunology Service at UoB have previously tested the impact of long-term storage at -20°C and of repeat freeze thaw cycles and found that neither significantly impact on the measurement of Igs, sFLC B2-M and CRP levels.

2.4 Statistical Analysis

2.4.1 Study Sample Sizes

Power Calculations were not performed, as the numbers of patients recruited into the three studies were determined by resource and time limitations. I had to consider the time period available to me to collect clinical data and perform sample analysis for each of the three studies. I aimed to recruit 100 PE and 100 control patients in chapter three, as I felt that this was a feasible figure to aim for within the time frame available, and would allow a suitable number of patients for meaningful comparison. I set a period of time to collect samples for the chapter four study and this determined the number of patients included in the study. Similarly, for chapter five I recruited patients up until a fixed date, to allow enough time to collect data and analyse samples within the time frame of my PhD.

2.4.2 Chapter Three

All analyses in the thesis were performed using IBM SPSS 22 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance throughout. For chapter three, initially, a range of factors were compared between the PE and control groups. For categorical variables, Chi squared tests were used to compare the relative proportions of patients in each category between two patient groups. Continuous variables were assessed for normality prior to the analysis using histograms. Those following skewed distributions were reported as medians with IQRs, with non-parametric Mann-Whitney tests used to compare groups. Normally distributed continuous variables were reported as means ± standard deviations (SD), with comparisons between groups made using independent samples t-tests. The same approach was also used to compare the markers being considered

between those patients that did and did not develop adverse events within the two cohorts. For variables which were significantly different between the two study groups, the diagnostic accuracy was then assessed using receiver operating characteristic (ROC) curves. As discussed in the introduction, obesity is associated with impaired immune response and levels of Igs vary with ethnicity and age [237-240]. Analysis of the relationship of these factors with Igs and sFLC levels were therefore explored. Associations between pairs of study markers and Igs were assessed using Spearman's correlation coefficients (rho) and these were also performed to explore the relationship of Igs and FLC with patient age and BMI. Comparison of Igs and sFLC with ehnicity was performed using one way ANOVA or Kruskal-Wallis tests, depending on whether or not the variables were normally distributed, with significant results followed by pairwise post hoc tests using Tukey's or Dunn's tests, respectively.

A multivariable analysis was then performed, to identify factors that were significantly independently associated with PE. This was based on a multivariable binary logistic regression model, using a forwards stepwise approach to variable selection. Study markers found to differ significantly with PE on univariable analysis were considered for inclusion in the model, alongside other factors known to be relevant in PE. The sFLT-1/PIGF ratio rather than these markers individually was included in the model. Separate models were produced to compare the diagnostic accuracy of the clinical factors and routine laboratory markers with and without the angiogenic factors. Odds ratios were reported, both for a one unit increase in the factor and for an increase of magnitude equivalent to the IQR of the distribution of the marker, in order to account for the different ranges of values across the

markers. The predictive accuracy of the resulting models was assessed by ROC curve analysis, with the areas under the ROC curves (AUROCs) compared using the "roccomp" command in Stata (College Station, TX: StataCorp LP).

2.4.3 Chapter Four

For categorical variables, Chi-square tests were used to compare the relative proportions of patients in each category between the PE and control group. Continuous variables were assessed for normality using visual assessment of normal Q-Q plots. For those following skewed distributions concentrations of markers were log–transformed, prior to statistical analysis. The matching criteria has been described earlier in this chapter. The primary analysis was performed using 1:2 matching on the cases matched with two controls, as this provided the largest overall sample size of patients. Comparisons of markers between cases and their controls was performed using repeated measures ANOVA (analysis of variance) and univariable conditional binary logistic regression models, in order to identify significant predictors of PE, whilst accounting for the pairing of patients. Repeated measures ANOVA was performed with a Helmert contrast to allow comparison for a specific marker between the mean of multiple controls and the value of their matched case. The mean (either arithmetic or geometric, as appropriate, with 95% CI) of controls and cases is reported. For the logistic regression analysis, the OR is reported per positive unit change for the marker considered with 95% CI. Multivariable conditional binary logistic regression analyses, using a forwards stepwise approach was then performed, which included all study markers as covariates, in order to identify significant independent predictors of PE. The analyses were repeated for the cases matched to three controls as a sensitivity analysis. It was also

repeated to specifically compare the subgroup of women who developed early onset PE (<34 weeks) to their matched controls.

Analyses were then performed to assess whether the study markers differed significantly between those women that did and did not develop adverse pregnancy outcomes. All the PE cases (i.e. matched and unmatched) were included in this analysis, alongside all available controls, with analyses performed separately for the PE and control subgroups. Comparisons were initially performed using independent samples t-test using the log-transformed or untransformed values of the markers, as applicable. The mean (either arithmetic or geometric, as appropriate, with 95% CI) of controls and cases is reported. Multivariable unconditional binary logistic regression analysis was then undertaken, using a forwards stepwise approach to variable selection, in order to identify markers that were significant independent predictors of adverse outcomes within the PE and control groups. The OR is reported per positive unit change for the marker considered with 95% CI.

2.4.4 Chapter Five

2.4.4.1 Patient Demographics

Initially, a range of demographic and clinical factors were compared between those patients that did and did not develop PE. Normally distributed continuous variables were reported as arithmetic mean±SD, and compared between groups using independent samples t-tests. Continuous variables that were not found to follow normal distributions were reported as medians and IQRs, and compared between groups using Mann-Whitney tests. Ordinal variables were also assessed using Mann-Whitney tests, whilst nominal variables were analysed using Chi-square tests. The PE group was then divided into early (<34 weeks) and

late onset (≥34 weeks), and subgroup analyses were performed to make comparisons between these groups.

A multivariable analysis was then performed, to identify factors that were independently associated with PE. Prior to the analysis, the goodness of fit of continuous variables was assessed using the Hosmer-Lemeshow test. Where poor fit was detected, variables were divided into categories, and treated as nominal in the model. Where nominal factors had categories with small numbers of patients, these were combined, in order that odds ratios were calculable. For groups of factors that were highly correlated, only one factor was considered for inclusion, in order to prevent issues with multicollinearity. A multivariable binary logistic regression model was then produced, with a forwards stepwise approach to variable selection. For factors relating to previous pregnancy outcomes, an additional "nulliparous" category was added, to allow these patients to be included in the model. Where a single previous pregnancy outcome was significant on univariable analysis, only this previous pregnancy outcome was considered for inclusion in the model. However, if multiple previous pregnancy outcomes were found to be significant, then all of these factors were considered for inclusion in the model. Where this occurred, a variable specifying whether a patient was nulliparous was forced into the model, in order to differentiate the effect of this factor from the effects of the previous pregnancy outcomes.

Factors selected for inclusion in the final model by the stepwise procedure were then entered into a new model, in order to prevent exclusions due to missing data on non-significant factors and maximise the included sample size. Factors with large quantities of missing data were excluded from the primary analysis, in order to maximise the included

sample size. However, a secondary model was then produced which considered these factors for inclusion, in order to assess whether this affected the conclusions of the analysis. The model was then evaluated for each patient, to produce predicted probabilities of developing PE. The resulting values were then analysed using a ROC curve, to assess the predictive accuracy of pre-pregnancy factors, with respect to PE. The multivariable analysis was then repeated for the composite adverse pregnancy outcome, using the approach previously described.

2.4.4.2 Change Over time in Markers

The rate of change in the markers was then estimated and compared between the PE and non-PE CKD groups. Prior to the analysis, the distribution of the markers was assessed, with $\log_{10}$-transformations applied to those that were skewed. In markers that had values of zero, a constant was added to all values, to make log-transformation possible. Patients had repeated samples taken throughout the pregnancy, which could potentially be taken close together, artificially inflating the effective sample size. As a result, the gestation was divided into five periods, with each pregnancy permitted to contribute a single value per period to the analysis. In cases where there were multiple samples within a period, the average value was used (geometric mean for skewed markers, and arithmetic mean in normal distributions), with the arithmetic mean of the gestations used as the timing of the sample.

Trends over time in marker concentrations were then assessed using a generalised estimating equation (GEE) model. The pregnancy ID was set as the subject effect, and the period of gestation was set as the within subject effect, with an auto-regressive (AR1) correlation structure assumed. As a result, the model estimated the correlation between

repeated measures of a marker within the same pregnancy and accounted for the degree of this non-independence. Each marker in turn was set as the dependent variable, with log_{10}-transformations applied, if required. The Pre-eclampsia status of the pregnancy, gestation of the sample and an interaction term were set as the independent variables in the model. The former compared the intercept of the model between the PE and non-PE pregnancies, to assess whether there was an overall difference in the marker between the groups. The coefficients of the sample gestation and interaction term were then combined, to estimate the rate of change over time in the PE and non-PE groups separately, and to make a comparison between these gradients. For normally distributed markers, the gradients were reported as average increases per 12 weeks of gestation. For markers that were log_{10}-transformed due to having skewed distributions, the coefficients from the model were anti-logged, and converted into percentage increases in the marker per 12 weeks' gestation.

Due to the shape of the trends in the angiogenic markers, it was not reasonable to produce a model assuming linear or log-linear relationships with gestation. As a result, the angiogenic values were gestation-adjusted, based on the reported normal (50^{th} percentile) values at the gestation at which the sample was taken [276, 277]. These normal values are reported for intervals of gestation between 10 weeks and the time of delivery (Table 2-2). For earlier gestations, the normal values were estimated by extrapolation, on the assumption that the log-linear change between 10-14(+6) and 15-19(+6) weeks would be similar to that between 5-9(+6) and 10-14(+6) weeks, and between 0-4(+6) and 5-9(+6) weeks.

Table 2-2: Normal pregnancy ranges for the angiogenic markers [276, 277]

Gestation (Weeks)	50th Percentile (pg/ml)		
	sFLT-1	***PIGF***	***Ratio***
0-4(+6)*	1276*	8*	138.3*
5-9(+6)*	1302*	20*	58.6*
10-14(+6)	1328	52.6	24.8
15-19(+6)	1355	135	10.5
20-23(+6)	1299	264	4.92
24-28(+6)	1355	465	3.06
29-33(+6)	1742	471	3.75
34-36(+6)	2552	284	9.03
37-Delivery	3485	191	19.6

**Values were extrapolated, based assuming that the rate of change across the interval would be consistent with that between 10-14 and 15-19 weeks' gestation.*

It was then assumed that the normal values for the angiogenic markers applied to the midpoint of the gestation intervals for which they were recorded, for example, that the normal value for 10-14(+6) weeks' gestation applied to 12.5 weeks. Based on this, normal values for all gestations between two midpoints were interpolated, based on a log-linear regression model. The observed values of each sample were then divided by the interpolated normal values at the gestation of sample collection, to produce a "gestation-adjusted" value, which represented the fold-difference from the normal value for the gestation. These gestation-adjusted values were then used as dependent variables in GEE models, as described for the non-angiogenic markers above.

2.4.4.3 Predictive accuracy of markers

As well as the overall comparisons between PE and non-PE groups, the predictive accuracies of the markers were assessed within each period of gestation, using ROC curves. In order to further quantify the difference between groups, the average values of the markers were reported at each gestation, using arithmetic or geometric means, as appropriate, along with 95% CIs.

2.4.4.4 Analysis for Outcomes by Pre-pregnancy CKD Stage

Initially, a range of patient factors were compared across the pre-pregnancy CKD stages. As the CKD stage is ordinal, comparisons across the groups were performed using Kendall's tau for dichotomous and ordinal factors, and Jonckheere-Terpstra tests for continuous factors. For nominal factors with more than two categories, Kruskal-Wallis tests were used to compare the "average" CKD stage between categories. Where significant differences between CKD stages were detected, pairwise post-hoc tests were performed, to identify the pairs of groups that differed. Dunn's test was used for ordinal and continuous factors, whilst nominal factors were analysed using pairwise Fisher's exact tests, with the p-values Bonferroni-corrected to adjust for three comparisons. Kaplan–Meier curves were then produced to visualise the timing of PE according to CKD stage. Patients without PE were treated as being censored at 40 weeks' gestation, to ensure that the assumption of non-informative censoring was met.

2.4.4.5 Postnatal Data

In addition to the antenatal samples, postnatal samples were collected for a subset of patients. For these, comparisons were made between the final antenatal sample, and the first postnatal sample using Wilcoxon's test, to assess how the markers changed after delivery. The PE and non-PE patients were then identified, and both the antenatal, postnatal and fold changes in the markers were compared between groups using Mann-Whitney tests.

2.4.4.6 Comparison of Chapter Cohorts

The cohorts from chapters three, four and five were compared in this analysis, representing early and late samples from women with and without CKD. For consistency across the

cohorts, “early” and “late” pregnancy was defined as gestations of <16 and 32+ weeks, respectively and therefore only patients with samples at these time points were included. Samples collected outside of these ranges were excluded from the analysis. For the cohort from chapter four only those women that were selected by the 1:2 matching process were included in the analysis. For the cohorts from chapter five, women with autoimmune disease were additionally excluded, for consistency with the non-CKD cohorts. Pregnancies ending in miscarriage were also excluded from this cohort, for consistency with the other cohorts.

2.4.5 Adjustment for multiple comparisons

Adjustment for multiple comparisons was not routinely applied to analyses across the study mainly because of the irrelevant null hypothesis [308-310]. For comparisons between more than three groups, such as across the CKD stages, significant analyses were followed by pairwise post-hoc tests, to identify the pairs of groups that differed. These post-hoc analyses were adjusted for multiple comparisons, either by using the appropriate post-hoc test (e.g. Dunn’s test), or by applying Bonferroni-correction to a standard two-group comparison (e.g. Fisher’s exact test).

For other analyses, it was not felt that adjustment for multiple comparisons was methodologically important. There were a few reasons for this decision. When comparing biomarkers between women with and without PE, applying adjustment for multiple comparisons would assume that the null hypothesis of interest would be that ‘women with and without PE have similar values of all biomarkers being analysed’, whilst the alternative hypothesis would be that ‘at least one biomarker differs between women with and without PE’. However, the aim of the study was to analyse each biomarker individually and test a

separate hypothesis, namely whether that specific biomarker differed between women with and without PE rather than applying overall analysis to each individual test.

Another factor in the decision not to apply adjustment for multiple comparisons was the resultant increase in the risk of type II errors. Whilst adjustment for multiple comparisons is effective in controlling the type I error (i.e. false-positive) rate, this is at the cost of a proportional increase in the type II error (i.e. false-negative) rate. Since this thesis was assessing a novel use of the biomarkers being analysed, it was felt that minimising the type II error rate should be prioritised, such that potentially clinically important associations were not missed. Hence, no adjustment for multiple comparisons was routinely applied to the analysis. This may have led to some tests returning false-positive findings and, as such, future validation studies in other cohorts would be recommended.

3 HUMORAL IMMUNITY IN PRE-ECLAMPSIA AND LINKAGE WITH ANGIOGENIC AND INFLAMMATORY MARKERS

In this chapter, I compared markers of humoral immunity in PE and their relationship to circulating markers of inflammation, angiogenic factors and renal function.

3.1 Objectives:

- To compare serum levels of intact immunoglobulin G, A and M, IgG subclasses 1-4 and free immunoglobulin light chains (sFLC) between women with Pre-eclampsia (PE) and healthy pregnant women at term.
- To correlate the above with levels of other serum proteins routinely measured in clinical laboratory practice and that have previously had some indication of association with PE. These are levels of C Reactive Protein down to the lower limits of the normal range (hs-CRP), beta2 microglobulin (B2-M), uric acid (UA), albumin and complement proteins (C3 and C4). Plus the anti-angiogenic profile by measuring levels of soluble fms-like tyrosine kinase-1 (sFLT-1) and the pro-angiogenic placental growth factor (PIGF) to derive the sFLT-1/PIGF ratio.
- Correlate differences in levels of the above proteins with renal function measured by serum creatinine and cystatin-C.
- To assess whether humoral system markers provide independent association with PE after control for angiogenic factors, inflammation and renal dysfunction.

3.2 Results

3.2.1 Comparison of Patient Demographics and Renal Function

A total of 195 women were recruited into the study, of whom 88 had PE and 107 were healthy pregnant controls. The proportion of twin pregnancies was similar in the PE and control group, with a small number of women with chronic hypertension recruited, therefore these patients remained in the study. A sensitivity analysis was nevertheless undertaken excluding those women with GDM and chronic hypertension, this had minimal impact on the results, which is explained by the small number of patients in these groups.

A comparison of baseline demographic and clinical features between the two groups is shown in Table 3-1. The women with PE were marginally younger (mean: 29.9 vs 31.9 years, p=0.012), enrolled at an earlier gestation (median: 37 vs. 39 weeks', p<0.001) and more likely to be experiencing their first pregnancy (53% vs. 16%, p<0.001) than those in the control group. The women with PE had significantly lower serum albumin (mean: 33.0 g/L vs 34.8 g/L) and higher serum creatinine (median: 64 vs. 55 umol/L) and cystatin-C (median: 1.27 vs 1.08 mg/L) than the healthy controls (all p<0.001). There were no significant differences in ethnicity, BMI or the prevalence of non-singleton pregnancies between the groups. Of the PE patients, five (6%) had gestational diabetes (GDM) and three (3%) had chronic essential hypertension, compared to no cases in the control patients (p=0.013 and 0.054 respectively).

3.2.2 Comparison of Humoral System Markers

Total serum IgG (mean:7.32 vs. 7.86 g/L, p=0.046) and IgG subclass 1 (mean:4.13 vs. 4.51 g/L, p=0.026) and subclass 3 (median:0.51 vs. 0.63 g/L, p=0.015) were significantly lower in the

PE group than in the control group, whilst no significant differences in IgG subclass 2 and 4 levels were detected (p=0.900,0.895 respectively). Serum levels of IgA and IgM were not found to differ significantly between the PE and control groups (p=0.300, 0.207 respectively). This data as well as for the other markers is represented in Table 3-2 and Figure 3-1.

Combined sFLC concentrations were found to be significantly raised in the PE versus control group, with medians of 29.7 versus 24.1 mg/L (p<0.001). When comparing isotypes, kappa FLC levels were significantly higher in the PE group (p<0.001), whereas levels of lambda FLC did not differ between the PE and control group (p=0.148). Seventeen (4%) of women had an abnormally high kappa lambda sFLC ratio (normal range 0.260-1.65), but serum immunofixation did not reveal monoclonal immunoglobulins or light chain in the five patients with the highest combined sFLC levels (66.5-85.0 mg/L). Figure 3-2 shows the immunofixation result for four of these samples.

3.2.3 Comparison of Other Immune System Markers

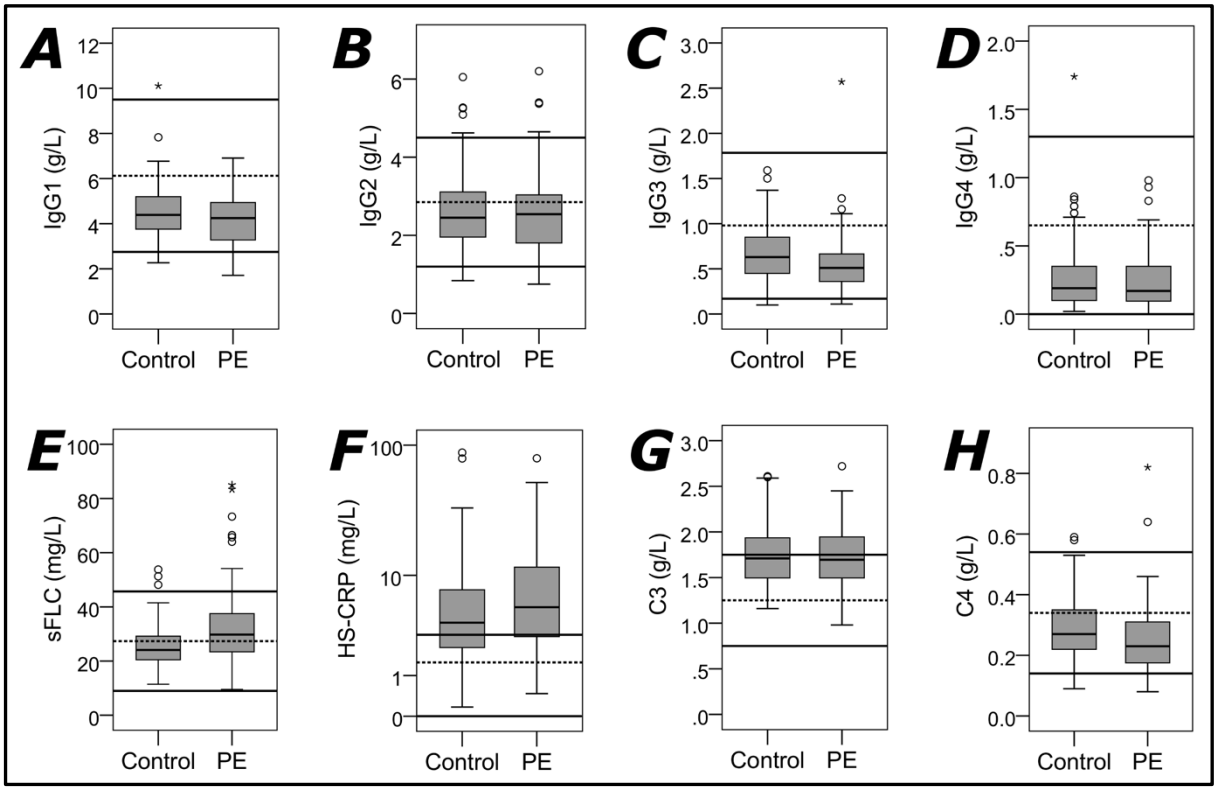
Both serum hs-CRP (median: 5.4 vs 3.9 mg/L, p=0.027) and B2-M (median: 2.14 vs 1.74 mg/L, p<0.001) were significantly higher in the PE than the control group. The C3 levels were similar (mean: 1.73 vs 1.74 g/L, p=0.838) in both study groups, whereas C4 levels were significantly lower in women with PE (median: 0.23 vs 0.27 g/L, p=0.001). Concentrations of hs-CRP and C3 in both groups were at the higher end of the non-pregnant normal reference range, in keeping with an acute phase response in pregnancy, whilst the measured C4 levels were clustered towards the middle-lower end of the non-pregnant reference range (Figure 3-1:F-H).

Table 3-1: Comparison of the demographics, clinical features and adverse clinical outcomes between groups

	PE (n = 88)	Control (n = 107)	p-Value
Demographics			
Age (years)	29.9 (±6.2)	31.9 (±4.6)	**0.012**
Gestation (weeks)†	37.0 (35.0-39.0)	39.0 (38.5-39.0)	**<0.001**
BMI (kg/m²)	28.0 (23.0-31.8)	26.1 (23.0-30.0)	0.310
Ethnicity (%)			0.262
White	57% (50)	69% (74)	
South Asian	24% (21)	19% (20)	
Black	10% (9)	5% (5)	
Mixed/Other	9% (8)	8% (8)	
First pregnancy (%)	53% (47)	16% (17)	**<0.001**
Twin pregnancy (%)	3% (3)	4% (4)	0.902
Gestational diabetes (%)	6% (5)	0% (0)	**0.013**
Chronic hypertension (%)	3% (3)	0% (0)	0.0543
Serum markers			
Serum albumin (g/L)	33.0 (±4.1)	34.8 (±2.5)	**<0.001**
Serum creatinine (umol/L)	64 (57-72)	55 (49-61)	**<0.001**
Cystatin-C (mg/L)	1.27 (1.11-1.54)	1.08 (0.91-1.24)	**<0.001**
Adverse clinical outcomes			
Pre-term birth	26% (23)	3% (3)	**<0.001**
Small for gestational age	22% (29)	7% (7)	**0.003**
Low baby weight	20% (18)	0% (0)	**<0.001**
HDU admission	35% (31)	5% (5)	**<0.001**
NNU admission	28% (25)	8% (9)	**<0.001**
Any of the above	57% (50)	18% (19)	**<0.001**

Non-categorical data are represented either are mean (±SD) or as median (IQR), with p-values derived from independent sample t-tests and Mann Whitney tests, respectively. Categorical data are reported as percentages, with p-values generated from Chi squared tests. Bold p-values are significant at p<0.05. †Refers to the gestation that the sample was collected at. Non-pregnant reference range for serum albumin is: 35-52 g/L, for serum creatinine: 44-80 µmol/L and for cystatin-C: 0.6-1.4 mg/L.

Figure 3-1: Box-Whisker plots comparing concentrations of A-D) IgG 1-4, E) combined sFLC, F) hs-CRP, G) C3 and H) C4 between control and PE Group



The vertical lines represent the values up to 1.5 times the IQR and the circles and stars are values outside this range (outliers). The normal reference range for the general non-pregnant population is illustrated as solid horizontal bars and a dashed line represents the middle of this reference range. In comparison to the control group, circulating levels of sFLC, hs-CRP, were significantly raised in the PE group and IgG1, IgG3 and C4 levels were lower. The concentrations of the remaining markers in this figure were similar in both groups.

3.2.4 Serum sFLT-1 and PIGF levels

Serum concentrations of sFLT-1 (median: 6103 vs. 3301 pg/mL, $p<0.001$) were significantly higher in the PE group, compared to the control group, whilst PIGF (median: 93.5 vs. 217.2 pg/mL, $p<0.001$) showed an opposite trend, resulting in a substantially higher sFLT-1/PIGF ratio in PE than controls (median: 68.6 vs. 14.0, $p<0.001$).

Table 3- 2: Comparison of immunoglobulins, sFLC levels, other immune and angiogenic markers between groups

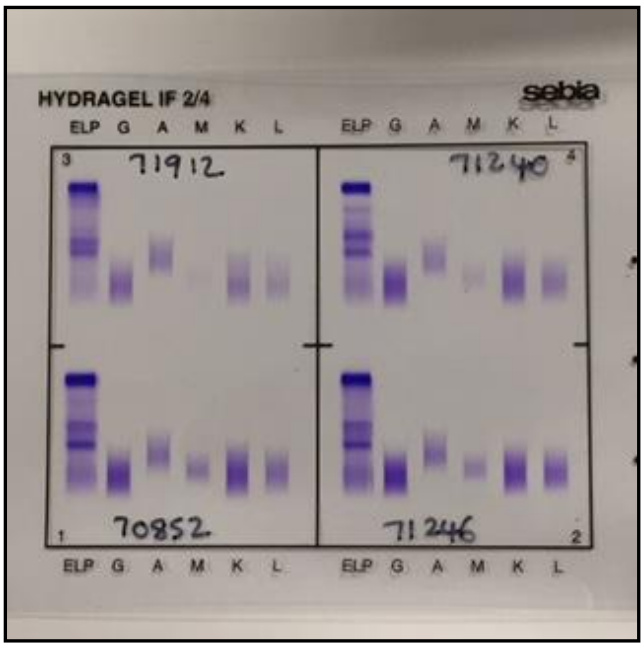
	Normal Range	PE (n = 88)	Control (n = 107)	p-Value
Immunoglobulins and FLCs*				
IgG g/L	6.00-16.00	7.32 (±1.92)	7.86 (±1.82)	**0.046**
IgA g/L	0.80-4.00	1.78 (±0.71)	1.68 (±0.64)	0.300
IgM g/L	0.50-2.00	1.13 (±0.62)	1.03 (±0.45)	0.207
IgG1 g/L	2.75-9.50	4.13 (±1.19)	4.51 (±1.20)	**0.026**
IgG2 g/L	1.20-4.50	2.58 (±1.03)	2.60 (±1.01)	0.900
IgG3 g/L	0.17-1.79	0.51 (0.36-0.67)	0.63 (0.45-0.85)	**0.015**
IgG4 g/L	0.00-1.30	0.17 (0.10-0.35)	0.19 (0.10-0.36)	0.895
Kappa mg/L (FL)	3.3-19.4	15.3 (11.1-22.1)	11.2 (9.1-14.2)	**<0.001**
Lambda mg/L (FL)	5.7-26.3	13.6 (11.5-15.7)	12.8 (10.4-15.1)	0.148
Combined FLC mg/L (FL)	9.0-45.7	29.7 (23.4-37.5)	24.1 (20.5-29.2)	**<0.001**
Other Immune markers				
C3 g/L	0.75-1.75	1.73 (±0.34)	1.74 (±0.31)	0.838
C4 g/L	0.14-0.54	0.23 (0.18-0.31)	0.27 (0.22-0.35)	**0.001**
hs-CRP mg/L	<3	5.4 (2.9-11.7)	3.9 (2.2-7.7)	**0.027**
Uric acid mg/dL	2.6-6.0	5.4 (4.3-6.7)	4.4 (3.7-5.1)	**<0.001**
B2-M mg/L	1.20-2.40	2.14 (1.86-2.65)	1.74 (1.53-1.93)	**<0.001**
Angiogenic Markers				
sFLT-1 pg/mL	-	6103 (3276-11097)	3301 (2120-4842)	**<0.001**
PIGF pg/mL	-	93.5 (59.7-166.6)	217.2 (142.1-417.8)	**<0.001**
sFLT-1/PIGF ratio	-	68.6 (32.3-170.0)	14.0 (6.3-28.5)	**<0.001**

Data are represented either are mean (±SD) or as median (IQR), with p-values derived from independent sample t-tests and Mann Whitney tests, respectively. Bold p-values are significant at p<0.05. The normal reference range for the non-pregnant population is reported in the second column.

3.2.5 Comparison of Diagnostic Accuracy for Pre-eclampsia

Figure 3-3 compares the ability of the serum biochemical markers, which were significantly different in the two groups, to distinguish between cases with PE and controls. The sFLT-1/PIGF ratio and B2-M provided the highest area under the ROC curve (AUROC) values, at 0.81 (95% CI 0.74-0.88) and 0.75 (95% CI 0.69-0.82), respectively, with no significant difference between the diagnostic accuracy of these two markers (p=0.184). Combined sFLC levels were also significantly (p<0.001) predictive of PE (AUROC: 0.67, 95% CI 0.59-0.75).

Figure 3-2: Immunofixation of four of the screened samples*

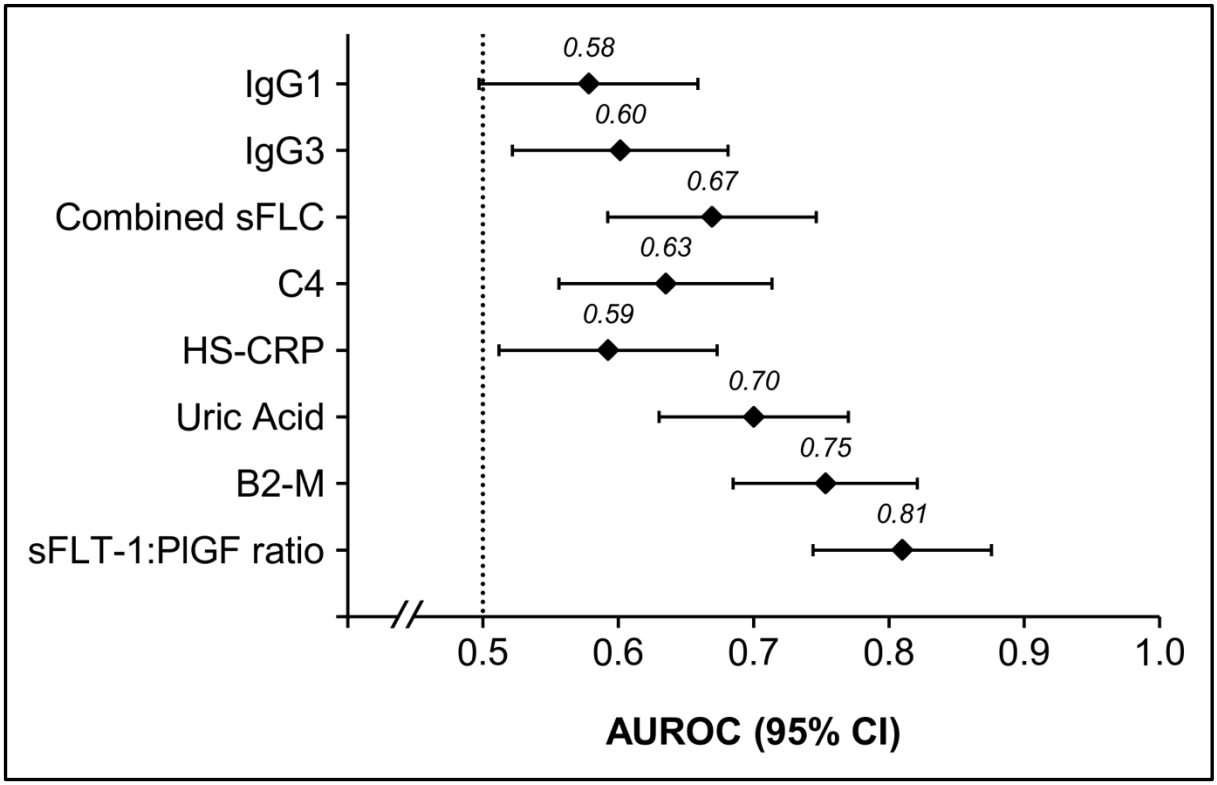


* confirming absence of monoclonal bands

3.2.6 Relationship between IgG and sFLCs with other immune markers

Spearman's rho correlation coefficients were performed to explore the relationship of Igs and FLC with the other study markers (Table 3-3). Combined sFLC levels were significantly positively correlated with total IgG (rho: 0.302, $p<0.001$), as well as with all IgG subclasses, except for IgG3 (rho=0.065, p=0.366). There are also other indicators that IgG3 may behave differently to the other classes in pregnancy. It was the only subclass to show significant (positive) correlation with C4 levels (p=0.010) and angiogenic markers (reported below), and was negatively correlated with B2-M (p=0.017), UA (p=0.021) and serum creatinine (p=0.023), suggesting a relationship with renal function also.

Figure 3-3 : Forest plot of the AUROCs for the differentiation between PE and controls by the markers identified as significant in table 3-2



The AUROC is displayed as diamond, and the error bars represent 95% confidence intervals. The sFLT-1/PlGF ratio and B2-M provided the highest area under the ROC curve (AUROC) values, at 0.81 (95% CI 0.74-0.88) and 0.75 (95% CI 0.69-0.82), respectively.

Total IgG (p=0.038) and specifically IgG1 (p<0.001) was positively correlated with C3 levels. Increased serum creatinine (p<0.001), cystatin-C (p=0.003), B2-M (p<0.001) and hs-CRP (p=0.005) were all associated with significantly increased total sFLC levels, in keeping with a relationship with kidney function and inflammatory response. This relationship was not observed with Ig levels. No significant correlations were detected between sFLC and UA (p=0.130) complement levels (C3: p=0.093, C4: p=0.590) or albumin (p=0.097).

3.2.7 Relationship between Humoral Markers and Patient Age, BMI and Ethnicity

Spearman's rho correlation coefficients were also performed to explore the relationship of Igs and FLC with patient age and BMI. Patient age was not found to be significantly correlated with levels of any of the Igs (including IgG subclasses), whereas a higher BMI was associated with significantly lower levels of IgG2 ($p=0.042$) and IgG3 ($p=0.039$). No significant correlations were detected between combined sFLC levels and the patient age ($p=0.192$) or BMI ($p=0.667$).

Combined sFLC levels and IgG levels were found to differ significantly with ethnicity (both $p<0.001$), with post-hoc analysis finding levels to be significantly higher in patients of South Asian origin, compared to patients of White Caucasian origin, with medians of 30.7 vs. 24.7 mg/L and 8.44 vs. 7.03 g/L respectively (both $p<0.001$). No significant association between ethnicity and IgA was detected ($p=0.063$), whilst IgM was significantly raised in the mixed/other group, relative to White Caucasian women (mean: 1.41 vs. 1.03 g/L, $p=0.037$).

Table 3-3: Correlations between immunoglobulins, sFLC levels and angiogenic markers with other study markers (all patients)

	IgG	IgA	IgM	sFLC	C3	C4	B2-M	Cystatin-C	UA	Creatinine	hs-CRP	Albumin	sFLT-1/PIGF
IgG		**0.302**	**0.162**	**0.323**	**0.149**	0.117	-0.060	-0.072	-0.022	0.041	-0.067	**0.223**	-0.052
IgA	**0.302**		**0.151**	**0.379**	0.005	-0.073	0.069	-0.001	0.065	0.078	-0.064	0.074	**0.160**
IgM	**0.162**	**0.151**		**0.149**	0.113	-0.076	0.099	-0.055	-0.056	0.020	-0.003	0.055	0.119
IgG1	**0.915**	**0.208**	0.078	**0.283**	**0.217**	0.112	-0.049	-0.120	-0.021	-0.003	-0.070	**0.282**	-0.074
IgG2	**0.712**	**0.262**	0.137	**0.264**	0.030	0.112	-0.075	0.039	0.040	0.062	-0.042	0.045	-0.008
IgG3	**0.434**	0.096	**0.143**	0.065	0.119	**0.183**	***-0.170***	-0.122	***-0.165***	***-0.163***	0.002	**0.210**	***-0.143***
IgG4	**0.467**	**0.157**	0.054	**0.195**	-0.008	0.002	-0.081	-0.048	0.024	-0.056	-0.032	0.037	-0.004
cFLC	**0.323**	**0.379**	**0.149**		0.121	0.039	**0.336**	**0.214**	0.068	**0.259**	**0.203**	-0.119	0.137
sFLT-1:PIGF	-0.052	**0.160**	0.119	0.137	***-0.145***	***-0.311***	**0.586**	**0.503**	**0.531**	**0.448**	0.073	***-0.291***	

Correlations are reported as Spearman's rho coefficients, with bold values significant at p<0.05, and significant negative correlations in italic. Serum FLC and Igs were also compared across ethnic groups using ANOVA and a Kruskal-Wallis test, as reported in the text.

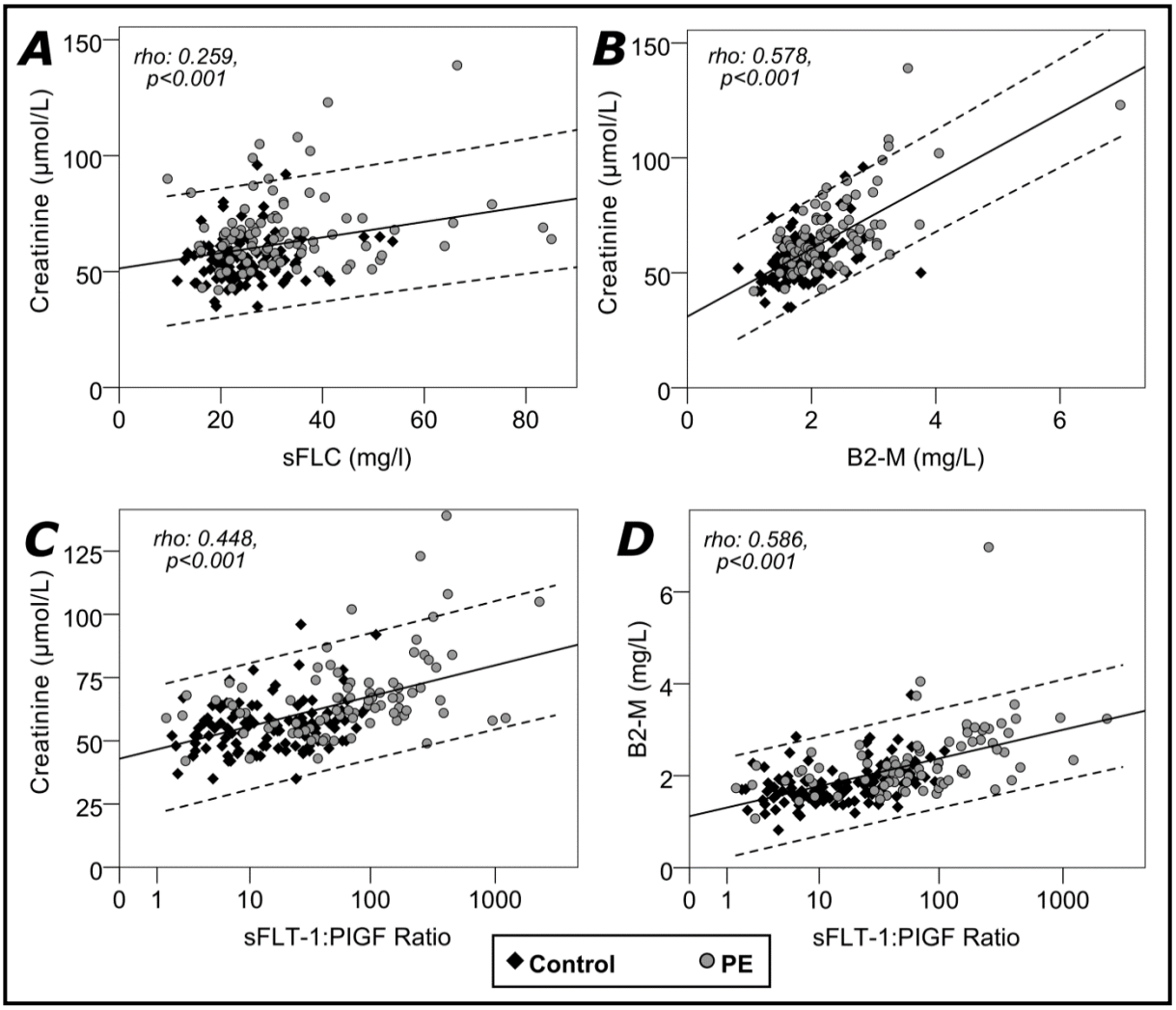
3.2.8 Relationship of degree of proteinuria with study markers

The degree of proteinuria, measured as a spot uPCR in the patients with PE, was not found to be significantly correlated with serum levels of IgG (rho=-0.130, p=0.250), IgA (rho=-0.043, p=0.707), IgM (rho=-0.122, p=0.281) or sFLC (rho=-0.095, p=0.401). A significant correlation was observed between increased levels of proteinuria and higher serum B2-M and sFLT-1/PIGF ratio (rho=0.287, p=0.010 and rho=0.442, p<0.001 respectively) and with lower C4 levels (rho=-0.425, p<0.001). Urine PCR was not significantly correlated with serum creatinine (p=0.209) nor UA levels (p=0.148). As table 3-3 highlights, there was a significant positive correlation between serum albumin concentrations and IgG (rho=0.223, p=0.002) but not with IgA (p=0.304) or IgM (p=0.442) levels.

3.2.9 Relationship of angiogenic factors to renal function and immune system markers

The associations between Igs and the sFLT-1/PIGF ratio were weak, with only serum IgA (rho = 0.160, p=0.028) and IgG3 (rho = -0.143, p=0.049) showing significant correlations (Table 3-3). There was no evidence of a significant correlation between sFLC concentrations and sFLT-1/PIGF ratio (rho=0.137, p=0.060). However, sFLT-1/PIGF ratio and B2-M levels were strongly correlated with each other (rho=0.586, p<0.001) and with both serum creatinine (rho=0.448, rho=0.578 respectively, both p<0.001, figure 1:J-K) and cystatin-C (rho=0.503, 0.717, both p<0.001), in keeping with a relationship of both markers with renal function. When split by study group (Table 3-4), the sFLT-1/PIGF ratio did not significantly correlate with serum C3 (p=0.600) or C4 (p=0.626) levels in the control group, but was significantly negatively correlated with both markers in women with PE (rho= -0.276, p=0.012, and rho= -0.409, p<0.001 respectively).

Figure 3-4: Scatter plots displaying significant correlations of serum creatinine against levels of a)sFLC b)B2-M c) sFLT-1/PIGF and d) sFLT-1/PIGF against B2-M levels



Spearman rho coefficients and p values are displayed and each graph. Solid lines represent linear regression models and broken lines are 95% prediction intervals. The sFLT-1/PIGF ratio has been log_{10} transformed and plotted against a logged x-axis. Renal function (creatinine) was strongly correlated with sFLC, B2-M and SFLT-1/PIGF ratio, the latter two markers were also strongly correlated with each other.

Table 3-4: Correlations between immunoglobulins, sFLC levels and angiogenic markers with other study markers split for control and PE Group

	IgG	IgA	IgM	sFLC	C3	C4	B2-M	Cystatin-C	UA	Creatinine	hs-CRP	Albumin	sFLT-1/PIGF
Controls													
IgG		**0.262**	0.126	**0.447**	0.015	-0.037	0.015	0.087	0.023	0.181	0.011	0.067	-0.016
IgA	**0.262**		0.043	**0.336**	-0.042	-0.068	0.013	-0.048	0.011	0.088	-0.072	0.084	0.116
IgM	0.126	0.043		0.136	0.078	-0.153	0.116	0.057	-0.058	0.052	-0.091	0.058	**0.228**
IgG1	**0.899**	0.109	0.054	**0.350**	0.079	-0.059	0.061	0.041	0.067	0.148	0.010	0.169	-0.042
IgG2	**0.718**	**0.302**	0.119	**0.405**	-0.072	-0.013	-0.029	0.140	-0.008	0.141	-0.036	-0.073	0.064
IgG3	**0.467**	0.185	0.041	**0.238**	0.097	0.108	-0.187	-0.091	**-0.199**	**-0.179**	-0.043	0.039	-0.027
IgG4	**0.430**	0.120	0.130	**0.260**	-0.115	-0.062	-0.047	0.007	-0.017	-0.011	0.042	-0.005	-0.025
cFLC	**0.447**	**0.336**	0.136		0.139	0.100	**0.204**	0.160	0.066	0.110	0.147	-0.107	0.026
sFLT-1/PIGF ratio	-0.016	0.116	**0.228**	0.026	-0.051	-0.048	**0.399**	**0.374**	**0.379**	**0.203**	0.050	-0.109	
PE Patients													
IgG		**0.364**	**0.211**	**0.299**	**0.300**	**0.225**	-0.050	-0.136	0.018	0.011	-0.121	**0.350**	0.016
IgA	**0.364**		**0.274**	**0.413**	0.049	-0.067	0.065	-0.018	0.037	0.023	-0.100	0.116	0.144
IgM	**0.211**	**0.274**		0.164	0.144	0.009	0.043	-0.193	-0.076	-0.014	0.060	0.081	0.020
IgG1	**0.937**	**0.332**	0.126	**0.317**	**0.371**	**0.225**	-0.062	-0.155	-0.015	-0.049	-0.132	**0.361**	0.009
IgG2	**0.719**	0.208	0.150	0.130	0.153	**0.265**	-0.173	-0.085	0.058	-0.015	-0.056	0.191	-0.115
IgG3	**0.384**	0.021	**0.293**	-0.007	0.167	0.181	-0.025	-0.049	-0.033	-0.007	0.102	**0.261**	-0.108
IgG4	**0.516**	0.191	-0.027	0.118	0.101	0.066	-0.154	-0.123	0.538	-0.105	-0.105	0.065	-0.013
cFLC	**0.299**	**0.413**	0.164		0.094	0.143	**0.232**	0.024	-0.163	0.195	0.166	-0.020	-0.045
sFLT-1/PIGF ratio	0.016	0.144	0.020	-0.045	***-0.276***	***-0.409***	**0.572**	**0.323**	**0.455**	**0.438**	-0.057	***-0.260***	

Correlations are reported as Spearman's rho coefficients, with bold values significant at p<0.05, and significant negative correlations in italic.

3.2.10 Independent Markers of Pre-eclampsia

Multivariable analysis was performed to determine whether the relationship of the humoral markers associated with PE was independent. The study markers associated with PE on univariable analysis, in addition to variables recognised as clinical risk factors for PE, such as patient BMI, were considered for inclusion in the model. Significant independent predictors of PE were, with OR reported as per unit change, sFLC concentration (OR: 1.11, p<0.001), IgG1 levels (OR: 0.62, p=0.024) and sFLT-1/PIGF (OR: 1.04, p<0.001) as shown in Table 3-5. First pregnancy (OR: 7.02, p<0.001) was an important independent risk factor for PE. After accounting for these factors, serum IgG3, creatinine, cystatin-C, B2-M, UA, C4, hs-CRP, patient age, BMI, ethnicity and twin pregnancy were not found to be significant independent markers of PE in this study population.

Table 3-5: Multivariable analyses to identify independent markers of Pre-eclampsia

	Odds Ratio (95% CI)		
Variable	***Per Unit Increase***	***Per IQR Increase***	**p-Value**
First Pregnancy	7.02 (2.90-16.99)	*N/A*	**<0.001**
IgG1 (g/L)	0.62 (0.41-0.94)	0.48 (0.26 - 0.91)	**0.024**
cFLC (mg/L)	1.11 (1.05-1.16)	3.07 (1.79 - 5.28)	**<0.001**
sFLT-1:PIGF ratio	1.04 (1.02-1.05)	7.37 (3.09 - 17.57)	**<0.001**

The factors considered for inclusion were patient age, BMI, ethnicity, first pregnancy, twin pregnancy, combined cFLC levels, serum albumin, B2-M, serum creatinine, UA, cystatin-C, C4, all four IgG subclasses and the sFLT-1b:PIGF. A binary logistic regression model was produced, with Pre-eclampsia as the dependent variable, and a forwards stepwise approach used to select variables for inclusion. Two sets of odds ratios are reported. The first are per unit increase in the factor (or for first pregnancy vs. previous pregnancies) and the second are for an increase of a magnitude equivalent to the interquartile range of the factor. Bold p-values are significant at p<0.05.

Without the addition of the angiogenic markers to the multivariable model, B2-M was found to be an independent predictor of PE (OR: 2.86, p<0.001) alongside sFLC (OR: 1.06, p=0.001), UA (OR 1.41, P= 0.045), serum albumin (OR 0.087, p=0.016) and first pregnancy (OR: 8.66, P<0.001). The change in the model with the introduction of the sFLT-1-1/PIGF ratio is likely explained by the ratio displaying a strong correlation with B2-M levels (rho: 0.586, p<0.001), but not sFLC (rho=0.137, p=0.060) as illustrated in Figure 3-4.

3.2.11 Relationship of Study Markers with Adverse Pregnancy Outcome

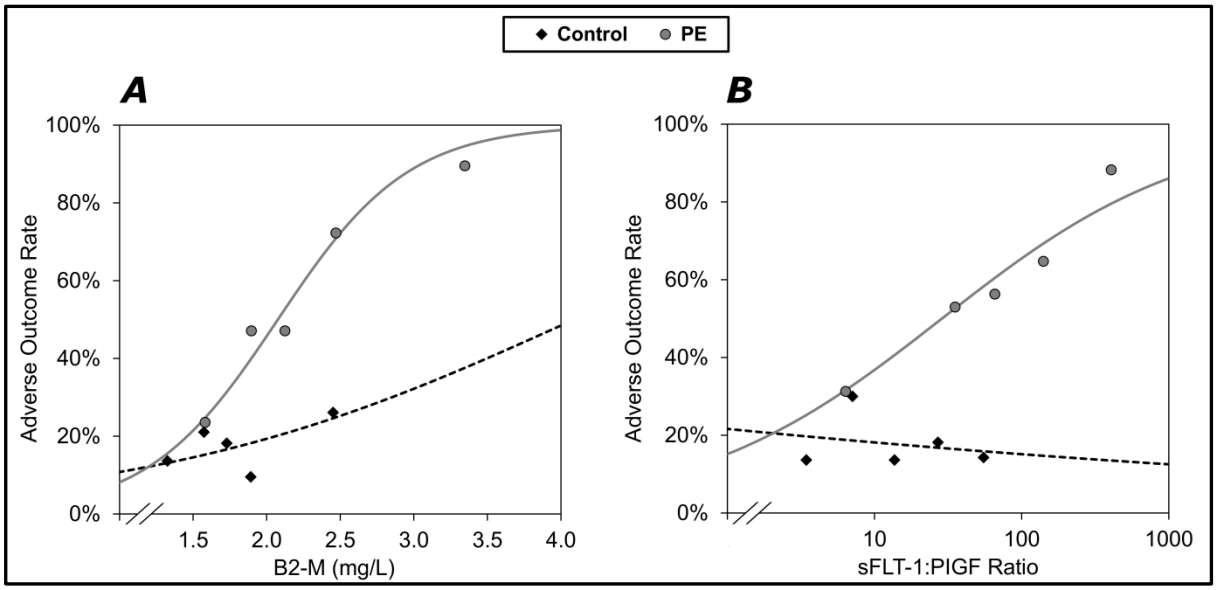
The overall rate of adverse clinical outcomes, defined in table 3-1, was significantly higher in PE than controls (57% vs. 18%, p<0.001), with each of the five events included in this composite outcome also being significantly more common in PE (Table 3-6). There were no intrauterine deaths or still births in either study group. Within the PE and control subgroups, associations between adverse clinical outcomes and the biomarkers previously found to be associated with PE were assessed. None of the factors considered were found to be associated with adverse outcomes in the control group. However, for patients with PE, serum creatinine (p=0.019), B2-M (p<0.001), UA (p=0.003) and sFLT-1/PIGF concentrations (p=0.001) were all significantly higher in those who developed adverse outcomes. The associations between adverse events and both B2-M and the sFLT-1/PIGF ratio are demonstrated graphically in Figure 3-5. In the PE group, there is a progressive increase in the rates of adverse outcome with increasing B2-M and sFLT-1/PIGF ratio. For B2-M, this trend is less pronounced in the control group, whilst no association between sFLT-1/PIGF ratio and adverse event is evident in the control group.

Table 3-6: Comparison of markers for those with and without adverse outcome

	PE group			Control group		
	Adverse (n=50)	***Non adverse (n=38)***	***p-Value***	***Adverse (n=19)***	***Non adverse (n=88)***	***p-Value***
Serum creatinine (umol/L)	67 (59-80)	62 (53-68)	**0.019**	58 (53-64)	55 (48-60)	0.122
Combined sFLC (mg/l)	28.8 (23.7-37.6)	30.3 (22.7-36.4)	0.876	24.9 (20.3-29.5)	24.0 (20.8-29.2)	0.702
UA (mg/dL)	5.8 (5.0-7.2)	4.7 (4.1-5.9)	**0.003**	4.1 (3.8-5.4)	4.4 (3.7-5.1)	0.935
B2-M (mg/L)	2.4 (2.1-3.0)	1.9 (1.7-2.2)	**<0.001**	1.7 (1.6-2.1)	1.7 (1.5-1.9)	0.517
Serum Albumin (g/L)	32.5 (±4.1)	33.6 (±4.1)	0.605	34.9 (±1.8)	34.8 (±2.7)	0.334
IgG1 (g/L)	3.97 (±1.19)	4.33 (±1.17)	0.603	4.56 (±1.22)	4.50 (±1.20)	0.805
sFLT-1/PIGF ratio	99.4 (44.3-251.7)	39.8 (10.0-95.4)	**0.001**	10.4 (5.6-25.6)	14.9 (6.3-28.9)	0.737

Data are reported as mean ((±SD) or median (IQR) with p-values from independent t-tests and Mann-Whitney tests respectively. Bold p-values are significant at p<0.05.

Figure 3-5: Association of clinical adverse events with levels of B2-M and the sFLT-1/PIGF ratio



Lines represent binary logistic regression models, treating the markers as continuous covariates. The sFLT-1:PIGF ratio was log10-transformed prior to the regression analysis to improve model fit. Points are plotted at the midpoints of the quintiles for each marker for reference. There is a progressive increase in the rates of adverse outcome in the PE group with increasing B2-M and sFLT-1/PIGF ratio.

3.3 Discussion

3.3.1 Summary of Findings

88 otherwise healthy women presenting with PE were compared with 107 women with normal term pregnancies. There were differences demonstrated in humoral system markers between the two groups with lower circulating IgG, IgG1, IgG3 and higher sFLC concentrations observed in women with PE compared to the control group. Levels of IgA and IgM were similar in both groups. Pre-eclampsia was also associated with other evidence of immune system disturbance indicated by raised hs-CRP and B2-M levels, low C4 (with normal C3). Furthermore, women with PE compared to controls had an increased sFLT-1/PIGF ratio (AUROC =0.81), thus demonstrating an anti-angiogenic profile in keeping with existing literature [91, 100, 255-259]. Immunoglobulin levels were in general not correlated with renal function, inflammatory nor angiogenic system markers, whereas sFLC levels showed a relationship with renal function and inflammatory markers.

Multivariable analysis identified first pregnancy, a higher sFLT-1/PIGF ratio, increased sFLC levels and lower serum IgG1 levels to be independently associated with PE. On a separate multivariable model, without the addition of angiogenic factors, increased B2-M, sFLC, UA and reduced serum albumin were also independent markers of PE. Neither serum creatinine nor cystatin-C were associated with PE in either multivariable model, suggesting that B2-M and UA are perhaps better markers of kidney function, than these two markers, in pregnancy complicated by PE.

The strongest diagnostic accuracy for PE was provided by sFLT-1/PIGF ratio and B2-M levels, with both providing a similar AUROC (p=0.184). IgG1 and sFLC were independently associated with PE on multivariable analysis and, thus, humoral markers along with B2-M,

may potentially, in combination with angiogenic proteins, improve the test performance of the angiogenic proteins as diagnostic biomarkers in PE. The diagnosis of PE was already established in our cohort on clinical criteria and, thus, there would be limited usefulness for diagnostic biomarkers in this particular population. However, they may aid earlier diagnosis in more challenging cases; leading to closer monitoring and earlier intervention to reduce risk of harm to mother and fetus. Further work on a separate validation cohort is required to explore this hypothesis.

As the blood samples in our cohort were taken at the time, and not before the onset of PE, it is not possible to establish whether these markers are predictive as well as associated with disease occurrence. Furthermore, it is also unclear whether the changes observed are a cause or consequence of PE. In chapter four, I look at whether the immune system and renal markers studied in this chapter are able to predict PE in early pregnancy. I will now describe the possible pathophysiological mechanisms for my findings, thereby supporting the role maternal immune dysregulation may play in PE.

3.3.2 Humoral Markers in Pre-eclampsia

3.3.2.1 Changes in sFLC levels

This chapter revealed a polyclonal increase in sFLC levels at the time of PE, compared to women experiencing a healthy pregnancy, which has not previously been reported. Concentrations of sLFCs are determined by the balance between production and renal clearance and, as discussed in the introduction, may act as a marker of inflammation [189] . Accordingly, levels of sFLC in this study were correlated with serum creatinine and cystatin-C (markers of renal function) and hs-CRP, which is a marker of inflammation. The relationship between sFLC and PE remained significant on multivariable analysis, after adjusting for both

factors. Potentially, this could mean that sFLC was a more sensitive marker of systemic inflammation in our patients than CRP. Alternatively, the increase in sFLC may reflect a pathophysiological mechanism independent of inflammation. Serum FLC levels and the sFLT-1/PIGF ratio were not significantly correlated with each other, and both remained significant predictors of PE when included together in a multivariable model, suggesting that they may represent independent disease pathways in PE.

Immunoglobulin light chains are catabolised by proximal tubules and thus an increase in levels seen in PE may be related to the consequence of tubular dysfunction [186]. Marked albuminuria which can occur in severe cases of PE is in keeping with disruption of the glomerular basement membrane as a result of glomerular endotheliosis [311]. The role that tubular dysfunction plays in PE is not well understood. There is published evidence supporting occurrence of tubular dysfunction at the time PE, with increased urinary levels of kidney injury molecule-1 (KIM-1); a marker of proximal tubule injury, demonstrated at the time of disease. Serum FLC may, therefore, be a better or earlier marker of tubular dysfunction compared to creatinine and cystatin-C, which would support the findings of the multivariable analysis.

Polyclonal sFLC secretion arises from B-cell activation and generalised immune system activation arising from infection, inflammation and autoimmune illness [187, 189, 312]. In this chapter, Kappa, not lambda free light chains, were found to be higher in the PE compared to control group. Why this is the case is unclear, as there was no evidence of monoclonal disease in the women with highest FLC levels. In patients with Multiple Sclerosis, levels of kappa but not lambda light chains have been found to be elevated and to have diagnostic and prognostic significance [313, 314]. Whether the selective increase in

the kappa FLC in PE is related to preferential overstimulation of cell types producing this isotype or reduced clearance of kappa light chains is unknown. Renal clearance is usually preferential for kappa over lambda light chains as kappa FLC are usually monomeric and lambda dimeric [186, 188]. As glomerular function reduces the kappa lambda ratio increases. As discussed in the introduction sFLC levels are associated with disease severity of a number of inflammatory conditions such as SLE [190-193]. Given their relationship with inflammation and renal dysfunction the potential for sFLC to act as a marker of adverse outcome in PE warrants further investigation.

3.3.2.2 Changes in Immunoglobulin levels

This chapter demonstrated similar levels of circulating IgM and IgA and a reduction of IgG (particularly subclasses IgG1 and IgG3) in PE versus healthy term pregnancy. As described in the introduction, there are very few studies comparing markers of humoral immunity in PE and healthy pregnancy [177-183]. These studies are not only limited in number, but also mostly old, recruited a small number of patients and often included women with gestational hypertension in the PE group. Nevertheless, they have also reported lower IgG concentrations in PE compared to healthy pregnancy, whereas findings for IgA and IgM levels have been more variable. Furthermore, these older studies used manual ELISA or radial diffusion techniques to measure Ig concentrations. In my study, concentrations of all markers, including Igs, were measured using a fully automated turbidimetry based technique. It has been reported that automated systems are perhaps more accurate when measuring Igs compared to radio- or immuno-assays [315].

An increase in sFLC levels would be expected to be observed in association with an increase in circulating levels of intact Ig. However, the findings of this chapter demonstrated a

reduction of IgG (particularly subclasses IgG1 and IgG3), with similar IgA and IgM levels, in women with PE compared to controls. Explanations for this discrepancy include a difference in half-life of sFLC compared to IgG, or increased placental transfer of the latter. The half-life of sFLC is hours compared to a 21-day half-life of the intact IgG molecule and, therefore, changes in sFLC may reflect more acute changes of the humoral system compared to Ig concentrations [186]. Lower IgG levels seen in the PE group may also be due to an increase in placental IgG transport in affected pregnancies, with IgG deposition reported in placentae from PE pregnancies [4]. There is preferential increase in maternal-fetal transfer of IgG1 over the other subclasses and IgG3 is known to have a shorter half-life compared to the other IgG subclasses [173, 316], which might explain why these specific IgG subclasses were reduced. The potential significance of IgG1 and IgG3 activating complement is discussed later on in this section.

Placental transfer of IgG is proposed to be dependent on levels of IgG in the maternal circulation, gestational age and placental integrity [170]. There is reduced efficiency of IgG transfer in low birth weight babies that are born at term and most IgG is transferred in the last 4 weeks of pregnancy. Since the PE cohort included women delivering before 36 weeks and the control cohort did not, this may partially explain some of the difference in maternal IgG concentrations observed. Women who miscarry with a history of previous recurrent miscarriages have also been shown to have lower levels of IgG compared to women who do not miscarry [317]. These findings suggest that difference in maternal humoral response maybe associated with adverse pregnancy outcome early on in pregnancy and not just in the setting of PE.

Both serum IgG and albumin are lost with heavy proteinuria, yet there was no evidence of a significant correlation between the degree of proteinuria and serum levels of any of the Igs in the PE group. Whilst some older studies have demonstrated IgG loss in urine in pregnancies complicated by proteinuria, this has not been a consistent finding [179, 181, 183]. This suggests alternative mechanisms could be involved in the low IgG levels observed in PE, which are independent of urinary protein loss. Another potential mechanism would be a reduction in systemic FcRN recycling and, thus, reduction in serum half-life of both albumin and IgG, as the half-life of both is extended by FcRN [164].

Although ethnicity was not found to be a significant risk factor for PE in my study, this may have been as a result of low statistical power, on account of the small sample size, as black ethnicity is recognised as a risk factor for PE [20]. There is also suggestion that black ethnicity is associated with more severe disease [318-321]. Interestingly, sFLC levels were higher in the non-white study subjects. Although post-hoc analysis only found this difference to be significant in the South Asian versus White population, this again maybe related to small study numbers in the other ethnic groups. Both healthy non-pregnant Black and South Asian subjects have been reported to have higher levels of circulating Igs compared to White individuals, in keeping with this pregnant study population [238, 322, 323]. Whether racial variations in Ig levels contribute to increased risk of inflammatory conditions, including PE, requires further investigation. There is little published literature on ethnicity and variation in sFLC levels. Liang et al. has reported a narrower reference range in a Han Chinese population [324]. Whereas Black patients are more pre-disposed to developing monoclonal gammopathy of undetermined significance (MGUS) and have been reported to have higher sFLC levels compared to their white counterparts [325].

3.3.3 C-reactive Protein and Inflammation in Pre-eclampsia

As discussed in the introduction, PE is associated with a pro-inflammatory state [60]. C-reactive protein is an acute phase reactant and increased production reflects inflammation and increased cytokine production [49]. A number of studies similar to mine have demonstrated pregnancies with PE are characterised by higher CRP levels compared to normotensive pregnancies, with further data suggesting that high levels of CRP also correlate with disease severity [49, 199, 200]. However, these previous studies have often been small, cross-sectional in design and often did not match women for appropriate confounding factors. For example, in a meta-analysis when difference in BMI between the PE and non-PE pregnancies was corrected, the relationship between PE and CRP was significantly modified [200]. The authors of the meta-analysis concluded that further high quality studies were required to assess the relationship of CRP with PE. In my study, BMI did not differ significantly between PE and control patients on group comparison, but women were not individually matched for this. The predictive accuracy was the lowest for hs-CRP of all the biomarkers considered, and it was not a significant marker of PE on multivariable analysis. This may be because sFLC and B2-M were, in comparison to CRP, superior markers of systemic inflammation.

There is evidence in the literature to support the potential involvement of CRP in the pathophysiology of PE; placentae from PE pregnancies produce more circulating CRP than those from non-PE pregnancies and injection of CRP in pregnant mice induces clinical features of PE (hypertension and proteinuria) [199]. This also results in increased sFLT-1 production in pregnant but not in non-pregnant mice. C-reactive protein has been demonstrated to directly cause endothelial damage [199, 326]. A correlation between

inflammatory and angiogenic system markers has been reported in another study, supporting a relationship between the two pathways in PE [71]. However, my study did not reveal a significant correlation between the sFLT-1/PIGF ratio and CRP, with the latter only correlated with sFLC levels.

3.3.4 Complement Activation in Pre-eclamspia

This study demonstrated lower C4 levels in the PE versus control group, as well as IgG1 and IgG3 levels, which are the IgG subclasses most strongly associated with complement activation. These findings lend support to activation of the complement pathway in PE with consumption of C4, IgG1 and IgG3 [171]. Low C4 levels at the time of PE have also been reported in other studies [226]. These findings are in keeping with an accumulating amount of evidence demonstrating increased maternal circulating concentrations of complement activation products C3a, C4a, Bb and C5a in PE versus non-PE pregnancies, indicative of activation of the complement system at the time of disease [217, 218, 223-226, 327-329]. Furthermore circulating and urinary concentrations of the terminal complement effector C5b-9, also known as the membrane attack complex and involved in cell lysis, are also raised. These findings support activation of the classical, alternative and possibly the lectin complement pathways at the time of disease. The findings in my study of reduced levels of C4 during PE favour activation of classical and lectin pathways, rather than the alternate pathway.

In contrast to my findings, Lynch et al. found that circulating angiogenic factors were not correlated with complement breakdown products, specifically complement-activation fragments Bb, C3a and C5b-9 [102]. However, their analysis was not split according to

controls and cases, as I found the correlation between the complement and angiogenic system markers was specific to the PE group. Guseh et al. did split their analysis by study group and reported urinary C5b-9 did not correlate with plasma-angiogenic factors, including sFLT-1 and PIGF, when cases and controls were combined [81]. However, they reported a negative correlation between plasma levels of C3a and sFLT-1 in PE cases, but not in the healthy controls, similar to my observations in this chapter. These findings suggest that the angiogenesis-related factors at the time of PE correlate with complement-mediated factors, which is not the case in healthy pregnancies. In a review on the subject, Denny et al. proposed that complement function is normal in pregnancy, but that excessive activation leads to poor pregnancy outcomes, including PE [330]. An acute and systemic inflammatory response, may also contribute to consumption of complement and thus reduced C4 levels [331].

3.3.5 B2-Microglobulin and Markers of Renal Function

B2-M along with sFLT-1/PIGF provided the strongest diagnostic accuracy for PE from the panel of biomarkers tested in this study and both were also predictive of adverse clinical outcome in the PE group. Both markers were also highly correlated with each other (rho: 0.586, $p<0.001$), which has not previously been reported. Other studies have also demonstrated higher B2-M concentrations at the time of PE [232-235]. Increased B2-M levels may just reflect worse kidney function in the PE group, as the molecule is freely filtered by the glomerulus and reabsorbed and catabolised by proximal tubular cells [235]. Thus B2-M maybe a superior marker of kidney function in pregnancy compared to the other renal markers. As expected I found that concentrations of B2-M were significantly correlated with serum creatinine, cystatin-C and UA. Saudan et al. demonstrated that

women with PE had higher levels of B2-M compared to controls [232]. However, fractional excretion of B2-M was similar, suggesting the difference may not be solely due to reduced β2-M excretion in PE [232]. Beta 2 microglobulin forms part of the FcRn receptor which is necessary for IgG recycling and placental transfer [164, 228]. Furthermore, serum levels of B2-M are increased in inflammatory states connected with activation of the lymphoid system [229-231]. Thus, other factors independent of renal function may lead to increased B2-M concentrations at the time of PE.

No markers were found to be associated with adverse outcome in the control group, although this may also have been a result of low statistical power, due to the low event rate in this group. The sFLT-1/PIGF ratio, UA and serum B2-M concentrations were associated with occurrence of adverse outcome in the PE group (maternal HDU admission, pre-term birth, SGA, low birth weight baby, or admission to the NNU). Since these markers were highly correlated with serum creatinine, the association of these markers with PE could be interpreted as surrogates of acute kidney injury. Whilst serum creatinine was only moderately higher in patients with adverse events in the PE group (median: 66.5 vs 62.0 umol/l respectively), the multivariable analysis suggests that creatinine and cystatin-C may not have been the best markers of kidney function in this study, as has been discussed already in the context of B2-M and sFLC levels.

Cystatin-C appeared to behave similarly to creatinine in my study, as both were associated with PE on univariate but not multivariate analysis. In a recent study the former was shown to correlate better with pre-term delivery in women with PE compared to serum creatinine levels [253]. In both the non-pregnant and pregnant setting, cystatin-C has not been definitively proven to be a superior marker of kidney function compared to creatinine.

However, it has the potential to be a promising marker in PE, as it is also a marker of inflammation and a predictor of cardiovascular disease independent of renal function in the non-pregnant population [253, 332, 333].

The relationship between sFLT-1 and PIGF levels and renal function has not been well established, with two studies showing higher levels of both markers in non-pregnant patients with CKD compared to those with normal renal function [288, 289]. This topic is further explored in chapter five. My study supports an increase in sFLT-1/PIGF ratio in the context of acute kidney injury in pregnancy in previously healthy women, without CKD.

Elevations in UA levels at the time of PE is an old and consistent finding, and was also seen in this chapter [250, 252]. However, UA lacks the required sensitivity and specificity to be used as a diagnostic marker [249]. It has been hypothesised that hyperuricaemia in PE arises from reduced renal clearance. I found UA levels were strongly correlated with renal function, with correlation coefficients of 0.54 with cystatin C, and 0.51 with creatinine (both $p<0.001$). Uric acid levels have also been shown to correlate with severity of glomerular endotheliosis [250, 334].

A number of studies, including a recent review by Bainbridge, have proposed that UA has a pathogenic role in PE through its ability to promote inflammation, oxidative stress and endothelial dysfunction, which have been demonstrated in in-vitro culture studies and hyperuricaemic animal models [252]. In keeping with the findings of this chapter, higher UA levels have been found to correlate with adverse clinical outcome in other studies also, namely pre-term birth, FGR and SGA [249, 250, 252, 335]. However, attempts at lowering

UA levels in PE, using pharmacological treatment, did not change the course of disease in two studies [249, 336, 337].

3.3.6 Biomarkers of a 'High-risk' Population?

Women with a history of hypertensive disorders of pregnancy have elevated UA concentrations decades after pregnancy, compared to women who had normotensive pregnancies, even after adjusting for traditional cardiovascular risk factors [251]. Hyperuricaemia and history of PE are both risk factors for hypertension, CKD and CVD, and both are also associated with insulin resistance and metabolic syndrome [17-19, 36, 252, 325, 338]. Elevated polyclonal sFLC levels have been found to be an independent risk factor for mortality and progression to end stage renal disease in patients with stages 3–5 CKD [189, 197] and are also associated increased mortality and cardiovascular events in patients without CKD [198]. Furthermore, polyclonal free light chain levels at the upper end of the normal range are predictive of mortality in the general population and this risk is independent of age, sex and renal impairment [339]. Similarly, outside pregnancy, elevated CRP is an independent predictor of CVD and is also a predictor of renal dysfunction [199, 340].

Metabolic syndrome is characterised by obesity, insulin resistance, dyslipidaemia and hypertension [60]. There is an overlap of inflammatory, metabolic, endothelial changes between metabolic syndrome and PE. A number of the markers that I tested may, therefore, characterise a 'high risk' population, pre-disposed to development of metabolic syndrome and related conditions, as well as PE. Underlying endothelial disease may be present before and/or accelerated by pregnancy, with pregnancy acting as a 'metabolic stress test'. It could also be the case that pregnancy temporarily exposes underlying subclinical disease, which

then resurfaces in later life [36]. This chapter has not assessed whether changes in these markers are present in early or pre-pregnancy; however, early pregnancy markers in women who develop PE will be covered in the next chapter.

3.3.7 Limitations of Study

The aim of this study was to use routinely available laboratory markers to characterise immunological changes at the time of onset of PE, and find support for their involvement in the pathophysiology of PE, specifically changes related to the humoral system, but also changes characteristic of other immune pathways. Since PE had already occurred in the study group, we were not able to look at predictive capability nor comment on causality. As there was a higher prevalence of PE in the overall study group compared to the general population, negative or positive predictive values were not calculated. However, relative comparisons between the study markers were performed, which is what the study set out to do.

The study design has limitations which are acknowledged. Whilst all women with a history of CKD or chronic illness were excluded from both groups; patients were not individually matched. It was a cross-sectional design rather than a prospective recruitment study. There was selection bias due to the nature of recruitment, with a higher number of primigravida women in the PE versus control group, and a small difference in gestation at recruitment between the two groups. Women in the control group were recruited at the time of their elective caesarean section which is usually booked from 38 weeks onwards. A significant proportion of women in the control group would also have elected for caesarean section deliveries due to factors related to earlier pregnancies, such as previous surgical deliveries and thus contribute to the higher proportion of multipara women in this group. The median

gestation in the PE group was 37 weeks versus 39 weeks, and whilst not ideal, this is not of huge clinical importance as both gestations are 'at term'.

Furthermore, PE cases were defined on the basis of hypertension and proteinuria and did not include women with atypical presentations of the disease for whom the findings may have differed. This allowed the study to focus on women in whom the diagnosis was clear, thereby preventing inclusion of women with potentially alternative diagnoses. It has also been suggested that atypical presentations of PE have different pathophysiological mechanisms, which may therefore mask differences between groups if included [341].

3.3.8 Conclusions

The findings of this chapter support changes in multiple immune system pathways in PE, specifically the humoral system, which appear to be independent of angiogenic disturbances. A better understanding of the timing and sequence of these changes could potentially lead to identification of supportive, diagnostic and possibly to novel treatment strategies. Further work is required to assess whether humoral markers can add to the predictive capability of sFLT-1/PIGF in PE and whether B2-M can be used as an alternative biomarker to angiogenic factors in this setting, given its strong correlation with the former.

4 PREDICTIVE BIOMARKERS IN EARLY PREGNANCY FOR DEVELOPMENT OF PRE-ECLAMPSIA

Chapter three demonstrated perturbations in several immunological, including humoral, system markers at the time of PE onset compared to non-PE pregnancies. However, I was unable to establish whether the differences were present before disease onset and, in particular, during early pregnancy. If differences in the marker concentrations are evident in early pregnancy, this may suggest that healthy women with a certain 'immune phenotype' are predisposed to developing PE. Alternatively these women may exhibit an abnormal immunological response in early pregnancy, which could predispose them to developing PE later on in pregnancy.

4.1 Objectives:

The purpose of this study was to determine whether healthy women who developed PE in their pregnancy have any differences in the clinical markers assessed in chapter three - Igs, IgG subclasses, albumin, hs-CRP, B2-M, UA, cystatin-C and creatinine - in early pregnancy (first trimester) compared to matched healthy pregnant women who do not develop PE.

4.2 Results

4.2.1 Demographics of Population and Validation of Coding

During the study period, 7354 pregnancies were screened, as detailed in chapter two. A total of 186 patients, following application of inclusion and exclusion criteria, were identified as cases (developed PE) and early pregnancy samples for these women were retrieved. These pregnancies were matched with pregnancies where PE did not develop for age, ethnicity, BMI and parity, as described in chapter two. 432 controls meeting the study

inclusion and exclusion criteria were identified. One case and one control required subsequent exclusion from the study, as these patients had a creatinine value above 90umol/l. This left a total of 185 women who developed PE and 431 controls. The gestation at which the samples were taken was median (IQR) 12.1 (11.4-12.9) weeks. Matching was not possible for 11 cases of PE. 174 cases of PE were matched with one or more control, 150 cases with two or more controls and 106 cases with at least 3 controls. Table 4-1 demonstrates that the overall PE and control group pregnancies did not differ significantly in age, ethnicity, BMI or parity.

Review of clinical notes for ten cases and ten controls was undertaken in order to validate the accuracy of coding of PE. One of the cases did not develop PE in the pregnancy pertinent to the study, her previous pregnancy was complicated by PE. All of the controls were correctly coded and had not developed PE during their pregnancy.

4.2.2 Predictors of Pre-eclampsia

Repeated measures ANOVA testing was performed to identify whether there was a difference in concentration of study markers in the pregnancies later complicated by PE compared to the matched healthy pregnancies. All study markers (IgG/A/M, IgG subclasses, cFLC, creatinine, B2-M, UA, cystatin-C, albumin and C3/4) were considered in the analysis. The results of this analysis are displayed in Table 4-2. All variables required log-transformation with the exception of IgG, IgG1, IgG3, UA and albumin, which were normally distributed. Only circulating IgM was found to be significantly different in the cohort where PE subsequently developed compared to the matched control group, with a lower mean concentration of 0.95 (95% CI 0.88-1.02) versus 1.06 (0.99-1.14)g/L (p=0.012) respectively.

Table 4-1: Comparison of age, ethnicity, BMI And parity between the PE And control group

	Subsequent PE (n=185)	**Controls (n=431)**	**P value**
Age (years)	29.1 ±6.1	28.8 ±5.5	0.536
Ethnicity % (n):			0.857
Gestation of sample (week)	12.1 (11.4-12.9)	12.1 (11.4-12.9)	0.416
White	56.3 % (104)	59.1% (255)	
Black	9.8% (18)	7.2% (31)	
South Asian	20. % (38)	22.9% (99)	
Other	7% (13)	5.9% (25)	
Unknown	6.4% (12)	4.9% (21)	
BMI (kg/m^2)	28.1% ±5.2	27.2%±5.3	0.115
Nulliparous % (n)	43.2% (80)	42.9% (185)	1.00

All pregnancies in both groups are included. Data are represented either are mean (±SD) or as median (IQR), with p-values derived from independent sample t-tests and Mann Whitney tests, respectively. Categorical data are reported as percentages, with p-values generated from Chi squared tests.

Conditional binary logistic regression analysis was then performed to determine whether any of the study markers were predictors of PE (Table 4-3). A lower IgM level was again the only marker that was significantly different on univariate regression analysis and was predictive of PE developing (OR 0.62, 95% CI 0.41-0.93, p=0.021). IgM remained an independent predictor of PE on multivariable regression analysis, where all markers were entered into the multivariable model (p=0.021). The analysis was repeated in the cohort of three matched controls per case as a sensitivity analysis, with the results not changing and IgM remaining an independent predictor of PE.

Table 4-2: Comparison of early pregnancy markers in the Pre-eclampsia versus matched control group (with two controls matched with each case)

Marker	Subsequent PE = 150	Control = 300	P value
IgG g/L	9.63 (9.23-10.03)	9.52(9.17-9.88)	0.621
IgA g/L	1.61 (1.49–1.72)	1.62 (1.52-1.74)	0.844
IgM g/L	0.95 (0.88-1.02)	1.06 (0.99-1.14)	**0.012**
IgG1 g/L	5.67 (5.43-5.92)	5.59 (5.34-5.84)	0.575
IgG2 g/L	3.28 (3.08-3.50)	3.35(3.15-3.55)	0.589
IgG3 g/L	0.77± (0.71-0.83)	0.76 (0.71-0.82)	0.868
IgG4 g/L	0.23 (0.20-0.27)	0.23 (0.20-0.27)	0.969
cFLC mg/L	23.12 (22.18-24.10)	23.67 (22.39-24.38)	0.640
Creatinine umol/l	52.97 (51.29-54.70)	52.31 (50.70-53.83)	0.535
B2-M mg/L	1.25 (1.21-1.29)	1.23 (1.19-1.27)	0.454
UA mg/dL	2.83 (2.71-2.94)	2.79 (2.66–2.91)	0.588
Cystatin-C mg/L	0.55 (0.53-0.57)	0.56 (0.54-0.57)	0.400
Serum albumin g/L	39.78 (38.91-40.65)	38.93 (38.93-40.40)	0.807
C3 g/L	1.77 (1.71-1.83)	1.73 (1.67-1.79)	0.275
C4 g/L	0.29 (0.27-0.31)	0.28 (0.27-0.30)	0.377
hs-CRP mg/L	4.57 (3.91-5.35)	4.42 (3.70-5.22)	0.644

Mean concentrations (with 95% CI) are reported in the PE versus matched control group with p values displayed (bold if significant), these were calculated using repeated measured ANOVA analysis. For variables which required log-transforming geometric rather than arithmetic means are reported.

Table 4-3: Early pregnancy predictors of subsequent Pre-eclampsia

Marker	OR	95% CI	P value
IgG	1.03	0.93-1.13	0.609
IgA	0.90	0.29-2.79	0.855
IgM	0.62	0.41-0.93	**0.021**
IgG1	1.04	0.91-1.20	0.572
IgG2	0.70	0.20-2.51	0.584
IgG3	1.05	0.59-1.89	0.860
IgG4	1.04	0.63-1.74	0.869
cFLC	0.67	0.10-4.35	0.672
Serum creatinine	2.52	0.20-23.25	0.526
B2-M	2.52	0.22-29.4	0.461
UA	1.07	0.82-1.39	0.612
Cystatin-C	0.32	0.02-4.66	0.403
Serum albumin	1.01	0.96-1.05	0.804
C3	1.41	0.77-2.57	0.264
C4	1.95	0.48-8.02	0.354
hs-CRP	1.12	0.68-1.86	0.663

Analysis was performed using univariable conditional binary logistic regression analysis. OR and 95% CI are displayed alongside p values (bold if significant).

4.2.3 Early-onset Pre-eclampsia

Early onset PE (<34 weeks' gestation) developed in 16 of the 1:2 matched pairings. These pregnancies were analysed separately as a subgroup analysis. There was no difference in the concentration of any of the markers in pregnancies subsequently complicated by early onset PE compared to their matched controls, nor were any of the markers predictive of this on regression analysis. IgM levels were lower, but not significantly so, in the cohort developing early onset PE compared to matched controls with mean concentrations of 0.89 (95% CI 0.71-1.12) vs. 1.03 (0.90-1.18) g/L (p=0.347).

4.2.4 Predictors of Adverse Pregnancy Outcome

Table 4-4 compares outcome data in the pregnancies complicated by PE and those that were not. There were no neonatal or in-utero deaths in either group. There were four still births, two each in group. There were more pre-term births in the pregnancies where PE developed compared to the cohort without PE developing (22.2% versus 6.0 % respectively, p=0.001). The composite adverse pregnancy outcome (delivery < 34 weeks, FGR, LBW, VLBW, still birth or neonatal death) occurred in 23.8% compared to 11.4% respectively in these two groups (p<0.001).

Independent sample t tests were performed to identity if the concentrations of any of the study markers were different in those with and without subsequent adverse pregnancy outcome occurring (Table 4-5). In the group in whom PE later developed, IgG concentrations were higher in the women with, compared to those without, a composite adverse outcome developing, with mean concentrations (95% CI) of 10.35 (9.47-11.23) compared to 9.37 (9.33-9.41)g/L (p=0.018). Furthermore, a higher IgG concentration was predictive of adverse pregnancy outcome on univariate regression analysis (OR 1.19, 95% CI 1.03-1.37,

p=0.020) as shown in Table 4-5. On multivariable regression analysis, with all markers considered, IgG remained an independent predictor (OR 1.28, 95% CI 1.08-1.50, p=0.004) of adverse outcome (Table 4-6). In the control group where PE did not develop, none of the study markers were significantly different or predictive in those who did or did not develop adverse pregnancy outcome.

Table 4-4: Comparison of adverse pregnancy outcomes in subsequent PE versus control patients

	Subsequent PE (n=185)	Control (n=431)	P value
Stillbirths % (n)	1.1% (2)	0.5% (2)	0.382
Neonatal death % (n)	0% (0)	0% (0)	-
Gestation at delivery (weeks')	38.0 (37-39)	39.0 (39-40)	**<0.001**
Pre-term < 37 weeks % (n)	22.2 (41)	6.0 (26)	**0.001**
Very pre-term < 34 weeks % (n)	11.4 (21)	2.6 (11)	**<0.001**
Composite adverse pregnancy outcome* % (n)	23.8 (44)	11.4 (49)	**<0.001**

** comprises delivery < 34 weeks, FGR, LBW, VLBW, in-utero or neonatal death. Gestation at delivery is represented as median with IQR in brackets with p-values derived from a Mann-Whitney test. Categorical data are reported as percentages, with p-values generated from chi-squared tests. Bold p-values are significant at p<0.05.*

Table 4-5: Difference in marker concentrations in those with and without adverse pregnancy outcome

	Subsequent PE without adverse outcome	Subsequent PE with adverse outcome	P value	Control without adverse outcome	Control with adverse outcome	P value
IgG g/L	9.37 (9.33-9.41)	10.35 (9.47-11.23)	**0.018**	9.51 (9.06-9.96)	9.41 (8.73-10.09)	0.778
IgA g/L	1.62 (1.50-1.75)	1.56 (1.38-1.75)	0.769	1.63 (1.57-1.70)	1.58 (1.52-1.76)	0.476
IgM g/L	0.96 (0.89-1.04)	0.97 (0.86-1.10)	0.906	1.04 (0.99-1.09)	1.16 (1.02-1.32)	0.102
IgG1 g/L	3.24 (2.97-3.48)	3.55 (3.05-4.05)	0.066	5.60 (5.45-5.75)	5.66 (5.13-6.19)	0.702
IgG2 g/L	3.45 (3.24-3.67)	3.93 (3.42-4.51)	0.169	3.32 (3.20-3.45)	3.05 (2.68-3.47)	0.137
IgG3 g/L	0.76 (0.70-0.82)	0.85 (0.72-0.98)	0.205	0.78 (0.74-0.82)	0.71 (0.61-0.81)	0.202
IgG4 g/L	0.22 (0.19-0.26)	0.26 (0.20-0.34)	0.304	0.22 (0.20-0.24)	0.25 (0.19-0.33)	0.304
cFLC mg/L	23.20 (22.30-23.14)	23.74 (21.95-25.68)	0.611	23.38 (22.78-23.99)	23.20 (21.49-25.04)	0.843
Creatinine umol/l	53.3 (51.43-55.24)	53.3 (50.01-56.80)	0.998	52.36 (51.41-53.32)	53.32 (50.40-56.41)	0.518
B2-M mg/L	1.25 (1.21-1.29)	1.26 (1.17-1.35)	0.911	1.23 (1.21-1.25)	1.22 (1.20-1.25)	0.696
UA mg/dL	2.80 (2.68-2.92)	2.88 (2.66-3.10)	0.546	2.77 (2.70-2.85)	2.88 (2.66-3.10)	0.345
Cystatin-C mg/L	0.55 (0.53-0.57)	0.56 (0.53-0.60)	0.460	0.56 (0.55-0.57)	0.57 (0.54-0.60)	0.302
Albumin g/L	39.41 (38.49-40.32)	39.45 (38.05-40.85)	0.966	39.89 (39.44-40.34)	39.57 (38.17-40.97)	0.647
C3 g/L	1.76 (1.70-1.82)	1.82 (1.69-1.95)	0.365	1.72 (1.68-1.76)	1.71 (1.61-1.81)	0.859
C4 g/L	0.30 (0.28-0.32)	0.28 (0.25-0.32)	0.475	0.28 (0.27-0.29)	0.29 (0.26-0.31)	0.298
hs-CRP mg/L	4.58 (3.92-5.36)	4.56 (4.02-5.17)	0.983	4.35 (3.91-4.84)	4.63 (3.57-6.00)	0.684

The two groups were considered separately and analysis was performed using independent sample t tests. Geometric means for logged variables and arithmetic means for non-logged variables are reported (with 95% CI).

Table 4-6: Predictors of adverse pregnancy outcome in the subsequent PE and control group

	OR (subsequent PE)	CI	P value	OR (controls)	CI	P value
IgG	1.19	1.03-1.37	**0.020**	0.98	0.85-1.13	0.777
IgA	0.76	0.13-4.63	0.767	0.52	0.09-3.11	0.475
IgM	1.11	0.20-6.28	0.906	3.64	0.77-17.20	0.103
IgG1	1.23	0.98-1.55	0.068	1.04	0.85-1.26	0.701
IgG2	4.24	0.54-33.28	0.170	0.26	0.04-1.55	0.138
IgG3	1.74	0.74-4.13	0.207	0.55	0.22-1.38	0.202
IgG4	1.58	0.66-3.76	0.302	1.46	0.70-3.21	0.303
cFLC	2.17	0.11-41.83	0.609	0.76	0.05-11.21	0.842
Creatinine	1.00	0.02-42.89	0.998	3.57	0.08-167.82	0.517
B2-M	1.25	0.91-1.25	0.910	0.49	0.01-17.41	0.695
UA	1.16	0.72-1.88	0.544	1.20	0.82-1.758	0.344
Cystatin-C	3.66	0.19-69.24	0.388	0.28	0.24-143.42	0.277
Albumin	1.00	0.94-10.66	0.966	0.99	0.92-1.05	0.646
C3	1.50	0.62-3.63	0.364	0.92	0.39-2.18	0.859
C4	0.44	0.45-4.20	0.474	3.24	0.36-29.48	0.297
hs-CRP	0.99	0.44-2.21	0.983	1.15	0.59-2.23	0.683

Analysis was performed using forward binary logistic regression analysis. OR and 95% CI are displayed alongside p values (bold if significant).

4.3 Discussion

4.3.1 Summary of Findings

In this chapter of the thesis, it has been demonstrated that in early pregnancy (average 12 weeks' gestation), healthy pregnant women who subsequently develop PE have lower levels of circulating IgM compared to matched healthy pregnant women who do not. There were no differences observed in the concentrations of IgG, IgA, IgG subclasses, renal function (measured with serum creatinine and cystatin-C), B2-M, UA, hs-CRP, C3/4 and albumin. On subgroup analysis, in the group of women who specifically developed early onset PE, the difference in IgM levels did not reach significance. Since this group was much smaller (16 matched pregnancies), the numbers may have been too small to detect differences of statistical significance. IgG levels were also higher and independently predictive of adverse pregnancy outcome developing in the cohort of women who developed subsequent PE.

In chapter three, it was discussed that increased baseline levels of sFLC, UA and CRP may highlight a high risk population predisposed to developing PE, as well as other related conditions associated with endothelial dysfunction. The findings in this chapter do not support this hypothesis and healthy pregnant women who later developed PE had similar levels of these markers compared to matched healthy women who did not develop PE. My results suggest that there may instead be early differences present in the humoral system in healthy pregnant women destined to develop PE compared to pregnant women who do not develop PE. This difference may exist prior to pregnancy or, alternatively, may reflect a difference between the two groups in their early immune response to pregnancy. Women with pre-existing comorbidities including autoimmune illness should have been excluded from this study and thus if differences in immune profile were present before pregnancy,

these are not thought to be associated with clinical disease. I will now discuss the markers in turn and compare my findings to those published in the literature.

4.3.2 Low IgM Levels – Potential Significance

I was able to demonstrate in chapter three, that immunoglobulin levels tend to run on the lower side of normal during pregnancy compared to outside pregnancy, and IgG levels are further reduced in PE in keeping with other studies [172, 174, 179, 180, 342]. This chapter identified low IgM as an independent predictor of development of PE. There does not appear to be published data on immunoglobulin levels in early pregnancy, in the context of later development of PE.

In a Nigerian study, samples were taken immediately following delivery, higher IgM levels were reported in women who developed hypertensive complications compared to those with healthy pregnancies [181]. Kestlereova, in their study from the Czech Republic, also demonstrated PE pregnancies were associated with higher IgM levels compared to healthy third trimester pregnancies [331]. These findings differ to my findings in chapter three, where IgM levels were not different between PE and control pregnancies. Furthermore, in a historical study from Taiwan, women with severe PE, including with eclampsia, were found to have lower concentrations of circulating IgM, in addition to IgG, compared to women with healthy term pregnancy [183].

Longitudinal trends of Ig levels in normal pregnancy have been reported. Miller et al. compared 16 healthy pregnant women from 7-37 weeks' gestation to 54 healthy non-pregnant women [342]. The authors reported that IgA and IgG levels started to rise in early pregnancy and decreased after the 17th week, with IgG levels falling below pre-pregnancy

levels. IgM levels in their study fell immediately in early pregnancy, more so than later on in pregnancy, and levels rose rapidly following delivery. In contrast, a Nigerian study of 75 pregnant women, 25 at each of the three trimesters, showed an increase in IgM levels from the first to third trimester compared to 25 non pregnant controls [174]. Ogbimi et al. in a separate Nigerian study, demonstrated that concentrations of IgM were lowest in the first trimester of healthy pregnancy, IgG levels fell throughout pregnancy and IgA levels remained stable. There is therefore data to suggest that an early fall in IgM levels maybe a normal response in healthy pregnancy, and this may be exaggerated in pregnancies where PE later develops. As was discussed in the introduction and chapter three, there are variations in Ig levels according to ethnicity and this may also contribute to differences in findings reported in the literature.

IgG transfer from the mother to the fetus is thought to occur from 13 weeks' gestation [170] and so any changes in Ig levels arising before then are unlikely to be due to placental transfer. The lower circulating IgM levels observed in this chapter may be related to increased urinary excretion, although this could not be determined in my study, as I did not collect urine samples. Higher urine IgM excretion, outside pregnancy, is associated with worse renal outcomes and increased cardiovascular disease [129]. In a recently published study based on a Mexican cohort of CKD pregnancies, higher urinary IgM levels, at an average gestational age of 20 weeks, were shown to be associated with increased risk of adverse pregnancy outcomes, including PE. These findings suggest that lower circulating IgM levels may be compatible with urinary losses; however, I did not include women with CKD in my study and, therefore, these study findings cannot be extrapolated to mine. Furthermore, in the Taiwanese study described above, concentrations of IgG but not IgM

were higher in urine samples taken from women with PE compared to healthy pregnant women at term [183]. Levels of IgM in this study were increased in cord blood of women with PE, whereas concentrations of IgG and IgA were not. The authors suggested that the reduction of circulating maternal IgM levels was perhaps due to 'active immunologic disease' and not due to increased transfer of IgM to neonates.

IgM is recognised to activate the complement system through the classical pathway [59, 180, 219]. It has been demonstrated in a study on rats that placental ischaemia is associated with increased placental IgM and C3 deposition [59]. In the same study, IgM deposition was also found to be increased in the kidney, which has been seen in other studies [343]. Furthermore, Buurma et al. showed complement deposition was present in half of the placentae taken from women with PE, with 60% of these samples revealing immune deposits, which were predominately IgM [219]. Therefore, IgM specifically may play a more important role in the pathogenesis of PE related to complement activation compared to the other subclasses of Ig.

4.3.3 C-Reactive Protein an Early Risk Marker in Pre-eclampsia

C-reactive protein is an early marker of the inflammatory process [199] and chapter three, along with other studies, demonstrated that levels of CRP are increased at the time of PE compared to healthy term pregnancies [49, 200, 206-208]. Higher CRP levels have also been shown to be correlated with disease severity and mean arterial blood pressure in PE [49, 199, 206, 344]. In this chapter, hs-CRP was not raised in the first trimester in women who subsequently developed PE, suggesting that the increase in inflammation associated with PE occurs after the first trimester.

A number of studies have examined the ability of CRP to predict PE, with conflicting results [49, 200, 208, 345-347]. In an Iranian study of 394 first trimester pregnancies, in which 10.7% developed PE, the authors reported women with PE had higher early pregnancy CRP levels compared to the normotensive group (7.06 vs. 3.6 mg/L respectively, $p<0.001$) [49]. Of note even at the highest CRP cut off of 7 mg/L, only 41.4% of women subsequently developed PE, suggesting a low predictive ability of CRP in this study. Cases and controls were not matched on demographics in this study, which may potentially explain why these findings differ to mine and others. Levels of CRP are recognised to be correlate with an individual's weight, yet few published studies have accounted for this [200].

A systematic literature review and meta-analysis has been previously undertaken, examining prospective studies which have assessed the ability of CRP to predict PE, taking weight taken into account [200]. A total of 727 women with PE and 3538 controls were included in this meta-analysis. Higher CRP levels were associated with an increased likelihood of PE developing with a pooled mean difference in CRP of 2.30mg/l between the two groups. However on meta-regression analysis, this relationship was modified when BMI were considered as a confounding factor. The difference in CRP concentrations was found to be smaller in studies where cases and controls had similar BMI (0.85 mg/L), compared to studies where BMI was higher in PE compared to the control group (2.01 mg/L). The failure of hs-CRP to act as a predictive marker for PE in my study, compared to others, is perhaps related to the matching that was undertaken between cases and controls, including for BMI. Nevertheless there are studies which have reported CRP concentrations are predictive of PE in lean women [347]. In a prospective study performed by Qiu et al. a hs-CRP level of ≥4.9 mg/L, at an average of 13 weeks' gestation, was found to be associated with an

increased risk of PE in lean women by up to 2.5 fold, whilst a similar relationship was not observed in overweight women. Further prospective studies, with appropriate matching are, therefore, required to better determine the ability of CRP to predict PE in early pregnancy. Levels of hs-CRP were not independently associated with PE in chapter three, and the findings of this chapter suggest that it is not a good first trimester predictive marker for disease, even when weight and other patient demographical factors are accounted for.

4.3.4 Renal Function Markers as Predictors of Pre-eclampsia

There are few published studies, which have examined suitability of markers of renal function, in the first trimester of pregnancy, to predict subsequent onset of PE. One such study demonstrated B2-M levels in early pregnancy were not different in women who subsequently developed PE versus those who remained normotensive; however the PE group was small, consisting of just seven cases [234]. Serum creatinine, cystatin-C, B2-M and UA are all endogenous markers of kidney function, levels of which increase as renal function declines. These markers have been studied during pregnancy in a study conducted in Switzerland by Risch et al. comprising of 144 control first trimester pregnancies and 39 first trimester pregnancies later complicated by PE [9]. Controls and cases were matched on the basis of maternal age, gestational age, smoking status, BMI and ethnicity, and all had normal serum creatinine values. Further matching was then undertaken to ensure that the difference in creatinine concentrations between cases and controls was no more than 6umol/l (median value 57.8 vs. 57.6 umol/l respectively). Women in the PE group had higher first trimester levels of cystatin-C (0.66 vs. 0.63 mg/L, p=0.015) and UA (2.47 vs. 2.24 mg/dL, p=0.011). Levels of B2-M were not significantly different between the two groups. These findings contrast to those from my study, where none of the renal markers were found to

differ significantly between cases and controls. There are differences in the study to mine which may contribute to differences in findings. For instance, women with co-morbidities were excluded from my study, but this was not the case in the Risch's study. There may therefore have been a greater proportion of women in the PE group with inflammatory conditions compared to the control group in the study by Rish et al. Furthermore, there was a higher proportion of women of White ethnicity (91.9 vs. 58.3%) in their study compared to mine. Second trimester levels of B2-M and both second and third trimester levels of cystatin-C, in some studies but not all, have been found to be predictive of PE [9, 233-236, 348-350]. It has also been suggested the second trimester B2-M levels may provide better predictive capability for PE than first trimester levels [235].

In the setting of PE, an increase in UA levels has been reported to occur prior to the development of hypertension and proteinuria [248, 249, 252]. Studies assessing the predictive capability of first trimester levels of UA in PE have yielded conflicting findings [248, 250, 252, 351], with a suggestion by some that this may work better in high risk populations [248, 352]. This was shown not to be the case in one study of pregnant women presenting with gestational hypertension, where first trimester UA levels were not predictive of the subsequent development of PE [338]. Nevertheless, even in studies reporting significant findings, the predictive ability of UA has not been demonstrated to be strong enough for it to be used as a predictive biomarker in clinical practice [248, 250]. In summary, the findings of this chapter support that, in women with normal kidney function, levels of renal markers in the first trimester do not predict the development of subsequent PE.

4.3.5 Complement System Proteins

In chapter three, it was demonstrated that there are reduced levels of complement component C4, in contrast to elevated levels of C3 and other acute phase reactants, at the time of PE in otherwise healthy women without pre-existing medical comorbidity or autoimmune illness. An increase in complement degradation products in PE pregnancies has been demonstrated by others, with increased levels in maternal circulation, urine, amniotic fluid and placental tissue samples compared to healthy pregnancies [81, 102, 214-224, 226]. This study did not demonstrate a difference in circulating C3 or C4 levels in the first trimester of pregnancies later complicated by PE compared to a matched control group where PE did not develop.

In contrast, a number of published studies have demonstrated an increase in complement activation fragments, before 20 weeks' gestation, in pregnancies later complicated by PE compared to normotensive pregnancies [102, 212, 214-216, 353]. Lynch et al. in a prospective study of 784 women enrolled at under 20 weeks' gestation (mean 11.9 ± 2.5 weeks), demonstrated that women with levels of Bb in the upper quartile were 4.7 fold more likely to have an adverse pregnancy outcome compared to women in the lowest quartile ($p<0.001$) [353]. Adverse pregnancy outcomes included PE, preterm birth and premature rupture of membranes. Women with chronic medical illness, but not autoimmune disease, were excluded in this study. Using the same cohort, the authors also looked at the predictive capability of Bb for PE, specifically in 701 of these pregnancies, which included some women with chronic medical illnesses, such as SLE. Pre-eclampsia developed in 4.6% of the cohort and they reported women with a Bb level over the 90th percentile were 3.3 times more likely to develop PE than women who had levels less than

the 90th decile [216]. Of note only 24% of samples were taken before 10 weeks' gestation in this group, with the remaining taken from 11-20 weeks' gestation, and so the gestation covered was a broader range compared to my study. The same research group in a further prospective study of 1002 pregnancies reported that women with C3a levels, taken before 20 weeks' gestation, at the upper quartile were 3 times more likely to experience an adverse pregnancy outcome which included hypertensive disorders of pregnancy, preterm birth, premature rupture of membranes, pregnancy loss and FGR [215]. Women with chronic medical illnesses or autoimmune disease were excluded from this study; however in contrast to my study, over half of the samples (54%) were taken after 12 weeks' gestation.

The authors of these studies have suggested that, since complement activation appears to occur before symptoms of PE develop, and complement factors have, in animal and in vitro studies, been shown to be involved in the dysregulation of angiogenic markers [101, 354], complement dysregulation likely plays a causal role in the development and pathology of PE [102]. It has also been hypothesised that placental ischaemia and local oxidative stress induces activation of the complement system, leading to a anti-angiogenic state and systemic maternal vascular disturbance [222]. In a further study, serum C3 levels taken at 14-20 weeks' gestation in healthy women provided similar predictive accuracy to detect PE compared to uterine artery Doppler imaging, with the authors reporting 100% sensitivity and 97.4% specificity being achieved when both methods were combined [355]. In contrast to my study, multiparous women were excluded from this study, as were those with a BMI > 26kg/m^2 and those aged under 18 years or older than 35 years of age.

There are a number of potential reasons as to why my study findings differ to the published studies which have reported a difference in complement proteins and activation products in early pregnancy in women destined to develop PE compared to normotensive pregnancies. In my study, PE and control cohort were matched for a number of factors including BMI and ethnicity which may otherwise act as a confounder for differences in complement factor levels. Lynch et al. reported obese pregnant women, with Bb and C3a concentrations in the top quartile taken at an average gestation of 11.6 weeks, were 10.0 and 8.8 times, respectively, more likely to develop PE compared to non-obese pregnant control individuals [214]. In addition, the inclusions of pregnant women with pre-existing medical and autoimmune illness may have acted as another confounder in these studies. It has been suggested that there is ethnic variation in predisposition to complement dysregulation [328]. I was able to match controls and cases for ethnicity, which has often not been performed in the published studies. The samples in my study were collected at earlier time points compared to many of the published studies and, therefore, it may have been too early to detect differences in complement factors. A longitudinal pregnancy study reported Bb levels were highest at 15-20 weeks' gestation in women without autoimmune disease who subsequently developed PE [212].

Furthermore, not all studies have demonstrated that complement break down products differ in early pregnancy in women who develop PE [222, 223]. Therefore, further prospective studies with appropriate matching of patients is required to assess the role of complement activation before the onset of PE. Whilst measuring circulating concentrations of complement factors provides an indication of systemic dysregulation of the complement system, it does not provide information at a local tissue level. For instance, increased C3a

concentrations have also been reported in amniotic fluid of PE pregnancies before symptoms appear [222]. Burwick et al. has suggested that complement markers in urine, rather than plasma, are better indicators of complement dysregulation [217]. It is therefore not proven how well circulating complement proteins and activations factors can predict PE, especially in early pregnancy, and future studies should take this into account, with paired urine and blood sample collection over a broader gestational period.

4.3.6 Conclusions and Limitations of Study

As discussed in chapter two, adjustment for multiple comparisons was not undertaken in this study, as the study hypothesis was to test the analysis of each biomarker individually, rather than take into account the results of the other tests/analyses. This increases the risk of risk of type II errors having been reported. The significant findings were limited in this study, and the ones that we have reported may be false-positive results and future validation studies in other cohorts would be recommended to further assess significant differences reported in this chapter.

The lack of positive predictors in my study may be related to study samples being taken too early on in pregnancy, as many of the studies I have compared my findings to included samples taken at the end of the first trimester/early second trimester. The median (IQR) for gestational age at sample collection in my study was 12.1(11.4-12.9) weeks. The samples in my study were taken at a convenient time for screening to be performed, as this is when pregnant women routinely have bloods taken for their antenatal care in the United Kingdom. First trimester screening studies in PE have been reported as not having been very successful and, therefore, whilst it may be a convenient time to screen, it may not be an effective time to screen [49].

The strength of my study compared to other early pregnancy studies is the detailed matching that was performed before cases and controls. Some of these studies may have detected differences in early pregnancy markers due to confounding clinical factors which are prevalent in PE versus healthy pregnancies, for instance differences in BMI, ethnicity and medical conditions such as autoimmune disease. However, there were some weaknesses to my study, I relied on coding data which in some cases may have recorded an incorrect or missed the diagnosis of PE. When validating the clinical coding, PE was incorrectly coded in one of the twenty records I reviewed. Furthermore, co-morbidities may also have been recorded in error or missed. I did not include twin pregnancies and, therefore, cannot extend findings to this group. Longitudinal measurements would provide a more detailed study into biomarkers in pregnancy related complications, for example, the rate in change may be more important than absolute values. This will be assessed in the next chapter in the context of CKD pregnancies. Finally, a comparison to a non-pregnant group would have provided additional important information and help better understand the early maternal immune response in normal and complicated pregnancy.

5 BIOMARKERS PREDICTIVE OF PRE-ECLAMPSIA IN WOMEN WITH CHRONIC KIDNEY DISEASE

The previous two results chapters have studied PE and normal pregnancies in healthy women, without pre-existing medical illness, CKD or autoimmune illness. Single point measurements in late (chapter three) and early pregnancy (chapter four) were undertaken, rather than longitudinal measurements. The latter would allow us to assess whether the rate in change in markers is more meaningful, than absolute values, to predict PE. The sFLT-1/PIGF ratio has not been studied in detail for SPE in CKD and a strong correlation between angiogenic system markers and kidney function was demonstrated in chapter three. Furthermore, it is not well established whether SPE is characterised by dysregulation of the maternal immune system as is evident in PE occurring in healthy women. In the current chapter, serial measurements of circulating markers of immunity, inflammation, renal function and angiogenic factors, studied in the previous chapters, were undertaken in pregnant women with CKD.

5.1 Objectives:

To explore:

- clinical risk factors for developing SPE and other adverse pregnancy outcomes in CKD pregnancies
- whether longitudinal antenatal measurements of the sFLT-1/PIGF ratio, clinical markers of the immune, including humoral, system and inflammation can predict SPE and other adverse pregnancy outcomes in CKD

- the impact of renal function on concentrations of angiogenic markers in pregnancy in the context of CKD
- which markers of renal function are most predictive of adverse pregnancy outcomes, including SPE, in CKD.

5.2 Results

5.2.1 Patient demographics

Data were available for a total of 169 pregnancies in 139 women. Of these, two ended in terminations, at 13 and 16 weeks' gestation, and so were excluded from further analysis. In addition, there were three miscarriages, occurring at 6, 8 and 10 weeks' gestation. These were excluded from the analysis of PE, but were included in analyses of the composite adverse pregnancy outcome, since miscarriage made up part of this outcome. Deliveries occurred at another hospital in four pregnancies and follow up data for these pregnancies was obtained through correspondence. There was a total of five twin pregnancies, making up 3% of each of the PE and non-PE group (p=1.000).

Of the 164 pregnancies resulting in live births, the mean age at last menstrual period (LMP) was 30.4 ± 5.6 years, with the majority of women being of White (62%) or South Asian (23%) ethnicity. Pre-pregnancy, 39% of women were at CKD stage 1, with 36%, 23% and 2% at stages 2, 3 and 4, respectively. There were no stage 5 CKD pregnancies captured in this study. Further details of the study cohort are reported in Tables 5-1a-e.

Superimposed Pre-eclampsia was diagnosed in a total of 37 pregnancies (23%). Of these, 21 (57%) were classified as "definite Pre-eclampsia", based on the study criteria used, with the remaining 16 (43%) diagnosed based on independent Obstetric and Nephrology consultant

consensus opinion as described in chapter two. Of those without SPE, the vast majority (n=110, 87%) were clearly deemed not to meet the study definition of PE, with the remaining 17 (13%) classified as without PE based on consensus opinion. In those that developed PE, the median gestation at diagnosis was 36.0 weeks (IQR: 32.9 – 37.0). Pre-eclampsia was classified as early onset (<34 weeks) in 7% (n=11) of the cohort, and late onset in 16% (n=26).

5.2.2 Demographic and Clinical Predictors of Pre-eclampsia

Comparisons were then made between patients that did and did not develop PE, the results of which are summarised in Table 5-1a-e. In addition, subgroup analyses were performed within the patients that developed PE to compare between those with early and late onset disease (Table 5-2a-e). However, due to the small sample size included in this comparison, statistical power was low, meaning that only large differences between groups could be detected, potentially resulting in an inflated false-negative rate.

Of the demographic factors considered (Table 5-1a/5-2a), no significant difference in the age at LMP (p=0.640), ethnicity (p=0.162) or BMI (p=0.165) was detected between the groups. However, pre-pregnancy smoking history was found to differ between patients with and without PE, with those that developed PE being significantly less likely to have smoked pre-pregnancy (p=0.045).

Table 5-1a: Associations Between Demographic Factors and Pre-eclampsia

	N	Overall (N=164)	Pre-eclampsia		p-Value
			No (N=127)	*Yes (N=37)*	
Age at LMP (Years)	164	30.4 ± 5.6	30.5 ± 5.5	30.0 ± 5.8	0.640
Ethnicity	162				0.162
White		100 (62%)	73 (58%)	27 (75%)	
South Asian		38 (23%)	31 (25%)	7 (19%)	
Black		13 (8%)	11 (9%)	2 (6%)	
Other		11 (7%)	11 (9%)	0 (0%)	
Booking BMI kg/m^2	155	26.4 (23.9 - 31.9)	26.0 (23.8 - 30.9)	27.0 (24.0 - 34.1)	0.165
Pre-Pregnancy Smoking History	153	39 (25%)	35 (29%)	4 (12%)	**0.045**
Known to Renal Team Pre-Pregnancy	164	147 (90%)	114 (90%)	33 (89%)	1.000
Attended Pre-Pregnancy Clinic	158	36 (23%)	30 (25%)	6 (17%)	0.373
Assisted Conception	162	8 (5%)	8 (6%)	0 (0%)	0.203

Continuous variables are reported as mean±SD, or as median (IQR), with p-values from independent sample t- tests or Mann-Whitney tests respectively. Categorical variables are reported as N (%), with p-values from Chi-square tests.

Table 5-1b: Associations between Pre-pregnancy Renal markers, Co-morbidity and Pre-eclampsia

	N	Overall (N=164)	Pre-eclampsia *No* (N=127)	Pre-eclampsia *Yes* (N=37)	*p-Value*
CKD History					
Pre-Pregnancy CKD Stage	161				**0.007***
1		63 (39%)	52 (42%)	11 (30%)	
2		58 (36%)	50 (40%)	8 (22%)	
3		37 (23%)	20 (16%)	17 (46%)	
4		3 (2%)	2 (2%)	1 (3%)	
Pre-Pregnancy Creatinine umol/l	125	83 (62 - 107)	79 (61 - 98)	101 (67 - 137)	**0.008**
Pre-Pregnancy eGFR**	126	74 (52 - *>90*)	79 (60 - *>90*)	54 (44 - *>90*)	**0.012**
Pre-Pregnancy urine ACR mg/mmol	120	7.5 (1.7 - 49.8)	4.9 (1.4 - 34.2)	32.6 (8.8 - 107.0)	**<0.001**
Renal Transplant	164	17 (10%)	13 (10%)	4 (11%)	1.000
Aetiology	146				0.522
ADPKD		16 (11%)	14 (12%)	2 (6%)	
Glomerular Disease		43 (29%)	32 (28%)	11 (34%)	
Lupus Nephritis		18 (12%)	12 (11%)	6 (19%)	
Structural		23 (16%)	19 (17%)	4 (13%)	
Tubulo-Interstitial Disease		28 (19%)	21 (18%)	7 (22%)	
Others		18 (12%)	16 (14%)	2 (6%)	
Single Functioning Native Kidney	164	17 (10%)	12 (9%)	5 (14%)	0.540
Duplex Kidney	164	7 (4%)	5 (4%)	2 (5%)	0.656
Pre-pregnancy UTI History	161	57 (35%)	47 (37%)	10 (29%)	0.545
Stone Disease	164	13 (8%)	11 (9%)	2 (5%)	0.734
Reflux	164	41 (25%)	32 (25%)	9 (24%)	1.000
Metabolic Disease	164	10 (6%)	9 (7%)	1 (3%)	0.459
Medical History					
SLE	164	18 (10%)	12 (9%)	6 (16%)	0.540
Other CTD	164	2 (1%)	2 (2%)	0 (0%)	1.000
Any Autoimmune Disease	164	43 (26%)	31 (24%)	12 (32%)	0.396
Chronic Hypertension	164	60 (37%)	37 (29%)	23 (62%)	**<0.001**
Diabetes Mellitus	164				0.413
No		160 (98%)	125 (98%)	35 (95%)	
Type 1		2 (1%)	1 (1%)	1 (3%)	
Type 2		2 (1%)	1 (1%)	1 (3%)	

*Continuous variables are reported as median (IQR), with p-values from Mann-Whitney tests. Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. **The laboratory reported eGFR levels that were truncated at 90 ml/min/1.73m^2, hence a value of 90 was assumed for these in the analysis. ADPKD = autosomal dominant polycystic kidney disease, UTI = urinary tract infection, CTD = connective tissue disease.*

Table 5-1c: Associations between Obstetric History and Pre-eclampsia

	N	Overall(N=164)	Pre-eclampsia		p-Value
			No(N=127)	*YesN=(37)*	
Pregnancy History					
Nulliparous	164	60 (37%)	45 (35%)	15 (41%)	0.567
Gravida	163				0.559*
1		48 (29%)	38 (30%)	10 (28%)	
2		52 (32%)	37 (29%)	15 (42%)	
3+		63 (39%)	52 (41%)	11 (31%)	
Number of Previous Miscarriages	163				0.322*
0		113 (69%)	91 (72%)	22 (61%)	
1		30 (18%)	20 (16%)	10 (28%)	
2+		20 (12%)	16 (13%)	4 (11%)	
Pre-Term birth (<37 Weeks) **	104	29 (28%)	20 (24%)	9 (41%)	0.179
Very Pre-Term birth (<34 Weeks) **	104	13 (13%)	8 (10%)	5 (23%)	0.142
Previous Pre-eclampsia**	104	23 (22%)	12 (15%)	11 (50%)	**<0.001**
Previous SGA/FGR**	104	37 (36%)	31 (38%)	6 (27%)	0.456
Previous NND**	104	5 (5%)	4 (5%)	1 (5%)	1.000
Previous Still Birth**	104	6 (6%)	4 (5%)	2 (9%)	0.604

*Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. **Excludes women that were nulliparous. SGA = small for gestational age infant, FGR- fetal growth restriction, NND = neonatal death.*

Of the CKD and co-morbidity related factors (Table 5-1b/5-2b), CKD stage was found to be significantly higher in those who developed PE, with 49% classified as stage 3-4, compared to 18% of those without PE (p=0.007). Correspondingly, PE patients were found to have significantly higher pre-pregnancy serum creatinine (p=0.008) and uACR (p<0.001) levels, and significantly lower eGFR (p=0.012) than those who did not develop PE. Subgroup analysis of the patients with early versus late onset PE found a tendency for those in the former group to have worse pre-pregnancy kidney function (higher creatinine and uACR), although none of these comparisons reached statistical significance. The distribution of aetiologies was found to be similar in those that did and did not develop PE (p=0.522). Of the additional comorbidities considered, only chronic hypertension was found to differ significantly between groups, being diagnosed in 62% of those who developed PE, compared to 29% of those who did not (p<0.001).

Table 5-1d: Associations between antenatal factors and Pre-eclampsia

	N	Overall(N=164)	Pre-eclampsia		p-Value
			No(N=127)	*Yes(N=37)*	
Antenatal Factors					
Twin Pregnancy	164	5 (3%)	4 (3%)	1 (3%)	1.000
Booking/Earliest Urine Dip Protein	157				0.156*
Negative		100 (64%)	81 (66%)	19 (54%)	
1+		24 (15%)	18 (15%)	6 (17%)	
2+		15 (10%)	11 (9%)	4 (11%)	
3+		18 (11%)	12 (10%)	6 (17%)	
Booking/Earliest Urine Dip Blood	157				0.362*
Negative		126 (80%)	100 (82%)	26 (74%)	
1+		12 (8%)	8 (7%)	4 (11%)	
2+		9 (6%)	6 (5%)	3 (9%)	
3+		10 (6%)	8 (7%)	2 (6%)	
Booking Systolic BP mmHg	159	125 ± 12	123 ± 12	129 ± 11	**0.037**
Booking Diastolic BP mmHg	159	77 ± 11	75 ± 11	82 ± 11	**<0.001**
AN Aspirin	154	139 (90%)	107 (88%)	32 (100%)	**0.042**
Static Growth Detected on USS	123	20 (16%)	18 (19%)	2 (8%)	0.240
Infections - 1st Trimester	154	24 (16%)	21 (18%)	3 (9%)	0.289
Infections - 2nd Trimester	156	27 (17%)	25 (20%)	2 (6%)	0.070
Infections - 3rd Trimester	156	22 (14%)	20 (16%)	2 (6%)	0.252
Recurrent UTI	157	18 (11%)	17 (14%)	1 (3%)	0.078
Gestational Diabetes**	153	14 (9%)	13 (11%)	1 (3%)	0.304
AN New/Worsening Hypertension	163	63 (39%)	28 (22%)	35 (95%)	**<0.001**
AN Renal Complication	162	59 (36%)	35 (28%)	24 (67%)	**<0.001**
Fetal Distress	164	12 (7%)	10 (8%)	2 (5%)	1.000
Growth Issue	154	33 (21%)	26 (22%)	7 (21%)	1.000
Uterine artery Doppler notching	130				**0.045**
No		62 (48%)	46 (45%)	16 (59%)	
Yes		18 (14%)	12 (12%)	6 (22%)	
*Unknown****		50 (38%)	45 (44%)	5 (19%)	
Umbilical Artery Flow Abnormality	145				0.114
No		121 (83%)	94 (83%)	27 (84%)	
Yes		14 (10%)	9 (8%)	5 (16%)	
*Unknown ****		10 (7%)	10 (9%)	0 (0%)	
Pre-Delivery Admission (1+)	160	59 (37%)	46 (37%)	13 (35%)	0.848

*Continuous variables are reported as mean±SD, with p-values from independent sample t-tests Categorical variables are reported as N (%) with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. **Patients with pre-pregnancy Diabetes Mellitus were excluded from this analysis. ***An "unknown" category was included to account for potential selection bias, for cases where it was unclear whether or not a test had been performed were excluded from the analysis. BP = blood pressure, AN= antenatal, USS= ultrasound scan.*

Table 5-1e: Associations between delivery/postnatal factors and baby outcomes and Pre-eclampsia

	N	Overall (N=164)	Pre-eclampsia		p-Value
			No (N=127)	*Yes (N=37)*	
Delivery and Postnatal					
Gestation at Delivery (Weeks')	164	37.6 (36.1 - 38.7)	38.0 (37.0 - 39.0)	36.1 (33.4 - 37.6)	**<0.001**
PROM	163	5 (3%)	4 (3%)	1 (3%)	1.000
Spontaneous Delivery	164	11 (7%)	11 (9%)	0 (0%)	0.072
Type of Delivery	164				**<0.001**
Normal Vaginal		65 (40%)	56 (44%)	9 (24%)	
Instrumental		20 (12%)	19 (15%)	1 (3%)	
Elective LSCS		21 (13%)	19 (15%)	2 (5%)	
Emergency LSCS		58 (35%)	33 (26%)	25 (68%)	
Postpartum Haemorrhage	143	19 (13%)	14 (13%)	5 (15%)	0.776
Birthweight (g)	158	2770 (2405 - 3240)	2850 (2480 - 3315)	2515 (2040 - 2785)	**<0.001**
Apgar (1 min)	156				**0.005***
<9		33 (21%)	20 (17%)	13 (37%)	
9		117 (75%)	95 (79%)	22 (63%)	
10		6 (4%)	6 (5%)	0 (0%)	
Apgar (5 min)	156				**0.039***
<9		11 (7%)	7 (6%)	4 (11%)	
9		98 (63%)	73 (60%)	25 (71%)	
10		47 (30%)	41 (34%)	6 (17%)	
Post-Delivery Length of Stay (Mother, days)	153	2 (2 - 5)	2 (2 - 4)	5 (3 - 7)	**<0.001**
Post-Delivery Length of Stay (Baby, days)	140	2 (2 - 5)	2 (1 - 4)	5 (3 - 7)	**<0.001**
PN Renal Complication	160	13 (8%)	4 (3%)	9 (25%)	**<0.001**
PN New/Worsening Hypertension	126	19 (15%)	15 (15%)	4 (15%)	1.000
Delivery at <34 Weeks	164	18 (11%)	8 (6%)	10 (27%)	**0.001**
Birthweight <1500g	158	10 (6%)	4 (3%)	6 (17%)	**0.010**
NNU/NICU	154	33 (21%)	18 (15%)	15 (43%)	**0.001**
NND	164	3 (2%)	1 (1%)	2 (5%)	0.128
FGR	164	20 (12%)	15 (12%)	5 (14%)	0.778

*Continuous variables are reported as median (IQR), with p-values from Mann-Whitney tests. Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. PROM = premature rupture of membranes, , LSCS = lower segment caesarean section, PN = postnatal, NICU = neonatal intensive care unit, NND = neonatal death and FGR = fetal growth restriction.*

The obstetric history was generally similar in those with and without PE (Table 5-1c/5-2c), with no significant difference in the proportion of women that were nulliparous (p=0.567) nor with gravidity (p=0.559). Patients that were nulliparous were excluded from analyses of previous pregnancy outcomes. Of the remainder, patients that developed PE were significantly more likely to have developed PE in a previous pregnancy (50% vs. 15% respectively, p<0.001).

Patients who developed PE were found to have significantly higher booking Systolic (p=0.037) and Diastolic BP (p<0.001) (Table 5-1d/5-2d) than those who did not develop PE. On subgroup analysis, within those who developed PE, both Systolic BP (p=0.039) and Diastolic BP (p=0.037) were also significantly higher in those with early onset compared to late onset PE. The presence of proteinuria (p=0.156) or haematuria (p=0.362) on urine dip analysis in early pregnancy was not predictive of PE developing later. In total, 90% of women received aspirin during pregnancy to reduce the risk of PE, although patients who developed PE were significantly more likely to have been treated with aspirin than those who did not develop PE (p=0.042). Infections occurred in the first trimester of 17% of pregnancies, with 14% and 11% developing infections in the second and trimester, respectively. After excluding those with diabetes pre-pregnancy (2%), gestational diabetes developed in 9% of pregnancies. None of these factors were found to be significantly associated with the development of PE. As expected, patients who developed PE were significantly more likely to experience a renal complication during the antenatal period (67% vs. 28%, p<0.001). 22% of women in the non-PE group developed new onset or worsening hypertension during the antenatal period.

Rates of documented fetal growth complications were almost identical in those that did and did not develop PE (21% vs. 22%, p=1.000). However, within the PE group, those with early onset PE had significantly higher rates of fetal growth issues than the late PE pregnancies (50% vs. 12%, p=0.037). Analysis of findings of uterine artery Doppler notching found this to differ significantly between patients with and without PE (p=0.045). However, this test was not performed on a considerable proportion of patients (38%), and there was an indication of selection bias, with 19% of women in the PE group being untested compared to 44% without PE. In light of this bias, it is difficult to give a reliable comparison of rates of uterine artery Doppler notching between the two groups, or to accurately comment on its predictive ability. Fetal distress was documented in 7% of the pregnancies and not significantly different between the PE and non-PE groups (p=1.000). Over a third of women (37%) had a separate hospital admission prior to their delivery admission, and this rate was comparable between the PE and non-PE groups (p=0.848).

Three neonatal deaths occurred, two in the PE group and one in the non-PE group (p=0.128). No IUD or still births occurred in either group. The type of delivery (Tables 5-1e/5-2e) differed between groups (p<0.001), with 68% of PE patients requiring an emergency lower segment caesarean section (LSCS) compared to 26% of those without PE, whilst normal vaginal delivery rates were 24% versus 44%, respectively. Women who developed PE, compared to those who did not, were found to have a significantly shorter

Table 5-2a: Associations between demographic factors and early onset Pre-eclampsia

	N	**Onset of Pre-eclampsia** ***Early (<34 Weeks)*** N = 11	***Late (34+ Weeks)*** N = 26	***p-Value***
Age at LMP (Years)	37	30.1 ± 5.3	29.7 ± 6.1	0.868
Ethnicity	36			0.099
White		10 (100%)	17 (65%)	
South Asian		0 (0%)	7 (27%)	
Black		0 (0%)	2 (8%)	
Other		0 (0%)	0 (0%)	
Booking BMI (kg/m^2)	35	26.5 (24.3 - 34.0)	27.5 (24.0 - 34.9)	0.776
Pre-Pregnancy Smoking History	34	3 (30%)	1 (4%)	0.067
Known to Renal Team Pre-Pregnancy	37	9 (82%)	24 (92%)	0.567
Attended Pre-Pregnancy Clinic	36	3 (27%)	3 (12%)	0.342

Continuous variables are reported as mean±SD, or as median (IQR), with p-values from independent sample t- tests or Mann-Whitney tests respectively. Categorical variables are reported as N (%), with p-values from Chi-square tests.

Table 5-2b: Associations between pre-pregnancy renal markers, co-morbidity and early onset Pre-eclampsia

	N	**Onset of Pre-eclampsia** *Early (<34 Weeks)* *N = 11*	*Late (34+ Weeks)* *N = 26*	*p-Value*
CKD History				
Pre-Pregnancy CKD Stage	37			0.284*
1		2 (18%)	9 (35%)	
2		2 (18%)	6 (23%)	
3		7 (64%)	10 (38%)	
4		0 (0%)	1 (4%)	
Pre-Pregnancy Creatinine umol/l	30	116 (104 - 143)	96 (66 - 120)	0.133
Pre-Pregnancy eGFR**	30	49 (36 - 54)	60 (44 - *>90*)	0.113
Pre-Pregnancy ACR mg/mmol	37	37.9 (30.5 - 64.0)	27.7 (8.3 - 128.7)	0.543
Renal Transplant	37	2 (18%)	2 (8%)	0.567
Aetiology	32			0.844
ADPKD		0 (0%)	2 (9%)	
Glomerular Disease		4 (44%)	7 (30%)	
Lupus Nephritis		1 (11%)	5 (22%)	
Structural		1 (11%)	3 (13%)	
Tubulo-Interstitial Disease		2 (22%)	5 (22%)	
Others		1 (11%)	1 (4%)	
Single Functioning Native Kidney	37	2 (18%)	3 (12%)	0.623
Duplex	37	0 (0%)	2 (8%)	1.000
Pre-pregnancy UTI History	34	5 (56%)	5 (20%)	0.085
Stone Disease	37	0 (0%)	2 (8%)	1.000
Reflux	37	4 (36%)	5 (19%)	0.404
Metabolic Disease	37	0 (0%)	1 (4%)	1.000
Medical History				
SLE	37	1 (9%)	5 (19%)	1.000
Other CTD	37	0 (0%)	0 (0%)	1.000
Any Autoimmune	37	2 (18%)	10 (38%)	0.279
Chronic Hypertension	37	9 (82%)	14 (54%)	0.150
Diabetes	37			0.245
No		10 (91%)	25 (96%)	
T1		1 (9%)	0 (0%)	
T2		0 (0%)	1 (4%)	

*Continuous variables are reported as median (IQR), with p-values from Mann-Whitney tests. Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. *p-Value from Mann-Whitney test, as the factor is ordinal. **The laboratory reported eGFR levels that were truncated at 90 ml/min/1.73m^2, hence a value of 90 was assumed for these in the analysis. ADPKD = autosomal dominant polycystic kidney disease, UTI = urinary tract infection, CTD = connective tissue disease.*

Table 5-2c: Associations between obstetric history and early onset Pre-eclampsia

	N	**Onset of Pre-eclampsia** *Early (<34 Weeks)* *N = 11*	*Late (34+ Weeks)* *N = 26*	*p-Value*
Pregnancy History				
Nulliparous	37	5 (45%)	10 (38%)	0.728
Gravida	36			0.274*
1		4 (40%)	6 (23%)	
2		4 (40%)	11 (42%)	
3+		2 (20%)	9 (35%)	
Number of Previous Miscarriages	36			0.541*
0		7 (70%)	15 (58%)	
1		2 (20%)	8 (31%)	
2+		1 (10%)	3 (12%)	
Pre-Term birth (<37 Weeks) **	22	3 (50%)	6 (38%)	0.655
Very Pre-Term birth (<34 Weeks) **	22	3 (50%)	2 (13%)	0.100
Previous Pre-eclampsia**	22	3 (50%)	8 (50%)	1.000
Previous SGA/FGR**	22	2 (33%)	4 (25%)	1.000
Previous NND**	22	0 (0%)	1 (6%)	1.000
Previous Still Birth**	22	2 (33%)	0 (0%)	0.065

*Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. *p-Value from Mann-Whitney test, as the factor is ordinal. **Excludes women that were nulliparous. SGA = small for gestational age infant, FGR-fetal growth restriction, NND = neonatal death.*

Table 5-2d: Associations between antenatal factors and early onset Pre-eclampsia

	N	**Onset of Pre-eclampsia** *Early (<34 Weeks) N = 11*	*Late (34+ Weeks) N = 26*	*p-Value*
Antenatal Factors				
Twin Pregnancy	37	0 (0%)	1 (4%)	1.000
Booking/Earliest Urine Dip Protein	35			0.841*
Negative		6 (60%)	13 (52%)	
1+		1 (10%)	5 (20%)	
2+		1 (10%)	3 (12%)	
3+		2 (20%)	4 (16%)	
Booking/Earliest Urine Dip Blood	35			0.651*
Negative		7 (70%)	19 (76%)	
1+		1 (10%)	3 (12%)	
2+		1 (10%)	2 (8%)	
3+		1 (10%)	1 (4%)	
Booking Systolic BP	35	134 ± 9	127 ± 11	**0.039**
Booking Diastolic BP	35	88 ± 12	79 ± 10	**0.037**
AN Aspirin	32	8 (100%)	24 (100%)	1.000
Static Growth Detected on USS	26	0 (0%)	2 (11%)	1.000
Infections - 1st Trimester	34	0 (0%)	3 (12%)	0.549
Infections - 2nd Trimester	34	0 (0%)	2 (8%)	1.000
Infections - 3rd Trimester	32	0 (0%)	2 (8%)	1.000
Recurrent UTI	35	1 (10%)	0 (0%)	0.286
Gestational Diabetes**	33	1 (13%)	0 (0%)	0.242
AN Renal Complication	36	6 (60%)	18 (69%)	0.700
Fetal Distress	37	0 (0%)	2 (8%)	1.000
Growth Issue	34	4 (50%)	3 (12%)	**0.037**
Uterine artery Doppler notching	27			0.318
No		5 (83%)	11 (52%)	
Yes		1 (17%)	5 (24%)	
*Unknown****		0 (0%)	5 (24%)	
Umbilical Artery Flow Abnormality	32			0.085
No		5 (63%)	22 (92%)	
Yes		3 (38%)	2 (8%)	
*Unknown****		0 (0%)	0 (0%)	
Pre-Delivery Admission (1+)	37	3 (27%)	10 (38%)	0.711

*Continuous variables are reported as mean±SD, with p-values from independent sample t-tests. Categorical variables are reported as N (%) with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. **Patients with pre-pregnancy Diabetes Mellitus were excluded from this analysis. ***An "unknown" category was included to account for potential selection bias, cases where it was unclear whether or not a test had been performed were excluded from the analysis. BP = blood pressure, AN= antenatal, USS= ultrasound scan, UTI = urinary tract infection.*

Table 5-2e: Associations between delivery/postnatal factors and baby outcomes and early onset Pre-eclampsia

	N	**Onset of Pre-eclampsia** *Early (<34 Weeks) N = 11*	*Late (34+ Weeks) N = 26*	*p-Value*
Delivery and Postnatal				
Gestation at Delivery (Weeks)	37	31.6 (27.3 - 33.1)	37.1 (36.0 - 37.6)	**<0.001***
PROM	36	0 (0%)	1 (4%)	1.000
Spontaneous Delivery	37	0 (0%)	0 (0%)	1.000
Type of Delivery	37			0.427
Normal Vaginal		1 (9%)	8 (31%)	
Instrumental		0 (0%)	1 (4%)	
Elective LSCS		1 (9%)	1 (4%)	
Emergency LSCS		9 (82%)	16 (62%)	
Postpartum Haemorrhage	34	2 (20%)	3 (13%)	0.618
Birthweight (g)	36	1390 (750 - 1860)	2753 (2430 - 2890)	**<0.001***
Apgar (1 min)	35			**0.001***
<9		8 (80%)	5 (20%)	
9		2 (20%)	20 (80%)	
10		0 (0%)	0 (0%)	
Apgar (5 min)	35			**0.002***
<9		4 (40%)	0 (0%)	
9		6 (60%)	19 (76%)	
10		0 (0%)	6 (24%)	
Post-Delivery Length of Stay (Mother, days)	35	9 (6 - 12)	4 (2 - 6)	**0.010***
Post-Delivery Length of Stay (Baby, days)	29	18 (12 - 21)	5 (2 - 6)	**<0.001***
PN Renal Complication	36	2 (20%)	7 (27%)	1.000
PN New/Worsening Hypertension	27	3 (50%)	1 (5%)	**0.025**
Delivery at <34 Weeks	37	10 (91%)	0 (0%)	**<0.001**
Birthweight <1500g	35	6 (60%)	0 (0%)	**<0.001**
NNU/NICU	35	10 (100%)	5 (20%)	**<0.001**
NND	37	2 (18%)	0 (0%)	0.083
FGR	37	4 (36%)	1 (4%)	**0.021**

*Continuous variables are reported as median (IQR), with p-values from Mann-Whitney tests. Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. PROM = premature rupture of membranes, LSCS = lower segment caesarean section, PN = postnatal, NNU = neonatal unit, NICU = neonatal intensive care unit, NND = neonatal death and FGR = fetal growth restriction.*

gestation at delivery (median: 36 vs. 38 weeks, p<0.001) and delivered a baby of significantly lower birthweight (median 2515g vs. 2850g, p<0.001). Subgroup analysis found that those with early onset PE had children of lower birthweights than those with late onset PE, as would be expected due to earlier deliveries. Apgar scores were significantly lower in patients who developed PE when assessed at both 1 (p=0.005) and 5 minutes (p=0.039) post-delivery. Subgroup analysis found that, within the PE patients, the Apgar scores were significantly lower in early vs. late onset PE at both times (p=0.001, 0.002, respectively).

Rates of postnatal renal complications were significantly higher in those who developed PE (25% vs. 3%, p<0.001), although similar rates were observed within early and late onset PE (20% vs. 27%, p=1.000). Whilst rates of postnatal hypertension were similar in patients with and without PE (both 15%, p=1.000), subgroup analysis found rates to be significantly higher in early versus late onset PE (50% vs. 5%, p=0.025).

Rates of early delivery defined as < 34 weeks (p=0.001), low birthweight defined as <1500g (p=0.010) and NNU/NICU admission (p=0.001) were all found to be significantly higher in patients who developed PE, with no significant differences in rates of NND (p=0.128) or FGR (p=0.778) detected. As a result of the above, the post-delivery length of stay for both the mother and baby were significantly higher in PE patients, with medians of 5 days for both, compared to 2 days in those without PE (p<0.001). Subgroup analysis found a median length of stay for early vs. late onset PE of 9 vs. 4 days (p=0.010) for the mother and 18 vs. 5 days (p<0.001) for the baby.

5.2.3 Multivariable Analysis Clinical Predictors of Pre-eclampsia

A multivariable analysis was then performed, to identify demographic, medical, pre-natal and antenatal patient factors that were independently predictive of PE. All of the demographic factors from Table 5-1a were considered for inclusion in this model, although ethnicity was categorised as White vs. Non-White, due to the small numbers of patients in the Black and Other groups. Of the factors in Table 5-1b, the pre-pregnancy CKD stage 3 and 4 categories were combined, due to the small number of patients in the latter group. Since pre-pregnancy creatinine and eGFR were highly correlated with CKD stage (rho >0.9), and had a greater quantity of missing data (>20%), these factors were not considered for inclusion in the model to prevent excessive exclusions of patients and issues with multicollinearity. Pre-pregnancy uACR was also excluded from the initial analysis, due to a large quantity of missing data (>25%), but was later considered in a sensitivity analysis. Significant poor fit was detected for this variable when it was treated as continuous (Hosmer–Lemeshow test p=0.012), hence an ordinal version of the variable was used, with categories of <3, 3-29 and 30+ mg/mmol. Of the obstetric history factors in Table 5-1c, due to correlations between the gravida, parity and number of miscarriages, only the former was considered for inclusion in the model. Nulliparous patients were treated as a separate group for the variables relating to previous pregnancy outcomes. Only the previous pre-eclampsia variable was considered for inclusion in the model, as this was the only factor found to be significantly associated with PE on univariable analysis.

The resulting model is reported in Table 5-3a. This identified chronic hypertension (OR: 7.95, p<0.001) to be associated with a significantly higher risk of PE, whilst Non-White ethnicity (OR: 0.17, p=0.002) and a pre-pregnancy smoking history (OR: 0.09, p=0.006) conferred a

lower risk of PE. Patients with a history of PE were also at higher risk of developing PE, with an OR of 8.43 (p=0.002), compared to those with previous pregnancies unaffected by PE. SLE was also found to be associated with a significantly higher risk of PE on multivariable analysis (OR: 6.48, p=0.015), despite not being found to be significantly associated with PE on univariable analysis (p=0.540). Further assessment of this factor found a significant association with ethnicity, with 5% of White patients having a SLE diagnosis, compared to 19% of Non-White patients (p=0.007). On subgroup analysis, the association between SLE and PE appeared to vary by ethnicity. In White patients, rates of PE were similar in those patients with and without SLE (20% vs. 27%, p=1.000). However, in the subgroup of Non-White patients, rates of PE tended to be higher in patients with SLE than in those without, although this did not reach statistical significance (33% vs. 10%, p=0.062). This relationship between ethnicity and SLE explains why both were found to be significant on multivariable analysis, despite non-significance on univariable analysis.

A sensitivity analysis was then performed, which additionally considered pre-pregnancy uACR for inclusion in the model. However, this was not selected by the stepwise procedure, with p=0.241 at the final step. The multivariable model was then used to produce predicted probabilities for each patient for whom data were available for the included factors (n=152). A ROC curve analysis found that the combination of the five factors in the multivariable model were significantly predictive of PE (p<0.001), with an AUROC of 0.852 (SE=0.035). However, in light of the relatively low ratio of PE cases to factors in the final model, the risk of overfitting of the model was high. As such, it is possible that the ROC analysis represents an overestimate of the true predictive accuracy of the model, and external validation would be required to assess the reliability of this finding.

Table 5-3a: Multivariable analysis of Pre-eclampsia

	OR (95% CI)	p-Value
Ethnicity (Non-White)	0.17 (0.05 - 0.53)	**0.002**
Pre-Pregnancy Smoking History	0.09 (0.02 - 0.51)	**0.006**
SLE	6.48 (1.44 - 29.10)	**0.015**
Chronic Hypertension	7.95 (2.87 - 22.05)	**<0.001**
History of Pre-eclampsia*	8.43 (2.14 – 33.16)	**0.002**

*Results are from a multivariable binary logistic regression model, using a forwards stepwise approach to variable selection. All factors from Table 5-1a were considered for inclusion in the model, as well as selected factors from Tables 5-1b-d as described in the text. Factors in the final model were then entered into a new model, to minimise exclusions due to missing data, with the final model being based on n=152 (n=33 PE). Bold p-values are significant at p<0.05. *History of PE is only applicable to those women who had previous pregnancies, hence the OR is relative to those with at least one previous pregnancy and no PE history. Nulliparous women were included as a separate category, to prevent exclusions, and this had an OR of 2.01 (95% CI: 0.71 – 5.75, p=0.194), relative to those with at least one previous pregnancy and no PE history.*

Table 5-3b: Multivariable analysis of Pre-eclampsia – adding pre-pregnancy creatinine

	OR (95% CI)	p-Value
Creatinine (per 10 umol/l)	1.14 (1.00 - 1.30)	0.056
Ethnicity (Non-White)	0.13 (0.03 - 0.54)	**0.005**
Pre-Pregnancy Smoking History	0.04 (0.00 - 0.54)	**0.015**
SLE	17.04 (2.59 - 112.10)	**0.003**
Chronic Hypertension	5.59 (1.71 - 18.26)	**0.004**
History of Pre-eclampsia*	6.11 (0.99 – 37.54)	0.051

*Results are from a multivariable binary logistic regression model, including all factors from Table 5-3a, alongside creatinine, and are based on n=115 (n=27 PE). Bold p-values are significant at p<0.05. *History of PE is only applicable to those women who had previous pregnancies, hence the OR is relative to those with at least one previous pregnancy and no PE history. Nulliparous women were included as a separate category, to prevent exclusions, and this had an OR of 3.18 (95% CI: 0.93 – 10.89, p=0.066), relative to those with at least one previous pregnancy and no PE history.*

The stepwise procedure of the multivariable analysis did not select the CKD stage for inclusion in the model, with this factor having p=0.180 at the final step, implying that the CKD stage is not a significant independent predictor of PE. A second model was produced, which included the pre-pregnancy creatinine levels alongside the factors identified by the previous multivariable analysis. Using actual creatinine levels had the benefit of giving greater detail than CKD staging, to more precisely quantify the kidney function of patients. However, the disadvantage of this approach was that creatinine levels were only recorded in 125 (76%) patients, reducing the sample size included in the model to 1150, after exclusions for missing data on the other factors. The resulting model is reported in Table 5-3b. This returned broadly similar results to the previous multivariable model, with non-White ethnicity and pre-pregnancy smoking history associated with lower rates of PE, whilst SLE and chronic hypertension were associated with higher rates of PE, although a history of PE was non-significant in this analysis (p=0.051). After accounting for these factors, pre-pregnancy creatinine levels were not found to be significantly associated with PE (p=0.056).

Due to the difficulty in accurately defining and diagnosing PE in patients with underlying CKD, analyses were also performed for a well-defined adverse pregnancy outcome. This comprised the following outcomes: miscarriage, delivery at < 34 weeks, FGR, birthweight <1500g, admission to NNU/NICU, neonatal death, IUD or still birth. Since miscarriage was a part of this outcome, the three pregnancies ending in miscarriage that had been excluded from the analysis of PE were included in the analysis of the composite adverse outcome, giving a total of 167 pregnancies. For 12 pregnancies, at least one of the components were missing, most commonly whether NNU/NICU admission was required (n=10), hence these pregnancies were excluded from the analysis of adverse events. For the remaining 155

pregnancies, adverse events occurred in 46 (30%). Most (87%) of the PE cases were included in this group.

A multivariable model was then produced to identify significant independent predictors of the composite outcome, the results of which are reported in Table 5-4. The approach to variable selection was as previously described. However, since multiple previous pregnancy outcomes were found to be significantly associated with the composite adverse pregnancy outcome on univariable analysis, all such factors were considered for inclusion in the model. This analysis found chronic hypertension (OR: 9.47, $p<0.001$) and a previous still birth (OR: 61.6, $p=0.005$) to be significant independent predictors of the composite adverse pregnancy outcome. A significant difference across CKD aetiologies was also detected ($p=0.006$), with lupus nephritis and the "others" category having the highest incidence of the adverse outcome. As a sensitivity analysis, the analysis was repeated with pre-pregnancy creatinine, eGFR and uACR levels considered for inclusion, but none of these factors were identified as significant independent predictors of the composite outcome, with $p=0.155$, 0.266 and 0.771, respectively.

Table 5-4: Multivariable analysis of composite adverse pregnancy outcome*

	OR (95% CI)	p-Value
Aetiology		**0.006**
ADPKD	-	-
Glomerular Disease	2.83 (0.27 - 30.02)	0.387
Lupus Nephritis	24.26 (1.84 - 319.97)	**0.015**
Structural	5.95 (0.44 - 80.11)	0.179
Tubulo-Interstitial Disease	1.85 (0.13 - 26.53)	0.650
Others	36.49 (2.81 - 474.31)	**0.006**
Chronic Hypertension	9.47 (2.82 - 31.77)	**<0.001**
Previous Still Birth**	61.56 (3.40 - 1114.78)	**0.005**

**This included miscarriage, delivery at < 34 weeks, FGR, birthweight <1500g, admission to NNU/NICU, neonatal death, IUD or still birth. Variables were selected using a forwards stepwise approach as described for Table 5-3. However, since multiple previous pregnancy outcomes were found to be significantly associated with the composite adverse outcome on univariable analysis, all such factors were considered for inclusion in this model. As these factors were not applicable to women who were nulliparous a nulliparous variable was forced into the model, so that the effect of this factor was isolated from the effect of the previous pregnancy outcomes (OR 2.83 (95% CI 0.92-8.63), p=0.068. The OR The final model was based on n=117 (29 outcomes) after exclusions for missing data. Bold p-values are significant at p<0.05. ADPKD = autosomal dominant polycystic kidney disease. **History of still birth is only applicable to those women who had previous pregnancies, hence the OR is relative to those with at least one previous pregnancy and no still births.*

5.2.4 Associations between Pre-Clinical Markers and Pre-eclampsia

At least one antenatal sample was available in 153 (93%) pregnancies in 127 patients, contributing a total of n=473 samples to the analysis, a median of 3 samples per pregnancy (range: 1-7). Of these samples, immune markers and sFLC were analysed in n=467-472, IgG subclasses in n=317 and angiogenetic markers in n=335-338. Two additional pregnancies, contributing a single sample each, ended in miscarriages, and were excluded from the analysis of PE.

In some pregnancies, data were available for multiple samples that were taken within a short period of time. In order to prevent these pregnancies having undue influence over the analysis, and to simplify the subgroup analyses within different gestations, the antenatal period was then divided into five categories, as per Table 5-5. For some pregnancies, data were available for multiple samples within a period of gestation. Where this occurred, the

average of the samples was taken, such that each pregnancy was only included once in each analysis. Geometric means were used for markers that followed skewed distributions, with arithmetic means used otherwise (namely for C3 and C4). After combining the samples in this way, a total of 398 data points were available for subsequent analysis.

Table 5-5: Number of samples by gestation

Gestation (Weeks+ days)	Number of Samples	
	All Samples	***From Unique Pregnancies***
< 16	96	73
16 – 21 +6	92	83
22 – 27 +6	100	79
28 – 31 + 6	83	81
32 +	102	82
Total	**473**	**398**

The resulting data were then analysed using two different approaches. The first assessed the trends over time in the markers using a regression-based approach, which fitted trendlines (either linear or log-linear) to the makers over the period of gestation, and compared these between the PE and non-PE groups (Table 5-6a). The second approach measured the predictive accuracy of the markers, with respect to PE, within the five periods of gestation previously described. This approach compared the average levels of the markers between the PE and non-PE in each gestational period, using a ROC curve approach (Table 5-7). The results of the analysis of both approaches are summarised below.

5.2.4.1 Angiogenic markers – Rate of Change

The angiogenic markers followed a complex trend over the period of gestation, which is visualised in Figure 5-1. Soluble FLT-1 levels remained relatively constant in both groups until around 30 weeks, before beginning to increase, whilst PIGF was also found to increase

in both groups progressively initially, before peaking at around 30 weeks, and then beginning to decline. As a result, the sFLT-1/PIGF ratio followed a "U-shaped" trend, with an initial decline to a trough level at around 30 weeks, followed by an increase over the remaining period of gestation in both PE and non-PE groups. These trends are similar to those reported in healthy pregnancy. Due to the complex shape of these trends, it would have been challenging to produce a reliable regression model that was a good fit to the data. Therefore, gestation-adjusted values were produced by dividing the values of the angiogenic markers by the reported normal (50^{th} percentile) values at the gestation at which the sample was taken [276, 277]. The gestation-adjusted values followed a log-linear trend over the period of gestation, making it possible to produce reliable models to compare between the PE and non-PE groups.

This analysis found no significant differences in baseline values between PE and non-PE groups for gestation-adjusted sFLT-1, PIGF or the sFLT-1/PIGF ratio (Table 5-6a). In addition, the trends over time were not found to differ significantly from those anticipated, based on the published normal ranges for these markers, with similar gradients observed in the PE and non-PE groups. The models used in the analysis are visualised in Figure 5-2.

Table 5-6a: Changes over time in the gestation-adjusted angiogenic markers by Pre-eclampsia

Marker	N	Baseline Difference: PE vs. Non-PE		Gradient per 12 Weeks					
		Average Difference	*p-Value*	Overall p-Value	Non-PE *Gradient (95% CI)*	*p-Value*	Pre-eclampsia *Gradient (95% CI)*	*p-Value*	Interaction p-Value
sFLT-1 (pg/ml)	297	12.4% (-37.3%, 101.7%)	0.695	0.424	1.4% (-13.0%, 18.2%)	0.859	10.2% (-12.0%, 38.2%)	0.398	0.548
PIGF (pg/ml)	300	-10.9% (-47.9%, 52.3%)	0.674	0.190	12.9% (-1.1%, 28.8%)	0.072	7.6% (-17.0%, 39.4%)	0.580	0.746
sFLT-1/PIGF	296	15.4% (-42.1%, 130.1%)	0.684	0.664	-11.9% (-28.8%, 9.0%)	0.244	4.2% (-24.3%, 43.5%)	0.801	0.392

Results are from GEE models described in chapter two. The log_{10}-transformed gestation-adjusted value of the marker set as the dependent variable.. The Pre-eclampsia status, gestation of the sample and an interaction term were included as factors in the model. The resulting coefficients were then anti-logged, and converted into percentage differences between groups, or percentage increases per 12 weeks of gestation. The 'baseline difference' section reports the average difference in the intercept between the PE and non-PE groups. In the 'gradient' section, the overall p-value is the significance of the change over time in the gestation-adjusted marker for the cohort as a whole. The gradients for the two groups are then reported separately, with the Interaction p-value representing a comparison between these gradients. Since the analysis is based on gestation-adjusted values, a gradient of zero does not imply that the marker was stable over time, rather it indicates that the rate of change over time is consistent with that of the published normal values.

Table 5-6b: Changes over time in non-angiogenic markers by Pre-eclampsia

Marker	N	Baseline Difference: PE vs. Non-PE		Gradient per 12 Weeks					
				Overall	Non-PE		Pre-eclampsia		Interaction
		Average Difference	*p-Value*	p-Value	*Gradient (95% CI)*	*p-Value*	*Gradient (95% CI)*	*p-Value*	p-Value
cFLC (mg/L)	396	25.2% (10.1%, 37.8%)	**0.002**	**<0.001**	4.8% (1.6%, 8.1%)	**0.003**	5.1% (1.9%, 8.4%)	**0.002**	0.892
κ FLC(mg/L)	396	42.8% (14.4%, 78.2%)	**0.002**	**<0.001**	6.8% (2.7%, 11.0%)	**<0.001**	6.2% (2.0%, 10.5%)	**0.003**	0.843
λ FLC (mg/L)	396	23.5% (5.1%, 45.1%)	**0.010**	**0.010**	2.5% (-0.1%, 5.2%)	0.064	3.6% (-0.2%, 7.5%)	0.064	0.642
IgG (g/L)	396	0.1% (-13.0%, 15.1%)	0.994	**<0.001**	-10.3% (-13.0%, -7.6%)	**<0.001**	-13.9% (-17.3%, -10.5%)	**<0.001**	0.103
IgA (g/L)	396	29.3% (2.6%, 62.9%)	**0.030**	**0.003**	-0.8% (-5.0%, 3.5%)	0.707	-8.2% (-12.2%, -4.0%)	**<0.001**	**0.015**
IgM (g/L)	396	14.5% (-10.7%, 46.7%)	0.286	0.123	-0.9% (-5.8%, 4.4%)	0.742	-4.2% (-7.9%, -0.3%)	**0.037**	0.305
IGG1 (g/l)	283	-9.6% (-24.8%, 8.8%)	0.286	**<0.001**	-11.6% (-14.2%, -8.9%)	**<0.001**	-9.0% (-14.4%, -3.3%)	**0.002**	0.416
IGG2 (g/l)	283	-11.7% (-35.7%, 21.1%)	0.440	**<0.001**	-12.4% (-16.1%, -8.7%)	**<0.001**	-9.5% (-19.0%, 1.0%)	0.076	0.585
IGG3 (g/l)	283	15.4% (-6.7%, 42.7%)	0.186	**<0.001**	-7.1% (-12.3%, -1.6%)	**0.013**	-11.0% (-16.9%, -4.5%)	**0.001**	0.361
IGG4 (g/l)	282	0.6% (-27.4%, 39.4%)	0.971	**<0.001**	-10.1% (-13.4%, -6.7%)	**<0.001**	-12.4% (-16.9%, -7.6%)	**<0.001**	0.447
C3 (g/L)*	396	-0.31 (-0.11, -0.52)*	**0.003**	**<0.001**	0.06 (0.03, 0.09)*	**<0.001**	0.19 (0.13, 0.25)*	**<0.001**	**<0.001**
C4 (g/L)*	396	0.00 (0.06, -0.06)*	0.932	0.158	0.00 (-0.01, 0.01)*	0.779	0.02 (0.00, 0.04)*	0.108	0.109
hsCRP (mg/L)	395	-27.2% (-74.9%, 111.0%)	0.558	0.822	-6.0% (-18.2%, 8.1%)	0.388	1.3% (-31.7%, 50.4%)	0.947	0.726
B2-M (mg/l)	391	27.9% (4.9%, 55.8%)	**0.015**	**<0.001**	12.9% (9.6%, 16.2%)	**<0.001**	17.0% (12.0%, 22.2%)	**<0.001**	0.182
Creatinine μmol/l	394	25.0% (2.8%, 52.0%)	**0.025**	**0.010**	1.6% (-0.8%, 4.0%)	0.190	4.0% (0.5%, 7.6%)	**0.025**	0.266
Cystatin-C (mg/L)	395	33.8% (9.0%, 64.2%)	**0.005**	**<0.001**	20.1% (16.4%, 23.9%)	**<0.001**	17.2% (10.4%, 24.4%)	**<0.001**	0.472
UA (mg/dL)	395	18.0% (0.8%, 38.2%)	**0.039**	**<0.001**	15.3% (12.6%, 18.0%)	**<0.001**	16.5% (10.4%, 22.8%)	**<0.001**	0.733

*Results are from GEE models, with the log_{10}-transformed value of the marker set as the dependent variable. The Pre-eclampsia status, gestation of the sample and an interaction term were included as factors in the model. The resulting coefficients were then anti-logged, and converted into percentage differences between groups, or percentage increases per 12 weeks of gestation. The 'baseline difference' section reports the average difference in the intercept between the Pre-eclampsia and non-pre-eclampsia groups. In the 'gradient' section, the overall p-value is the significance of the change over time in the marker for the cohort as a whole. The gradients for the two groups are then reported separately, with the Interaction p-Value representing a comparison between these gradients. Bold p-values are significant at p<0.05. *C3 and C4 were normally distributed, and had linear trends over time, hence were not log-transformed prior to analysis, meaning that the coefficients represent absolute differences between groups and over time.*

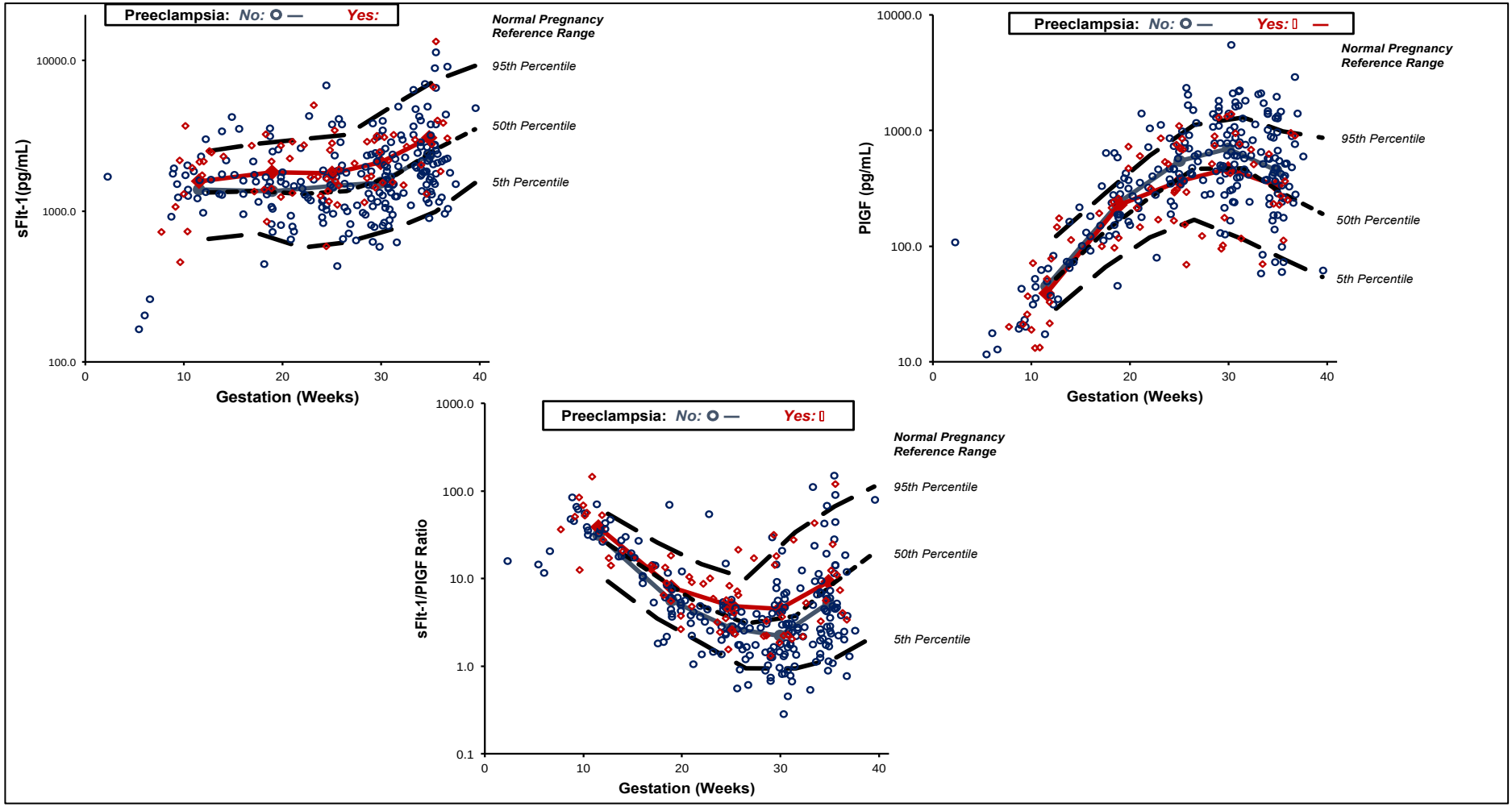
Table 5-7: Associations between markers and Pre-eclampsia by gestation

Marker/ Gestation (Weeks)	Pre-eclampsia: No N	No Average (95% CI)	Yes N	Yes Average (95% CI)	AUROC (SE)	p-Value
cFLC (mg/L)						
<16	54	37.7 (33.2 - 42.7)	17	48.7 (39.2 - 60.4)	0.679 (0.075)	**0.036**
16-21	68	37.6 (34.3 - 41.2)	15	54.9 (43.5 - 69.2)	0.745 (0.070)	**0.003**
22-27	60	38.0 (33.9 - 42.6)	19	54.5 (43.9 - 67.6)	0.721 (0.068)	**0.004**
28-31	66	41.1 (36.7 - 46.2)	15	52.1 (41.2 - 65.9)	0.642 (0.070)	0.086
32+	70	39.5 (35.3 - 44.3)	12	52.5 (41.5 - 66.4)	0.693 (0.066)	**0.034**
κ FLC (mg/L)						
<16	54	19.5 (16.9 - 22.5)	17	25.9 (20.1 - 33.4)	0.653 (0.074)	0.059
16-21	68	19.8 (17.9 - 22.0)	15	30.4 (23.3 - 39.5)	0.743 (0.071)	**0.003**
22-27	60	19.8 (17.4 - 22.6)	19	30.8 (23.9 - 39.6)	0.725 (0.068)	**0.003**
28-31	66	21.6 (18.9 - 24.7)	15	29.3 (22.3 - 38.4)	0.671 (0.070)	**0.040**
32+	70	21.2 (18.5 - 24.2)	12	30.2 (22.6 - 40.4)	0.692 (0.065)	**0.035**
λ FLC (mg/L)						
<16	54	17.9 (16.0 - 20.0)	17	22.2 (18.5 - 26.6)	0.663 (0.076)	**0.043**
16-21	68	17.5 (16.1 - 19.1)	15	24.2 (19.8 - 29.6)	0.734 (0.069)	**0.005**
22-27	60	17.8 (16.1 - 19.7)	19	23.1 (19.2 - 27.7)	0.683 (0.071)	**0.017**
28-31	66	19.1 (17.3 - 21.1)	15	22.1 (17.9 - 27.3)	0.589 (0.076)	0.285
32+	70	18.0 (16.3 - 19.8)	12	21.4 (17.6 - 26.1)	0.644 (0.077)	0.112
IgG (g/L)						
<16	54	9.5 (8.8 - 10.4)	17	8.7 (7.4 - 10.3)	0.600 (0.080)	0.218
16-21	68	9.1 (8.5 - 9.6)	15	7.7 (6.4 - 9.2)	0.668 (0.079)	**0.042**
22-27	60	8.9 (8.3 - 9.5)	19	7.8 (6.4 - 9.3)	0.601 (0.079)	0.187
28-31	66	7.9 (7.2 - 8.5)	15	7.9 (7.1 - 8.8)	0.513 (0.073)	0.879
32+	70	7.5 (7.0 - 8.0)	12	8.1 (6.9 - 9.5)	0.533 (0.090)	0.713
IgA (g/L)						
<16	54	1.53 (1.32 - 1.79)	17	2.11 (1.67 - 2.65)	0.678 (0.076)	**0.028**
16-21	68	1.65 (1.50 - 1.81)	15	1.71 (1.30 - 2.25)	0.507 (0.088)	0.934
22-27	60	1.49 (1.29 - 1.72)	19	1.59 (1.27 - 1.98)	0.506 (0.080)	0.936
28-31	66	1.55 (1.38 - 1.74)	15	1.58 (1.36 - 1.84)	0.504 (0.074)	0.961
32+	70	1.53 (1.39 - 1.69)	12	1.94 (1.53 - 2.47)	0.629 (0.075)	0.155
IgM (g/L)						
<16	54	1.13 (0.94 - 1.35)	17	1.17 (0.94 - 1.46)	0.516 (0.077)	0.845
16-21	68	1.03 (0.91 - 1.17)	15	1.21 (0.95 - 1.54)	0.588 (0.083)	0.287
22-27	60	1.06 (0.93 - 1.21)	19	1.17 (0.91 - 1.51)	0.532 (0.084)	0.675
28-31	66	0.99 (0.88 - 1.12)	15	0.93 (0.68 - 1.26)	0.502 (0.081)	0.981
32+	70	1.04 (0.92 - 1.17)	12	0.90 (0.60 - 1.36)	0.546 (0.094)	0.609
IGG1 (g/l)						
<16	42	5.66 (5.27 - 6.09)	13	5.19 (4.30 - 6.26)	0.603 (0.101)	0.267
16-21	44	5.25 (4.91 - 5.62)	9	4.53 (3.79 - 5.41)	0.654 (0.096)	0.148
22-27	38	5.09 (4.67 - 5.56)	12	4.85 (3.78 - 6.22)	0.503 (0.099)	0.973
28-31	44	4.48 (4.04 - 4.96)	10	5.01 (4.30 - 5.84)	0.570 (0.089)	0.490
32+	61	4.40 (4.11 - 4.73)	10	4.79 (3.95 - 5.81)	0.525 (0.103)	0.804
IGG2 (g/l)						
<16	42	3.29 (2.96 - 3.65)	13	3.23 (2.44 - 4.26)	0.502 (0.096)	0.984
16-21	44	3.04 (2.70 - 3.41)	9	2.45 (2.01 - 3.00)	0.657 (0.088)	0.142
22-27	38	3.07 (2.72 - 3.46)	12	2.77 (2.28 - 3.37)	0.552 (0.086)	0.593
28-31	44	2.54 (2.27 - 2.85)	10	2.53 (2.12 - 3.02)	0.518 (0.095)	0.859
32+	61	2.48 (2.26 - 2.72)	10	2.64 (2.09 - 3.35)	0.525 (0.090)	0.804
IGG3 (g/l)						
<16	42	0.68 (0.57 - 0.81)	13	0.78 (0.68 - 0.90)	0.516 (0.076)	0.866
16-21	44	0.64 (0.55 - 0.74)	9	0.59 (0.43 - 0.81)	0.549 (0.101)	0.644
22-27	38	0.68 (0.57 - 0.80)	12	0.64 (0.50 - 0.82)	0.546 (0.087)	0.633
28-31	44	0.65 (0.57 - 0.75)	10	0.59 (0.43 - 0.80)	0.558 (0.098)	0.570
32+	61	0.57 (0.50 - 0.66)	10	0.65 (0.49 - 0.87)	0.561 (0.090)	0.541
IGG4 (g/l)						
<16	42	0.21 (0.15 - 0.28)	13	0.31 (0.20 - 0.45)	0.634 (0.083)	0.148
16-21	44	0.23 (0.17 - 0.30)	9	0.09 (0.04 - 0.16)	0.763 (0.080)	**0.014**
22-27	38	0.21 (0.15 - 0.28)	11	0.24 (0.10 - 0.46)	0.520 (0.102)	0.839
28-31	44	0.14 (0.11 - 0.19)	10	0.13 (0.04 - 0.29)	0.600 (0.104)	0.327
32+	61	0.18 (0.14 - 0.22)	10	0.14 (0.05 - 0.30)	0.607 (0.108)	0.279

C3 (g/L)*						
<16	54	1.30 (1.24 - 1.37)*	17	1.12 (0.90 - 1.33)*	0.601 (0.083)	0.213
16-21	68	1.41 (1.35 - 1.48)*	15	1.37 (1.16 - 1.57)*	0.524 (0.093)	0.772
22-27	60	1.43 (1.36 - 1.51)*	19	1.30 (1.10 - 1.51)*	0.521 (0.080)	0.787
28-31	66	1.44 (1.37 - 1.52)*	15	1.50 (1.34 - 1.65)*	0.531 (0.083)	0.706
32+	70	1.45 (1.38 - 1.51)*	12	1.53 (1.36 - 1.70)*	0.596 (0.087)	0.288
C4 (g/L)*						
<16	54	0.27 (0.24 - 0.30)*	17	0.32 (0.24 - 0.40)*	0.564 (0.085)	0.427
16-21	68	0.28 (0.26 - 0.31)*	15	0.33 (0.27 - 0.39)*	0.633 (0.077)	0.109
22-27	60	0.26 (0.23 - 0.29)*	19	0.28 (0.23 - 0.34)*	0.567 (0.074)	0.383
28-31	66	0.29 (0.26 - 0.32)*	15	0.33 (0.26 - 0.39)*	0.583 (0.076)	0.319
32+	70	0.27 (0.24 - 0.29)*	12	0.29 (0.21 - 0.37)*	0.540 (0.100)	0.660
hsCRP (mg/L)						
<16	54	3.01 (2.30 - 3.92)	17	2.58 (1.08 - 6.21)	0.520 (0.096)	0.808
16-21	68	4.34 (3.52 - 5.34)	15	4.58 (2.61 - 8.05)	0.550 (0.085)	0.546
22-27	60	3.59 (2.83 - 4.55)	19	2.46 (1.38 - 4.41)	0.600 (0.085)	0.191
28-31	65	3.17 (2.56 - 3.93)	15	2.75 (1.54 - 4.90)	0.535 (0.091)	0.671
32+	70	2.74 (2.20 - 3.43)	12	2.33 (1.22 - 4.46)	0.504 (0.101)	0.969
B2-M (mg/l)						
<16	54	1.82 (1.63 - 2.03)	17	2.40 (1.87 - 3.08)	0.662 (0.073)	**0.045**
16-21	68	1.86 (1.70 - 2.04)	14	3.17 (2.45 - 4.08)	0.809 (0.062)	**<0.001**
22-27	58	1.93 (1.75 - 2.13)	19	2.99 (2.36 - 3.78)	0.751 (0.066)	**0.001**
28-31	64	2.25 (2.00 - 2.54)	15	3.02 (2.41 - 3.79)	0.692 (0.071)	**0.021**
32+	70	2.30 (2.08 - 2.55)	12	2.95 (2.20 - 3.95)	0.642 (0.091)	0.118
Creatinine μmol/l						
<16	54	65.4 (59.5 - 71.9)	17	74.4 (58.6 - 94.4)	0.560 (0.089)	0.455
16-21	67	61.8 (56.9 - 67.1)	15	93.9 (76.7 - 115.1)	0.793 (0.070)	**<0.001**
22-27	60	57.6 (52.3 - 63.4)	18	86.4 (68.3 - 109.4)	0.739 (0.072)	**0.002**
28-31	66	63.6 (57.1 - 70.9)	15	84.1 (64.7 - 109.3)	0.668 (0.086)	**0.043**
32+	70	63.7 (58.1 - 69.8)	12	77.3 (56.8 - 105.3)	0.576 (0.110)	0.405
Cystatin-C (mg/L)						
<16	54	0.82 (0.73 - 0.91)	17	1.05 (0.84 - 1.32)	0.655 (0.076)	0.055
16-21	68	0.80 (0.73 - 0.88)	15	1.29 (1.05 - 1.57)	0.810 (0.062)	**<0.001**
22-27	60	0.85 (0.77 - 0.94)	19	1.18 (0.98 - 1.41)	0.725 (0.064)	**0.003**
28-31	65	1.05 (0.94 - 1.17)	15	1.38 (1.13 - 1.69)	0.701 (0.074)	**0.016**
32+	70	1.20 (1.10 - 1.31)	12	1.49 (1.19 - 1.85)	0.644 (0.086)	0.112
UA(mg/dL)						
<16	54	3.85 (3.57 - 4.16)	17	4.42 (3.77 - 5.19)	0.629 (0.086)	0.112
16-21	68	4.25 (3.97 - 4.55)	15	5.72 (5.06 - 6.47)	0.791 (0.056)	**<0.001**
22-27	60	4.30 (3.97 - 4.66)	19	5.03 (4.39 - 5.76)	0.650 (0.074)	**0.050**
28-31	65	4.71 (4.36 - 5.08)	15	6.12 (4.82 - 7.78)	0.689 (0.089)	**0.023**
32+	70	5.09 (4.77 - 5.44)	12	5.22 (4.42 - 6.17)	0.542 (0.092)	0.641
sFLT-1 (pg/ml)						
<16	29	1392 (1058 - 1831)	14	1574 (1157 - 2142)	0.579 (0.101)	0.407
16-21	33	1354 (1152 - 1591)	12	1805 (1420 - 2296)	0.649 (0.093)	0.130
22-27	40	1459 (1223 - 1741)	19	1783 (1442 - 2203)	0.641 (0.074)	0.081
28-31	59	1541 (1361 - 1744)	13	2101 (1728 - 2554)	0.701 (0.071)	**0.024**
32+	67	2348 (2050 - 2689)	12	3053 (2116 - 4405)	0.629 (0.086)	0.155
PIGF (pg/ml)						
<16	29	45 (34 - 59)	15	39 (25 - 60)	0.543 (0.096)	0.647
16-21	35	242 (194 - 302)	12	227 (156 - 332)	0.536 (0.102)	0.714
22-27	40	544 (442 - 669)	19	366 (258 - 519)	0.632 (0.082)	0.105
28-31	59	695 (572 - 845)	13	466 (268 - 811)	0.592 (0.091)	0.302
32+	67	454 (362 - 569)	12	334 (211 - 529)	0.601 (0.083)	0.269
sFLT-1/PIGF Ratio						
<16	29	31.1 (25.8 - 37.5)	14	38.5 (26.5 - 55.9)	0.601 (0.103)	0.288
16-21	33	5.6 (4.3 - 7.2)	12	7.9 (5.7 - 11.1)	0.669 (0.092)	0.085
22-27	40	2.7 (2.1 - 3.4)	19	4.9 (3.5 - 6.7)	0.728 (0.070)	**0.005**
28-31	59	2.2 (1.8 - 2.8)	13	4.5 (2.4 - 8.3)	0.668 (0.077)	0.060
32+	66	5.3 (3.9 - 7.1)	12	9.1 (4.7 - 17.9)	0.643 (0.075)	0.118

*Data are reported as geometric means and 95% CIs, unless stated otherwise. N represents the number of samples included in the analysis of the stated gestation. AUROC = Area under the ROC curve. Bold p-values are significant at p<0.05.*Arithmetic mean and 95% CI.*

Figure 5-1: Trends in angiogenic markers by gestation in patients that did and did not develop Pre-eclampsia

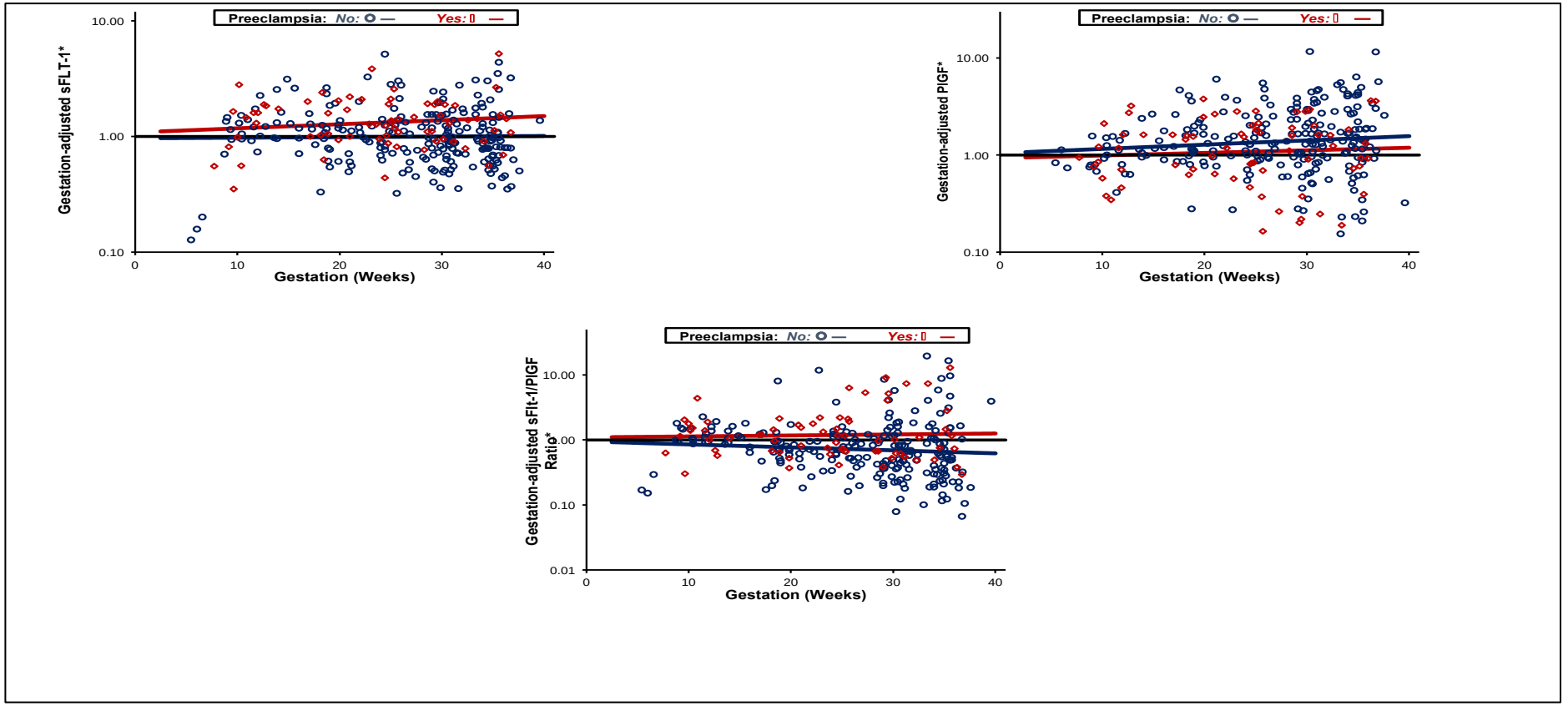


Individual points represent the observed values of all samples with available data. For pregnancies with multiple samples within the same period of gestation in table 5-5 the samples were combined using a geometric mean, and plotted at the arithmetic mean gestation. Trend lines are based on the geometric means within the study gestation periods (Table 5-7) and are plotted at the arithmetic mean gestation of this period. Normal ranges are those published by Roche [276, 277]. During pregnancy, women with CKD showed a similar trend for the angiogenic markers as reported in the general population and this was generall comparable in the PE and non-PE group.

5.2.4.2 Angiogenic Markers – Predictive Accuracy

The predictive accuracy of the angiogenic markers was also assessed using a ROC curve approach (Table 5-7). In this analysis, whilst sFLT-1 tended to be higher throughout pregnancy in those patients that developed PE, the difference between groups was only found to be statistically significant at 28-31 weeks' gestation, with a geometric mean of 2101 vs. 1541pg/ml in those that did vs those that did not develop PE, and a corresponding AUROC of 0.701 (p=0.024). PIGF levels tended to be lower throughout pregnancy in patients that developed PE, but no significant differences between groups were detected within any of the periods of gestation considered. When the markers were combined to form the sFLT-1/PIGF ratio, levels were found to differ significantly between the groups at 22-27 weeks' gestation, with geometric means of 4.9 vs. 2.7 in patients that did vs those that did not develop PE (AUROC=0.728, p=0.005). There was also a tendency for the sFLT-1/PIGF ratio to be higher in PE during 16-21 and 28-31 weeks' gestation, but neither of these comparisons reached significance, with p=0.085 and 0.060, respectively. Comparison of the early onset PE group in isolation versus the late onset PE and non-PE group combined did not show evidence of better predictive performance of the angiogenic markers, either individually or combined as a ratio.

Figure 5-2: Trends in gestation-adjusted values of angiogenic markers by gestation in patients that did and did not develop Pre-eclampsia



**fold-difference, individual points represent the gestation-adjusted values of all samples with available data, consisting of the fold-difference between the observed value and the 50th percentile for the gestation, as published by Roche[276, 277]. For pregnancies with multiple samples within the same period of gestation* (Table 5-5)*, the samples were combined using a geometric mean, and plotted at the arithmetic mean gestation. Trend lines are based on the generalised estimating equation model in Table 5-6a. There was no significant differences in baseline values between PE and non-PE groups for gestation-adjusted sFLT-1, PlGF or the sFLT-1/PlGF ratio (Table 5-6a). In addition, the trends over time were not found to differ significantly from those anticipated, based on the published normal ranges for these markers, with similar gradients observed in the PE and non-PE groups.*

5.2.4.3 Non-angiogenic Markers – Rate of Change

The regression-based analysis (Table 5-6b) of the non-angiogenic study markers being considered found significant differences in the baseline measurements of combined sFLC, IgA, B2-M, serum creatinine, cystatin-C and UA, all of which were higher in the PE versus non-PE CKD group, whilst C3 was significantly lower in the PE cohort. The levels of the majority of markers were found to change significantly over the course of pregnancy for the cohort as a whole. However, the interaction term was significant for only two of the markers, implying that the rate of change differed significantly between the PE and non-PE groups for these two markers.

The first of these was IgA which, as previously reported, was significantly higher in the PE group at baseline, by an average of 29.3% (p=0.030). Over the course of the pregnancy, IgA levels remained relatively constant in the non-PE group, with an average reduction of 0.8% per 12 weeks of gestation (p=0.707). However, the PE patients had a significantly higher rate of decline (p=0.015), at an average of 8.2% per 12 weeks of gestation (p<0.001). The other marker with a significant interaction was C3. Levels were found to be significantly lower in PE pregnancies at baseline, by an average of 0.31g/L (p=0.003). Both groups then saw significant increases in C3 levels over time, but the rate of change was significantly greater in PE patients than in the non-PE group (0.19 vs. 0.06g/L per 12 weeks, p<0.001).

5.2.4.4 Non-angiogenic markers – Predictive Accuracy

Starting with those markers where significant interaction terms were identified above, IgA levels were found to be significantly higher in PE at <16 weeks' gestation (geometric mean: 2.11 vs. 1.53g/L, p=0.028) (Table 5-7). However, since the PE group then showed a more rapid decline than the non-PE group, levels then became more similar, with no significant

differences detected between groups at subsequent periods of gestation. Analysis of C3 did not find this marker to be significantly predictive of PE within any of the periods of gestation considered. However, as per the regression analysis, the tendency was for levels to be lower in PE initially, with 1.12 vs. 1.30g/L at <16 weeks (p=0.213), thereafter, this difference was reversed later in pregnancy (1.53 vs. 1.45g/L at 32+ weeks, p=0.288).

Of the remaining factors, all of those previously identified as differing between the PE and non-PE groups at baseline were found to be significantly predictive of PE within at least one of the periods of gestation considered. Predictive accuracy was consistently the greatest at 16-21 weeks' gestation, at which time combined sFLC, B2-M, creatinine, cystatin-C and UA were all significantly predictive of PE. Overall, the markers with the greatest AUROCs were those related to kidney function, with cystatin-C and B2-M returning values of 0.810 and 0.809 (both p<0.001) at 16-21 weeks of gestation. The other two markers of renal function, creatinine and UA at the same gestational period, provided AUROCs of 0.793 and 0.791, respectively. Only combined sFLC was found to be significantly predictive of PE at the latest gestational period (32 weeks onwards) and the renal markers at this stage were not.

5.2.5 Correlations between Angiogenic System and Other Study Markers

The correlations between the angiogenic system markers with the other study markers were then assessed (Table 5-8a). This found sFLT-1 to be significantly positively correlated with combined sFLC, B2-M, creatinine, cystatin-C and UA, whilst negative correlations were detected with C3, C4 and hsCRP. PIGF was also significantly negatively correlated with C4 and hsCRP, with an additional significant negative correlation with IgG4.

The sFLT-1/PIGF ratio was found to be significantly positively correlated with creatinine, cystatin-C, UA and IgG4. The sFLT-1/PIGF ratio did not demonstrate a significant correlation with the complement system components, even when the study group was split according to PE status as was demonstrated in chapter three (Table 5-8b). Whilst levels of sFLT-1 and the sFLT-1/PIGF ratio did demonstrate a significant positive correlation with markers of renal function, including B2-M, the correlation was less strong in comparison to what was observed in chapter three. Furthermore, the significant negative correlation with renal function markers and PIGF observed in chapter three was not demonstrated in these results. The relationship between pre-pregnancy renal function and angiogenic factors is further explored in Table 5-9, which reports the average values of the angiogenic factors according to CKD stage in all pregnancies. This demonstrates that whilst sFLT-1 concentrations generally appear to be higher in the CKD stage 3-4 versus stage 1, PIGF concentrations and the sFLT-1/PIGF ratio does not demonstrate a relationship with CKD stage.

Table 5-8a: Correlations between angiogenic markers and other markers

	sFLT-1	PIGF	sFLT-1/PIGF Ratio
cFLC	**0.118 (p=0.041)**	-0.003 (p=0.956)	0.086 (p=0.138)
IgG	-0.084 (p=0.148)	-0.088 (p=0.125)	0.064 (p=0.268)
IgA	-0.095 (p=0.100)	-0.081 (p=0.161)	0.053 (p=0.363)
IgM	0.031 (p=0.594)	-0.056 (p=0.330)	0.074 (p=0.205)
IGG1	0.010 (p=0.884)	-0.085 (p=0.211)	0.101 (p=0.138)
IGG2	-0.079 (p=0.244)	-0.122 (p=0.070)	0.089 (p=0.189)
IGG3	-0.063 (p=0.355)	0.102 (p=0.132)	-0.107 (p=0.113)
IGG4	-0.050 (p=0.460)	**-0.213 (p=0.002)**	**0.188 (p=0.005)**
hsCRP	**-0.191 (p=0.001)**	**-0.168 (p=0.003)**	0.062 (p=0.289)
B2-M	**0.217 (p<0.001)**	0.011 (p=0.849)	0.099 (p=0.090)
Creatinine	**0.138 (p=0.017)**	-0.100 (p=0.084)	**0.176 (p=0.002)**
Cystatin-C	**0.325 (p<0.001)**	0.035 (p=0.548)	**0.125 (p=0.032)**
UA	**0.255 (p<0.001)**	-0.018 (p=0.757)	**0.142 (p=0.014)**

Data are reported as Spearman's rho correlation coefficients with p-values, bold values are significant at p<0.05.

Table 5-8b: Correlations between angiogenic markers and C3/C4 by subgroup

	sFLT-1	PIGF	sFLT-1/PIGF Ratio
C3			
Whole Cohort	**-0.203 (p<0.001)**	0.001 (p=0.985)	-0.101 (p=0.081)
Non-PE	**-0.228 (p=0.001)**	-0.008 (p=0.910)	-0.112 (p=0.092)
PE	-0.103 (p=0.394)	0.013 (p=0.915)	-0.052 (p=0.671)
C4			
Whole Cohort	**-0.153 (p=0.008)**	**-0.121 (p=0.036)**	0.037 (p=0.525)
Non-PE	**-0.158 (p=0.017)**	-0.069 (p=0.299)	-0.029 (p=0.660)
PE	-0.193 (p=0.110)	-0.225 (p=0.059)	0.174 (p=0.149)

Data are reported as Spearman's rho correlation coefficients with p-values, and bold

Table 5-9: Medians and interquartile ranges of the angiogenic markers by gestation and CKD stage

Gestation (Weeks)	Overall N	Overall Median (IQR)	CKD 1 N	CKD 1 Median (IQR)	CKD 2 N	CKD 2 Median (IQR)	CKD 3-4 N	CKD 3-4 Median (IQR)
sFLT-1 (pg/ml)								
<16	43	1724 (1233 - 2162)	8	1798 (1297 - 2470)	16	1320 (820 - 1704)	19	1930 (1293 - 2306)
16-21	45	1462 (1064 - 2134)	15	1373 (808 - 1676)	9	1504 (1032 - 2241)	20	1577 (1300 - 2588)
22-27	59	1518 (1092 - 1895)	26	1458 (911 - 1866)	14	1461 (1177 - 2344)	19	1662 (1168 - 2729)
28-31	72	1550 (1182 - 2359)	26	1296 (973 - 1966)	27	1688 (1312 - 2370)	17	1551 (1435 - 2459)
32+	79	2221 (1611 - 3204)	35	2197 (1494 - 4760)	29	2270 (1801 - 2976)	14	2064 (1328 - 3176)
PIGF (pg/ml)								
<16	44	40 (21 - 73)	8	63 (41 - 102)	17	35 (20 - 72)	19	37 (21 - 82)
16-21	47	215 (151 - 354)	16	229 (171 - 358)	9	215 (187 - 258)	21	241 (146 - 425)
22-27	59	466 (308 - 754)	26	394 (302 - 788)	14	535 (340 - 701)	19	539 (316 - 849)
28-31	72	739 (409 - 1282)	26	693 (335 - 999)	27	790 (443 - 1382)	17	739 (425 - 1299)
32+	79	472 (242 - 880)	34	431 (186 - 865)	30	573 (260 - 1132)	14	418 (250 - 762)
sFLT-1/PIGF Ratio								
<16	43	35.3 (20.4 - 51.8)	8	28.4 (15.4 - 40.2)	16	33.9 (17.8 - 44.6)	19	40.6 (26.1 - 65.8)
16-21	45	6.0 (3.9 - 10.4)	15	4.4 (3.1 - 6.0)	9	8.3 (5.5 - 10.4)	20	6.2 (4.5 - 8.9)
22-27	59	2.8 (2.0 - 4.6)	26	3.5 (1.5 - 4.6)	14	2.7 (2.3 - 4.9)	19	2.6 (2.4 - 4.2)
28-31	72	2.2 (1.3 - 3.8)	26	2.3 (1.3 - 3.5)	27	2.2 (1.2 - 3.6)	17	2.5 (1.6 - 5.8)
32+	78	4.6 (2.3 - 11.3)	34	4.6 (2.2 - 23.5)	29	4.2 (2.2 - 10.9)	14	5.7 (3.7 - 7.7)

As per previous analyses, only one sample per pregnancy was included in each period of gestation, with the geometric mean value used where multiple samples were available. Subgroup analysis by CKD stage only includes those patients were this was known pre-pregnancy.

5.2.6 Analysis of Clinical and Late Pregnancy Samples

Analyses were then performed to assess the predictive accuracy of the markers when measured within two weeks of delivery. For this analysis, an additional 7 samples that were taken from patients who were symptomatic with PE were considered for inclusion. The final recorded antenatal sample was within two weeks of delivery for 47 (29%) pregnancies. Of these 17 (36%) patients developed PE, and samples were taken after symptoms presented in 7 (41%) of these pregnancies. The timing of these samples, relative to delivery, were similar in PE and non-PE groups (p=0.478), with medians of 5 days (IQR: 2-8) and 6 days (IQR: 1-11), respectively.

Comparisons of the markers between the PE and non-PE groups are reported in Table 5-10. None of the study markers, as measured within two weeks of delivery, were significantly different between the PE and non-PE groups. A comparison was also undertaken in Table 5-10b, looking specifically at samples taken at the time of clinical manifestation of PE compared to non-PE patients within 2 weeks of delivery. The only marker found to be significantly different between the two groups was a higher level of UA (7.80 vs. 5.74 mg/dL, AUROC 0.771, p= 0.027) in the PE versus non-PE CKD group respectively. The sFLT-1/PIGF ratio was not significantly different between the two groups (22.2 versus 10.6, p=0.385) in this analysis, which contrasts with the findings presented in chapter three.

Table 5-10a: Predictive accuracy of samples taken within two weeks of delivery

	Non-PE		PE			
	N	*Average (95% CI)*	*N*	*Average (95% CI)*	AUROC (SE)	p-Value
sFLC (mg/L)	30	49.3 (40.1 - 60.6)	17	52.5 (43.7 - 63.2)	0.551 (0.084)	0.565
IgG (g/L)	30	7.4 (6.5 - 8.5)	17	7.0 (6.2 - 7.9)	0.653 (0.084)	0.084
IgA (g/L)	30	1.68 (1.46 - 1.92)	17	1.85 (1.48 - 2.31)	0.546 (0.089)	0.603
IgM (g/L)	30	1.19 (1.00 - 1.42)	17	1.02 (0.83 - 1.27)	0.609 (0.085)	0.219
IGG1 (g/l)	25	4.20 (3.61 - 4.89)	15	4.33 (3.86 - 4.84)	0.552 (0.091)	0.581
IGG2 (g/l)	25	2.57 (2.18 - 3.04)	15	2.25 (1.85 - 2.73)	0.646 (0.088)	0.121
IGG3 (g/l)	25	0.59 (0.46 - 0.76)	15	0.55 (0.43 - 0.70)	0.562 (0.089)	0.512
IGG4 (g/l)	25	0.14 (0.10 - 0.19)	15	0.08 (0.05 - 0.13)	0.657 (0.087)	0.096
C3 (g/L)*	30	1.40 (1.28 - 1.52)*	17	1.56 (1.41 - 1.70)*	0.632 (0.082)	0.135
C4 (g/L)*	30	0.29 (0.22 - 0.35)*	17	0.31 (0.24 - 0.37)*	0.564 (0.086)	0.472
hsCRP (mg/L)	30	2.95 (2.02 - 4.29)	17	2.99 (1.51 - 5.91)	0.535 (0.100)	0.690
B2-M (mg/l)	30	2.97 (2.37 - 3.72)	17	3.47 (2.85 - 4.22)	0.626 (0.083)	0.153
Creatinine (μmol/l)	30	77.1 (63.3 - 94.0)	17	93.5 (74.0 - 118.2)	0.600 (0.088)	0.259
Cystatin-C (mg/L)	30	1.54 (1.30 - 1.83)	17	1.67 (1.44 - 1.94)	0.574 (0.085)	0.406
UA (mg/dL)	30	5.74 (5.09 - 6.48)	17	6.79 (5.68 - 8.12)	0.642 (0.084)	0.108
sFLT-1 (pg/ml)	27	3073 (2448 - 3858)	16	3593 (2682 - 4812)	0.567 (0.093)	0.466
PIGF (pg/ml)	26	293 (197 - 438)	16	265 (149 - 470)	0.500 (0.097)	1.000
sFLT-1/PIGF Ratio	26	10.6 (6.4 - 17.5)	16	13.6 (6.5 - 28.3)	0.512 (0.096)	0.897

*Data are reported as geometric means and 95% CIs, unless stated otherwise. AUROC = Area under the ROC curve. Bold p-values are significant at p<0.05.*Arithmetic mean and 95% CI.*

Table 5-10b: Predictive accuracy of samples taken within two weeks of delivery for non-PE versus those with clinical (symptomatic) PE

	Non-PE		Symptomatic PE			
	N	*Average (95% CI)*	*N*	*Average (95% CI)*	AUROC (SE)	p-Value
sFLC (mg/L)	30	49.3 (40.1 - 60.6)	7	42.5 (37.7 - 47.9)	0.548 (0.090)	0.698
IgG (g/L)	30	7.4 (6.5 - 8.5)	7	6.5 (5.7 - 7.4)	0.705 (0.084)	0.095
IgA (g/L)	30	1.68 (1.46 - 1.92)	7	1.62 (1.18 - 2.22)	0.507 (0.137)	0.954
IgM (g/L)	30	1.19 (1.00 - 1.42)	7	0.98 (0.76 - 1.26)	0.626 (0.099)	0.304
IGG1 (g/l)	25	4.20 (3.61 - 4.89)	7	4.02 (3.59 - 4.51)	0.616 (0.093)	0.349
IGG2 (g/l)	25	2.57 (2.18 - 3.04)	7	2.00 (1.57 - 2.55)	0.714 (0.091)	0.085
IGG3 (g/l)	25	0.59 (0.46 - 0.76)	7	0.60 (0.44 - 0.83)	0.532 (0.110)	0.798
IGG4 (g/l)	25	0.14 (0.10 - 0.19)	7	0.07 (0.02 - 0.13)	0.698 (0.112)	0.110
C3 (g/L)*	30	1.40 (1.28 - 1.52)*	7	1.60 (1.34 - 1.87)*	0.664 (0.120)	0.181
C4 (g/L)*	30	0.29 (0.22 - 0.35)*	7	0.33 (0.26 - 0.39)*	0.640 (0.100)	0.253
hsCRP (mg/L)	30	2.95 (2.02 - 4.29)	7	4.29 (1.51 - 12.18)	0.590 (0.134)	0.461
B2-M (mg/l)	30	2.97 (2.37 - 3.72)	7	3.39 (2.64 - 4.36)	0.648 (0.090)	0.229
Creatinine (umol/l)	30	77.1 (63.3 - 94.0)	7	92.6 (65.9 - 130.2)	0.626 (0.104)	0.304
Cystatin-C (mg/L)	30	1.54 (1.30 - 1.83)	7	1.70 (1.39 - 2.08)	0.614 (0.088)	0.352
UA (mg/dL)	30	5.74 (5.09 - 6.48)	7	7.80 (6.60 - 9.22)	0.771 (0.081)	**0.027**
sFLT-1 (pg/ml)	27	3073 (2448 - 3858)	6	4654 (3047 - 7110)	0.679 (0.129)	0.176
PIGF (pg/ml)	26	293 (197 - 438)	6	210 (65 - 678)	0.551 (0.149)	0.699
sFLT-1/PIGF Ratio	26	10.6 (6.4 - 17.5)	6	22.2 (5.3 - 92.8)	0.615 (0.132)	0.385

*Data are reported as geometric means and 95% CIs, unless stated otherwise. AUROC = Area under the ROC curve. Bold p-values are significant at p<0.05. *Arithmetic mean and 95% CI.*

5.2.7 Postnatal Samples

In 37 pregnancies (8 with PE, 22%), sFLT-1 and/or PIGF levels were measured in both the antenatal and postnatal periods. Where multiple samples were available for one of the periods, the one closest to the date of delivery was used in the analysis. The included antenatal samples were collected a median of 27 days (IQR: 10 – 48) before delivery, and this was similar in the PE and non-PE groups (p=0.769). Postnatal samples were collected a median of 24 days (IQR: 17 – 37) after delivery, with PE patients tending to have earlier postnatal samples (median: 18 vs. 27 days post-delivery, p=0.028).

Analyses of the postnatal samples are reported in Table 5-11. Both sFLT-1 and PIGF were found to decrease significantly post-delivery, from a median of 2370 to 92pg/ml and 423 to 12pg/ml, respectively (both p<0.001). However, no significant change in the sFLT-1/PIGF ratio was detected, with a median of 5.9 for the antenatal samples, compared to 9.1 in the postnatal samples (p=0.133). Comparisons between the PE and non-PE groups found the postnatal sFLT-1 to be significantly higher in PE, with medians of 113 vs. 78pg/ml (p=0.042). However, neither PIGF nor the sFLT-1/PIGF ratio were found to differ significantly between groups, either in the antenatal or postnatal samples, or the fold change between these.

Table 5-11: Comparisons of angiogenic markers in the antenatal and postnatal periods

	Overall (N=37)	Pre-eclampsia		p-Value
		No (N=29)	*Yes (N=8)*	(PE vs. non-PE)**
Antenatal Sample to Delivery (Days)	27 (10, 48)	27 (10, 48)	27 (9, 56)	0.769
Delivery to Postnatal Sample (Days)	24 (17, 37)	27 (21, 41)	18 (12, 21)	**0.028**
sFLT-1 (pg/ml)				
Last Antenatal Sample	2370 (1715, 3424)	2370 (1842, 3424)	2560 (1512, 3505)	0.854
First Postnatal Sample	92 (72, 114)	78 (69, 101)	113 (94, 162)	**0.042**
Fold Change	0.04 (0.03, 0.06)	0.04 (0.03, 0.06)	0.06 (0.04, 0.10)	0.150
*p-Value (Ante vs. Postnatal)**	**<0.001**	**<0.001**	0.123	-
PIGF (pg/ml)				
Last Antenatal Sample	423 (189, 861)	423 (238, 888)	204 (112, 812)	0.238
First Postnatal Sample	12 (8, 15)	12 (8, 16)	12 (10, 13)	0.796
Fold Change	0.03 (0.01, 0.07)	0.03 (0.01, 0.06)	0.07 (0.02, 0.15)	0.060
*p-Value (Ante vs. Postnatal)**	**<0.001**	**<0.001**	**0.012**	-
sFLT-1/PIGF Ratio				
Last Antenatal Sample	5.9 (3.0, 14.0)	4.9 (2.5, 11.3)	8.2 (5.0, 19.6)	0.210
First Postnatal Sample	9.1 (6.4, 13.0)	8.4 (6.0, 13.0)	11.3 (8.3, 14.8)	0.253
Fold Change	1.48 (0.87, 3.44)	1.75 (0.87, 3.94)	1.40 (0.67, 2.56)	0.438
*p-value (Ante vs. Postnatal)**	0.133	0.107	0.674	-

*Data are reported as median (IQR), and bold p-values are significant at p<0.05 . *p-Values from Wilcoxon's tests, comparing the ante- vs. postnatal values. **p-Values from Mann-Whitney tests, comparing the PE vs. non-PE groups.*

5.2.8 Biochemical Predictors of PE and/or Composite Adverse Pregnancy Outcomes

The previously described composite adverse pregnancy outcome was then combined with PE, to produce an outcome which occurred in 66/155 (43%) of pregnancies. Comparisons of a range of factors between those pregnancies with and without this composite outcome is reported in Tables 5-12a-b. The comparison yielded very similar results to the analysis of clinical PE predictors, with similar demographic, CKD-related, obstetric and antenatal factors associated with both outcomes.

Furthermore, the predictive accuracies of the study markers for this composite outcome over the five gestational periods were also compared. These were also comparable to findings for the PE analysis, with markers of kidney function (cystatin-C, UA, B2-M, creatinine) and cFLC higher at multiple gestational periods in those with the composite outcome compared to those without. However, in this analysis the sFLT-1/PIGF ratio was a stronger predictor than it was for PE alone as it was significantly higher in the adverse outcome group at all four gestational periods after 16 weeks with AUROC of 0.688-0.726 which was comparable to the AUROCs generated to the kidney function markers.

5.2.9 Pregnancy Outcomes by Pre-pregnancy CKD Stage

The distribution of CKD aetiologies was not found to vary significantly with CKD stage ($p=0.160$, Table 5-13b). However, the proportions of patients with renal transplant and chronic hypertension both increased progressively with CKD stage, from 2% to 26% ($p<0.001$) and 27% to 57% ($p=0.003$) in CKD stage 1 vs. 3-4, respectively. Patients with higher CKD staging were significantly more likely to have attended a pre-pregnancy clinic ($p=0.015$). The numbers of previous pregnancies ($p=0.264$) and miscarriages ($p=0.238$) were not found to differ significantly between the groups (Table 5-13c). In those with previous

pregnancies, no significant differences in the rates of previous adverse obstetric events were detected across the CKD stages, although there was a tendency for higher rates of previous pre-term birth in CKD stage 3-4 (48% vs. 23% in stage 1, p=0.079).

Comparisons across the CKD stages found a significant, progressive increase in booking diastolic BP, with means of 74, 77 and 79 mmHg in stages 1, 2 and 3-4, respectively (p=0.025, Table 5-14d). Rates of antenatal complications were similar across the CKD stages, with the exception of renal complications and new onset/worsening hypertension, both of which increased significantly with CKD stage, from 24% to 72% (p<0.001) and 30% to 56% (p=0.017) between CKD stages 1 and 3-4, respectively.

Table 5-12a: Associations between demographic factors and Pre-eclampsia and/or composite adverse pregnancy outcomes

	N	Pre-eclampsia/Adverse Outcome *No (N=88)*	*Yes (N=64)*	*p-Value*
Demographics/Pre-Pregnancy				
Age at LMP (Years)	155	30.4 ± 5.2	30.5 ± 6.0	0.723
Ethnicity	155			0.881
White		51 (57%)	42 (64%)	
South Asian		23 (26%)	15 (23%)	
Black		8 (9%)	5 (8%)	
Other		7 (8%)	4 (6%)	
Booking BMI kg/m^2	149	26.0 (23.8 – 30.8)	27.9 (23.9 – 33.2)	0.270
Pre-Pregnancy Smoking History	146	25 (29%)	11 (19%)	0.178
Known to Renal Team Pre-Pregnancy	155	81 (91%)	59 (89%)	0.788
Attended Pre-Pregnancy Clinic	150	21 (24%)	11 (17%)	0.319
Assisted Conception	155	5 (6%)	3 (5%)	1.000

Continuous variables are reported as mean±SD, or as median (IQR), with p-values from independent sample t-tests and Mann-Whitney tests respectively. Categorical variables are reported as N (%), with p-values from Chi-square.

Table 5-12b: Associations between pre-pregnancy renal markers, co-morbidity and Pre-eclampsia and/or composite adverse pregnancy outcome

	N	Pre-eclampsia/Adverse Outcome *No(N=88)*	*Yes(N=64)*	*p-Value*
CKD History				
Pre-Pregnancy CKD Stage	152			**0.005***
1		39 (44%)	21 (33%)	
2		37 (42%)	17 (27%)	
3		11 (13%)	24 (38%)	
4		1 (1%)	2 (3%)	
Pre-Pregnancy Creatinine umol/l	118	77 (60 – 92)	92 (65 – 118)	**0.016**
Pre-Pregnancy eGFR**	118	81 (63 - *>90*)	63 (45 - *>90*)	**0.025**
Pre-Pregnancy urine ACR mg/mmol	113	4.4 (1.5 – 43.0)	15.0 (2.9 – 64.0)	**0.020**
Aetiology	138			0.086
ADPKD		12 (15%)	4 (7%)	
Glomerular Disease		25 (31%)	15 (26%)	
Lupus Nephritis		5 (6%)	11 (19%)	
Structural		14 (18%)	7 (12%)	
Tubulo-Interstitial Disease		17 (21%)	11 (19%)	
Others		7 (9%)	10 (17%)	
Single Functioning Native Kidney	155	9 (10%)	7 (11%)	1.000
Duplex	155	4 (4%)	3 (5%)	1.000
Pre-pregnancy UTI History	152	35 (39%)	19 (30%)	0.303
Stone Disease	155	7 (8%)	6 (9%)	0.779
Reflux	155	24 (27%)	14 (21%)	0.454
Metabolic	155	5 (6%)	4 (6%)	1.000
Medical History				
SLE	155	5 (6%)	11 (17%)	**0.033**
Other CTD	155	0 (0%)	2 (3%)	0.180
Any Autoimmune	155	18 (20%)	24 (36%)	**0.029**
Renal Transplant	155	7 (8%)	9 (14%)	0.290
Chronic Hypertension	155	19 (21%)	37 (56%)	**<0.001**
Diabetes	155			0.063
No		89 (100%)	62 (94%)	
T1		0 (0%)	2 (3%)	
T2		0 (0%)	2 (3%)	

*Continuous variables are reported as median (IQR), with p-values from Mann-Whitney tests. Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. **The laboratory reported eGFR levels that were truncated at 90 ml/min/1.73m^2, hence a value of 90 was assumed for these in the analysis. ADPKD = autosomal dominant polycystic kidney disease, UTI = urinary tract infection, CTD = connective tissue disease.*

Table 5-12c: Associations between obstetric history and Pre-eclampsia and/or composite adverse pregnancy outcomes

	N	Pre-eclampsia/Adverse Outcome *No(N=88)*	*Yes(N=64)*	*p-Value*
Pregnancy History				
Nulliparous	155	28 (31%)	30 (45%)	0.094
Gravida	154			0.199*
1		25 (28%)	21 (32%)	
2		24 (27%)	23 (35%)	
3+		40 (45%)	21 (32%)	
Number of Previous Miscarriages	154			0.644*
0		62 (70%)	42 (65%)	
1		14 (16%)	15 (23%)	
2+		13 (15%)	8 (12%)	
Pre-Term delivery (<37 Weeks)**	97	10 (16%)	14 (39%)	**0.016**
Very Pre-Term delivery (<34 Weeks)**	97	2 (3%)	8 (22%)	**0.005**
Previous Pre-eclampsia**	97	7 (11%)	13 (36%)	**0.008**
Previous SGA/FGR**	97	19 (31%)	15 (42%)	0.379
Previous NND**	97	4 (7%)	1 (3%)	0.648
Previous Still Birth**	97	1 (2%)	5 (14%)	**0.025**

*Categorical variables are reported as N (%), with p-values Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. **Excludes women that were nulliparous. SGA = small for gestational age infant, FGR –fetal growth restriction, NND = neonatal death.*

Table 5-12d: Associations between antenatal factors and Pre-eclampsia and/or composite adverse pregnancy outcomes

	N	Pre-eclampsia/Adverse Outcome		p-Value
		No(N=88)	*Yes(N=64)*	
Antenatal Factors				
Twin Pregnancy	155	1 (1%)	4 (6%)	0.164
Booking/Earliest Urine Dip Protein	147			0.167*
Negative		59 (69%)	36 (59%)	
1+		12 (14%)	9 (15%)	
2+		8 (9%)	6 (10%)	
3+		7 (8%)	10 (16%)	
Booking/Earliest Urine Dip Blood	147			0.732*
Negative		69 (80%)	47 (77%)	
1+		5 (6%)	7 (11%)	
2+		6 (7%)	3 (5%)	
3+		6 (7%)	4 (7%)	
Booking SBP	149	122 ± 12	127 ± 12	**0.025**
Booking DBP	149	74 ± 11	80 ± 11	**0.001**
AN Aspirin	146	77 (90%)	55 (92%)	0.780
Static Growth Detected on USS	118	10 (15%)	10 (19%)	0.625
Infections - 1st Trimester	145	16 (18%)	8 (14%)	0.504
Infections - 2nd Trimester	146	19 (22%)	7 (12%)	0.185
Infections - 3rd Trimester	147	15 (17%)	7 (12%)	0.482
Recurrent UTI	147	14 (16%)	3 (5%)	0.064
Gestational Diabetes**	143	11 (13%)	3 (5%)	0.249
AN Renal Complication	152	20 (22%)	34 (54%)	**<0.001**
AN New/Worsening Hypertension	151	17 (19%)	39 (63%)	**<0.001**
AN Fetal Distress	155	9 (10%)	3 (5%)	0.238
Growth Issue	145	9 (11%)	23 (38%)	**<0.001**
Uterine artery Doppler notching	124			**0.017**
No		33 (43%)	27 (57%)	
Yes		7 (9%)	9 (19%)	
*Unknown****		37 (48%)	11 (23%)	
Umbilical Artery Flow Abnormality	138			**0.002**
No		68 (85%)	46 (79%)	
Yes		3 (4%)	11 (19%)	
*Unknown****		9 (11%)	1 (2%)	
Pre-Delivery Admission (1+)	151	30 (34%)	21 (34%)	1.000

*Continuous variables are reported as mean±SD, with p-values from independent t-tests. Categorical variables are reported as N (%), with p-values from Chi-square tests unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney test, as the factor is ordinal. **Patients with pre-pregnancy DM were excluded from this analysis. ***An "unknown" category was included to account for potential selection bias. Cases where it was unclear whether or not a test had been performed were excluded from the analysis., BP=blood pressure, AN = antenatal, USS = ultrasound scan.*

Table 5-13: Associations between markers and Pre-eclampsia and/or composite adverse pregnancy outcome by gestation

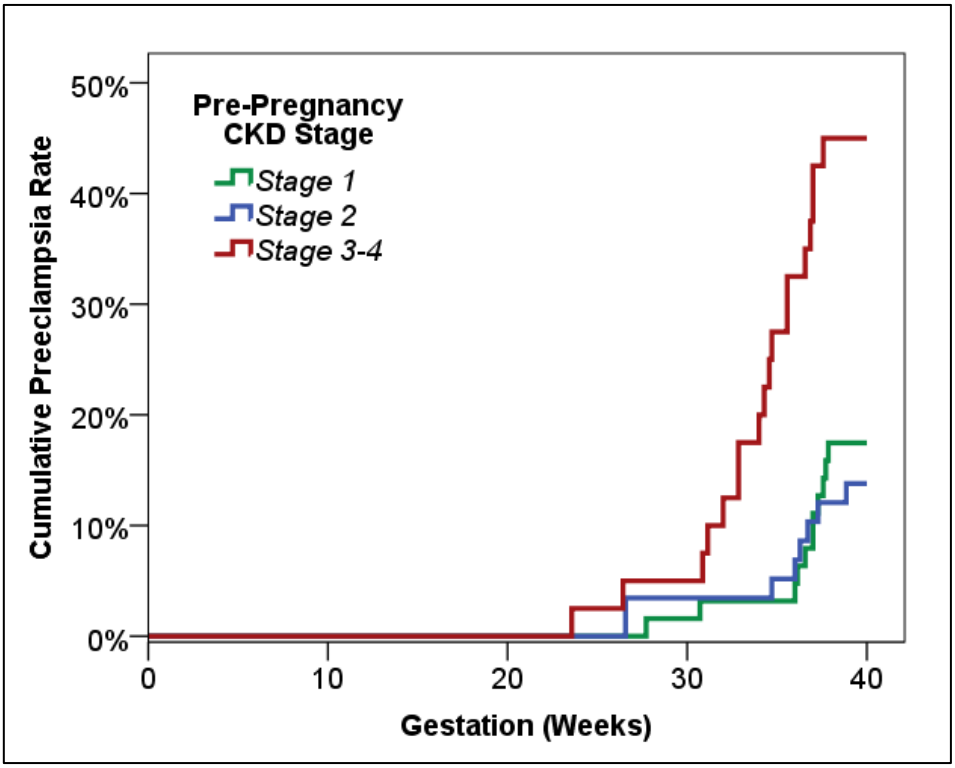
Marker/ Gestation (Weeks)	Pre-eclampsia / Adverse Outcome: No *N*	No *Average (95% CI)*	Yes *N*	Yes *Average (95% CI)*	AUROC (SE)	p-Value
cFLC (mg/L)						
<16	33	34.6 (29.9 - 40.1)	35	48.9 (42.2 - 56.6)	0.731 (0.062)	**0.001**
16-21	50	36.3 (32.7 - 40.3)	29	47.9 (40.5 - 56.5)	0.677 (0.062)	**0.009**
22-27	41	35.9 (31.5 - 40.8)	34	51.6 (43.9 - 60.7)	0.724 (0.060)	**<0.001**
28-31	49	39.4 (34.8 - 44.6)	27	53.3 (44.2 - 64.2)	0.667 (0.065)	**0.016**
32+	55	39.0 (34.3 - 44.3)	22	50.5 (41.7 - 61.1)	0.669 (0.064)	**0.021**
IgG (g/L)						
<16	33	9.2 (8.5 - 10.0)	35	10.1 (9.1 - 11.3)	0.635 (0.069)	0.056
16-21	50	9.0 (8.5 - 9.6)	29	8.6 (7.6 - 9.8)	0.568 (0.069)	0.314
22-27	41	8.4 (7.8 - 9.0)	34	8.8 (7.7 - 10.1)	0.549 (0.068)	0.463
28-31	49	7.9 (7.3 - 8.6)	27	8.0 (7.1 - 9.1)	0.525 (0.067)	0.720
32+	55	7.4 (6.9 - 7.9)	22	8.6 (7.6 - 9.7)	0.636 (0.072)	0.064
IgA (g/L)						
<16	33	1.56 (1.39 - 1.75)	35	1.82 (1.44 - 2.32)	0.662 (0.066)	**0.021**
16-21	50	1.64 (1.48 - 1.82)	29	1.76 (1.47 - 2.11)	0.553 (0.071)	0.434
22-27	41	1.53 (1.37 - 1.71)	34	1.51 (1.18 - 1.93)	0.540 (0.069)	0.551
28-31	49	1.60 (1.44 - 1.78)	27	1.57 (1.28 - 1.94)	0.527 (0.070)	0.696
32+	55	1.57 (1.43 - 1.73)	22	1.78 (1.41 - 2.26)	0.629 (0.072)	0.080
IgM (g/L)						
<16	33	1.22 (0.95 - 1.58)	35	1.01 (0.86 - 1.20)	0.605 (0.069)	0.136
16-21	50	1.08 (0.93 - 1.25)	29	1.02 (0.85 - 1.22)	0.521 (0.068)	0.760
22-27	41	1.08 (0.92 - 1.26)	34	1.08 (0.90 - 1.29)	0.508 (0.069)	0.907
28-31	49	0.98 (0.83 - 1.14)	27	0.97 (0.80 - 1.17)	0.523 (0.067)	0.737
32+	55	1.06 (0.93 - 1.22)	22	0.87 (0.68 - 1.11)	0.595 (0.073)	0.197
IGG1 (g/l)						
<16	28	5.44 (5.03 - 5.89)	26	5.90 (5.24 - 6.65)	0.594 (0.079)	0.236
16-21	34	5.38 (5.01 - 5.78)	17	4.90 (4.37 - 5.49)	0.586 (0.083)	0.323
22-27	26	4.76 (4.32 - 5.24)	22	5.31 (4.51 - 6.25)	0.629 (0.081)	0.126
28-31	31	4.63 (4.14 - 5.18)	20	4.51 (3.82 - 5.32)	0.505 (0.082)	0.954
32+	46	4.31 (4.00 - 4.65)	20	5.05 (4.44 - 5.74)	0.617 (0.080)	0.134
IGG2 (g/l)						
<16	28	3.15 (2.75 - 3.61)	26	3.47 (2.96 - 4.05)	0.563 (0.080)	0.431
16-21	34	3.16 (2.78 - 3.60)	17	2.62 (2.20 - 3.12)	0.608 (0.084)	0.212
22-27	26	2.97 (2.53 - 3.47)	22	3.05 (2.66 - 3.50)	0.530 (0.088)	0.725
28-31	31	2.55 (2.23 - 2.91)	20	2.56 (2.17 - 3.03)	0.516 (0.085)	0.847
32+	46	2.45 (2.20 - 2.74)	20	2.81 (2.45 - 3.24)	0.596 (0.073)	0.217
IGG3 (g/l)						
<16	28	0.63 (0.50 - 0.78)	26	0.84 (0.72 - 0.98)	0.631 (0.076)	0.098
16-21	34	0.62 (0.53 - 0.73)	17	0.65 (0.51 - 0.83)	0.533 (0.088)	0.704
22-27	26	0.63 (0.53 - 0.74)	22	0.67 (0.54 - 0.84)	0.543 (0.086)	0.612
28-31	31	0.65 (0.56 - 0.75)	20	0.60 (0.48 - 0.74)	0.533 (0.085)	0.692
32+	46	0.55 (0.47 - 0.64)	20	0.69 (0.55 - 0.88)	0.641 (0.076)	0.070
IGG4 (g/l)						
<16	28	0.22 (0.15 - 0.32)	26	0.27 (0.19 - 0.36)	0.573 (0.079)	0.354
16-21	34	0.25 (0.18 - 0.33)	17	0.15 (0.10 - 0.21)	0.644 (0.079)	0.097
22-27	26	0.22 (0.14 - 0.33)	21	0.20 (0.12 - 0.31)	0.535 (0.086)	0.684
28-31	31	0.16 (0.11 - 0.23)	20	0.11 (0.06 - 0.18)	0.629 (0.080)	0.123
32+	46	0.18 (0.14 - 0.23)	20	0.16 (0.09 - 0.25)	0.542 (0.079)	0.586
C3 (g/L)*						
<16	33	1.35 (1.27 - 1.43)	35	1.21 (1.09 - 1.34)	0.582 (0.070)	0.244
16-21	50	1.40 (1.32 - 1.48)	29	1.42 (1.30 - 1.54)	0.574 (0.070)	0.276
22-27	41	1.44 (1.34 - 1.53)	34	1.35 (1.22 - 1.48)	0.509 (0.069)	0.898
28-31	49	1.47 (1.38 - 1.55)	27	1.43 (1.30 - 1.56)	0.555 (0.072)	0.431
32+	55	1.47 (1.40 - 1.55)	22	1.45 (1.34 - 1.56)	0.524 (0.071)	0.739
C4 (g/L)*						
<16	33	0.29 (0.25 - 0.33)	35	0.28 (0.23 - 0.33)	0.551 (0.071)	0.469
16-21	50	0.28 (0.25 - 0.32)	29	0.30 (0.25 - 0.34)	0.546 (0.067)	0.502
22-27	41	0.28 (0.25 - 0.32)	34	0.25 (0.21 - 0.29)	0.589 (0.067)	0.189
28-31	49	0.30 (0.26 - 0.33)	27	0.30 (0.25 - 0.36)	0.515 (0.072)	0.832
32+	55	0.28 (0.24 - 0.31)	22	0.25 (0.20 - 0.30)	0.581 (0.076)	0.269

hsCRP mg/L						
<16	33	3.49 (2.66 - 4.59)	35	3.13 (1.90 - 5.19)	0.501 (0.072)	0.985
16-21	50	4.11 (3.18 - 5.31)	29	4.89 (3.46 - 6.90)	0.577 (0.067)	0.257
22-27	41	3.40 (2.52 - 4.59)	34	3.12 (2.14 - 4.54)	0.513 (0.069)	0.844
28-31	48	3.13 (2.42 - 4.04)	27	2.90 (1.98 - 4.26)	0.519 (0.072)	0.783
32+	55	2.94 (2.31 - 3.74)	22	2.24 (1.36 - 3.69)	0.579 (0.077)	0.282
B2-M (mg/l)						
<16	33	1.72 (1.50 - 1.98)	35	2.25 (1.94 - 2.61)	0.685 (0.064)	**0.009**
16-21	50	1.78 (1.61 - 1.97)	28	2.49 (2.06 - 3.01)	0.699 (0.064)	**0.004**
22-27	39	1.86 (1.65 - 2.09)	34	2.62 (2.22 - 3.10)	0.706 (0.061)	**0.003**
28-31	48	2.11 (1.87 - 2.38)	26	3.13 (2.56 - 3.83)	0.724 (0.064)	**0.002**
32+	55	2.32 (2.06 - 2.62)	22	2.67 (2.20 - 3.24)	0.586 (0.074)	0.239
Creatinine(μmol/l)						
<16	33	62.3 (55.8 - 69.5)	35	74.3 (64.1 - 86.1)	0.607 (0.070)	0.130
16-21	50	59.4 (54.4 - 64.8)	28	79.7 (67.5 - 94.0)	0.697 (0.067)	**0.004**
22-27	41	56.1 (50.2 - 62.7)	33	74.4 (62.5 - 88.6)	0.660 (0.065)	**0.019**
28-31	49	60.0 (53.6 - 67.0)	27	84.3 (68.9 - 103.2)	0.685 (0.070)	**0.008**
32+	55	64.3 (57.9 - 71.5)	22	68.9 (56.4 - 84.2)	0.541 (0.078)	0.573
Cystatin-C (mg/L)						
<16	33	0.77 (0.67 - 0.89)	35	0.99 (0.86 - 1.14)	0.682 (0.065)	**0.010**
16-21	50	0.77 (0.69 - 0.85)	29	1.08 (0.91 - 1.26)	0.724 (0.059)	**<0.001**
22-27	41	0.83 (0.74 - 0.93)	34	1.07 (0.93 - 1.24)	0.683 (0.061)	**0.007**
28-31	48	0.99 (0.88 - 1.10)	27	1.39 (1.17 - 1.65)	0.719 (0.063)	**0.002**
32+	55	1.21 (1.09 - 1.34)	22	1.35 (1.16 - 1.58)	0.592 (0.070)	0.209
UA (mg/dL)						
<16	33	3.65 (3.36 - 3.97)	35	4.43 (4.02 - 4.89)	0.689 (0.064)	**0.007**
16-21	50	4.12 (3.82 - 4.44)	29	5.12 (4.59 - 5.71)	0.717 (0.060)	**0.001**
22-27	41	4.16 (3.79 - 4.58)	34	5.02 (4.56 - 5.54)	0.674 (0.062)	**0.010**
28-31	48	4.47 (4.13 - 4.84)	27	6.07 (5.20 - 7.09)	0.737 (0.064)	**<0.001**
32+	55	5.12 (4.77 - 5.50)	22	5.24 (4.65 - 5.91)	0.544 (0.074)	0.550
sFLT-1 (pg/ml)						
<16	16	1431 (1102 - 1859)	26	1251 (891 - 1755)	0.510 (0.089)	0.917
16-21	22	1209 (981 - 1491)	22	1764 (1504 - 2070)	0.699 (0.081)	**0.024**
22-27	23	1435 (1169 - 1760)	33	1749 (1457 - 2099)	0.603 (0.078)	0.191
28-31	45	1454 (1262 - 1674)	24	2145 (1880 - 2447)	0.757 (0.058)	**<0.001**
32+	55	2247 (1935 - 2609)	20	3144 (2429 - 4070)	0.676 (0.068)	**0.020**
PIGF (pg/ml)						
<16	16	39 (29 - 52)	27	39 (28 - 54)	0.528 (0.089)	0.763
16-21	24	251 (193 - 327)	22	224 (168 - 299)	0.519 (0.087)	0.826
22-27	23	603 (468 - 777)	33	405 (312 - 528)	0.639 (0.074)	0.079
28-31	45	730 (578 - 921)	24	524 (367 - 750)	0.585 (0.072)	0.246
32+	54	475 (373 - 604)	21	343 (231 - 509)	0.598 (0.072)	0.190
sFLT-1/PIGF Ratio						
<16	16	36.7 (29.1 - 46.2)	26	31.7 (24.7 - 40.7)	0.587 (0.088)	0.351
16-21	22	4.8 (3.6 - 6.4)	22	7.9 (5.8 - 10.7)	0.688 (0.082)	**0.033**
22-27	23	2.4 (2.0 - 2.9)	33	4.3 (3.2 - 5.8)	0.726 (0.068)	**0.004**
28-31	45	2.0 (1.5 - 2.6)	24	4.1 (2.7 - 6.1)	0.705 (0.064)	**0.005**
32+	54	4.7 (3.4 - 6.5)	20	9.7 (5.7 - 16.7)	0.683 (0.067)	**0.016**

*Data are reported as geometric means and 95% CIs, unless stated otherwise. N represents the number of samples included in the analysis of the stated gestation. Bold p-values are significant at p<0.05.*Arithmetic mean and 95% CI.*

When analysing PE outcomes by CKD stage, pregnancies that ended in miscarriages were excluded. The rates of PE were found to differ significantly by the pre-pregnancy CKD stage (p=0.011, Figure 5-3), increasing from 17% at stage 1 to 45% at CKD stage 3-4. The composite adverse pregnancy outcome rate without PE was also found to differ significantly across the CKD stages (p=0.010), rising from 22% at CKD stage 1 to 50% in stage 3-4. Further interrogation of the components of this outcome found that the observed difference was largely a result in the rates of NNU/NICU admission, which were 43% in CKD stage 3-4, compared to 15% and 11% in stages 1 and 2, respectively (p=0.011). The median gestation at delivery was found to decline significantly with CKD stage, from 38.0 weeks in those at stage 1 to 35.8 weeks in stage 3-4 (p<0.001), with a corresponding decline in the median birthweight from 2918 to 2560g (p<0.001, Table 5-14e).

Figure 5-3: Kaplan-Meier curve of the timing of Pre-eclampsia by pre-pregnancy CKD stage



Patients without Pre-eclampsia were treated as being censored at 40 weeks' gestation. Rates of Pre-eclampsia are shown to increase with higher CKD stage.

Table 5-14a: Associations between demographic factors and CKD stage

	N	Overall	Pre-Pregnancy CKD			*Overall p-Value*	Post-Hoc Tests		
			Stage 1	*Stage 2*	*Stage 3-4*		*1 vs. 2*	*1 vs. 3-4*	*2 vs. 3-4*
Demographics/Pre-Pregnancy									
Age at LMP (Years)	167	30.5 ± 5.6	29.5 ± 5.1	30.8 ± 6.3	31.3 ± 5.1	0.058	-	-	-
Ethnicity	165					0.104*	-	-	-
White		101 (61%)	33 (52%)	36 (62%)	31 (76%)				
South Asian		39 (24%)	19 (30%)	10 (17%)	9 (22%)				
Black		14 (8%)	5 (8%)	9 (16%)	0 (0%)				
Other		11 (7%)	6 (10%)	3 (5%)	1 (2%)				
Booking BMI kg/m^2	156	26.5 (24.0 - 31.8)	26.0 (23.8 - 29.0)	26.0 (23.9 - 31.6)	30.0 (24.0 - 34.6)	0.114	-	-	-
Pre-Pregnancy Smoking History	154	39 (25%)	15 (24%)	17 (32%)	7 (20%)	0.939	-	-	-
Known to Renal Team Pre-Pregnancy	167	150 (90%)	57 (90%)	53 (90%)	39 (93%)	0.718	-	-	-
Attended Pre-Pregnancy Clinic	160	36 (23%)	8 (13%)	16 (28%)	12 (32%)	**0.015**	0.198	0.114	1.000
Assisted Conception	165	8 (5%)	1 (2%)	4 (7%)	2 (5%)	0.246	-	-	-

*Continuous variables are as median (IQR), with overall p-values from Jonckheere-Terpstra tests and post-hoc p-values from Dunn's test. Categorical variables are reported as N (%), with overall p-values from Kendall's tau, and post-hoc p-values from Bonferroni-corrected Chi-square tests, unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Kruskal-Wallis test, comparing the CKD grade across the groups.*

Table 5-14b: Associations between pre-pregnancy renal markers, co-morbidity and CKD stage

	N	Overall	Pre-Pregnancy CKD			*Overall*	Post-Hoc Tests		
			Stage 1	*Stage 2*	*Stage 3-4*	*p-Value*	*1 vs. 2*	*1 vs. 3-4*	*2 vs. 3-4*
CKD History									
Pre-Pregnancy Creatinine umol/l	128	84 (62 - 110)	59 (54 - 64)	86 (78 - 91)	120 (111 - 140)	N/A	-	-	-
Pre-Pregnancy eGFR**	129	73 (52 - *>90*)	*All >90****	70 (66 - 81)	45 (37 - 49)	N/A	-	-	-
Pre-Pregnancy uACR mg/mmol	123	7.6 (1.7 - 48.6)	9.2 (1.6 - 46.2)	3.5 (1.4 - 18.7)	12.2 (4.0 - 83.5)	0.117	-	-	-
Renal Transplant	167	18 (11%)	1 (2%)	6 (10%)	11 (26%)	**<0.001**	0.168	**<0.001**	0.171
Aetiology	149					0.160*	-	-	-
ADPKD		18 (12%)	9 (16%)	6 (12%)	3 (8%)				
Glomerular Disease		43 (29%)	16 (28%)	11 (21%)	13 (36%)				
Lupus Nephritis		18 (12%)	8 (14%)	7 (13%)	3 (8%)				
Structural		23 (15%)	9 (16%)	10 (19%)	4 (11%)				
Tubulo-Interstitial Disease		29 (19%)	5 (9%)	15 (29%)	9 (25%)				
Others		18 (12%)	11 (19%)	3 (6%)	4 (11%)				
Single Functioning Native Kidney	167	17 (10%)	4 (6%)	8 (14%)	5 (12%)	0.254	-	-	-
Duplex	167	7 (4%)	5 (8%)	1 (2%)	1 (2%)	0.147	-	-	-
Pre-pregnancy UTI History	167	59 (36%)	18 (29%)	26 (46%)	15 (36%)	0.277	-	-	-
Stone Disease	167	13 (8%)	7 (11%)	5 (8%)	1 (2%)	0.091	-	-	-
Reflux	167	41 (25%)	11 (17%)	19 (32%)	11 (26%)	0.181	-	-	-
Metabolic	167	10 (6%)	5 (8%)	3 (5%)	2 (5%)	0.474	-	-	-
Medical History									
SLE	167	17 (10%)	8 (13%)	7 (12%)	3 (7%)	0.352	-	-	-
Other CTD	167	3 (2%)	2 (3%)	0 (0%)	1 (2%)	0.667	-	-	-
Any Autoimmune	167	44 (26%)	15 (24%)	12 (20%)	15 (36%)	0.275	-	-	-
Chronic Hypertension	167	62 (37%)	17 (27%)	20 (34%)	24 (57%)	**0.003**	1.000	**0.006**	0.078
Diabetes Mellitus	167	4 (2%)	2 (3%)	0 (0%)	2 (5%)	0.849	-	-	-

*Continuous variables are reported as median (IQR), with overall p-values from Jonckheere-Terpstra tests and post-hoc p-values from Dunn's test. Categorical variables are reported as N (%), with overall p-values from Kendall's tau, and post-hoc p-values from Bonferroni-corrected Chi-square tests, unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Kruskal-Wallis test, comparing the CKD grade across the groups. **The laboratory reported eGFR levels that were truncated at 90 ml/min/1.73m^2, hence a value of 90 was assumed for these in the analysis. ***All patients had an eGFR value that was >90. ADPKD = autosomal dominant polycystic kidney disease, UTI = urinary tract infection, CTD = connective tissue disease.*

Table 5-14c: Associations between obstetric history and CKD stage

	N	Overall	Pre-Pregnancy CKD			*Overall*	Post-Hoc Tests		
			Stage 1	*Stage 2*	*Stage 3-4*	*p-Value*	*1 vs. 2*	*1 vs. 3-4*	*2 vs. 3-4*
Pregnancy History									
Nulliparous	167	61 (37%)	19 (30%)	23 (39%)	17 (40%)	0.238	-	-	-
Gravida	166					0.264	-	-	-
1		48 (29%)	16 (25%)	19 (32%)	12 (29%)				
2		52 (31%)	18 (29%)	18 (31%)	15 (37%)				
3+		66 (40%)	29 (46%)	22 (37%)	14 (34%)				
Number of Previous Miscarriages	166					0.152	-	-	-
0		113 (68%)	46 (73%)	41 (69%)	24 (59%)				
1		31 (19%)	11 (17%)	7 (12%)	12 (29%)				
2+		22 (13%)	6 (10%)	11 (19%)	5 (12%)				
Previous Pregnancy Outcomes*									
Pre-Term birth (<37 Weeks) *	106	29 (27%)	10 (23%)	7 (19%)	12 (48%)	0.079	-	-	-
Very Pre-Term birth (<34 Weeks)*	106	13 (12%)	4 (9%)	3 (8%)	6 (24%)	0.168	-	-	-
Pre-eclampsia*	106	23 (22%)	9 (20%)	7 (19%)	7 (28%)	0.561	-	-	-
SGA/FGR*	106	37 (35%)	17 (39%)	14 (39%)	6 (24%)	0.279	-	-	-
NND*	106	5 (5%)	2 (5%)	3 (8%)	0 (0%)	0.483	-	-	-
Still Birth*	106	6 (6%)	1 (2%)	2 (6%)	3 (12%)	0.138	-	-	-

*Categorical variables are reported as N (%), with overall p-values from Kendall's tau, and post-hoc p-values from Bonferroni-corrected Chi-square tests, unless stated otherwise. Bold p-values are significant at p<0.05. *Excludes women that were nulliparous. SGA = small for gestational age infant, FGR = fetal growth restriction, NND = neonatal death.*

Table 5-14d: Associations between antenatal factors and CKD stage

	N	Overall	Pre-Pregnancy CKD			*Overall p-Value*	Post-Hoc Tests		
			Stage 1	*Stage 2*	*Stage 3-4*		*1 vs. 2*	*1 vs. 3-4*	*2 vs. 3-4*
Twin Pregnancy	167	5 (3%)	2 (3%)	1 (2%)	1 (2%)	0.752	-	-	-
Booking/Earliest Urine Dip Protein	158					0.194	-	-	-
Negative		101 (64%)	39 (65%)	42 (74%)	20 (53%)				
1+		24 (15%)	11 (18%)	9 (16%)	4 (11%)				
2+		15 (9%)	7 (12%)	2 (4%)	4 (11%)				
3+		18 (11%)	3 (5%)	4 (7%)	10 (26%)				
Booking/Earliest Urine Dip Blood	158					0.182	-	-	-
Negative		127 (80%)	44 (73%)	49 (86%)	32 (84%)				
1+		12 (8%)	7 (12%)	3 (5%)	1 (3%)				
2+		9 (6%)	6 (10%)	1 (2%)	2 (5%)				
3+		10 (6%)	3 (5%)	4 (7%)	3 (8%)				
Booking Systolic BP	160	125 ± 12	123 ± 14	125 ± 10	127 ± 11	0.190	-	-	-
Booking Diastolic BP	160	77 ± 11	74 ± 13	77 ± 10	79 ± 10	**0.025**	0.365	0.054	0.271
AN Aspirin	156	140 (90%)	55 (90%)	50 (91%)	34 (92%)	0.774	-	-	-
Static Growth Detected on USS	123	20 (16%)	7 (15%)	6 (13%)	6 (21%)	0.569	-	-	-
Infections - 1st Trimester	155	25 (16%)	9 (15%)	13 (23%)	3 (8%)	0.689	-	-	-
Infections - 2nd Trimester	156	27 (17%)	12 (19%)	11 (20%)	4 (11%)	0.384	-	-	-
Infections - 3rd Trimester	156	22 (14%)	10 (16%)	10 (18%)	2 (6%)	0.234	-	-	-
Recurrent UTI	157	18 (11%)	7 (11%)	8 (15%)	3 (8%)	0.728	-	-	-
Gestational Diabetes**	153	14 (9%)	7 (12%)	5 (9%)	2 (6%)	0.293	-	-	-
AN Renal Complication	162	59 (36%)	15 (24%)	13 (23%)	28 (72%)	**<0.001**	1.000	**<0.001**	**<0.001**
New/Worsening Hypertension	163	63 (39%)	19 (30%)	19 (33%)	22 (56%)	**0.017**	1.000	**0.036**	0.069
Fetal Distress	167	12 (7%)	2 (3%)	6 (10%)	4 (10%)	0.136	-	-	-
Growth Issue	154	33 (21%)	10 (17%)	14 (25%)	7 (19%)	0.552	-	-	-
Uterine artery Doppler notching	130					0.280*	-	-	-
No		62 (48%)	23 (44%)	22 (46%)	17 (59%)				
Yes		18 (14%)	7 (13%)	5 (10%)	6 (21%)				
*Unknown ****		50 (38%)	22 (42%)	21 (44%)	6 (21%)				
Umbilical Artery Flow Abnormality	145					0.643*	-	-	-
No		121 (83%)	51 (88%)	41 (79%)	28 (85%)				
Yes		14 (10%)	3 (5%)	7 (13%)	3 (9%)				
*Unknown ****		10 (7%)	4 (7%)	4 (8%)	2 (6%)				
Pre-Delivery Admission (1+)	160	59 (37%)	23 (37%)	21 (38%)	15 (38%)	0.895	-	-	-

*Continuous variables are reported as mean±SD, with overall p-values from Jonckheere-Terpstra tests and post-hoc p-values from Dunn's test. Categorical variables are reported as N (%), with overall p-values from Kendall's tau, and post-hoc p-values from Bonferroni-corrected Chi-square tests, unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Kruskal-Wallis test, comparing the CKD grade across the groups. **Patients with pre-pregnancy Diabetes Mellitus were excluded from this analysis. ***An "unknown" category was included to account for cases where it was unclear whether or not a test had been performed and these cases were excluded from the analysis. BP= blood pressure, UTI = urinary tract infection, AN = antenatal.*

Table 5-14e: Associations between delivery/postnatal factors and CKD stage

	N	Overall	Pre-Pregnancy CKD			*Overall p-Value*	Post-Hoc Tests		
			Stage 1	*Stage 2*	*Stage 3-4*		*1 vs. 2*	*1 vs. 3-4*	*2 vs. 3-4*
Delivery and Postnatal									
Gestation at Delivery (Weeks')	164	37.6 (36.1 - 38.7)	38.0 (37.3 - 39.3)	37.6 (36.7 - 39.0)	35.8 (34.1 - 37.6)	**<0.001**	0.454	**<0.001**	**0.001**
Pre-eclampsia	164	37 (23%)	11 (17%)	8 (14%)	18 (45%)	**0.011**	1.000	**0.009**	**0.003**
PROM	163	5 (3%)	3 (5%)	2 (3%)	0 (0%)	0.148	-	-	-
Spontaneous Delivery	164	11 (7%)	5 (8%)	6 (10%)	0 (0%)	0.113	-	-	-
Type of Delivery	164					**0.010***	1.000	**0.018**	**0.045**
Normal Vaginal		65 (40%)	31 (49%)	24 (41%)	10 (25%)				
Instrumental		20 (12%)	6 (10%)	10 (17%)	3 (8%)				
Elective LSCS		21 (13%)	10 (16%)	8 (14%)	3 (8%)				
Emergency LSCS		58 (35%)	16 (25%)	16 (28%)	24 (60%)				
Postpartum Haemorrhage	143	19 (13%)	6 (11%)	5 (10%)	6 (17%)	0.478	-	-	-
Birthweight	158	2770 (2405 - 3240)	2918 (2510 - 3330)	2813 (2470 - 3290)	2560 (2044 - 2770)	**<0.001**	0.829	**<0.001**	**0.009**
Apgar (1 min)	156					0.239	-	-	-
<9		33 (21%)	10 (17%)	9 (17%)	13 (33%)				
9		117 (75%)	49 (82%)	42 (78%)	24 (62%)				
10		6 (4%)	1 (2%)	3 (6%)	2 (5%)				
Apgar (5 min)	156					0.258	-	-	-
<9		11 (7%)	3 (5%)	3 (6%)	5 (13%)				
9		98 (63%)	39 (65%)	31 (57%)	26 (67%)				
10		47 (30%)	18 (30%)	20 (37%)	8 (21%)				
Length of Stay (Mother, days)	153	2 (2 - 5)	2 (1 - 4)	2 (1 - 4)	5 (3 - 7)	**<0.001**	1.000	**<0.001**	**<0.001**
Length of Stay (Baby, days)	140	2 (2 - 5)	2 (1 - 4)	2 (1 - 3)	5 (2 - 7)	**0.005**	1.000	**<0.001**	**<0.001**
PN Renal Complication	163	13 (8%)	3 (5%)	3 (5%)	6 (15%)	0.126	-	-	-
PN Hypertension	128	19 (15%)	9 (18%)	4 (8%)	5 (17%)	0.686	-	-	-

*Pregnancies ending in miscarriages are excluded. Continuous variables are reported as median (IQR), with overall p-values from Jonckheere-Terpstra tests and post-hoc p-values from Dunn's test. Categorical variables are reported as N (%), with overall p-values from Kendall's tau, and post-hoc p-values from Bonferroni-corrected Chi-square tests, unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Kruskal-Wallis test, comparing the CKD grade across the groups. LSCS = lower segment caesarean section, PROM = premature rupture of membranes, PN= postnatal.*

Table 5-14f: Associations between composite adverse pregnancy outcomes and CKD stage

	N	Overall	Pre-Pregnancy CKD			*Overall p-Value*	Post-Hoc Tests		
			Stage 1	*Stage 2*	*Stage 3-4*		*1 vs. 2*	*1 vs. 3-4*	*2 vs. 3-4*
Pregnancy Outcomes									
Delivery at <34 Weeks	164	18 (11%)	4 (6%)	4 (7%)	8 (20%)	0.069	-	-	-
Birthweight <1500g	158	10 (6%)	1 (2%)	4 (7%)	3 (8%)	0.113	-	-	-
NNU/NICU	154	33 (21%)	9 (15%)	6 (11%)	16 (43%)	**0.011**	1.000	**0.012**	**0.003**
NND	164	3 (2%)	0 (0%)	1 (2%)	1 (3%)	0.237	-	-	-
Miscarriage	167	3 (2%)	0 (0%)	1 (2%)	2 (5%)	0.119	-	-	-
FGR	164	20 (12%)	5 (8%)	11 (19%)	3 (8%)	0.681	-	-	-
Any of the Above (composite)*	155	46 (30%)	13 (22%)	12 (22%)	19 (50%)	**0.010**	1.000	**0.012**	**0.021**
Composite Outcome and/or PE*	152	64 (42%)	21 (35%)	17 (32%)	26 (68%)	**0.005**	1.000	**0.005**	**0.002**

Pregnancies ending in miscarriages are excluded, except for in the analysis of the miscarriage outcome and the composite adverse pregnancy outcome. Categorical variables are reported as N (%), with overall p-values from Kendall's tau, and post-hoc p-values from Bonferroni-corrected Chi-square tests. Bold p-values are significant at p<0.05.

**Excludes patients with missing data for any of the components of the composite outcome. NNU = neonatal unit, NICU = neonatal intensive care unit, NND = neonatal death, FGR = fetal growth restriction.*

Pre-pregnancy CKD stage was not identified by the stepwise procedure as a significant independent predictor of either PE or the composite adverse pregnancy outcome (Table 5-3 and 5-4). Entering this factor alongside the factors in the final multivariable model for composite adverse pregnancy outcome found a tendency for increasing composite outcome rates with CKD stage, with odds ratios of 1.37 (p=0.589) and 2.66 (p=0.120) for stages 2 and 3-4 vs stage 1, respectively, but this effect did not reach statistical significance (p=0.290). This lack of significance is likely a reflection of the previously identified correlation between CKD stage and chronic hypertension, with the latter found to be the better predictor of adverse pregnancy outcome.

The types of delivery also differed significantly with CKD stage (p=0.010), with emergency LSCS required in 25% of stage 1 pregnancies, compared to 60% of stage 3-4 (Table 5-13e). However, no significant differences in the rates of PROM (p=0.148), spontaneous delivery (p=0.113), postpartum haemorrhage (p=0.478), or postnatal renal complications (p=0.126) or new/worsening hypertension (p=0.686) were detected between the CKD stages. Apgar scores, measured at 1 (p=0.239) and 5 (p=0.258) minutes, were also similar across the CKD stages. However, the lengths of stay of both the mother (p<0.001) and the baby (p=0.005) increased significantly with CKD stage, with both having medians of 2 vs 5 days for CKD stages 1 vs 3-4.

For all of the outcomes that were found to differ significantly with CKD stage, post-hoc pairwise comparisons were then performed, to identify the pairs of groups between which the differences were largest. In each case, comparisons between CKD stages 1 and 2 returned non-significant results, whilst patients at CKD stage 3-4 had significantly worse outcomes than those in either stage 1 or 2. This implies that, rather than outcomes

becoming progressively worse across the CKD stages, there is a hard cut-off above CKD stage 2, such that outcomes are similar for CKD stages 1 and 2, but appear to worsen considerably in those patients with CKD stage 3-4.

5.2.10 Comparison of Thesis Chapter Cohorts

A comparison of the study markers, excluding angiogenic proteins, was undertaken in the study populations in the three thesis chapters. This enabled comparison of immunological markers in CKD and non-CKD pregnancy. The cohorts are summarised in Table 5-15. After exclusions as described in chapter two, data were available for n=418 in the 'Early non-CKD' cohort, n=47 for 'Early CKD', n=187 for 'Late, non-CKD' and n=65 for 'Late CKD'. The early pregnancy samples revealed that women with CKD had significantly higher cystatin-C, UA, B2-M and cFLC levels but lower C3 and hs-CRP concentrations than those without CKD (Table 5-16). These differences persisted in the analysis of the late pregnancy samples, except for cystatin-C levels, which were not significantly different between the two groups.

Table 5-15: Key to cohorts for comparison

	Early, Non-CKD	Early, CKD	Late, Non-CKD	Late, CKD
Thesis Chapter	4	5	3	5
Original Data Collection	Early pregnancy samples in women without pre-existing chronic illness. Those who developed PE were matched in a 1:2 ratio with those that did not.	Longitudinal antenatal blood samples in women with CKD	Samples at the time of PE in women without pre-existing chronic illness. Women without PE were recruited from those undergoing elective caesarean sections	Longitudinal antenatal blood samples in women with CKD
Sample Gestations	<16 Weeks	<16 Weeks	32+ Weeks	32+ Weeks
Additional Exclusions*	• Women without PE that were not included in the final 1:2 matching	• Women with autoimmune diseases. • Pregnancies ending in miscarriage		• Women with autoimmune diseases • Pregnancies ending in miscarriage

**Exclusion criteria that were not applied in the original chapter*

Table 5-16: Comparison of biomarkers between CKD and non-CKD cohorts

	Early Pregnancy					Late Pregnancy				
	Non-CKD		CKD		p-	Non-CKD		CKD		p-
	N	*Median (IQR)*	*N*	*Median (IQR)*	**Value**	*N*	*Median (IQR)*	*N*	*Median (IQR)*	**Value**
Creatinine	418	53.0 (47.0 – 60.0)	47	63.0 (51.5 – 83.3)	0.157	187	58.0 (52.0 – 66.0)	65	63.0 (50.0 – 79.0)	0.062
Cystatin-C	417	0.56 (0.50 - 0.62)	47	0.76 (0.61 - 1.23)	**<0.001**	187	1.16 (0.99 - 1.35)	65	1.18 (0.99 – 1.80)	0.233
Uric Acid	417	2.80 (2.30 - 3.30)	47	4.05 (3.40 – 4.70)	**<0.001**	187	4.70 (3.90 – 5.70)	65	5.30 (4.40 – 6.80)	**0.009**
hs-CRP	417	4.59 (2.52 - 8.92)	47	2.73 (1.21 – 5.71)	**0.001**	187	4.62 (2.58 – 9.05)	65	3.58 (1.37 – 5.64)	**0.003**
B2-M	417	1.23 (1.09 - 1.38)	47	1.70 (1.40 - 2.40)	**<0.001**	187	1.87 (1.63 - 2.27)	65	2.20 (1.80 – 3.40)	**0.001**
C3	418	1.73 (1.47 – 1.97)	47	1.24 (1.08 - 1.44)	**<0.001**	187	1.70 (1.49 - 1.94)	65	1.46 (1.28 - 1.66)	**<0.001**
C4	418	0.28 (0.23 - 0.35)	47	0.27 (0.20 - 0.35)	0.538	187	0.26 (0.20 - 0.34)	65	0.27 (0.21 - 0.35)	0.518
cFLC	418	22.6 (19.1 - 27.5)	47	34.1 (26.6 – 45.1)	**<0.001**	187	26.0 (21.3 – 32.4)	65	36.1 (29.4 – 51.5)	**<0.001**
IgA	417	1.63 (1.26 - 2.17)	47	1.64 (1.25 - 2.00)	0.736	187	1.62(1.23 - 2.19)	65	1.70 (1.22 - 2.10)	0.747
IgG	417	9.53 (8.02 - 10.89)	47	9.11 (8.15 – 10.48)	0.276	187	7.50 (6.47 - 8.77)	65	7.27 (6.34 – 8.16)	0.257
IgM	417	1.04 (0.77 - 1.38)	47	1.24 (0.79 - 1.97)	0.083	187	0.93 (0.71 - 1.30)	65	0.96 (0.69 - 1.54)	0.260
IgG1	418	5.54 (4.56 - 6.60)	36	5.41 (4.79 – 6.03)	0.739	187	4.31 (3.51 – 5.06)	57	4.13 (3.85 – 5.02)	0.847
IgG2	418	3.44 (2.65 - 4.33)	36	3.14 (2.80 - 3.72)	0.152	187	2.49 (1.82 - 3.07)	57	2.25 (1.96 - 2.97)	0.748
IgG3	418	0.71 (0.51 - 0.96)	36	0.69 (0.48 - 1.05)	0.921	187	0.56 (0.39 - 0.76)	57	0.61 (0.37 - 0.90)	0.297
IgG4	417	0.24 (0.14 - 0.45)	36	0.23 (0.08 - 0.46)	0.258	187	0.17 (0.10 - 0.34)	57	0.17 (0.07 - 0.27)	0.179

Data are reported as median (IQR), with p-values from Mann-Whitney tests comparing between patients with and without CKD. Bold p-values are significant at p<0.05.

5.3 DISCUSSION

5.3.1 Summary of Findings

The findings of this results chapter demonstrate rates of maternal and fetal complications affected a significant proportion (43%) of my cohort of CKD pregnancies. It was shown that independent clinical predictors of developing SPE in this group were White ethnicity, non-smoking history, SLE, chronic hypertension and a previous history of PE. Clinical predictors of composite adverse pregnancy outcome, which were based on more objectively-defined end points, were chronic hypertension, lupus nephritis and previous still birth. Other CKD related factors such as pre-pregnancy kidney function and proteinuria, whilst significantly associated with a higher risk of composite adverse pregnancy outcome and PE on univariate analysis, were not independent predictors of either outcome. I have also shown that rates of PE and other adverse pregnancy outcome are similar in women with pre-pregnancy CKD stages 1 and 2, but higher in those at CKD stages 3-4.

Concentrations of circulating sFLC, B2-M, serum creatinine, cystatin-C and UA were higher in early pregnancy and at later gestational periods in women who subsequently developed PE compared to CKD pregnancies not complicated by PE. The greatest predictive accuracy for PE was observed at 16-21 weeks for cystatin-C and B2-M (AUROC 0.810, 0.805 respectively), followed closely by creatinine and UA (AUROC 0.793, 0.791 respectively), all of which are markers closely associated with kidney function. The sFLT-1/PIGF ratio was predictive for the subsequent development of PE at 22-27 weeks' gestation (AUROC 0.728), but not at earlier or later gestations including at the time of PE. This contrasts to my findings in chapter three where the sFLT-1/PIGF ratio was higher at the time of PE in previously healthy women

compared to uncomplicated pregnancies at term. The rate of change for the angiogenic markers and also for the other study markers did not differ in the PE and non-PE group of CKD pregnancies with the exception of C3 which increased more rapidly and IgA concentrations increasing more slowly in the PE pregnancies.

The performance of B2-M as a marker and predictor of PE has been discussed in the previous results chapters and in the introduction. In chapter three, B2-M showed a strong association with PE in healthy pregnant women, which was similar to that of sFLT-1/PIGF and both markers were also highly correlated with each other (rho: 0.586, p<0.001). Beta-2-M levels were not predictive of PE in the first trimester in chapter four. In this chapter, there appeared to be differences in how B2-M behaved compared to the cohorts in previous chapters. Firstly, in pregnant women with CKD, B2-M levels were predictive of PE from early pregnancy up until 32 weeks' gestation but not thereafter. Secondly, whilst a positive correlation was observed between B2-M levels and sFLT-1 (rho:0.217, P<0.001), a negative correlation was not seen with PIGF (rho: 0.011, p=0.849), as was demonstrated in chapter three. The relationship of the angiogenic factors with renal function in this chapter is revisited later in the discussion.

Increased B2-M levels in the PE group may reflect worse baseline kidney function at the start of pregnancy in these women, as levels were increased even at < 16 weeks' gestation. As has been described previously in this thesis, B2-M is related to kidney function as it is freely filtered by the glomerulus and reabsorbed and catabolised by proximal tubular cells [235]. Unlike creatinine, B2-M levels do not fall in early pregnancy in women with normal or mildly impaired kidney function. As reviewed in the introduction and chapter three, B2-M forms

part of the FcRn receptor which is required for IgG recycling and placental transfer [164, 228]. It also forms part of the MHC I molecule and levels of B2-M are increased in inflammatory states associated with activation of the lymphoid system [229-231]. Thus, other factors independent of renal function may lead to increased B2-M concentrations in association with PE. The potential role of B2-M in antenatal monitoring of renal function and risk prediction in pregnant women with CKD warrants further research.

5.3.2 Clinical Predictors of SPE

Factors associated with PE in the general population, such as increased BMI, age and first pregnancy [18] were not predictors of PE in my CKD cohort, even on univariate analysis. This may suggest that other CKD-related factors override these traditional risk factors. Alternatively, the pathophysiological mechanisms may differ in superimposed PE, which is reflected in a difference in clinical and biochemical predictors of PE in this subset of patients, compared to the general population. Another possible explanation is that as PE which is defined on clinical grounds, is easier to define and recognise in the general population. Therefore, cases of PE may have been incorrectly diagnosed or missed in my study cohort, which is discussed later.

This chapter demonstrated that the risk of adverse outcomes increased with CKD stage 3 and 4 compared to 1-2. Whilst the study did not compare outcomes in mild CKD with non-CKD pregnancies, I was able to demonstrate that there was no difference in adverse outcomes between CKD stages 1 and 2. While a higher pre-pregnancy serum creatinine, CKD stage and uACR level were all associated with an increased risk of PE and composite adverse pregnancy outcome developing, none of the factors were found to be independent

predictors of either outcome on multivariable analysis (Table 5-3). Underlying chronic hypertension was a strong independent predictor of both outcomes in the multivariable models. This suggests that this is a greater risk factor in CKD-pregnancy outcome than baseline renal function. Furthermore, the presence of hypertension may also be a surrogate of CKD severity. Table 5-14b demonstrates that the presence of hypertension was increased proportionally with higher CKD stages ($p<0.003$). There is strong evidence in the literature to support the presence of underlying hypertension as an important determinant of pregnancy outcome in CKD [122, 125, 127, 144, 148]. As covered in chapter one, pre-pregnancy renal function has been shown to be strong predictor of adverse pregnancy outcome but in my study, this was not an independent predictor. This may be related to small numbers of women with advanced renal impairment included in my study as the majority had CKD stage 1 and 2 and only 2% of women had stage 4 CKD.

A higher blood pressure reading (both Systolic and Diastolic independently) taken at the time of the first antenatal (booking) visit was associated with increased likelihood of developing composite adverse pregnancy outcome and PE, on univariate analysis but not on multivariable analysis. This was likely related to chronic hypertension being a stronger predictor and displacing booking blood pressure from the final model. In the general pregnant population, women who subsequently develop PE have a higher initial blood pressure and a steeper increase after the mid-pregnancy nadir [356]. Addition of routine antenatal blood pressure measurements, taken after 28 weeks' gestation, to maternal baseline characteristics (BMI, height, age, parity, diabetes, ethnicity and previous gestational hypertension) has been demonstrated to improve prediction of PE compared to using the

clinical factors alone. In our study, maternal characteristics were found to be significantly predictive of PE with an AUROC of 0.852 and, therefore, addition of blood pressure readings and baseline kidney function and proteinuria measurements may improve predictive accuracy further. There is advantage in using a risk model which uses readily available data in terms of cost implications, practicality and also universal appeal, as such a model can also be used in low resource settings.

A higher pre-pregnancy uACR was a predictor of PE and composite adverse pregnancy outcome on univariable analysis, which is in keeping with established literature [127, 357]. A positive urinalysis in early pregnancy, either for blood or protein was not associated with adverse pregnancy outcome. Whilst urine dipstick analysis is cheap, easy to use and provides a rapid result available at bedside assessment, it is known to have low sensitivity and specificity when correlated with 24 hour and spot urine protein quantification [358]. In pregnancy results of urinalysis also vary according to maternal hydration status. Nevertheless false positive rates are reported to decrease with increased amounts of proteinuria and less common with $\geq$ 2+ protein on urine dip [357]. In my study urine protein quantification as measured by pre-pregnancy uACR provided superior risk prediction compared to urinalysis in early pregnancy.

The rates of antenatal and fetal complications were high in this chapter cohort with 23% of women developing PE and 43% developing at least one of the following - PE, delivery < 34 weeks, spontaneous pregnancy loss, NND, FGR, low birth weight baby or NNU/NICU admission. There were no still births or IUDs. These figures are comparable with existing literature, as noted in the introduction [127, 136, 141, 143, 158]. We also demonstrated that

the majority of adverse pregnancy outcomes in this group occurs in the context of PE developing. Earlier deliveries were, as expected, associated with increased rate of lower birth weight babies, lower APGAR scores, increased admissions to NNU/NICU and longer hospital inpatient stays for mother and child. The number of admissions pre-delivery were not different in the PE and non-PE group, which suggests that symptoms of PE likely developed suddenly in patients, prompting admission and timely delivery.

A significant proportion of my study cohort had an adverse obstetric history, with a third having delivered babies that were small and 28% having experienced a preterm birth. Whilst most women were known to the renal team pre-pregnancy, only a quarter had been seen in the combined renal-obstetric pre-pregnancy clinic for counselling. Given the high rate of maternal and fetal complications observed, pre-pregnancy counselling should be offered routinely to women with CKD contemplating pregnancy, with particular focus on those with hypertension, advanced renal impairment, SLE and adverse obstetric history. Only 10% of women in my study were not known to the renal team prior to their pregnancy, suggesting a lower rate of newly diagnosed CKD, identified during pregnancy, compared to the reported rate of 18-28% in published cohorts [129].

Black ethnicity is a risk factor for developing PE in the general population [18] but White ethnicity was a predictor of PE in my study. Further assessment of this relationship on multivariable analysis, found rates of PE were similar in White patients with and without SLE, whereas in non-White patient's rates of PE were higher if they had SLE. This is in keeping with published literature, as Black and Hispanic women with SLE have been shown to have disproportionally higher rates of PE, preterm deliveries, and FGR compared to White women

with lupus [359].

My findings also reported a positive smoking history as a protective risk factor against the development of PE, which is similar to findings in the general population [7]. A positive smoking history is reported to halve the risk of developing PE, although the relationship is likely dose-dependent, and smoking remains a protective factor in women who continue to smoke during pregnancy [7, 360-362]. Pregnant smokers have reduced concentrations of sFLT-1 and cigarette smoke has been shown to reduce placental production of sFLT-1, which suggests that the protective effect is related to its ability to modify the balance of angiogenic factors. It has also been suggested that the protective effects of smoking arise from generation of carbon monoxide, which may have an effect on the endothelial system and/or immune system, as well as angiogenic factors.

The underlying disease process causing CKD did not appear to be a significant factor in predicting adverse pregnancy outcome, including PE, with the exception of SLE. Whilst literature on the topic is lacking, lupus nephritis and diabetic nephropathy are acknowledged to be associated with a higher rate of adverse pregnancy outcome compared to other CKD aetiologies [125, 142, 144]. There is also a higher rate of perinatal death associated with diabetic nephropathy [122]. In women with lupus, a history of lupus nephritis specifically is associated with a higher risk of adverse pregnancy outcome, including PE occurring earlier in gestation [144, 363]. Maternal hypertension is reported to occur in 16% of pregnancies in women with lupus and premature births in 39%, with a history of lupus nephritis and anti-phospholipid antibodies increasing the risk of developing both complications [364].

5.3.3 Immunological Factors

In chapter three it was noted that PE, at the time of clinical manifestation, is associated with an acute phase response, a change in humoral markers and complement factor consumption. These changes were not observed in the pre-clinical or clinical samples in my SPE-CKD cohort. This suggests that the abnormal maternal immune and inflammatory response postulated to occur in PE [68] is different or not-apparent in superimposed PE and is an area which warrants further research. First pregnancy in the general population is a well-recognised risk factor to develop PE in the general population and this was demonstrated in chapter three [1, 32]. This is hypothesised to be related to immunological factors and possibly the Mother having less exposure to her partner's sperm [7]. The absence of nulliparity in this cohort as a risk factor for PE could therefore support immune mechanisms being less important in the pathophysiology of developing PE in CKD pregnancy. Interestingly, comparison of CKD and non-CKD cohorts revealed that women with CKD had lower C3 and hs-CRP levels compared to the non-CKD group, both in early and late pregnancy. This analysis is limited, as the cohorts were from studies with methodological heterogeneity and, thus, not directly comparable. Further work is required to assess whether there are differences in the immune profile during pregnancy in women with CKD compared to healthy pregnant women, and whether this is associated with increased risk of adverse pregnancy outcome.

As described in chapter four, increased IgM excretion during pregnancy as determined by 24-hour urine collections has in one study been demonstrated to be associated with increased adverse outcomes (PE, preterm birth or SGA infant) in CKD pregnancies [129]. The

authors of this study also reported that urinary IgM were more closely associated with adverse pregnancy risk compared to CKD stage or amount of proteinuria, and in CKD stage 1 pregnancies the relationship was independent of proteinuria. Circulating level of immunoglobulins were not measured in this study. In my study, where Ig levels were assessed, concentrations of circulating IgM were not related to adverse outcome. Furthermore, IgA levels were higher in the PE group at the start of pregnancy and levels declined more rapidly as pregnancy progressed.

Future studies with measurement of circulating and paired urine concentrations of Ig levels would enable us to determine whether these trends are related to increase urinary losses in the PE group. Published data regarding IgA levels in PE are conflicting, with no change, increase and a reduction in levels at the time of disease onset reported [180, 181]. Measurement of circulating Ig levels in CKD pregnancies has rarely been reported. A Chinese study has recently identified higher IgA levels in pregnant women with CKD who develop PE compared to those who do not [365]. As discussed in chapter three, levels of Ig vary according to ethnicity and this may explain why the trend of IgA levels in my PE-CKD group differ from those of the Chinese study, and likewise provide an explanation as to why studies reporting Ig levels in normal and PE pregnancies in the general population have also been inconsistent in their findings.

I did not demonstrate a difference in complement factors at the time of PE in this study, which was demonstrated in chapter three. Levels of C3 did rise more rapidly in the PE group compared to non-PE group. The significance of this change is uncertain, but could suggest an acute phase response, rather than a complement-pathway mediated change. As was

discussed in chapter four, measurement of complement breakdown products in different tissues, such as urine, would provide a greater overview of complement dysregulation. Levels of sFLT-1, but not the sFLT-1/PIGF ratio, were inversely correlated with both C3 and C4 levels, and, when split by study group, the correlation was not specific to the PE group, which has been observed in other studies and in chapter three [81]. This suggests that the interaction between the complement and angiogenic system pathways maybe different in CKD versus non-CKD pregnancy and not related to development of PE. While sFLC were increased at multiple gestational intervals in the PE group, this was also the case in the earliest gestational period (<16 weeks) and, so, it is likely the increase was related to worse baseline kidney function in the PE versus non-PE group. As was shown in chapter four, first trimester levels of sFLC were not a predictor of PE in the general population.

5.3.4 Markers of Kidney Function in CKD Pregnancy

The markers associated with the greatest AUROCs for PE and composite adverse outcome were markers closely related to kidney function - cystatin-C and B2-M, followed closely by creatinine and UA. Of interest, these markers were mostly predictive of PE in the gestational periods between 16-31 weeks. In addition, as is shown in Table 5-6, these markers were higher at baseline in the PE group versus non-PE group, but the rate of change for these markers did not differ significantly between the groups. In both the PE and non-PE groups in this chapter, the rate of change for creatinine was lower than for the other renal markers, suggesting that it may be a less sensitive marker for change in kidney function during pregnancy compared to UA, B2-M and cystatin-C. In the normal pregnant population, the levels of the these markers have been reported to increase at the end of pregnancy [209,

253, 366]. In my non-PE CKD group a similar trend was apparent. However, in the PE-CKD group, B2-M and UA levels were not at their highest level at the end of pregnancy.

At 15-20 weeks' gestation, both cystatin-C and B2-M levels have been shown to predict PE in the general pregnant population and this was also the case in this chapter cohort of CKD pregnancies, with early third trimester levels also predictive of subsequent disease development [235]. A difference in first trimester concentrations of cystatin-C and UA in the context of normal creatinine values have also been demonstrated to be predictive of PE in the general pregnant population [9]. While cystatin-C was higher at < 16 weeks' gestation in the PE versus non-PE group (1.05 and 0.82 mg/L respectively), the p value was just below level of significance (p= 0.055). Nevertheless, these results suggest cystatin-C maybe the strongest marker of renal function assessed in this study, as it appears to have behaved most consistently in my non-PE and PE cohort when compared with published literature available for normal and PE pregnancies in healthy women.

Towards the end of pregnancy (>32 weeks' gestation), none of the renal markers differed significantly in the PE and non-PE group, whilst all of these markers were higher in the PE group in healthy women in chapter three. It is unclear why we did not find these markers to differ at the end of pregnancy in this chapter. It could be related to women with CKD, regardless of PE status, displaying a different renal physiological response at the end of pregnancy compared to women without CKD. This would, therefore, have an impact on differences observed in these markers when comparing the two groups.

5.3.5 Angiogenic Factors

Alteration in the sFLT-1/PIGF ratio at the time of PE onset is very well established and has also been demonstrated in the weeks prior to onset of symptoms developing [15, 33, 100, 260]. More recent focus has been on incorporating the ratio into clinical practice to predict or exclude imminent PE developing in women suspected of having the condition and a ratio cutoff of 38 has been demonstrated to have accurate predictive capability in the general pregnant population [91, 367, 368]. Fewer studies have assessed these markers in superimposed PE and even fewer in the CKD population specifically. To my knowledge mine is the first study that has assessed longitudinal changes in both sFLT-1 and PIGF in CKD pregnancy.

I discovered that the sFLT-1/PIGF ratio was higher in the PE group during 16-21, 22-27 and 28-31 weeks' gestation, but the difference was only statistically significant at 22-27 weeks' gestation. The predictive accuracy of the angiogenic factors was superior nonetheless for composite adverse outcome and/or PE, rather than for PE alone, and the ratio was significantly higher at all gestational periods, with the exception of <16 weeks' gestation, for this broader adverse outcome. I also demonstrated that the difference in sFLT-1/PIGF at the time of PE in women with CKD compared to non-PE samples, taken close to the time of delivery, was not significant. The number of samples tested for this comparison (6 and 26 samples respectively) was small and could have had an impact on the ability to detect differences reaching statistical difference. The predictive accuracy of the sFLT-1/PIGF ratio in the general pregnant population, as discussed in chapter one, has been demonstrated to reduce after 34 weeks, which is related to 'normal' changes in concentrations of both

markers in healthy pregnancy, with an increase in the ratio towards the end of healthy pregnancy [5]. This may also explain why I did not identify differences in the angiogenic proteins in the two groups during late pregnancy. I did not find the sFLT-1/PIGF ratio to be a better predictor in early versus late onset disease, which is in contrast to other studies in the general population [259, 260, 264, 369]. However, splitting the groups for subgroup analysis would have required large differences to be present to allow detection, due to the small study numbers involved.

Previous studies have reported a higher sFLT-1/PIGF ratio in CKD pregnancies complicated by PE compared to those uncomplicated by PE [92, 139, 286]. Bramham et al. reported a high sFLT-1/PIGF ratio has a high diagnostic accuracy for the development of SPE requiring delivery within 14 days, with an AUROC of 0.83 in women with CKD presenting with symptoms suspicious of PE [139]. Furthermore, the diagnostic accuracy of the test for the same outcome was similar in the pregnant CKD group compared to pregnant women without pre-existing disease and also with pregnant women with pre-existing chronic hypertension. The difference in my findings compared to published literature could be related to a number of reasons.

Some of the previous studies defined PE in the CKD group on the basis of new onset hypertension and proteinuria and, therefore, women in whom these were pre-existing may have been excluded [92, 286]. Therefore, the study cohort in these studies would have differed to mine. In the study by Bramham et al., women with pre-existing hypertension and/or proteinuria were enrolled [139]. Pregnant women with chronic hypertension, without CKD, were combined in the CKD group and, so, the cohort was not entirely

comparable to mine. Furthermore, the PIGF and sFLT-1 measurements in this study were performed using the Triage assay, and not the Eclecys. A strength of the study by Bramham et al. was that their study findings were validated on a separate cohort. This validation cohort comprised of women presenting with symptoms suggestive of PE. I did not include a similar cohort in my study and, therefore, the study methodology differed, as did the nature and aims of the primary analysis in the two studies. Nevertheless, the study by Bramham et al. alongside others suggest the most suitable role of the angiogenic factors in a clinical setting is as a triage test to predict or exclude short-term onset of disease in women presenting with symptoms suspicious of PE [33, 91]. Its predictive accuracy in asymptomatic stages of superimposed PE warrants further study.

It may also be possible that angiogenic factors are not as strong predictors for superimposed PE as they are for PE. Costa et al. in a study comparing SPE in women with chronic hypertension, reported that a higher sFLT-1/PIGF ratio was observed only at 32 weeks' gestation compared to an increase in ratio observed at 26, 32 and 36 gestational weeks in previously healthy women with PE [283]. Dwyer and Powers et al., in their separate studies, reported no difference in angiogenic markers in women with SPE compared to those without PE [284, 370]. In a further study by Perni et al. SPE was associated with a higher sFLT-1/PIGF ratio only at the time of disease in women with late onset disease [285].

In the general pregnant population, a high sFLT-1/PIGF ratio has been shown to be predictive of adverse outcome associated with PE, such as FGR and pre-term birth [5, 33, 291-293]. The angiogenic factors displayed better diagnostic accuracy when outcome was extended to include a broader composite adverse outcome. This may also suggest that cases

of PE were not correctly identified in my cohort. Diagnostic criteria for superimposed PE varies in studies, and this is likely related to lack of clear diagnostic criteria in published guidelines [26, 28, 127]. In one study, kidney biopsies performed on women with CKD following a diagnosis of PE, revealed that only 58% had evidence of renal histological changes consistent with PE [371]. Changes in renal function, blood pressure and proteinuria during CKD pregnancy may be due to PE developing, but can also be physiological or related to underlying renal pathology [139]. In clinical practice diagnosis of superimposed PE is made on clinical grounds and usually by senior Obstetricians and Physicians, which is what was done in this study.

The 'U-shaped' trend of sFLT-1/PIGF observed in my CKD cohort is similar to published trend in the general pregnant population, including those who develop PE [260, 276, 277]. There was an initial decline to a trough level at around 30 weeks' gestation, followed by an increase over the remaining period of gestation. A number of studies have demonstrated that multiple measurements of the sFLT-1/PIGF ratio can also be used to improve predictive accuracy by measuring the rate of change [5, 260, 279, 281, 282] and this has not previously been undertaken in CKD pregnancies. I did not find that the rate of change of either angiogenic proteins individually nor the sFLT-1/PIGF ratio to differ in the PE versus non-PE group. Post-delivery concentrations of angiogenic factors have to my knowledge not been previously reported. I have shown that levels of both sFLT-1 and PIGF decreased significantly pre to post-delivery after a median of 24 days. This is in keeping with production of these factors in pregnancy being placental. When comparing postnatal values to literature reporting non-pregnant ranges in CKD, these appeared to be comparable, suggesting that

values return to 'normal' within 3-4 weeks of delivery [287-290].

5.3.5.1 Relationship of Angiogenic Markers to Kidney Function

The results of chapter three and, to an extent, this chapter also support a relationship between kidney function and concentrations of the sFLT-1/PIGF ratio, which may impact on its predictive accuracy of PE in women with CKD. In chapter three, I demonstrated that markers of kidney function were highly correlated with higher sFLT-1 and lower PIGF concentrations, and a relationship of these angiogenic markers with proteinuria was also observed. In this chapter, the relationship between sFLT-1 and renal function was less strong, with lower but still statistically significant correlation coefficients (Table 5-7). As is seen Table 5-6, levels of sFLT-1 ran higher throughout pregnancy in the PE versus non-PE group, although the difference only reached statistical significance at 28-31 weeks' gestation. Outside pregnancy, increased sFLT-1 levels in CKD has been linked to endothelial dysfunction and atherosclerosis [287]. These observations suggest that pregnant women with CKD with higher baseline levels of sFLT-1, may already have or are more predisposed to developing endothelial dysfunction in pregnancy. Higher levels of sFLT-1 have also been reported in association with other risk factors for PE, such as first versus second pregnancies, twin versus singleton pregnancies and hydatidiform mole pregnancies [10].

In the non-pregnant CKD population, levels of both sFLT-1 and PIGF have been reported to be increased [287, 288, 290, 372]. I found negative correlations between PIGF levels and kidney function in pregnant women without pre-existing illness (chapter three), but did not find a relationship between PIGF levels and renal function in pregnant women with CKD. Bramham et al. also reported no significant correlation between creatinine and PIGF levels in

their study of pregnant women with and without CKD. It is unclear why the relationship between renal function and sFLT-1/PIGF was less strong in this chapter compared to chapter three. Changes in renal function in the chapter three cohort were likely to be a result of AKI, rather than longstanding renal dysfunction which may have differing degrees of impact on circulating concentrations of angiogenic factors. Further studies are required to assess whether a relationship between renal function and angiogenic factors does exist in pregnancy and, if one does exist, this may require revision of the 'normal ranges' of angiogenic factors during pregnancy for women with CKD.

5.3.6 Limitations and Strengths

This is the first longitudinal study of CKD pregnancies which has measured a variety of immune system markers and the sFLT-1/PIGF ratio. It is also one of the larger biomarker studies of CKD pregnancies and we were able to provide comprehensive demographic, antenatal, delivery and postnatal, including baby follow up data. All patients were managed by the same clinicians in the same hospital, and so care would have been similar and diagnostic thresholds for PE consistent. Women were followed up from an early stage, allowing miscarriage data to be captured which is often not the case in pregnancy outcome studies.

In my study, PE was not defined on the basis of severity due to small patient numbers, but this may have yielded further differences. The possibility of incorrectly diagnosing patients has already been discussed; however, by including a composite adverse pregnancy outcome, I was able to use a more objective outcome definition and was able to capture clinically relevant complications. Furthermore, a history of previous PE was a risk factor for PE in the

current pregnancy which may also lend support to reliability of diagnosis, although clinicians may have been more likely to have made the diagnosis of PE on the basis of obstetric history. I was not able to validate the multivariable model findings of clinical predictors of PE and composite adverse pregnancy outcome on a separate cohort as my study numbers were too small to have done this.

In addition, delivery <34 weeks' gestation was included as part of the composite adverse outcome in the chapter and also classified women into the early onset PE group. This at times may have been influenced by increased anxiety by clinicians about the Mother's condition due to her CKD rather than solely due to obstetric indications. There may, therefore, be a subjective bias by clinicians influencing this as an outcome measure. Nevertheless, this outcome occurred in only 11% of the cohort and was not a primary outcome measure. It is, therefore, unlikely to have had a significant influence on the results of this chapter, even if subjective bias was present.

A limitation of the longitudinal analysis is the potential impact of selection bias on the analysis. The data collection used a 'convenience sample' approach, with blood samples taken when patients attended routine clinic appointments. The issue with this approach is that patients who had been identified by clinicians as potentially being high risk may have attended clinic and had samples taken more regularly than lower risk patients. As a result, these high-risk patients will have had greater contribution to the data than the lower risk patients, and so may have unduly influenced the analysis, making the results less generalisable to the cohort as a whole. In an attempt to mitigate the impact of this selection bias on the longitudinal analysis of samples, the period of gestation was divided into five

intervals, with each pregnancy only permitted to contribute a single sample to each period. This prevented those patients with disproportionately large numbers of samples from having excessive influence on the results of the analysis, as patients were limited to a maximum of five samples spread across the gestation of their pregnancy. In addition, in the regression analyses that included multiple samples per pregnancy, a generalized estimating equation approach was used to account for the correlations between repeated measurements from the same pregnancy, further preventing the patients with the greatest number of samples having undue influence over the results.

5.3.7 Conclusions

Pregnancy in women with CKD is associated with high rates of antenatal complications and pre-term deliveries, which are most often related to superimposed PE having developed. Chronic hypertension is a strong predictor of adverse pregnancy outcome in CKD, more so than pre-pregnancy kidney function and degree of proteinuria. There is potential for risk models using maternal characteristics to be created to identify which women with CKD are at most risk of developing complications, so that they can be counselled and followed up appropriately. My study demonstrated that immunological and angiogenic markers were not strongly predictive of PE developing in CKD pregnancies, suggesting that these pathways may not be as important in the pathophysiology of PE in CKD and other, non-placental, factors may be more important. Markers of kidney function appear to be stronger than immunological and angiogenic markers at predicting PE, with a suggestion that cystatin-C may be the best of these. Angiogenic markers were better at predicting composite adverse pregnancy outcome and their levels, especially of sFLT-1, may be related to baseline kidney

function. Future studies should attempt to harmonise definitions of superimposed PE in CKD, which will aid discovering the most accurate diagnostic and predictive tests for this condition, and by doing so, we can hopefully reduce the substantial impact Pre-eclampsia has on maternal and fetal health in this population.

6 FUTURE DIRECTIONS

In this thesis, I have demonstrated that within the general population Pre-eclampsia is manifest by a greater acute phase response, a shift towards an anti-angiogenic profile, an increase in circulating polyclonal free light chains and a reduction in IgG concentrations compared to healthy pregnancies at term. In the first trimester of pregnancy, concentrations of IgM are lower in healthy women who later develop PE compared to those that do not. Furthermore, women who develop PE with adverse clinical outcome have higher circulating IgG concentrations in the first trimester compared to the women who developed less severe disease. The markers of humoral immunity in healthy pregnancy and that complicated by PE were not correlated with angiogenic or other clinical immune system markers. These findings support a possible independent humoral mediated response associated with PE in healthy women, separate from angiogenic and inflammatory pathways both of which are thought to play an important role in the pathology of PE.

In pregnancy in women with chronic kidney disease, immune system and angiogenic markers were not associated with, or strongly predictive, of the development of SPE. This is in contrast to findings in the healthy pregnant cohort and suggests that the pathophysiological mechanisms pertinent to development of PE differs between the two groups. However further work is required to explore this hypothesis and to establish the strongest clinical and biochemical markers of PE in women with CKD. This would potentially identify a group in need of enhanced antenatal monitoring and facilitate early diagnosis and intervention in difficult cases, where hypertension and/or proteinuria may pre-date pregnancy.

The findings generated from screening of first-trimester samples yielded few differences in the markers tested for between women who later developed PE compared to those who had healthy pregnancies. Sampling at later time points, for instance from 15-20 weeks' gestation onwards, may generate more positive findings. I also demonstrated evidence of complement activation in serum samples at the time of PE in healthy women. It would be interesting to assess whether complement activation is also present in other tissue samples, such as urine, placenta and cord samples, and to assess for differences in other complement components, including terminal complement activation such as C5b-9.

The work of this thesis has highlighted the importance of accurately diagnosing SPE cases in CKD cohorts as, without objective diagnostic tests, cases may be misdiagnosed or simply missed. Current universal diagnostic criteria for PE lack clarity to help distinguish between SPE and worsening CKD during pregnancy. Studies that include women with CKD and/or chronic hypertension should utilise standardised diagnostic criteria for the diagnosis of PE, to allow amalgamation of study findings, which would greatly facilitate developments in this understudied field.

It is also important that large studies into PE include women who have underlying CKD, hypertension and other major risk factors, in addition to healthy women. A diverse range of ethnic groups should, where possible, be included as mechanisms of disease and diagnostic predictors and biomarkers of PE may well vary between ethnic groups. Moreover, any findings or risk models generated from studies in healthy pregnant women should be separately validated in high risk cohorts, including in women with CKD. This would aid in

determining whether PE has a different aetiological basis to SPE, and may offer some insights into its pathophysiology.

Future studies which examine changes in concentrations of immunoglobulins should ideally measure these in paired serum and urine samples to determine urinary losses of these proteins. Furthermore, inclusion of a non-pregnant cohort would also allow further comparison and understanding of changes occurring in samples collected during early pregnancy. Many women of child bearing age with CKD are under outpatient follow up, allowing the potential for sample collection prior to and after conception, further increasing our understanding of the immune-mediated and angiogenic factor changes that occur in pregnancy. The relationship between angiogenic markers and renal function during and outside pregnancy also needs to be better established, especially important if these markers are to be introduced into clinical practice. Lastly, long-term follow up studies would establish the long-term complications of SPE, in mothers and their offspring, in women with CKD and allow comparison with PE outcomes reported in the general population.

APPENDIX

Appendix 1: Published Paper (Chapter Three)

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ORIGINAL ARTICLE

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Humoral immunity in late--onset Pre--eclampsia and linkage with angiogenic and inflammatory markers

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Problem: Pre--eclampsia (PE) is a leading cause of maternal and foetal morbidity worldwide. Given the implication of immune mechanisms, we compared markers of humoral immunity in PE and their relationship to circulating markers of inflammation, angiogenic factors, and renal function.

Method of study: Serum samples from 88 previously healthy women admitted to hospital with PE and 107 healthy pregnant controls at term were analysed for serum immunoglobulins (Ig), including IgG subclasses and free light chain (sFLC) levels, beta--2 microglobulin (B2--M), high--sensitivity C--reactive protein (HS--CRP), albumin, complement proteins (C3 & C4), creatinine, cystatin--C and the ratio of soluble fms-- like tyrosine kinase--1 (sFLT--1) and placental growth factor (PlGF).

Results: Compared to the controls, women with PE had significantly reduced renal function, serum IgG (subclass 1 & 3), albumin, and C4 levels, whilst concentrations of total sFLC, HS--CRP, B2--M, and sFLT--1:PlGF were raised. On multivariable analysis, sFLT--1:PlGF ratio ($P < 0.001$), sFLC ($P < 0.001$) and IgG1 ($P < 0.024$) were found to be independently associated with PE, after accounting for renal function, patient age, BMI, ethnicity, and parity. B2--M and sFLT--1:PlGF had comparable diagnostic associa-tion with PE ($P = 0.184$), and correlated strongly with each other ($\rho = 0.588$, $P < 0.001$) as well as with renal function and adverse clinical outcome.

Conclusion: We describe for the first time that PE is independently associated with activation of the humoral immune system independent of deranged kidney function and angiogenic markers. The role of B2--M as a potential predictive marker of PE re-mains to be determined.

KEYWORDS
angiogenic proteins, complement system proteins, humoral, immunity, inflammation, Pre-eclampsia

1 | INTRODUCTION

The aetiology of Pre--eclampsia (PE), a leading cause of maternal mortality and morbidity, is unknown, with treatment options limited to delivery.[1,2] Shallow placentation and subsequent maternal endo-thelial dysfunction are currently accepted as pathological hallmarks of the condition.[3,4] A better understanding of the disease processes involved could lead to the identification of new therapeutic targets and biomarkers useful for the prediction of PE and clarification where there is diagnostic uncertainty.

There is strong evidence to support underlying immunological disturbance in PE. Pre--existing autoimmune illness, primigravidity and first pregnancy with a new partner have been shown to in-crease the risk of PE developing.[1,5,6] There is a shift towards a Th1

vs Th2 lymphocyte response, with an increase in circulating pro-inflammatory cells and cytokines, compared to healthy pregnancies, in addition to activation of numerous other immune pathways and cell types.[7-9]

Intact Immunoglobulin (Ig) levels change in healthy pregnancy, with reduction in IgG concentrations as pregnancy progresses, whereas conflicting trends for IgA and IgM levels have been reported.[10-12] Limited, and now mostly outdated studies which have examined serum antibody levels in PE compared to healthy pregnancies have also reported a difference (reduction) in IgG concentrations in PE, whereas findings for IgA and IgM levels have again been more variable.[13-18] However, these studies have often included women with gestational hypertension in the PE group, used small patient numbers and rarely examined the relationship between Ig concentration and proteinuria. Furthermore, to our knowledge, differences in IgG subclasses have not been investigated in PE and may help aid understanding of changes in the humoral response in PE, given their difference in function.

Immunoglobulin free light chains (FLCs) are by-products of antibody synthesis and circulate as serum FLCs (sFLCs), before being cleared by the kidneys.[19] They have a half-life of just a few hours, rather than days to weeks (Igs), so may allow identification of acute changes in antibody secretion. Thus, measurement of FLCs and the IgG subclasses, which have not previously been reported in PE, would allow for a more detailed assessment of humoral immunity in PE.

Pre-eclampsia is associated with a shift towards an antiangiogenic profile, with increased circulating levels of the antiangiogenic factor, soluble fms-like tyrosine kinase-1 (sFlt-1) and reduction in the pro-angiogenic placental growth factor (PlGF). An interaction between angiogenic and inflammatory pathways is recognized and PE has been described as an exaggerated inflammatory response to normal pregnancy.[20-22] Serum levels of sFLT-1, PlGF and their ratio, when measured in the third trimester, are strong predictors of the subsequent development of PE.

However, whilst these markers provide an extremely important insight into the pathogenesis of PE, they are not widely adopted in routine clinical practice.

1.1 | Objectives

- To compare serum levels of intact immunoglobulin G, A and M, IgG subclasses and sFLC between women with PE and healthy pregnant women at term.
- To correlate the above with other immune system markers previously shown to be associated with PE[30-32]; high-sensitive C-reactive protein (HS-CRP), Beta-2 microglobulin (B2-M), serum albumin and complement proteins (C3 and C4) and with the sFLT-1:PlGF ratio.
- To assess whether humoral system markers provide independent association with PE after control for angiogenic factors, inflammation, and renal dysfunction.

2 | METHODS

2.1 | Study design

This was a single-centre cross-sectional study with patients re-cruited over an 8-month period (February-September 2014). Following written, informed consent, antenatal serum samples were collected from pregnant women with a clinical diagnosis of PE (BP ≥140/90 mm Hg and urine protein to creatinine ratio (PCR) >30 mg/mmol) admitted to Birmingham Women's Hospital (BWH), a tertiary obstetric service in the United Kingdom.[33] Those with known underlying Chronic Kidney Disease (CKD) or pre-existing chronic illness, including autoimmune or inflammatory disease, were excluded. Healthy pregnant control subjects undergoing elective caesarean section delivery for obstetric indications, with-out co-morbid illness, previous history of PE or poor obstetric his-tory (defined as ≥3 consecutive miscarriages) were enrolled and consented from the preoperative assessment clinic. Women with pre-existing chronic essential hypertension were not excluded. Demographic and clinical details were collected from patient notes, electronic records and BWH medical informatics data. Ethical ap-proval was obtained through the University of Birmingham Human Biomaterials Resource Centre (HBRC) (reference: 15/NW/0079 with date of approval 21/01/2014). The study conformed to the ethical principles contained in the Declaration of Helsinki.

Adverse clinical outcome was defined as occurrence of one of the following: admission to maternal high dependency unit (HDU), preterm birth (<37 weeks gestation), small for gestational age baby, low birth weight baby or admission to the neonatal unit (NNU).

2.2 | Laboratory analyses

All serum samples were spun at 2120 g for 10 minutes (at room temperature) within 3 hours of collection and frozen at −20°C until analysis. Samples were pseudo-anonymized at the time of storage and analysis was performed in batches, with the circulating immune system and angiogenic markers analysed separately. Sample testing commenced in July 2015 and was completed in August 2016, with both PE and control samples analysed in the same batches. Samples were thawed on the day of analysis and repeated thaw-freeze cycles were avoided.

All immune system markers were measured on the Hitachi Cobas 6000 Turbidimeter (Roche diagnostics, West Sussex, UK). The angiogenic factors sFLT-1 and PlGF were measured using the fully automated Elecsys assays on the Cobas e411 analyser. All reagents were supplied by Roche diagnostics, except for those used in analysis of the IgG subclasses and the Freelite® FLC assay (The Binding Site Group Ltd, Birmingham, UK).

When applying sFLC measurements to management of plasma cell dyscrasias, different sFLC assays correlate, but give different absolute sFLC values. Furthermore, in advanced renal disease, the kappa:lambda sFLC ratio is increased when using polyclonal method of detection (Freelite®), whilst this does not happen in a monoclonal-based assay.[34] Because of these known differences between sFLC

TA B LE 1 Comparison of the demographics, clinical features and adverse clinical outcomes between groups

	PE (n = 88)	Control (n = 107)	*P*--value
Demographics			
Age (y)	29.9 (±6.2)	31.9 (±4.6)	**0.012**
BMI	28.0 (23.0-31.8)	26.1 (23.0-30.0)	0.310
Ethnicity (%)			
White	57% (50)	69% (74)	0.262
South Asian	24% (21)	19% (20)	
Black	10% (9)	5% (5)	
Mixed/ Other	9% (8)	8% (8)	
First pregnancy (%)	53% (47)	16% (17)	**<0.001**
Twin pregnancy (%)	3% (3)	4% (4)	0.902
Gestational diabetes (%)	6% (5)	0% (0)	**0.013**
Chronic hyperten-sion (%)	3% (3)	0% (0)	0.054
Gestation (wk)[a]	37.0 (35.0-39.0)	39.0 (38.5-39.0)	**<0.001**
Blood markers			
Serum albumin (g/L)[b]	33.0 (±4.1)	34.8 (±2.5)	**<0.001**
Serum creatinine (μmol/L)[b]	64 (57-72)	55 (49-61)	**<0.001**
Cystatin-C (mg/L)[b]	1.27 (1.11-1.54)	1.08 (0.91-1.24)	**<0.001**
Adverse clinical outcomes			
Preterm birth (<37 wk)	26% (23)	3% (3)	**<0.001**
Small for gestational age	22% (29)	7% (7)	**0.003**
Low baby weight	20% (18)	0% (0)	**<0.001**
HDU admission	35% (31)	5% (5)	**<0.001**
NNU admission	28% (25)	8% (9)	**<0.001**
Any of the above	57% (50)	18% (19)	**<0.001**

Noncategorical data are represented either as mean (±SD) or as median (IQR), with *P*--values derived from independent sample *t* tests and Mann--Whitney tests, respectively. Categorical data are reported as percent-ages, with *P*--values generated from Chi--squared tests. Bold *P*-values are significant at $P < 0.05$.

[a]This refers to the gestation at which the serum sample was collected at.

[b]Nonpregnant reference range for serum albumin is: 35-52 g/L, for serum creatinine: 44--80 μmol/L and for cystatin--C: 0.6--1.4 mg/L.

assays, we used two assays in this study. Primarily, the Freelite® assay was used, which is based on polyclonal anti--FLC antibody detec-tion. However, samples were also tested on in--house luminex assay, which uses monoclonal antibodies (Abingdon Health, York, UK).[35,36] Immunofixation was performed using Sebia Hydrays System (Sebia UK Limited, Surrey, UK), as per manufacturer's instructions.

2.3 | Statistical analysis

Statistical analyses were performed in IBM SPSS 22 (IBM Corp., Armonk, NY, USA), unless stated otherwise, with $P < 0.05$ set as the level for statistical significance. For categorical variables, Chi--squared tests were used to compare the relative proportions of patients in each category between the two patient groups. Continuous variables were assessed for normality prior to the anal-ysis using histograms. Normally distributed variables were reported as mean ± SD, and compared using independent sample *t* tests, with medians and interquartile ranges (IQRs) and Mann--Whitney tests used otherwise. The diagnostic accuracy of variables which were significantly different between the two study groups was then assessed using receiver operating characteristic (ROC) curves. Comparison of the sFLC assays was performed using Wilcoxon signed ranks test. Associations between pairs of continuous vari-ables were assessed using Spearman's correlation coefficients (ρ). Comparisons of study markers by ethnicity were performed using one way ANOVA or Kruskal--Wallis tests, depending on whether or not the variables were normally distributed. Significant results were followed by post hoc analyses using Tukey's or Dunn's tests, respectively. A multivariable analysis was then performed, to iden-tify factors that were significantly associated with PE. This used a binary logistic regression model, with PE as the dependent variable, and a forwards stepwise approach to select clinical and biochemi-cal variables for inclusion.

3 | RESULTS

3.1 | Patient demographics, obstetric history and renal function

A total of 195 women were recruited into the study, of whom 88 had PE and 107 were healthy pregnant controls. A comparison of baseline demographic and clinical features between the two groups is shown in Table 1. The women with PE were marginally younger (mean: 29.9 vs 31.9 years, $P = 0.012$), enrolled at an earlier gestation (median: 37 vs 39 weeks, $P < 0.001$) and more likely to be experiencing their first pregnancy (53% vs 16%, $P < 0.001$) than those in the control group. The women with PE had significantly lower serum albumin (mean: 33.0 g/L vs 34.8 g/L) and higher serum creatinine (median: 64 vs 55 μmol/L) and cystatin--C (me-dian: 1.27 vs 1.08 mg/L) than the healthy controls (all $P < 0.001$). There were no significant differences in ethnicity, BMI or the prev-alence of nonsingleton pregnancies between the groups. Of the PE patients, five (6%) had gestational diabetes (GDM) and three

TA B LE 2 Comparison of Immunoglobulins, sFLC levels, other immune and angiogenic markers between groups

	Normal range[a]	PE (n = 88)	Control (n = 107)	*P*--value
Immunoglobulins and sFLCs[b]				
IgG g/L	6.00-16.00	7.32 (±1.92)	7.86 (±1.82)	**0.046**
IgA g/L	0.80-4.00	1.78 (±0.71)	1.68 (±0.64)	0.300
IgM g/L	0.50-2.00	1.13 (±0.62)	1.03 (±0.45)	0.207
IgG1 g/L	2.75-9.50	4.13 (±1.19)	4.51 (±1.20)	**0.026**
IgG2 g/L	1.20-4.50	2.58 (±1.03)	2.60 (±1.01)	0.900
IgG3 g/L	0.17-1.79	0.51 (0.36-0.67)	0.63 (0.45-0.85)	**0.015**
IgG4 g/L	0.00-1.30	0.17 (0.10-0.35)	0.19 (0.10-0.36)	0.895
Kappa mg/L (FL)	3.3-19.4	15.3 (11.1-22.1)	11.2 (9.1-14.2)	**<0.001**
Lambda mg/L (FL)	5.7-26.3	13.6 (11.5-15.7)	12.8 (10.4-15.1)	0.148
Combined sFLC mg/L (FL)	9.0-45.7	29.7 (23.4-37.5)	24.1 (20.5-29.2)	**<0.001**
Kappa mg/L (Lx)	4.4-19.4	10.0 (8.7-11.9)	9.3 (8.1-10.5)	**0.005**
Lambda mg/L (Lx)	4.1-19.2	10.3 (8.3-12.2)	8.2 (7.4-9.7)	**<0.001**
Combined sFLC mg/L (Lx)	8.5-38.6	19.8 (17.8-24.5)	17.7 (16.0-20.1)	**<0.001**
Other immune markers				
C3 g/L	0.75-1.75	1.73 (±0.34)	1.74 (±0.31)	0.838
C4 g/L	0.14-0.54	0.23 (0.18-0.31)	0.27 (0.22-0.35)	**0.001**
HS-CRP mg/L	<3	5.4 (2.9-11.7)	3.9 (2.2-7.7)	**0.027**
B2-M mg/L	1.20-2.40	2.14 (1.86-2.65)	1.74 (1.53-1.93)	**<0.001**
Angiogenic markers				
sFLT-1 pg/mL	--	6103 (3276-11 097)	3301 (2120-4842)	**<0.001**
PIGF pg/mL	--	93.5 (59.7-166.6)	217.2 (142.1-417.8)	**<0.001**
sFLT-1:PIGF ratio	--	68.6 (32.3-170.0)	14.0 (6.3-28.5)	**<0.001**

Data are reported as per Table 1.

[a]The normal reference range for the nonpregnant population.

[b]Measurement of FLC levels was undertaken using two methods, the polyclonal Freelite® (FL) assay and monoclonal Luminex (Lx) assay – results for both methods are reported.

(3%) had chronic essential hypertension, compared to no cases in the control patients (P = 0.013 and 0.054, respectively). Excluding the GDM and hypertension patients as a sensitivity analysis had minimal impact on the demographic and clinical data provided for the PE group.

3.2 | Serum IgG levels but not IgA or IgM levels, are reduced in PE

Total serum IgG (mean: 7.32 vs 7.86 g/L, P = 0.046), and IgG subclass 1 (mean: 4.13 vs 4.51 g/L, P = 0.026) and subclass 3 (median: 0.51 vs 0.63 g/L, P = 0.015) were significantly lower in the PE group than in the control group, whilst no significant differences in IgG subclass 2 and 4 levels were detected (P = 0.900,0.895, respectively) (Table 2 and Figure 1). Serum levels of IgA and IgM were not found to differ significantly between the PE and control groups (P = 0.300, 0.207, respectively).

3.3 | Serum FLC levels are increased in PE

Serum concentrations of combined (sum of kappa and lambda) sFLC were measured using both Freelite® and luminex assays. Results from the two assays were highly correlated (Spearman's ρ = 0.75, P < 0.001), although the magnitude of measurements on the Freelite® was significantly higher than on the luminex (P < 0.001) (Table 2 and Figure 1E). On both assays, the sFLC concentrations were found to be significantly raised in the PE vs control groups, with medians of 29.7 vs 24.1 mg/L using Freelite® and correspond-ing values of 19.8 vs 17.7 mg/L using the luminex assay (both P < 0.001).

Seventeen (4%) of women had abnormally high kappa lambda sFLC ratios (normal range 0.260--1.65) by the Freelite test, but serum immunofixation did not reveal monoclonal immunoglobulins or sFLC in the five patients with the highest combined FLC levels (Freelite® values 66.5--85.0 mg/L).

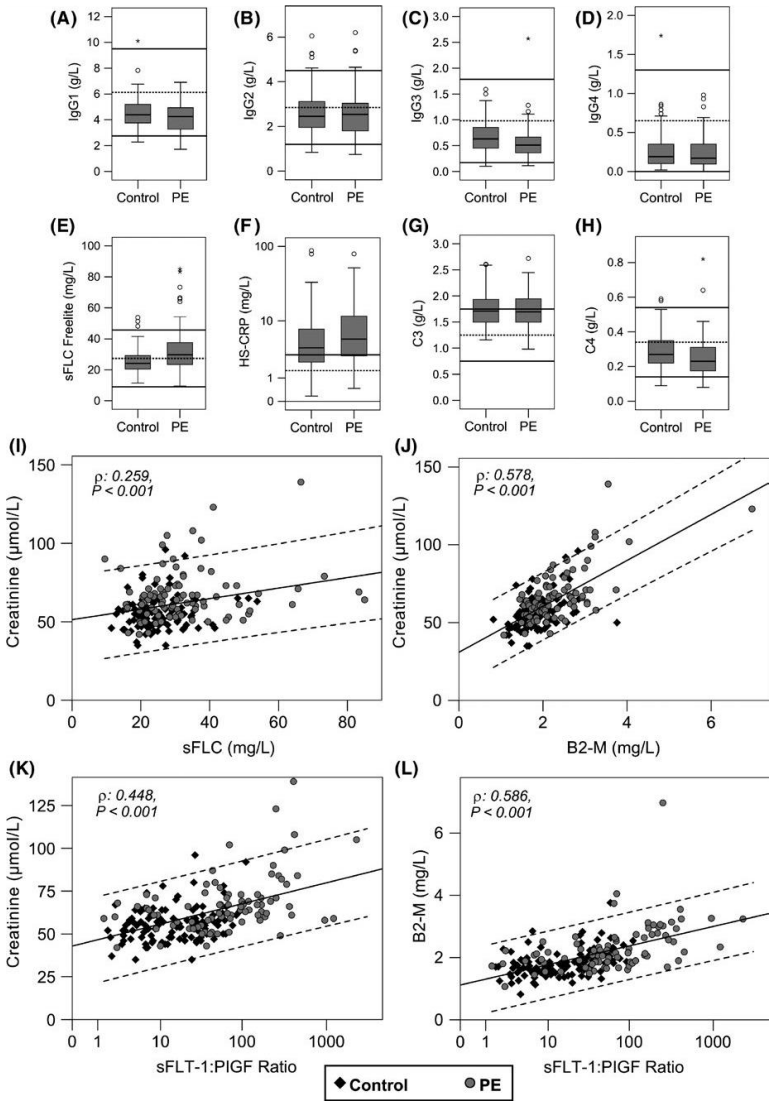


FIGURE 1 (A-G) Box-whisker plots comparing concentrations of (A--D) IgG 1--4, (E) total sFLC (Freelite®), (F) HS--CRP, (G) C3 and (H) C4 between control and PE groups. The normal reference range for the general nonpregnant population is also illustrated as solid horizontal bars and a dashed line represents the middle of this reference range. (I--L) Scatterplots of Serum Creatinine against levels of (I) sFLC (Freelite®), (J) B2--M, (K) sFLT--1:PlGF and (L) sFLT--1:PlGF against B2--M levels. Spearman rho coefficients and *P*-values are displayed. Solid lines represent linear regression models, and broken lines are 95% prediction intervals. The sFLT-1:PlGF ratio was $\log_{10}$- transformed for the regression analysis to improve model fit, and is plotted on a logged x-axis

3.4 | Pregnancy is associated with an acute phase response (elevated serum HS--CRP and C3) that is greater in PE patients, who also have reduction in C4 levels and raised B2--M

Both serum HS--CRP (median: 5.4 vs 3.9 mg/L, $P = 0.027$) and B2--M (median: 2.14 vs 1.74 mg/L, $P < 0.001$) were significantly higher in the PE than the control group (Table 2 and Figure 1). The C3 levels were similar (mean: 1.73 vs 1.74 g/L, $P = 0.838$) in both study groups, whereas C4 levels were significantly lower in women with PE (me-dian: 0.23 vs 0.27 g/L, $P = 0.001$). Concentrations of HS--CRP and C3 in both groups were at the higher end of the nonpregnant normal reference range, in keeping with an acute phase response in preg-nancy, whilst the measured C4 levels were clustered towards the middle--lower end of the nonpregnant reference range (Figure 1F--H).

3.5 | Serum sFLT--1 and PlGF in women with normal pregnancy and those complicated by PE

Serum concentrations of sFLT--1 (median: 6103 vs 3301 pg/mL, $P < 0.001$) were significantly higher in the PE group, compared to the control group, whilst PlGF (median: 93.5 vs 217.2 pg/mL, $P < 0.001$) showed an opposite trend, resulting in a substantially higher sFLT--1:PlGF ratio in PE than controls (median: 68.6 vs 14.0, $P < 0.001$) (Table 2).

3.6 | Comparison of diagnostic accuracy

Figure 2 compares the ability of the serum biochemical markers which were significantly different in the two groups to distinguish between cases with PE and controls. The sFLT--1:PlGF ratio and B2--M

provided the highest area under the ROC curve (AUROC) values, at 0.81 (95% CI 0.74-0.88) and 0.75 (95% CI 0.69-0.82), respectively, with no significant difference between the diagnostic accuracy of these two markers (P = 0.184). The AUROCs for sFLC measured on the Freelite® and luminex assays were also comparable (0.67 vs 0.68, P = 0.655), hence only the Freelite® assay data is considered in subsequent analysis.

3.3 | Relationship between serum IgG and FLCs with other immune markers

Significant positive correlations were detected between Igs and sFLC levels. Total IgG and IgG1 showed a weak correlation with C3, and a correlation between IgG3 and C4 levels was also ob- served. Increased serum creatinine (P < 0.001, Figure 1L), cysta- tin-C (P = 0.003), B2-M (P < 0.001) and HS-CRP (P = 0.005) were all associated with significantly increased total sFLC levels, in keeping with a relationship with kidney function and inflamma- tory response (Table 3). This relationship was not observed with Ig levels.

3.4 | Ethnicity and sFLC and Igs levels

Total sFLC levels and IgG levels were found to differ significantly with ethnicity (both P < 0.001), with post hoc analysis finding lev- els to be significantly higher in patients of South Asian origin, com- pared to patients of White European origin, with medians of 30.7 vs 24.7 mg/L and 8.44 vs 7.03 g/L, respectively (both P < 0.001). No significant association between ethnicity and IgA was detected (P = 0.063), whilst IgM was significantly raised in the Mixed/Other group, relative to White Caucasian women (mean: 1.41 vs 1.03 g/L, P = 0.037).

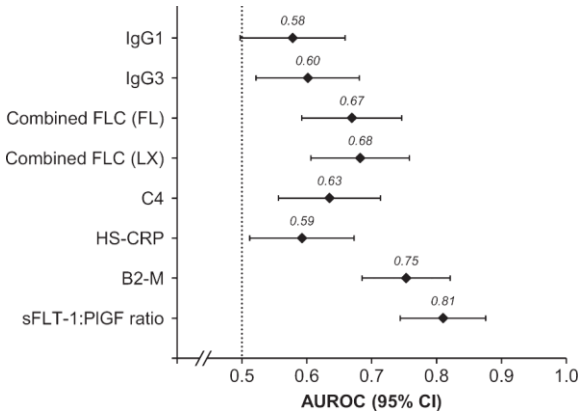


FIGURE 2 Forest plot of the AUROCs for the differentiation between PE and controls by the markers identified as significant in Table 2. The AUROC is displayed as diamond, and the error bars represent 95% confidence intervals. As per Table 2, results for both the polyclonal Freelite® (FL) assay and monoclonal Luminex (Lx) assay are displayed for measurement of sFLC

TABLE 3 Correlations between Immunoglobulins, sFLC levels (Freelite®) and angiogenic markers with other study markers (all patients)

	IgG	IgA	IgM	sFLC	C3	C4	B2-M	Cystatin-C	Creatinine	HS-CRP	Albumin	sFLT-1: PlGF ratio
IgG	N/A	**0.302**	**0.162**	**0.323**	**0.149**	0.117	−0.060	−0.072	0.041	−0.067	**0.223**	−0.052
IgA	**0.302**	N/A	**0.151**	**0.379**	0.005	−0.073	0.069	−0.001	0.078	−0.064	0.074	**0.160**
IgM	**0.162**	**0.151**	N/A	**0.149**	0.113	−0.076	0.099	−0.055	0.020	−0.003	0.055	0.119
IgG1	**0.915**	**0.208**	0.078	**0.283**	**0.217**	0.112	−0.049	−0.120	−0.003	−0.070	**0.282**	−0.074
IgG2	**0.712**	**0.262**	0.137	**0.264**	0.030	0.112	−0.075	0.039	0.062	−0.042	0.045	−0.008
IgG3	**0.434**	0.096	**0.143**	0.065	0.119	**0.183**	***−0.170***	−0.122	***−0.163***	0.002	**0.210**	***−0.143***
IgG4	**0.467**	**0.157**	0.054	**0.195**	−0.008	0.002	−0.081	−0.048	−0.056	−0.032	0.037	−0.004
sFLC	**0.323**	**0.379**	**0.149**	N/A	0.121	0.039	**0.336**	**0.214**	**0.259**	**0.203**	−0.119	0.137
sFLT-1:PlGF ratio	−0.052	**0.160**	0.119	0.137	***−0.145***	***−0.311***	**0.586**	**0.503**	**0.448**	0.073	***−0.291***	N/A

Correlations are reported as Spearman's rho coefficients, with bold values significant at P < 0.05, and significant negative correlations in italic. Serum FLC and Igs were also compared across ethnic groups using ANOVA and a Kruskal-Wallis test, as reported in the text.

3.9 | Relationship of degree of proteinuria with study markers

The degree of proteinuria, measured as a urine PCR in the patients with PE, was not found to be significantly correlated with serum lev-els of IgG (ρ = −0.130, P = 0.250), IgA (ρ = −0.043, P = 0.707), IgM (ρ = −0.122, P = 0.281) or sFLC (ρ = −0.095, P = 0.401). A significant correlation was observed between increased levels of proteinuria and higher serum B2-M and sFLT-1:PlGF ratio (ρ = 0.287, P = 0.010 and ρ = 0.442, P < 0.001, respectively) and with lower C4 levels (ρ = −0.425, P < 0.001). As Table 3 highlights, there was a significant positive cor-relation between serum albumin concentrations and IgG (ρ = 0.223, P = 0.002) but not with IgA (P = 0.304) or IgM (P = 0.442) levels.

3.10 | Serum angiogenic factors were correlated with renal function, B2-M levels, and complement proteins - but not with serum immunoglobulins

The associations between Igs and the sFLT-1:PlGF ratio were weak, with only serum IgA (ρ = 0.160, P = 0.028) and IgG3 (ρ = −0.143, P = 0.049) showing significant correlations (Table 3). There was no ev-idence of a significant correlation between sFLC concentrations and sFLT-1:PlGF ratio (ρ = 0.137, P = 0.060). However, sFLT-1:PlGF ratio and B2-M levels were strongly correlated with each other (ρ = 0.586, P < 0.001) and with both serum creatinine (ρ = 0.448, ρ = 0.578, re-spectively, both P < 0.001, Figure 1J-K) and cystatin-C (ρ = 0.503, 0.717, both P < 0.001), in keeping with a relationship of both mark-ers with renal function. When split by study group, the sFLT:PlGF ratio did not significantly correlate with serum C3 (P = 0.600) or C4 (P = 0.626) levels in the control group (Table S1), but was signif-icantly negatively correlated with both markers in women with PE (ρ = −0.276, P = 0.012, and ρ = −0.409, P < 0.001, respectively)..

3.11 | Serum FLCs, IgG1 and sFLT-1:PlGF are independently associated with PE

On multivariable analysis, significant independent predictors of PE were increased sFLC concentration (OR: 1.11 per mg/L, P < 0.001), sFLT-1:PlGF (OR: 1.04 per unit, P < 0.001) and IgG1 levels (OR: 0.62 per g/L, P = 0.024) (Table 4). After accounting for these factors, serum creatinine, cystatin-C, B2-M, serum C4, IgG subclasses 2-4, HS-CRP, patient age, BMI, ethnicity and twin pregnancy were not found to be significant independent mark-ers of PE in our study population. As expected, first pregnancy (OR: 7.02, P < 0.001) was an important independent risk factor for PE. As per Figure 1L, B2-M was highly correlated with sFLT-1:PlGF and, without the addition of the angiogenic markers to the multivariable model, B2-M was found to be an independent predictor of PE (P < 0.001) alongside sFLC (Table S2).

3.12 | Increased sFLT-1:PlGF ratio and B2-M levels are associated with adverse clinical outcome

The overall rate of adverse clinical outcomes was significantly higher in PE than controls (57% vs 18%, P < 0.001), with each of the five events in-cluded in this composite outcome also being significantly more common in PE (Tables 1 and 5). There were no intrauterine deaths or still births in our cohort. Within the PE and control subgroups, associations between adverse clinical outcomes and the biomarkers previously found to be associated with PE were assessed (Table 5). None of the factors considered were found to be associated with adverse outcomes in the control group. However, for patients with PE, serum creatinine (P = 0.019), B2-M (P < 0.001) and sFLT-1:PlGF concentrations (P = 0.001) were all significantly higher in those that developed adverse outcomes. The associations between adverse events and both B2-M and the sFLT-1:PlGF ratio are demonstrated graphically in Figure 3.

4 | DISCUSSION

4.1 | Main findings

We report, to our knowledge for the first time, changes in sFLC (measured by two analytical methods) and IgG1 concentrations at the time of PE. These changes appear to be independent of other markers of immune system activation, inflammation, angiogenic markers and renal function. The current study has also provided a normal range of sFLC levels in late third trimester for healthy pregnant women. Concentrations of sFLT-1:PlGF (arguably the strongest

TA B LE 4 Multivariable analyses to identify independent markers of pre-eclampsia

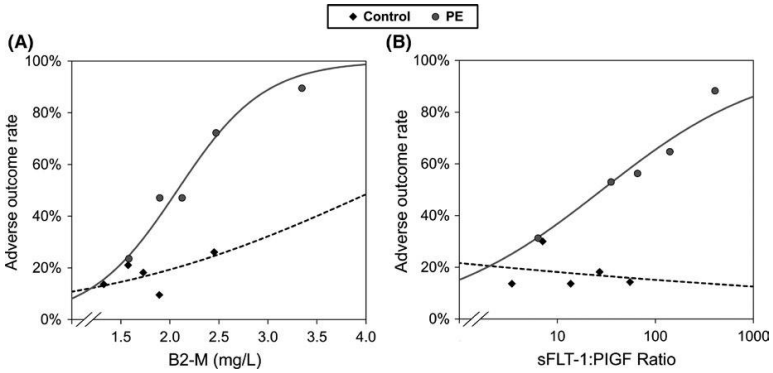
Variable	Odds ratio (95% CI)		*P*-value
	Per unit increase	Per IQR increase	
First pregnancy	7.02 (2.90-16.99)	*N/A*	**<0.001**
IgG1 (g/L)	0.62 (0.41-0.94)	0.48 (0.26-0.91)	**0.024**
sFLC (mg/L)	1.11 (1.05-1.16)	3.07 (1.79-5.28)	**<0.001**
sFLT1:PlGF ratio	1.04 (1.02-1.05)	7.37 (3.09-17.57)	**<0.001**

The factors considered for inclusion were patient age, BMI, ethnicity, first pregnancy, twin preg-nancy, combined FLC levels (Freelite®), serum albumin, B2-M, serum creatinine, cystatin-C, C4, all four IgG subclasses and the sFLT-1:PlGF. A binary logistic regression model was produced, with pre-eclampsia as the dependent variable, and a forwards stepwise approach used to select variables for inclusion. Two sets of odds ratios are reported. The first are per unit increase in the factor (or for first pregnancy vs previous pregnancies) and the second are for an increase of a magnitude equivalent to the interquartile range of the factor. Bold *P*-values are significant at P < 0.05.

TA B LE 5 Comparison of markers for those with and without adverse outcome (defined in methods section)

	PE group			Control group		
	Adverse (n = 50)	Non adverse (n = 38)	*P*--value	Adverse (n = 19)	Non adverse (n = 88)	*P*--value
Serum creatinine (μmol/L)	67 (59-80)	62 (53-68)	**0.019**	58 (53-64)	55 (48-60)	0.122
sFLC (mg/L) Freelite®	28.8 (23.7-37.6)	30.3 (22.7-36.4)	0.876	24.9 (20.3-29.5)	24.0 (20.8-29.2)	0.702
B2-M (mg/L)	2.4 (2.1-3.0)	1.9 (1.7-2.2)	**<0.001**	1.7 (1.6-2.1)	1.7 (1.5-1.9)	0.517
Serum albumin (g/L)	32.5 (±4.1)	33.6 (±4.1)	0.605	34.9 (±1.8)	34.8 (±2.7)	0.334
IgG1 (g/L)	3.97 (±1.19)	4.33 (±1.17)	0.603	4.56 (±1.22)	4.50 (±1.20)	0.805
sFLT-1:PlGF ratio	99.4 (44.3-251.7)	39.8 (10.0-95.4)	**0.001**	10.4 (5.6-25.6)	14.9 (6.3-28.9)	0.737

Data are reported as mean ± SD or as median and IQR, with *P*--values from Mann--*Whitney tests*. Bold *P*--values are significant at $P < 0.05$.



FI G U R E 3 Markers of adverse outcome in the PE and control groups. Lines represent binary logistic regression models, treating the markers as continuous covariates. The sFLT--1:PlGF ratio was $\log_{10}$--transformed prior to the regression analysis to improve model fit. Points are plotted at the midpoints of the quintiles for each marker for reference

current biomarker for prediction of PE) were significantly correlated with renal function, B2--M levels, and complement proteins, but not with serum Igs or sFLCs, raising the possibility of independent hu-moral immunity and angiogenic pathways in the setting of PE. The diagnostic accuracy of serum B2--M levels and the sFLT--1:PlGF ratio for PE were comparable (AUROC 0.79 vs 0.81, respectively) and both markers were closely correlated (ρ = 0.572), resulting in sFLT-- 1:PlGF displacing B2--M in multivariable analysis, further supporting a close association between the two markers in the setting of PE.

4.2 | Strengths and limitations

As PE was already present in our study group, we have not been able to look at predictive capability, nor can we comment on causality. However, our findings lend support to future study directions, with the aim of better understanding of the complex biological pathways involved in PE.

The study design has limitations which we acknowledge. Whilst all women with a history of CKD or chronic illness were excluded from both groups; patients were not individually matched. There was selection bias due to the nature of recruitment. A significant proportion of women in the control group would have elected for caesarean section deliveries due to factors related to earlier pregnancies, which may partially account for the difference in the rate first pregnancies between the two groups. However, this difference was accounted for on multivariable analysis. The median gestation in the PE group was 37 weeks vs 39 weeks in controls and, whilst not ideal, this is not of huge clinical importance, as both gestations are "at term." Pre--eclampsia is a heterogeneous condition; we defined all PE cases on the basis of hypertension and proteinuria, and did not consider women with atypical presentations of PE or those with early onset for whom the findings may have differed.

4.3 | Interpretation

There has been little published previously on the humoral re-sponse in normal pregnancy or in those women who develop PE, and reported alterations in Ig levels have been inconsistent.[10-18] This study demonstrated similar IgM and IgA levels and a reduc-tion of total IgG (particularly subclasses IgG1 and IgG3), in the context of PE vs healthy term pregnancy. The reduction in IgG lev-els could be related to proteinuria, reduced half--life as a result of lower systemic neonatal Fc receptor (FcRN) recycling, increased transport through the placenta to the foetus, or a combination of these factors.

Although serum IgG and albumin are lost with heavy protein-uria, there was no evidence of a significant correlation between the degree of proteinuria and serum levels of any of the Igs in the PE

group. Whilst some historical studies have demonstrated IgG loss in urine in pregnancies complicated by proteinuria, this has not been a consistent finding.[15,17] This suggests alternative mechanisms could be involved in the low IgG levels observed in PE, which are independent of urinary protein loss. A unifying mechanism would be a reduction in systemic FcRN recycling and, thus, reduction in serum half--life of both albumin and IgG. There is preferential increase in placental transfer of IgG1 over the other subclasses and IgG3 is known to have a shorter half--life, compared to the other IgG subclasses,[11,37] which might explain why these IgG subclasses are the most reduced. Both of these subclasses are also most strongly associated with complement activation, and the low C4 levels seen in our PE cohort could be in keeping with activation of the complement pathway through these two IgG subclasses and, thus, consumption of C4, IgG1 and IgG3.[38] Complement consumption in PE through the classical pathway is supported by an increasing body of evidence finding enhanced complement breakdown products in a variety of tissue samples in PE.[32,39-47]

Serum FLC levels can be elevated as a result of increased secretion from polyclonal or monoclonal plasma cell clones and reduced glomerular filtration.[19] The elevated polyclonal sFLC levels in this study were independent of markers of inflammation and renal function, supporting a humoral response in PE. Normal serum half--life is of only a few hours and is therefore shorter than the half--life of intact Igs (5--21 days). Polyclonal sFLC is elevated in a proportion of the normal population, including patients with CKD, and are associated with poor long--term survival and morbidity.[48,49] Women with PE have a higher risk of developing renal and cardiovascular disease in the future.[50] Further studies are required to determine the time course of the rise in sFLC levels in PE and whether this also exists prepregnancy.

Although ethnicity was not found to be a significant risk factor for PE in our study, this may have been as a result of low statistical power, as black ethnicity is recognized as a risk factor for PE.[51] This study found higher Ig and sFLC levels in the nonwhite study subjects. Both healthy nonpregnant Black and South Asian subjects have been reported to have higher levels of circulating Igs, compared to Caucasian subjects, in keeping with this pregnant study population.[52-54] Whether racial variations in Ig levels contribute to increased risk of inflammatory conditions including PE requires further investigation. There is little--published literature on ethnicity and variation in sFLC levels, with some differences reported.[55,56]

The sFLT--1:PlGF ratio and serum B2--M were both associated with the occurrence of adverse clinical outcome in the PE group. As both of these markers were highly correlated with serum creatinine (Table 3), the association of these markers with PE could be interpreted as surrogates of pregnancy--associated acute kidney injury. However, serum creatinine was only moderately higher in patients with adverse events in the PE group (median: 67 vs 62 μmmol/L, respectively). The relationship between sFLT--1 and PlGF levels and renal function has not been well established, with two studies showing higher levels in patients with CKD compared to those with normal renal function.[57,58]

Beta 2 microglobulin (B2--M) forms part of the FcRn receptor which, as mentioned earlier, is necessary for IgG recycling and placental transfer.[59,60] Serum levels of B2--M are increased in inflammatory states connected with activation of the lymphopoietic system.[61-63] Saudan et al demonstrated that women with PE had higher levels of B2--M compared to controls. However, fractional excretion of B2--M was similar, suggesting the difference may not be solely due to re-duced β2--M excretion in PE.[64] A further study showed B2--M levels in early pregnancy were not different in women who subsequently developed PE vs those who remained normotensive; however, the PE group was small, consisting of just seven women.[65]

5 | CONCLUSION

This current study supports activation of multiple immune system pathways in PE, specifically the humoral system, which appears to be independent to angiogenic disturbances. A better understanding of the timing and sequence of these changes could potentially lead to the identification of supportive, diagnostic and possibly to novel treatment strategies. Further work is required to assess whether hu-moral markers can add to the predictive capability of sFLT--1:PlGF in PE and also whether B2--M can be used as an alternative biomarker to angiogenic factors in this setting.

AC K N OW L E D G M E N T S

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DISCLOSURES OF INTERESTS

University of Birmingham and MD have shares in Abingdon Health Ltd that manufactures sFLC assays and MD is an advisor to Abingdon Health.

AUTHOR CONTRIBUTIONS

NS: study design, patient recruitment, sample collection, conduct-ing experiments, data interpretation, analysing data and writing. JH: analysing data and writing. MTD: study design, data interpretation, writing and manuscript review. EK: study design and manuscript review. TP: conducting experiments. CJD: manuscript review. GWL: study design, data interpretation, writing, and manuscript review.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Sarween N, Drayson MT, Hodson J, et al. Humoral immunity in late--onset Pre--eclampsia and linkage with angiogenic and inflammatory markers. *Am J Reprod Immunol*. 2018;e13041.

Appendix 2: HBRC Ethics Approval

College of Medical and Dental Sciences
Research and Knowledge Transfer Office

21st January 2014

Dr Graham Lipkin
Queen Elizabeth Hospital Birmingham
Mindelsohn Way
Edgbaston
Birmingham B15 2WB

Dear Dr Lipkin

Application Number:	**13-160**
Project Title:	**Identification of biomarkers predictive and diagnostic of preeclampsia in women with chronic kidney disease**
Application Received:	**14th October 2013**

I am pleased to confirm that your application to obtain human biomaterials from the Human Biomaterials Resource Centre has now been approved by the Access Review Panel.

I would be grateful if you could facilitate signing of the Tissue Transfer Agreement (attached to the email) and its return to me as soon as possible.

I would like to remind you that the materials released to you should only be used for the project described in your application, which has been approved by the Access Review Panel, and there should be no transfer of materials to other researchers unless this has been agreed in writing. We should be informed promptly of any changes to the original application.

A hard copy of this correspondence will be sent to you.

Yours sincerely

Dr Jane Steele
Director, Human Biomaterials Resource Centre
cc. Research Governance Team

North West 5 Research Ethics Committee - Haydock Park
North West Centre for Research Ethics Committees
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone:
Facsimile:

28 April 2010

Dr Jane C Steele
Director, Human Biomaterials Resource Centre
College Medical & Dental Sciences
University of Birmingham
Edgbaston
Birmingham B15 2TT

Dear Dr Steele

Title of the Research Tissue Bank: University of Birmingham Human Biomaterials

REC reference:
Designated Individual: Resource Centre
09/H1010/75
Professor Lawrence S Young

Thank you for your letter of 19 April 2010, responding to the Committee's request for further information on the above research tissue bank and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair (Dr Donal Manning – Consultant Paediatrician).

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation as revised.
The Committee has also confirmed that the favourable ethical opinion applies to all research projects conducted in the UK using tissue or data supplied by the tissue bank, provided that the release of tissue or data complies with the attached conditions. It will not be necessary for these researchers to make project-based applications for ethical approval. They will be deemed to have ethical approval from this committee. You should provide the researcher with a copy of this letter as confirmation of this. The Committee should be notified of all projects receiving tissue and data from this tissue bank by means of an annual report.

Duration of ethical opinion
The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter: From Dr Jane C Steele		02 November 2009
REC application	IRAS Version 2.5	02 November 2009
Human Tissue Authority Licence: Licensing Number: 12358		11 June 2008
Response to Request for Further Information: From: Dr Jane C Steele		03 March 2010
Protocol for Management of the Tissue Bank	2	03 March 2010
Participant Information Sheet: Taking part in research at University Hospitals Birmingham	2	03 March 2010
Participant Information Sheet: Donation of Human Tissue for Research	2	03 March 2010
Participant Consent Form: Donation of Human Tissue for Research	2	03 March 2010
Participant Information Sheet: Donation of Placenta, Umbilical Cord and Cord Blood for Research	2	03 March 2010
Participant Consent Form: Donation of Placenta, Umbilical Cord and Cord Blood for Research	2	03 March 2010
Response to Request for Further Information: From Dr Jane C Steele		19 April 2010
Participant Consent Form: CF#1; Participant Consent Form: Patient Agreement to Investigation or Treatment	3	19 April 2010
Letter to Dr Jane C Steele from Mr Bob Hibberd, Head of Governance, University Hospitals Birmingham NHS Foundation Trust		14 April 2010

Licence from the Human Tissue Authority

Thank you for providing a copy of the above licence.

Research governance

A copy of this letter is being sent to the R&D office responsible for for University Hospital Birmingham NHS Foundation Trust. You are advised to check their requirements for approval of the research tissue bank.

Under the Research Governance Framework (RGF), there is no requirement for NHS research permission for the establishment of research tissue banks in the NHS. Applications to NHS R&D offices through IRAS are not required as all NHS organisations are expected to have included management review in the process of establishing the research tissue bank.

Research tissue bank managers are advised to provide R&D offices at all TCCs with a copy of the REC application for information, together with a copy of the favourable opinion letter when available. All TCCs should be listed in Part C of the REC application.

NHS researchers undertaking specific research projects using tissue or data supplied by a research tissue bank must apply for permission to R&D offices at all organisations where the research is conducted, whether or not the research tissue bank has ethical approval.

Site-specific assessment (SSA) is not a requirement for ethical review of research tissue banks. There is no need to inform Local Research Ethics Committees.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

Here you will find links to the following:

- Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
- Annual Reports. Please refer to the attached conditions of approval.
- Amendments. Please refer to the attached conditions of approval.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email:

referencegroup@nres.npsa.nhs.uk

09/H1010/75	**Please quote this number on all correspondence**

Yours sincerely

Dr Donal Manning
Chair
E-mail:

Appendix 3: Patient Information Leaflet

UNIVERSITY OF BIRMINGHAM

Birmingham Women's NHS
NHS Foundation Trust

DONATION OF SAMPLES TO THE HUMAN BIOMATERIALS RESOURCE CENTRE

Adult Patient Information Sheet

Introduction

The Human Biomaterials Resource Centre (HBRC) collects and stores human samples in a secure environment for ethically approved medical research. Scientists need human tissue, cells and body fluids for research into finding out how a disease starts, and to find new ways of diagnosing and treating the illness.

This information sheet explains what happens when a sample is donated to the HBRC. Please take time to read it carefully and ask if anything is not clear. If you have any questions at a later date please contact us using the details at the bottom of this sheet.

What are we asking you to do?

- We would like to invite you to donate a sample to the HBRC. The sample may be tissue, blood or another body fluid. Your sample(s) may be stored until release to researchers for use in ethically and scientifically approved research projects, or on some occasions they may be released directly to an approved project.
- Sometimes samples are removed for routine tests and during surgery or clinical procedures. They may be disposed of, or they may be sent to the Pathology laboratories in the hospital to help with diagnosis. In many cases there will be a surplus of the sample after the diagnosis is complete. With your agreement, these "waste" and "leftover" samples can be donated to the HBRC. The Pathology laboratories may also have kept your sample(s) from previous tests, surgery or procedures, and these can still be very useful for researchers. Again, with your agreement, some of this material could become part of the HBRC collection.
- On some occasions and only if it is safe and easy to do so, we may ask whether we can take a small additional sample, for example, an extra blood sample or extra small bit of tissue. These samples can usually be taken as part of treatment so should not involve any extra pain, discomfort or inconvenience.
- Some research projects require serial blood or urine samples, or need samples to be taken at a certain time point, so that they can monitor disease progress. In these instances you may be asked to donate a sample at a different time to when you are having samples taken for routine hospital tests.
- Information about your medical condition, other disease(s) and treatments (now, and in the longer term) is scientifically useful to researchers. This includes long term follow up information held, for example, by the National Cancer Registry and the Office of National Statistics. We therefore ask you to agree to allow us to access your health records if you agree to donate a sample(s).

What will happen if you agree?

- First you need to give your written permission by signing the consent form. You will be given a copy of the signed consent form to keep. Please also keep this information sheet to remind you of what you were asked to do.
- When your sample arrives at the HBRC it will be given a unique code linked to your NHS or hospital number. A link to your identity will be retained within the hospital. All information about you will remain confidential and will be stored in accordance with the UK Data Protection Act 1998. No information will ever be released to an insurance company. Researchers receiving your samples will NOT be provided with any personal information such as your name, address or phone number. The information they will be given for their work will relate to your disease, treatment and medical history only and will NOT be directly linked to your identity.
- Your samples may be released for genetic studies but only to research projects aimed at providing clues to the nature of disease, or if it is known already that genes are important.

- Your sample(s) may also be used to support ethically approved medical research which uses animals, but only when this is absolutely necessary and experiments cannot be performed in any other way. Animal models can be invaluable for increasing our understanding of disease and advancing treatments.
- Sometimes researchers may grow your cells in a culture dish for a long time and again, this type of approach can be invaluable for medical research.
- Your samples and associated information from you health records will be used mainly by local researchers but may also be made available to researchers outside of Birmingham, elsewhere in the UK or overseas. They may work in universities, hospitals or private/commercial companies that do medical research. Commercial collaborations are vital for the development of suitable drugs and treatments.
- You will not receive any personal financial reward for donating your samples and the samples you have gifted will never be sold for profit. However, we may ask researchers to cover some of the cost incurred in sample collection and storage.

Do you have to say yes?

NOT AT ALL. It is entirely up to you. You do not have to donate a sample, or give a reason if you choose not to. Your decision will not affect your care or treatment in any way now, or in the future.

What happens if you change your mind?

If you consent to donate samples to the HBRC, this will be lasting unless you change your mind. You can change your mind at any time by contacting your hospital doctor or the hospital Research and Development Office (details below). You do not have to give a reason why.

If you tell us you have changed your mind, this will not affect your care or treatment in any way now or in the future. All samples held in the HBRC will be destroyed in the way human samples are normally destroyed by hospitals. Similarly, the information we store about you will be deleted so that it can never be used again. We will also contact any research groups using your samples and ensure that they destroy any that are unused and associated data.

If you change your mind after a long period of time, the samples may already have been used. We cannot recall samples or information from researchers if this is the case.

What are the benefits to you?

It is unlikely that there will be any direct benefit to you since it takes many years for research to produce advances in the way diseases are diagnosed, treated or prevented. You can benefit from the knowledge that research will make faster progress if more human samples are studied and you are personally contributing to this.

What are the risks to you?

As far as we know there are NO risks associated with the donation of samples to the HBRC. Samples will only be collected when it is safe to do so during your routine hospital visits. Your identity will remain confidential and researchers are bound by a strict agreement to use the samples only for the research they said they would.

Can you find out the results?

The development of reliable new clinical tests takes many years so the HBRC will not routinely report individual research results. You can find out more generally about the types of research projects using your samples by contacting us using the details below.

If a research project which uses your samples generates clinically important information then your doctor, or another member of your healthcare team, will contact you. They may wish to discuss how the information could be used to guide treatment for your current condition or for other conditions (possibly hereditary) which may affect you and your family.

Contact details: Research and Development Department, Birmingham Women's Hospital, K13 Norton Court, Metchley Park Road, Edgbaston, Birmingham B15 2TG.

Tel: [redacted]

Appendix 2: Patient Consent Form

UNIVERSITY OF BIRMINGHAM

Birmingham Women's NHS
NHS Foundation Trust

DONATION OF HUMAN TISSUE FOR RESEARCH

Patient Consent Form

Please initial each box if you agree with the statement and then sign the bottom of the form

I have received the Information Sheet entitled 'DONATION OF HUMAN TISSUE FOR RESEARCH' dated 26-Jul-2010 (Version 3).	*Please initial*
I consent to the storage of my tissues in the Human Biomaterials Resource Centre and their use for ethically approved research projects, including genetic studies.	*Please initial*
I understand that donated tissue samples may sometimes be used in ethically approved medical research which uses animals, but only when this is absolutely necessary and no alternative approach is available.	*Please initial*
I understand that giving my tissue for research is completely voluntary and that I am free to withdraw my consent at any time without giving a reason, and without my medical care being affected.	*Please initial*
I understand that my health records may be accessed for research but that all extracted information will be anonymised.	*Please initial*
I understand that my tissues may be used by researchers in Birmingham and elsewhere.	*Please initial*

Name of Patient	Date	Signature

Name of Person taking Consent	Role	Date	Signature

Version 3
26-Jul-2010

Pink copy to be given to the patient
White copy to go in patient's notes
Yellow copy to be retained by the Human Biomaterials Resource Centre

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