UNIVERSITY OF BIRMINGHAM

Investigating the role of complement in the pathogenesis of pre-eclampsia in previously healthy pregnant women, and in high-risk groups.

Student: Dr Hannah Blakey [redacted]

Specialist Registrar in Renal Medicine,

Queen Elizabeth Hospital Birmingham.

Supervisors:

Academic: Professor Mark Drayson

Professor of Clinical Immunodiagnostics, University of Birmingham

Clinical: Dr Graham Lipkin

Consultant Nephrologist, Queen Elizabeth Hospital Birmingham

Dr Ellen Knox

Consultant Obstetrician, Birmingham Women's Hospital

Abstract

Pre-eclampsia (PE) is a leading cause of obstetric morbidity and mortality. Certain groups of women, including those with chronic kidney disease (CKD) and those of sub-Saharan African (SSA) ethnicity, are at particularly high risk. There remains no definitive treatment other than expedited delivery of baby and placenta. Previous studies suggest a role for complement dysregulation in the pathogenesis of PE, but results are often conflicting, and it remains unclear whether changes in circulating complement concentrations reflect a general heightened inflammatory state in PE or are directly associated with placental complement-mediated injury.

This thesis tested the hypothesis that PE is associated with excessive complement activation within placental tissue, with concurrent complement activation within the maternal and fetal circulation, and that groups with a high prevalence of PE, and of PE with severe features (women with CKD and women of SSA ethnicity) would exhibit a greater degree of systemic complement activation. Three arms of research were conducted, and I report:

- In a cohort of previously healthy women, PE was associated with significant placental complement deposition, associated with concurrent changes in maternal and fetal circulating complement markers (reduced maternal properdin and C4, and elevated maternal and fetal Ba). Placental C4d deposition was strongly correlated with maternal properdin and C4, suggesting that those patients with the most excessive changes in circulating markers of complement activation also have the greatest extent of placental complement-mediated damage.
- There was no evidence of excessive complement activation in the maternal circulation in superimposed PE in a cohort of women with CKD. However, raised Ba levels were associated with adverse pregnancy outcomes in women with CKD.
- There was no evidence of excessive complement activation in PE in a Ghanaian cohort of women of SSA ethnicity when compared to healthy pregnant controls. However, pregnant women of

SSA ethnicity did have significantly elevated levels of C5b-9, serum free light chains, and immunoglobulin G, when compared to the UK-recruited cohorts; suggestive of a baseline elevated inflammatory state.

The results suggest that inhibition of complement activation is a potential therapeutic target for certain groups of women with PE. However, PE is a heterogenous syndrome and additional pathophysiological mechanisms may contribute to the development of disease in women with CKD and women of SSA ethnicity.

Acknowledgments

There are a great many people who have helped to make this work possible. I am especially grateful to my supervisors: Graham Lipkin, Mark Drayson, and Ellen Knox for their guidance, support, and encouragement; especially after a large covid-shaped spanner was thrown in the works! Thank you also to Nadia Sarween for the help and valuable words of advice over the last few years.

I am indebted to everyone who collaborated with me for this research. To Claire Harris, Ruyue Sun, Long Xie, Rebecca Russell, Neil Sheerin, and Edwin Wong – I am really grateful for their expertise and assistance with the complement analysis, and for the excellent hospitality in Newcastle. Also to Kate Bramham, Jo Adu, and Nana Yaa Agyemang, for generously contributing samples from London and Ghana, and for their enthusiasm and support for this work from the outset.

I am also really grateful to James Hodson for the statistics Zoom calls; Desley Neil, Beata Hargitai and Tamas Marton for teaching me how to interpret placental histopathology; the CIS staff (especially Tim Plant, Dave Birch and Zaheer Afzal) for assisting me with the complement and immunology assays; and to the HBRC staff (especially Michael Russell and Joe Flint) for supporting me with processing all the tissue samples.

I am hugely thankful to the Queen Elizabeth Hospital Kidney Patient Association and Renal Research Fund for helping to fund the project, and to all the patients who enthusiastically participated in this research.

Finally, I owe a special thank you to my husband Paul for his love, support, and constant encouragement over the last few years. There is not a lot life hasn't thrown at us since I started my research fellowship and I couldn't have done it without him.

Table of Contents

List of Figures

List of Tables

Abbreviations

AA	African American
ACOG	American College of Obstetrics and Gynecology
ACR	Albumin creatinine ratio
ADPKD	Autosomal dominant polycystic kidney disease
aHUS	Atypical haemolytic uraemic syndrome
AKI	Acute kidney injury
ANOVA	Analysis of variance
ALT	Alanine transaminase
AO	Adverse outcome
APOL-1	Apolipoprotein L1
APS	Antiphospholipid syndrome
AST	Aspartate aminotransaminase
AT1-R	Angiotensin II type 1 receptor
AUROC	Area under receiver operating curve
B2M	Beta-2 microglobulin
BMI	Body Mass Index
BP	Blood pressure
BSA	Bovine serum albumin
BWH	Birmingham Women's Hospital
C4BP	C4b-binding protein
CfB	Complement factor B
CIS	Clinical Immunology Service
CK	Creatine kinase
CKD	Chronic kidney disease
CI	Confidence interval
CIS	Clinical Immunology Service
CR3	Complement receptor 3
CR4	Complement receptor 4
Crry	Complement receptor 1-related gene/protein y

CS	Caesarean section
CV	Coefficient of variation
DAF	Decay Accelerating Factor
DB	Dilution buffer
DBP	Diastolic blood pressure
dNK	decidual Natural Killer
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
ELISA	Enzyme-linked immunosorbent assay
GFR	Glomerular filtration rate
GPI	Glycosylphosphatidylinositol
HBRC	Human Biomaterials Resource Centre
HELLP	Haemolysis, Elevated Liver enzymes and Low Platelets
HLA	Human lymphocyte antigen
HS-CRP	High-sensitivity C-reactive protein
HTN	Hypertension
IFNγ	Interferon gamma
Ig	Immunoglobulin
ISSHP	International Society for the Study of Hypertension in Pregnancy
IQR	Interquartile range
IRAS	Integrated Research Application System
IUGR	Intra-uterine growth restriction
KIR	Killer IgG-like receptor(s)
LDH	Lactate dehydrogenase
mAb	Monoclonal antibody
MAC	Membrane attack complex
MASP	MBL-associated serine proteases
MBL	Mannose-binding lectin
MBRRACE	Mothers and Babies: Reducing Risk through Audits and Confidential Enquiries

MCP	Membrane Cofactor Protein
mRNA	Messenger ribonucleic acid
MSD	MesoScale Diagnostics
MTA	Materials transfer agreement
NICE	National Institute for Health and Care Excellence
NICU	Neonatal intensive care unit
NNU	Neonatal unit
OR	Odds ratio
pAb	Polyclonal antibody
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Protein creatinine ratio
PE	Pre-eclampsia
PlGF	Placental growth factor
PNH	Paroxysmal nocturnal haemoglobinuria
PP	Polypropylene
PTB	Preterm birth
QEHB	Queen Elizabeth Hospital Birmingham
QEKPA	Queen Elizabeth Hospital Kidney Patient Association
R&D	Research and Development
REC	Research Ethics Committee
Rec St	Reconstituted standard
RGC	Response gene to complement
ROC	Receiver operating characteristic
RR	Relative risk
RRT	Renal replacement therapy
RUPP	Reduced utero-placental perfusion pressure
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
sENG	Soluble endoglin

sFLC	Serum free light chain(s)
sFlt-1	Soluble fms-like tyrosine kinase-1
SGA	Small for gestational age
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphism
SPE	Superimposed pre-eclampsia
SSA	Sub-Saharan Africa(n)
STAT-8	Isoprostane 8-iso-prostaglandin
STB	Syncytiotrophoblast
TCC	Terminal complement complex
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
TRIS	Trisaminomethane
UoB	University of Birmingham
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VUR	Vesico-ureteric reflux
UK	United Kingdom
US	United States

CHAPTER 1

INTRODUCTION AND BACKGROUND

1 Introduction and background

1.1 Summary introduction

Pre-eclampsia (PE) is a multi-system disorder of pregnancy, characterised by new onset hypertension, and proteinuria or maternal end-organ dysfunction after 20 weeks' gestation. It is among the commonest causes of obstetric morbidity both within the UK and globally, and carries significant long-term implications for the health of mother and child. Despite the high prevalence of PE, its pathophysiological mechanisms remain poorly understood, and the only definitive treatment remains delivery of baby and placenta. This is often required preterm. Some groups of women – including those with chronic kidney disease (CKD), and those of sub-Saharan African (SSA) ethnicity – have an unusually high incidence of PE.

The complement system plays an essential role in the human innate immune system, and there is growing interest among research communities around its potential contribution to the pathogenesis of PE. Animal studies have demonstrated the importance of a tightly regulated complement system in healthy placental development. Human studies have reported placental complement deposition in women with PE and healthy pregnancy, and other studies report evidence of complement activation within the maternal circulation in cases of PE. These studies suggest a role for complement dysregulation in PE, but the findings are based on small cohorts and results are conflicting. It remains unclear whether raised concentrations of circulating complement reflect a general heightened inflammatory state in PE or are directly associated with placental complement-mediated injury. Little is known about the relative contribution of the complement system to the high rates of PE seen in women with CKD and women of SSA ethnicity.

This chapter will review the current evidence base for the role of complement in healthy pregnancy, as well as in adverse pregnancy outcomes including PE. Further assessment will be made of the potential role of the complement system in the pathogenesis of PE in high-risk groups (including women with CKD, and of SSA ethnicity).

The research within this thesis will, for the first time, examine samples of placental tissue, maternal blood, and umbilical cord blood from a cohort of previously healthy women to assess which complement components are associated with PE, and to explore how placental complement deposition might be related to increased levels of circulating complement in the fluid phase. This is the first study to simultaneously examine for evidence of complement activation in placental tissue *and* maternal and fetal circulation. The results would potentially support a role for complement activation in the pathogenesis of pre-eclampsia, rather than complement being present secondary to systemic inflammation.

Further analysis of longitudinally collected blood samples from pregnant women with CKD (some of whom developed PE) will enable a unique assessment of the changes in complement markers during pregnancy in this high-risk group. Finally, maternal blood samples will be analysed from a cohort of pregnant Ghanaian women, to provide a novel review of complement biomarkers in PE and healthy pregnancy in women of SSA ethnicity.

Together, the research findings will enrich the evidence base for the role of complement in the pathogenesis of PE in previously healthy women and in high-risk groups. This has potentially important clinical implications. Complement proteins may have a role as novel biomarkers for PE and help to identify pregnancies at risk. Furthermore, complement-modifying medications are undergoing rapid development, and many are currently being trialled for treating a variety of complement-mediated diseases, although there is not yet enough evidence for their use in PE. If a role for complement in the pathogenesis of PE can be firmly established, complement-modifying agents could provide a vital means of treating PE and improve global health outcomes for mothers and babies.

1.2 The complement system

The complement system is comprised of a series of proteins found widely within the circulation and on cell surfaces. It forms an essential part of the innate immune system through defence against bacterial infection and elimination of immune complexes, inflammatory waste products, and apoptotic and necrotic cells (1, 2).

Complement proteins can become activated through one of three pathways: the classical pathway, alternative pathway, and lectin pathway (see ***Figure 1.1***). The classical pathway is activated following antigen-antibody binding and immune complex formation (3). The C1q molecule can be activated by IgG or IgM immune complexes, or by apoptotic and necrotic cells, to cleave C4 and C2, resulting in the formation of C3 convertase (2).

Similarly, the lectin pathway is initiated in response to an exogenous stimulus. In this instance, mannose-binding lectins (MBLs) and ficolins bind to distinct carbohydrate or glycoprotein molecules on a pathogen's surface, activating MBL-associated serine proteases (MASPs). These in turn cleave C4 and C2, leading to the formation of C3 convertase (2).

In contrast, the alternative pathway operates constantly at a low-level steady state ('tickover') in the fluid phase from spontaneous hydrolysis of C3. Factor B is able to bind to hydrolysed C3 in the fluid phase. Interaction of this compound with Factor D then cleaves Factor B into activation fragments Ba and Bb, resulting in the formation of the alternative pathway C3 convertase ($C3(H_2O)Bb$). This subsequently cleaves C3 into C3a and C3b (2).

Amplification of the alternative pathway can then occur by C3b binding to factor B on foreign cell surface components including bacteria and viruses to form an amplification loop C3 convertase (C3bBb) (1). This enzyme is stabilised by properdin, which significantly prolongs its half-life (2).

The activation of each complement pathway results in the formation of a C3 convertase. The binding of further C3b leads to C5 convertase formation. All pathways thus converge to the final common

pathway: C5 convertases cleave C5 into C5a and C5b, resulting in subsequent formation of the membrane attack complex (MAC), C5b-9. C3a and C5a are potent anaphylatoxins; activating leucocytes and enhancing inflammatory responses. C3b is crucial in opsonisation, by forming covalent bonds with foreign pathogens or immune complexes, effectively marking them for phagocytosis. The MAC forms a pore in the plasma membrane, leading to lysis of invading pathogens and damaged cells (1, 2).

Meticulous regulation of the complement system is essential in order to prevent inappropriate activation and injury to the host, whilst at the same time responding appropriately to clear foreign pathogens or damaged cells. C3b is constantly deposited on cell surfaces that come into contact with plasma (4). Without regulation, amplification of C3b deposition occurs, followed by further downstream production of anaphylatoxins C3a and C5a, ultimately leading to cell destruction. In humans, a number of complement regulatory proteins protect host cells from excessive complement activation. C1 inhibitor acts as a regulator of the initial steps in both the classical and lectin pathways through inhibiting C1r, C1s, and MASPs. In the alternative pathway, Factors H and I and properdin are required for regulation. Inappropriate alternative pathway activation occurs where one of these factors is absent or non-functioning, causing overconsumption of downstream complement components and relative complement deficiency. Membrane Cofactor Protein (MCP; encoded by CD46) binds to C3b and interacts with factor I, to produce the inactivated iC3b, thus preventing further downstream complement activation and damage to host cells. Other important complement regulators include Decay Accelerating Factor (DAF; CD55), which limits C3 convertase formation by competing with factor B, and CD59, which regulates MAC formation by inhibiting C9 (2).

Deficiency or dysregulation of complement proteins and/or their regulators has been linked to various disease processes, including IgA nephropathy (5), C3 glomerulopathy (6), atypical haemolytic uraemic syndrome (aHUS) (7), systemic lupus erythematosus (SLE) (3) and overwhelming bacterial infection; particularly from Neisseria species (1). Furthermore, an emerging body of evidence has

shown a potential link between complement dysregulation and adverse outcomes in pregnancy, including PE (8-10). The research within this thesis will explore this association in greater detail.

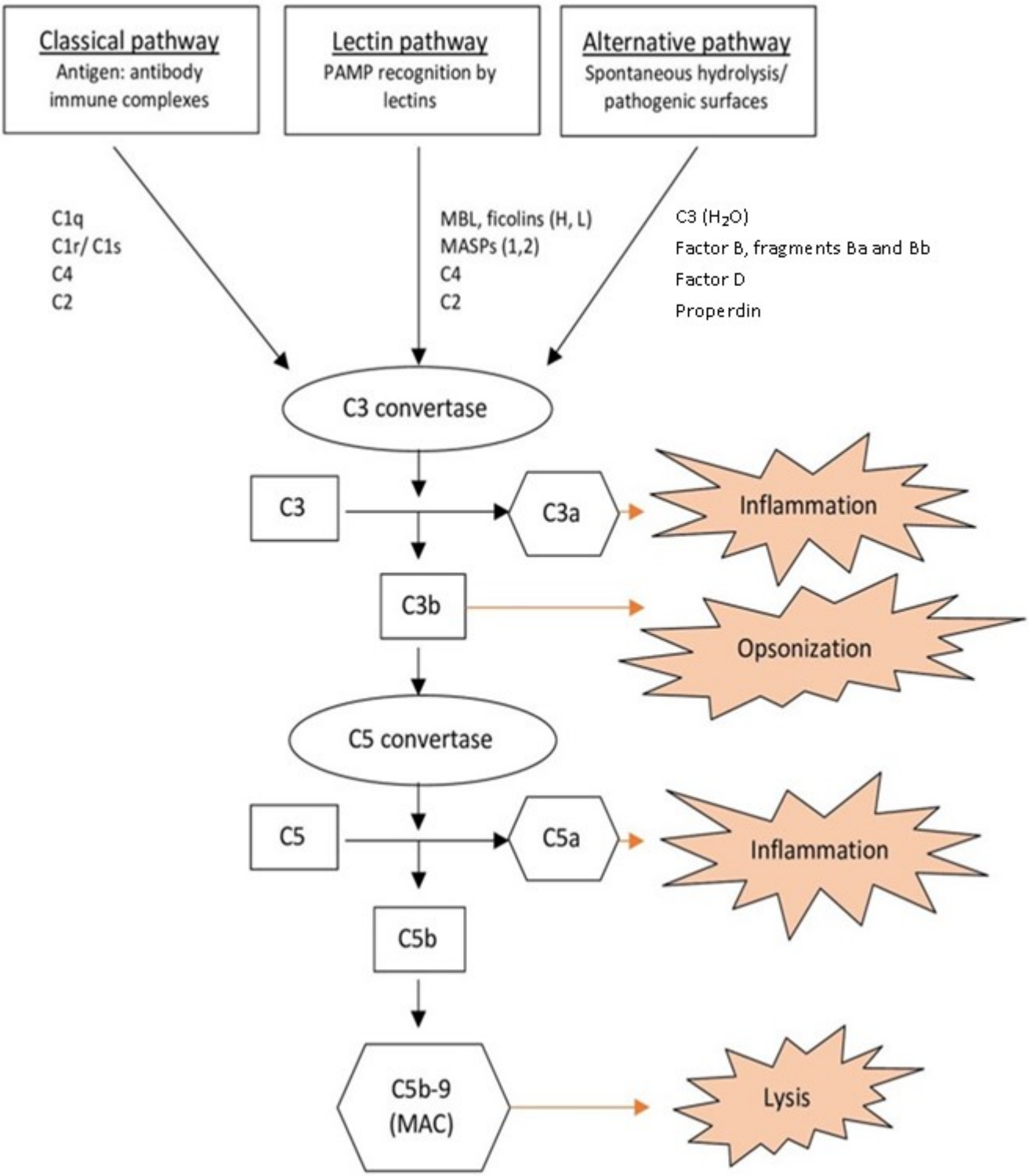


Figure 1.1: Complement activation pathways

Reproduced and adapted from (11). Published by Frontiers Media (open access).

PAMP: Pathogen-associated Molecular Pattern, MBL: Mannose-binding Lectin, MASPs: MBL-associated Serine Proteases, MAC: Membrane Attack Complex.

1.3 The placenta: structure and function

The human placenta provides a unique interface between fetal and maternal circulations, allowing nutritional exchange, protection from infection, and secretion of hormones involved in regulating pregnancy, metabolism and birth. However, the placenta and developing fetus express foreign antigens and therefore represent potential targets for the maternal immune system (4). Precise control is required to prevent cellular injury and adverse pregnancy outcomes. Before examining how the complement system is implicated in both normal and adverse pregnancy outcomes, it is first important to understand the structure and function of the placenta in healthy pregnancy.

The placenta is comprised of maternal tissue (arising from the endometrium; termed the decidua basalis), and fetal tissue (arising from the chorionic sac; sometimes termed the 'chorion'). These layers are separated by the intervillous space, which acts as the interface between maternal and fetal circulation, and is where the exchange of nutrients and gases takes place (12, 13). This is shown in greater detail in ***Figure 1.2***.

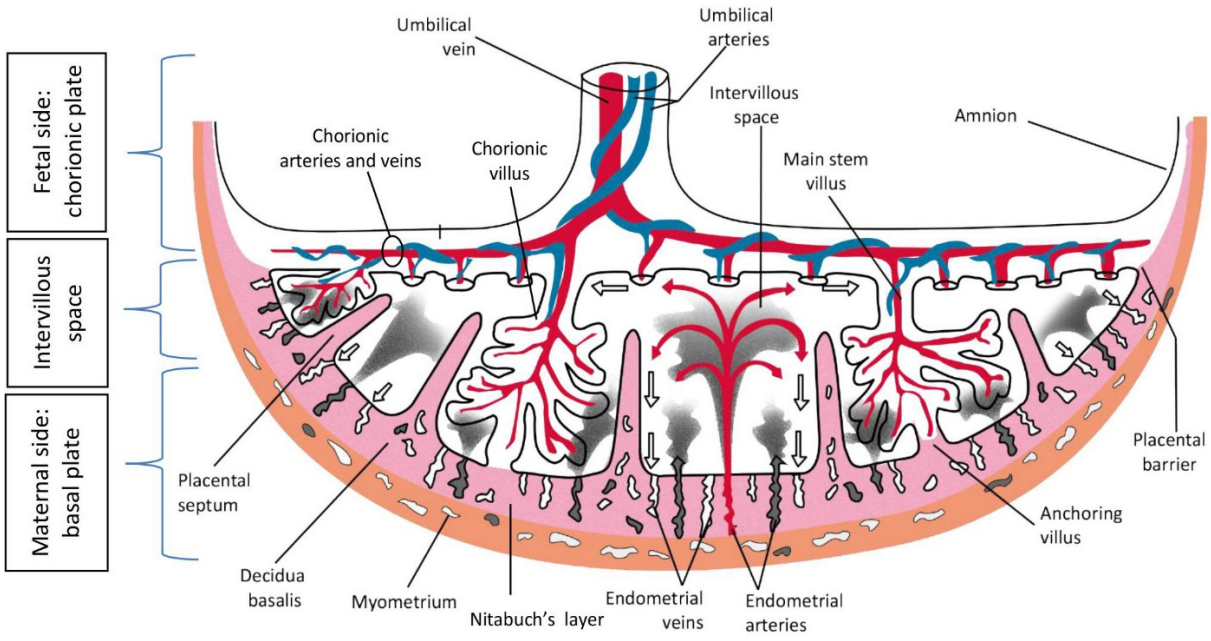


Figure 1.2: Structure of human placenta

Reproduced from (14). Published by Blackwell Munksgaard (open access).

Placental development is initiated following successful implantation of the blastocyst. Fetal trophoblast cells thereafter undergo rapid proliferation. Some differentiate into extravillous cells that invade the maternal decidua and contribute to uterine spiral artery remodelling (see ***Figure 1.3***). The resultant dilated, compliant blood vessels enhance the blood supply to the placenta. Other trophoblast cells differentiate to form syncytiotrophoblast (STB) cells, which form the outer epithelial cell layer of the chorionic villi (12). The chorionic villi protrude into the intervillous space, where they are bathed in maternal blood, allowing transfer of oxygen and nutrients from the maternal to fetal circulation, and reciprocal removal of waste products.

The human placenta is haemochorial in structure, meaning that fetal blood (contained within the chorionic villi) comes into almost direct contact with maternal blood in the intervillous space, separated by only a few layers of placental membrane. Meticulous regulation of the maternal immune system is therefore of crucial importance to prevent a harmful immune response to the semi-allogeneic fetus, while at the same protecting mother and fetus from infection (4).

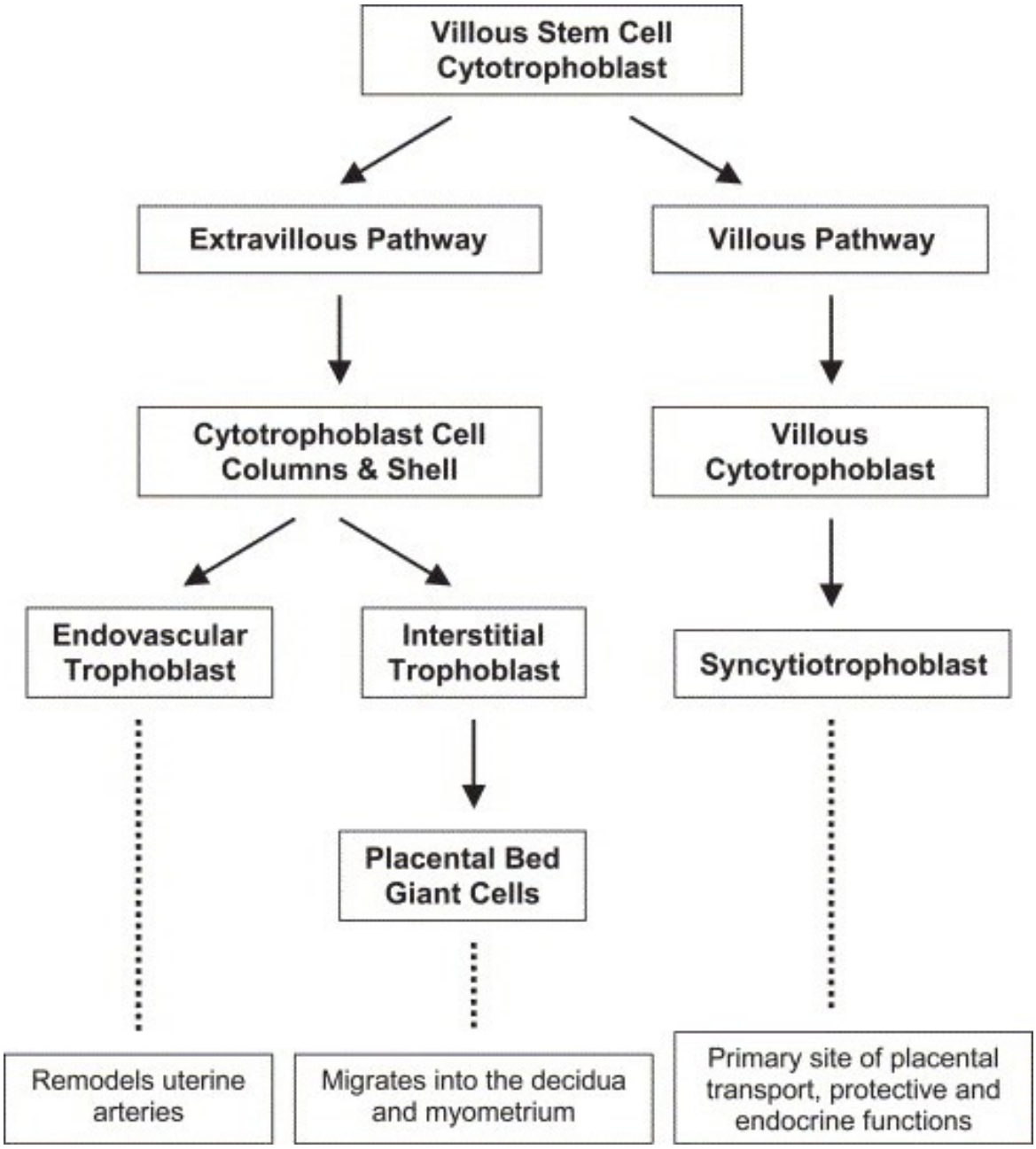


Figure 1.3: Schematic of fetal trophoblast cell differentiation

Reproduced from (12) with permission from Elsevier Ltd.

1.4 The role of complement in healthy pregnancy

1.4.1 Introduction: the role of complement in healthy pregnancy

The developing fetus expresses paternal as well as maternal antigens and can therefore be regarded as semi-allogeneic. Careful regulation of the maternal innate immune system, including complement activation pathways, is necessary to prevent adverse pregnancy outcomes and harm to the fetus (4).

Some degree of complement activation occurs in normal pregnancy; with a role in enhancing placental development and protecting mother and baby from pathogens. However, evidence from animal and human studies has shown that defects in complement pathways (leading either to complement deficiencies or excessive complement activation) have been associated with adverse pregnancy outcomes (15-18).

It is important to first consider the role of the complement system in normal healthy pregnancy before examining pathogenic conditions, including PE, where this system may be disrupted. This may in turn enable the identification of biomarkers of disease and targets for potential therapeutic intervention. The role of complement in healthy pregnancy is discussed in further detail below.

1.4.2 Complement and healthy placental development

From the onset of pregnancy, placental tissue undergoes rapid development and continuous remodelling. Extensive cell debris is produced, causing activation of the complement cascade. The developing placenta is therefore inevitably exposed to complement activation, which must be carefully regulated in order to prevent damage to the semi-allogeneic fetus at a site where the maternal and fetal circulation are in close contact (4, 19, 20).

1.4.2.1 The role of complement in placental development: animal and in vitro human studies

A number of animal studies and in vitro studies of human pregnancy have sought to investigate the role of complement in normal placental development.

During the early stages of healthy placental development, fetal trophoblast cells perform a crucial role in invading the maternal decidual tissue and endometrial vasculature to allow successful implantation. Murine studies have demonstrated the contribution of the complement system to this process. Albieri and colleagues showed, in an in vitro study of mouse trophoblast cells, that phagocytosis of erythrocyte cells was enhanced in the presence of activated C3 (21). Furthermore, in C3-deficient plasma, phagocytic activity of trophoblast cells was very low. These findings suggest that C3 plays a role in trophoblast invasion (phagocytosis) of the maternal decidua in healthy placental development.

Following implantation, differentiated endovascular trophoblast cells migrate to the endometrial spiral arteries and adhere to decidual endothelial cells to bring about their remodelling (see ***Figure 1.3***). The resulting compliant blood vessels which are non-responsive to vasoconstriction ensure a continuous blood supply to the placenta. There is a significant body of evidence highlighting the importance of the complement component C1q in this step of healthy placental formation (4). Studies of human placental tissue in early pregnancy have demonstrated that decidual endothelial cells express C1q, and C1q is found at contact sites between endovascular trophoblast cells and decidual endothelial cells (22). However, no evidence of immunoglobulins or C4 were found to co-localise with C1q, suggesting that complement activation was not responsible for this finding. This is in contrast to other endothelial cells (such as blood vessels of skin and brain), which only express C1q in pathological conditions, in the presence of complement activation. In vitro studies of human placenta have demonstrated that C1q may act as a 'molecular bridge', allowing adhesion between endovascular trophoblasts and decidual endothelial cells (22), thus promoting spiral artery remodelling and healthy placentation.

Agostinis et al. performed histopathological analysis of placental tissue from C1q knockout mice and found evidence of reduced trophoblast invasion and vascular remodelling, and deficient matrix development, as compared to wild type controls (23). Furthermore, C1q-deficient mice had smaller

litter sizes and an increased incidence of fetal death (23, 24). These findings help illustrate why acquired or inherited deficiencies of complement present an increased risk of adverse pregnancy outcomes (see ***Section 1.5.2***).

1.4.2.2 Placental complement regulators in healthy pregnancy

The haemochorial structure of the human placenta necessitates precise regulation of the complement system in order to prevent excessive complement activation at the sites where maternal and fetal circulation are in close contact. Several studies have examined for the presence of membrane-bound complement regulatory proteins MCP, DAF and CD59, typically in early placental tissue following elective termination of pregnancy, or in healthy placenta taken following delivery at term. These studies consistently report evidence of all three complement regulators at the STB surface (25-27) during all three trimesters of normal pregnancy. Furthermore, the presence of MCP, DAF and CD59 has variably been reported on villous and extravillous cytotrophoblast cells, depending on the stage of pregnancy (26, 27). Overall, DAF appeared to stain less strongly within placental tissue than MCP and CD59 (25, 26). Further evidence of the presence of complement inhibitors within healthy term placenta has been reported by Lokki et al, who found classical pathway inhibitor C4 binding protein (C4BP) deposited on the apical syncytium, and alternative pathway inhibitor Factor H deposited on the STB membrane (25).

It is interesting to note that complement regulatory proteins are most prevalent within the most superficial layers of the placenta. STB cells and extravillous trophoblasts that invade the maternal decidua form the closest contact points between maternal and fetal circulations, so it would follow that they require the greatest degree of protection from excessive maternal complement activation in response to trophoblast cells which bear paternal antigens.

1.4.2.3 Placental complement deposition and secretion in healthy pregnancy

Several studies have employed immunohistochemical and immunofluorescent analysis of healthy term placenta, demonstrating deposition of components from early and late in the complement cascade within placental tissue (8, 19, 20).

Among the earliest studies of placental complement deposition, Faulk et al. reported positive staining for C3d and C9 in the trophoblast basement membrane (28), and Wells et al. reported expression of C3d and C9 in decidual spiral arteries of healthy placental tissue (29). C1q, C4 and C6 were also deposited, although the staining was less intense. Furthermore, Tedesco et al. demonstrated MAC deposition in the decidual basal plate and chorionic villi stroma in term placentae (30). The findings from these early studies are corroborated by more recent reports of positive staining for C3d (25, 31) and C9 (25), deposited at the basal membrane of the STB in healthy placental tissue.

Although the majority of complement synthesis takes place in the liver, other tissues, including the placenta, are able to synthesise complement too. This may enhance the immune response locally at a tissue level, in order to improve immune complex clearance, and elimination of invading pathogens and other cell debris (20). Bulla et al. demonstrated that human trophoblast cells (taken following elective termination of pregnancy between 8 and 12 weeks' gestation) secrete C3 and C4 (20). The authors postulate that this enhances host defences against infection and inflammation by promoting phagocytosis of pathogens. The same research laboratory also demonstrated that decidual endothelial cells are able to secrete C1q, unlike endothelial cells derived from other organs (22). Furthermore, Goldberg et al. reported that chorion-derived cells were able to secrete a number of components from the classical and alternative complement pathways, including factor B, C3, C1r, C1s, C1 inhibitor, factor H, C4 and C2 (32). This study investigated placental tissue taken at full term, from both vaginal and elective Caesarean deliveries. There were no significant differences in concentrations of complement components found at 4 hours, or after being cultured overnight, and

components were still expressed after 5 days in culture. These findings supported the view that expression of complement was not solely a result of inflammatory stimulation from giving birth.

Collectively, these findings provide evidence of complement activation at the maternal-fetal interface within placental tissue in physiological pregnancy. It is important to understand these patterns of complement activity in healthy pregnancy so that comparisons can be drawn with pathological processes, including PE.

1.4.3 Circulating complement in healthy pregnancy

A number of studies have sought to characterise the profile of circulating complement components and their activation products during healthy pregnancy, as compared to the non-pregnant state. The results are summarised in ***Table 1.1*** by complement component tested and stage of gestation.

In a cross-sectional study comparing 134 pregnant women between 20 weeks' gestation and full term with 40 non-pregnant women, Richani et al. reported significantly higher levels of anaphylatoxins C3a (median 2364.7 ng/ml versus 1340.4 ng/ml), C4a (median 10125.4 ng/ml versus 2625.4 ng/ml), and C5a (median 12.4 ng/ml versus 4.1 ng/ml) in the pregnant group versus the non-pregnant group, respectively; all $p<0.001$ (33).

Another cross-sectional study found that circulating concentrations of C4d, C3a, C5b-9, C3, C9 and factor H antigen were significantly higher in blood drawn from pregnant women at 36-37 weeks' gestation, compared to non-pregnant women (34). This study also reported significantly reduced levels of C1 inhibitor in healthy pregnant women, when compared to non-pregnant controls (34). The authors argue that their data demonstrate increased classical and/or lectin pathway activity in normal pregnancy, leading to increased terminal pathway activation. They postulate that the classical and/or lectin complement pathways become activated during normal pregnancy as a result of cellular debris and apoptosis of trophoblast cells during placental growth and remodelling.

It has been shown that concentrations of complement components change throughout gestation, although the evidence base is still developing. An historic study by Baines et al. reported that C3 levels decreased in the first trimester, and then gradually increased throughout pregnancy thereafter (35). A more recent prospective study by He et al. sought to define gestational complement changes in greater detail, analysing peripheral blood samples taken longitudinally throughout pregnancy (36). This study found that C3 levels in early pregnancy were comparable to non-pregnant women but began to rise from the second trimester onwards, in keeping with the earlier findings from Baines et al. (35). In contrast, C4 levels were elevated from early pregnancy, and increased gradually throughout pregnancy thereafter. This is consistent with earlier in vitro research findings (discussed in ***Section 1.4.2.3***), which demonstrated trophoblast cells being able to secrete C4, which may play an important role in early placental development (20). Furthermore, He et al. reported that levels of factors B and H rise from early pregnancy until the end of the second trimester, plateauing from 28 weeks' gestation. Concentrations of C1q, C5a and C5b-9 did not change significantly during pregnancy (36). High levels of factor B indicate alternative complement pathway activity, even from early in normal pregnancy. However, the authors postulate that the concomitant high concentration of factor H prevents excessive alternative pathway activation, and therefore levels of late complement components (such as C5b-9) are relatively unchanged. Whilst this study provided the first detailed longitudinal insight into gestational complement changes, it is important to note that less than one-third of subjects had more than three blood samples drawn during pregnancy.

Another recent longitudinal study compared circulating complement concentrations in 100 healthy pregnant women with normal laboratory reference ranges (37). This found that C1q levels peaked in the first trimester then levelled off later in pregnancy. Conversely, concentrations of C3 and C4 rose throughout pregnancy, to peak in the third trimester; in keeping with findings from other cohorts (36). Of the complement components tested, none of the mean concentrations fell outside the normal laboratory reference ranges. Despite this, however, the 95th percentile concentrations for C3

in trimester 2 and at term were above the upper limit reference range, as well as the 95th percentile C4 concentrations at term in the pregnancy group. This study also only measured one blood sample from each trimester of pregnancy. Almost all of the cohort tested were of Caucasian ethnic origin, so the results may not be generalisable to the wider population.

In conclusion, the evidence presented above suggests that complement concentrations differ between healthy pregnancy and non-pregnant individuals, with pregnancy itself being a 'complement-active' state. Patterns in complement activation and deposition appear to differ according to gestational age. Adaptive immunity is often compromised even in healthy pregnancy, so increased complement activation during pregnancy may counterbalance this effect and help protect mother and fetus from external pathogens (33).

Table 1.1: Complement component changes by trimester in healthy pregnancy, compared to non-pregnant groups

Complement pathway	Complement component	Trimester 1	Trimester 2	Trimester 3
Classical	C1q	No difference: (36): in pregnant women (n=362) in 1st trimester, vs. non-pregnant controls (n=65) (37): in pregnant women (n=100) in 1st trimester, vs. normal lab reference ranges. C1q highest in 1st trimester then levels off.	No difference: (36): in pregnant women (n=362) in 2nd trimester vs. non-pregnant controls (n=65) (37): in pregnant women (n=100) in 2nd trimester, vs. normal lab reference ranges.	No difference: (36): in pregnant women (n=362) in 3rd trimester, vs. non-pregnant controls (n=65). Static levels through pregnancy (37): in pregnant women (n=100) in 3rd trimester, vs. normal lab reference ranges.
Classical / lectin	C4	Elevated levels: (36): in pregnant women (n=362) in 1st trimester, vs. non-pregnant controls (n=65) No difference: (37): in pregnant women (n=100) in 1st trimester, vs. normal lab reference ranges.	Elevated levels: (36): in pregnant women (n=362), vs. non-pregnant controls (n=65). C4 levels rise during pregnancy No difference: (37): in pregnant women (n=100) in 2nd trimester, vs. normal lab reference ranges.	Elevated levels: (36): in pregnant women (n=362), vs. non-pregnant controls (n=65). C4 levels rise during pregnancy No difference: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59) (37): in pregnant women (n=100) in 1st trimester, vs. normal lab reference ranges. C4 levels rise during pregnancy
	C4d			Elevated levels: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59)
	C1 inhibitor			Reduced levels: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59)
Lectin	MBL	Elevated levels: (36): in pregnant women (n=362) in 1st trimester, vs. non-pregnant controls (n=65)	Elevated levels: (36): in pregnant women (n=362) in 2nd trimester, vs. non-pregnant controls (n=65)	Elevated levels: (36): in pregnant women (n=362) in 3rd trimester, vs. non-pregnant controls (n=65). Static levels through pregnancy
Alternative	C3	Reduced levels: (35): C3 levels fall in 1st trimester normal pregnancy (longitudinal study; n=478 pregnant women) No difference: (36): in pregnant women (n=362) at 6-13 weeks gestation, vs. non-pregnant controls (n=65) (37): in pregnant women (n=100) in 1st trimester, vs. normal lab reference ranges.	Elevated levels: (35): C3 gradually rises from 2nd trimester onwards (longitudinal study: n=478 pregnant women) (36): in pregnant women (n=362), vs. non-pregnant controls (n=65). C3 rises from 2nd trimester onwards No difference: (37): in pregnant women (n=100) in 2nd trimester, vs. normal lab reference ranges.	Elevated levels: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59) (36): in pregnant women (n=362), vs. non-pregnant controls (n=65). Levels rise throughout pregnancy No difference: (37): in pregnant women (n=100) in 1st trimester, vs. normal lab reference ranges. C3 levels rise during pregnancy
	FH	Elevated levels: (36): in pregnant women (n=362) in 1st trimester, vs. non-pregnant controls (n=65)	Elevated levels: (36): FH levels rise during 2nd trimester in pregnant women (n=362)	Elevated levels: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59)

				(36): FH levels remain high in pregnant women (n=362), vs. non-pregnant controls (n=65), but level off between 2nd and 3rd trimester
	FB	Elevated levels: (36): in pregnant women (n=362) in 1st trimester, vs. non-pregnant controls (n=65)	Elevated levels: (36): FB levels rise during 2nd trimester in pregnant women (n=362)	Elevated levels: (36): FB levels remain high in pregnant women (n=362), vs. non-pregnant controls (n=65), but level off between 2nd and 3rd trimester
	Bb			No difference: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59)
Anaphylatoxins	C3a	No difference: (36): in pregnant women (n=362), in 1st trimester vs. non-pregnant controls (n=65).		Elevated levels: (33): in pregnant women (n=134) median gestation 35.5 weeks, vs. non-pregnant controls (n=40) (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59)
	C5a	No difference: (36): in pregnant women (n=362), in 1st trimester vs. non-pregnant controls (n=65).	No difference: (36): in pregnant women (n=362), in 2nd trimester vs. non-pregnant controls (n=65).	Elevated levels: (33): in pregnant women (n=134) vs. non-pregnant controls (n=40), median gestation 35.5 weeks No difference: (36): in pregnant women (n=362), in 3rd trimester vs. non-pregnant controls (n=65). Static levels during pregnancy
Terminal	C5b-9	No difference: (36): in pregnant women (n=362), in 1st trimester vs. non-pregnant controls (n=65).	No difference: (36): in pregnant women (n=362), in 2nd trimester vs. non-pregnant controls (n=65).	Elevated levels: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59) No difference: (36): in pregnant women (n=362), in 3rd trimester vs. non-pregnant controls (n=65). Static levels during pregnancy
	C9			Elevated levels: (34): in pregnant women (n=60) median gestation 36 weeks, vs. non-pregnant controls (n=59)

MBL: mannose-binding lectin; FH: complement factor H; FB: complement factor B. Numbers in tables relate to reference list / publications from where data was derived.

1.4.4 Complement and the fetal circulation

Relatively little is known about complement activation within the fetal circulation during healthy pregnancy. Earlier studies have instead examined circulating complement markers in preterm infants and compared them with infants born at term. One such study reported very low concentrations of C1q, C2-C9, factor B and properdin in infants born between 28-33 weeks, with higher levels in infants born between 34-36 weeks, and higher again in those born at 37-42 weeks (38). Similarly, another study reported increasing neonatal blood concentrations of complement factors H and I, as well as increasing classical pathway (CH50) activity, as gestational age increased (39). Interestingly, neonatal complement concentrations were not correlated with other birth outcomes, such as birth weight. There was also no reported association with mode of delivery (vaginal versus Caesarean), or with gender (38). In all cases, neonatal complement concentrations were significantly lower than adult reference ranges (38, 39).

These findings have been replicated in a recent study, showing reduced umbilical cord blood C1q, C3 and C4 concentrations compared to maternal blood complement concentrations in healthy pregnancies (37). Globally depressed umbilical cord blood complement concentrations are indicative of the immaturity of the neonatal complement system. This presents a significant risk of infection; particularly to those babies born more prematurely.

1.5 Complement and adverse pregnancy outcomes

The evidence presented in ***Section 1.4*** highlights the pivotal role of a well-functioning and precisely regulated complement system in physiological pregnancy. However, there is a growing body of evidence linking complement deficiency, or inappropriate stimulation and activity, to disease and adverse pregnancy outcomes.

1.5.1 Animal studies of complement genetics and pregnancy

In a landmark study investigating the importance of complement regulation in pregnancy, Xu et al. developed a mouse model of Crry (complement receptor 1-related gene/protein y) knockout (40). The Crry gene has a similar mode of action in mice to the human membrane-bound complement regulator proteins MCP and DAF, limiting excessive complement activation (4). Xu et al. demonstrated that when mice with heterozygous Crry deficiency (Crry +/-) were mated, no Crry -/- offspring were born (from 245 births). They hence concluded that the absence of complement regulatory genes was lethal to embryos. Histological analysis found that the placental tissue of Crry -/- mice demonstrated defective formation of vasculature (41).

Further studies demonstrated that when Crry +/- mice that were also deficient in either C3 (C3-/-) (40), factor B (fB-/-) (41), or properdin (P-/-) were mated, pregnancies were rescued and an expected proportion of Crry -/- live births were seen (42). This is presumably because the absence of C3, factor B, or properdin resulted in limited alternative complement pathway activity. In contrast, deficiency of C4 or C5 did not result in rescue of pregnancy (40). These results imply that uncontrolled alternative complement pathway activation is responsible for embryo lethality in the absence of complement regulatory genes. Inhibition of excessive alternative complement pathway activation (via complement regulatory proteins) may therefore be crucial in facilitating successful pregnancy outcomes.

1.5.2 Complement-mediated diseases and adverse pregnancy outcomes

A number of acquired and inherited complement-mediated diseases have observed associations with adverse pregnancy outcomes. Research into these diseases has strengthened the evidence base for the role of complement in pregnancy pathologies, and its relationship with disease states including PE.

1.5.2.1 Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS)

SLE is a multi-system autoimmune disease, typically presenting in females of childbearing age. Formation of immune complexes cause complement activation, predominantly via the classical pathway. Low levels of complements C3 and C4 are typically seen during disease flares; indicative of complement consumption. Systematic reviews have consistently reported adverse pregnancy outcomes in the setting of lupus, including PE, intra-uterine growth restriction (IUGR), and premature birth (43-45). The greatest risks are conferred in women with active or flaring disease (44).

APS occurs in a proportion of patients with SLE but may also arise independently ('primary APS'). It is characterised by arterial or venous thrombosis, and in the context of pregnancy is associated with recurrent miscarriage, unexplained fetal death after 10 weeks' gestation, and preterm birth (PTB) (46, 47).

Girardi et al. developed a mouse model which demonstrated that complement activation is implicated in fetal tissue injury and adverse pregnancy outcomes in the setting of APS (48). Mice injected with human IgG containing antiphospholipid antibodies demonstrated high rates of embryo resorption, fetal growth restriction, and evidence of placental tissue necrosis with significant C3 deposition. Interestingly, mice that were deficient in C5, or that were treated with an antagonist of C5a receptor, were protected from fetal loss or growth restriction. Similarly, C4 deficient mice injected with antiphospholipid antibodies were also protected from adverse pregnancy outcomes (48). The authors postulate that antiphospholipid antibodies initiate classical complement pathway

activation, leading to placental tissue damage via C3 deposition. The subsequent production of C5 and C5a appears to cause further amplification of complement activation via the alternative pathway (19, 48).

Studies of human placental tissue in patients with SLE and APS have reinforced the evidence base. Placental tissue from patients with SLE has been reported to show significantly greater levels of C4d deposition when compared to healthy control pregnancies (49). Increased deposition of C4d (50) and C5b-9 (46) has been reported in the placental tissue of APS patients. The intensity of C4d deposition was significantly correlated with the presence of pathological placental lesions ($p<0.001$) (50).

The prospective, multi-centre 'PROMISSE' study sought to examine adverse pregnancy outcomes in patients with SLE and APS in further detail. Nearly 1 in 5 of those women who went on to develop PE were found to have mutations in genes encoding complement regulatory proteins CD46, complement factor I or factor H (n=7) (18). This study identified variants of CD46 and factor I that have previously been linked to aHUS, and a novel CD46 mutation that was linked to defective C4b binding (18). Of the 7 cases of PE, 3 were complicated by very preterm delivery (prior to 34 weeks gestation), and 3 women had PE with severe features. It is notable, however, that in over 80% of PE cases within the PROMISSE cohort, no complement risk variant was identified. The majority of women with autoimmune disease and PE in this cohort therefore did not have a clearly defined complement-mediated risk.

Analysis of maternal blood samples taken longitudinally throughout pregnancy from the same 'PROMISSE' cohort found that at 12-15 weeks' gestation, levels of Bb and C5b-9 were significantly higher in women who went on to have an adverse pregnancy outcome (including fetal death, preterm delivery and fetal growth restriction) as compared to women with SLE and APS without an adverse outcome. After controlling for confounding factors, a significant association remained between levels of Bb and C5b-9 at 12-15 weeks, and adverse pregnancy outcome (OR 1.41, 95% CI

1.06-1.89, p=0.019 for each standard deviation increase in Bb, and OR 1.37, 95% CI 1.05-1.80, p=0.022 for C5b-9) (45).

1.5.2.2 Paroxysmal nocturnal haemoglobinuria (PNH)

PNH is caused by an acquired mutation of the gene encoding the production of glycosylphosphatidylinositol (GPI). GPI anchors the complement regulators DAF and CD59 in erythrocytes, protecting them from complement-mediated damage. Where this process is defective, the clinical syndrome of PNH results; typically featuring complement-mediated haemolysis and thrombosis. Historically, pregnancy outcomes in women with PNH were extremely poor, and pregnancy was generally discouraged among this patient cohort (51, 52).

The advent of the novel anti-C5 monoclonal antibody eculizumab has revolutionised the treatment of PNH in more recent times, leading to reduced incidence of intravascular haemolysis (53), reduced dependence on blood transfusions, and improved overall survival (54).

There is encouraging evidence for the successful use of eculizumab during pregnancy, with improved pregnancy outcomes for patients with PNH. In a retrospective multicentre analysis of 75 pregnancies in 61 women with PNH, there were 69 recorded live births, 6 first trimester miscarriages and 3 stillbirths (55). There were no reported cases of maternal death. One-third of births occurred preterm (< 37 weeks), but all offspring achieved normal developmental milestones at follow up. This study may have been vulnerable to selection bias however, as it was administered in survey format to clinical centres and relied on individual reporting of relevant pregnancies. The dose and/or frequency of eculizumab had to be escalated in 54% of pregnancies; reinforcing the view that pregnancy itself is a 'complement-active' state. Interestingly, rates of invasive meningococcal disease are significantly reduced in pregnancy when compared to non-pregnant women of childbearing age, which would further support this view (56).

1.5.2.3 Atypical Haemolytic Uraemic Syndrome (aHUS)

aHUS is a rare thrombotic microangiopathy, characterised by microangiopathic haemolytic anaemia, thrombocytopenia, and acute kidney injury (AKI). Mutations in genes encoding complement factors H and I, MCP, factor B and C3 have all been linked to aHUS (7). It is believed that uncontrolled alternative complement pathway activity results, leading to widespread endothelial dysfunction (57). This process may be triggered by various stimuli, including pregnancy.

Up to 20% of aHUS cases are diagnosed during pregnancy or in the postpartum period (58). Diagnosis of pregnancy-associated aHUS is challenging to differentiate from HELLP syndrome (Haemolysis, Elevated Liver enzymes and Low Platelets), which has a similar phenotype, although tends to occur in tandem with PE (8, 59). HELLP syndrome is discussed further in ***Section 1.6.1.1.***

Complement mutations have been reported in a large proportion of cases of pregnancy-associated aHUS; particularly in genes encoding complement factors H and I (57, 60). Pregnancy complications are common in this patient cohort, including gestational hypertension or PE in 57% of cases, reported in a recent systematic review (58).

Eculizumab was licensed as a treatment for aHUS in 2011, and appears to be effective in inducing remission (58). Furthermore, there were no reports of renal failure, dialysis or death in patients treated with eculizumab for a first episode of aHUS, as compared to an incidence of 24% in patients not treated with eculizumab (p=0.04) (58). One case report however did demonstrate placental transfer of eculizumab from a mother receiving treatment for aHUS to a newborn baby, with suppression of both the classical and alternative complement pathways in the neonatal circulation (61). The higher doses of eculizumab used in aHUS (as compared to PNH), together with the relatively later gestational age of the baby may have contributed to this finding.

1.5.2.4 Summary: complement-mediated diseases and adverse pregnancy outcomes

Collectively, these research findings highlight a relationship between complement abnormalities and adverse pregnancy outcomes, including PE. The evidence presented suggests a potential opportunity

for using complement components as biomarkers for adverse pregnancy outcomes in certain disease conditions. Furthermore, targeted inhibition of C5 using eculizumab appears to be safe during pregnancy and has been linked to improved obstetric outcomes (55, 58, 59). Existing data are however based on retrospective case series, and there remains no randomised controlled trial evidence for the use of eculizumab in pregnancy. Longer term outcomes of this medication and the potential effects of neonatal immunosuppression are not yet known (61).

1.5.3 Complement and miscarriage

Through mouse modelling, Girardi et al. demonstrated that complement activation is implicated in recurrent early pregnancy loss in the setting of APS (48) (discussed previously in ***Section 1.5.2.1***). Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS)However, complement abnormalities have also been linked to recurrent miscarriage in women without a known autoantibody-mediated disease. Between 20 and 30% of first trimester losses in women affected by recurrent miscarriage have been associated with hypocomplementaemia (low levels of C3 and Factor B; indicative of a consumptive process via excessive alternative pathway activity) (62).

Abnormalities of complement regulatory proteins have also been linked with miscarriage. A three-fold decrease in expression of MCP and DAF was reported in the placental tissue of women with spontaneous abortion, as compared to cases of elective abortion (63), together with significantly elevated plasma concentrations of C5a. Furthermore, a genetics study from a French cohort reported mutations in MCP and C4BP in women with recurrent unexplained miscarriage (64). These findings were contradicted, however, by a later study of Iranian women in whom no genotype differences in MCP were found between fertile women and those with recurrent miscarriage (65).

1.5.4 Complement and preterm birth (PTB)

Several studies have reported complement abnormalities in association with PTB (delivery prior to 37 weeks' gestation). Significantly increased concentrations of Bb have been reported in cases of PTB in early pregnancy maternal plasma samples (66, 67), and in third trimester maternal plasma (68), as compared to pregnancies ending in spontaneous labour at term. The differences persisted even in the absence of intra-amniotic infection. Increased early pregnancy concentrations of C3a have also been reported in cases of PTB (16).

Collectively these findings support a role for inappropriate complement activation in the pathogenesis of PTB, but also as potential biomarkers for predicting preterm labour.

1.5.5 Complement and the hypertensive disorders of pregnancy

1.5.5.1 Definitions and classification

Hypertensive disorders of pregnancy are common, affecting around 10% of women at some stage during their pregnancy (69, 70). Historically, they were classified according to the gestation of onset and presence or absence of significant proteinuria (urine protein to creatinine ratio (PCR) ≥30 mg/mmol, or albumin to creatinine ratio (ACR) ≥8 mg/mmol, or urine dip protein ≥2+) (70, 71). The diagnostic criteria for pre-eclampsia have recently been updated; in the absence of proteinuria, new onset hypertension after 20 weeks gestation and evidence of maternal end organ dysfunction or uteroplacental dysfunction is sufficient to make the diagnosis (72) (see ***Table 1.2***).

Table 1.2: Definition and severity classification of hypertensive disorders of pregnancy

Definition	Gestation at Presentation	Significant Proteinuria
Chronic hypertension	<20 weeks	No
Gestational hypertension	>20 weeks	No
Pre-eclampsia (PE)	>20 weeks	Yes*
PE superimposed on chronic hypertension	>20 weeks	Yes*

Hypertension severity	Systolic BP (mmHg)	Diastolic BP (mmHg)
Hypertension	140-159	90-109
Severe hypertension	>160	>110

*Adapted from (72). Significant proteinuria is defined as urine protein to creatinine ratio (PCR) ≥30 mg/mmol, or albumin to creatinine ratio (ACR) ≥8 mg/mmol, or urine dip protein ≥2+. *In the absence of significant proteinuria, new onset hypertension after 20 weeks' gestation and evidence of maternal end organ dysfunction (thrombocytopenia, haemolysis, renal or liver insufficiency, pulmonary oedema, cerebral symptoms) or uteroplacental dysfunction is sufficient to make the diagnosis.*

1.5.5.2 Epidemiology and health outcomes

In Western populations, trends towards increasing maternal age and body mass index (BMI) have led to a rising incidence of chronic hypertension in the pregnant population (73). This has potentially serious implications for the health outcomes of mother and baby.

A meta-analysis of almost 800,000 pregnancies in women with chronic hypertension reported strong associations with a variety of adverse pregnancy outcomes (73). When compared to a United States (US) national dataset of healthy pregnancies, women with chronic hypertension had a significantly higher incidence of superimposed PE (relative risk (RR) 7.7; 95% CI 5.7-10.1), Caesarean section (1.3; 1.1-1.5), delivery prior to 37 weeks' gestation (2.7; 1.9-3.6), low birth weight below 2500 g (2.7; 1.9-3.8) and perinatal death (4.2; 2.7-6.5) (73).

In the United Kingdom (UK), hypertensive disorders of pregnancy account for between 2 to 8% of maternal deaths each year (74, 75), and almost 40% of obstetric admissions to intensive care units (76). Although the proportion of deaths attributable to maternal hypertension has been decreasing

over recent decades in the UK, many of these deaths are preventable. Accurate monitoring with early detection and treatment of hypertension in pregnancy is therefore of paramount importance.

The body of research within this thesis will focus on the role of complement in the pathogenesis of PE; one of the major hypertensive disorders of pregnancy. This will be discussed in greater detail in the sections that follow.

1.6 Pre-eclampsia

1.6.1 Pre-eclampsia: definitions and classification

PE is a common multi-system disorder of pregnancy, characterised by the de novo onset of elevated blood pressure (BP) (> 140/90 mmHg) and significant proteinuria (urine protein to creatinine ratio (PCR) ≥ 30 mg/mmol, or albumin to creatinine ratio (ACR) ≥ 8 mg/mmol, or urine dip protein ≥ 2+) after 20 weeks' gestation. In the absence of proteinuria, evidence of maternal end-organ compromise, including uteroplacental dysfunction, in tandem with new onset hypertension is sufficient to make the diagnosis (72). Recent international consensus diagnostic criteria are detailed in ***Table 1.3***, and will be used throughout this thesis when defining or referring to PE.

PE is a heterogenous syndrome, with wide variations seen in its presentation. It can be classified according to the timing of onset during pregnancy (early PE < 34 weeks' gestation, late PE ≥ 34 weeks' gestation) (77), and the presence or absence of severe features (including severe hypertension ≥ 160/110 mmHg, acute renal or liver dysfunction, haematological complications, respiratory symptoms, or cerebral disturbance including seizures, severe headache, or visual scotomata) (70, 71, 78, 79).

1.6.1.1 HELLP Syndrome

Some women present atypically with features of HELLP syndrome which does not always fit the standard diagnostic criteria but is widely accepted as a severe form of PE (77). Many, but not all, of this subset of patients have evidence of hypertension and proteinuria. Interestingly, HELLP syndrome is phenotypically similar to pregnancy-associated aHUS (a complement-mediated thrombotic microangiopathy; see ***Section 1.5.2.3***), and it can be extremely challenging to distinguish between the two conditions (59, 80). In contrast to aHUS, HELLP syndrome tends to resolve quickly following delivery of baby and placenta (80).

Table 1.3: Pre-eclampsia diagnostic criteria

Blood pressure	• ≥ 140 mmHg systolic or ≥ 90 mmHg diastolic on two occasions at least 4 hours apart after 20 weeks gestation in a woman with previously normal blood pressure • ≥ 160 mmHg systolic or ≥ 110 mmHg diastolic (severe hypertension)
and	
Proteinuria	• Urine protein to creatinine ratio ≥ 30 mg/mmol, *or* • Urine albumin to creatinine ratio ≥ 8 mg/mmol, *or* • Dipstick reading of ≥ 2+ (used only if other quantitative methods not available)
In the absence of proteinuria, new-onset hypertension with the new onset of any of the following:	
Haematological complications	• Platelet count < 100 x 10^9/L • Haemolysis or disseminated intravascular coagulation
Renal insufficiency	• Creatinine ≥ 90 µmol/L or a doubling of the serum creatinine concentration in the absence of other renal disease
Impaired liver function	• Rise in liver transaminases (ALT or AST > 40 IU/L)
Respiratory symptoms	• Pulmonary oedema
Cerebral or visual symptoms	• Severe headache / altered mental status / visual scotomata • Clonus • Seizures (eclampsia)
Uteroplacental dysfunction	• Fetal growth restriction • Placental abruption • Abnormal umbilical artery Doppler waveform • Angiogenic imbalance (low PlGF, or increased sFLt-1:PlGF ratio) • Stillbirth

Table adapted from ISSHP 2021 guideline (72). ISSHP: International Society for the Study of Hypertension in Pregnancy. ALT: alanine aminotransferase; AST: aspartate aminotransferase; PlGF: placental growth factor; sFlt-1: soluble fms-like tyrosine kinase-1.

1.6.2 Epidemiology and health outcomes

PE is a major global obstetric problem and a leading cause of maternal and fetal morbidity and mortality, causing significant burden to healthcare services (81). PE affects up to 5% of all pregnancies (82), and is implicated in around 40,000 maternal deaths per year (83) - the vast majority of which occur in developing countries (84, 85). The large regional variations reflect in part the more advanced antenatal surveillance and treatment in the healthcare systems of more economically developed countries (74, 75).

In addition to the immediate clinical risk to pregnant women, PE can also pose significant long-term health implications (86). One large meta-analysis found that women previously diagnosed with PE have a substantially increased risk of developing cerebrovascular disease (odds ratio (OR) 1.77, 95% CI 1.43-2.21), and cardiovascular disease (OR 2.28, 95% CI 1.87-2.78) later in life (87). These findings are corroborated by another large meta-analysis which reported on vascular outcomes between 10-14 years after PE. The relative risks of stroke, ischaemic heart disease, and hypertension were 1.8, 2.1, and 3.7, respectively (88). Another potential sequela of PE is an increased lifetime risk of maternal CKD (79, 89, 90). One meta-analysis reported an up to 4-fold increased risk of microalbuminuria following PE at a mean of 7 years postpartum, with the risk increasing to 8-fold in cases of severe PE (91). Two large Scandinavian cohort studies have established links between PE and an increased future risk of end stage renal disease, with the relative risks higher in women with early-onset PE, or with PE in more than one pregnancy (92, 93).

Adverse fetal outcomes in pregnancies affected by PE are commonplace and have potentially devastating consequences. One in three cases of PE occurs before 35 weeks of pregnancy (94). Deteriorating maternal health and fetal growth restriction (resulting from defective placentation) often necessitate preterm delivery. In the UK, up to 10% of all premature births result from maternal hypertensive disease, and as many as 25% of preterm births in pre-eclamptic women are small for gestational age (birth weight below the tenth percentile) (95). Many will require admission to a neonatal unit (NNU). Beyond the neonatal period, premature birth also confers an elevated risk of long-term adverse health outcomes, such as cardiorespiratory disease and neurodevelopmental disability (86, 96).

1.6.3 Pathophysiology of pre-eclampsia

The pathophysiological mechanisms underpinning PE are complex and remain a subject of debate. Redman first proposed a two-stage model of PE (97), which has since been widely adopted by academic and clinical communities. Stage 1 PE comprises the ‘pre-clinical’ phase, characterised by

poor placental formation in early pregnancy. Stage 2 PE is the consequent 'clinical' phase during which the maternal clinical syndrome becomes evident (98). See ***Figure 1.4***.

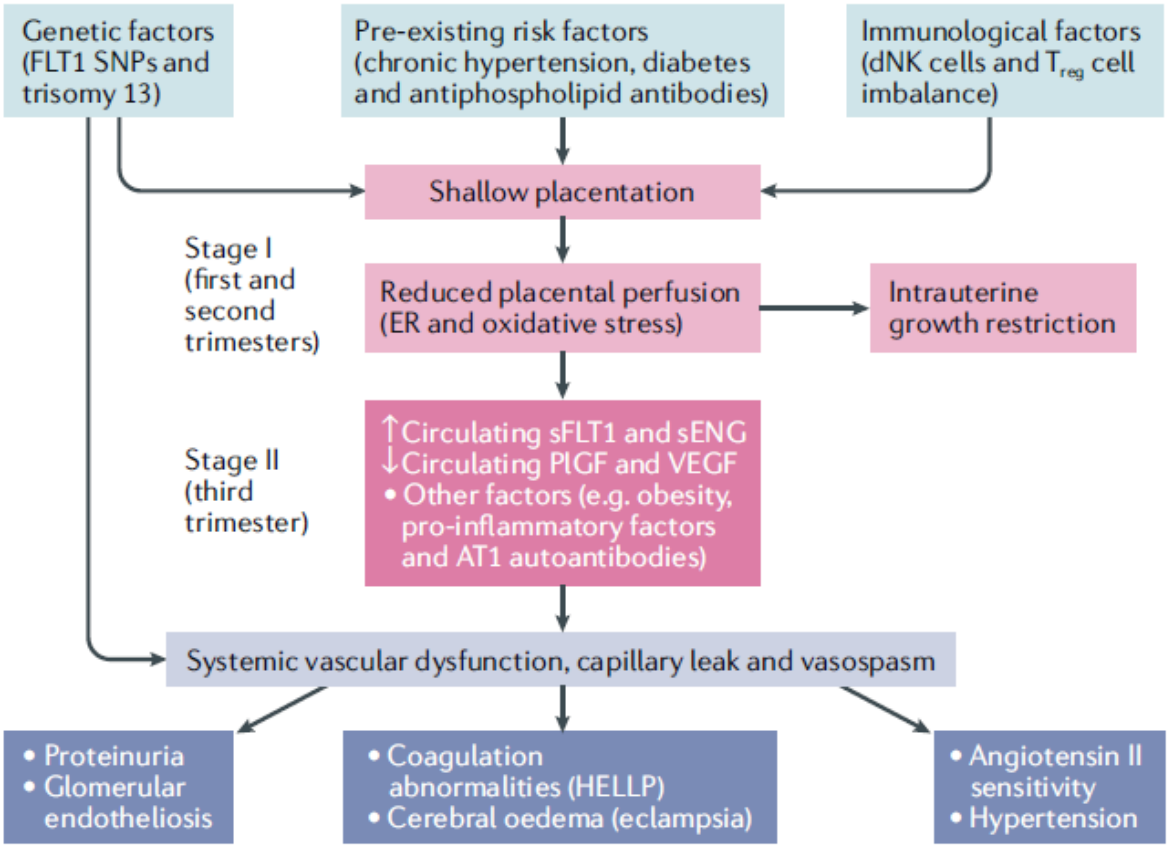


Figure 1.4: Pathogenesis of pre-eclampsia

Reproduced from (86) with permission from Springer Nature.

sFLT1: soluble fms-like tyrosine kinase 1; SNP: single-nucleotide polymorphism; dNK: decidual natural killer; ER: endoplasmic reticulum; sENG: soluble endoglin; PlGF: placental growth factor; VEGF: vascular endothelial growth factor; AT1: angiotensin II type 1 receptor; HELLP: haemolysis, elevated liver enzymes and low platelets.

1.6.3.1 Stage 1 ('pre-clinical') pre-eclampsia

Stage 1 PE is thought to arise from defective placentation in the early stages of pregnancy. In healthy placental development, myometrial invasion by differentiated fetal trophoblast cells allows spiral artery remodelling, ensuring an adequate placental blood supply throughout pregnancy (see ***Section 1.3***). This process is disrupted in PE, leading to shallow invasion of maternal decidua and inadequate

spiral artery modulation (77). A shallow placenta results, susceptible to recurrent ischaemia-reperfusion injuries which cause oxidative stress. There is an inadequate utero-placental circulation to meet the demands of the developing fetus, hence later resulting in IUGR (77, 98).

Pre-existing environmental and genetic risk factors mean that certain individuals are more susceptible to developing PE than others (discussed later in ***Section 1.6.4***). However, it is hypothesised that an abnormal maternal immune response to pregnancy is a crucial contributory factor in disrupting the normal physiological processes of placental formation (77, 86, 98-100).

Decidual natural killer (dNK) cells play an important part in healthy placentation (86, 98). Cavalli et al. demonstrated that when immunocompromised mice with a raised uterine artery resistive index (mimicking PE) were injected with human dNK cells, uterine artery resistance decreased, and placental perfusion was thus enhanced (101).

The interaction between human lymphocyte antigen (HLA) molecules, expressed by trophoblasts, and dNK cell receptors (killer IgG-like receptors; KIRs) is another crucial aspect of normal placental development. Extravillous trophoblasts express a combination of HLA molecules, but only HLA-C is inherited from both parents, and therefore expresses both maternal and paternal antigens. During invasion of the myometrium, HLA-C expressed by trophoblasts interacts with KIRs on maternal dNK cells (102), producing haplotypes which either enhance or inhibit NK cell function.

It has been demonstrated that in pregnancies affected by PE, specific subtypes of HLA-C and haplotypes of KIR are expressed more frequently (86, 98, 102). It is possible that the interaction between maternal KIRs and paternally-derived HLA-C is crucial in successful trophoblast invasion and thus in determining the likelihood of a successful pregnancy outcome (103). Studies showing PE to be more common in primiparity and first pregnancies with a new partner (presumed due to a shorter 'coital interval', a reduced period of exposure to paternal semen, and therefore a reduced timeframe for developing maternal immune tolerance) appear to support this hypothesis (104, 105).

Imbalances in T-cell subsets may also contribute to PE. Unlike in healthy pregnancy, there is a predominance of T helper 1 (Th1) lymphocytes over Th2 in PE, leading to an excess of proinflammatory cytokines including interferon gamma (IFNy) and tumour necrosis factor alpha (TNFα) (106). This in turn may contribute to poor placental development.

I have earlier presented evidence for the role of complement in normal placental formation in healthy pregnancy, and in adverse pregnancy outcomes including recurrent miscarriage and PTB. There is a growing body of evidence linking abnormal complement activity in early pregnancy to poor placentation and subsequent development of PE (4, 9). This will be discussed in detail in ***Section 1.7***.

1.6.3.2 Stage 2 ('clinical') pre-eclampsia

Stage 2 PE develops in the second half of pregnancy as a direct result of the significant oxidative stress placed upon the poorly vascularised placenta. This is thought to stimulate a systemic inflammatory response leading to the release of pro-inflammatory cytokines and autoantibodies (77, 86, 99). This in turn leads to an imbalance in expression of angiogenic factors (77, 107), and widespread endothelial dysfunction. The clinical manifestations of PE (including IUGR, hypertension, and maternal end organ damage) thus become evident.

In a case-control study of PE, anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1) was found to be significantly upregulated when compared to healthy control pregnancies (108). sFlt-1 is a powerful antagonist of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). The same study reported significantly lower PlGF concentrations in women with PE as compared to controls (108). Maynard et al. demonstrated that administering sFlt-1 to pregnant rats induces endothelial dysfunction, hypertension and proteinuria; the hallmark features of the clinical syndrome of PE (109).

There is increasing recognition of the implications for angiogenic factor imbalance in the development of stage 2 PE within the clinical environment. Assessment of angiogenic factor

concentrations may have utility in the diagnosis of PE, as abnormal levels can precede clinically detectable symptoms by several weeks (108). A low sFlt-1:PlGF ratio has been associated with a low risk of developing PE in the short term in cases where PE is clinically suspected (110). Furthermore, a recent multicentre randomised controlled trial reported that testing PlGF in women with suspected PE hastened diagnosis and reduced adverse maternal outcomes (111).

There is significant interest in the temporal relationship between complement activation and angiogenic factor imbalance in women with PE, with evidence suggestive of the two processes being intrinsically linked (15, 112-115). This is explored in greater detail in ***Section 1.7.1.1***.

1.6.3.3 Early- and late-onset pre-eclampsia

There remains some debate as to whether early- and late-onset PE are the same disease entity. Some authors postulate that early-onset PE (prior to 34 weeks' gestation) has a different underlying pathophysiological mechanism to late-onset PE (98, 107, 116, 117). The model described above relating to stage 1 (pre-clinical) and stage 2 (clinical) disease has thus been termed 'placental PE' (116). Placental PE is said to develop early in pregnancy as a result of defective trophoblast invasion and subsequent placental dysfunction, which later drives disordered angiogenesis and maternal endothelial dysfunction. Such cases tend to be early onset, more severe forms of PE characterised by small volume placentae and resulting in fetal growth restriction (98).

In contrast, a phenotypically distinct form of the disease has been described as 'maternal PE' (116). Such cases tend to occur later in pregnancy, with fewer placental lesions present, and are not as commonly associated with fetal growth restriction (118). It is proposed that women with certain underlying health conditions such as obesity, hypertension and CKD are predisposed to systemic vascular inflammation, thus lowering their threshold to develop PE during pregnancy (99). Revisions to this model have since been proposed, with one group postulating that 'maternal PE' in fact results from placental hypoxia in the latter stages of pregnancy, caused by the uterus being unable to sustain the demands of the growing placenta (107). More recently, Staff et al. argue that existing

medical conditions predisposing to vascular inflammation may increase the risk of developing *either* placental (early-onset) *or* maternal (late-onset) PE, and can accelerate either of the pathways involved (119). In either case, inadequate placental perfusion occurs and maternal endothelial dysfunction results (120).

1.6.4 Risk factors for pre-eclampsia

A complex interplay of environmental, genetic and clinical risk factors is believed to contribute to the development of PE (77). PE has been observed more commonly in nulliparous women, and those with a short interval between first coitus with a new partner and conception (104). It is postulated that a reduced period of maternal exposure to paternal antigens in semen leads to reduced maternal immune tolerance, and a risk of developing PE when maternal dNK cells are exposed to paternally derived HLA-C on fetal trophoblasts (104) (see ***Section 1.6.3.1***). Other environmental risk factors for PE include multiple pregnancy, conferred by a larger placental mass (86).

Heritability may be culpable in over half of PE cases (121). Alterations in both the maternal and fetal genome are thought to be contributory (86). A large genome-wide study recently reported variations in the fetal genome near the FLT1 gene that encodes sFlt-1 in the offspring of women with PE (122). Another area of interest is the high incidence of PE in women of African ethnic origin (86), and the potential contributory role of alterations in the gene encoding apolipoprotein L1 (APOL-1), which is only found in those with recent African ancestry (123, 124). This will be discussed in detail in ***Section 1.8.2***.

A number of pre-existing medical conditions increase the risk of women developing PE in pregnancy. These include CKD, chronic hypertension, and autoimmune diseases including SLE and APS (77, 86). It is postulated that these conditions predispose to ‘sensitive’ vasculature, lowering the threshold for systemic inflammation and development of PE (99) (see ***Section 1.6.3.3***).

1.6.5 Management of pre-eclampsia

There remains no definitive treatment for established PE, other than delivery of baby and placenta. The focus of care is centred upon primary prevention through regular antenatal surveillance of BP and proteinuria. Once PE is detected, treatment with antihypertensive medication may delay the progression of symptoms. However, induced preterm delivery of the baby is frequently necessary due to deterioration in maternal and/or fetal health. The recent multicentre randomised controlled 'PHOENIX' trial recommended earlier planned delivery over expectant management in the setting of late-onset preterm PE (125). Earlier planned delivery was associated with reduced incidences of maternal morbidity and severe hypertension when compared to expectant management and was not associated with an increase in adverse fetal outcomes (although admissions to NNUs did rise as a result of prematurity).

In high-risk women, the prescription of low dose aspirin from 12 weeks gestation is effective in reducing the incidence of early-onset PE and fetal growth restriction (126). A large randomised controlled trial reported an OR of 0.38 for developing pre-term PE in women taking prophylactic low dose aspirin as compared to women taking a placebo (95% CI 0.20 to 0.74; P=0.004) (127).

There remains a critical unmet need for additional treatment options to delay the onset of PE and prolong pregnancy, especially in the setting of early-onset PE (128).

1.7 Complement and pre-eclampsia

There is a growing body of evidence implicating excessive activity of complement pathways in the development of PE. The precise mechanisms of injury remain a subject of debate, however, as it is unclear how systemic complement activation within the circulation relates to complement deposition and tissue damage within the placenta. Furthermore, the evidence published to date is often conflicting and features small cohorts of patients. The basis of this thesis will be to examine the role of complement in the pathogenesis of PE in greater detail.

1.7.1 The role of complement in 'stage 1' pre-eclampsia (placental dysfunction)

Defective placentation in early pregnancy is thought to be the central driver for the development of PE (previously discussed in ***Section 1.6.3.1***, and sometimes termed 'stage 1 PE' (98)). Defective angiogenesis results in a poorly vascularised and shallow placenta which is vulnerable to hypoxic damage and repeated ischaemia-reperfusion injury (86). There is increasing evidence for the role of complement dysregulation in this process.

1.7.1.1 Animal studies of placental complement deposition in pre-eclampsia

A number of animal studies have investigated placental complement deposition in the setting of PE. These studies have provided mechanistic insights into the pathogenesis of PE by suggesting a contributory role for complement deposition in placental dysfunction, rather than complement purely being present as a consequence of PE-driven inflammation.

The CBA/J x DBA/2 mouse model is characterised by poor placentation, maternal proteinuria, recurrent miscarriage, and growth restriction in surviving offspring, and has therefore been used for investigating PE (4, 9, 11). However, hypertension is not a feature of this model and thus any inferences relating to PE pathophysiology must be treated with some caution.

Girardi and colleagues have conducted extensive experiments with CBA/J x DBA/2 mice, and report increased deposition of C3 in early pregnancy placental tissue, associated with embryonic death (15) when compared to controls. Treatment with anti-C5 monoclonal antibody resulted in rescue of pregnancy, and subsequent immunohistochemical analysis found minimal placental C3 deposition or inflammation. The research group hypothesised that early placental inflammation in diseased mice triggered further alternative pathway amplification. Interestingly, treatment with anti-factor B monoclonal antibody prevented fetal demise and growth restriction. Further experiments by this group showed that mice with diseased placentas had disordered angiogenesis, with depressed levels of VEGF, and increased anti-angiogenic sFlt-1. Complement inhibition with C3 and anti-C5 inhibitors during early pregnancy prevented sFlt-1 concentrations from rising, and from subsequent fetal demise or growth restriction (15).

Another study of the CBA/J x DBA/2 mouse model reported elevated circulating concentrations of C3a compared to control mice, together with increased placental C3 deposition (129). Treatment with C3 convertase inhibition was associated with reductions in placental oxidative stress (measured by isoprostane 8-iso-prostaglandin (STAT-8)), and circulating sFlt-1 levels. Furthermore, CBA/J x DBA/2 mice that had been treated with C3 convertase inhibitors or anti-C5 monoclonal antibody (mAb) had reductions in urea and urine ACR when compared to mice that had not received complement inhibition, and reduced renal endothelial damage on histological examination.

More recent studies of the CBA/J x DBA/2 model have shown disordered lectin pathway activity when compared to healthy pregnant control mice (130). MBL is found deposited at implantation sites in early pregnancy in diseased mice, but pregnancy loss is prevented by administering anti-C5 antibody, and in MBL-A deficient mice.

The other major mouse model used to investigate PE is BPH/5. In this model, mice are mildly hypertensive prior to pregnancy, but hypertension and proteinuria increase as the pregnancy progresses. Rates of early pregnancy loss and growth restriction are high, and litter sizes tend to be

small. Gelber and colleagues reported significant placental C3 deposition in early pregnancy (day 6) of BPH/5 mice, which preceded neutrophil infiltration and subsequent complement amplification. Treatment with complement inhibition blocked placental neutrophil infiltration and resulted in increased concentrations of pro-angiogenic VEGF. Furthermore, placental spiral artery diameter normalised, and placental weights increased (112).

Lillegard and colleagues investigated the role of complement in PE using the reduced utero-placental perfusion pressure (RUPP) model in rats, in which a procedure inducing placental ischaemia is performed, leading to reduced uteroplacental blood flow and maternal hypertension (131). RUPP treated rats had reduced circulating concentrations of C3 and increased C3a when compared to controls; indicative of placental ischaemia resulting in complement activation. Subsequent complement inhibition treatment in the RUPP treated group led to a reduction in C3a levels and restored maternal blood pressure to normal levels.

Collectively, these research studies provide important evidence directly linking inappropriate complement activation to abnormal placentation and tissue damage from very early in pregnancies affected by PE. Crucially, these studies also demonstrate improvement of pregnancy outcomes when complement activation is blocked.

Sones et al. sought to clarify the link between complement activation and angiogenic factor imbalance in PE, using the BPH/5 mouse model. They established that very early changes in VEGF pathway gene expression occur during the peri-implantation and decidualisation stages. Increased messenger ribonucleic acid (mRNA) expression of complement components including C3 and factor B then follows at the peak of decidualisation and during placental formation (114). A schematic illustrating this is shown in ***Figure 1.5***. Analysis of implantation sites found C3 deposition, along with compromised decidual angiogenesis. The authors postulate that very early imbalances in angiogenic factors precede complement activation before the placenta begins to develop. This in turn leads to

defective myometrial trophoblast invasion, and inadequate placentation in pregnancies affected by PE, stimulating further complement activation and amplification.

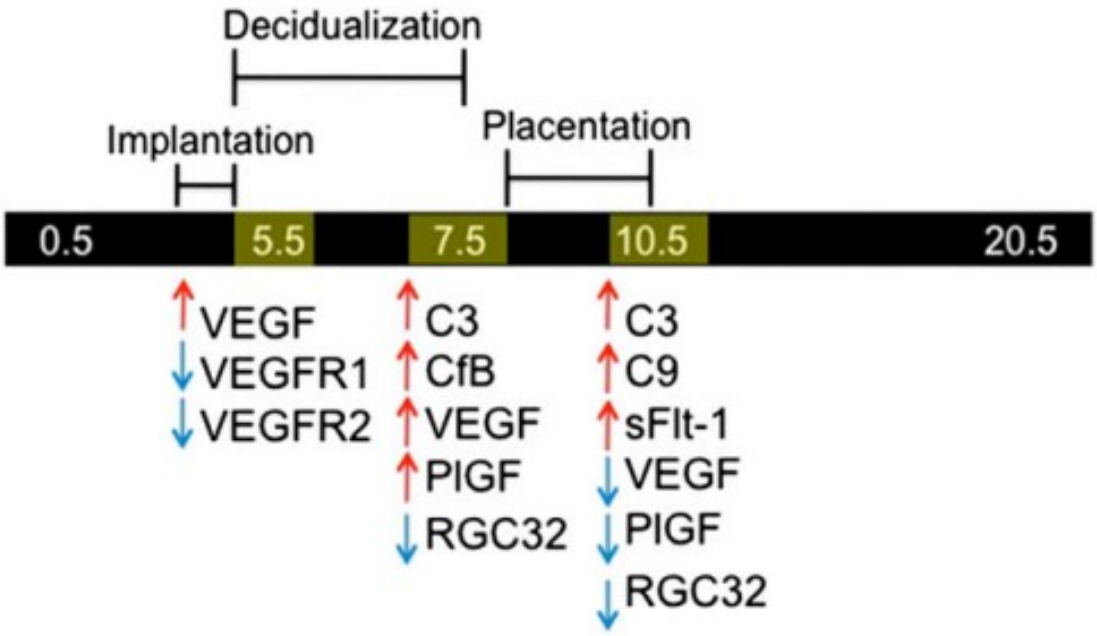


Figure 1.5: Changes in angiogenic factor and complement component mRNA expression in BPH/5 mice, compared to controls

Reproduced from (114) with permission from John Wiley and Sons.

Numbers in figure relate to embryonic days. VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; CfB: complement factor B; PlGF: placental growth factor; RGC: response gene to complement; sFlt-1: soluble fms-like tyrosine kinase-1.

1.7.1.2 Human studies of placental complement deposition in pre-eclampsia

Studies of human pregnancy have built upon evidence gathered from animal studies of placental complement deposition in pregnancies affected by PE. The inherent difficulties in collecting human placental tissue mean that much of the existing research base is observational in nature, has limited cohort sizes, and features placental tissue taken after delivery (often some time after the clinical features of PE became apparent).

Excess deposition of C4d at the STB membrane has been consistently reported in women with PE, when compared to healthy pregnant controls (31, 132-134). In a study of 28 women with PE and 44

pregnant controls, Buurma et al. reported placental C4d deposition in half of PE cases, versus just 3% of controls, p=0.001. Furthermore, in women with PE, diffuse C4d staining was associated with significantly earlier gestation at delivery (albeit with only 5 subjects categorised as having diffuse deposition) (31). Kim et al. reported increased placental C4d deposition in 21 cases of PE with fetal growth restriction, as compared to 20 non-growth restricted PE cases; C4d deposition was seen on the STB membrane in 76.2% versus 10.0% of cases, respectively, p<0.001 (132). Another study examining placental tissue sections from 111 subjects with PE reported significant associations between C4d deposition and earlier gestation at delivery, and with reduced birth weight (134). The results from these studies are suggestive of a potential link between placental complement deposition and disease severity.

Buurma et al. argue that placental C4d deposition in the context of PE is driven by classical (rather than lectin) complement pathway activation (31). Immunohistochemical analysis of placental tissue taken from subjects with PE found no evidence of MBL deposition, but co-localisation of C1q with diffuse C4d deposition. Further analysis found a trend towards IgM immune deposits (rather than other immunoglobulin isotypes) in placentas exhibiting diffuse C4d deposition (31). The authors suggest that this is indicative of classical pathway activation, driven by IgM binding to damaged endothelium. This hypothesis is supported by Kim et al, who found that expression of maternal HLA class I or II antibodies does not appear to correlate with placental C4d deposition (132). In this study, C4d deposition was more commonly observed in cases of PE with fetal growth restriction, with evidence of placental malperfusion (132). Thus, an inflammatory / ischaemia-driven model of classical pathway activation is favoured in PE, rather than by antibody-mediated ‘rejection’ to fetal alloantigens.

Further studies of human placental tissue offer evidence of alternative complement pathway activity, with increased deposition of C3d at the basal plate (135) and within villous epithelial cells (136). The evidence is sometimes conflicting however, with other studies reporting no significant

differences in placental deposition of C3d (25) or properdin (31) – another marker of alternative complement pathway activity – between cases of PE and healthy pregnant controls.

Reports of MAC (C5b-9) deposition are similarly variable. Whilst one study found no significant differences in placental C9 deposition between PE cases and healthy pregnant controls (25), other research groups have reported increased MAC deposition in PE (30, 133). In a study comparing placental tissue from 18 healthy controls, 25 women with PE, and 6 women with HELLP syndrome, Collier et al. reported significantly increased immunofluorescence intensity for MAC in PE placentas compared to controls ($p=0.003$), and in HELLP syndrome placentas compared to controls ($p=0.02$) (133). There were no significant differences in staining intensity however between cases of PE and HELLP syndrome. These findings suggest that placental complement activation in PE (and HELLP syndrome) may extend all the way to the terminal complement pathway.

Upregulated mRNA expression of placental membrane-bound complement regulatory proteins DAF and CD59 has been demonstrated in PE when compared to healthy control pregnancies (31). Their increased expression may be necessary to prevent complement-mediated damage at the STB membrane, and subsequent amplification of the complement cascade, in the setting of excessive complement activation at the placental surface in women with PE. Again, the evidence is sometimes conflicting. Another study reported profuse expression of MCP, DAF and CD59 at the STB membrane in placental tissue taken from healthy control pregnancies *and* from PE, with no significant differences between groups, or between cases of early-onset and late-onset PE (25).

Finally, recent studies have shown a link between placental complement deposition and angiogenic factor expression in women with PE. One group demonstrated a significant positive correlation between placental C4d deposition and placental expression of sFlt-1 (Spearman's $R=0.72$; $p<0.001$), and between placental C9 and sFlt-1 expression ($R=0.59$; $p=0.01$) (133). Another study examined the effect of in vitro complement activation on trophoblast cells isolated from human placentas. Complement activation stimulated upregulation of sFlt-1 mRNA, as well as inducing sFlt-1 secretion

from trophoblast cells. MAC was also associated with release of sFlt-1 (137). Together, the findings from these studies imply that complement activation within placental tissue is associated with angiogenic dysregulation (via upregulation of anti-angiogenic sFlt-1) in pregnancies affected by PE. Defective angiogenesis, poor placentation, and adverse pregnancy outcomes result.

1.7.2 The role of complement in 'stage 2' pre-eclampsia: vascular inflammation and endothelial dysfunction

1.7.2.1 Complement in maternal circulation in pre-eclampsia

A number of studies have investigated biomarkers of complement activation within the maternal circulation. A prospective study of 701 pregnant women examined blood samples taken during the first half of pregnancy, and found that subjects with elevated concentrations of Bb (within the top decile) were significantly more likely to develop PE later in pregnancy than those women with Bb concentrations beneath the 90th decile (RR 3.3, 95% CI 1.6-7.0, p<0.001) (17). This is suggestive of alternative pathway dysregulation from early in pregnancy in PE as the placenta is developing, and also that plasma Bb may be a potentially useful biomarker in identifying women at risk of PE. This hypothesis is corroborated by a more recent study by He et al. who report significantly elevated first trimester factor B and factor H concentrations in women who later developed PE, compared to healthy pregnancies (138).

Bb concentrations have also been investigated at the time of PE diagnosis in cross-sectional studies. Research groups have reported significant increases in plasma Bb in women with PE compared to healthy pregnant controls, although with no significant differences reported between early-onset and late-onset PE (139, 140). Contrary to this however, Derzsy et al. found no significant difference in Bb concentration between women with PE and healthy pregnant controls (34). Potential differences with the Derzsy study were that it featured an entirely Caucasian cohort, whereas other

studies had a more diverse mix of ethnicities, and that it included subjects with less severe, later onset PE (139, 141).

There are consistent reports of reduced concentrations of C4 in maternal blood in cases of PE, when compared to healthy control pregnancies (140, 142, 143). This is indicative of C4 consumption via classical or lectin pathway activity in the setting of PE. Jia et al. also found reduced concentrations of C1q in PE, in a gestationally-matched study comparing PE with healthy pregnancy (140). There were however no significant associations found between C1q or C4 and PE severity, with comparable levels of both complement components in early-onset and late-onset PE (140).

Data on lectin pathway activation in PE is conflicting. In one study comparing 99 women with PE with 187 healthy pregnant controls, PE was associated with significantly elevated plasma MBL concentrations (144). In contrast, two other studies report comparable MBL levels in women with PE, when compared to healthy pregnant controls (145, 146). Overall, there is weaker evidence for lectin pathway activation in the context of PE, than for classical and alternative complement pathway activation.

There is evidence of increased production of anaphylatoxins in PE, with raised levels of C3a (34, 147, 148) and C5a (149) reported in women with PE, when compared to healthy pregnant controls. C3a concentration may be associated with disease severity, with significantly increased levels reported in early-onset versus late-onset PE (147, 148).

Complement activation in the maternal circulation may extend as far as the terminal complement pathway, with reports of increased C5b-9 concentrations in women with PE when compared to healthy pregnant controls (34, 147, 148, 150). C5b-9 activity has been linked to disease severity, with one group reporting in subgroup analysis elevated C5b-9 concentrations in PE pregnancies complicated by IUGR, compared to non-growth restricted PE pregnancies (34). However, in other studies, plasma C5b-9 levels did not distinguish between early-onset (severe) PE and late-onset PE cases (148, 150). In early pregnancy blood testing, there were no significant differences in C5b-9

concentrations between women with a healthy pregnancy, and those who later developed PE (115, 138). Downstream terminal pathway activation may therefore develop later in pregnancy once the clinical signs of PE become established.

1.7.2.2 Complement in fetal circulation in pre-eclampsia

There is a paucity of evidence for complement activation in the fetal circulation in cases of PE. Denny et al. compared umbilical cord blood samples from 29 pregnancies affected by PE, with 14 gestational hypertension pregnancies, and 35 healthy pregnancies. They reported elevated concentrations of the activation fragment C5a in PE, and levels positively correlated with maternal plasma C5a concentrations (149). The authors suggest that this may reflect diffusion of C5a across the placenta from maternal to fetal circulation. There was however no significant difference in cord C3a levels between the study groups.

There is conflicting evidence on umbilical cord plasma Bb in the setting of PE. Hoffman et al. compared umbilical cord blood samples from 15 cases of early-onset PE with 15 healthy control pregnancies (139). They reported significantly elevated levels of Bb in PE cord plasma and postulated that this represented alternative complement pathway activation occurring within the fetal circulation as well as the maternal circulation in cases of PE. In contrast, in a larger study of 154 subjects of predominantly African American (AA) ethnicity, there was no reported difference in cord plasma Bb concentration between PE and healthy pregnancy (141).

The data presented above is sometimes contradictory, but there is some evidence for complement activation occurring within the fetal compartment in pregnancies affected by PE. Alternatively, complement proteins may diffuse across the placenta, and simply reflect complement activation within the maternal circulation, rather than activation within the fetal circulation per se. Regardless, it is possible that fetal exposure to complement and subsequent inflammatory mediators has an injurious effect, and may contribute to adverse events including miscarriage and fetal death (149).

1.7.2.3 Complement in renal tissue and urine in pre-eclampsia

Penning et al. examined kidney tissue taken from autopsy samples from women diagnosed with PE during pregnancy, and from pregnant controls with no history of hypertension. They reported evidence of classical complement pathway activation, with increased staining for C4d and C1q in the renal tissue of women with PE (151). There were no significant differences in MBL, properdin, C3d, or C5b-9 staining between groups.

Renal endothelial dysfunction is a key clinical manifestation of PE, and therefore there is also interest in examining urine complement component levels during pregnancy. Urine C5b-9 concentrations have consistently been reported to be raised in women with PE, compared to healthy pregnant controls (150, 152-154). Urine C5b-9 concentrations appear to be associated with end-organ injury (defined in one study as thrombocytopenia and/or renal dysfunction) (154), and were also associated with raised plasma sFlt-1 and reduced plasma PlGF concentrations (153). Furthermore, urine C5b-9 levels appear to differentiate PE from chronic hypertension and from healthy pregnancy, and are better indicators of PE severity than plasma C5b-9 levels (150, 152, 153). Overall, the findings suggest that terminal complement pathway activation within the kidneys is a feature of severe PE.

1.7.3 Complement pathway genetics and susceptibility to pre-eclampsia

Certain individuals may be more susceptible to complement dysregulation than others and have been shown to have an increased risk of developing PE during pregnancy. Salmon and colleagues investigated genes encoding complement regulatory proteins (CD46 and complement factors I and H) in women with SLE and/or APS who developed PE in pregnancy. They found that approximately one fifth of the 40 subjects with PE had complement regulator gene mutations encoding CD46 and factor I (18). These mutations have previously been shown to be risk variants for aHUS. A novel CD46 mutation was also found which was linked to defective C4b binding (18). All but one of the PE

cases with a gene mutation had a severe phenotype (preterm delivery, fetal growth restriction, or HELLP syndrome). It is notable, however, that the majority of PE cases within this cohort did not have an identifiable complement risk variant (of the complement regulators that were sequenced).

A later study examining polymorphisms in the gene encoding CD46 did not report any association between CD46 variants and PE; with the authors refuting the suggestion that CD46 single nucleotide polymorphisms (SNPs) bear functional consequences (155). Demographic differences between cohorts may contribute to the contrasting findings. The study by Salmon et al. solely included women with autoimmune disease, who may have had a lower threshold for complement dysregulation and developing PE (18).

Another study reported a significantly increased prevalence of SNPs in the genes encoding maternal C3 and factor H, in addition to *fetal* CD46, in pregnancies affected by PE compared to healthy pregnancies (156). Interestingly, specific fetal CD46 variants were associated with reduced placental CD46 expression (suggestive of a lack of complement regulation at the maternal-fetal interface). In addition, specific factor H variants were associated with increased alternative pathway (AP50) activity (156). The authors argue that, in combination, these mutations predispose to a risk of PE.

Inherited mutations in complement genes have also been associated with PE severity. A Chinese cohort study found an association between polymorphisms in the genes encoding C6 and MASP1, and an increased risk of developing early-onset PE (157). Lokki and colleagues identified several SNPs in the gene encoding C3 that were associated with PE with severe features (158). Another case-control study compared rates of mutations in alternative complement pathway genes in women with HELLP syndrome and healthy pregnant controls. Mutations in alternative pathway genes were far more common in the HELLP group (48% versus 8%, $p<0.01$) (159).

Lokki and colleagues sought to clarify the link between complement polymorphisms and the risk of PE, through functional studies within a Finnish cohort. They demonstrated that specific mutations affecting complement receptor 3 (CR3) and complement receptor 4 (CR4) were linked to an overall

increased risk of PE, through their altered adhesion to iC3b (160). iC3b is a crucial opsonin, marking cells for phagocytosis through binding to CR3 and CR4. Where this process is defective, disordered clearance of cellular debris and inflammation may result (161).

A recent study of the same Finnish cohort identified 5 variants in factor H that were associated with severe PE (162). Affected cases were characterised by early-onset PE, small for gestational age babies, and HELLP syndrome, and none of the subjects were later diagnosed with aHUS (10-year follow up period). 4 of the 5 polymorphisms were found to have deleterious functional consequences, through impaired C3b and C3d binding, and dysfunctional factor H activity.

Collectively, these findings highlight links between polymorphisms in complement and complement regulatory genes, and PE. Some, but not all, of these variants have demonstrable deleterious consequences, highlighting the complexity of PE pathogenesis. The findings reinforce the importance of a functioning complement system in pregnancy and may also provide therapeutic targets for complement blockade treatments in certain groups of patients with PE.

1.7.4 Summary and proposed model for the role of complement in pre-eclampsia pathogenesis

The evidence presented above supports the theory of excessive complement activation being an important factor in the pathogenesis of PE. However, despite the growing body of evidence, published reports are sometimes contradictory and are predominantly derived from cross-sectional studies of limited size. Furthermore, the precise mechanisms of PE pathogenesis and potential involvement of the complement system remains unclear, with no studies in human subjects demonstrating how systemic complement activation within the fluid phase relates to complement deposition, and injury, at a placental tissue level.

Animal studies do provide clear supporting evidence of complement activation during early pregnancy leading to poor placental formation and causing harmful outcomes including failure of

pregnancy (15, 112, 114). Subsequent complement inhibition has been shown to improve placental vascularisation and rescue pregnancy (15, 112), with reduced angiogenic dysregulation or maternal endothelial dysfunction (129).

Studies of human pregnancy report excess complement deposition in placental tissue in subjects with PE; particularly involving components of the classical pathway (31, 132). In addition, there is evidence of increased alternative pathway activity from early pregnancy in the maternal circulation of women who later develop PE (17). Downstream complement activation leading to increased production of anaphylatoxins and MAC is present at the point of PE presentation later in pregnancy, and raised plasma and urine concentrations may be associated with severe PE phenotypes (34, 152, 154).

It has been debated whether this complement activation *causes* PE to develop, or whether complement is present as a *result* of PE-driven inflammation. Evidence from both human and murine studies has established a link between complement and angiogenic factor imbalance in PE, which may help answer this question.

A landmark study in a mouse model of PE found abnormal angiogenesis from very early in pregnancy preceded complement activation during decidualisation (114). Abnormal trophoblast invasion and poor placental formation followed, with growth restriction and fetal death. Another animal study found that complement blockade treatment led to a reduction in anti-angiogenic sFlt-1 concentrations in mice and prevented features of PE developing (129). In vitro studies of human placental tissue have attempted to establish a temporal relationship, with one group showing that complement activated trophoblast cells induce sFlt-1 production (137). These findings are reflected in other mouse studies which show that complement activation fragments can also stimulate the release of sFlt-1 (15, 112).

It has therefore been postulated that abnormal sFlt-1 expression stimulates complement activation during early placental development in women with PE. Complement activation and amplification

may also in turn trigger further sFlt-1 production as the pregnancy progresses, which leads to increasing inflammation and maternal endothelial dysfunction (133, 163). A proposed model for the role of complement, and its relationship with angiogenic dysregulation, is shown in ***Figure 1.6***.

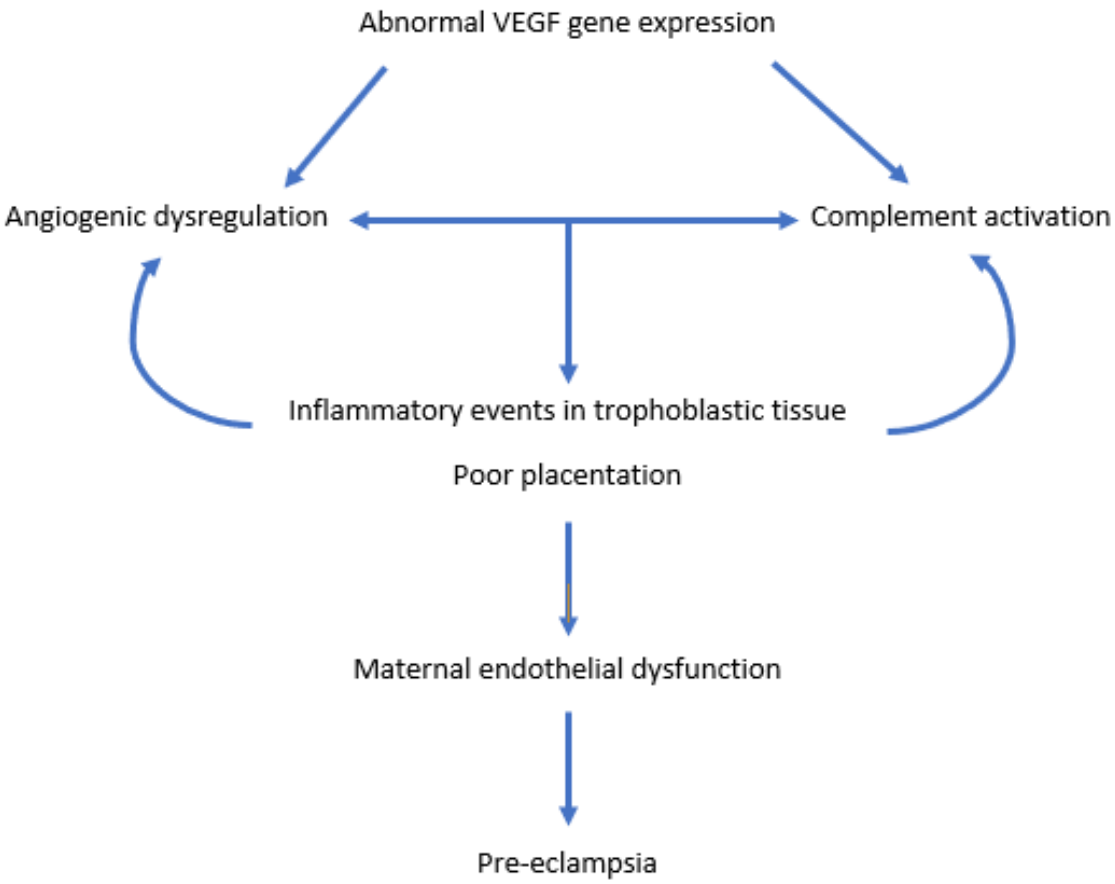


Figure 1.6: Proposed role of complement in pre-eclampsia pathogenesis

Adapted from (66) and (163), with permission from Elsevier Ltd.

1.7.5 Clinical implications and potential therapeutic interventions

Complement-modifying agents are currently undergoing rapid development and may provide novel opportunities to treat complement-mediated diseases (164-167). The monoclonal antibody eculizumab is a C5 inhibitor and has revolutionised the treatment of aHUS and PNH over the last decade. Evidence for its use in PE is limited to isolated case reports. In one report, eculizumab was

used to treat a patient presenting with HELLP syndrome at 26 weeks gestation (168). In the period following administration, the patient's full blood count and liver function normalised, and the pregnancy was prolonged by 17 days before delivery took place. In another case, eculizumab was commenced in a pregnant patient at 20 weeks' gestation following a diagnosis of aHUS. The patient was subsequently diagnosed with PE at 24 weeks and eculizumab was continued until delivery at 27 weeks (169). Interestingly, post hoc analysis found that plasma sFlt-1:PlGF ratio fell after the initiation of complement blockade treatment, which may have contributed to the successful prolongation of pregnancy. One further case report describes a patient with HELLP syndrome who delivered at 34 weeks' gestation, then developed postpartum aHUS which was successfully treated with eculizumab, leading to recovery of renal failure (170).

A recent in-vitro study demonstrated reduced cytotoxicity when serum containing eculizumab was mixed with serum from women with HELLP syndrome (171). These results indicate that eculizumab is an effective agent in blocking alternative pathway-mediated cell killing and may be a useful treatment for women with HELLP syndrome. C5 inhibition has been demonstrated to be safe when used for pregnant women with PNH, with low rates of adverse maternal and fetal events (55). However, the long-term effects of using complement inhibition therapy during pregnancy and potential immunosuppressant effects on newborns are not yet known (61), and whether this treatment could be extrapolated from HELLP syndrome to women with severe PE remains to be seen.

Many more complement modifying drugs targeting other components of the complement cascade are in development, with clinical trials particularly in the setting of glomerular and ophthalmic disease already in progress (164, 166, 167). A major concern in the delivery of complement inhibiting drugs is the risk of infection and over-immunosuppression. New drugs under development are aimed at reducing the dose of medication administered through 'recycling' antibodies, and also at targeting specific local sites for inhibition (167). Furthermore, drugs targeted towards the

amplification loop may allow attenuation of complement activation rather than total blockade, which could reduce adverse treatment effects (167). Medications which can be delivered subcutaneously or orally are also under development and may facilitate easier administration than eculizumab, which can only be given intravenously (164, 167).

This raises the question of whether complement inhibition therapy could offer a potential alternative treatment strategy to preterm delivery of the fetus in cases of severe PE (172). Potential benefits to patients and healthcare systems are significant because no other definitive treatment strategies for PE are currently available. If pregnancy could be prolonged by inhibiting excessive complement activation and the downstream inflammatory and anti-angiogenic effects, this could result in fewer preterm deliveries and fewer subsequent adverse pregnancy outcomes including fetal growth restriction and requirement for NNU care.

1.8 Pre-eclampsia in high-risk groups

Epidemiological data highlight cohorts of women that are at a particularly elevated risk of PE when compared to the general pregnant population. This includes women with CKD (79, 173), and women of SSA ethnicity (174-176). The work within this thesis will aim to investigate the role of complement in the pathophysiology of PE in previously healthy women, as well as in high-risk groups (women with CKD, and women of SSA ethnicity). It is hoped this will provide greater insight into the mechanisms involved in PE pathogenesis, with potentially more pronounced changes in cohorts with particularly high disease incidence. A comparison of patterns of complement activation between high-risk groups and the previously healthy general pregnant population will also enable analysis of whether more than one mechanism of PE pathogenesis exists.

1.8.1 Chronic kidney disease and pregnancy

1.8.1.1 Epidemiology

CKD has an estimated prevalence of 3% among women of childbearing age (177, 178). In some cases, renal disease is detected for the first time during routine antenatal screening. Pregnancy is less common in women with more advanced renal dysfunction, with an estimated 1 in 750 pregnancies occurring in women with CKD stages 3-5 (178, 179). The advent of kidney transplantation has led to rapid improvements in renal function and restoration of fertility for many women with CKD (180). This, coupled with the rising incidence of obesity, diabetes and cardiovascular disease among the general population, mean that rates of CKD in pregnant women are predicted to increase further in the coming years (179).

1.8.1.2 Impact of CKD on pregnancy – maternal and fetal outcomes

Cohort studies and meta-analyses have shown that rates of adverse pregnancy outcomes in women with CKD are higher than for the general population (173, 181). This includes an increased risk of

adverse maternal outcomes, such as Caesarean section and PE, as well as fetal complications including preterm delivery, growth restriction and requirement for NNU admission. Absolute rates of adverse pregnancy outcomes vary between cohorts due to differences in ethnicities, healthcare systems, and heterogeneity in cause and severity of CKD; with the majority of reported data derived from women with CKD stages 1-3. In general, however, the risk of adverse pregnancy outcome rises incrementally with worsening baseline renal function (see ***Figure 1.7***). Despite this trend, data suggest that even 'mild' renal disease (CKD stage 1), in the absence of proteinuria, chronic hypertension or systemic disease, confers a 'baseline risk' of adverse pregnancy outcome; (OR 1.88, 95% CI 1.27-2.79) (181). Other risk factors for adverse pregnancy outcome include pre-pregnancy proteinuria (179, 182), chronic hypertension (73, 179), previous kidney transplantation (183, 184), and lupus nephritis (43, 44, 185).

Despite the higher rates of adverse outcome, successful pregnancy in CKD is common, with live birth rates reported to be 98% for women with CKD stages 3-5 in a recent UK-wide cohort study (179). Specialist pre-pregnancy counselling and regular antenatal review in a dedicated renal-obstetric clinic is recommended to ameliorate concerns and detect any potential complications early on (186, 187).

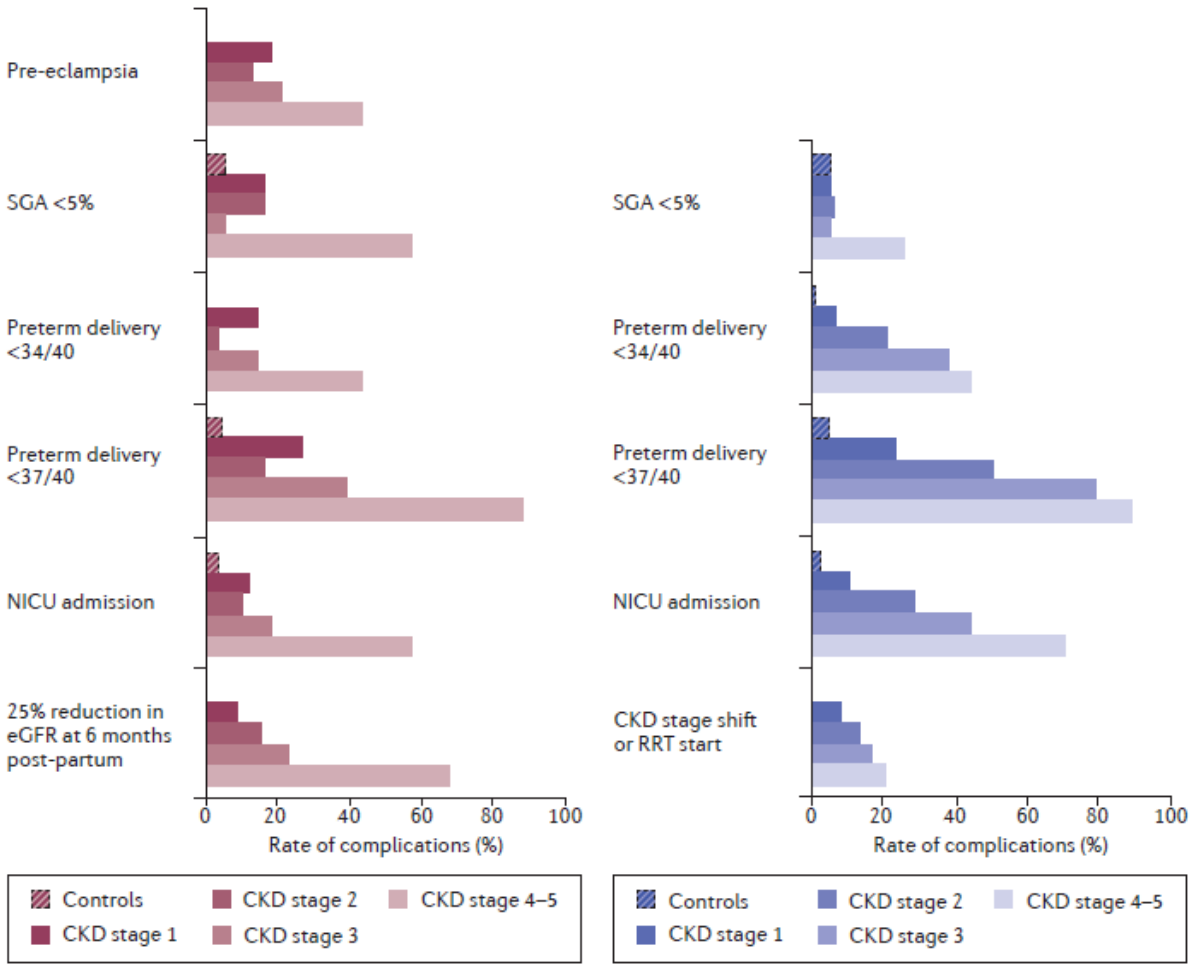


Figure 1.7: Rates of adverse pregnancy outcome by CKD stage

Reproduced from (180), with permission from Springer Nature. Figure shows results from two cohort studies: Bramham et al. (red) (188) and Piccoli et al. (blue) (181). SGA: small for gestational age; NICU: neonatal intensive care unit; eGFR: estimated glomerular filtration rate; RRT: renal replacement therapy; CKD: chronic kidney disease.

1.8.1.3 Impact of pregnancy on CKD

Pregnancy is *not* associated with an accelerated postnatal decline in renal function in women with mild pre-pregnancy renal disease (CKD stages 1 and 2), well-controlled blood pressure, and low-level proteinuria (173). The long-term impact on maternal renal function is greater for those with more advanced pre-pregnancy renal impairment (CKD stages 3-5) (189). A UK-based cohort study reported that almost half of pregnant women with CKD stages 3-5 had lost more than 25% of their GFR at 1 year postpartum or required renal replacement therapy (RRT) (179). The risk of pregnancy-

associated decline in renal function in this cohort increased with CKD stage, the presence of chronic hypertension, and a gestational fall in creatinine of less than 10% of pre-pregnancy baseline.

1.8.1.4 Chronic kidney disease and superimposed pre-eclampsia

1.8.1.4.1 Superimposed pre-eclampsia: definition and statistics in renal patients

The standard diagnostic criteria for PE (71) do not easily apply to patients with CKD, who often have impaired excretory renal function, proteinuria and/or chronic hypertension which pre-date the pregnancy and can cloud the diagnosis of PE. The term superimposed pre-eclampsia (SPE) is often used in this setting, representing the changes of PE superimposed upon CKD.

SPE affects up to 40% of women with CKD during pregnancy (79). This equates to a 10-fold greater prevalence than in women without pre-existing renal disease (OR 10.4, 95% CI 6.3-17.1) (173). Women with more severe pre-pregnancy renal impairment, proteinuria, or chronic hypertension are at greater risk of developing SPE during pregnancy (73, 173, 179, 188, 190), as well as women with a kidney transplant (adjusted OR of SPE 6.3; 95% CI 3.0-13.4 as compared to healthy women) (191).

There are no consensus criteria for the diagnosis of SPE in women with CKD. For women without pre-existing hypertension or proteinuria, the standard diagnostic criteria for PE can be used (see ***Table 1.3***). Where chronic hypertension and proteinuria are present, previous cohorts have suggested their own diagnostic criteria for SPE in women with CKD (79, 192). These criteria are detailed in ***Table 1.4***, and have been used to define cases of SPE for the body of work within this thesis.

Table 1.4: Diagnostic criteria for superimposed pre-eclampsia in chronic kidney disease

Gestational hypertension (if chronic hypertension present)	• De novo hypertension ≥ 160 mmHg systolic or ≥ 110 mmHg diastolic OR • Increase in antihypertensive treatment after 20 weeks' gestation to maintain BP <160/110 mmHg
and	
Gestational proteinuria (if chronic proteinuria present)	• Doubling in uPCR after 20 weeks' gestation compared to pre-pregnancy levels AND • uPCR > 30 mg/mmol
Or at least one of the following if only one of the above indicators present:	
Acute kidney injury	• Rapidly worsening kidney function: ≥ 50% increase in serum creatinine in 7 days not attributable to an alternate diagnosis AND • Creatinine ≥ 27 µmol/L above pre-pregnancy concentration
Uteroplacental dysfunction	• Small for gestational age (birth weight <10th centile) • Abnormal umbilical artery Doppler waveform
Severe features	• Platelet count < 100 x 10^{9}/L • Haemolysis or disseminated intravascular coagulation • Rise in liver transaminases (ALT or AST > 40 IU/L) • Severe headache / altered mental status / visual scotomata • Clonus • Seizures (eclampsia) • Pulmonary oedema not attributable to an alternate diagnosis

Table adapted from (79, 192). BP: blood pressure; uPCR: urine protein to creatinine ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

1.8.1.4.2 Superimposed pre-eclampsia: diagnostic dilemma and pathophysiology theories

The diagnosis of SPE in women with CKD is challenging because hypertension, proteinuria and renal dysfunction characterise both conditions. Renal physiological changes in normal pregnancy (including a fall in blood pressure in early and mid-pregnancy, followed by a rise in the third trimester, and a gestational rise in proteinuria as a result of hyperfiltration (193)) cause further diagnostic uncertainty and can lead to difficult decision-making around timing of delivery for women with CKD (194). In the absence of standard diagnostic criteria for SPE, relative changes in blood pressure, creatinine and proteinuria during pregnancy are analysed in addition to expert consensus review (79).

The pathophysiological mechanisms by which CKD patients are disproportionately affected by SPE remain a subject of investigation. Endothelial dysfunction characterises both CKD and PE, leading to proteinuria, chronic hypertension, adverse pregnancy outcomes, and long-term cardiovascular and renal disease in both conditions. Disrupted angiogenesis (195, 196) and up-regulation of renin-angiotensin system markers (angiotensin II type 1 receptor (AT1-R) and AT1-R auto antibody) (197) contribute to endothelial dysfunction in both CKD and in PE. Complement activation has also been shown to mediate endothelial dysfunction and lead to progressive disease in both PE and CKD (11, 198). Finally, PE and CKD have both been associated with renal tissue injury, which contributes to endothelial damage. Kidney biopsy specimens taken from women with PE show glomerular endotheliosis (199) and recent turnover of podocytes (200). Furthermore, one study showed that women with a resolved episode of AKI were more likely to develop PE in future pregnancy (23% versus 4% rate of PE in controls, $p<0.001$) (201). The relationship between CKD and PE is summarised in ***Figure 1.8*** (79).

It is postulated that pre-existing endothelial dysfunction, through a combination of the mechanisms outlined above, 'sensitises' the maternal vasculature to circulating antiangiogenic factors in women with CKD. This accounts for the increased incidence of PE seen in women with CKD, as there is a lower threshold to develop angiogenic dysregulation, and subsequent SPE, than for women without renal disease (79, 201). This model will typically manifest as later-onset 'maternal' SPE, as the demands of the growing fetus outstrip the capacity of the placenta (107); see ***Section 1.6.3.3.*** In contrast to 'maternal' SPE, 'placental' SPE classically presents with early-onset disease and fetal growth restriction, driven by defective placentation early in pregnancy (118). It may therefore be particularly important to identify, define and separate 'maternal' SPE cases from 'placental' SPE when researching pregnancy in CKD as the pathophysiology between disease subtypes may be very different.

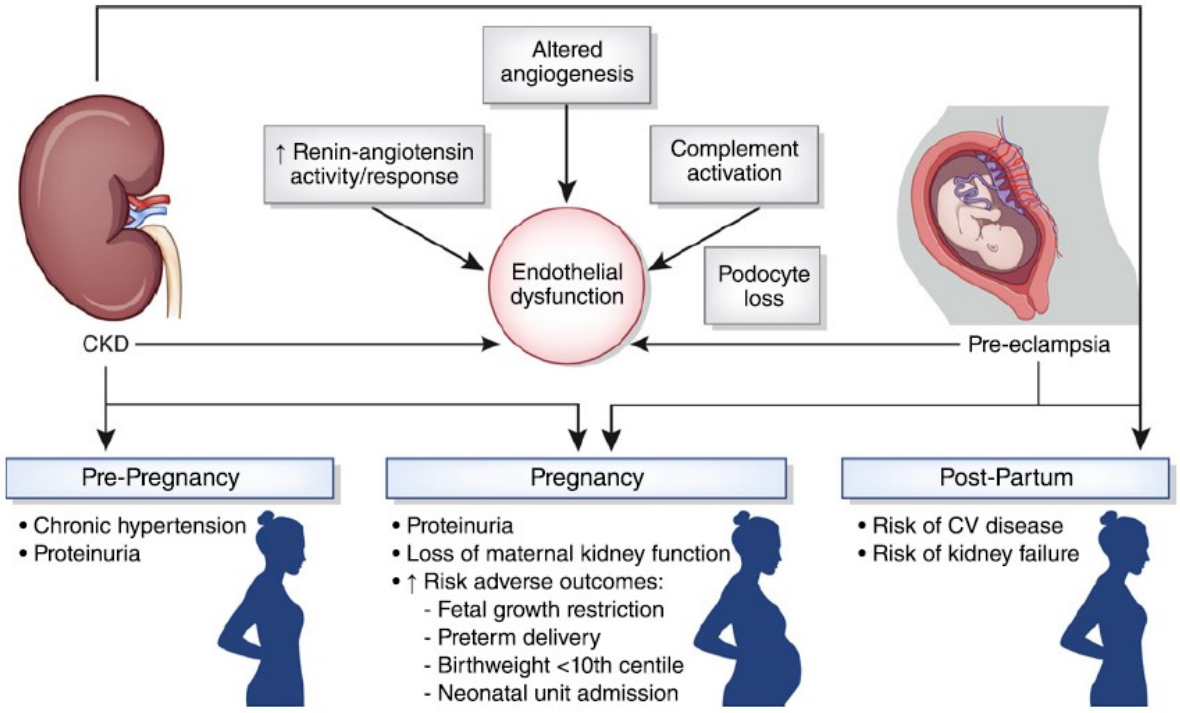


Figure 1.8: Relationship between chronic kidney disease and pre-eclampsia

Reproduced from (79), with permission from Wolters Kluwer Health, Inc.

1.8.1.4.3 Biomarkers of superimposed pre-eclampsia in patients with chronic kidney disease

There is growing interest in identifying biomarkers to assist in the diagnosis of SPE in women with CKD, given the shared phenotype of both conditions. This would allow earlier identification of SPE, and timely monitoring and treatment. The ability to discriminate more accurately between SPE and pregnancy-induced CKD progression could also lead to improved pregnancy outcomes through the avoidance of iatrogenic prematurity.

In recent years, testing of angiogenic markers PlGF and sFlt-1:PlGF ratio has been shown to aid PE diagnosis in previously healthy women where there is diagnostic uncertainty (108, 110, 111). In the last 2 months of healthy normotensive pregnancy, sFlt-1 levels rise and PlGF levels fall, but this pattern occurs earlier and with more a pronounced change in pregnancies affected by PE (108). Testing of PlGF or sFlt-1:PlGF ratio is now recommended in UK clinical practice as a diagnostic adjunct in ruling in and excluding preterm PE (between 20 to 36+6 weeks gestation) in cases where

there is high clinical suspicion (202). PlGF testing can help inform clinicians of the likely need for delivery due to suspected PE in the short term. Very low PlGF levels are suggestive of severe placental dysfunction and high risk of preterm delivery. Conversely, normal PlGF levels indicate normal placental function, and a low likelihood of requiring delivery within the next 14 days (202). Similarly, sFlt-1:PlGF testing can aid in the short term prediction and diagnosis of PE. A low sFlt-1:PlGF ratio rules out a PE diagnosis within 7 days, whereas an elevated sFlt-1:PlGF ratio rules in a diagnosis of PE within the next 4 weeks (202). The specific cut off values depend on the assay used.

The major caveat to testing for angiogenic markers is that the presence of pre-existing medical conditions characterised by endothelial damage might affect the diagnostic accuracy of these biomarkers. Therefore, the utility of PlGF or sFlt-1:PlGF assays in women with CKD is disputed.

In a prospective UK cohort study of pregnant women with CKD (232 pregnancies in 221 women), Wiles et al. reported significantly reduced PlGF concentrations from 21 to 37 weeks' gestation in women who developed SPE versus those that did not (192). Reduced PlGF concentration had high predictive accuracy of the need for delivery within 14 days due to SPE (AUROC 0.80; 95% CI 0.66-0.94). Predictive accuracy was lower, however, for those with CKD stages 3-5. Further research is therefore required to assess the impact of impaired excretory renal function on angiogenic markers, and their utility in diagnosing SPE in women with more advanced renal disease. Additionally, in contrast to healthy women, measurement of sFlt-1:PlGF ratio in pregnant women with CKD does not predict the need for delivery due to SPE (192).

Another smaller cohort study reported raised plasma concentrations of endothelial glycocalyx components hyaluronan and vascular cell adhesion molecule (VCAM) in women with CKD who developed SPE (203). These markers were significantly negatively correlated with PlGF concentration; underlining the role of endothelial dysfunction in SPE in women with CKD, and a potential link with disordered angiogenesis.

1.8.1.4.4 Complement and superimposed pre-eclampsia in women with chronic kidney disease

Although there is mounting evidence for the role of complement in PE pathogenesis – discussed earlier within this thesis – very little is known about whether the same mechanisms apply in SPE. Furthermore, certain groups of patients with CKD may already have conditions affecting complement activity (such as lupus and aHUS) or take medications that suppress their immune system, which may confound results.

One small cohort study measured anaphylatoxins C3a and C5a, alternative pathway regulator factor H, and C5b-9 in pregnant women with CKD; a proportion of whom had SPE. There were no significant differences in any of the complement components between pregnant CKD patients with and without SPE. Therefore, none of the complement components measured were deemed useful adjuncts in diagnosing SPE (203).

One further study analysed renal tissue specimens taken from previously healthy women with PE, for evidence of complement deposition. There was significant C4d and C1q deposition seen in PE samples, as compared to healthy pregnant controls. This suggests that classical complement pathway activity occurs within the kidney itself in PE, although this study did not examine any samples from women with pre-existing renal disease (151).

Further research is therefore required to assess the potential utility of complement components as biomarkers of SPE in women with CKD, and as a potential therapeutic target in this specific cohort.

1.8.2 Sub-Saharan African ethnicity and pre-eclampsia

1.8.2.1 Pregnancy outcomes in women of sub-Saharan African ethnicity

Although there have been significant improvements in global maternal mortality rates over the last three decades, maternal mortality does remain disproportionately high in the world's least developed countries (84). Around two-thirds of worldwide maternal deaths in 2017 occurred in SSA

alone (84), often as a result of obstetric complications including haemorrhage and hypertensive disorders of pregnancy, including PE and eclampsia (204). The problem is exacerbated in some settings by limited access to emergency healthcare, including antenatal monitoring and treatment (175, 205, 206). However, Black ethnicity itself appears to confer an increased risk of adverse pregnancy outcome, independent of socioeconomic status. In a recent international study of over 2 million pregnancies in high- and upper-middle income countries, the risk of neonatal death in babies born to Black mothers was twice as high as the risk for White mothers (OR 2.0, 95% CI 1.4-2.8). The differences were similarly as stark for preterm birth and stillbirth (207). The authors cite barriers such as structural racism as a key area to be addressed.

Racial disparities in pregnancy outcomes are also seen within the UK, with the recent MBRRACE (Mothers and Babies: Reducing Risk through Audits and Confidential Enquiries across the UK) report highlighting that from 2017-2019, Black women were four times more likely to die in pregnancy than White women (RR 4.5; 95% CI 2.8 to 7.0) (208). Further to this, a UK national cohort study of more than 1 million pregnancies found that women of Black ethnic backgrounds had significantly higher rates of stillbirth, preterm birth and fetal growth restriction than White women, even after controlling for socioeconomic deprivation, smoking status and BMI (209).

1.8.2.2 Pre-eclampsia in women of Sub-Saharan African ethnicity

The exact prevalence of PE in SSA is difficult to pinpoint, as existing data are largely drawn from English-speaking nations and from hospital admissions in urban areas only. There is a lack of accurate clinical records, particularly from home births and from less economically developed regions and nations (204, 210), introducing likely selection biases. Despite this, recent systematic reviews report high prevalence rates of hypertensive disorders of pregnancy in SSA, with up to 1 in 10 of all pregnancies affected, which is significantly higher than in Europe and North America (210, 211). See ***Figure 1.9***.

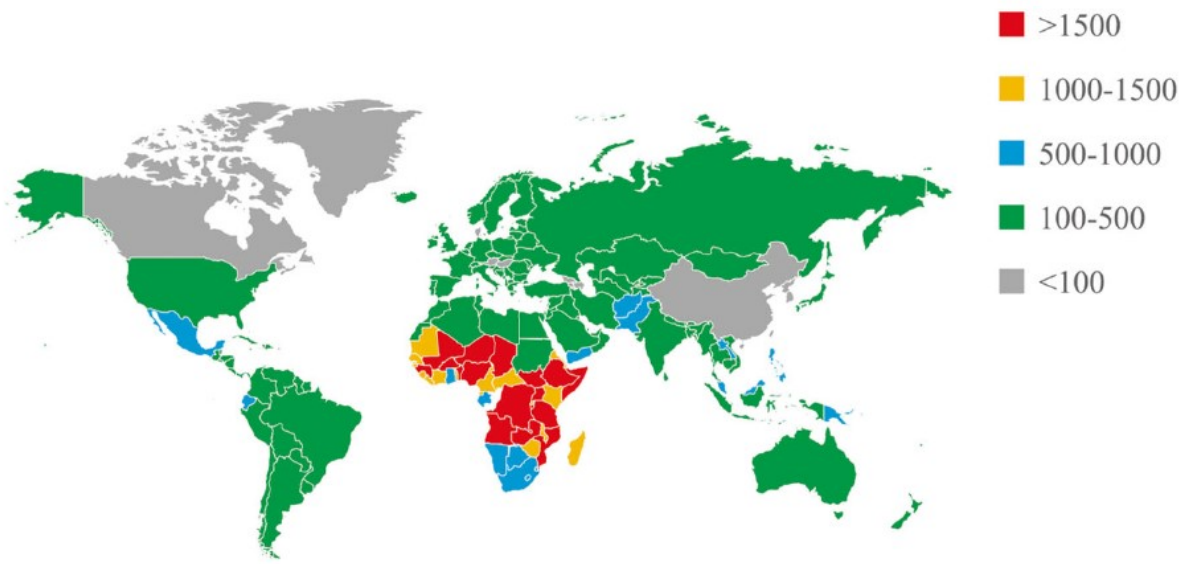


Figure 1.9: Global age standardised incidence rates of hypertensive disorders of pregnancy per 100,000 population in 2019

Reproduced from (81). Published by Springer Nature (open access article)

The prevalence rate of PE in SSA has been reported as 4.1% (95% CI 3.2-5.1) (210), which is comparable to global rates. Significantly, however, rates of PE with severe features appear to be particularly raised in SSA. A systematic review of pregnancies across Africa found that half of all cases of PE were diagnosed with severe features (211). Another review reported a prevalence rate of eclampsia in SSA of 1.5%, which is more than 5 times higher than estimated global rates (212). The reasons for the high rates of hypertensive disorders of pregnancy across SSA are complex. Increasing development and urbanisation in some regions over recent years has led to rising rates of chronic hypertension and obesity among the general population (213). Low rates of hypertension awareness, diagnosis, or medical treatment across SSA further exacerbate the problem (214). In less economically developed and rural areas, there is limited access to routine obstetric care, meaning that late presentation for emergency care is more common (211). High rates of PE with severe features may therefore reflect later presentation to obstetric care, rather than more severe disease phenotypes per se.

In addition to the socioeconomic complexities discussed, it has been postulated that Black ethnicity itself presents an inherent risk of PE. In a US-based study, women of AA heritage had substantially higher rates of PE when compared to those of European American background (OR 1.67, 95% CI 1.64-1.71) (174). This difference persisted after accounting for maternal age and socioeconomic factors. In contrast however, another US-based cohort study reported higher age-adjusted rates of PE in Black women, compared to Hispanic and non-Hispanic White women, but the differences did not persist after controlling for socioeconomic and cardiovascular risk factors (215).

Further studies of ethnically diverse populations have found SSA ethnicity to be a risk factor for early-onset or severe PE. A French cohort study compared characteristics of PE in White European women with women of North-West African (Maghrebian) ethnicity, and women of SSA ethnicity. This study reported that women of SSA ethnicity were significantly more likely to develop early-onset PE (diagnosis prior to 28 weeks' gestation); with rates of 22.3%, versus 7.1% in White European women, and 7.0% in Maghrebian women ($p<0.01$). The median gestational age of PE diagnosis was 32.4 weeks for SSA women, versus 33.8 weeks for White European women, and 34.3 weeks for Maghrebian women ($p=0.02$). However, there were no significant differences in birth weight or rates of fetal growth restriction between ethnic groups (176).

It is postulated that there may be a genetic component responsible for the higher rates of PE and PE with severe features seen in women of SSA ethnicity, which cannot be fully explained by differences in access to obstetric care, demographic, or cardiovascular characteristics (175, 176, 211). As earlier discussed in ***Section 1.6.3.1***, particular combinations of maternal KIR and fetal HLA-C alleles confer an increased risk of PE. Research has shown high rates of risk-conferring KIR and HLA-C variants in Ugandan (216) and Ghanaian (217) populations, which may contribute to the high rates of PE seen in SSA populations.

Another area of interest is the APOL-1 gene. 'High risk' variants of G1 and G2 alleles encoding the APOL-1 gene are only found in those with recent African ancestry and are known to confer an

increased risk of kidney disease in Black populations (218). A study of two US cohorts reported an association between high-risk *fetal* APOL-1 genotype and PE, with an OR in excess of 1.8 (123). Fetal high risk APOL-1 genotype was also associated with an increased sFlt-1:PlGF ratio. Interestingly, *maternal* APOL-1 high-risk genotype was not associated with PE. These findings were replicated in a later cohort study, which also found a significant association between fetal APOL-1 high-risk genotype and PE (OR 1.41, 95% CI 1.04-1.93; p=0.029) (124). However, neither study found an association between fetal APOL-1 genotype and gestational age, early-onset PE, birth weight, or placental pathological features. These findings indicate that fetal (and therefore paternal) genetic factors are important in determining the risk of developing PE, and the mechanism of injury conferred by high-risk fetal APOL-1 variants is not solely linked to defective placentation.

One further study reported an association between the presence of the maternal APOL-1 G1 allele and the risk of developing early-onset PE (OR 2.2, p=0.03) (219). However, this study found no association between maternal APOL-1 G1 *or* G2 risk allele positivity and the development of late-onset PE. Furthermore, in keeping with the aforementioned research, maternal APOL-1 high risk genotype (G1 *and* G2 allele positivity) was not associated with the overall risk of developing of PE.

1.8.2.3 The role of complement in pre-eclampsia in women of Sub-Saharan African ethnicity

There is a paucity of published data on the role of complement in the pathogenesis of PE in high-risk SSA populations. The evidence base is limited to women of AA heritage in the US. One study tested maternal and umbilical cord blood samples for alternative pathway activation fragment Bb from a cohort of 291 women, of whom 227 (78%) identified as AA (141). This found significantly elevated Bb concentrations in maternal blood samples taken from AA women with PE (1.26 μg/ml in PE vs. 0.96 μg/ml in AA controls, p=0.007). This study however did not report a subgroup analysis of Bb levels in AA women with PE, versus women from other ethnic groups. There were no reported differences in cord Bb concentrations between groups.

An earlier prospective study reported an association between AA ethnicity and high levels of Bb in blood samples taken from women in the first half of pregnancy, who later developed PE (17). From the overall patient cohort, women with a top decile Bb measurement were at a greater than 3-fold increased risk of developing PE later in pregnancy. Subgroup analysis showed that AA women had a 2-fold increased risk of having a Bb concentration in the top decile, when compared to non-AA women (RR 2.2 (95% CI 1.2-4.0; p=0.008)). However, this study only included relatively small numbers of women of AA ethnicity: 51 (7%) from a total cohort of 701 women.

Further research is needed into the potential contribution of complement activation to the high rates of PE seen in SSA women, to identify which pathways may be involved in disease pathogenesis.

1.9 Summary and hypothesis

PE is a common obstetric disorder, with potentially devastating adverse effects for mother and baby. There is a critical unmet need for alternative treatment options for PE, other than preterm delivery. The evidence presented above suggests a key role for complement dysregulation in the pathogenesis of PE, but the exact mechanisms and pathways involved remain a subject of debate, with findings based on small cohorts and with conflicting results. The ongoing development of complement-modifying agents raises the question of promising potential therapeutic targets, but there is currently insufficient evidence to support the widespread use of complement inhibition therapy in pregnant women with PE.

One of the key gaps in the current evidence base is whether abnormal concentrations of circulating complement reflect a general heightened inflammatory state in PE or are directly associated with placental complement-mediated injury. There are no studies that simultaneously compare complement components in the maternal and fetal circulation, and within placental tissue, for the same patient. A study of this nature would potentially provide further support for the role of complement activation in the pathogenesis of PE and may also provide insight into sequence of events leading to complement activation on a local and systemic level. This could help build a more compelling case for a future trial of complement inhibition therapy for women with PE. The body of research within this thesis will attempt to strengthen the current evidence base by addressing some of these shortfalls.

SPE disproportionately affects women with CKD; likely due in part to pre-existing endothelial damage, rendering the maternal vasculature more ‘sensitive’ to the demands of pregnancy. Although complement has been implicated in the pathogenesis of PE, the pathways involved and timing of complement dysregulation during pregnancy is not well understood. It also remains unclear if the same mechanisms apply in SPE, with very little published data on the role of complement in the pathogenesis of SPE in women with CKD.

SSA ethnicity has been identified as another risk factor for developing PE. This may be due in part to socioeconomic, cardiovascular, and genetic factors. There is little published data for the role of complement in PE pathogenesis in African women, but evidence suggests increased concentrations of complement activation fragments in AA women with PE.

An investigation into a wider panel of complement pathway components across a range of gestations in women considered at high risk of PE (women with CKD, and women of SSA ethnicity) would allow an assessment of the relative role of complement dysregulation in the high rates of PE seen in these specific populations. The results would also enable an evaluation of the utility of complement as a biomarker for PE and SPE, and as a potential therapeutic target in high-risk groups.

This thesis aims to test the hypothesis that PE is associated with excessive complement deposition within placental tissue, with concurrent complement activation within the maternal and fetal circulation; and therefore, that abnormal levels of circulating complement are reflective of complement-mediated placental tissue damage. Excessive complement activation may be triggered during very early pregnancy, leading to defective placentation, and further potentiated later in pregnancy by placental inflammation, disordered angiogenesis, and amplification of systemic (circulating) complement activation. The clinical syndrome of PE results.

A secondary hypothesis was that groups with a high prevalence of PE, and of PE with severe features, (women with CKD and women of SSA ethnicity) would exhibit a greater degree of systemic complement activation. Complement markers identified to be associated with PE may be potential therapeutic targets, and/or biomarkers of disease.

1.9.1 Thesis aims and objectives

The aims of this thesis are to investigate the precise complement pathways involved in the pathogenesis of PE in previously healthy women, and also in high-risk cohorts (women with CKD, and

women of SSA ethnicity). The identification of complement components and pathways associated with PE will build the evidence base towards potential future therapeutic targets.

The specific aims of the research are to:

- Investigate biomarkers of complement activation in the maternal and fetal circulation (C3, iC3b, Ba, properdin, C4, and C5b-9), and placental tissue (C1q, C4d, C3d and C9) in concurrently collected samples from previously healthy pregnant women with PE, and from healthy pregnant controls.
- Correlate placental complement deposition with complement markers in the maternal and fetal bloodstream, to determine the relative changes in tissue and circulatory complement activation in PE and in healthy pregnancy.
- Investigate biomarkers of complement activation in the maternal circulation (Ba, properdin and C5b-9) in a cohort of pregnant women with CKD; a proportion of whom developed SPE during pregnancy.
- Investigate markers of complement activation in maternal circulation (Ba, properdin, C3, C4 and C5b-9) in a Ghanaian cohort of pregnant women, and to compare the relative changes in PE, gestational hypertension, and healthy controls.
- Compare and contrast the patterns of complement pathways and activation seen in PE between women with no pre-existing medical conditions, and with 'high-risk' cohorts (women with CKD, and women of SSA ethnicity).

CHAPTER 2

METHODOLOGY

2 Methodology

2.1 Thesis overview

The aims and objectives of this thesis have been discussed in Chapter 1. In order to investigate the role of complement in the pathogenesis of PE, 3 distinct groups of women will be studied:

- Pregnant women with no pre-existing medical conditions: 'Birmingham Cohort'.

  Samples of maternal blood, umbilical cord blood, and placental tissue to be tested from women with PE (cases) and healthy pregnant controls.

  An additional validation cohort 'London Cohort' with maternal blood samples to be measured for complement components to validate the findings from this arm of the study.
- Pregnant women with CKD: 'CKD Cohort'.

  Women with CKD with samples of maternal blood collected longitudinally throughout pregnancy. A proportion of these women developed SPE during pregnancy, to be compared to those who did not.
- Pregnant women of SSA ethnicity: 'Ghana Cohort'.

  Pregnant women recruited in Accra, Ghana, who were all of SSA ethnicity. Samples of maternal blood to be tested from women with PE, women with gestational hypertension, and healthy pregnant controls.

A summary of the study designs and cohort details is shown in ***Figure 2.1***.

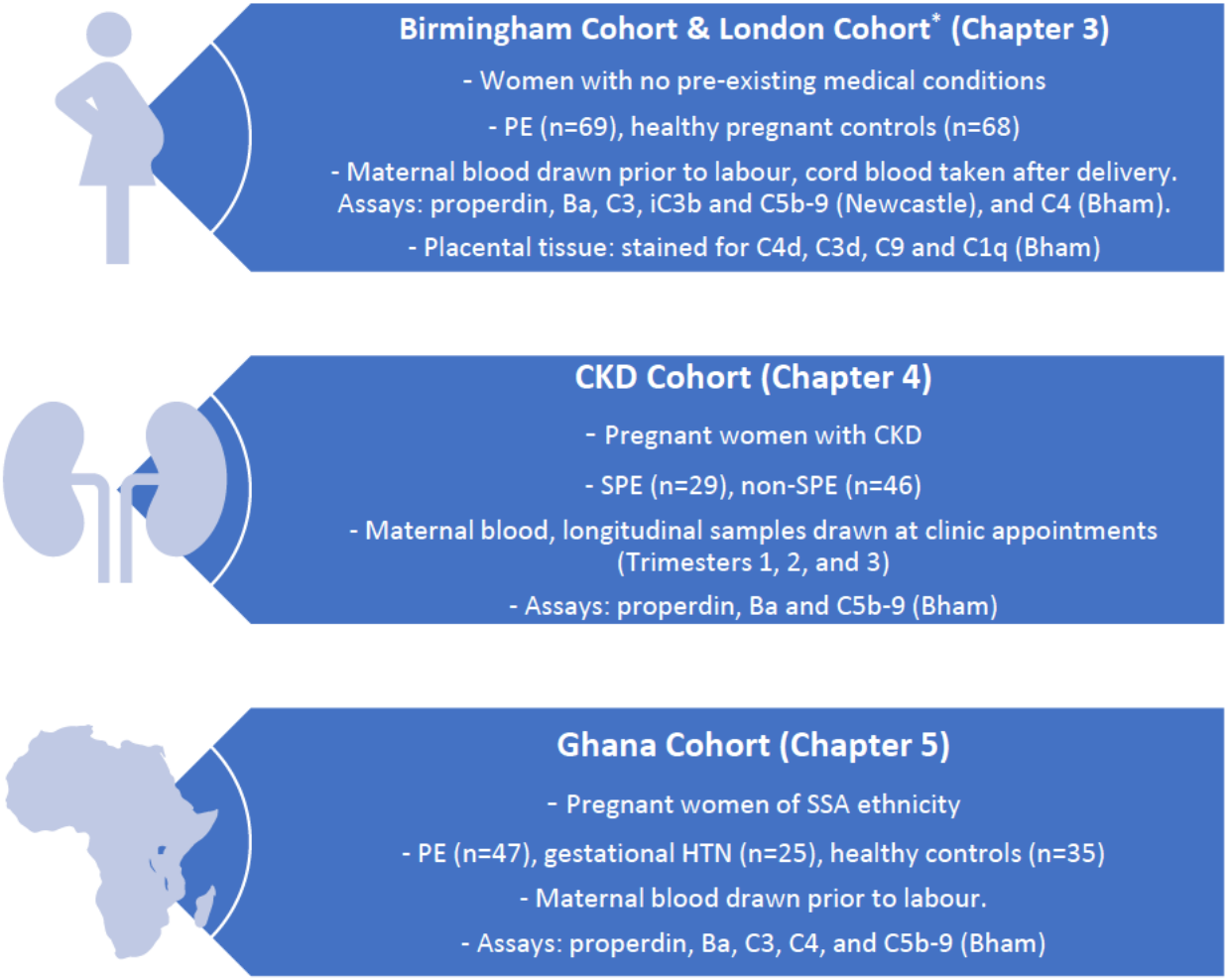


Figure 2.1: Summary of cohort details and study designs

*PE: pre-eclampsia; CKD: chronic kidney disease; SPE: superimposed pre-eclampsia; SSA: sub-Saharan Africa; HTN: hypertension. *London Cohort: maternal blood only, tested for properdin and Ba in Newcastle as a validation cohort.*

2.2 Statement of own work

The author of this thesis designed and planned the study methodology, and completed applications for ethical approval, sponsorship, and materials transfer agreement (MTA) documentation. The author exclusively recruited the patients from the Birmingham Cohort and collected all blood and placental tissue samples. Blood samples from the other study cohorts had previously been collected and were kindly donated for use in this thesis by collaborators.

Blood sample analysis for the Birmingham and London cohorts was completed by trained laboratory staff in Newcastle. The author was present at the time of testing and has a detailed understanding of the methods used. Complement assays for the CKD and Ghana cohorts were conducted in Birmingham by the author with supervision from a member of laboratory staff. Placental immunohistochemistry staining was conducted by expert histopathology staff. Evaluation and scoring of the stained tissue sections was conducted solely by the author, after receiving training in placental histology interpretation from histopathologists. Statistical analysis was carried out by the author with guidance from a statistician.

2.3 Investigating the role of complement in the pathophysiology of pre-eclampsia in previously healthy women

2.3.1 Study design overview

This was a cross-sectional study designed to examine the role of complement in the pathophysiology of PE in women with no pre-existing medical conditions.

Samples of maternal blood, umbilical cord blood and placental tissue were collected from study participants (women with a confirmed diagnosis of PE, and healthy pregnant controls): 'Birmingham Cohort'. Complement components were measured in each tissue type to enable a comparison of complement concentration in PE and healthy pregnancy, and for a comparison of complement activity across multiple tissue types for the same patient.

Additional samples of maternal blood were tested from a separate, pre-existing cohort of pregnant women recruited from St Thomas' Hospital, London ('London Cohort'). Blood samples were tested from women with no pre-existing medical conditions, with and without PE.

The primary outcome measure for this study was the complement concentration in maternal and umbilical cord plasma, and complement deposition in placental tissue, in pregnancies affected by PE as compared to healthy pregnant controls.

Secondary outcome measures were:

i) Correlation between placental complement deposition (immunoreactivity score) with circulating complement concentrations in maternal and fetal blood. This would allow an analysis of whether complement activation in maternal blood is reflective of complement activation in the fetal circulation, and of deposition at a tissue level within the placenta.

ii) Diagnostic value of circulating complement components in differentiating between PE and normal healthy pregnancy (using area under receiver operator curve (AUROC) analysis).

iii) Subgroup analyses to determine whether time of disease onset (early-onset versus late-onset PE) or mode of delivery (vaginal versus Caesarean section) is associated with complement dysregulation in maternal and fetal circulation, and in placental tissue.

iv) Subgroup analysis to determine whether Black ethnicity (Black versus non-Black ethnic group) is associated with complement dysregulation in the maternal circulation.

v) Correlation between circulating complement biomarkers, and biochemical and immune markers of inflammation.

Subgroup analysis of SSA versus non-SSA ethnicity was not possible for the Birmingham or London cohorts. Ethnicity was self-reported by study participants at the time of recruitment, using only the groups White, Asian, Black, or Mixed/Other. As such, it was not possible to identify ethnic subgroups within these broader categories, and analysis could only be conducted for Black versus non-Black women. There are likely to be multiple ethnic groups represented within those women who identified as Black, and specific differences between these groups might contribute to their individual risk of PE.

2.3.2 Ethical approval and Good Clinical Practice

Ethical approval for the research project was obtained via the University of Birmingham (UoB) Human Biomaterials Resource Centre (HBRC), Research Ethics Committee (REC) reference: 15/NW/0079, date of approval 8/5/2018 (see ***Appendix 2: REC approval*** and ***Appendix 3: HBRC study approval***).

The principles of Good Clinical Practice were adhered to throughout this research study (220). All specimens collected locally ('Birmingham Cohort') were stored securely in the HBRC and assigned an

anonymised patient and sample number for identification purposes. Clinical data was linked to the anonymised patient-specific number and held on a secure password-protected database. Where specimens were transferred between collaborators, MTA documents were completed and signed off by the research and development (R&D) teams of the institutions involved.

2.3.3 Funding

A research grant was awarded by Queen Elizabeth Hospital Kidney Patient Association (QEKPA) in January 2018, which helped to fund this study, and both other arms of the research (see ***sections 2.4 and 2.5***). Further funding was provided by the Renal Research Fund, Queen Elizabeth Hospital Birmingham (QEHB).

2.3.4 Study population and recruitment

Study participants were recruited from Birmingham Women's Hospital (BWH) between August 2018 and July 2019. The full study inclusion and exclusion criteria are detailed in ***Table 2.1***.

Women with a confirmed clinical diagnosis of PE (according to international statement consensus criteria (70-72, 78); see ***Table 1.3***) were recruited as cases at the point of hospital admission. Healthy pregnant women with no pre-existing medical conditions were recruited as controls from a pre-operative elective Caesarean section clinic and induction of labour suite. This only included subjects undergoing planned delivery for non-emergency reasons. Study participants were recruited by convenience sampling, with no deliberate matching between cases and controls.

The above locations were chosen for recruitment of controls to achieve a balance of patients delivering vaginally and by Caesarean section. It is unclear whether mode of delivery could impact upon complement activation, so ensuring a mixture of delivery types would allow subgroup analysis, and a representative comparison between PE cases and healthy controls. An additional

consideration was that healthy pregnant women, unlike women with PE, seldom attend hospital prior to delivery and are usually managed under midwifery-led care in the community. Attending pre-operative clinics and induction of labour suite was therefore the most practicable way of recruiting healthy women in late gestation who were not yet in established labour.

Eligible women were interviewed about the aims of the research study and given a Patient Information Sheet (see ***Appendix 4***). After providing time to consider the written information, study participants were required to sign a Patient Consent Form (see ***Appendix 5***) to indicate their informed consent for participation.

A flow chart detailing the Birmingham Cohort recruitment is displayed in ***Figure 2.2***.

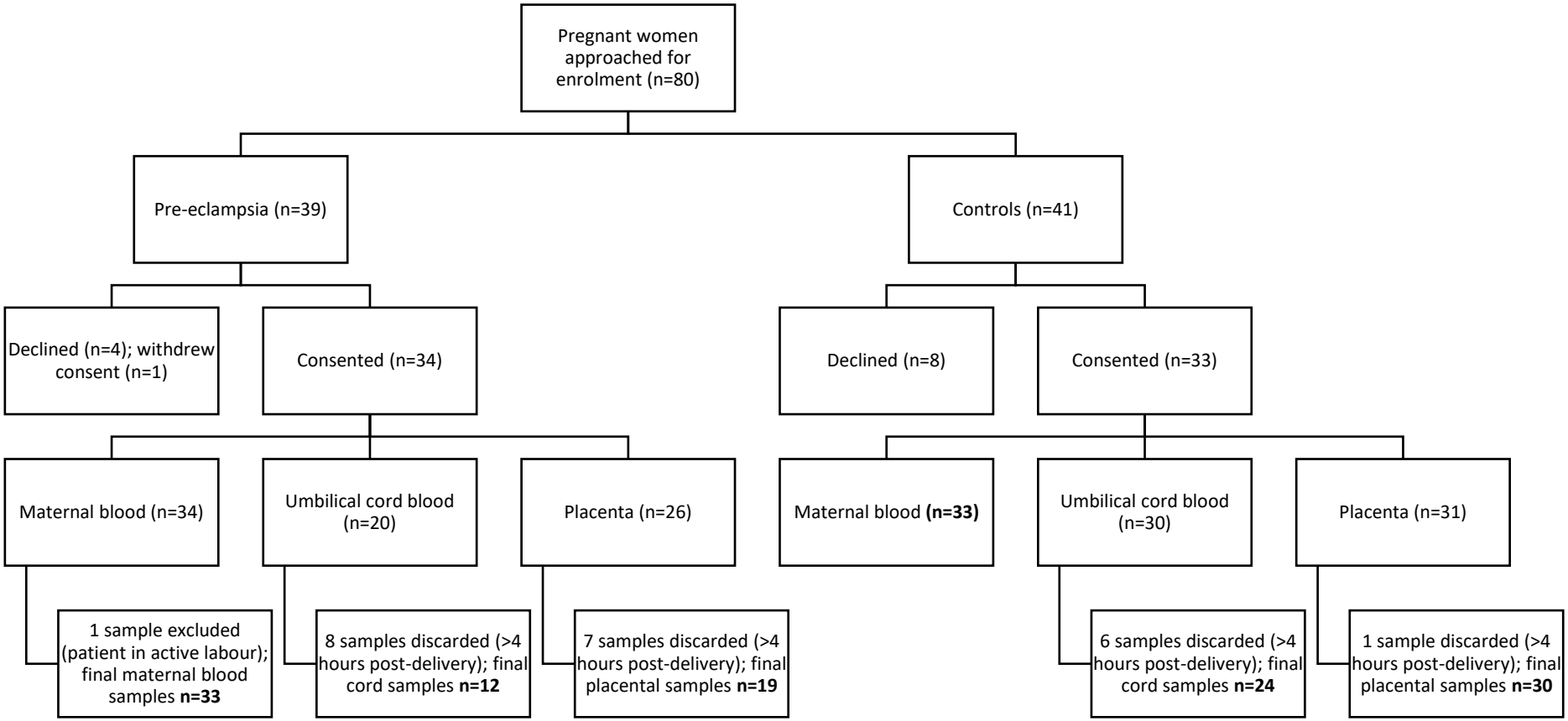


Figure 2.2: Birmingham Cohort recruitment flow chart

2.3.4.1 Inclusion and exclusion criteria

Study inclusion and exclusion criteria are summarised in ***Table 2.1***. Women with a known history of CKD and chronic hypertension or other co-morbidities that can affect BP control (including endocrine conditions and other secondary causes of hypertension) were excluded from this study. These conditions predispose to proteinuria and elevated BP, thus complicating and sometimes masking the diagnosis of PE (79).

Subjects with a history of conditions known to be associated with complement dysregulation, including recurrent miscarriage (4, 15), chronic autoimmune disease (221, 222), and/or known disorders of complement regulation (including SLE and APS) (45, 49) were also excluded, to avoid introducing confounding factors. Recurrent miscarriage was defined as "the spontaneous loss of three or more consecutive pregnancies with the same biological father in the first trimester" (223).

Control patients were only recruited from pre-operative clinic or induction of labour suite where Caesarean section or induced delivery was planned for non-emergency reasons (such as breech presentation, previous Caesarean delivery, and post-dates pregnancy). Patients undergoing expedited delivery for obstetric complications including IUGR, reduced fetal movements, obstetric cholestasis, and gestational diabetes were excluded.

Table 2.1: Birmingham Cohort study inclusion and exclusion criteria

Inclusion criteria	*Exclusion criteria*
Cases: Women with a confirmed diagnosis of PE (70, 71, 78)	Women with a known history of: • CKD • chronic hypertension • recurrent miscarriage • chronic autoimmune disease
Controls: Healthy pregnant women with no pre-existing medical conditions	Women with known abnormalities of complement regulatory genes or a known disorder related to complement dysregulation
	Subjects unable to consent to participation in the study because of language or physical barriers and/or learning difficulties (translators will be allowed)
	Age < 18 years

2.3.5 Sample collection

Samples of maternal plasma (EDTA) and serum were collected prospectively from cases and controls at the point of hospital admission, within seven days prior to delivery, and before established labour. Two EDTA bottles and two serum bottles (7.5 ml volume Sarstedt: S-Monovette® tubes) were collected from each patient. Umbilical cord blood plasma (EDTA) and serum samples were collected immediately following birth (one bottle of each, 2.7 ml volume Sarstedt: S-Monovette® tubes). Two full thickness tissue sections measuring 1 cm^3 were cut from a central portion of each placenta using a scalpel, under aseptic conditions. All samples were transported directly thereafter to the HBRC, UoB, for anonymisation, processing and storage.

Maternal and umbilical cord blood samples were centrifuged at 3000 rpm for 10 minutes, separated into 0.5 mL aliquots of plasma and serum, and frozen at -80^{0}C within four hours of collection to minimise ex vivo complement activation. Placental tissue was formalin-fixed and paraffin-embedded (FFPE) to produce a tissue block using a Leica ASP300S Advanced Tissue Processor (Leica Biosystems, Nussloch, Germany). Fresh placental tissue was placed in 10% formalin for fixation, then placed into a processing cassette for 12 hours to dehydrate. Processed tissue was then placed into an embedder containing liquefied wax at 67^{0}C, then left to cool on a cold plate to form a FFPE placental tissue block.

2.3.6 Laboratory analyses: complement

Maternal and umbilical cord blood samples were tested for:

- properdin and Ba (specific to the alternative complement pathway),
- C4 (indicative of classical and lectin pathway activity),
- C3 and iC3b (indicative of both alternative and classical pathway activity), and
- C5b-9 (MAC; resulting from activation of any of the three complement pathways).

Properdin acts as a positive regulator of the alternative complement pathway by stabilising the amplification loop C3 convertase, significantly prolonging in half life, and therefore potentiating its action (224). Ba is an activation fragment formed from the cleavage of Factor B, and elevated levels are indicative of alternative pathway activation (225). Properdin and Ba were selected for testing, given the existing evidence indicating increased alternative pathway activity in PE as outlined in Chapter 1. This includes high early pregnancy concentrations of Bb and Factor B in women who later developed PE (17, 138), and increased levels of Bb in women with PE with severe features (140). Furthermore, other diseases such as aHUS are known to be mediated by alternative pathway activation (7), have some overlap in clinical presentation with HELLP syndrome and severe PE (58), and have been successfully treated using complement inhibition medication (80). Thus, detailed investigation of alternative pathway activity in the present study is warranted. Properdin is a complement pathway regulator (rather than an activation fragment) and may therefore be a more stable measure of alternative pathway activity. Ba may be a more reliable measure of factor B split products than Bb, which binds to C3 convertases in the fluid phase and on cell surfaces (2).

Testing of C4, iC3b and C3 would provide an indication of activation of all three complement pathways. A nephelometry assay was used for C4, which detects both native and activated forms of the protein.

In contrast, an in-house C3 assay was designed to detect native, non-activated C3 which may differ from nephelometry assays that detect both native C3 and activation fragments. iC3b is a complement split product of C3b, and is a biomarker of specific complement activation, often driven by a pathogenic process. Sandwich assays were used to generate antibody specificity (see ***section 2.3.6.1*** for detailed methods). For the C3 assay, both capture and detection antibodies bind to native C3. However, clone 2898 (capture antibody) only binds the C3a domain of C3, and Clone 3 (detection antibody) binds C3, C3b, iC3b, and C3d. In combination, only antigens with BOTH the C3a domain and the C3b domain are detected (hence native C3). In the case of the iC3b assay, the capture

antibody mAb807 binds iC3b and C3dg, and the detection antibody BH6 binds C3b, iC3b and C3c. Therefore, the only analyte that can bind both antibodies is iC3b. The measurement of iC3b:C3 ratio can provide a more accurate indicator of complement production (iC3b) relative to consumption (C3) (226).

Samples were tested for C5b-9, to assess whether there was evidence of complement activation in PE that extended all the way to formation of the terminal complement complex (TCC). This has clinically relevant implications, due to eculizumab (a C5 inhibitor) already being licensed for use in the treatment of other complement-mediated diseases (55, 58, 167, 168).

2.3.6.1 Newcastle University in-house complement assays

Maternal and umbilical cord plasma samples were transferred to Newcastle University (Translational and Clinical Research Institute, under the supervision of Professor Claire Harris), and tested for Ba, properdin, iC3b, C3, and C5b-9. This laboratory works closely with the National Renal Complement Therapeutics Centre and has extensive expertise in researching complement-mediated disease. Complement assays for the components listed above had been developed in-house, and the Newcastle laboratory offered to test maternal and umbilical cord plasma samples for the Birmingham Cohort free of charge. Therefore, for cost and pragmatic reasons, no additional complement markers were tested for; except C4 which was tested using serum samples in Birmingham (see ***section 2.3.6.2***).

Complement biomarker concentrations were determined using electrochemiluminescent immunoassays using MesoScale Diagnostics (MSD; Rockville, MD) Gold 96 well Streptavidin QuickPlex plates (Ba, properdin, C3, and C5b-9), or Small Spot Streptavidin plates (for iC3b). Streptavidin QuickPlex plates were blocked with 150 µl block buffer (phosphate buffered saline (PBS) + 3% bovine serum albumin (BSA) + 10mM EDTA, pH 7.4). Plates were incubated for 1 hour at room temperature at 800 rotations per minute on a horizontal plate shaker. Small Spot Streptavidin plates were not blocked. All capture antibodies were biotinylated prior to the experiment using Pierce EZ-

Link Sulfo-NHS-Biotin according to the manufacturer's instructions (227). Capture antibodies were prepared according to ***Table 2.2***.

Table 2.2: Capture antibody preparation

Assay	Capture antibody	Stock concentration of biotinylated antibody (mg/ml)	Coating concentration (µg/ml)
Ba	Clone D22/3 (Hycult)	0.5	2
Properdin	Clone SIM 295 (Hycult)	1.6	2
C3	Clone 2898 (Hycult)	0.45	2
iC3b	Ab807 (HuCal, in-house)	1.5	2
C5b-9	aE11 (Hycult)	0.36	1

Assay diluent (PBS pH 7.4 + 1% BSA + 10 mM EDTA) required to generate the final coating concentration.

Blocked plates were washed 3 times, droplets were removed by tapping on laboratory paper towel, and 25 µl capture solution was added rapidly to the singleplex assays. At no point during the assay were the plates allowed to air dry. Plates were incubated overnight at 4^0C.

Plasma samples and calibrators were thawed rapidly at 37°C, briefly vortexed, centrifuged to recover droplets and immediately stored on ice. Samples were diluted in assay diluent according to ***Table 2.3***. Plasma samples were diluted in assay diluent (PBS pH 7.4 + 1% BSA + 10 mM EDTA) in a stepwise fashion to generate the final dilution on a 96-well polypropylene (PP) plate, later to be transferred to the MSD plate. Samples were mixed on the PP plate after each dilution for either 15 seconds at 1500 rpm (dilution 1: properdin) or for all other dilutions for 15 seconds at 1000 rpm.

Table 2.3: Plasma sample dilution plan

Assay	Plasma Dilution 1	Plasma Dilution 2	Plasma Dilution 3	Final Dilution
Ba	1/5	-	-	1/5
Properdin	1/100	1/20	-	1/2,000
C3	1/50	1/50	1/8	1/20,000
iC3b	1/5	1/4	1/10	1/200
C5b-9	1/5	1/4	-	1/20

Calibrators were processed according to ***Table 2.4***. A pre-determined volume of assay diluent (PBS pH 7.4 + 1% BSA + 10 mM EDTA) was added to each of the calibrator aliquots to generate the top point on the standard curve. Each of the top standards was diluted in a 1:4 serial dilution for 6 further dilutions to create 7 dilutions for the standard curve. An eighth point contained a blank (buffer only). Calibrator solutions were transferred to the 96 well PP plate. The sample/calibrator plate was stored on ice for no more than 30 minutes prior to transfer to the MSD plate.

Table 2.4: Calibrator preparation

Assay	Volume of calibrator stock (µl)	Volume of assay diluent to add to stock (µl)	Top standard concentration (ng/ml)
Ba	10 (40 µg/ml)	190	2000
Properdin	10 (9.16 µg/ml)	190	458
C3	10 (40 µg/ml)	190	2000
iC3b	10 (20 µg/ml)	190	1000
C5b-9	10 (50 µg/ml)	190	2500

MSD plates were washed 3 times, and 25 µl of assay diluent was added to each well. Diluted samples and calibrators on the PP plate were transferred using an electronic multichannel pipette and reverse pipetting (25 µl for all assays) onto specific wells of the MSD plate. Plates were incubated for 1 hour at room temperature at 800 rpm.

Detection antibodies were prepared according to ***Table 2.5*** in assay diluent (singleplex). Assay plates were washed as described above and 25 µl volume was transferred to the washed plates as described above.

Table 2.5: MSD detection antibody preparation

Assay	Antibody	Stock Concentration (mg/ml)	Final Dilution (µg/ml)
Ba	P21 (Hycult)	1.4	2
Properdin	Clone 2.9 (Hycult)	1.6	2
C3	mAb3 (HM2198; Hycult)	0.72	2
iC3b	BH6 (Hycult)	0.77	2
C5b-9	E2 anti-C8 (gift from Cardiff University)	1.2	2

Plates were washed as described above and 150 µl of 2 x read buffer was added to each well. The plates were 'read' immediately on the MSD QuickPlex SQ 120 instrument. To avoid inconsistency in read time, wash times and buffer addition was staggered.

The concentration of each analyte was determined by interpolation from the standard curves using the MSD Workbench software v15.0. Plasma samples were tested in duplicate, with the average value taken.

Internal quality control procedures were used to measure the performance of the assays used. Intra-assay coefficient of variation (CV) was measured by testing plasma samples from an in-house control cohort in replicate preparations on one plate. The intra-assay coefficients of variation (CVs) were 0.9% (properdin), 3.3% (Ba), 2.3% (iC3b), 2.0% (C3), and 3.3% (C5b-9). A minimum of two control plasma samples (low/high for analyte) were used to measure inter-assay variance over multiple plates, and inter-assay CVs were 12% (properdin and Ba), 7% (iC3b), 8% (C3), and 12.5% (C5b-9).

2.3.6.2 C4 assay, University of Birmingham

Serum samples of maternal and umbilical cord blood were tested singly for C4 at UoB Clinical Immunology Service (CIS) using a Hitachi Cobas 6000 Turbidimeter (c501 module), (Roche Diagnostics, West Sussex, UK). The Cobas 6000 analyser is fully automated and has ready to use reagents included with test kits. C4 concentrations were measured using an immunoturbidimetry

approach. 1:10 dilutions of serum samples (maternal and umbilical cord blood) were made and pipetted into a cuvette. A reagent was then added, which causes clustering of antibodies and antigens to form an immune complex precipitate. Light is passed through the sample, and the absorbency is measured using immunoturbidimetry and compared with a standard curve to calculate the concentration of C4. The intra-assay CV for the C4 assay used was 0.7-1.1%, and inter-assay CV 1.4-1.6%. See ***Table 2.6***.

2.3.7 Laboratory analyses: biochemical and immunological markers

Maternal and umbilical cord serum samples were tested for the following biochemical and immunological markers:

- Creatinine
- Cystatin C
- Urea
- Uric acid
- Beta-2 microglobulin (B2M)
- Serum free light chains (sFLCs) - kappa and lambda
- Immunoglobulins (Ig) G, A and M
- Lactate dehydrogenase (LDH)
- High-sensitivity C-reactive protein (HS-CRP)

Markers of excretory kidney function (creatinine, urea, cystatin C, uric acid), haemolysis (LDH) and inflammation (HS-CRP) were selected for testing to provide additional clinical context. Renal dysfunction and haemolysis are indicative of PE with severe features (71, 78). Measurement of these markers would allow correlation with markers of complement activation, and an assessment of whether complement activation is associated with PE severity. Measurement of B2M, SFLCs, and Ig

G, A and M was performed to provide an assessment of adaptive immune system activity in PE. Previous research has shown increased concentrations of sFLCs and B2M in PE, as well as reduced IgG (142). Furthermore, this study found that sFLCs and IgG were independently associated with PE; and raised B2M levels were associated with adverse clinical outcomes, raising the possibility of its use as a biomarker for PE.

It was not possible to test samples for PlGF or sFlt-1:PlGF ratio due to the cost, time and laboratory staffing constraints of the research project.

Serum samples were tested at the CIS laboratory, UoB, using the same equipment and methodology as outlined for C4 assays in ***Section 2.3.6.2***. All samples were measured together on the same day as C4, to minimise free-thaw cycles. An overview of the assays used and associated CVs are detailed in ***Table 2.6***.

Table 2.6: Summary of biochemical and immunological assays tested with associated CVs

Assay	Product ID	Catalogue no.	Intra-assay CV	Inter-assay CV
C4	C4-2	03001962 322	0.7-1.1%	1.4-1.6%
Creatinine	CREP2	03263991 190	0.6-0.9%	1.1-1.4%
Cystatin C	CYSC2	06600239 190	0.7-1.7%	1.4-2.2%
Urea	UREAL	04460715 190	0.9-1.0%	1.1-1.2%
Uric acid	UA2	03183807 190	0.7-0.9%	1.5-1.6%
B2M	B2MG	11660551 216	0.8-1.2%	1.4-1.5%
IgG	IGG-2	03507432 190	1.0-1.2%	1.5%
IgA	IGA-2	03507343 190	0.7-1.1%	1.4-1.8%
IgM	IGM-2	03507190 190	1.3-1.6%	2.0-3.8%
Kappa	KAPP2	06749976 190	0.9-1.9%	2.4-2.9%
Lambda	LAMB2	06749992 190	1.1-1.7%	1.4-1.9%
HS-CRP	CRPHS	04628918 190	1.0-1.2%	1.3-2.6%
LDH	LDHI2	03004732 122	0.7-0.8%	0.9-1.0%

Product ID, catalogue numbers and CV information available from Roche Diagnostics (228). CV: coefficient of variation.

2.3.8 Placental immunohistochemistry

FFPE placental tissue blocks were placed on wet ice to cool for 20 minutes before being cut to 4 μm thickness (except 1 section cut at 2 μm thickness for C1q staining) using a microtome at HBRC, UoB. Sections were then placed on VWR SuperFrost® slides and heat-treated at 65^0C for 30 minutes.

Placental tissue sections were stained for the following complement components:

- C3d (a degradation product of C3 in all three complement pathways)
- C4d (a marker of classical and lectin pathway activity)
- C9 (a component of MAC; also termed TCC)
- C1q (a marker of classical pathway activity)

For each subject, a slide was also stained for haematoxylin and eosin (H&E), to provide a histological reference point.

The above complement markers were selected for testing, as they would allow evaluation of activity of all 3 complement pathways, as well as the TCC, in PE and healthy pregnancy. These immunohistochemical stains were already being widely used by the histopathology laboratory for renal, liver and dermatology specimens, and thus protocols for their use had already been firmly established.

Immunohistochemical staining was carried out on placental tissue sections in the Histopathology Department (QEHB), using the Dako Autostainer Link 48 (Agilent, CA, USA). Placental tissue sections were deparaffinised and rehydrated, then incubated with a trisaminomethane (TRIS)/EDTA buffer solution to enable antigen retrieval. Sections were washed in PBS, then endogenous peroxidase was blocked in 3% hydrogen peroxide solution for 10 minutes, to reduce background artefact staining. Sections were washed again and antibodies applied for 30 minutes at room temperature – see ***Table 2.7.*** Prior to C1q staining, slides were protease-digested (using Dako Proteinase K S3020) so as to enhance antigen retrieval; in line with established local laboratory protocol in use for C1q staining of

kidney tissue specimens. For C4d, a renal transplant nephrectomy specimen with antibody mediated rejection was used as a positive control. Normal kidney and brain tissue were used as negative controls.

Table 2.7: Placental immunohistochemistry antibodies

Antibody	Origin	Dilution	Supplier	Role in complement cascade
C3d	Rabbit pAb	1:200	DBBiotech DB106-01	Alternative pathway component
C4d	Rabbit pAb	1:40	Biomedica BI-RC4d	Classical and MBL pathway component
C9	Mouse mAb	1:2000	abcam ab17931	TCC component
C1q	Rabbit pAb	1:60000	Dako A0136	Classical pathway component

pAb: polyclonal antibody; mAb: monoclonal antibody; MBL: mannose-binding lectin; TCC: terminal complement complex.

2.3.8.1 Immunohistochemical analysis of placental tissue sections

Immunostained slides were scanned using high resolution digital software to an online virtual repository (Aperio eSlide Manager; Leica Biosystems, Nussloch, Germany). Placental sections were scored semi-quantitatively by the author, blinded to clinical data, using a modified placental immunoreactivity score (229, 230) based upon staining intensity and distribution at the STB membrane. Intensity was scored as 0 = negative, 1 = weak (visible at 40x magnification), 2 = intermediate (visible at 10-20x magnification), or 3 = strong (visible at 4x magnification). The distribution was scored according to the proportion of cells staining positive, with 0 = negative, 1 = 1-10% cells staining positive, 2 = 11-50%, 3 = 51-80%, and 4 = 81-100%. Scores were multiplied to calculate a composite placental immunoreactivity score (range 0-12). A score of zero was classified as “absent” immunoreactivity, with scores of 1-8 classified as “focal” and 9-12 as “diffuse” (229).

Each slide was evaluated in ten random fields and scored on three separate occasions by the author. The median composite score was determined as representative for that slide. A random sample of

slides were verified independently by experienced histopathologists, to ensure consistency and accuracy of scoring.

2.3.9 Clinical outcomes

Demographic information and clinical outcome data were collated from patients' obstetric records. The following data points were recorded:

Maternal age (years)

Ethnicity

BMI at antenatal booking (kg/m^2)

Gravidity and parity

Pregnancy outcomes:

- Live birth
- Twin pregnancy
- Birth weight (g)
    - Low birth weight (<2500 g)
- Gestation at delivery (weeks)
    - PTB (gestation <37 weeks)
    - Small for gestational age (SGA) baby (birth weight <10^{th} centile for gestation)
- Mode of delivery (Caesarean section / vaginal delivery)
- Admission to neonatal unit (NNU)
- Presence or absence of PE
    - Gestation at PE diagnosis (weeks)

Laboratory data:

- BP (peak recorded systolic and diastolic BP prior to onset of labour)
- Peak urinary protein (urine albumin to creatinine ratio (ACR))

The above clinical outcome points were recorded to allow a comparison of pregnancy outcomes between PE and control groups, and to control for any potential confounding factors or significant demographic differences between groups. Many of the data points relate to adverse pregnancy outcomes and would put into the context the severity of disease in the PE group. Furthermore, this would allow a subgroup analysis of how complement markers correlated with PE severity.

2.3.10 Sample size and statistical analysis

Based on reported incidence rates (31), a placental tissue sample size of 19 patients per arm would be sufficient to detect a 50% difference in C4d deposition between cases of PE and controls. Further to this, a blood sample size of 50 patients per arm would be sufficient to detect a statistically significant difference in C5b-9 concentrations between cases of PE and pregnant controls; based on published data (150, 152). In both instances, these calculations assumed α=0.05 and 80% power. Despite these targets, patients were recruited by convenience sampling within a fixed timeframe of 1 year, given the relative limitations in time, resources, and funding available. This would allow sufficient time to collect, process and analyse blood and placental tissue samples and to prepare data for thesis submission.

For comparisons between PE and control groups, nominal variables were assessed using Fisher's exact tests. The distributions of ordinal and continuous variables were assessed graphically using Q-Q plotting prior to analysis. Those that were approximately normally distributed were reported as means ± standard deviation (SD), with p-values derived from independent samples t-tests. Medians, interquartile ranges (IQRs) and Mann-Whitney U tests were used otherwise.

Blood markers found to differ significantly between groups were assessed using a receiver operating characteristic (ROC) curve approach, with diagnostic accuracy for PE summarised using area under the curve (AUROC) and associated standard error (SE). Optimal cut-off values were identified using Youden's J statistic, with associated specificity and sensitivity reported.

In order to adjust for the effect of gestational age at the time of blood draw, maternal plasma complement markers were also assessed using a linear regression approach. These models initially included the group (PE vs. control), gestational age at the time of the blood sample, and an interaction term as covariates. Where this interaction term was non-significant, it was removed from the model, and the analysis repeated. Goodness-of-fit of the resulting models was then assessed graphically. Where poor fit was detected due to skew in the dependent variables, values were $\log_{10}$-transformed to reduce the degree of skew, and the analysis repeated. The resulting coefficients were then anti-logged and converted into percentage differences, in order to simplify interpretation.

Correlations between variables were quantified using Spearman's correlation coefficients (rho).

For analysis of biochemical and immunological markers in PE and healthy control pregnancy, biomarkers known to be affected by excretory renal function were entered into binary logistic regression models, with PE as the dependent variable, and creatinine as a continuous covariate. This allowed an assessment of which biomarkers were independently associated with PE after correcting for differences in excretory renal function.

In order to assess which complement components were independently associated with PE after controlling for differences in biochemical and immunological markers, multivariate logistic regression analysis was conducted. A binary logistic regression model was initially produced with PE as the dependent variable, and a forward stepwise approach used to select biochemical / immunological variables for inclusion and sequentially remove interacting terms. Complement components were then added in alongside these variables in subsequent models. The Hosmer-Lemeshow test was used to assess goodness-of-fit of the resulting models, and results were reported as OR for PE diagnosis per unit increase in biomarker, with associated 95% confidence intervals.

All analyses were performed using IBM SPSS 28 (IBM Corp. Armonk, NY). Significance was primarily assessed using the standard threshold of $p<0.05$ throughout this thesis. In some sections, due to the

number of analyses being performed, there is an increased risk of obtaining false-positive results. However, applying adjustment for multiple comparisons would be at the cost of a grossly inflated false-negative rate, given the relatively small sample size (231). Therefore, in an attempt to mitigate the impact of multiple comparisons in those analyses with large numbers of comparisons, the Bonferroni-corrected significance threshold is indicated in the tables, where appropriate.

2.3.11 London Cohort

A separate cohort of patients with PE and healthy pregnant controls had been recruited at St Thomas' Hospital, London between 2015-18. Ethical approval was granted through the Integrated Research Application System (IRAS; study ID 83429, October 2014). Samples of maternal plasma and serum from women with no pre-existing medical conditions were already held within a biobank for use in cohort studies of pregnancy outcomes in PE. Permission was granted to use a random selection of samples from PE cases and healthy pregnant controls, to serve as a validation cohort for the present research study. Subjects were selected at random from the database.

Maternal blood samples were collected at the time of PE diagnosis (cases), and at routine antenatal hospital visits (controls). The same inclusion and exclusion criteria were applied as for the Birmingham Cohort, with the addition of multifetal pregnancies being excluded for both cases and controls. Blood samples had been centrifuged, separated into 0.5ml aliquots, and frozen at -80^{0}C within 4 hours of collection in a local research facility.

Plasma samples were transferred to Newcastle University, where they were tested for properdin and Ba using the same methodology as described in ***Section 2.3.6.1***. Due to limitations including costs and laboratory staffing, only complement components that had shown significant differences between PE and healthy pregnancy for the Birmingham Cohort were tested. A total sample size of 70 was tested (35 PE cases and 35 controls), so that a single MSD plate could be used for each assay. Samples were again tested in duplicate, with the average value taken.

Initially, statistical analyses of complement component concentrations were performed separately for Birmingham and London cohorts. However, analyses were also performed after combining the cohorts, to maximise the sample size, according to the statistical analysis methods outlined in ***Section 2.3.10***.

2.4 Investigating the role of complement in the pathophysiology of superimposed pre-eclampsia in women with chronic kidney disease

2.4.1 Study design overview

This was a single-centre, cross-sectional study designed to investigate the role of complement in the pathophysiology of SPE in women with CKD: 'CKD Cohort'. Serial samples of blood had historically been collected from women with CKD attending a specialist renal-antenatal clinic during pregnancy. A proportion of these women developed SPE during pregnancy. Maternal blood samples had been stored in a biobank and were tested for markers of complement activation in the present study.

The CKD Cohort has already been described in a thesis by Dr Nadia Sarween, UoB (232) for a previous study examining immunological and angiogenic markers in SPE. For the present body of research, blood samples from a proportion of women within this original cohort were tested for markers of complement activation. Not all patients included in the original research study by Sarween et al. had remaining aliquots of blood available for analysis in the present study. However, subjects with remaining blood samples were representative of the original study cohort.

Where available, three blood samples were tested for markers of complement activation for each patient:

- one drawn during early pregnancy (0 to 16 weeks gestation),
- one from mid-pregnancy (16+1 to 27+6 weeks gestation), and
- one from late pregnancy (28+ weeks gestation).

A proportion of the study participants had blood samples available for only 1 or 2 of these defined timepoints.

The primary outcome measure for this study was maternal plasma complement concentrations in women with CKD and SPE, as compared to women with CKD who did not develop SPE, in early, mid and late pregnancy.

Secondary outcome measures were:

i) The predictive value of each complement component for SPE, for each of the 3 specified timepoints, using AUROC analysis.

ii) Subgroup analysis of the association between complement component concentration and composite adverse pregnancy outcome in women with CKD.

2.4.2 Ethical approval

Health Research Authority approval for this study was obtained through the UoB HBRC (REC reference: 15/NW/0079; date of original approval 21/1/2014; renewed for the present study 8/5/2018). See ***Appendix 2 and 3***.

2.4.3 Study population and recruitment

Women with pre-existing CKD and new diagnoses of CKD during pregnancy were recruited prospectively from a specialist renal-obstetric antenatal clinic during routine monthly clinic visits between 2011 and 2016. Patients were interviewed about the aims of the research study and given a Patient Information Sheet (see ***Appendix 4***). After providing time to consider the written information, study participants were required to sign a Patient Consent Form (see ***Appendix 5***) to indicate their informed consent.

Convenience sampling was employed to recruit patients, with all clinic attendees being invited to participate. Women were enrolled at all stages of pregnancy. Patients with all causes and stages of CKD were recruited, including those with renal transplants, underlying autoimmune disease, and

with chronic hypertension, so as to maximise the potential sample size. Inclusion and exclusion criteria are shown in ***Table 2.8***.

A flow chart detailing the recruitment for the CKD Cohort is shown in ***Figure 2.3***.

Table 2.8: Inclusion and exclusion criteria: CKD Cohort study

Inclusion criteria	*Exclusion criteria*
Confirmed diagnosis of CKD as per NICE definition (233): • Persistent reduction in kidney function > 3 months with: • GFR <60 ml/min/1.73m^2 *or* • markers of kidney damage (albuminuria, structural abnormality)	Subjects unable to consent to participation in the study because of language or physical barriers and/or learning difficulties (translators will be allowed)
CKD stages 1 to 5, including those with a kidney transplant or on dialysis	Age < 18 years
CKD of any cause, including • Autoimmune disease • Chronic hypertension	Resolved acute kidney injury

CKD: chronic kidney disease; NICE: National Institute for Health and Care Excellence; GFR: glomerular filtration rate.

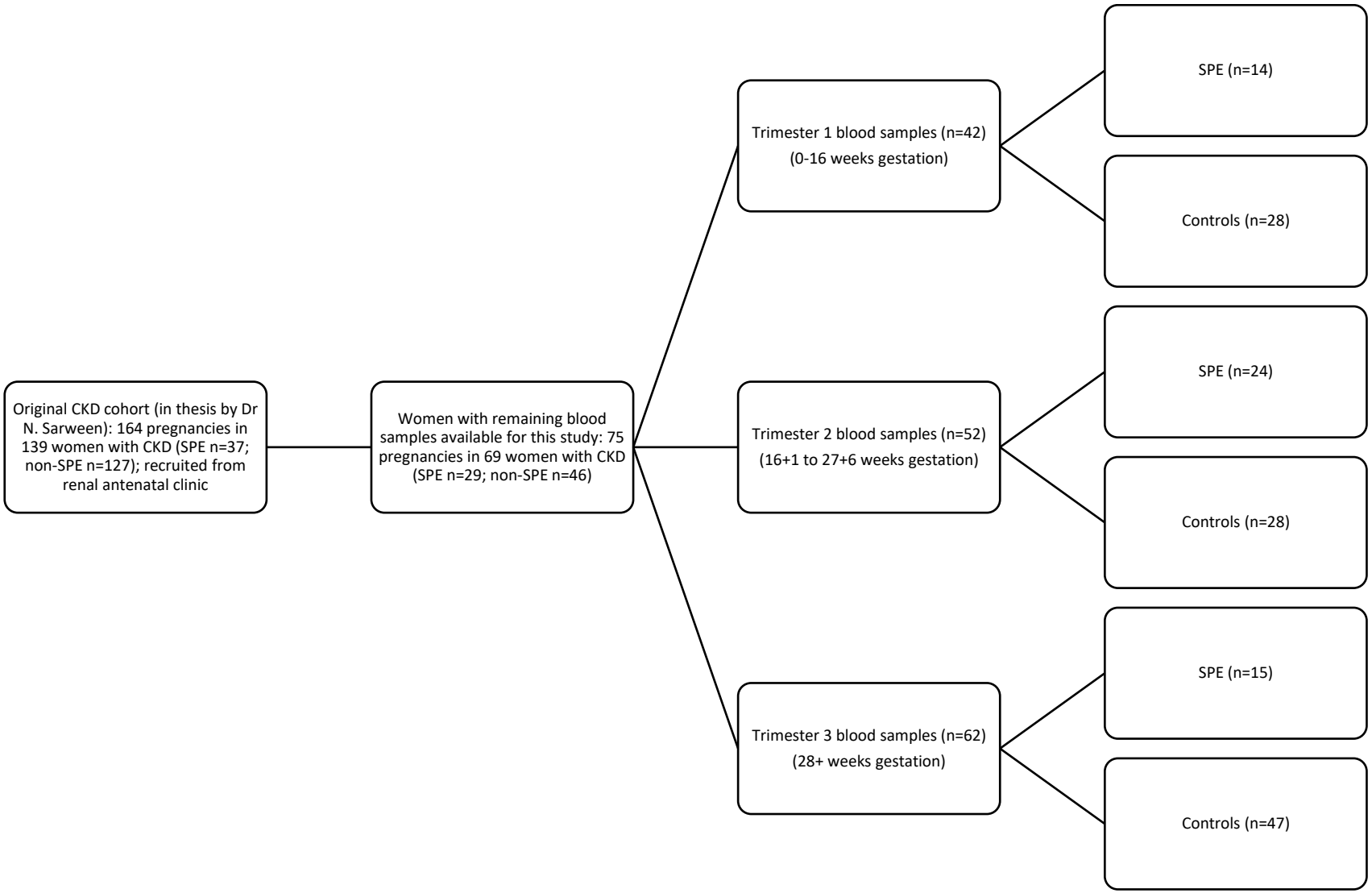


Figure 2.3: CKD Cohort recruitment flow chart

2.4.4 Sample collection

Serial samples of maternal plasma (EDTA) were drawn at antenatal clinic visits throughout pregnancy. All blood samples were drawn prior to established labour. Blood samples were transferred to HBRC (UoB) where they were centrifuged at 3000 rpm for 10 minutes, separated into 0.5 ml aliquots, and frozen at -80^{0}C within 4 hours of collection to minimise ex vivo complement activation. All blood samples were anonymised at the point of arrival at HBRC by being assigned a unique patient number (P-number) and specimen number (S-number).

2.4.5 Laboratory analyses

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to test maternal plasma samples for the following complement proteins:

- Properdin
- Ba
- C5b-9

Properdin and Ba were selected for testing because significant differences were found in these complement components between women with PE and healthy pregnant controls in earlier work (Birmingham and London cohorts). It would therefore be interesting to evaluate how results compared in a cohort of women with CKD, and whether the potential mechanisms of complement activation and PE pathogenesis differed from those observed in healthy pregnant women.

C5b-9 was tested in order to assess whether complement activation in SPE extends all the way to the TCC. This could have important therapeutic implications due to complement-modifying agents, including a C5 inhibitor, eculizumab, already being licensed for use in diseases such as aHUS and PNH and reported to be safe in pregnancy (55, 167, 168). Due to funding and laboratory time constraints,

as well as blood sample availability, it was not feasible to test any further complement components for this cohort.

Complement assays for this cohort were carried out by the author in the CIS laboratory, UoB. A Dynex DSX ® Automated ELISA System machine was used to analyse the plasma samples (Dynex Technologies, Chantilly, VA). Assay details are summarised in ***Table 2.9***.

Table 2.9: Summary of complement ELISAs carried out at CIS, UoB

Assay	Supplier / product code	Antibody	Sample dilutions	Standards / controls
Properdin	Hycult Biotech HK334-01	Mouse mAb specific for human properdin	1:5000	8 standards 1 blank
Ba	Quidel MicroVue A034	Murine mAb specific for human Ba	1:1000	5 standards High / low control 1 blank
C5b-9	Quidel MicroVue A029	Mouse mAb specific for human C5b-9	1:10	5 standards High / low control 1 blank

mAb: monoclonal antibody

2.4.5.1 Properdin ELISA assay

Properdin ELISA kits were purchased from Hycult Biotech (Uden, Netherlands). Plasma samples were brought to room temperature while reagent preparation took place:

- Wash buffer was prepared by mixing 60 ml of wash buffer concentrate with 1140 ml distilled water to make a 1:20 solution.
- Dilution buffer was prepared by mixing 20 ml diluent with 180 ml distilled water, with care taken not to shake the solution to avoid formation of bubbles.
- Standard solution was prepared according to ***Figure 2.4***, to make a total of 8 standards. The standard vial was reconstituted with 1.220 ml of dilution buffer to make Human Properdin

Standard 1 with concentration 20 ng/ml. Serial dilutions of the reconstituted standard were then made by adding 225 µl of the previous solution to 225 µl of dilution buffer. Standard 8 was a blank.

- The tracer was reconstituted by adding 1 ml distilled water and 11 ml dilution buffer.
- Streptavidin-peroxidase solution was prepared by mixing 0.25 ml streptavidin-peroxidase with 24.75 ml dilution buffer.

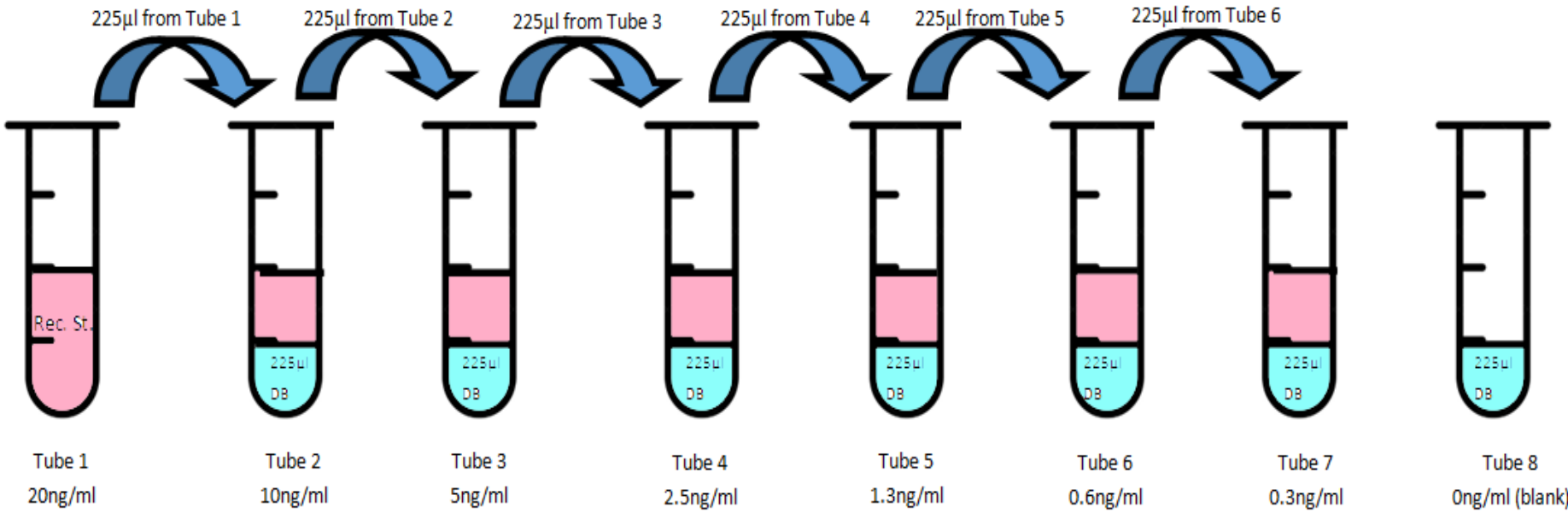


Figure 2.4: Properdin standard solution preparation

Rec St: reconstituted standard, DB: dilution buffer

The concentration of standard solution is shown beneath each tube by serial dilution (ng/ml).

Plasma samples were then diluted by hand pipetting, mixing 10 µl of sample with 490 µl of dilution buffer. A second dilution was later made by the Dynex DSX ® machine, mixing 10 µl of diluted sample with 990 µl of dilution buffer, to make a final dilution of 1:5000. Plasma samples and substrates were then placed into the machine, which was programmed to run the automated ELISA test:

- 100 µl of diluted samples and prepared standards were transferred onto the 96-well test plate and incubated for 1 hour at room temperature.
- Plates were washed 4 times with 200 µl wash buffer.
- 100 µl of diluted tracer was added to each well and incubated for 1 hour at room temperature. The wash procedure was then repeated as above.
- 100 µl of diluted streptavidin-peroxidase was added to each well, followed by another 1 hour incubation at room temperature and wash procedure as above.
- 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was then added to each well. The plate was then covered to avoid exposure to sunlight and incubated at room temperature for 30 minutes.
- 100 µl of stop solution was added to each well and the plate read using a 450 nm plate reader.

Samples were tested singly due to restrictions in laboratory space / time and costs. The inter-assay CV was <15% and intra-assay CV <10% (specifications set by Hycult during original validation of the assay (234)).

2.4.5.2 Ba and C5b-9 ELISA assays

Quidel MicroVue™ ELISA kits were purchased for Ba and C5b-9 assays (Quidel, San Diego, CA). These assays were performed together on a second Dynex DSX ® machine at the same time as properdin assays, to minimise repeated freeze-thawing of samples. Reagents, standards, and controls were supplied in kits with no additional preparation required. For each assay, a wash buffer solution was

made by mixing 50 ml of wash buffer concentrate with 950 ml distilled water to make a 1:20 solution. For Ba assays, plasma samples were prepared by hand pipetting 20 µl of sample and mixing with 180 µl of diluent. A further dilution was later made by the Dynex DSX ® machine, of 5 µl diluted sample with 495 µl diluent, to make a final dilution of 1:1000. For C5b-9 assays, the Dynex DSX ® machine was programmed to mix 50 µl sample with 450 µl diluent, to achieve a final dilution of 1:10. The Dynex DSX ® was then programmed to run the ELISAs as follows:

- 300 µl wash solution was added to 96 well ELISA plates and incubated for 1 minute at room temperature (2 minutes for C5b-9). Plates were then washed twice with wash buffer.
- 100 µL specimen diluent (for blanks), standards, controls (high and low), and diluted specimens were pipetted into wells and incubated for 1 hour at room temperature. Plates were then washed 5 times with wash buffer.
- Conjugate was then added to wells (100 µl for Ba and 50 µl for C5b-9) and incubated at room temperature (1 hour for Ba and 30 minutes for C5b-9).
- 100 µl substrate solution was added to wells, and the plate incubated at room temperature for 15 minutes.
- 100 µl stop solution added and the optical density read at 450 nm.

All samples were tested singly, due to restrictions in costs and laboratory space / time. For Ba, the intra-assay CV was reported in product literature as 3.3%, and inter-assay CV 2.4% (235). For C5b-9, intra-assay CV was reported as 1.9-6.8% and inter-assay CV 5.2-13.1% (from original validation of the assay (236).

2.4.6 Clinical outcomes

For women without pre-existing chronic hypertension or proteinuria, the standard diagnostic criteria for PE were used (see ***Table 1.3***). For women with pre-existing hypertension or proteinuria, the diagnostic criteria for SPE are detailed in ***Table 1.4***.

A diagnosis of SPE was defined as:

- Gestational hypertension (if chronic hypertension present):
    - De novo hypertension ≥ 160 mmHg systolic or ≥ 110 mmHg diastolic, or
    - Worsening chronic hypertension requiring escalation of antihypertensive medication doses after 20 weeks' gestation to maintain BP <160/110 mmHg

AND

- Gestational proteinuria (if chronic proteinuria present):
    - Doubling in uPCR after 20 weeks' gestation compared to pre-pregnancy levels, and
    - uPCR > 30 mg/mmol

OR at least one of the following if only one of the above indicators present:

- Acute kidney injury
    - Rapidly worsening kidney function: ≥ 50% increase in serum creatinine in 7 days not attributable to an alternate diagnosis
- Uteroplacental dysfunction
    - Small for gestational age (birth weight <10th centile), or
    - Abnormal umbilical artery Doppler waveform
- Severe features
    - Abnormal liver function tests (ALT or AST > 40 IU/L)
    - Haematological complications (including platelet count < 100 x 10^9/L, haemolysis, or disseminated intravascular coagulation)
    - Neurological symptoms (including eclampsia, visual disturbance / scotomata, and clonus)
    - Pulmonary oedema not attributable to an alternate diagnosis

There are no consensus criteria for the diagnosis of SPE in women with CKD, so these indicators were adapted from previous research cohorts of SPE in women with CKD (79, 192). Where diagnostic uncertainty remained, a diagnosis of SPE was made postnatally following expert consensus review by an obstetrician and a nephrologist, considering the rate of change of maternal factors including BP, proteinuria, and excretory renal function. Clinical records were reviewed independently, and a diagnosis of SPE only made if both parties were in agreement.

Demographic details and clinical outcome data were collated from patient handheld antenatal notes and electronic healthcare records. The same clinical outcomes were recorded as for the Birmingham Cohort (see ***Section 2.3.9***), with the addition of the following parameters:

- Pre-pregnancy creatinine and GFR
- Pre-pregnancy proteinuria (urine ACR)
- Pre-pregnancy CKD stage (according to NICE criteria (233))
- Cause of CKD
- Presence or absence of chronic hypertension

2.4.7 Sample size and statistical analysis

No formal sample size calculation was conducted for this study, given that maternal blood samples were retrospectively tested from an historically collected patient cohort. All available maternal plasma samples for each specified gestation were selected for testing, and therefore the final sample size utilised the maximum available number of samples held in the biobank.

For comparisons between SPE and non-SPE groups, nominal variables were compared using Fisher's exact tests. Differences between ordinal and continuous variables were reported as mean +/- SD where variables were normally distributed, and as median and IQR otherwise. Q-Q plots were used

to assess for normality of data distribution. p-values were derived from independent samples t-tests, and Mann-Whitney U tests, respectively.

Complement concentrations in SPE and non-SPE groups were compared for blood samples collected in early pregnancy (0-16 weeks gestation), mid-pregnancy (16+1 to 27+6 weeks gestation), and late pregnancy (28+ weeks gestation).

In addition, patients were grouped according to the presence or absence of adverse pregnancy outcome, and statistical analysis was repeated. Adverse pregnancy outcome was a composite measure, defined as any of:

- neonatal death,
- PTB (delivery prior to 37 weeks' gestation),
- admission to NNU,
- low birth weight (<2500 g), or
- SGA (birth weight <10th centile)

Complement markers found to differ significantly between groups were assessed using ROC curve analysis, with predictive accuracy for SPE (or composite adverse pregnancy outcome) reported using AUROC and associated SE. Optimal cut-off values were identified using Youden's J statistic, with associated specificity and sensitivity reported.

In addition, binary logistic regression analyses were conducted in order to assess which complement components were independently associated with adverse pregnancy outcome after controlling for differences in pre-pregnancy renal function and proteinuria. Adverse pregnancy outcome was used as the dependent variable, and pre-pregnancy creatinine and urine ACR were entered into the model as continuous covariates. The Hosmer-Lemeshow test was used to assess goodness-of-fit of the resulting models. Where poor fit was detected due to skew in the dependent variables, values were $\log_{10}$-transformed to reduce the degree of skew, and the analysis repeated. Results were

reported as OR for adverse pregnancy outcome per unit increase in biomarker, with associated 95% confidence intervals.

A separate statistical analysis was conducted for subjects who had one blood sample collected within each specified timepoint (early, mid, and late pregnancy) to allow an assessment of how complement markers change longitudinally during pregnancy.

All analyses were performed using IBM SPSS version 28 (IBM Corp. Armonk, NY). Statistical significance was determined as $p<0.05$ throughout.

2.5 Investigating the role of complement in the pathophysiology of pre-eclampsia in women of Sub-Saharan African ethnicity

2.5.1 Study design overview

This was a single-centre, cross-sectional study designed to investigate the role of complement in the pathophysiology of PE in women of SSA ethnicity.

The primary outcome measure for this study was circulating complement biomarker concentrations in women of SSA ethnicity with PE, compared to those with gestational hypertension, and healthy pregnant controls.

The secondary outcome measures were:

i) Subgroup analysis: comparison of circulating complement biomarker concentrations in women with a hypertensive disorder of pregnancy (PE *or* gestational hypertension), and healthy pregnant controls.

ii) Correlation between circulating complement biomarkers, and biochemical and immune markers of inflammation in PE, gestational hypertension, and healthy control pregnancy.

2.5.2 Ethical approval

Ethical approval for this arm of research was granted by Korle-Bu Teaching Hospital Institutional Review Board (KBTH IRB). Study approval number KBTH-IRB/00056/2017; date of approval 25/10/2017 (see ***Appendix 6***).

All subjects provided informed consent for their participation in the study, and the study was performed according to the principles of Good Clinical Practice (220). Patients were originally recruited for a study of 'genetic determinants of hypertensive disease in pregnancy'. They consented to having blood specimens stored indefinitely for use in future unrelated studies; hence research

collaborators donated blood samples for use in this study on the role of complement in the pathogenesis of PE. A copy of the consent form is shown in ***Appendix 7***.

2.5.3 Study population and recruitment

Participants were recruited from Korle-Bu Teaching Hospital, Accra, Ghana, between 2017 and 2018. Healthy pregnant controls and cases (women with any hypertensive disorder of pregnancy, including: PE, eclampsia, HELLP syndrome, or gestational hypertension) were recruited purposively at the point of hospital admission for delivery.

PE was defined as:

- new onset hypertension (BP ≥140/90 mmHg) after 20 weeks gestation, AND
- proteinuria: > 1+ protein on urine dip (resource-limited setting, so laboratory urine protein quantification with ACR/PCR not routinely available); OR
- evidence of eclampsia, thrombocytopenia, liver impairment, renal insufficiency, or pulmonary oedema; according to international statement consensus criteria (70-72, 78). See ***Table 1.3***.

Gestational hypertension was defined as new-onset hypertension (BP ≥140/90 mmHg) after 20 weeks gestation, in the absence of proteinuria.

A recruitment flow chart detailing the breakdown of the Ghana Cohort is shown in ***Figure 2.5***.

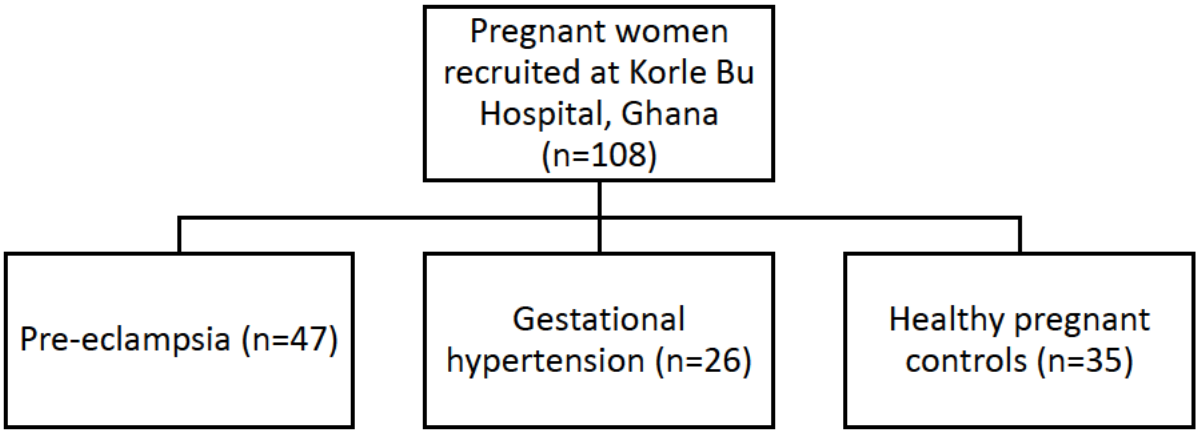


Figure 2.5: Ghana Cohort recruitment flow chart

A full list of inclusion and exclusion criteria are detailed in ***Table 2.10***. Women with chronic hypertension were not excluded from this study. Similarly, women with CKD were not explicitly excluded from recruitment, although no subjects with known CKD were present in the final Ghana Cohort (cases or controls).

Table 2.10: Inclusion and exclusion criteria: Ghana Cohort study

Inclusion criteria	*Exclusion criteria*
Cases: • Women with a confirmed diagnosis of PE (70, 71, 78), including those with eclampsia and HELLP syndrome • Women with gestational hypertension	Subjects unable to consent to participation in the study because of language or physical barriers and/or learning difficulties (translators will be allowed)
Controls: Healthy pregnant women without gestational hypertension of at least 30 weeks gestation at time of delivery	Maternal age < 18 years
Women with chronic hypertension not excluded	Women with co-morbid conditions including diabetes mellitus, and other endocrine diagnoses

2.5.4 Sample collection

Samples of maternal blood (1 x plasma in EDTA tube, and 1 x serum tube) were collected prior to delivery by trained phlebotomists. Blood samples were centrifuged, separated into 0.5 ml aliquots, and frozen at -80^{0}C within 4 hours of collection. Samples were assigned a unique study identification and tube number at the point of arrival in the research laboratory for anonymisation purposes.

2.5.5 Laboratory analyses

Following completion of MTA documentation, blood samples were flown from Ghana on dry ice and brought to CIS, UoB. On arrival in the laboratory, samples were immediately placed back into a -80^{0}C freezer.

Maternal plasma samples were tested for the same complement components as the CKD Cohort (properdin, Ba and C5b-9) in the CIS laboratory, UoB. Samples were only defrosted once on the day of analysis, to eliminate any freeze-thaw degradation. The same ELISA kits and methodology were used as detailed in ***section 2.4.5***, and ***Table 2.9***.

Maternal serum samples were tested for C4, creatinine, cystatin C, urea, uric acid, B2M, sFLCs, immunoglobulins G, A and M, LDH and HS-CRP. In addition, maternal serum was tested for C3 (Roche Diagnostics assay; product ID C3C-2; catalogue number 03001938 322; intra-assay CV 0.8-1.2% and inter-assay CV 1.3-2.0%) (228). A fully automated Hitachi Cobas 6000 Turbidimeter (c501 module; Roche Diagnostics, West Sussex, UK) was used to test all serum samples, using the same methodology outlined in ***sections 2.3.6.2 and 2.3.7***.

2.5.6 Clinical outcomes

Demographic data were collated from a structured questionnaire completed by study investigators and trained research assistants with the study participant at the time of their recruitment into the

study. Clinical outcome data were collected from patient records following delivery. The following data points were recorded:

Antenatal history:

- Maternal age (years)
- Parity
- Gravidity
- Chronic hypertension
- Previous gestational hypertension
- Regular antenatal clinic attendance

Maternal outcomes:

- Gestational hypertension
- PE
- Eclampsia
- Proteinuria (presence or absence on dipstick testing)
- Peak recorded BP (systolic and diastolic in mmHg)
- Mode of delivery (vaginal / emergency Caesarean / elective Caesarean)

Fetal outcomes:

- Neonatal death
- Gestation (weeks)
- PTB (<37 weeks)
- Birth weight (g)
- Low birth weight (<2500 g)
- SGA (birth weight < 10^{th} centile)

2.5.7 Sample size and statistical analysis

No formal sample size calculation was conducted for this study, as blood samples were tested retrospectively from a previously recruited patient cohort, and all available blood samples were used. Based on published data, a sample size of 50 patients per arm would be sufficient to detect a statistically significant difference in C5b-9 concentrations between cases of PE and pregnant controls (150, 152), assuming α=0.05 and 80% power (see ***Section 2.3.10***).

For comparisons between PE, gestational hypertension and control groups, categorical variables were assessed using Fisher's exact tests. Q-Q plots were then used to assess for normality of continuous data distribution. Continuous variables were reported as mean ± SD, with p-values derived from one-way Analysis of Variance (ANOVA) tests where normally distributed, or as median (IQR), with p-values taken from Kruskal-Wallis tests otherwise. Post hoc analyses were then carried out using Tukey's test for multiple comparisons between groups for normally distributed variables, and Bonferroni-adjusted pairwise comparisons between groups for non-normally distributed variables.

The association between variables (complement markers, and biochemical / immunological tests) were quantified using Spearman's correlation coefficients (rho). Binary logistic regression analysis was conducted in order to assess whether Ba was independently associated with PE after controlling for serum creatinine. PE was entered into the model as the dependent variable, with creatinine as a continuous covariate. Goodness-of-fit was assessed, and results reported as odds ratio of PE per unit increase in complement marker, with associated 95% confidence intervals.

All analyses were performed using IBM SPSS 28 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance throughout. In analyses with large numbers of comparisons, the Bonferroni-corrected significance threshold was indicated in the tables, where appropriate.

2.6 Comparison of the role of complement in the pathogenesis of pre-eclampsia between cohorts (previously healthy women, versus CKD, versus SSA ethnicity)

2.6.1 Comparison of complement assays tested in Newcastle and Birmingham

Complement component tests for properdin, Ba and C5b-9 were repeated for a small number of randomly selected patients from the Birmingham Cohort using commercially purchased ELISA kits, and testing was performed in the CIS laboratory, UoB (as for the CKD and Ghana Cohorts, using the methods outlined in ***section 2.4.5***). The rationale for this was to ensure that the results were comparable to the tests carried out in Newcastle using MSD assays. This would allow a reliable comparison of complement results between the 3 cohorts, and inferences to be made about the relative similarities and differences between the groups.

In addition, maternal serum samples from the Birmingham Cohort were tested for C3 and C4 using the same Roche Diagnostics assays as for the Ghana Cohort (***section 2.3.6.2 and 2.5.5***), to allow a comparison of C3 and C4 between the patient groups. The MSD C3 assay only detected native, non-activated C3 and results could therefore not be compared to those from a traditional nephelometry assay.

2.6.2 Statistical analysis

Bland-Altman plots were constructed to compare the performance of properdin, Ba and C5b-9 assays conducted using MSD assays in Newcastle, versus commercially available ELISA kits tested in Birmingham. Where there was evidence of a proportional bias between Newcastle and Birmingham assay results, linear regression analysis was conducted using the Newcastle result as the independent variable, and Birmingham result as the independent variable. A conversion factor between assays was then determined from the regression equation, with associated R^2 and p-values (derived from ANOVA).

For comparisons between Birmingham, CKD and Ghana Cohort complement markers (tested using commercially available ELISA kits in Birmingham), Q-Q plots were used to assess for normality of data distribution. Variables were then reported as mean ± SD, with p-values derived from one-way ANOVA tests where normally distributed, or as median (IQR), with p-values taken from Kruskal-Wallis tests otherwise. Post hoc analyses were then carried out using Tukey's test for multiple comparisons between groups for normally distributed variables, and Bonferroni-adjusted pairwise comparisons between groups for non-normally distributed variables.

All analyses were performed using IBM SPSS 28 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance throughout.

CHAPTER 3

INVESTIGATING THE ROLE OF COMPLEMENT IN THE PATHOGENESIS OF PRE-ECLAMPSIA IN PREVIOUSLY HEALTHY WOMEN

3 Investigating the role of complement in the pathogenesis of pre-eclampsia in previously healthy women

3.1 Introduction and overview

PE is a multisystem disorder that affects around 5% of all pregnancies, and is a leading contributor to maternal and fetal morbidity and mortality (82). There remains no definitive therapy other than delivery of the baby and placenta, which is often necessitated preterm due to declining maternal and/or fetal wellbeing. In addition to the immediate clinical risks, long-term sequelae include an increased lifetime risk of maternal CKD (79) and cardiovascular disease (87, 237).

There is increasing evidence, presented in Chapter 1, implicating complement dysregulation in the pathogenesis of PE. Complement-modifying agents are undergoing rapid development and may provide novel opportunities to treat complement-mediated diseases (164, 165), although there is currently insufficient evidence to support their widespread use in PE. The evidence base is often conflicting, and it remains unclear whether raised concentrations of circulating complement reflect a general heightened inflammatory state in PE or are directly associated with placental complement-mediated injury.

As such the objective of this study was, for the first time, to simultaneously compare markers of local placental complement deposition with systemic complement activation in the maternal and fetal circulation between women with PE and healthy pregnancy. The results will potentially provide further support for the role of complement activation in the pathogenesis of PE and may also provide insight into the sequence of events leading to complement activation on a local and systemic level.

The specific aims of this study were to:

- Investigate biomarkers of complement activation in the maternal and fetal bloodstream (C3, iC3b, Ba, properdin, C4, and C5b-9), and placental tissue (C1q, C4d, C3d and C9) in women with no known pre-existing medical conditions (PE and healthy pregnant controls).
- Correlate placental complement deposition (immunoreactivity score) with markers of complement activation in the maternal and fetal bloodstream, to determine the relative changes in tissue and circulatory complement in PE and normal healthy pregnancy.

Samples of maternal and umbilical cord blood were tested for iC3b, C3, C4, properdin, Ba and C5b-9 using electrochemiluminescent immunoassays from women with PE (n=34) and healthy pregnant controls (n=33): 'Birmingham Cohort'. Placental tissue was stained for C3d, C4d, C9 and C1q and ascribed a composite immunoreactivity score, based on the intensity and distribution of staining at the STB membrane. Demographic and clinical outcome data were collated from obstetric records following delivery.

A secondary aim of the study was to evaluate biochemical and immunological markers in PE and healthy pregnancy, and to assess their association with circulating markers of complement activation. Samples of maternal and umbilical cord serum were therefore tested for creatinine, cystatin C, urea, uric acid, B2M, LDH, HS-CRP, sFLCs, and immunoglobulins G, A and M.

Maternal plasma properdin and Ba tests were repeated in a separate validation cohort ('London Cohort'): 35 women with PE, and 35 healthy pregnant controls.

The body of work within this chapter has now been published in a peer-reviewed journal (238) (see ***Appendix 8***).

3.2 Results

3.2.1 Cohort characteristics

A total of 67 women were recruited in the Birmingham Cohort (34 PE cases and 33 healthy pregnant controls). The London Cohort comprised a further 70 subjects (35 PE cases and 35 healthy pregnant controls). Baseline demographics and clinical outcome data for each cohort are presented in ***Table 3.1.*** There were no significant differences in age, BMI, ethnicity, parity, or mode of delivery between PE and control groups for either cohort.

Subjects with PE, by definition, had significantly higher peak recorded blood pressures than controls (Birmingham Cohort: median peak BP 163/105 mmHg for PE, vs. 128/79 mmHg for controls, $p<0.001$; and London Cohort: median peak BP 173/106 mmHg for PE, vs 135/85 mmHg for controls, $p<0.001$). PE patients in the Birmingham Cohort had heavier proteinuria than those in the London Cohort: median peak urinary PCR 259 mg/mmol (133-429), and 85 mg/mmol (54-200), respectively. However, more than half of the London Cohort PE patients had early-onset disease (diagnosis prior to 34 weeks), compared to approximately one-third of the Birmingham Cohort. The median gestation at PE diagnosis was 35.9 weeks (33.4-36.6) for the Birmingham Cohort, and 33.9 weeks (32.6-35.3) for the London Cohort.

In both cohorts, PE was characterised by significantly earlier gestation at delivery: median 36.9 weeks (IQR 36.0-37.7), vs. 39.3 weeks (39.0-40.6) for controls in the Birmingham Cohort, $p<0.001$; and 36.1 weeks (34.6-37.1) for PE, vs. 39.6 weeks (39.0-41.1) for controls in the London Cohort, $p<0.001$. This can be visualised in ***Figure 3.1.*** In the Birmingham Cohort, maternal blood samples were drawn at a median gestation of 36.6 weeks (range: 29.1-38.3) in the PE group, which was significantly earlier than for controls (39.0 weeks, range: 38.7-42.0), $p<0.001$; also shown in ***Figure 3.1***. This difference was a result of the earlier gestation at delivery in the PE group, as patients were recruited to the study at the time of their hospitalisation. However, in the London Cohort, blood

samples tended to be drawn earlier in pregnancy, and at a similar gestation for PE and control groups (median 34.3 vs. 34.0 weeks respectively, p=0.837).

The PE group in both cohorts had significantly lower mean birth weights than controls, and increased rates of adverse pregnancy outcomes, including PTB (50% for PE, vs. 0% of controls, p<0.001), low birth weight <2500 g (56% for PE, vs. 0% of controls, p<0.001), SGA babies weighing less than the 10th centile (65% for PE, vs. 15% of controls, p<0.001), and requirement for NNU care (38% for PE, vs. 12% of controls, p=0.023) – all statistics for Birmingham Cohort.

Table 3.1: Demographic and clinical outcome data: Birmingham and London Cohorts

	Birmingham Cohort				London Validation Cohort			
	N	***Pre-eclampsia***	***Control***	***p-Value***	***N***	***Pre-eclampsia***	***Control***	***p-Value***
Maternal age at delivery (years)	67	30.6 ± 6.6	29.6 ± 5.6	0.527	70	33.7 ± 6.5	33.0 ± 4.5	0.617
BMI (kg/m^2)	67	28.5 ± 5.1	28.9 ± 5.1	0.741	70	28.8 ± 7.4	26.3 ± 4.5	0.094
Ethnicity	67			0.530	70			0.051
White		15 (44%)	19 (58%)			15 (43%)	24 (69%)	
Asian		12 (35%)	9 (27%)			1 (3%)	0 (0%)	
Black		6 (18%)	3 (9%)			12 (34%)	4 (11%)	
Mixed/Other		1 (3%)	2 (6%)			7 (20%)	7 (20%)	
Parity	67			0.082*	70			0.722*
0		25 (74%)	16 (48%)			22 (63%)	22 (63%)	
1		4 (12%)	12 (36%)			5 (14%)	9 (26%)	
2+		5 (15%)	5 (15%)			8 (23%)	4 (11%)	
Peak SBP (mmHg)	65	163 ± 14	128 ± 9	**<0.001**	70	173 ± 12	135 ± 13	**<0.001**
Peak DBP (mmHg)	65	105 ± 9	79 ± 7	**<0.001**	70	106 ± 8	85 ± 10	**<0.001**
Peak uPCR (mg/mmol)	34	259 (133-429)	*NA*	-	35	85 (54-200)	*NA*	-
Mode of delivery	67			0.242	70			0.227
Caesarean		24 (71%)	28 (85%)			23 (66%)	17 (49%)	
Vaginal		10 (29%)	5 (15%)			12 (34%)	18 (51%)	
Gestation at (weeks):								
Delivery	66	36.9 (36.0-37.7)	39.3 (39.0-40.6)	**<0.001**	70	36.1 (34.6-37.1)	39.6 (39.0-41.1)	**<0.001**
Blood Draw	65	36.6 (35.6-37.4)	39.0 (38.8-40.4)	**<0.001**	70	34.3 (32.7-35.9)	34.0 (32.1-35.9)	0.837
PE Diagnosis	34	35.9 (33.4-36.6)	*NA*	-	35	33.9 (32.6-35.3)	*NA*	-
Early-onset PE (<34 weeks)	34	10 (29%)	*NA*	-	35	19 (54%)	*NA*	-
Twin Pregnancy	67	1 (3%)	0 (0%)	1.000	70	0 (0%)	0 (0%)	1.000
Preterm Birth (<37 weeks)**	67	17 (50%)	0 (0%)	**<0.001**	70	24 (69%)	2 (6%)	**<0.001**
Birth Weight (g)***	67	2324 ± 670	3543 ± 491	**<0.001**	70	2397 ± 863	3592 ± 531	**<0.001**
Low Birth Weight (<2500g)**	67	19 (56%)	0 (0%)	**<0.001**	70	20 (57%)	0 (0%)	**<0.001**
SGA (<10th centile)**	67	22 (65%)	5 (15%)	**<0.001**	70	17 (49%)	2 (6%)	**<0.001**
Neonatal Unit Care**	67	13 (38%)	4 (12%)	**0.023**	70	22 (63%)	1 (3%)	**<0.001**

*Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests, unless stated otherwise. Bold p-values are significant at p<0.05. *p-Value from Mann-Whitney U test, as the factor is ordinal. **For the twin pregnancy, outcomes were the same for both babies; hence these were combined for analysis. ***For the twin pregnancy, the average weight of the two babies was assumed.* NA=data were not available in the cohort for the stated variable.

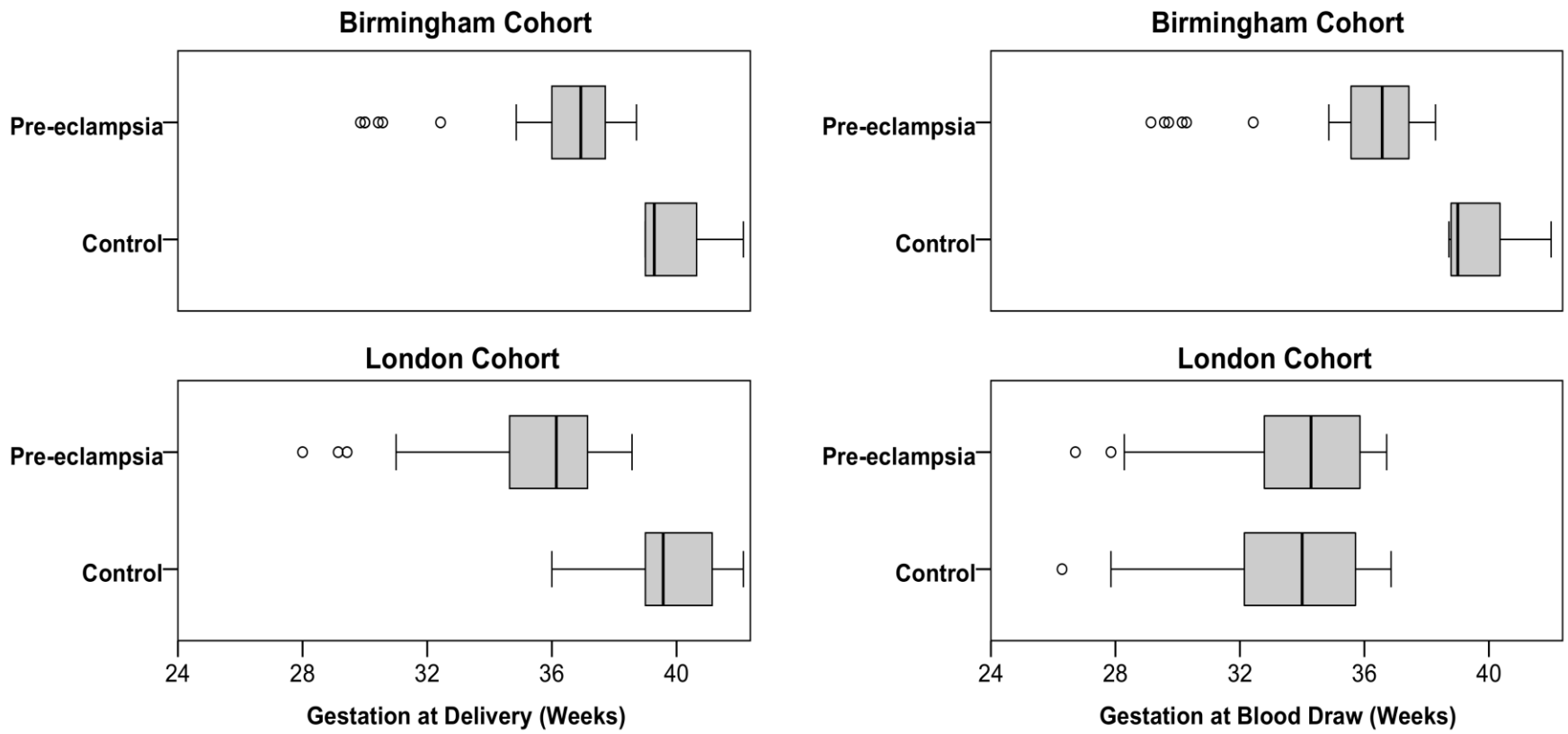


Figure 3.1: Gestations at delivery and blood draw by cohort and pre-eclampsia status

Figure shows box-and-whisker plots, with vertical lines representing the median, boxes representing the interquartile range, and whiskers representing the total range.

3.2.2 Maternal blood complement

Maternal blood complement concentrations in the PE and control groups are reported in ***Table 3.2.*** In the Birmingham Cohort, women with PE had significantly lower concentrations of properdin (mean: 4828 vs. 6877 ng/ml, p<0.001), C4 (mean: 0.20 vs. 0.31 g/l, p<0.001), iC3b (mean: 489 vs. 606 ng/ml, p=0.003), and C3 (mean: 1.9 vs. 2.4 g/l, p<0.001), and significantly higher Ba (median: 150 vs. 113 ng/ml, p=0.012), compared to the control group. However, no significant differences in the iC3b:C3 ratio (p=0.734) or C5b-9 (p=0.753) were detected between groups. Analysis of the London Cohort returned consistent results, with significantly reduced properdin concentrations in women with PE compared to controls (mean: 5282 vs. 7021 ng/ml, p<0.001). However, the difference in maternal Ba levels did not reach statistical significance in this cohort (median: 165 in PE vs. 151 ng/ml in controls, p=0.310).

The analysis was repeated using a regression approach, to account for the observed differences in gestational age at blood sampling between PE and control groups (see ***Table 3.3***). For the pooled Birmingham and London cohorts, the difference in maternal properdin remained significant, with concentrations being an average of 1945 ng/ml lower in the PE vs. control groups (95% CI: 1487-2402, p<0.001, ***Figure 3.2 A***). The differences in C3 and C4 in the Birmingham Cohort also persisted after adjustment for sample gestation, with concentrations being an average of 0.5 g/l (95% CI: 0.2-0.8, p<0.001) and 0.07 g/l (95% CI: 0.02-0.13, p=0.014) lower, respectively, in PE vs. control groups. However, after adjustment for the effect of gestation, the differences in iC3b (p=0.252) and Ba (p=0.194) between PE and controls were not statistically significant.

The three complement components found to differ significantly between PE and control groups, after adjustment for gestation, were further assessed using a ROC curve approach. This found maternal properdin to be the strongest biomarker of PE diagnosis, with an AUROC of 0.87 (SE: 0.03) when applied to the combined Birmingham and London cohorts. This effect is visualised in ***Figure 3.2 B***. This was followed by maternal C4 (AUROC 0.82; SE 0.05) and C3 (AUROC: 0.80; SE: 0.06).

ROC curves for maternal properdin, C4 and C3 are displayed in ***Figure 3.3***. Optimal cut-off points for the diagnosis of PE for each biomarker are also shown. This identified that a maternal properdin concentration of < 5764 ng/ml was suggestive of a diagnosis of PE (sensitivity and specificity both 81%). Maternal C4 < 0.25 g/l (sensitivity and specificity both 73%) and maternal C3 < 2.07 g/l (sensitivity 76% and specificity 82%) were also indicative of a PE diagnosis. These results are only applicable to the timepoint that the blood tests were taken (late in the pregnancy, prior to delivery). As such, they represent the cut-off value for diagnosing PE, rather than predicting it.

Table 3.2: Maternal and umbilical cord blood complement results: Birmingham and London cohorts

	Birmingham Cohort				London Validation Cohort			
Blood Marker	*N*	*Pre-eclampsia*	*Control*	*p-Value*	*N*	*Pre-eclampsia*	*Control*	*p-Value*
Maternal Blood								
iC3b (ng/ml)	66	489 ± 153	606 ± 157	**0.003**	*NA*	-	-	-
C3 (g/l)	66	1.90 ± 0.39	2.36 ± 0.39	**<0.001**	*NA*	-	-	-
iC3b:C3 ($x10^6$)	66	245 (192-301)	251 (219-290)	0.734	*NA*	-	-	-
C4 (g/l)	66	0.20 ± 0.08	0.31 ± 0.08	**<0.001**	NA	-	-	-
Properdin (ng/ml)	66	4828 ± 806	6877 ± 1421	**<0.001**	70	5282 ± 1467	7021 ± 1317	**<0.001**
C5b-9 (ng/ml)	66	237 (198-335)	237 (185-334)	0.753	*NA*	-	-	-
Ba (ng/ml)	66	150 (119-223)	113 (89-148)	**0.012**	70	165 (117-268)	151 (113-198)	0.310
Umbilical Cord Blood								
iC3b (ng/ml)	36	740 (496-1179)	774 (631-1009)	0.788	*NA*	-	-	-
C3 (g/l)	36	0.81 ± 0.29	0.77 ± 0.20	0.610	*NA*	-	-	-
iC3b:C3 ($x10^6$)	36	840 (502-1901)	1133 (734-1468)	0.546	*NA*	-	-	-
C4 (g/l)	31	0.12 ± 0.04	0.13 ± 0.05	0.551	*NA*	-	-	-
Properdin (ng/ml)	36	2943 ± 894	3321 ± 1170	0.333	*NA*	-	-	-
C5b-9 (ng/ml)	36	85.5 (58.2-111.8)	64.8 (41.2-97.5)	0.214	*NA*	-	-	-
Ba (ng/ml)	36	337 (273-370)	233 (155-261)	**0.004**	*NA*	-	-	-

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Bold p-values are significant at p<0.05. For the Birmingham cohort, maternal blood concentrations were available for N=33/N=33 pre-eclampsia/control patients, whilst umbilical blood concentrations were available for N=12/N=24, with the exception of C4 (N=10/N=21). NA=data were not available in the cohort for the stated variable.

Table 3.3: Regression analysis of maternal plasma complement concentrations by gestation and pre-eclampsia status

Blood Marker	N	Difference PE vs. Control		Gradient per Week of Gestation		Interaction
		Coefficient (95% CI)	*p-Value*	*Coefficient (95% CI)*	*p-Value*	p-Value**
iC3b (ng/ml)	65	-63 (-171, 45)	0.252	14 (-4, 33)	0.133	0.167
C3 (g/l)	65	-0.50 (-0.77, -0.22)	**<0.001**	0.00 (-0.05, 0.04)	0.887	0.067
iC3b:C3 (x10^6)	65	50 (-8, 109)	0.090	9 (-1, 19)	0.069	0.735
C4 (g/l)	65	-0.07 (-0.13, -0.02)	**0.014**	0.01 (0.00, 0.02)	0.068	0.818
Properdin (ng/ml)	135	-1945 (-2402, -1487)	**<0.001**	-24 (-91, 43)	0.474	0.417
C5b-9 (ng/ml)*	65	-4% (-35%, 40%)*	0.812	-3% (-9%, 3%)*	0.316	0.337
Ba (ng/ml)*	135	14% (-7%, 40%)*	0.194	-2% (-4%, 1%)*	0.292	0.393

*Results are from linear regression models, with the stated marker as the dependent variable, and both the gestation at blood sampling and the group (PE or control) as covariates. As such, the coefficient for the former represents the estimated increase in the marker per week of gestation, whilst the latter represents the difference between the PE vs. control groups, after adjustment for differences in the gestation that the sample was collected. Analyses of properdin and Ba pooled data from the Birmingham and London cohorts, whilst the other markers were assessed for the Birmingham cohort only. Bold p-values are significant at p<0.05. *Values were found to follow a skewed distribution, hence were log_{10}-transformed before analysis; the resulting coefficients were then anti-logged and converted to percentage differences for ease of interpretation. **To test for potential interactions between PE-status and gestation, a second set of models were produced which additionally included an interaction term, the p-values of which are reported; none of these interaction terms were found to be statistically significant, hence they were excluded from the main models.*

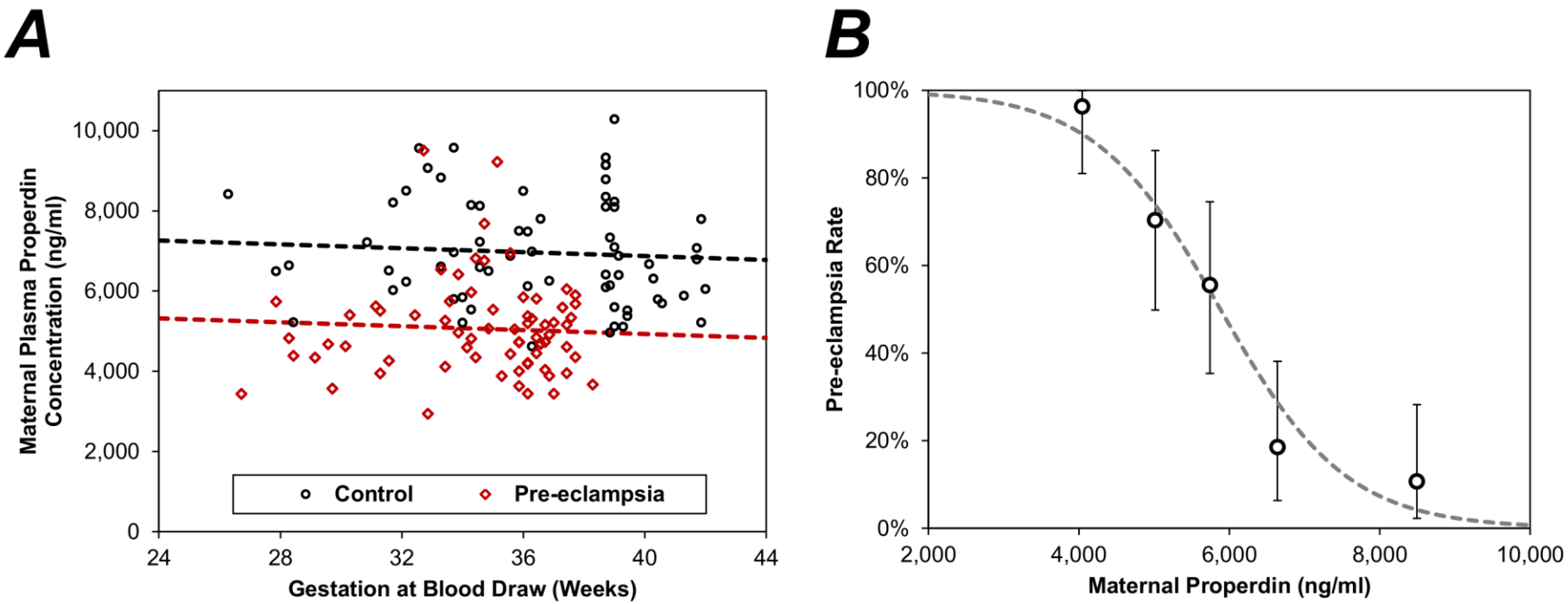
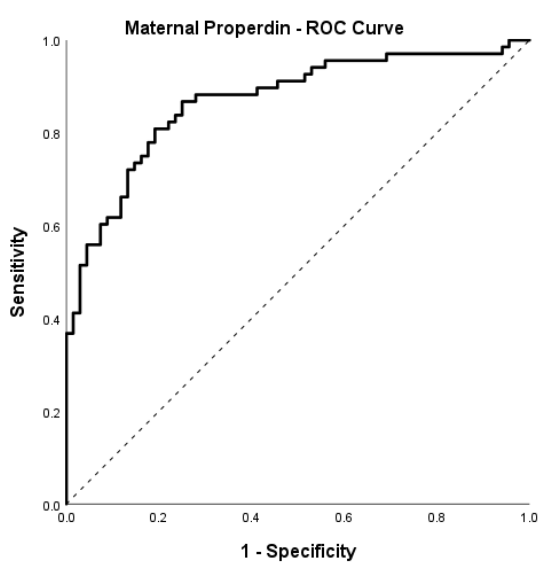
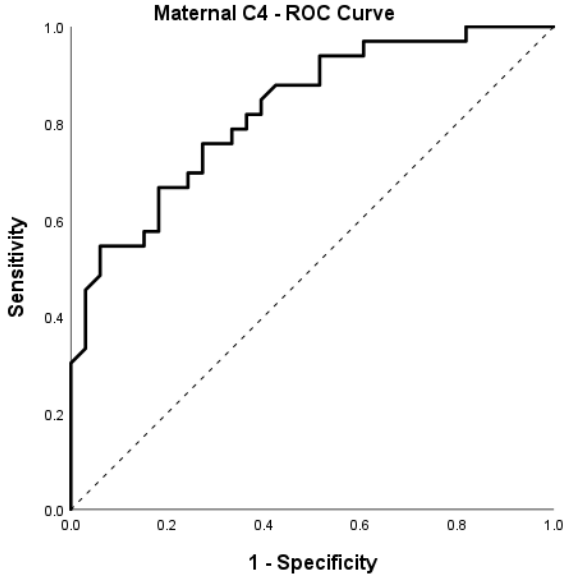
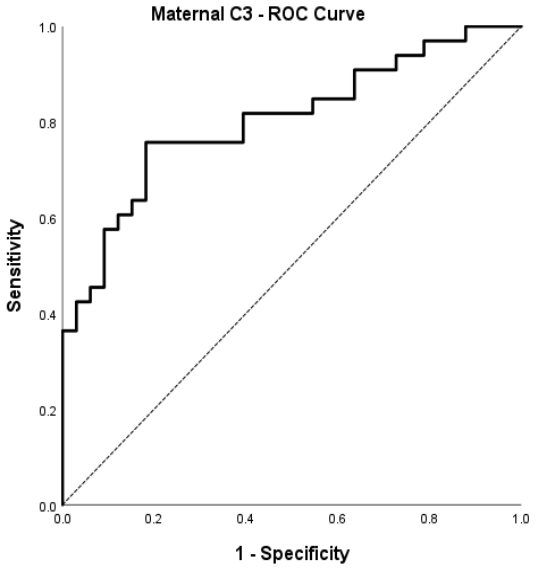


Figure 3.2: Associations between pre-eclampsia and maternal properdin concentration

Both figures include patients from both the Birmingham and London cohorts, with the exception of those with no properdin result recorded (N=1). Figure A additionally excludes one patient for whom the gestation at blood draw was not recorded. In Figure A, points represent the data for individual patients, and broken lines are from a linear regression model, with gestation and pre-eclampsia status as covariates. In Figure B, points represent the observed pre-eclampsia rates within quintiles of the distribution, which are plotted at the mean of the interval, and whiskers represent 95% confidence intervals. The broken line is from a binary logistic regression model on the patient-level data, with the maternal properdin concentration as a continuous covariate.







Properdin: AUROC 0.87 (0.03)
Reference

C4: AUROC 0.82 (0.05)
Reference

C3: AUROC 0.80 (0.06)
Reference

Biomarker	N	Sensitivity	Specificity
Maternal Properdin < 5764 ng/ml	136	81%	81%
Maternal C4 < 0.25 g/l	66	76%	73%
Maternal C3 < 2.07 g/l	66	76%	82%

Figure 3.3: Diagnostic accuracy of maternal properdin, C4 and C3 concentration for pre-eclampsia: ROC analysis

Images show ROC curves for the performance of maternal properdin, C4 and C3 concentration in the diagnosis of PE. AUROC values are quoted, with associated standard error. Optimal cut off points for the diagnosis of PE were then identified using Youden's J statistic, with associated sensitivity and specificity.

3.2.3 Umbilical cord blood complement

Data for umbilical cord blood complement concentrations were available for 36 patients from the Birmingham Cohort (12 with PE, and 24 controls; see ***Table 3.2***), with one patient not having the corresponding maternal blood measurements.

Comparisons between PE and control groups found Ba to be the only umbilical cord measurement to differ significantly between groups (median: 337 vs. 233ng/ml respectively, p=0.004*)*, returning an AUROC of 0.80 (SE: 0.09) for the prediction of PE.

Maternal and umbilical cord blood complement results were also compared (see ***Table 3.4*****).** Significant positive correlations between maternal and fetal circulation results were observed for C4 (rho: 0.39, p=0.033) and Ba (rho: 0.44, p=0.008). No significant correlations were observed for the other complement markers assessed. Absolute values of iC3b and Ba (both complement split products / activation fragments) were higher in umbilical cord blood than maternal blood values. Conversely, cord blood concentrations of properdin, C3, C4, and C5b-9 were all lower than maternal concentrations and followed the same pattern of difference between PE and control groups.

Table 3.4: Correlation between maternal and umbilical cord blood complement concentrations

	Correlation between Maternal and Cord Blood		
	N	*Rho*	*p-Value*
Properdin	35	0.132	0.450
Ba	**35**	**0.442**	**0.008**
C5b-9	35	0.226	0.191
iC3b:C3	35	0.199	0.251
C4	**30**	**0.391**	**0.033**

For each marker, correlations between concentrations for maternal and cord blood were assessed using Spearman's correlation coefficients (rho), with associated p-values. Analyses are based on N=35 (11 with PE, and 24 healthy controls), other than for C4 (N=9/N=21). Bold values are significant at p<0.05.

3.2.4 Placental immunohistochemistry

Examples of placental immunohistochemistry for two patients with PE and one healthy control are shown in ***Figure 3.4***. ***Figure 3.5*** depicts examples of diffuse staining for C4d, C3d, and C9 in separate cases of PE (there were no cases of diffuse staining for C1q). ***Figure 3.6*** demonstrates focal staining examples for C4d, C3d, C9, and C1q. ***Figure 3.7*** shows examples of absent staining for C4d, C9, and C1q (there were no cases with absent C3d staining).

Comparisons of placental immunoreactivity scores between PE and control groups are reported in ***Table 3.5***, with scores at the STB membrane visualised in ***Figure 3.8.***

C4d staining was present on the STB membrane in almost all PE placentas (17/19, 89%), as compared to only 17% (5/30) of controls, with median immunoreactivity scores of 3 vs. 0, respectively ($p<0.001$). None of the healthy control placental tissue demonstrated diffuse staining for C4d. When present, C4d was observed in a linear staining pattern on the STB surface and deposited circumferentially around placental villi (see ***Figure 3.4*** images B and F and ***Figure 3.5*** image A).

There were no cases of absent C3d staining at the STB membrane in the PE group or in healthy subjects. However, the PE group had significantly increased C3d deposition compared to controls (median immunoreactivity score 6 vs. 4, $p=0.004$). ***Figure 3.4*** image A and ***Figure 3.5*** image B show examples of diffuse C3d staining on the STB membrane in separate cases of PE. Overall, there was no apparent relationship between C3d and C4d staining at the STB membrane, with co-localisation observed infrequently.

C9 was also detected more intensely at the STB surface in PE patients when compared to controls (median immunoreactivity score 4 vs. 1, $p<0.001$). C9 staining was observed most frequently in areas of villous injury and did not show consistent co-localisation with sites of C4d staining. An example of C9 deposition on the STB membrane is shown in ***Figure 3.5*** image C.

C1q was not strongly detected in the placental tissue of PE patients or of controls, with the majority of subjects exhibiting absent or weak staining at the maternal-fetal interface (see ***Figure 3.4*** images I, J, K and L). There were no significant differences seen between groups in C1q staining on the STB membrane (p=0.698).

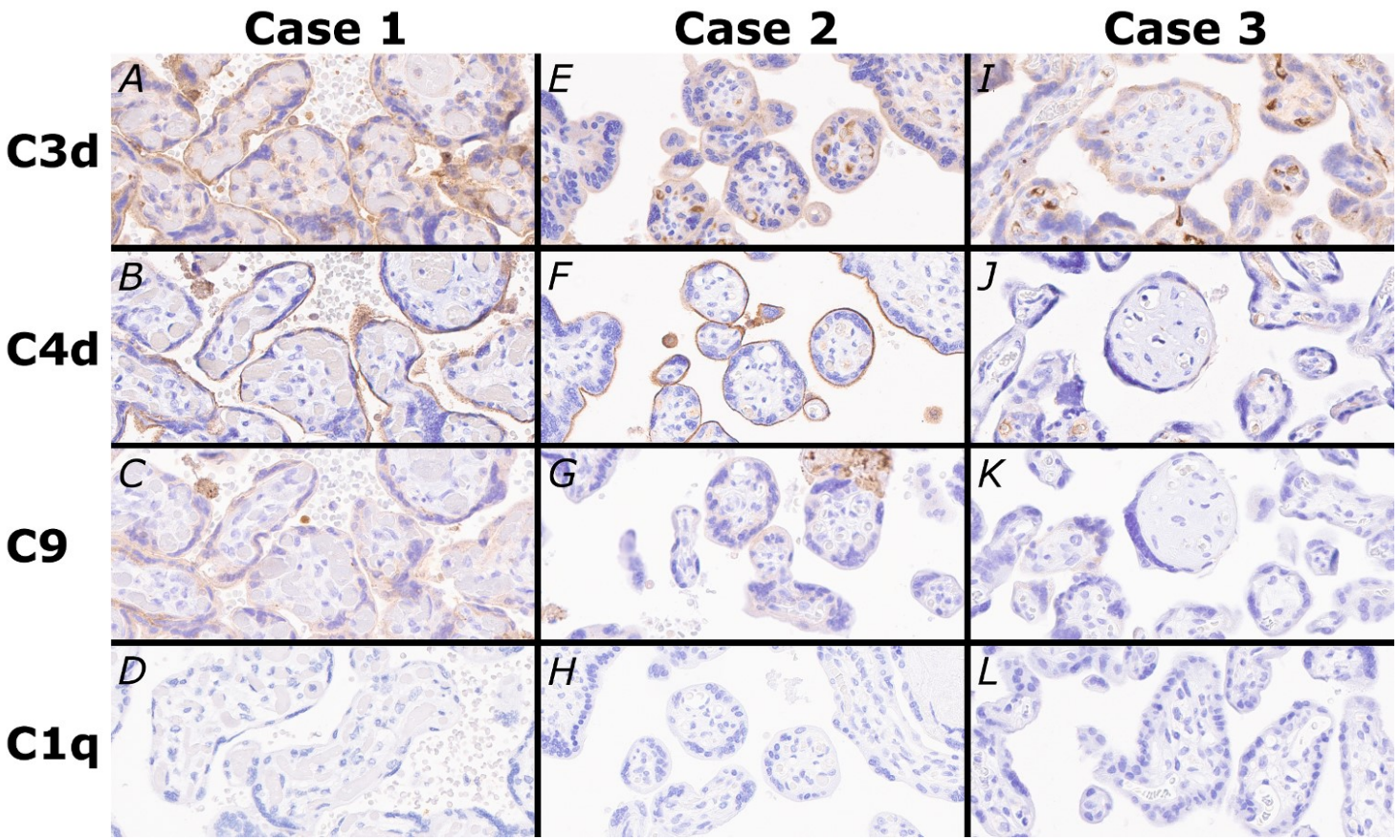


Figure 3.4: Placental staining of C3d, C4d, C9 and C1q in pre-eclampsia and healthy pregnancy

Case 1 (Subject with PE): *Diffuse C3d staining of the STB membrane [A]. Diffuse C4d staining localized to the STB membrane (strong, linear staining with syncytial cytoplasmic protrusions and membrane sheds) [B]. C9 staining shows weak membrane immunolocalization in STBs [C]. No immunostaining was detected for C1q [D].* ***Case 2 (Subject with PE):*** *C3d: weak staining of STB membrane (co-localizes with C4d staining pattern) [E]. Strong C4d staining localized to the STB membrane in a circumferential, linear pattern around the villi [F]. C9 and C3d: non-specific background staining of stromal-mesenchymal cells and villous capillary plasma [G and E]. C1q: no immunostaining detected [H].* ***Case 3 (Healthy control):*** *C3d: weak, non-specific background staining [I]. C4d, C9 and C1q: absent immunostaining [J, K, L]. All images are shown at high power (40 x magnification).*

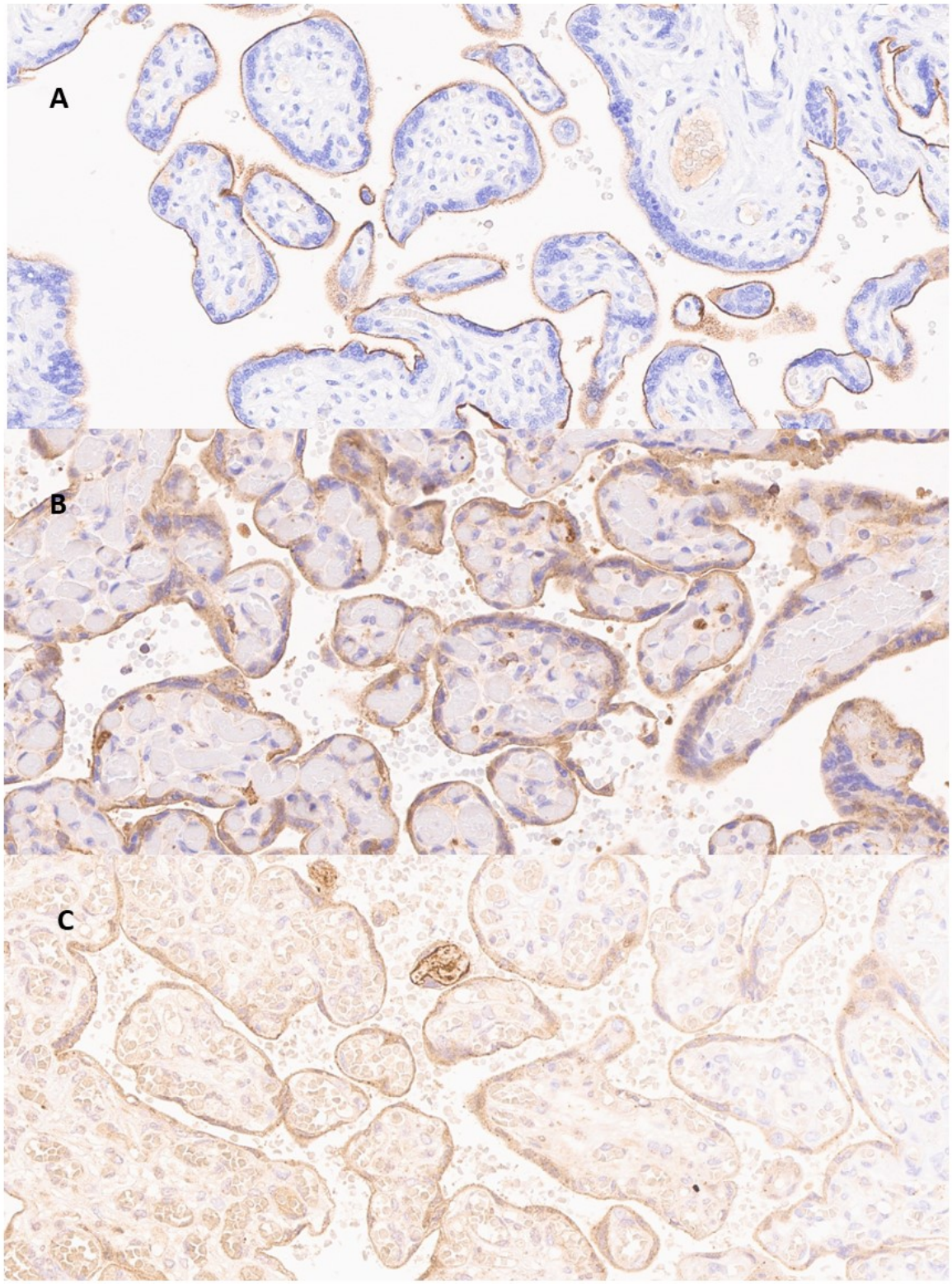


Figure 3.5: Placental tissue exhibiting diffuse staining of C4d, C3d and C9 in pre-eclampsia

Placental tissue shows diffuse staining on the STB membrane for C4d [A], C3d [B], and C9 [C]. Images are taken from separate cases of pre-eclampsia and are shown at 20 x magnification. There were no cases of diffuse C1q staining.

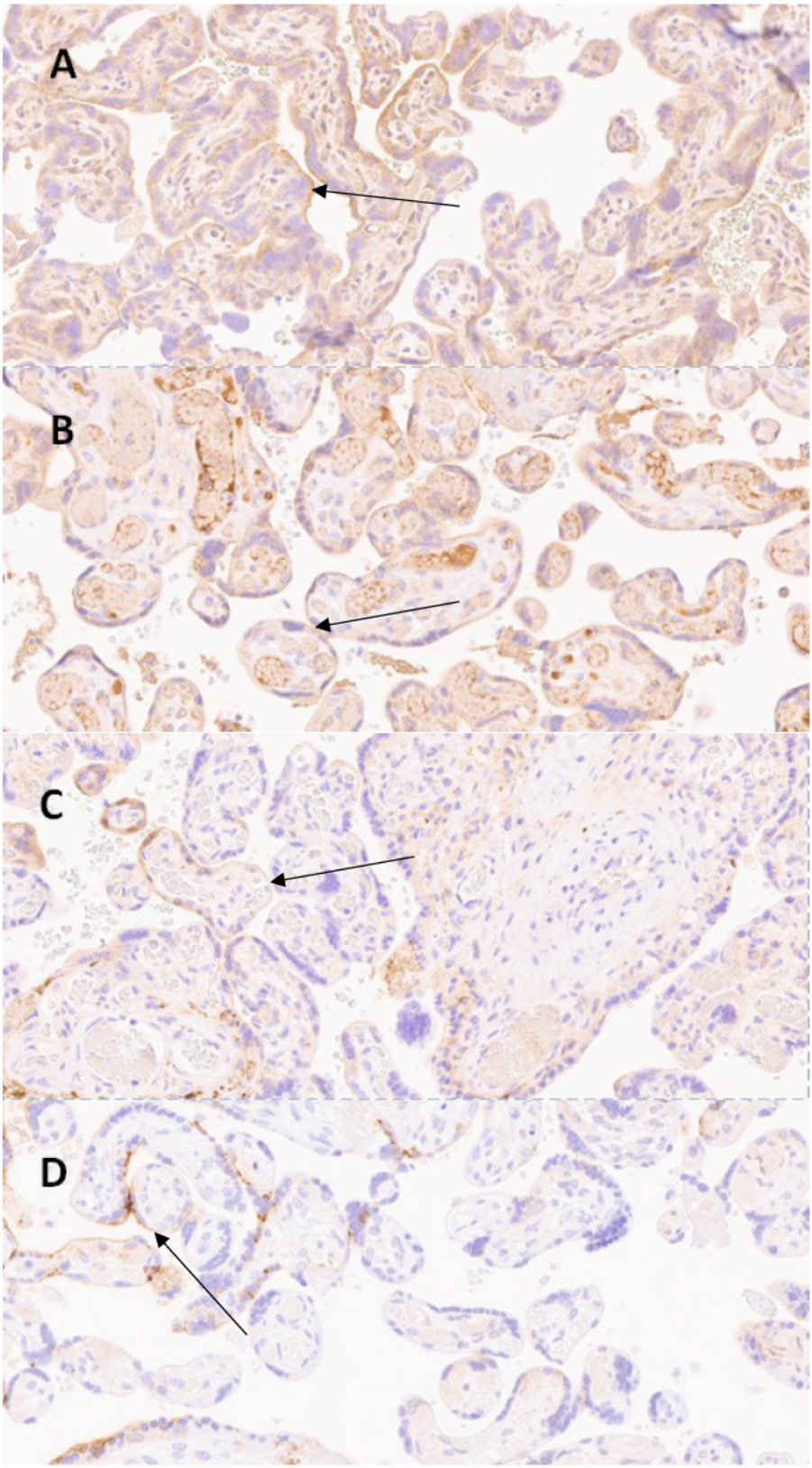


Figure 3.6: Placental tissue exhibiting focal staining of C4d, C3d, C9 and C1q in pre-eclampsia

Placental tissue shows focal staining on the STB membrane for C4d [A], C3d [B], C9 [C] and C1q [D], indicated by arrows. Images are taken from separate cases of pre-eclampsia and are shown at 20 x magnification.

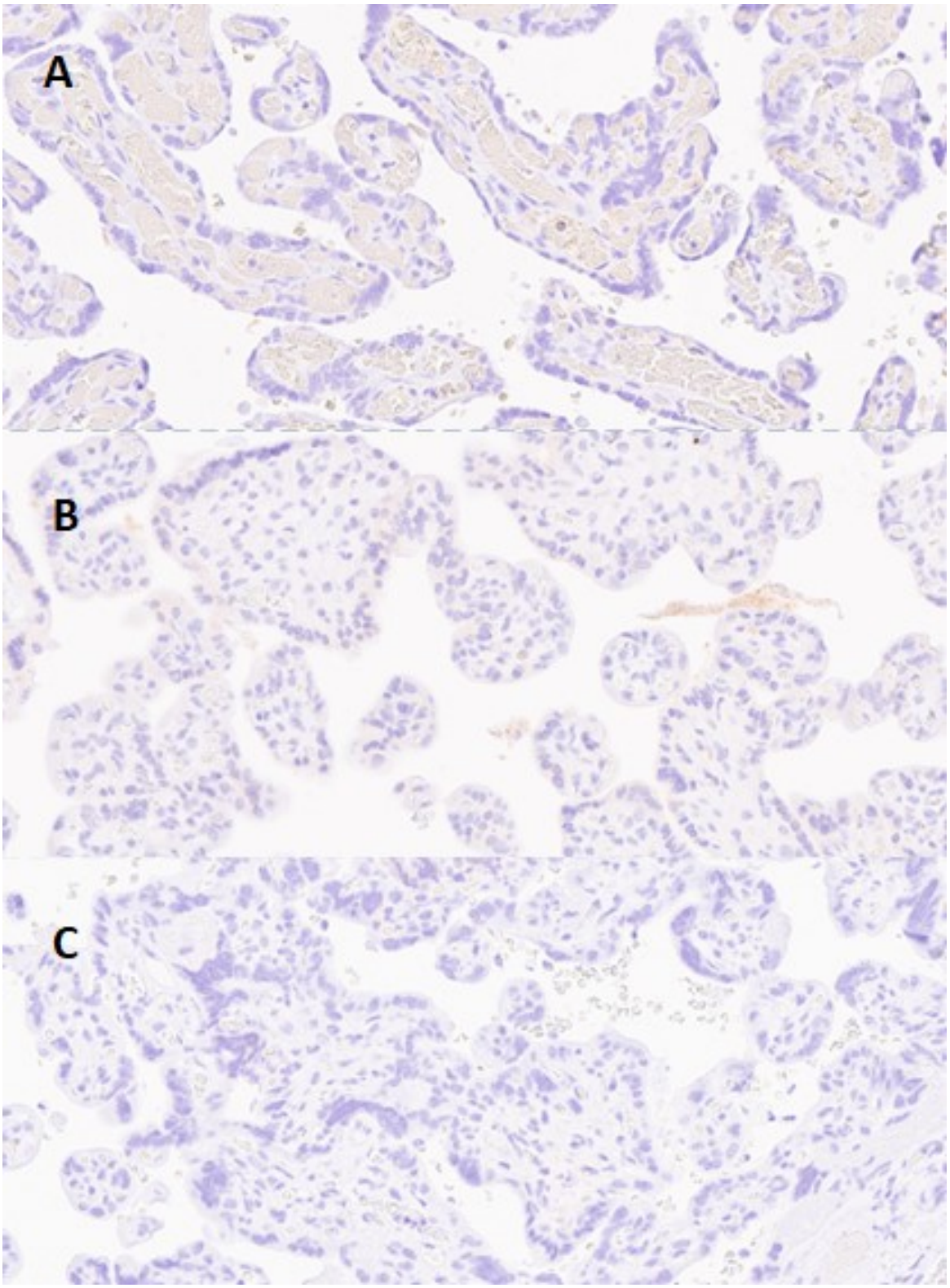


Figure 3.7: Placental tissue exhibiting absent staining of C4d, C9 and C1q in healthy controls

Placental tissue shows absent staining for C4d [A}, C9 [B], and C1q [C]. Images are taken from separate healthy controls and are shown at 20 x magnification. There were no cases of absent C3d staining.

Table 3.5: Placental immunoreactivity score by stain and cell type

Cell Type	PE	Control	p-Value	Cell Type	PE	Control	p-Value
Stain = C3d				Stain = C9			
STB	*6 (4-9)*	*4 (3-6)*	**0.004**	STB	*4 (2-6)*	*1 (0-3)*	**<0.001**
Absent	0 (0%)	0 (0%)		*Absent*	1 (5%)	13 (43%)	
Focal	11 (58%)	27 (93%)		*Focal*	15 (79%)	17 (57%)	
Diffuse	8 (42%)	2 (7%)		*Diffuse*	3 (16%)	0 (0%)	
Stain = C4d				Stain = C1q			
STB	*3 (2-6)*	*0 (0-0)*	**<0.001**	STB	*0 (0-1)*	*0 (0-1)*	0.698
Absent	2 (11%)	25 (83%)		*Absent*	12 (63%)	20 (67%)	
Focal	13 (68%)	5 (17%)		*Focal*	7 (37%)	10 (33%)	
Diffuse	4 (21%)	0 (0%)		*Diffuse*	0 (0%)	0 (0%)	

Results are based on N=19 PE and N=30 controls, with the exception of C3d, which only included N=29 controls, due to missing data in one case. Data are reported as both median (IQR), and the N (%) within intervals of the score. "Absent" is defined as a placental immunoreactivity score of 0, with "focal" and "diffuse" defined as scores of 1-8 and 9-12, respectively. p-Values are from Mann-Whitney U tests, and bold p-values are significant at p<0.05. PE=Pre-eclampsia; STB=Syncytiotrophoblast.

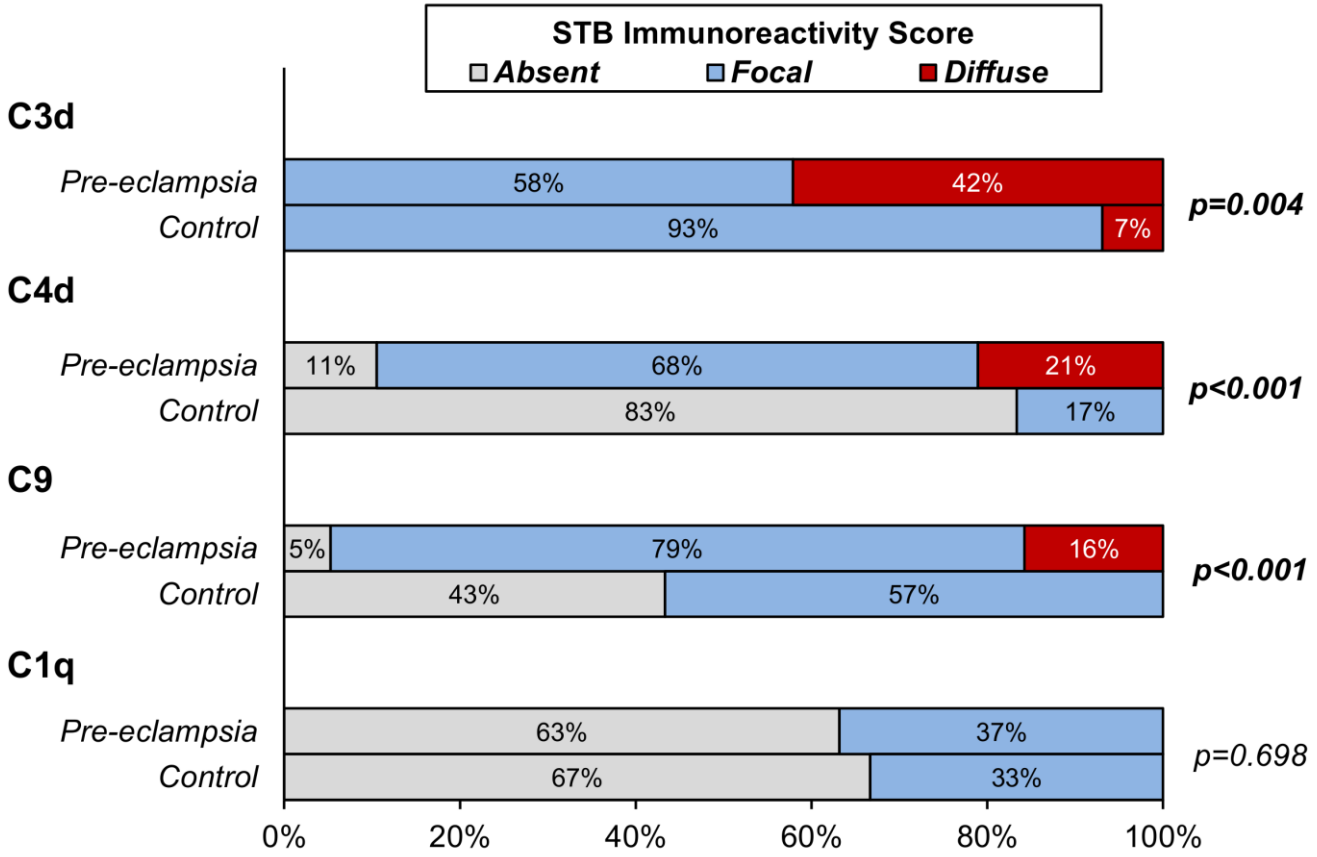


Figure 3.8: Placental immunoreactivity scores by pre-eclampsia status

*Results are based on N=19 pre-eclampsia and N=30 controls, with the exception of C3d, which only included N=29 controls, due to missing data in one case. "Absent" is defined as a score of 0, with "focal" and "diffuse" defined as scores of 1-8 and 9-12, respectively. p-Values are taken from Mann-Whitney U tests comparing the median immunoreactivity scores between groups (from **Table 3.5**); bold p-values are significant at p<0.05. STB=Syncytiotrophoblast.*

3.2.5 Relationship between circulating complement and placental complement

Associations between placental immunoreactivity scores and complement concentrations from maternal and umbilical cord blood were assessed (see ***Table 3.6***). This identified a significant negative correlation between maternal properdin concentration and C4d STB deposition (Spearman's rho: -0.524, $p<0.001$). Specifically, mean maternal properdin concentrations were 6839 ± 1559 ng/ml in those with absent C4d STB deposition; and 5393 ± 1095 and 4367 ± 820 ng/ml in those with focal (immunoreactivity score: 1-8) and diffuse (immunoreactivity score: 9-12) C4d STB deposition, respectively. Maternal C4 concentration was also significantly negatively correlated with placental C4d STB deposition (rho: -0.337, $p=0.019$). In addition, there was a significant positive correlation between maternal Ba and C4d deposition at the STB membrane (rho: 0.337, $p=0.019$). However, using a Bonferroni-corrected threshold for statistical significance of $p<0.005$, only the correlation between maternal properdin and placental C4d deposition remained significant.

There were no significant correlations found between umbilical cord blood complement concentration and placental complement staining at the STB membrane.

Table 3.6: Correlation between maternal / cord blood complement concentration, and placental immunoreactivity score

	Maternal Blood					Umbilical Cord Blood				
	Properdin	*Ba*	*C5b-9*	*iC3b:C3*	*C4*	*Properdin*	*Ba*	*C5b-9*	*iC3b:C3*	*C4*
C3d – STB	r: -0.130 p=0.385	r: 0.156 p=0.296	r: 0.240 p=0.104	**r: 0.291** **p=0.047**	r: -0.217 p=0.143	r: -0.233 p=0.216	r: 0.257 p=0.171	r: 0.032 p=0.867	r: -0.115 p=0.546	r: -0.200 p=0.326
C4d – STB	**r: -0.524** **p<0.001***	**r: 0.337** **p=0.019**	r: 0.216 p=0.141	r: -0.013 p=0.931	**r: -0.337** **p=0.019**	r: -0.181 p=0.329	r: 0.308 p=0.091	r: 0.233 p=0.208	r: -0.238 p=0.198	r: 0.027 p=0.893
C9 – STB	r: -0.184 p=0.210	r: 0.146 p=0.322	r: 0.264 p=0.070	r: 0.088 p=0.550	r: -0.267 p=0.066	r: -0.104 p=0.578	r: 0.337 p=0.064	r: 0.157 p=0.399	r: 0.054 p=0.771	r: -0.068 p=0.738
C1q – STB	r: -0.038 p=0.796	r: 0.001 p=0.995	r: 0.070 p=0.636	r: 0.127 p=0.388	r: -0.106 p=0.475	r: 0.013 p=0.947	r: -0.158 p=0.396	r: -0.156 p=0.401	r: -0.346 p=0.057	r: 0.026 p=0.897

*Analyses are based on N=48 for maternal blood and N=31 for umbilical cord blood, unless stated otherwise. Correlations between C3d and maternal/umbilical cord blood are based on N=47/N=30, due to missing data for one case. Correlations with maternal/umbilical cord blood are based on N=48/N=27. Associations between variables are reported as Spearman's correlation coefficients, with associated p-values. Bold values are significant at p<0.05. *p<0.005 (Bonferroni corrected threshold for multiple comparisons). r: Spearman's correlation coefficient; STB: syncytiotrophoblast.*

3.2.6 Biochemical and immunological markers

Maternal serum samples from the Birmingham Cohort were tested for a range of biochemical and immunological markers. The results are presented in ***Table 3.7***. There were significant differences between PE and controls in almost all the markers tested, apart from IgM. Specifically, biomarkers of excretory renal function (creatinine, urea, cystatin C and uric acid) were elevated in PE when compared to healthy pregnant controls (all p<0.001). B2M, LDH, kappa and lambda light chains, combined sFLCs and IgA levels were also significantly higher in PE than for healthy pregnant controls, whereas HS-CRP and IgG concentrations were significantly lower in PE.

Table 3.7: Biochemical and immunological marker results: Birmingham Cohort

	Birmingham Cohort			
Blood Marker	*N*	*Pre-eclampsia*	*Control*	*p-Value*
Creatinine (μmol/L)	66	59.5 ± 13.9	46.3 ± 9.5	**<0.001**
Urea (mmol/L)	66	3.9 (3.1-4.9)	2.7 (2.2-2.9)	**<0.001**
Cystatin C (mg/L)	66	1.5 (1.3-1.7)	1.1 (0.9-1.2)	**<0.001**
Uric acid (mg/dl)	66	6.5 ± 1.3	4.7 ± 1.0	**<0.001**
B2M (mg/L)	66	2.5 (2.3-3.0)	1.8 (1.6-2.0)	**<0.001**
Kappa (mg/L)	66	17.2 (14.8-20.6)	11.9 (10.2-13.3)	**<0.001**
Lambda (mg/L)	66	12.9 (11.5-15.1)	11.1 (10.0-12.8)	**0.010**
Combined sFLC (mg/L)	66	30.7 (26.9-33.9)	23.2 (20.4-25.2)	**<0.001**
IgG (g/L)	66	7.1 ± 2.1	8.4 ± 1.9	**0.012**
IgA (g/L)	66	1.9 ± 0.6	1.5 ± 0.8	**0.026**
IgM (g/L)	66	1.2 ± 0.5	1.2 ± 0.6	0.995
HS-CRP (mg/L)	66	5.8 (2.9-8.1)	8.6 (4.7-13.7)	**0.019**
LDH (u/L)	66	371.4 ± 103.8	311.6 ± 44.2	**0.003**

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests where data was normally distributed, or as median (IQR), with p-values for Mann-Whitney U tests otherwise. Bold p-values are significant at p<0.05. sFLC: serum free light chains.

B2M and sFLC concentrations are known to be affected by excretory renal function and are elevated in those with CKD (239, 240). Binary logistic regression analysis was therefore conducted to adjust for serum creatinine concentration. The results are presented in ***Table 3.8***. B2M, kappa, and combined sFLCs all remained independently associated with PE. However, lambda light chains were no longer significant associated with PE after controlling for the effect of serum creatinine (OR for PE 1.18, 95% CI 0.98-1.43, p=0.087).

Table 3.8: Logistic regression analysis to identify biomarkers independently associated with PE after controlling for serum creatinine

Factor	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	*p-value*	*OR (95% CI)*	*p-value*	*OR (95% CI)*	*p-value*	*OR (95% CI)*	*p-value*
B2M (per 0.1 mg/L increase)	1.56 (1.21-2.01)	**<0.001**						
Kappa (per 1mg/L increase)			1.22 (1.05-1.41)	**0.008**				
Lambda (per 1mg/L increase)					1.18 (0.98-1.43)	0.087		
Combined sFLCs (per 1mg/L increase)							1.12 (1.02-1.22)	**0.016**
Creatinine (per 10µmol/L increase)	1.75 (0.66-4.68)	0.263	3.89 (1.64-9.21)	**0.002**	4.22 (1.84-9.66)	**<0.001**	4.01 (1.71-9.37)	**<0.001**

Binary logistic regression analyses were conducted, using PE as the dependent variable. Creatinine was entered into each model as a continuous covariate, along with the biochemical / immunological marker being tested. Odds ratios for PE are reported, with associated 95% confidence intervals. P-values are significant at p<0.05.

The biochemical and immunological markers found to differ significantly between PE and control groups were then assessed using ROC curve analysis to determine the diagnostic accuracy for PE of each blood test. The results are displayed in ***Figure 3.9***, which provides a visual representation of the diagnostic accuracy of biomarkers that were significantly associated with PE (and the relative strength of the association of each biomarker with PE). B2M was the strongest predictor of PE diagnosis, with an AUROC of 0.92 (95% CI 0.85-0.99). This was followed by markers of excretory renal function: urea (AUROC 0.87, 95% CI 0.79-0.95), uric acid (AUROC 0.87, 95% CI 0.78-0.95), cystatin C (AUROC 0.85, 95% CI 0.75-0.94), and creatinine (AUROC 0.83, 95% CI 0.72-0.93).

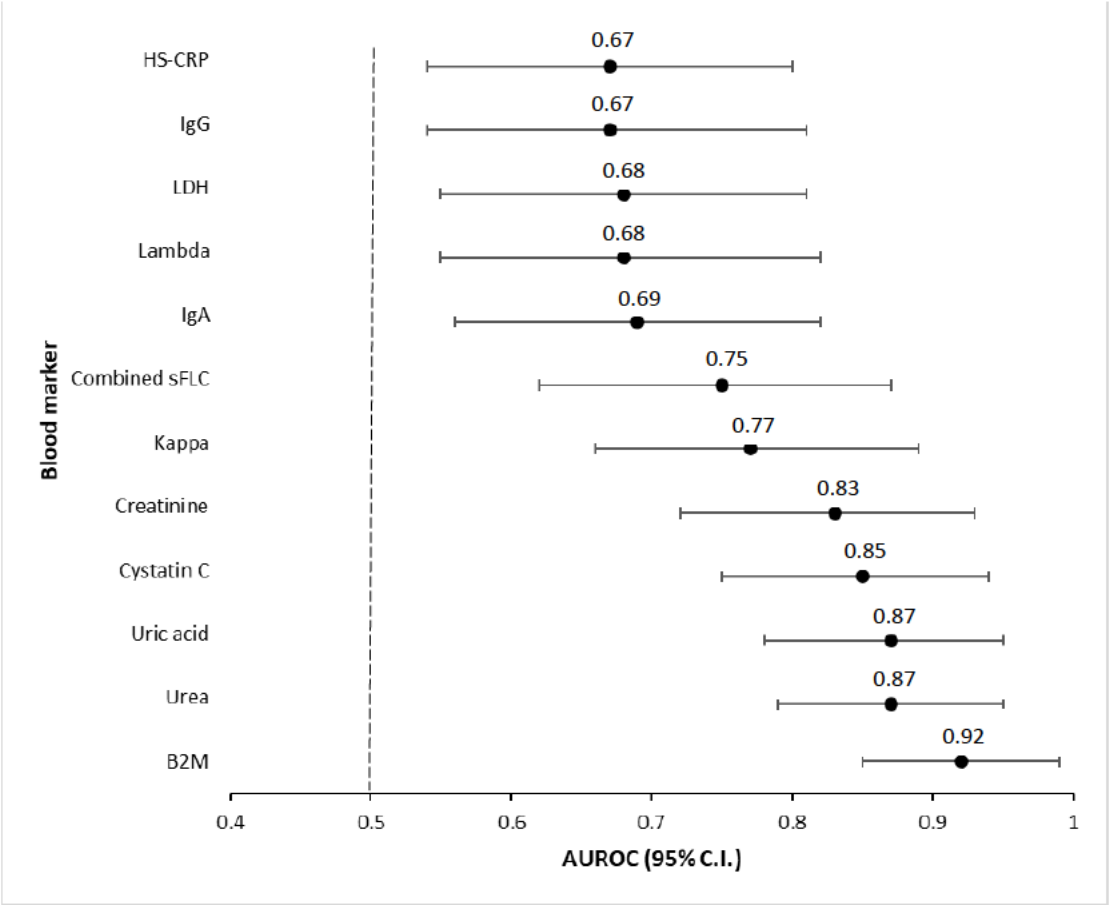


Figure 3.9: Forest plot of AUROC results for diagnostic accuracy of pre-eclampsia by biomarker

AUROC values are displayed as black circles, with corresponding error bars representing 95% confidence intervals. AUROC: area under receiver operating curve; CI: confidence interval.

3.2.7 Relationship between complement and biochemical / immunological markers

The relationship between complement and biochemical and immunological markers in maternal blood was assessed in order to characterise the cohort in greater detail, to assess the relationship between complement and other markers of immunity and inflammation, and to be able to identify and control for potential confounding factors in subsequent analysis.

Correlations between complement components and biochemical / immunological markers are shown in ***Table 3.9***. Given that multiple comparisons were performed, a Bonferroni-corrected threshold for statistical significance of $p<0.004$ is also indicated.

Using the corrected threshold for multiple comparisons, there were significant negative correlations between properdin and biomarkers of excretory renal function (creatinine, urea, cystatin C, and uric acid), as well as with B2M, kappa and combined sFLCs, and IgA. Properdin concentrations were significantly positively correlated with HS-CRP.

Ba concentrations were significantly positively correlated with B2M, kappa and combined sFLCs, but with none of the other biochemical or immunological biomarkers tested.

There were significant negative correlations between C4 and creatinine, cystatin C, uric acid, and B2M. None of the other markers tested were significantly correlated with C4 concentrations using the Bonferroni-corrected threshold.

Similarly, there were no significant relationships between iC3b:C3 ratio, or C5b-9, and any of the biochemical and immunological markers tested.

Table 3.9: Correlation between maternal blood complement, and biochemical and immunological markers

		Maternal blood complement marker				
		Properdin	*Ba*	*iC3b:C3*	*C4*	*C5b-9*
Biochemical / immunological marker	*Creatinine*	**r: -0.530 p<0.001***	r: 0.225 p=0.070	r: 0.050 p=0.691	**r: -0.474 p<0.001***	r: -0.013 p=0.917
	Urea	**r: -0.458 p<0.001***	r: 0.168 p=0.177	r: -0.004 p=0.976	**r: -0.344 p=0.005**	r: -0.034 p=0.789
	Cystatin C	**r: -0.609 p<0.001***	**r: 0.332 p=0.007**	r: 0.188 p=0.131	**r: -0.390 p=0.001***	r: 0.139 p=0.267
	Uric acid	**r: -0.633 p<0.001***	**r: 0.264 p=0.032**	r: 0.132 p=0.290	**r: -0.375 p=0.002***	r: 0.057 p=0.652
	HS-CRP	**r: 0.367 p=0.002***	r: -0.026 p=0.837	r: 0.152 p=0.224	**r: 0.294 p=0.017**	r: 0.309 p=0.012
	LDH	r: -0.170 p=0.173	**r: 0.344 p=0.005**	**r: 0.257 p=0.037**	r: -0.222 p=0.073	r: 0.208 p=0.094
	B2M	**r: -0.700 p<0.001***	**r: 0.367 p=0.002***	r: 0.210 p=0.091	**r: -0.449 p<0.001***	r: 0.167 p=0.181
	Kappa	**r: -0.435 p<0.001***	**r: 0.413 p<0.001***	r: 0.206 p=0.097	**r: -0.288 p=0.019**	r: 0.138 p=0.270
	Lambda	**r: -0.305 p=0.013**	**r: 0.316 p=0.010**	**r: 0.308 p=0.012**	**r: -0.268 p=0.029**	r: 0.070 p=0.578
	Combined sFLCs	**r: -0.402 p<0.001***	**r: 0.397 p<0.001***	**r: 0.261 p=0.034**	**r: -0.278 p=0.024**	r: 0.104 p=0.406
	IgG	**r: 0.307 p=0.012**	r: -0.046 p=0.712	r: -0.036 p=0.776	r: 0.104 p=0.405	r: 0.176 p=0.158
	IgA	**r: -0.360 p=0.003***	r: 0.172 p=0.167	r: 0.136 p=0.277	r: -0.219 p=0.077	r: 0.087 p=0.485
	IgM	r: -0.211 p=0.089	r: 0.187 p=0.133	r: -0.031 p=0.802	**r: -0.260 p=0.035**	r: 0.123 p=0.326

*Analyses are based on N=66. Associations between variables are reported as Spearman's correlation coefficient (r), with associated p-values. Bold values are significant at p<0.05. *p<0.004 (Bonferroni corrected threshold for multiple comparisons).*

3.2.7.1 Logistic regression analysis

Multiple logistic regression analysis was conducted, to determine whether maternal properdin, C4, C3, and Ba – which had been found to differ significantly between women with PE and healthy pregnant controls – were independently associated with PE, after accounting for biochemical and immunological marker concentrations.

The factors considered for inclusion were creatinine, urea, cystatin-C, uric acid, B2M, kappa, lambda, combined sFLCs, IgG, IgA, IgM, HS-CRP, and LDH. A binary logistic regression model was produced, with PE as the dependent variable, and a forward stepwise approach used to sequentially remove

interacting terms. Urea, HS-CRP and LDH were selected as the variables for inclusion. Properdin, Ba, C4, and C3 were then added in alongside these variables in subsequent models, to assess the independent association of each complement marker with PE, after adjusting for the effects of biochemical and immunological markers. The results are presented in ***Table 3.10***.

Maternal properdin remained independently associated with a diagnosis of PE after controlling for the effects of biochemical and immunological markers (OR for PE 0.70 per 100 ng/ml increase in properdin; 95% CI 0.50-0.97, p=0.032). Similarly, C4 was independently associated with PE (OR 0.20 per 0.1 g/L increase in C4, 95% CI 0.06-0.73, p=0.015). Finally, C3 was also independently associated with PE (OR 0.77 per 0.1 g/L increase in C3, 95% CI 0.61-0.97, p=0.026)

In contrast, maternal Ba no longer had a statistically significant association with PE after controlling for the effects of the biochemical and immunological markers tested.

Table 3.10: Multiple logistic regression analysis to identify independent markers of pre-eclampsia

Factor	Model 1		Model 2		Model 3		Model 4		Model 5	
	OR (95% CI)	*p-value*	*OR (95% CI)*	*p-value*	*OR (95% CI)*	*p-value*	*OR (95% CI)*	*p-value*	*OR (95% CI)*	*p-value*
Properdin (per 100 ng/ml increase)			0.70 (0.50-0.97)	**0.032**						
Ba (per 10 ng/ml increase)					1.01 (0.96-1.05)	0.690				
C4 (per 0.1 g/l increase)							0.20 (0.06-0.73)	**0.015**		
C3 (per 0.1 g/l increase)									0.77 (0.61-0.97)	**0.026**
Urea (per 0.1 mmol/l increase)	1.29 (1.11-1.50)	**<0.001**	1.65 (1.08-2.50)	**0.020**	1.30 (1.11-1.51)	**<0.001**	1.29 (1.09-1.52)	**0.002**	1.31 (1.10-1.55)	**0.002**
HS-CRP (per 1 mg/l increase)	0.83 (0.70-0.99)	**0.034**	0.90 (0.71-1.16)	0.424	0.83 (0.69-0.99)	**0.035**	0.89 (0.72-1.10)	0.279	0.86 (0.70-1.06)	0.146
LDH (per 10 u/l increase)	1.27 (1.07-1.52)	**0.007**	1.74 (1.01-3.00)	**0.046**	1.27 (1.06-1.52)	**0.009**	1.20 (1.02-1.42)	**0.029**	1.26 (1.05-1.50)	**0.012**

Results in table are from multiple logistic regression analysis. The factors considered for inclusion were creatinine, urea, cystatin-C, uric acid, B2M, kappa, lambda, combined sFLCs, IgG, IgA, IgM, HS-CRP, and LDH. A binary logistic regression model was produced, with PE as the dependent variable, and a forward stepwise approach used to select variables for inclusion (Model 1). Properdin, Ba, C4, and C3 were then added in alongside these variables (Models 2-5, respectively). Odds ratios for diagnosis of PE are reported, with associated 95% confidence intervals. Bold p-values are significant at p <0.05.

3.2.8 Subgroup analyses

Subgroup analyses were conducted for the Birmingham Cohort to assess the impact of potential confounding factors on systemic and placental complement activation, and to review whether complement concentration was associated with PE severity.

An additional subgroup analysis was conducted for the combined Birmingham and London cohorts to assess the effect of ethnicity on circulating complement concentrations.

3.2.8.1 Effect of mode of delivery on complement activity

Subjects were categorised into groups by mode of delivery to allow a comparison between vaginal and Caesarean section deliveries. Although maternal blood samples were all drawn prior to the onset of labour, umbilical cord blood and placental tissue was collected following delivery, and therefore, mode of delivery could in theory have affected complement expression in both.

Baseline demographic and pregnancy outcome data by PE status and mode of delivery group is presented in ***Table 3.11***. There were no significant differences in maternal age, BMI, ethnicity, parity, blood pressure, or gestational age between Caesarean and vaginal deliveries for PE pregnancies, or for healthy control pregnancies. However, blood samples were drawn later in pregnancy for vaginal deliveries in the control group. Pregnancies that were delivered by Caesarean section in the PE group had significantly lower birth weights and higher rates of NNU admission than PE pregnancies delivered vaginally. Furthermore, more than 40% of the Caesarean deliveries had early onset PE, compared with none in the PE vaginal delivery group.

Table 3.11: Demographic and clinical outcome data by pre-eclampsia status and mode of delivery

	Birmingham Cohort						
		Pre-eclampsia			*Controls*		
	N	***Caesarean (n=24)***	***Vaginal delivery (n=10)***	***p-value***	***Caesarean (n=28)***	***Vaginal delivery (n=5)***	***p-value***
Maternal age at delivery (years)	67	31.4 ± 6.5	28.6 ± 6.6	0.261	30.1 ± 5.5	27.2 ± 6.2	0.301
BMI (kg/m^2)	67	28.3 ± 4.8	28.9 ± 6.0	0.766	28.7 ± 5.2	30.4 ± 4.1	0.485
Ethnicity	67			0.442			0.677
White		10 (42%)	5 (50%)		15 (54%)	4 (80%)	
Asian		10 (42%)	2 (20%)		8 (29%)	1 (20%)	
Black		3 (13%)	3 (30%)		3 (11%)	0 (0%)	
Mixed/Other		1 (4%)	0 (0%)		2 (7%)	0 (0%)	
Parity	67			0.448			0.214
0		18 (75%)	7 (70%)		12 (43%)	4 (80%)	
1		3 (13%)	1 (1%)		12 (43%)	0 (0%)	
2+		3 (13%)	2 (2%)		4 (14%)	1 (20%)	
Peak SBP (mmHg)	65	165 ± 14	158 ± 13	0.236	128 ± 10	129 ± 8	0.877
Peak DBP (mmHg)	65	106 ± 9	102 ± 9	0.227	79 ± 8	79 ± 3	0.947
Peak uPCR (mg/mmol)	34	254.5 (171.3-419.3)	264.0 (112.3-471.3)	0.910	NA	NA	NA
Gestation at (weeks):							
Delivery	66	36.8 (34.3-37.7)	37.3 (36.9-37.4)	0.205	39.2 (39.0-39.9)	41.9 (40.6-42.0)	0.063
Blood Draw	65	36.4 (33.6-37.2)	36.8 (36.3-37.4)	0.182	39.0 (38.7-39.4)	41.7 (40.3-41.9)	**0.031**
PE diagnosis	34	35.1 (32.8-36.5)	36.2 (35.6-36.8)	0.135	NA	NA	NA
Early-onset PE (<34 weeks)	34	10 (42%)	0 (0%)	**0.017**	NA	NA	NA
Twin Pregnancy	67	1 (4%)	0 (0%)	1.000	0 (0%)	0 (0%)	1.000
Preterm Birth (<37 weeks)*	67	13 (54%)	4 (40%)	0.708	0 (0%)	0 (0%)	1.000
Birth Weight (g)**	67	2162 ± 678	2714 ± 482	**0.026**	3528 ± 521	3624 ± 293	0.694
Low Birth Weight (<2500 g)*	67	17 (71%)	2 (20%)	0.010	0 (0%)	0 (0%)	1.000
SGA (<10th centile)*	67	18 (75%)	4 (40%)	0.112	4 (14%)	1 (20%)	0.743
Neonatal Unit Care*	67	13 (54%)	0 (0%)	**0.005**	2 (7%)	2 (40%)	0.099

*Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *For the twin pregnancy, outcomes were the same for both babies; hence these were combined for analysis. **For the twin pregnancy, the average weight of the two babies was assumed. NA=data were not available in the cohort for the stated variable.*

Maternal and umbilical cord blood complement concentrations were then compared by mode of delivery within PE and control groups (***Table 3.12***), and between groups (***Table 3.13***).

There were no significant differences in maternal or umbilical cord blood complement levels, or placental complement immunostaining, between vaginal and Caesarean section deliveries in women with PE. Similarly, mode of delivery appeared to have no impact on maternal blood and placental complement levels in the healthy control group. However, umbilical cord Ba and C5b-9 concentrations were significantly higher in healthy control pregnancies delivered vaginally, when compared to controls delivered by Caesarean (see ***Table 3.12***).

Assessment of just those pregnancies delivered by Caesarean section found that PE was associated with reduced C4 and properdin in maternal blood, elevated Ba in maternal and umbilical cord blood, and increased deposition of C3d, C4d and C9 on the STB membrane, when compared to healthy controls. These results are reflective of the findings from the overall Birmingham Cohort. Analysis of just vaginal deliveries found reduced maternal blood concentrations of properdin and C4, and increased placental deposition of C4d and C1q in pregnancies affected by PE, compared to healthy controls. However, there were no significant differences in Ba in maternal or umbilical cord blood between PE and controls, or of placental C3d or C9 deposition (see ***Table 3.13***).

Table 3.12: Comparison of complement markers by mode of delivery within pre-eclampsia / control group

		PE			Controls		
	N	**Caesarean**	**Vaginal delivery**	**p-value**	**Caesarean**	**Vaginal delivery**	**p-value**
Maternal blood	66						
iC3b (ng/ml)		472 ± 126	527 ± 207	0.353	605 ± 167	611 ± 97	0.939
C3 (g/l)		1.90 ± 0.43	1.89 ± 0.31	0.938	2.38 ± 0.41	2.21 ± 0.23	0.358
iC3b:C3 ($x10^6$)		245 (194-284)	252 (183-356)	0.802	239 (213-289)	284 (251-301)	0.338
C4 (g/l)		0.20 ± 0.08	0.20 ± 0.09	0.933	0.31 ± 0.09	0.32 ± 0.04	0.776
Properdin (ng/ml)		4834 ± 817	4816 ± 823	0.956	6954 ± 1490	6447 ± 943	0.471
Ba (ng/ml)		161 (130-228)	126 (96-204)	0.207	119 (88-166)	109 (82-111)	0.514
C5b-9 (ng/ml)		237 (200-372)	240 (202-259)	0.893	236 (184-335)	290 (216-334)	0.639
Umbilical cord blood	36						
iC3b (ng/ml)		898 (537-1179)	663 (496-817)	0.465	769 (627-922)	1154 (951-2532)	0.145
C3 (g/l)		0.74 ± 0.26	0.91 ± 0.34	0.344	0.75 ± 0.20	0.88 ± 0.16	0.318
iC3b:C3 ($x10^6$)		1283 (650-1901)	622 (537-846)	0.465	1114 (715-1461)	1215 (984-3428)	0.505
C4 (g/l)		0.12 ± 0.04	0.14 ± 0.03	0.477	0.14 ± 0.05	0.13 ± 0.03	0.873
Properdin (ng/ml)		2619 ± 698	3396 ± 1013	0.145	3339 ± 1248	3197 ± 377	0.849
Ba (ng/ml)		354 (315-393)	298 (253-338)	0.062	224 (136-246)	356 (306-391)	**0.016**
C5b-9 (ng/ml)		60 (52-82)	102 (90-121)	0.223	53 (41-72)	138 (120-151)	**0.016**
Placental STB immunoreactivity score	49						
C3d		6 (6-9)	5 (4-7)	0.299	4 (4-6)	4 (3-5)	0.433
C4d		4 (2-8)	2 (2-2)	0.089	0 (0-0)	0 (0-0)	0.346
C9		4 (2-6)	4 (3-5)	0.960	1 (0-3)	1 (0-3)	0.898
C1q		0 (0-1)	2 (1-2)	0.092	0 (0-1)	0 (0-0)	0.143

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Maternal blood results were available for N=33/N=33 pre-eclampsia/control patients, whilst umbilical blood concentrations were available for N=12/N=24, with the exception of C4 (N=10/N=21). Placental results are based on N=19/N=30, with the exception of C3d, which only included N=29 controls, due to missing data in one case. Bold p-values are significant at p<0.05. PE=Pre-eclampsia; STB=Syncytiotrophoblast.

Table 3.13: Comparison of complement markers by mode of delivery between pre-eclampsia / control groups

		Caesarean delivery			Vaginal delivery		
	N	**PE**	**Control**	**p-value**	**PE**	**Control**	**p-value**
Maternal blood	66						
iC3b (ng/ml)		472 ± 126	605 ± 167	**0.03**	527 (207)	611 (97)	0.412
C3 (g/l)		1.90 ± 0.43	2.38 ± 0.41	**<0.001**	1.89 (0.31)	2.21 (0.23)	0.063
iC3b:C3 ($x10^6$)		245 (194-284)	239 (213-289)	0.910	252 (183-356)	284 (251-301)	0.768
C4 (g/l)		0.20 ± 0.08	0.31 ± 0.09	**<0.001**	0.20 (0.09)	0.32 (0.04)	**0.017**
Properdin (ng/ml)		4834 ± 817	6954 ± 1490	**<0.001**	4816 (823)	6447 (943)	**0.004**
Ba (ng/ml)		161 (130-228)	119 (88-166)	**0.012**	126 (96-204)	109 (82-111)	0.327
C5b-9 (ng/ml)		237 (200-372)	236 (184-335)	0.545	240 (202-259)	290 (216-334)	0.462
Umbilical cord blood	36						
iC3b (ng/ml)		898 (537-1179)	769 (627-922)	0.730	663 (496-817)	1154 (951-2532)	0.180
C3 (g/l)		0.74 ± 0.26	0.75 ± 0.20	0.895	0.91 ± 0.34	0.88 ± 0.16	0.886
iC3b:C3 ($x10^6$)		1283 (650-1901)	1114 (715-1461)	0.691	622 (537-846)	1215 (984-3428)	0.180
C4 (g/l)		0.12 ± 0.04	0.14 ± 0.05	0.429	0.14 ± 0.03	0.13 ± 0.03	0.827
Properdin (ng/ml)		2619 ± 698	3339 ± 1248	0.162	3396 ± 1013	3197 ± 377	0.761
Ba (ng/ml)		354 (315-393)	224 (136-246)	**0.002**	298 (253-338)	356 (306-391)	0.297
C5b-9 (ng/ml)		60 (52-82)	53 (41-72)	0.577	102 (90-121)	138 (120-151)	0.297
Placental STB immunoreactivity score	49						
C3d		6 (6-9)	4 (4-6)	**0.006**	5 (4-7)	4 (3-5)	0.180
C4d		4 (2-8)	0 (0-0)	**<0.001**	2 (2-2)	0 (0-0)	**0.046**
C9		4 (2-6)	1 (0-3)	**0.002**	4 (3-5)	1 (0-3)	0.137
C1q		0 (0-1)	0 (0-1)	0.520	2 (1-2)	0 (0-0)	**0.046**

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Maternal blood results were available for N=33/N=33 pre-eclampsia/control patients, whilst umbilical blood concentrations were available for N=12/N=24, with the exception of C4 (N=10/N=21). Placental results are based on N=19/N=30, with the exception of C3d, which only included N=29 controls, due to missing data in one case. Bold p-values are significant at p<0.05. PE=Pre-eclampsia; STB=Syncytiotrophoblast.

3.2.8.2 Effect of PE timing of onset on complement activity

Subjects with PE were categorised according to timing of PE onset to serve as a marker of disease severity: early-onset PE (diagnosis of PE prior to 34 weeks gestation), versus late-onset PE (diagnosis of PE at or after 34 weeks' gestation). Baseline demographic and clinical outcome data are shown in ***Table 3.14***. There were no significant differences in maternal age, BMI, ethnicity or parity between groups. However, early-onset PE was characterised by significantly higher blood pressure, lower gestation at blood draw and delivery, lower birth weight, and higher rates of Caesarean section delivery, preterm birth and NNU admission than the late-onset PE group.

Table 3.14: Demographic and clinic outcome data: early-onset versus late-onset pre-eclampsia

	N	***Early-onset PE (n=10)***	***Late-onset PE (n=24)***	***p-value***
Maternal age at delivery (years)	34	29.4 ± 6.7	31.1 ± 6.6	0.504
BMI (kg/m^2)	34	27.9 ± 3.9	28.8 ± 5.6	0.650
Ethnicity:	34			0.923
White		5 (50%)	10 (42%)	
Asian		3 (30%)	9 (38%)	
Black		2 (20%)	4 (17%)	
Mixed/Other		0 (0%)	1 (4%)	
Parity:	34			0.544
0		6 (60%)	19 (79%)	
1		2 (20%)	2 (8%)	
2+		2 (20%)	3 (13%)	
Peak SBP (mmHg)	34	173 ± 15	159 ± 11	**0.005**
Peak DBP (mmHg)	34	110 ± 12	103 ± 7	**0.043**
Peak uPCR (mg/mmol)	34	363.5 (234.3-772.8)	241.5 (100.5-397.8)	0.050
Mode of delivery:	34			**0.017**
Caesarean		10 (100%)	14 (58%)	
Vaginal		0 (0%)	10 (42%)	
Gestation at (weeks):				
Delivery	34	31.5 (30.1-35.7)	37.4 (36.9-37.7)	**<0.001**
Blood Draw	34	31.4 (29.8-35.5)	37.0 (36.4-37.4)	**<0.001**
PE diagnosis	34	30.9 (29.4-33.1)	36.4 (35.8-36.9)	**<0.001**
Twin Pregnancy	34	0 (0%)	1 (4%)	1.000
Preterm Birth (<37 weeks)*	34	10 (100%)	7 (29%)	**<0.001**
Birth Weight (g)**	34	1676 ± 726	2594 ± 423	**<0.001**
Low Birth Weight (<2500 g)*	34	9 (90%)	10 (42%)	**0.020**
SGA (<10th centile)*	34	9 (90%)	13 (54%)	0.061
Neonatal Unit Care*	34	7 (70%)	6 (25%)	**0.022**

*Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values from Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *For the twin pregnancy, outcomes were the same for both babies; hence these were combined for analysis. **For the twin pregnancy, the average weight of the two babies was assumed.*

Complement component markers in the maternal blood, umbilical cord blood, and placental tissue were compared between early-onset and late-onset PE groups (see ***Table 3.15***). Maternal blood C4 concentrations were significantly lower in the early-onset PE group than the late-onset group (0.16 vs. 0.22 g/L, p=0.048). There were no other significant differences in maternal blood complement, or in any of the umbilical cord blood complement markers, between groups. Analysis of placental immunoreactivity scores showed significant C9 deposition in late-onset PE placentas compared to early-onset PE (median immunoreactivity score 6 vs. 2, p=0.023). There were no other significant differences between groups for the other placental complement stains.

Table 3.15: Complement component markers: early-onset versus late-onset pre-eclampsia

		Pre-eclampsia group		
	N	**Early-onset PE**	**Late-onset PE**	**p-value**
Maternal blood	33			
iC3b (ng/ml)		414 ± 99	521 ± 163	0.063
C3 (g/l)		1.82 ± 0.32	1.93 ± 0.42	0.475
iC3b:C3 (x10^6)		214 (189-243)	259 (202-308)	0.117
C4 (g/l)		0.16 ± 0.08	0.22 ± 0.08	**0.048**
Properdin (ng/ml)		4678 ± 591	4894 ± 887	0.243
Ba (ng/ml)		148 (109-181)	161 (124-236)	0.638
C5b-9 (ng/ml)		223 (204-299)	237 (198-349)	0.829
Umbilical cord blood	12			
iC3b (ng/ml)		918 (664-1171)	740 (517-923)	0.830
C3 (g/l)		0.79 ± 0.19	0.82 ± 0.32	0.911
iC3b:C3 (x10^6)		1316 (880-1753)	840 (559-1531)	0.830
C4 (g/l)		0.07 ± 0.00	0.13 ± 0.03	0.141
Properdin (ng/ml)		2504 ± 477	3030 ± 949	0.473
Ba (ng/ml)		323 (308-338)	337 (264-377)	0.830
C5b-9 (ng/ml)		44 (37-50)	89 (78-117)	0.053
Placental STB immunoreactivity score	19			
C3d		6 (4-6)	9 (6-9)	0.054
C4d		2 (2-11)	4 (2-5)	0.794
C9		2 (2-3)	6 (4-8)	**0.023**
C1q		0 (0-1)	0 (0-1)	0.769

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values from Mann-Whitney U tests. Maternal blood results were available for N=10/N=23 early-onset PE/late-onset PE patients, whilst umbilical blood concentrations were available for N=2/N=10. Placental results are based on N=7/N=12. Bold p-values are significant at p<0.05. PE=Pre-eclampsia; STB=Syncytiotrophoblast.

3.2.8.3 Effect of ethnicity on circulating complement activity

Subjects from the combined Birmingham and London cohorts were categorised into 'Black' and 'non-Black' ethnic groups to enable an assessment of the potential effect of Black ethnicity on circulating complement concentrations. Baseline demographic and clinical outcome data by group and PE status are shown in ***Table 3.16***. There were no significant differences between ethnic group categories in any of the recorded maternal demographic factors or pregnancy outcome parameters.

Maternal blood complement component concentrations by ethnic group category and PE status are shown in ***Table 3.17***. Data from a total of 136 pregnancies from combined Birmingham and London cohorts were available for properdin and Ba, whereas data from 66 pregnancies from the Birmingham Cohort alone were available for the remaining complement markers (iC3b, C3, iC3b:C3 ratio, C4, and C5b-9).

There were no significant differences between Black and non-Black ethnic groups in any of the complement components measured for women with PE or for healthy pregnant controls. However, in the PE group, there was a possible signal for elevated C5b-9 levels in women of Black ethnicity, compared with women of non-Black ethnicity (median C5b-9 concentration 390 ng/ml (IQR 261-590) in Black women, vs. 232 ng/ml (IQR 198-277) in non-Black women, p=0.059). Although the difference between groups did not reach statistical significance, there was very little overlap in the IQR between ethnic groups.

Table 3.16: Demographic and clinical outcome data: Black versus non-Black ethnicity

	Combined Birmingham and London cohorts						
		Pre-eclampsia			*Controls*		
	N	***Black (n=18)***	***non-Black (n=51)***	***p-value***	***Black (n=7)***	***Non-Black (n=61)***	***p-value***
Maternal age at delivery (years)	137	31.6 ± 4.5	32.3 ± 7.3	0.660	33.2 ± 5.8	31.2 ± 5.3	0.348
BMI (kg/m^2)	137	29.6 ± 5.8	28.3 ± 6.5	0.470	31.2 ± 5.3	27.8 ± 7.3	0.232
Mode of delivery:	137			1.000			1.000
Vaginal		6 (33%)	16 (31%)		2 (29%)	21 (34%)	
Caesarean section		12 (66%)	35 (69%)		5 (71%)	40 (66%)	
Parity	137			0.367			0.422
0		10 (56%)	37 (73%)		3 (43%)	35 (57%)	
1		4 (22%)	5 (10%)		3 (43%)	18 (30%)	
2+		4 (22%)	9 (18%)		1 (14%)	8 (13%)	
Peak SBP (mmHg)	135	173 ± 15	166 ± 13	0.074	132 ± 7	132 ± 12	0.986
Peak DBP (mmHg)	135	107 ± 11	105 ± 7	0.323	81 ± 7	82 ± 10	0.854
Peak uPCR (mg/mmol)	69	161.5 (55.3-401.8)	164.0 (84.0-317.5)	0.662	NA	NA	NA
Gestation at (weeks):							
Delivery	136	36.1 (34.5-36.8)	36.9 (35.4-37.4)	0.063	39.7 (39.1-40.5)	39.4 (39.0-40.9)	0.885
Blood Draw	135	34.5 (31.6-36.1)	35.8 (33.9-36.8)	0.075	34.6 (32.9-38.9)	36.7 (33.9-39.0)	0.460
PE diagnosis	69	33.7 (31.8-35.8)	34.7 (33.2-36.1)	0.229	NA	NA	NA
Early-onset PE (<34 weeks)	69	10 (56%)	19 (37%)	0.267	NA	NA	NA
Twin Pregnancy	137	1 (6%)	0 (0%)	0.261	0 (0%)	0 (0%)	1.000
Preterm Birth (<37 weeks)*	137	14 (78%)	27 (53%)	0.094	1 (14%)	1 (2%)	0.197
Birth Weight (g)**	137	2204 ± 847	2416 ± 741	0.317	3615 ± 492	3563 ± 514	0.800
Low Birth Weight (<2500 g)*	137	12 (67%)	27 (53%)	0.410	0 (0%)	0 (0%)	1.000
SGA (<10th centile)*	137	11 (61%)	28 (55%)	0.784	0 (0%)	7 (11%)	1.000
Neonatal Unit Care*	137	11 (61%)	24 (47%)	0.413	0 (0%)	5 (8%)	1.000

*Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *For the twin pregnancy, outcomes were the same for both babies; hence these were combined for analysis. **For the twin pregnancy, the average weight of the two babies was assumed. NA=data were not available in the cohort for the stated variable.*

Table 3.17: Complement component markers: Black versus non-Black ethnicity

		Pre-eclampsia			Controls		
Complement marker	**N**	**Black**	**non-Black**	**p-value**	**Black**	**non-Black**	**p-value**
Maternal blood							
iC3b (ng/ml)	66	521 ± 276	482 ± 119	0.582	544 ± 231	612 ± 152	0.486
C3 (g/l)	66	1.74 ± 0.37	1.93 ± 0.40	0.298	2.44 ± 0.54	2.35 ± 0.38	0.703
iC3b:C3 ($x10^6$)	66	273 (200-303)	244 (194-299)	0.674	206 (193-235)	252 (221-294)	0.133
C4 (g/l)	66	0.23 ± 0.09	0.19 ± 0.08	0.322	0.38 ± 0.00	0.30 ± 0.08	0.111
Properdin (ng/ml)	136	5151 ± 1491	5030 ± 1102	0.717	7462 ± 1744	6892 ± 1314	0.298
Ba (ng/ml)	136	147 (109-206)	163 (122-238)	0.532	144 (127-179)	129 (97-176)	0.552
C5b-9 (ng/ml)	66	390 (261-590)	232 (198-277)	0.059	254 (172-307)	236 (221-294)	0.791

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Bold p-values are significant at p<0.05. For women with pre-eclampsia, maternal blood concentrations were available for N=18/N=50 Black/non-Black ethnicity for properdin and Ba and N=6/N=27 Black/non-Black ethnicity for the remaining complement markers. For healthy control pregnancies, maternal blood concentrations were available for N=7/N=61 Black/non-Black ethnicity for properdin and Ba and N=3/N=30 Black/non-Black ethnicity for the remaining complement markers.

3.2.9 Comparison between complement assays tested in Newcastle and Birmingham

Complement assays for properdin, Ba and C5b-9 were repeated for a small number of randomly selected patients from the Birmingham Cohort using commercially purchased ELISA kits (Hycult / Quidel), and testing was performed in Birmingham (CIS, UoB) (see Methodology ***section 2.6***). The rationale for this was to ensure that the results were comparable to MSD assays carried out in Newcastle. This would allow reliable complement testing to be undertaken in Birmingham using commercially available ELISA kits for cohorts of women with CKD and of SSA ethnicity for chapters 4 and 5 of this thesis, respectively.

A total of 32 subjects had blood tests repeated for properdin (16 with PE, and 16 healthy pregnant controls), and 20 subjects had repeat blood testing for Ba and C5b-9 (10 with PE, and 10 healthy controls). Demographics are presented in ***Table 3.18.*** There were no significant differences in age, BMI, ethnicity, or mode of delivery between groups. A greater proportion of the PE group were nulliparous (81%, versus 38% of controls, p=0.025). Furthermore, the PE group delivered earlier in pregnancy (median 36.9 weeks (IQR 36.0-37.5), versus 39.3 weeks in controls (39.0-40.6), p<0.001), and had blood samples drawn earlier in gestation (36.7 weeks (35.5-37.1), versus 39.0 weeks (38.7-40.4), p<0.001). Rates of preterm birth, low birth weight, and SGA deliveries were all higher in the PE group than in controls (all p<0.001).

Table 3.18: Demographic and clinical outcome data for repeat blood samples tested in Birmingham

	N	***PE***	***Controls***	***p-value***
Maternal age at delivery (years)	32	28.5 (7.4)	30.1 (6.0)	0.517
BMI (kg/m^2)	32	27.2 (5.0)	27.9 (4.4)	0.696
Ethnicity:	32			0.753
White		9 (56%)	9 (56%)	
Asian		4 (25%)	4 (25%)	
Black		3 (19%)	2 (13%)	
Mixed/Other		0 (0%)	1 (6%)	
Parity	32			**0.025**
0		13 (81%)	6 (38%)	
1		2 (13%)	6 (38%)	
2+		1 (6%)	4 (25%)	
Peak blood pressure (mmHg)				
SBP	32	150 (157-163)	128 (123-136)	**<0.001**
DBP	32	104 (101-108)	80 (71-86)	**<0.001**
Peak uPCR (mg/mmol)	16	379.0 (244.3-491.8)	NA	NA
Mode of delivery	32			1.000
Caesarean		12 (75%)	13 (81%)	
Vaginal		4 (25%)	3 (19%)	
Gestation at (weeks):				
Delivery	31	36.9 (36.0-37.5)	39.3 (39.0-40.6)	**<0.001**
Blood draw	31	36.7 (35.5-37.1)	39.0 (38.7-40.4)	**<0.001**
PE diagnosis	16	35.7 (33.5-36.6)	NA	NA
Early-onset PE (<34 weeks)	16	5 (31%)	NA	NA
Preterm birth (<37 weeks)	32	10 (63%)	0 (0%)	**<0.001**
Birth weight (g)	32	2311 (725)	3457 (344)	**<0.001**
Low Birth Weight (<2500g)	32	10 (63%)	0 (0%)	**<0.001**
SGA (<10th centile)	31	9 (56%)	0 (0%)	**<0.001**
Neonatal unit care	32	6 (38%)	2 (13%)	0.110

Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values from Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05.

A comparison of complement component concentration by location tested / assay used is shown in ***Table 3.19***. Although absolute values differ between groups, the results show that properdin concentrations were significantly lower in women with PE using either MSD assays in Newcastle, or Hycult assays in Birmingham (p<0.001 for both, when compared to healthy pregnant controls). Similarly, Ba levels were significantly elevated in women with PE compared to controls, for both Newcastle MSD assays (p<0.001), and Birmingham Quidel assays (p=0.019). There were no

significant differences in C5b-9 between women with PE and healthy controls for either of the assays used.

Bland-Altman plots are displayed in ***Figure 3.10***, to enable further comparison of complement assays carried out in Newcastle and Birmingham.

The plot for properdin assays is indicative of a proportional bias being present. The higher the mean properdin concentration, the greater the degree of difference between the Newcastle and Birmingham assay result. There appeared to be a linear relationship between the two variables, so linear regression analysis was conducted to identify the conversion factor between assay results. This found that: Birmingham assay properdin (ng/ml) = (1.77 x Newcastle assay properdin) + 5226; R^2 0.549, $p<0.001$.

Similarly, the plot for Ba was also indicative of a proportional bias, with a linear relationship between mean assay result and difference between assay results. Linear regression analysis found that: Birmingham assay Ba (ng/ml) = (3.91 x Newcastle assay Ba) + 472; R^2 0.670, $p<0.001$. The higher R^2 value indicates that this model is more accurately able to predict Ba values than the preceding properdin model.

In contrast, the C5b-9 Bland Altman plot showed fixed bias (a consistent difference between Newcastle and Birmingham assay results, regardless of the mean C5b-9 concentration). The mean difference between C5b-9 assay results was 16.5 ng/ml (95% limits of agreement -123.5 to 156.5).

The absolute concentrations of complement components were generally higher in magnitude in the assays conducted in Birmingham, compared to those tested in Newcastle. The cause of the difference in results between assays may be related to the assays being calibrated to different reference material, in particular with the standards used for properdin and Ba differing between the assays conducted in Newcastle, and those tested in Birmingham.

Table 3.19: Complement component results by location tested / assay used

Blood Marker (ng/ml)	N	Newcastle Results (MSD assays)			Birmingham Results (Hycult / Quidel assays)		
		Pre-eclampsia	Control	p-Value	Pre-eclampsia	Control	p-Value
Maternal Properdin	32	4275 ± 568	7481 ± 1393	**<0.001**	12605 ± 3052	18669 ± 3915	**<0.001**
Maternal Ba	20	188 (146-245)	89 (79-108)	**<0.001**	1286 (977-1544)	853 (626-1001)	**0.019**
Maternal C5b-9	20	234 (183-391)	200 (176-257)	0.149	262 (196-378)	204 (175-356)	0.481

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values from Mann-Whitney U tests. Maternal properdin results were available for N=16/N=16 PE/control patients, whereas maternal Ba and C5b-9 concentrations were available for N=10/N=10. Bold p-values are significant at p<0.05.

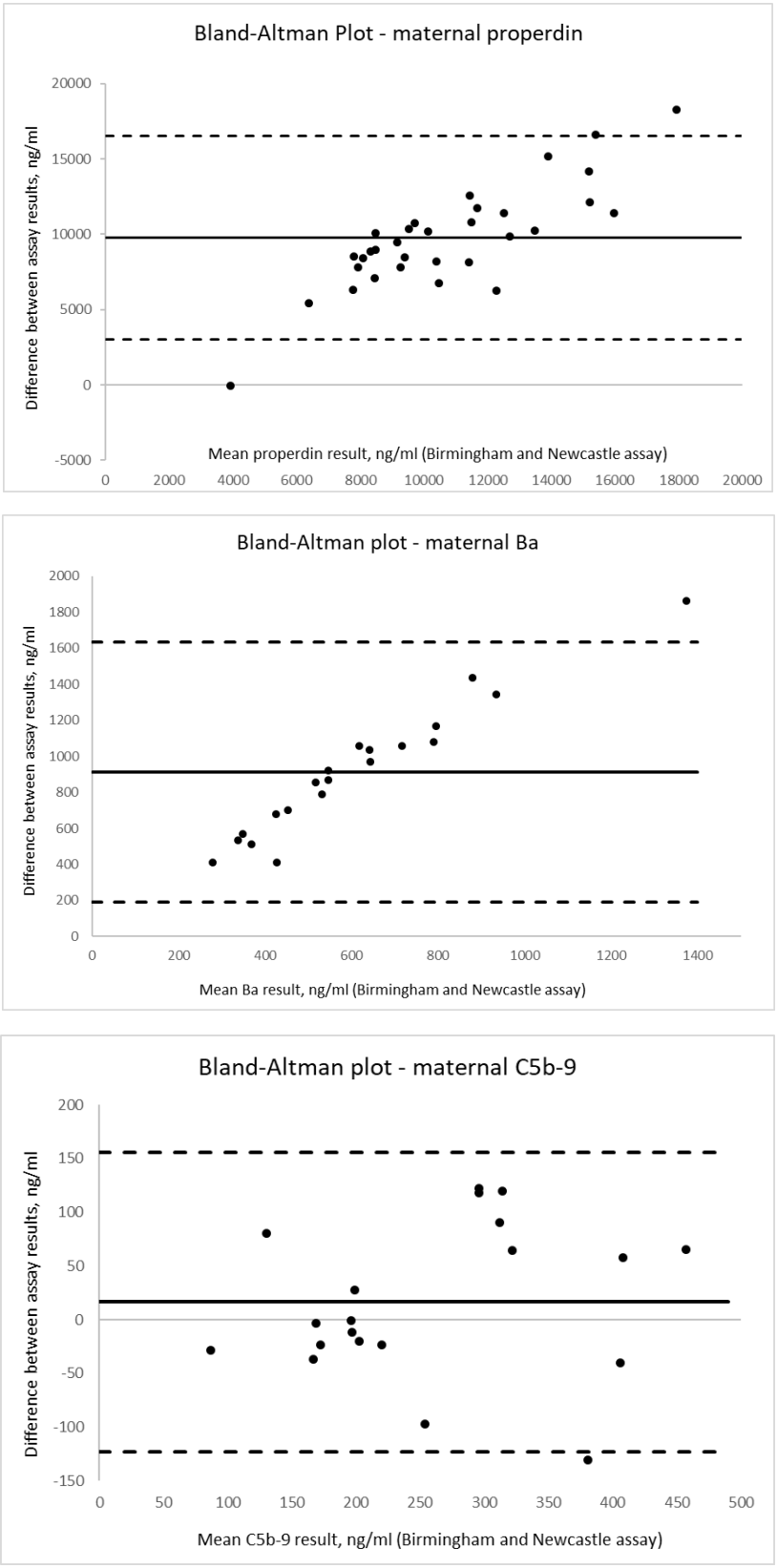


Figure 3.10: Bland-Altman plots comparing complement assays conducted in Newcastle and Birmingham

Bold horizontal line indicates the mean difference between assay results, and dashed lines indicate upper and lower 95% limits of agreement.

3.3 Discussion

3.3.1 Summary of findings

There is accumulating evidence supporting the role of complement dysregulation in the pathogenesis of PE, but the precise mechanisms are debated (4, 11), and the relationship between circulating complement and placental complement deposition is unclear. This study reports novel evidence of simultaneous placental complement deposition, associated with significant changes in complement biomarkers in the maternal and fetal circulation, in women with PE.

3.3.1.1 Complement in the maternal and fetal circulation

In this study, women with PE had significantly lower plasma properdin concentrations when compared to healthy pregnant controls. This is the first time a reduction in properdin in the setting of PE has been reported. The findings were replicable in a separate validation cohort, and significant differences in properdin concentration persisted after controlling for gestational age at blood draw, and after controlling for excretory renal function and other immunological markers including B2M and sFLCs. Furthermore, maternal plasma properdin concentration appears to provide excellent diagnostic accuracy at distinguishing between cases of PE from healthy pregnancy.

Properdin acts as a positive regulator of the alternative complement pathway by stabilising surface-bound C3 convertase and prolonging its half-life, potentiating alternative pathway activity. In addition, it has been shown that properdin is able to bind to self-surfaces (including apoptotic and necrotic cells, and platelets), to directly trigger alternative pathway activation (224, 241, 242). Significantly reduced plasma properdin levels have previously been reported in the context of C3 glomerulopathy (243), ANCA vasculitis and active lupus nephritis (244), but never before in PE. A reduction in circulating properdin is indicative of excessive alternative complement pathway activity through properdin consumption from deposition in tissues (224).

Elevated concentrations of Ba (an activation fragment of Factor B) in maternal and umbilical cord blood provided further evidence of excessive alternative pathway activity in PE. However, the difference between PE and healthy control groups was not statistically significant in the London Cohort, or after adjusting for the effect of gestational age at blood draw. The Birmingham Cohort results may have been influenced by the earlier gestation at blood draw in PE subjects. However, a longitudinal study found that Factor B concentration rises during the first and second trimesters, and levels off after 28 weeks in normal pregnancy (36). Thus, gestational differences may not be a significant confounding factor for Ba results drawn from the third trimester.

Excessive alternative complement pathway activation in PE has been reported in existing gestationally matched cross-sectional studies. There is no published data on Ba concentrations in the setting of PE, but several studies report changes in Bb, another split product of Factor B. Increased levels of Bb have been found in PE when compared to healthy control pregnancies (139, 140, 148), as well as decreased Factor H levels (140), although one study reported no differences in Bb concentrations between PE, healthy control pregnancies, and healthy non-pregnant women (34). The cases involved in this study were less severe, later-onset PE, with blood samples tending to be drawn later in pregnancy (34). In a large prospective study of pregnant women, subjects with a top decile Bb level at 10-20 weeks' gestation were significantly more likely to develop PE later in pregnancy (adjusted OR 6.1, 95% CI 2.2-17.0, $p<0.001$) (17). The authors inferred that excessive alternative pathway activation was a feature of PE from as early as the first trimester, and that Bb could be a useful biomarker for pregnancies at risk of PE. This theory was corroborated by a more recent longitudinal study of pregnancy, which reported elevated first trimester Factor B and Factor H levels in women who went on to develop PE (138).

In the present study, subjects with PE had reduced maternal blood concentrations of C4 when compared to healthy pregnant controls. This is in keeping with previous research, which has reported reduced circulating C4 in women with PE (142), and high levels of split product C4d (34),

and is indicative of C4 consumption through activation of the classical or lectin complement pathways. C4 remained independently associated with PE after controlling for gestational age and other potential confounding factors including renal function. The assay used in this study detected both native and activated forms of C4, so reduced concentrations in subjects with PE are likely to represent significant consumption.

C3 concentrations were reduced in the maternal plasma of patients with PE. This could indicate consumption due to excessive complement activation in PE. However, iC3b, a split product of C3b and biomarker of C3 activation, was not increased. This could indicate that C3 activation is highly localised, or that levels reflect background 'tickover' of complement; a possibility supported by the stable iC3b:C3 ratios. Alternatively, this could be a gestational effect, as differences in iC3b between groups did not persist after adjusting for gestational age at blood draw. Note that earlier research has not reported significant differences in C3 concentrations in subjects with PE, although assays used measured both native and activated forms of the protein (142, 147).

The present study did not report any significant differences in C5b-9 between PE and control groups. Published data on C5b-9 concentrations in the setting of PE are conflicting (245). Some studies report elevated maternal blood concentrations of C5b-9 in women with PE compared to control pregnancies (148, 150, 152), with associations with fetal growth restriction (34), early-onset disease (147), and HELLP syndrome (246). These reports are contradicted by other studies that found no difference in plasma C5b-9 levels in women with PE versus healthy controls (138), and no association with early onset PE (148), or PE with severe features (150, 154). There were no cases of HELLP syndrome in the Birmingham Cohort, and relatively small numbers of early-onset, severe PE, which may have contributed to the lack of difference seen in C5b-9 seen between groups. Buurma and colleagues reported increased deposition of the membrane-bound complement regulator CD59 in the placental tissue of women with PE (31). Upregulation of CD59 may help mediate terminal

complement pathway activation and may explain why circulating C5b-9 levels are not always raised, particularly at the milder end of the PE disease spectrum.

Ba was the only umbilical cord complement marker found to differ significantly between PE and healthy control groups. In addition, there was significant positive correlation between maternal and umbilical cord plasma Ba concentrations. This finding is consistent with earlier research that found Bb to be elevated in the maternal and cord blood of patients with severe PE (139), although a later larger study refutes this, with no reported difference in cord blood Bb between women with PE and healthy control pregnancies (141). Elevated cord blood Ba concentrations in pregnancies affected by PE might indicate diffusion of this relatively small molecule across the maternal-fetal interface (225), rather than fetal circulatory complement activation per se. This is particularly the case, given that no other umbilical cord blood complement components significantly differed between PE and controls, together with the lack of correlation between umbilical cord blood complement and placental complement deposition. Ba is a relatively small molecule, weighing 30 kDa (225), whereas the other complement components measured have relatively larger molecular weights and may not have such propensity to diffuse from the maternal to fetal circulation.

Umbilical cord blood complement concentrations tended to be lower in magnitude than their equivalent complement biomarkers in the maternal circulation. A study by Saleh at al. has previously shown this pattern in healthy pregnancy, which is reflective of the immature fetal innate immune system (37). The only exceptions to this in the present study were Ba and iC3b concentrations, which were higher in umbilical cord blood than in maternal blood. Both are unstable complement activation fragments, and therefore may have been more liable to activation during sample collection, freezing, and thawing.

3.3.1.2 Placental complement deposition

The present study, for the first time, examined placental tissue for the same subjects from which maternal and fetal blood samples were drawn. Immunohistochemical analysis demonstrated that

C4d was present on the STB membrane in almost all PE placentas, whereas it was observed rarely in healthy controls. These findings are in keeping with previous studies reporting increased placental C4d deposition in women with PE (31, 132, 133). In their study comparing placental tissue staining from 28 cases of PE and 44 healthy controls, Buurma et al. found no evidence of increased MBL deposition in PE, but co-localisation of C1q staining with areas of diffuse C4d deposition (31). Thus, C4d deposition in PE is likely to be driven by classical (rather than lectin) pathway activation. Further analysis found a trend towards IgM immune deposits (rather than fetally-derived IgG) in placental tissue exhibiting increased C4d deposition. The authors argue that this is suggestive of ischaemia-reperfusion injury driving classical pathway activation (driven by IgM binding to damaged endothelium), rather than activation driven by antibodies directed at fetal antigens (31, 247).

Immunohistochemical analysis in the present study also demonstrated excessive deposition of C3d and C9 on the STB membrane in PE placentas. Although there are some reports of increased placental C3d deposition in PE in the current literature (135, 136), the evidence base is sometimes conflicting, with other studies reporting no significant differences in deposition of C3d (25) or properdin (31) – another marker of alternative complement pathway activity – between cases of PE and healthy pregnant controls. Reports of MAC deposition are similarly variable; probably related in part to the relatively small sample sizes that existing studies are based upon. Some research groups have reported increased MAC deposition in PE placentas (30, 133), particularly at sites of villous injury (248), although this finding is refuted by others (25). The presence of C9 is indicative of MAC assembly on the surface of villi. This implies that placental complement activation extends downstream as far as the terminal part of the complement cascade in PE.

No significant differences were found in the intensity and distribution of placental C1q deposition between PE and controls. It is possible that the protease pre-treatment method may have removed bound C1q, leading to false negative results. The current evidence base, albeit small, has variably reported reduced C1q deposition at the STB surface in women with PE (25); and no difference in C1q

deposition between PE cases and healthy controls (31). It has been postulated that C1q deposition is an important feature of normal placentation, but the present study is unable to confirm this.

Importantly, the data from this study show that placental C4d deposition at the STB surface was negatively correlated with maternal blood properdin and C4 concentrations. This indicates that those patients with the greatest degree of complement consumption within the maternal circulation also had concurrent high complement deposition within placental tissue.

3.3.1.3 Relationship between complement and biochemical / immunological markers

Women with PE had significantly elevated biomarkers of excretory renal dysfunction (creatinine, cystatin C, urea and uric acid), compared to healthy pregnant controls. This was an expected finding, given that abnormal renal function is one of the hallmark diagnostic features of PE. After controlling for biochemical and immunological markers in multivariate analysis, maternal properdin, C4, and C3 remained independently associated with PE. However, the difference in Ba between women with PE and controls did not remain significant.

Ba is known to be renally excreted and in previous research was found to be significantly elevated in patients with advanced CKD, compared to the normal assay range (249). Therefore, the increased levels of Ba seen in PE compared to controls in the Birmingham Cohort may be reflective of poorer renal function rather than complement activation. The caveat to this is that the Birmingham PE group had a median creatinine of 59.5 μmol/L, which is considered to be within the normal range in pregnancy (250). Although there were more patients with abnormal renal function (creatinine > 77 μmol/L (250)) in the PE group as compared to controls, the difference in proportion between groups was not significant (9% versus 0% respectively, p=0.238). Therefore, any effect on excretion of small and middle molecules would be expected to be minimal. Overall, the potential confounding effect of renal dysfunction on circulating complement marker concentration in PE should be borne in mind when reviewing and interpreting the current evidence base.

B2M and sFLC concentrations were raised in the context of PE in this study. B2M, kappa, and combined sFLC concentrations (but not lambda light chains) remained significantly associated with PE after controlling for serum creatinine concentration. Previous research also reports an association between elevated B2M levels and adverse pregnancy outcomes in women with PE, leading the authors to suggest that B2M could be a potentially useful biomarker for PE (142). The same study also found significantly reduced IgG concentrations in women with PE. This finding was replicated in the present study, and may represent IgG consumption as a result of complement activation (142).

3.3.1.4 Complement activation and mode of delivery

Little is known about whether mode of delivery impacts upon complement activation. It seems plausible that vaginal delivery could cause a more prolonged inflammatory state than a planned Caesarean section. Recent evidence has reported increased concentrations of endothelial markers in umbilical cord blood following spontaneous vaginal delivery, as compared to planned Caesarean section delivery (251). This is suggestive of endothelial cell activation, which can be stimulated by excessive complement activation (252). Therefore, theoretically, parturition could also be associated with a greater degree of complement activation than surgical (Caesarean) delivery. This could have confounded study results for umbilical cord blood and placental tissue (but not for maternal blood complement, as maternal blood samples were all drawn prior to the onset of labour).

Subgroup analysis did not highlight any impact of mode of delivery on complement deposited within placental tissue. This is in accordance with the findings from an in vitro study of human placental cells taken from healthy pregnancies, which showed that mode of delivery and the process of giving birth had no significant effect on complement expression (32). There was evidence, however, of increased Ba and C5b-9 levels in the umbilical cord blood of healthy control pregnancies delivered vaginally, as compared to Caesarean deliveries. This may indicate that excess complement activation took place during vaginal delivery. However, this trend was not seen in the pre-eclamptic pregnancies, nor in one small study of neonatal complement levels which showed no impact of

mode of delivery upon results (38). Overall, there is no conclusive evidence from this study for mode of delivery impacting upon umbilical cord blood or placental complement expression.

3.3.1.5 Complement activation and pre-eclampsia timing of onset

Subgroup analysis of early-onset versus late-onset PE showed that of all the circulating complement markers tested, only maternal C4 levels significantly differed between groups. Reduced C4 concentrations are suggestive of increased complement activation in earlier onset (typically placentally-driven) disease. However, the results may be impacted by significantly earlier gestation at blood draw in the early-onset PE group. A longitudinal study of pregnancy found that C4 levels rise until delivery (37), and therefore the lower concentrations seen in the early-onset PE group could be a result of gestational differences, rather than excessive complement activation.

This subgroup analysis involved a relatively small number of cases. Any inferences must be drawn with some caution, and a larger sample size would be needed to accurately adjust for the effect of gestation. For this reason, logistic regression analysis was not conducted due to the risk of overfitting the regression model, and misinterpretation of any potential associations.

Previous research examining the effect of PE time of onset on maternal blood complement levels found no significant difference in Bb concentrations when comparing early-onset with late-onset PE (148). Similarly, maternal plasma C5b-9 levels do not appear to differentiate between early- and late-onset cases of PE (148, 150). However, one group did report an association between elevated maternal plasma C5b-9 levels and fetal growth restriction, suggesting that terminal pathway activation could be a feature of more severe, placentally-driven forms of PE (34). Interestingly, several studies have demonstrated that urine C5b-9 levels are superior to plasma C5b-9 in identifying cases of PE with severe features (150, 152, 153). This suggests that PE with severe features is characterised by renal complement activation.

Prior studies have reported associations between placental C4d deposition and adverse pregnancy outcomes, including lower gestational age (31) and fetal growth restriction (132) in women with PE.

It is postulated that poor placentation with associated oxidative stress and ischemia-reperfusion insults lead to excessive complement activation in more severe forms of PE (132). No significant differences in C4d deposition between early-onset and late-onset PE cases were observed in the present study. However, C9 immunoreactivity scores were actually higher in the late-onset PE group than the early-onset group. This subgroup analysis may be underpowered to detect significant differences in placental complement staining between PE subgroups.

3.3.1.6 Complement activation and ethnicity

High rates of PE have been reported in women of Black ethnicity (175, 207, 253, 254). In subgroup analysis of combined cohorts there was no evidence of excessive complement activation in the maternal circulation in women of Black ethnicity, compared to those of non-Black ethnicity. This might suggest that complement activation is not responsible for the high rates of PE seen in Black populations. There was however a potential signal for C5b-9, which appeared to be elevated in Black women with PE when compared to non-Black women with PE, although the difference between groups did not reach statistical significance.

There were relatively few women in the Birmingham and London cohorts who identified as Black, and as such this analysis is underpowered to detect significant differences between groups. Furthermore, there are likely to be multiple ethnic groups represented within those women who identified as Black; including women of Black African, Black Caribbean, and mixed heritage. There may be specific differences between these groups contributing to their individual risk of PE.

The effect of ethnicity on PE will be discussed in depth in Chapter 5.

3.3.2 Proposed mechanism of complement activation in pre-eclampsia pathogenesis

The findings from this research support a hypothesis that in PE, properdin binds to activated complement in placental tissue, resulting in depleted plasma concentrations. Depleted circulating

properdin concentrations have previously been reported in studies of glomerular disease and are thought to represent a movement of properdin from the fluid phase to sites of surface-bound complement activation within tissue (224).

Classical pathway activation is thought to predominantly drive C4d deposition within the placenta in cases of PE and is likely to be triggered by oxidative stress and repeated ischemia-reperfusion injury caused by defective placental development (2, 31, 131, 132). The amplification loop then exacerbates the cycle of activation via the alternative complement pathway.

This hypothesis is reflective of pathogenetic mechanisms suggested from murine models of PE, in which early placental inflammation in diseased mice triggered further alternative pathway amplification, and alternative pathway blockade resulted in rescue of pregnancy (15). Interestingly, human studies also report alternative pathway dysregulation from early in pregnancy, suggesting that inappropriate complement activation begins early in PE as the placenta is developing, before clinical symptoms are detectable (17, 138).

Animal studies and in vitro studies of human pregnancy reporting links between complement activation and disordered angiogenesis directly implicate aberrant complement activity in the pathogenesis of the clinical syndrome of PE. Murine studies of PE have demonstrated that complement activation can stimulate the release of antiangiogenic sFlt-1 (15), and that complement blockade can reduce sFlt-1 production (15, 129) and restore placental mean arterial pressure to normal levels (131). This results in improved placental vascularisation, a reduction in placental oxidative stress, and rescue of pregnancy (15, 112, 129). In humans, complement activation of placental trophoblast cells leads to upregulation and secretion of sFlt-1 (137), and placental deposition of C4d and MAC are strongly correlated with placental sFlt-1 in women with PE (133). It is postulated that abnormal sFlt-1 expression stimulates complement activation during early placental development in women with PE (114). Complement activation and amplification may in turn trigger

further sFlt-1 production as the pregnancy progresses, which leads to increasing inflammation and maternal endothelial dysfunction (133, 163).

Inhibition of complement activation could be a viable treatment option for women PE: particularly for those with severe or early-onset disease who suffer the greatest maternal and obstetric morbidity and premature iatrogenic delivery. Complement blockade could allow better pregnancy outcomes through preventing cumulative placental tissue damage, and the endothelial dysfunction that results.

3.3.3 Strengths and limitations

This study's major strength is the simultaneous analysis of complement across multiple tissue types: this is the first time an analysis of concurrently collected maternal blood, umbilical cord blood, and placental tissue complement has been reported. The findings are strengthened by being replicable in a separate validation cohort, and after controlling for gestational age at blood draw, as well as for biochemical and immunological biomarkers. Whilst this study cannot prove direct cause and effect, the results do provide new mechanistic insights into the role of complement in the pathogenesis of PE across different tissue types, and enhance the evidence base towards potential much-needed therapeutic targets in the identification and treatment of PE.

This study also features some other important novel findings. Maternal properdin is identified as a particularly significant marker of PE, and is strongly associated with placental complement deposition, which has never been reported before in the existing literature. Furthermore, the study reports on a wide panel of umbilical cord complement components in PE and healthy pregnancy, strengthening the limited evidence base in this specific area.

Although not individually matched, PE and control groups were comparable for maternal age, BMI and parity, and included a diverse mix of ethnicities which reflect the local populations of the

cohorts. Analysis of potential confounding factors including mode of delivery was conducted, and any women with known pre-existing medical conditions including chronic hypertension and autoimmune disease were excluded.

The primary limitation of the blood sample analysis was the difference in gestation at which samples were collected between the PE and control groups. This was largely a consequence of sample collection logistics, as women with PE tended to present to hospital and deliver earlier in pregnancy than controls. Consequently, if complement concentrations varied over the course of the pregnancy, this difference in gestational age would confound comparisons of PE vs. controls. For maternal blood complement results, a regression analysis was used to account for any effect of gestation. However, the lack of crossover in gestational ages between the PE and control groups for the Birmingham cohort meant that these models needed to extrapolate the trends for later gestations in the PE group, and for earlier gestations in the control group. Therefore, the accuracy of the adjustment cannot be confirmed, and over-adjustment or residual confounding may have been present in the final models.

Whilst the introduction of a validation cohort strengthened the maternal blood complement marker analysis, the absolute numbers of umbilical cord blood and placental tissue samples were relatively smaller. There were inherent difficulties in collecting these samples – with some being discarded or lost during night-time deliveries, or if clinical / emergency need superseded the research requirements. Further tests, including circulating angiogenic markers, and placental properdin immunostaining would have strengthened the research further, but were not possible within the time and cost constraints of the study.

When comparing complement component results with biochemical and immunological markers, there is likely an inflated false positive rate, due to the number of comparisons being performed. However, applying adjustment for multiple comparisons would likely lead to a grossly inflated false negative rate, given the relatively small sample size (231). Attempts to mitigate the impact of

multiple comparisons were made by additionally identifying comparisons that remained significant after Bonferroni-correction, in analyses with large numbers of comparisons. However, the potential for false positives throughout the study must be considered when interpreting the results.

3.4 Conclusions

In conclusion, this study for the first time demonstrates evidence of excessive placental complement deposition (particularly C4d), associated with significant concurrent changes in complement biomarkers (consumption of properdin, C4, and C3, and production of Ba in maternal circulation, and elevated Ba in fetal circulation) in pregnancies affected by PE. Classical complement pathway activity may drive placental C4d deposition, and the amplification loop potentiates further complement activation via the alternative pathway. Placental complement deposition is strongly correlated with complement activation within the maternal circulation, suggesting that those patients with the most excessive changes in circulating markers of complement activation also have the greatest extent of placental complement-mediated damage. Inhibition of complement activation might be a viable treatment option for women with PE, particularly for those with early-onset or severe forms of disease. This could allow improved pregnancy outcomes through blocking placental tissue damage and the endothelial dysfunction that results. Furthermore, longitudinal monitoring of complement biomarkers during pregnancy (such as a decrease in maternal properdin concentration) could be a useful adjunct in PE diagnosis and prognostication.

CHAPTER 4

INVESTIGATING THE ROLE OF COMPLEMENT IN THE PATHOGENESIS OF SUPERIMPOSED PRE-ECLAMPSIA IN WOMEN WITH CHRONIC KIDNEY DISEASE

4 Investigating the role of complement in the pathogenesis of superimposed pre-eclampsia in women with chronic kidney disease

4.1 Summary and overview

Superimposed pre-eclampsia (SPE) affects up to 40% of women with CKD during pregnancy (79). This equates to a 10-fold greater prevalence than for PE in women without pre-existing renal disease (82, 173). Women with more severe pre-pregnancy renal impairment, proteinuria, or chronic hypertension are at greater risk of developing SPE during pregnancy (73, 173, 179, 188, 190), as well as those with a prior kidney transplant (191). Putative mechanisms for the increased risk include disrupted angiogenesis and complement activation, which can lead to endothelial dysfunction in both CKD and SPE (11, 79, 195, 196, 198).

The diagnosis of SPE in women with CKD is challenging because hypertension, proteinuria and renal dysfunction characterise both conditions. Renal physiological changes in normal pregnancy can cause further diagnostic uncertainty. In the absence of standard diagnostic criteria for SPE, relative changes in blood pressure, creatinine and proteinuria during pregnancy are analysed in addition to expert consensus review (79, 192, 203). The management of SPE is limited to prophylactic aspirin for at-risk individuals and regular antenatal surveillance of blood pressure and proteinuria. There is no definitive treatment other than expedited delivery of baby and placenta, which is often necessitated preterm. There is therefore a need for biomarkers to aid in the earlier diagnosis of SPE for women with CKD, and a need for identification of potential therapeutic targets to provide an alternative to premature delivery.

Together with a growing body of evidence in published literature, the findings from Chapter 3 of this thesis implicate complement in the pathogenesis of PE in women with no pre-existing medical conditions (4, 9, 11). There is however a scarcity of evidence for the role of complement in SPE pathogenesis for women with CKD, despite the very high prevalence of SPE in this patient cohort.

Data are limited to a single small study, which reported no difference in C3a, C5a, Factor H, or C5b-9 concentrations between women with CKD and SPE, and those without SPE (203). It is therefore unclear if the same complement pathways identified in PE pathogenesis research apply to SPE, or if different mechanisms may be responsible for the high rates of disease seen in women with CKD.

The specific aims of this arm of research were to:

- Investigate biomarkers of complement activation in the maternal circulation (Ba, properdin and C5b-9) in a cohort of pregnant women with CKD; a proportion of whom developed SPE during pregnancy.
- Analyse whether complement dysregulation is associated with adverse pregnancy outcome in women with CKD.
    - Adverse pregnancy outcome was a composite measure, defined as any of:
        - neonatal death,
        - PTB (delivery prior to 37 weeks' gestation),
        - admission to neonatal unit,
        - low birth weight (< 2500 g), or
        - SGA baby (birth weight < 10th centile).

For this research project, SPE was defined as per the diagnostic criteria listed in ***section 2.4.6***. Maternal blood samples were compared between SPE and non-SPE groups at 3 specified timepoints: early pregnancy (samples collected at 0-16 weeks gestation), mid-pregnancy (16+1 to 27+6 weeks) and late pregnancy (28+ weeks). For the purposes of this study, these timeframes will be referred to as 'trimester 1', 'trimester 2', and 'trimester 3'.

4.2 Results

4.2.1 Cohort characteristics

A total of 75 pregnancies in 69 women with CKD were included in this study: 6 women had 2 pregnancies, with the remaining 63 women having a single pregnancy each. Of the 75 pregnancies, 29 were assessed to have been complicated by SPE. Cohort characteristics are displayed in ***Table 4.1***. Age, BMI, ethnicity, and parity were comparable between SPE and non-SPE groups. There were no significant differences between groups in the timing of blood draw during each trimester. The median gestation at blood draw 1 was 13.7 weeks (IQR 11.8-14.2) in SPE pregnancies, vs. 13.1 weeks (10.3-14.2) in the non-SPE group, p=0.612. For blood draw 2, the median gestations were 24.6 weeks (22.7-27.1) vs. 24.9 weeks (22.9-26.5), p=0.927, and for blood draw 3, 32.4 weeks (29.8-35.6) vs. 34.0 weeks (31.6-35.1), p=0.330 for SPE vs. non-SPE pregnancies, respectively.

The SPE group had increased rates of adverse pregnancy outcomes, including Caesarean delivery (79% in SPE vs 46% in non-SPE, p=0.004), PTB (median gestation at delivery 36.0 weeks in SPE vs 37.9 weeks in non-SPE, p<0.001), lower mean birth weight (2369 g in SPE vs. 2824 g in non-SPE, p=0.002), and higher rates of NNU admission (36% in SPE vs. 13% in non-SPE, p=0.048). Despite this, there were no significant differences in SGA babies between groups (29% of SPE pregnancies vs. 27% non-SPE, p=1.000).

4.2.1.1 Renal characteristics

Women who developed SPE during pregnancy tended to have poorer pre-pregnancy renal function when compared to non-SPE pregnancies, although the differences between groups did not reach statistical significance. Median pre-pregnancy creatinine in the SPE group was 99.0 μmol/L, and GFR 58.5 ml/min/1.73m^2, compared to 80.5 μmol/L and 73.0 ml/min/1.73m^2 in the non-SPE group (p=0.075 and p=0.114, respectively). Both study groups included patients with pre-pregnancy CKD stages 1-4, although a significant proportion of participants had ‘mild’ disease (CKD stages 1 and 2) (233). Patients who developed SPE tended to have a more advanced pre-pregnancy CKD stage (50%

of the SPE group had a pre-pregnancy CKD stage of 3 or 4, compared to 23% of the non-SPE group, p=0.062).

There were no significant differences in the cause of CKD between groups, although of note, there were higher proportions of subjects with glomerulonephritis and lupus in the SPE group (54% vs. 33% of the non-SPE cohort, p=0.366). A comparable proportion of patients had a kidney transplant in SPE and non-SPE pregnancies (10% vs. 7% respectively, p=0.671). The SPE group was characterised by increased pre-pregnancy proteinuria: median urine albumin creatinine ratio (uACR) 35.3 mg/mmol in SPE vs. 2.7 mg/mmol in non-SPE (p<0.001), and higher rates of chronic hypertension (66% in SPE vs. 26% in non-SPE, p<0.001).

Table 4.1: Demographic and clinical outcome data: CKD cohort

	N	***SPE***	***Non-SPE***	***p-value***
Maternal age at delivery (years)	75	29.2 ± 5.7	30.2 ± 5.8	0.496
BMI (kg/m^2)	72*	28.5 ± 5.6	26.3 ± 5.7	0.120
Ethnicity:**	69			0.411
White		19 (73%)	16 (60%)	
Asian		6 (23%)	10 (23%)	
Black		1 (4%)	4 (9%)	
Mixed/Other		0 (0%)	3 (7%)	
First pregnancy	75	12 (41%)	16 (35%)	0.628
Mode of delivery	75			**0.004**
Caesarean		23 (79%)	21 (46%)	
Vaginal		6 (21%)	25 (54%)	
Gestation at (weeks):				
Delivery	75	36.0 (33.4-37.4)	37.9 (37.0-38.9)	**<0.001**
Blood draw 1	42	13.7 (11.8-14.2)	13.1 (10.3-14.2)	0.612
Blood draw 2	52	24.6 (22.7-27.1)	24.9 (22.9-26.5)	0.927
Blood draw 3	62	32.4 (29.8-35.6)	34.0 (31.6-35.1)	0.330
Fetal outcomes:				
Birth weight (g)	72*	2369 ± 707	2824 ± 471	**0.002**
SGA (<10th centile)	72*	8 (29%)	12 (27%)	1.000
Neonatal unit care	62*	8 (36%)	5 (13%)	**0.048**
Pre-pregnancy renal status:				
Creatinine (µmol/L)	68*	99.0 (66.3-132.8)	80.5 (65.3-96.8)	0.075
GFR (ml/min/1.73m^2)	69*	58.5 (44.0-90.0)	73.0 (61.5-90.0)	0.114
uACR (mg/mmol)	65*	35.3 (9.4-89.4)	2.7 (0.8-30.7)	**<0.001**
Chronic hypertension	75	19 (66%)	12 (26%)	**<0.001**
Kidney transplant	75	3 (10%)	3 (7%)	0.671
Pre-pregnancy CKD stage:	69*			0.062
1		8 (31%)	12 (28%)	
2		5 (19%)	21 (49%)	
3		12 (46%)	9 (21%)	
4		1 (4%)	1 (2%)	
Cause of CKD:**	69			0.366
Glomerular disease		8 (31%)	9 (21%)	
Tubulo-interstitial disease		5 (19%)	6 (14%)	
Lupus nephritis		6 (23%)	5 (12%)	
ADPKD		2 (8%)	7 (16%)	
Structural disease / VUR		1 (4%)	5 (12%)	
Other		1 (4%)	7 (16%)	
Unknown		3 (12%)	4 (9%)	

*Analyses are based on N=29 SPE pregnancies, and N=46 non-SPE pregnancies. Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values from Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available for from total of 75 pregnancies). **75 pregnancies in 69 women were recorded. 6 women had 2 pregnancies each: for these women, ethnicity and cause of CKD is only counted once, with the remaining data relating to each individual pregnancy. ADPKD: autosomal dominant polycystic kidney disease; VUR: vesico-ureteric reflux.*

4.2.2 Complement results

Complement blood results are shown in ***Table 4.2.*** Variable numbers of samples were tested for each trimester and complement component, since not all participants had a blood sample drawn within each specified timeframe. A total of 42 blood samples were available for testing from the first trimester, 52 from the second trimester, and 62 from the third trimester. In some cases, not enough plasma volume was available to test for all 3 complement components. In this instance, testing for properdin was prioritised, followed by Ba (based upon the significant differences found between PE and healthy pregnant controls in Chapter 3 of this thesis). The ELISA test for one properdin plate was unsuccessful (due to suspected substrate contamination), causing a loss of some second and third trimester properdin results.

Trends in complement component concentrations over the course of pregnancy are displayed in ***Figure 4.1.*** There were no significant differences seen in first and third trimester properdin concentrations between groups. However, second trimester properdin was significantly higher in pregnancies affected by SPE when compared to non-SPE pregnancies (21438 ng/ml vs. 14668 ng/ml, p=0.003). In SPE pregnancies, properdin concentrations appeared to rise in the second trimester, before falling to a nadir in the third trimester. However, the opposite trend was the case for non-SPE pregnancies.

Plasma Ba concentrations were higher in SPE pregnancies than in women who did not develop SPE in all 3 trimesters, but none of the differences between groups were statistically significant. Ba concentrations appeared to rise to reach a peak during the second trimester, before falling again in the third trimester.

No significant differences were observed in C5b-9 concentrations between SPE and non-SPE pregnancies in any of the 3 trimesters measured. In non-SPE pregnancies, C5b-9 concentrations rose throughout pregnancy to reach a peak in the third trimester. In pregnancies affected by SPE, C5b-9 concentrations fell slightly during the second trimester but then rose to a peak in the third trimester.

Table 4.2: Complement results by trimester of blood draw: CKD Cohort

Blood test (ng/ml)	*N*	*SPE*	*Non-SPE*	*p-value*
Trimester 1 Properdin	42	19370 ± 4283 (n=14)	20032 ± 6595 (n=28)	0.735
Trimester 2 Properdin	43	21438 ± 7504 (n=23)	14668 ± 6242 (n=20)	**0.003**
Trimester 3 Properdin	53	19065 ± 8341 (n=14)	17805 ± 5495 (n=39)	0.526
Trimester 1 Ba	41	1066 (800-2280) (n=14)	917 (663-1162) (n=27)	0.169
Trimester 2 Ba	51	1899 (1215-3179) (n=24)	1340 (985-2266) (n=27)	0.213
Trimester 3 Ba	42	1608 (1260-2581) (n=15)	1251 (891-2186) (n=27)	0.312
Trimester 1 C5b-9	40	265 (230-307) (n=14)	226 (151-283) (n=26)	0.100
Trimester 2 C5b-9	51	259 (222-309) (n=24)	278 (236-344) (n=27)	0.509
Trimester 3 C5b-9	42	295 (258-415) (n=15)	281 (254-400) (n=27)	0.783

Normally distributed variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values from Mann-Whitney U tests otherwise. Bold p-values are significant at p<0.05.

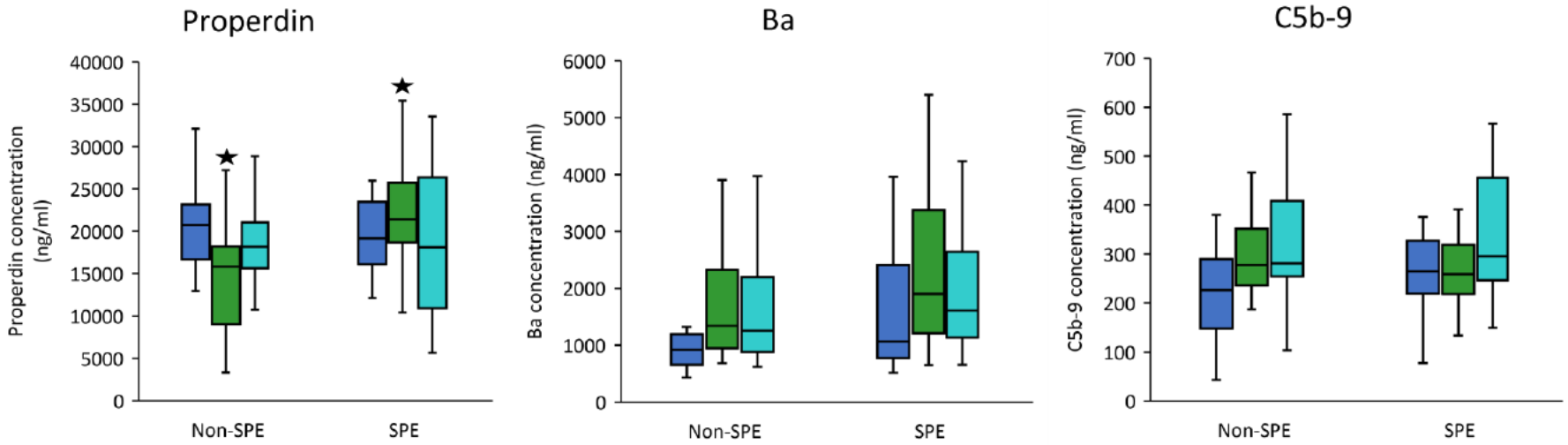


Figure 4.1: Plasma complement concentrations in non-SPE and SPE pregnancy by trimester

Image shows box-and-whisker plots, with horizontal lines representing the median, boxes representing interquartile range, and whiskers representing the total range. Outliers >1.5 standard deviations from upper quartiles are excluded for display purposes. ★ = significant difference between groups (Non-SPE vs. SPE) by trimester measured ($p<0.05$).

Legend:

Trimester 1 *Trimester 2* *Trimester 3*

4.2.2.1 Subgroup analysis: CKD patients with non-immune-mediated disease

The analysis was repeated after removing all patients with a background of lupus nephritis or glomerular disease, so as to eliminate any potential confounding factors including autoimmune or complement-mediated disease. This left a comparison between 12 SPE pregnancies, and 30 non-SPE pregnancies. Demographic and clinical outcome data are presented in ***Table 4.3***. After removing all patients with lupus and glomerular disease, pre-pregnancy creatinine and GFR were now significantly different between SPE and non-SPE groups (median creatinine 139.0 μmol/L for SPE, vs. 79.0 μmol/L for non-SPE, $p<0.001$, and median eGFR 39.0 ml/min/1.73m^2 for SPE, vs. 73.5 ml/min/m^2 for non-SPE pregnancies, $p<0.001$). SPE pregnancies were also characterised by higher urine ACR, and lower mean birth weights and gestational age than pregnancies not affected by SPE.

Complement analysis is presented in ***Table 4.4***, and shows that, in line with the results from the overall CKD Cohort, second trimester properdin was again significantly higher in SPE pregnancies compared to the non-SPE group (20850 ng/ml, vs. 13018 ng/ml, respectively, $p=0.011$). In addition, trimester 3 Ba concentrations were now significantly raised in SPE pregnancies, compared to non-SPE (2581 ng/ml, vs. 1217 ng/ml, $p=0.027$), as well as being elevated for trimesters 1 and 2 in women with SPE – albeit without reaching a statically significant difference. There were no significant differences between groups in C5b-9 at any of the timepoints measured.

Table 4.3: Demographic and clinical outcome data: CKD Cohort with lupus and glomerulonephritis patients removed

	N	***SPE (n=12)***	***Non-SPE (n=30)***	***p-value***
Maternal age at delivery (years)	42	30.5 ± 5.9	30.8 ± 6.2	0.891
BMI (kg/m^2)	40*	28.2 ± 5.7	25.7 ± 4.8	0.172
Ethnicity:	42			0.802
White		10 (83%)	20 (67%)	
Asian		2 (17%)	6 (20%)	
Black		0 (0%)	1 (3%)	
Mixed/Other		0 (0%)	3 (10%)	
First pregnancy	42	6 (50%)	10 (33%)	0.483
Mode of delivery	42			0.300
Caesarean		9 (75%)	16 (53%)	
Vaginal		3 (25%)	14 (47%)	
Gestation at (weeks):				
Delivery	42	35.8 (34.4-37.3)	38.0 (36.9-39.0)	**0.003**
Blood draw 1	23	13.9 (13.1-14.0)	13.9 (10.4-14.7)	0.973
Blood draw 2	29	23.3 (21.8-26.8)	25.0 (22.4-26.6)	0.647
Blood draw 3	37	35.1 (29.6-35.6)	33.9 (31.3-34.8)	0.985
Fetal outcomes:				
Birth weight (g)	40*	2337 ± 809	2872 ± 492	**0.014**
SGA (<10th centile)	40*	4 (33%)	4 (14%)	0.211
Neonatal unit care	33*	3 (43%)	3 (12%)	0.093
Pre-pregnancy renal status:				
Creatinine (µmol/L)	36*	139.0 (99.0-172.0)	79.0 (63.0-91.0)	**<0.001**
GFR (ml/min/1.73m^2)	37*	39.0 (30.0-60.0)	73.5 (62.8-90.0)	**<0.001**
uACR (mg/mmol)	36*	51.0 (3.7-195.5)	1.6 (0.3-6.9)	**0.005**
Chronic hypertension	42	7 (58%)	7 (23%)	0.067
Kidney transplant	42	2 (17%)	2 (7%)	0.565
Pre-pregnancy CKD stage:	37*			0.053
1		0 (0%)	8 (29%)	
2		3 (33%)	14 (50%)	
3		5 (56%)	5 (18%)	
4		1 (11%)	1 (4%)	
Cause of CKD:	42			0.460
Tubulo-interstitial disease		5 (42%)	6 (20%)	
ADPKD		2 (17%)	8 (27%)	
Structural disease / VUR		1 (8%)	5 (17%)	
Other		1 (8%)	7 (23%)	
Unknown		3 (25%)	4 (13%)	

*Analyses are based on N=12 SPE pregnancies, and N=30 non-SPE pregnancies. Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values from Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available for from total of 42 pregnancies)*

Table 4.4: Complement results by trimester of blood draw: CKD Cohort with lupus and glomerulonephritis patients removed

Blood test (ng/ml)	*N*	*SPE*	*Non-SPE*	*p-value*
Trimester 1 Properdin	23	21843 ± 3000 (n=6)	20615 ± 6750 (n=17)	0.675
Trimester 2 Properdin	23	20850 ± 6174 (n=11)	13018 ± 7212 (n=12)	**0.011**
Trimester 3 Properdin	30	22760 ± 7917 (n=5)	18310 ± 4593 (n=25)	0.092
Trimester 1 Ba	22	1917 (1174-3614) (n=6)	707 (622-1212) (n=16)	0.083
Trimester 2 Ba	28	1972 (1446-3456) (n=12)	1320 (1002-1735) (n=16)	0.133
Trimester 3 Ba	22	2581 (1816-3182) (n=6)	1217 (951-2052) (n=16)	**0.027**
Trimester 1 C5b-9	22	211 (157-235) (n=6)	158 (136-266) (n=16)	0.747
Trimester 2 C5b-9	28	259 (240-307) (n=12)	244 (223-323) (n=16)	0.599
Trimester 3 C5b-9	22	328 (288-432) (n=6)	280 (259-427) (n=16)	0.494

Normally distributed variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values from Mann-Whitney U tests otherwise. Bold p-values are significant at p<0.05.

4.2.3 Serial samples results

The analysis was repeated to review complement component results for only those patients who had a blood sample drawn in each trimester of pregnancy. This would allow an accurate review of the relative differences in complement concentrations by trimester in women with CKD who went on to develop SPE, and in those who did not.

From the original cohort of 75 pregnancies in women with CKD, data were available for 34 pregnancies in 33 women who had had serial blood samples drawn in each trimester of pregnancy. Of the 34 pregnancies, 7 were complicated by SPE. Baseline demographic and pregnancy outcome data are presented in ***Table 4.5***. There were no significant differences in maternal age, BMI, ethnicity, or parity between groups. Similarly, there were no significant differences in pre-pregnancy renal parameters between groups (creatinine, GFR, urine ACR, CKD stage, or aetiology of CKD). Furthermore, pregnancy outcomes, including mode of delivery, birth weight, gestational age, rates of growth restriction, and requirement for NNU care were comparable between groups. Finally, there were no significant differences in the timing of blood draw between groups in any of the 3 trimesters tested.

Table 4.5: Demographic and clinical outcome data: CKD cohort with serial blood samples

	N	***SPE***	***Non-SPE***	***p-value***
Maternal age at delivery (years)	34	30.3 ± 5.2	30.0 ± 5.6	0.886
BMI (kg/m^2)	32*	28.9 ± 4.9	26.5 ± 5.6	0.321
Ethnicity:**	33			0.740
White		5 (71%)	15 (58%)	
Asian		2 (29%)	7 (27%)	
Black		0 (0%)	2 (8%)	
Mixed/Other		0 (0%)	2 (8%)	
First pregnancy	34	4 (57%)	10 (37%)	0.410
Mode of delivery	34			
Caesarean		5 (71%)	14 (52%)	0.426
Vaginal		2 (29%)	13 (48%)	
Gestation at (weeks):				
Delivery	34	36.4 (35.5-37.4)	37.6 (36.9-38.9)	0.105
Blood draw 1	34	13.9 (13.2-14.3)	13.0 (10.1-14.1)	0.259
Blood draw 2	34	23.7 (23.2-25.9)	24.7 (22.8-26.2)	0.670
Blood draw 3	34	35.6 (29.6-35.9)	34.7 (31.1-36.4)	0.482
Fetal outcomes:				
Birth weight (g)	32*	2680 ± 461	2817 ± 525	0.536
SGA (<10th centile)	32*	1 (14%)	7 (28%)	0.646
Neonatal unit care	31*	0 (0%)	4 (16%)	0.561
Pre-pregnancy renal status:				
Creatinine (µmol/L)	31*	93.5 (71.5-112.5)	88.0 (66.0-103.0)	0.671
GFR (ml/min/1.73m^2)	32*	65.0 (48.0-84.5)	66.0 (57.3-89.3)	0.769
uACR (mg/mmol)	30*	30.3 (9.0-159.4)	5.9 (0.8-34.8)	0.161
Chronic hypertension	34	5 (71%)	8 (30%)	0.079
Kidney transplant	34	1 (14%)	3 (11%)	1.000
Pre-pregnancy CKD stage:	32*			0.653
1		2 (33%)	7 (27%)	
2		2 (33%)	12 (46%)	
3		1 (14%)	6 (23%)	
4		1 (14%)	1 (4%)	
Cause of CKD:**	33			0.202
Glomerular disease		1 (14%)	7 (27%)	
Tubulo-interstitial disease		4 (57%)	3 (12%)	
Lupus nephritis		1 (14%)	3 (12%)	
ADPKD		0 (0%)	5 (19%)	
Structural disease / VUR		1 (14%)	3 (12%)	
Other		0 (0%)	3 (12%)	
Unknown		0 (0%)	2 (8%)	

*Analyses are based on N=7 SPE pregnancies, and N=27 non-SPE pregnancies. Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values for Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests, unless stated otherwise. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available for from total of 34 pregnancies. **34 pregnancies in 33 women were recorded. 1 woman had 2 pregnancies: in this instance, ethnicity and cause of CKD is only counted once, with the remaining data relating to each individual pregnancy.*

Complement concentrations from serially collected blood samples are shown in ***Table 4.6.*** Trends in complement component concentrations by trimester of blood sampling are presented graphically in ***Figure 4.2.***

When compared to pregnancies not complicated by PE, women who developed SPE had significantly elevated plasma concentrations of Ba in the third trimester (2524 ng/ml vs. 1251 ng/ml, p=0.025). Ba concentrations were higher throughout pregnancy when comparing SPE to non-SPE pregnancies, although the differences did not reach statistical significance for first or second trimesters. Ba concentration appeared to rise between the first and second trimesters, and then level off in the third trimester, in both groups. The predictive accuracy of third trimester Ba concentration was assessed, returning an AUROC of 0.778 (SE 0.08) for distinguishing between cases of SPE and non-SPE.

There were no significant differences in properdin or C5b-9 concentrations between study groups across any of the trimesters tested. Properdin concentrations were of higher magnitude in SPE pregnancies than in non-SPE pregnancies for each trimester tested. There was no clear trend shown in properdin levels during pregnancy in this arm of the study. In the non-SPE group, properdin decreased between the first and second trimesters, before rising again in the third trimester. Conversely, in the SPE group, properdin levels rose between the first and second trimesters before falling in the third trimester.

C5b-9 concentration appeared to rise during pregnancy, with a higher rate of increase in third trimester SPE pregnancies, and a separation from non-SPE values. The difference between groups did not reach statistical significance however.

Table 4.6: Complement biomarker results: CKD Cohort with serial blood samples

Blood test (ng/ml)	*N*	*SPE*	*Non-SPE*	*p-value*
Trimester 1 Properdin	34	20359 ± 4631	20184 ± 6670	0.949
Trimester 2 Properdin	25*	20614 ± 7355	16317 ± 5531	0.069
Trimester 3 Properdin	26**	18945 ± 8471	16781 ± 6789	0.523
Trimester 1 Ba	34	1143 (809-1917)	917 (663-1162)	0.297
Trimester 2 Ba	34	2315 (1347-3830)	1340 (985-2266)	0.154
Trimester 3 Ba	34	2524 (2040-3001)	1251 (891-2186)	**0.025**
Trimester 1 C5b-9	34	234 (208-254)	226 (151-283)	0.481
Trimester 2 C5b-9	34	270 (232-296)	278 (236-344)	0.594
Trimester 3 C5b-9	34	361 (307-415)	281 (254-400)	0.120

*Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values for Mann-Whitney U tests. Bold p-values are significant at p<0.05. Maternal plasma complement concentrations were available for N=7/N=27 SPE/non-SPE pregnancies, with the exception of * (N=6/N=19) for Trimester 2 properdin, and ** (N=6/N=20) for Trimester 3 properdin, due to the failure of an ELISA plate.*

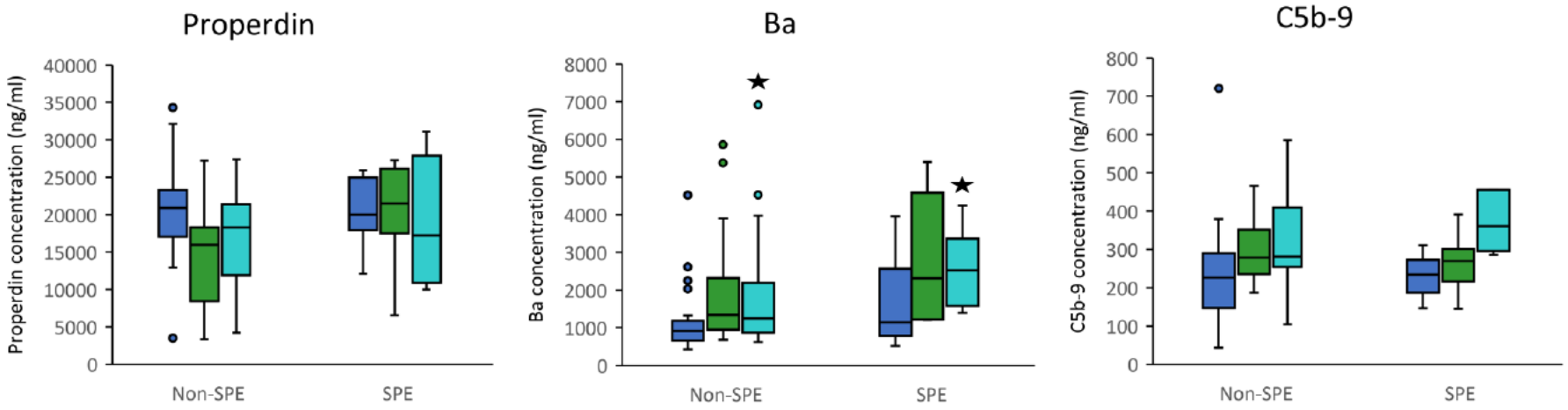


Figure 4.2: Plasma complement concentrations by SPE status in CKD Cohort with serial blood samples

Image shows box-and-whisker plots, with horizontal lines representing the median, boxes representing interquartile range, and whiskers representing the total range.

★ *= significant difference between groups (Non-SPE vs. SPE) by trimester measured ($p<0.05$).*

Legend:

Trimester 1 *Trimester 2* *Trimester 3*

4.2.4 Complement and adverse pregnancy outcomes in women with CKD

There are no established diagnostic criteria for SPE in women with CKD, and diagnosis is often complicated by women having abnormal renal function, proteinuria and/or chronic hypertension prior to pregnancy. There was consequently a risk that the analysis presented in ***Sections 4.2.2*** and ***4.2.3*** could have mis-classified SPE and/or non-SPE pregnancies. Patients were therefore grouped according to the presence or absence of composite adverse pregnancy outcome (see definition in ***section 4.1***), and the results analysis repeated.

Cohort characteristics by adverse pregnancy outcome group are presented in ***Table 4.7.*** A total of 74 pregnancies were classified by composite adverse pregnancy outcome (1 pregnancy from the original cohort had missing data so was unable to be classified). Of these, 42 pregnancies met the criteria for adverse outcome classification. There were no significant differences in maternal age, BMI, ethnicity, parity, or mode of delivery between groups. There were no significant differences in gestation at blood draw for samples collected in the second trimester of pregnancy, but first and third trimester blood samples were drawn significantly earlier in the adverse outcome group vs. non-adverse outcome group (median 12.0 vs. 13.9 weeks, p=0.024 in first trimester, and 31.7 vs. 34.1 weeks in third trimester, p=0.021, respectively).

The adverse outcome group had a higher median pre-pregnancy creatinine than the non-adverse outcome group (95.5 μmol/L vs. 76.0 μmol/L, p=0.013), and lower median pre-pregnancy GFR (61.5 ml/min/1.73m^2 vs. 83.0 ml/min/1.73m^2, p=0.012), respectively. The adverse outcome group also had more significant pre-pregnancy proteinuria (ACR 32.6 mg/mmol, vs. 4.8 mg/mmol in non-adverse outcome pregnancies, p=0.006). Rates of chronic hypertension were higher in the adverse outcome group (55% vs. 25% in non-adverse outcome pregnancy, p=0.017). There were no statistically significant differences in the stage or cause of CKD between groups, but the adverse outcome group featured a higher proportion of women with lupus nephritis (23%; vs. 7% in the non-adverse outcome group).

Table 4.7: Demographic and clinical outcome data by composite adverse pregnancy outcome status

	N	*Adverse outcome (n=42)*	*Non-adverse outcome (n=32)*	*p-value*
Maternal age at delivery (years)	74	29.5 ± 6.6	30.0 ± 4.5	0.711
BMI (kg/m^2)	72*	27.1 ± 6.2	27.3 ± 5.2	0.884
Ethnicity:**	68			0.853
White		26 (67%)	18 (62%)	
Asian		9 (23%)	7 (24%)	
Black		3 (8%)	2 (7%)	
Mixed/Other		1 (3%)	2 (7%)	
First pregnancy	74	17 (40%)	11 (34%)	0.635
Mode of delivery	74			0.351
Caesarean		27 (64%)	17 (53%)	
Vaginal		15 (36%)	15 (47%)	
Gestation at (weeks):				
Delivery	74	35.9 (34.1-37.1)	38.1 (37.7-39.0)	**<0.001**
Blood draw 1	41	12.0 (9.6-13.9)	13.9 (12.8-14.7)	**0.024**
Blood draw 2	51	25.4 (22.8-26.6)	24.3 (22.6-26.9)	0.770
Blood draw 3	61	31.7 (30.3-34.9)	34.1 (33.0-35.9)	**0.021**
Birth weight (g)	72*	2259 ± 511	3132 ± 304	**<0.001**
Pre-pregnancy renal status:				
Creatinine (µmol/L)	68*	95.5 (75.5-119.8)	76.0 (59.3-91.0)	**0.013**
GFR (ml/min/1.73m^2)	68*	61.5 (44.5-84.8)	83.0 (62.3-90.0)	**0.012**
uACR (mg/mmol)	64*	32.6 (3.2-71.2)	4.8 (0.3-18.7)	**0.006**
Chronic hypertension	74	23 (55%)	8 (25%)	**0.017**
Kidney transplant	75	5 (12%)	1 (3%)	0.226
Pre-pregnancy CKD stage:	68*			0.069
1		7 (18%)	13 (43%)	
2		14 (37%)	11 (37%)	
3		16 (42%)	5 (17%)	
4		1 (3%)	1 (3%)	
Cause of CKD:**	68			0.097
Glomerular disease		11 (28%)	6 (21%)	
Tubulo-interstitial disease		6 (15%)	5 (17%)	
Lupus nephritis		9 (23%)	2 (7%)	
ADPKD		2 (5%)	7 (24%)	
Structural disease / VUR		5 (13%)	1 (3%)	
Other		3 (8%)	5 (17%)	
Unknown		3 (8%)	3 (10%)	

*Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values for Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests, unless stated otherwise. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available for from total of 74 pregnancies. **74 pregnancies in 68 women were recorded. 6 women had 2 pregnancies each: for these women, ethnicity and cause of CKD is only counted once, with the remaining data relating to each individual pregnancy.*

Complement concentration results by pregnancy outcome group are shown in ***Table 4.8***, with the distribution by trimester shown in box-and-whisker plots in ***Figure 4.3***. Ba concentrations appeared to rise from early to mid-pregnancy, before levelling off in the third trimester for both adverse outcome and non-adverse outcome groups. Ba levels were significantly elevated in CKD pregnancies with adverse outcomes at all three timepoints when compared to non-adverse outcome pregnancies: median Ba 1133 vs. 737 ng/ml, p=0.008 in first trimester, 2203 vs. 1188 ng/ml, p=0.001 in second trimester, and 2186 vs. 1048 ng/ml, p=0.001 in third trimester.

No significant differences were observed in properdin or C5b-9 concentrations between adverse outcome and non-adverse outcome pregnancies at any of the timepoints measured. Properdin and C5b-9 concentrations were higher in adverse outcome pregnancies than non-adverse outcome pregnancies in first and second trimester blood samples, and lower in third trimester samples, but the differences between groups were not statistically significant.

Table 4.8: Complement biomarker results by composite adverse pregnancy outcome status

Blood test (ng/ml)	*N*	*Adverse outcome*	*Non-adverse outcome*	*p-value*	*AUROC*
Trimester 1 Properdin	41	20127 ± 6074 (n=23)	19673 ± 5841 (n=18)	0.810	0.527 (0.09)
Trimester 2 Properdin	42	20040 ± 7560 (n=27)	15940 ± 6932 (n=15)	0.091	0.662 (0.09)
Trimester 3 Properdin	52	17085 ± 6034 (n=25)	19121 ± 6623 (n=27)	0.253	0.625 (0.08)
Trimester 1 Ba	40	1133 (880-2145) (n=23)	737 (624-930) (n=17)	**0.008**	**0.747 (0.08)**
Trimester 2 Ba	50	2203 (1332-3555) (n=32)	1188 (796-1599) (n=18)	**0.001**	**0.781 (0.07)**
Trimester 3 Ba	41	2186 (1317-3184) (n=22)	1048 (824-1642) (n=19)	**0.001**	**0.797 (0.07)**
Trimester 1 C5b-9	39	235 (200-327) (n=23)	208 (152-276) (n=16)	0.278	0.603 (0.09)
Trimester 2 C5b-9	50	279 (237-328) (n=32)	248 (223-329) (n=18)	0.701	0.533 (0.09)
Trimester 3 C5b-9	41	284 (254-372) (n=22)	308 (258-494) (n=19)	0.229	0.390 (0.09)

Normally distributed variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values for Mann-Whitney U tests otherwise. AUROC = area under receiver operating curve, reported with associated standard error. Bold p-values are significant at p<0.05.

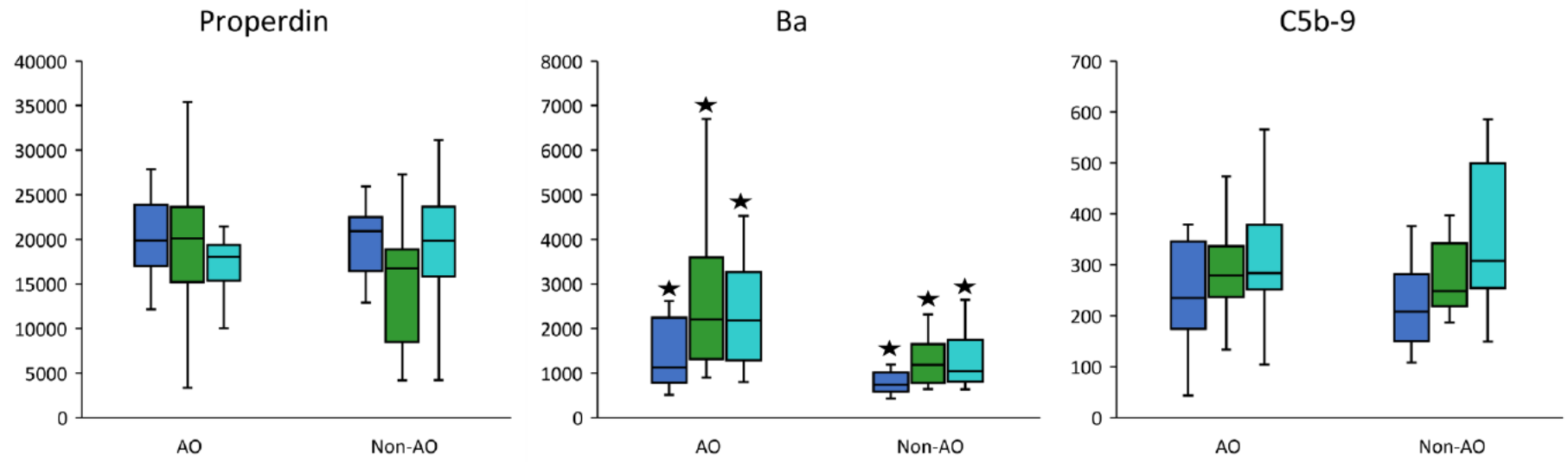


Figure 4.3: Plasma complement concentrations (ng/ml) in adverse outcome and non-adverse outcome pregnancy by trimester

Image shows box-and-whisker plots, with horizontal lines representing the median, boxes representing interquartile range, and whiskers representing the total range. Outliers >1.5 standard deviations from upper quartiles are excluded for display purposes. ★= significant difference between groups (AO vs. non-AO) by trimester measured ($p<0.05$). AO: adverse outcome pregnancy; Non-AO: non-adverse outcome pregnancy.

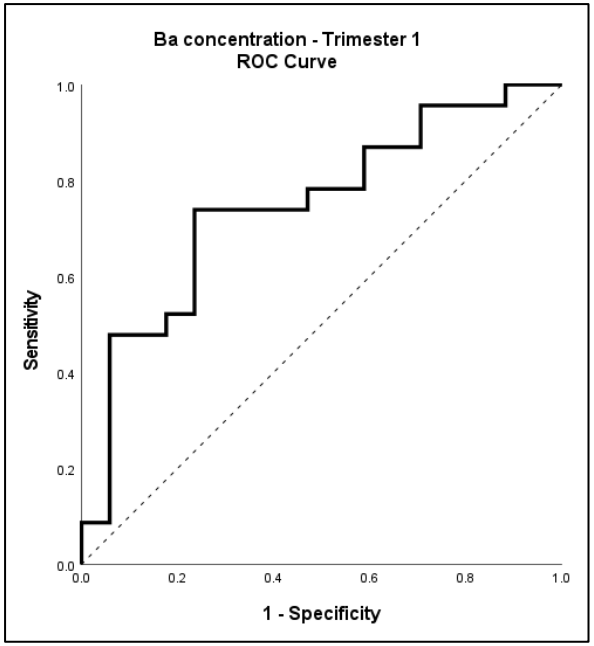
Legend:

Trimester 1 *Trimester 2* *Trimester 3*

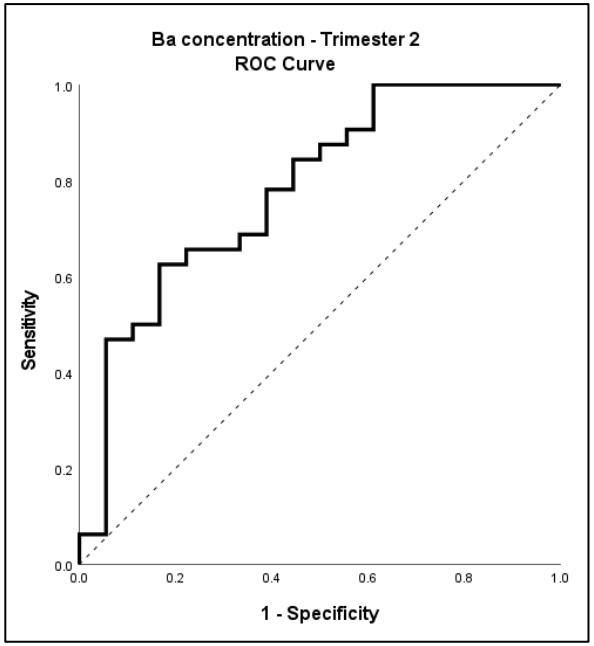
4.2.4.1 Predictive accuracy of plasma Ba for adverse pregnancy outcomes

ROC curve analysis found third trimester Ba to be the strongest predictor of adverse pregnancy outcome (AUROC 0.797, SE 0.07). This was followed by second trimester Ba (AUROC 0.781, SE 0.07), and first trimester Ba (AUROC 0.747, SE 0.08). See ***Table 4.8***, with ROC curves shown in ***Figure 4.4***.

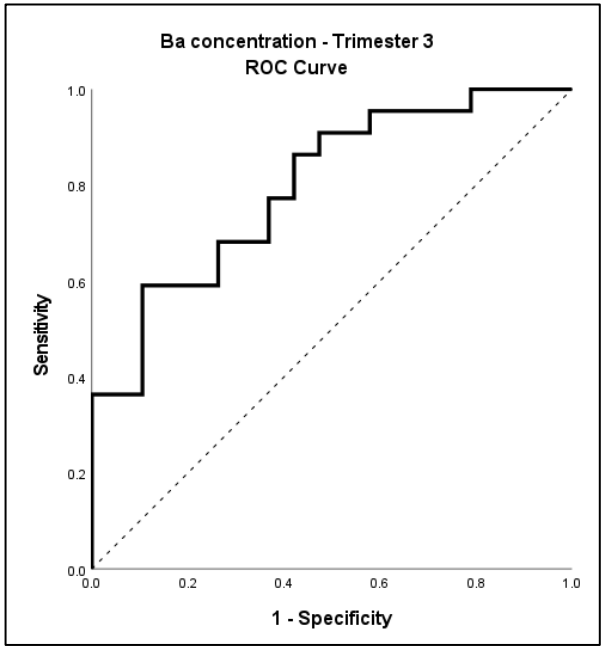
Further ROC curve analysis sought to determine cut-off values for Ba concentration in each trimester to successfully identify pregnancies at risk of adverse outcome. This found that a Ba concentration above 949 ng/ml (from a maternal blood sample taken from 0-16 weeks of pregnancy); 1731 ng/ml (16+1 to 27+6 weeks of pregnancy); and 2027 ng/ml (28 weeks of pregnancy and beyond) would be indicative of a pregnancy at risk of adverse outcome in women with CKD (see also ***Figure 4.4***).



Ba trimester 1: AUROC 0.747 (0.08)
Reference



Ba trimester 2: AUROC 0.781 (0.07)
Reference



Ba trimester 3: AUROC 0.797 (0.07)
Reference

Biomarker	N	Sensitivity	Specificity
Trimester 1 Ba > 949 ng/ml	40	74%	77%
Trimester 2 Ba > 1731 ng/ml	50	63%	83%
Trimester 3 Ba > 2027 ng/ml	41	59%	90%

Figure 4.4: Predictive accuracy of Ba concentration in determining adverse pregnancy outcome: ROC analysis

Images show ROC curves for the performance of Ba in the prediction of adverse outcome by trimester of blood draw. AUROC values are reported with associated standard error. Optimal cut off points are then identified using Youden's J statistic with associated sensitivity and specificity

4.2.4.2 Logistic regression analysis

Although Ba was consistently elevated across all 3 trimesters in the adverse pregnancy outcome group, this group was characterised by significantly poorer pre-pregnancy renal function and higher levels of pre-pregnancy proteinuria than the non-adverse outcome group.

In Chapter 3, Ba was elevated in previously healthy pregnant women with PE compared to pregnant controls in univariate analysis, but the difference between groups became non-significant after controlling for biochemical and immunological markers in multivariate analysis. This raises the question of whether the elevation of Ba in PE (and SPE) is actually a reflection of impaired excretory renal function and AKI, rather than being a result of systemic complement activation.

Data for renal function at the time of delivery were not available for the CKD Cohort, but logistic regression analysis was performed after controlling for pre-pregnancy creatinine and pre-pregnancy proteinuria (urine ACR). The results are displayed in ***Table 4.9.***

Trimester 3 Ba concentration remained independently associated with composite adverse pregnancy outcome in women with CKD after controlling for pre-pregnancy creatinine and proteinuria (odds of adverse outcome 1.20 per 100 ng/ml increase in Ba, 95% CI 1.04-1.39, p=0.016).

However, neither Trimester 1 nor Trimester 2 Ba concentrations were independently associated with adverse pregnancy outcome in women with CKD, after controlling for pre-pregnancy creatinine and urine ACR.

Table 4.9: Logistic regression analysis to identify independent markers of adverse pregnancy outcome in women with CKD

Factor	Model 1		Model 2		Model 3	
	OR (95% CI)	*p-value*	*OR (95% CI)*	*p-value*	*OR (95% CI)*	*p-value*
Trimester 1 Ba (per 100 ng/ml increase)	1.08 (0.93-1.26)	0.323				
Trimester 2 Ba (per 100 ng/ml increase)			1.08 (0.99-1.17)	0.095		
Trimester 3 Ba (per 100 ng/ml increase)					1.20 (1.04-1.39)	**0.016**
Pre-pregnancy creatinine (per 10 µmol/l increase)	0.92 (0.66-1.30)	0.647	0.94 (0.74-1.20)	0.635	0.77 (0.56-1.06)	0.109
Pre-pregnancy uACR (per 10-fold increase, mg/mmol)*	1.98 (0.69-5.68)	0.202	2.03 (0.77-5.40)	0.155	2.86 (0.79-10.38)	0.111

*Binary logistic regression analyses were conducted, using adverse pregnancy outcome as the dependent variable. Pre-pregnancy creatinine and urine ACR were entered into each model as continuous covariates, and odds ratios for adverse pregnancy outcome are reported, with associated 95% confidence intervals. *OR for pre-pregnancy uACR reported per 10-fold increase, because values were log-transformed to improve goodness-of-fit of the regression model. P-values are significant at p<0.05.*

4.3 Discussion

4.3.1 Summary of findings

Despite the growing body of evidence supporting a role for complement dysregulation in the pathogenesis of PE (4, 9, 11), the results from this study do *not* conclusively confirm evidence of increased complement activation in pregnant women with CKD and SPE. This is contrary to the findings presented in Chapter 3, demonstrating excessive complement activation in the placental tissue and maternal and fetal circulation in women with PE with no pre-existing medical conditions.

4.3.1.1 Complement biomarkers in CKD: SPE versus non-SPE pregnancy

In this study, comparisons between groups showed that Ba levels were consistently higher in SPE than non-SPE pregnancies, albeit with the only significant difference between groups in third trimester Ba in the serially collected samples cohort. This is potentially suggestive of excessive alternative complement pathway activation in SPE; a phenomenon which has previously been described in the setting of PE (17, 139), and is demonstrated in the Chapter 3 results. Contrary to this, however, was the lack of evidence of properdin consumption in SPE pregnancies. In fact, second trimester properdin concentrations were significantly raised in SPE when compared to non-SPE pregnancies and were generally higher in SPE pregnancies across the time points measured (albeit with no other statistically significant differences between groups). To date, there are no published reports of Ba or properdin in the setting of SPE secondary to CKD. Data are limited to a single cohort study by Wiles et al., of 60 pregnant women with CKD; 15 of whom developed SPE (203). The study reported no evidence of any difference in alternative pathway regulator Factor H concentrations between SPE and non-SPE pregnancies.

No significant differences in C5b-9 were observed between groups at any stage of pregnancy in the present study. This is in keeping with the results from the study by Wiles et al., in which third trimester C5b-9 concentrations did not differentiate SPE from non-SPE pregnancy in women with CKD (203). In the serially collected samples group, there did appear to be an uplift in C5b-9 levels in

third trimester samples taken from women who developed SPE, but the difference between groups was not statistically significant.

Analyses were repeated after removing subjects with a background of lupus nephritis or glomerular disease, so as to eliminate potential confounding factors caused by underlying autoimmune or complement-mediated disease. This had no significant effect on the overall results, other than third trimester Ba levels now being statistically significantly higher in the SPE group, compared to non-SPE pregnancies. The caveat to this is the very small numbers of patients included in this analysis, meaning that results should be treated with an element of caution, and the significantly poorer pre-pregnancy renal function in the SPE group.

4.3.1.2 Interpretation of findings

There are several potential reasons why the results from this study differ from those seen in previously healthy women with PE in Chapter 3. One theory is that the pathogenesis of SPE is different to PE, and therefore may be thought of as a separate disease entity. A proportion of women develop early-onset PE, which appears to be a primarily placentally-driven disease, typified by malplacentation, complement dysregulation, and significant angiogenic imbalance (4). This often results in adverse pregnancy outcomes including low birth weight and growth-restricted babies. This is in contrast to late-onset PE, which is less commonly associated with placental lesions, angiogenic imbalance, or fetal growth restriction (118, 120, 255). Instead, it is postulated that late-onset PE develops as a result of the rising metabolic demands of later pregnancy outstripping the perfusion capacity of the placenta (107). This process is intensified in those with metabolic or cardiovascular disease, including women with CKD, which is characterised by pre-existing endothelial dysfunction (79), and already 'inflamed' tissue. Some writers cite the 'sensitive vasculature' theory as the predominant driver of SPE pathogenesis in women with CKD. It is postulated that pre-existing endothelial dysfunction sensitises the maternal vasculature to circulating antiangiogenic factors,

creating a lower threshold to develop angiogenic dysregulation, and subsequent SPE, than for women without renal disease (79, 201).

This theory is supported by the findings from a study by Wiles et al., who report increased expression of markers of endothelial dysfunction (hyaluronan and VCAM) in pregnant women with CKD who developed SPE, compared to those who did not (203). In contrast, there were no differences in maternal plasma complement concentrations between groups (C3a, C5a, Factor H, or C5b-9). The authors argue that this indicates a significant maternal component to the pathogenesis of SPE, driven by endothelial dysfunction (203). This is in contrast to the complement-mediated, placentally-driven process, described in 'classical' PE (4, 9, 11), and also suggested by the Chapter 3 results.

Another potential explanation for the lack of difference seen in complement component concentrations between women with CKD who did and did not develop SPE is the more advanced renal dysfunction in the SPE group. Complement components are renally excreted, and previous studies have found positive correlations between urine complement concentrations, including properdin and C5b-9, and serum creatinine (256). C5b-9 and properdin are both relatively high molecular weight proteins, with properdin forming polymers of approximately 220 kDa (257). It is conceivable that their excretion may be reduced in subjects with more advanced renal impairment, and therefore elevated plasma concentrations are seen. This may explain why properdin consumption, which was consistently seen in PE in previously healthy women in Chapter 3, was not observed in this study in women with CKD and SPE. Another study reported significantly elevated Ba concentrations in patients with advanced chronic kidney disease and renal failure when compared to the assay normal range (249). The utility of other renally-excreted biomarkers in predicting SPE (including hyaluron, VCAM, and PlGF), was found to be reduced in women with more advanced renal disease (CKD stages 3-5), compared to women with CKD stages 1-2 (192).

4.3.1.3 Complement biomarkers in CKD: adverse outcome versus non-adverse outcome pregnancy

A final contributory factor to the lack of difference seen in complement concentrations between groups could be the inherent difficulties in diagnosing SPE in women with pre-existing CKD (192, 203). Although clearly defined criteria for the diagnosis of SPE were used (see section 2.4.6), proteinuria, hypertension, and impaired renal function characterise both pregnancy-related CKD progression and SPE. Baseline CKD parameters may have a confounding effect, with the effects of physiological adaptations to pregnancy triggering arbitrary thresholds in the absence of disease. Thus, some patients in this study could have been misclassified.

The distinction between SPE and pregnancy-related CKD progression is particularly blurred in milder forms of disease when proteinuria and hypertension occur later in pregnancy (258). Conversely, early-onset PE (prior to 34 weeks gestation) has a similar biochemical and clinical profile in healthy women and those with CKD, and has led some commentators to question whether SPE actually exists, or if "PE is always PE" (258).

To alleviate this problem, the analysis was repeated using a composite adverse pregnancy outcome measure. In this analysis, Ba concentration was significantly elevated across all 3 trimesters of pregnancy in women who had an adverse pregnancy outcome, compared to those who did not. Ba concentrations were consistently higher in adverse outcome pregnancies at all timepoints measured, with a particular separation in values during the second trimester, before the symptoms of SPE would usually be clinically detectable. Furthermore, in all 3 trimesters, an elevated Ba concentration appeared to offer strong predictive accuracy of the development of adverse pregnancy outcome.

One potential confounder was the significantly poorer pre-pregnancy renal function and elevated pre-pregnancy proteinuria in the adverse outcome group. In Chapter 3 results, Ba was found not to be independently associated with PE after controlling for biochemical and immunological markers. However, in this chapter, third trimester Ba concentration *did* remain independently associated with

adverse pregnancy outcome in women with CKD after controlling for pre-pregnancy creatinine and urine ACR (but not first or second trimester Ba levels).

This is suggestive of increased alternative complement pathway activation in women with CKD who later developed adverse pregnancy outcomes. Raised Ba levels may also indicate activation of any of the 3 complement pathways, triggering the alternative pathway amplification loop. These findings are corroborated by previous studies that report elevated levels of Bb in the first half of pregnancy in healthy women who later developed PE (17) or experienced spontaneous preterm birth (66). The authors hypothesise that inflammatory and/or ischaemic events during early placental development trigger complement activation (and production of complement activation fragments). This in turn leads to defective placental angiogenesis, maternal endothelial dysfunction, and adverse pregnancy outcomes including PE.

Overall, the results raise the possibility of third trimester plasma Ba levels being a useful biomarker for 'at-risk' pregnancies in women with CKD. The potential clinical utility would include informing decisions around increased antenatal surveillance, and in timing of delivery. Furthermore, the results raise the question of using complement inhibition therapies, particularly through blockade of excessive alternative pathway activation, before adverse pregnancy outcomes develop.

There were no significant differences in properdin or C5b-9 between adverse outcome and non-adverse outcome pregnancy groups. The more advanced renal dysfunction in the adverse outcome group may again have been a contributory factor in this observation (192), as well as earlier gestation of blood draw in trimester 1 and 3 samples.

4.3.2 Strengths and limitations

This study offers a novel review of circulating complement component activity in pregnant women with CKD across a wide range of aetiologies and disease severity (including CKD stages 1 to 4, and

those with a prior kidney transplant). This represents the first time maternal plasma Ba or properdin have been reported on in the context of CKD and SPE, and provides a detailed overview of the potential role of circulating complement in the pathogenesis of SPE.

The analysis included a range of timepoints measured during pregnancy, allowing an assessment of how complement biomarker expression changes during gestation in women who did and did not go on to develop SPE or an adverse pregnancy outcome.

Furthermore, the study aimed to reduce the impact of potential confounding factors, by repeating analyses after removing patients with lupus nephritis and glomerular disease, and by re-classifying pregnancies by composite adverse outcome. Logistic regression analysis also aimed to control for the potential confounding effect of differences in pre-pregnancy renal function and proteinuria. However, this analysis was based on a relatively small sample size, and a risk of over-adjustment of the regression model or residual confounding exists. Furthermore, only data on pre-pregnancy creatinine and proteinuria were available. Renal function can change during pregnancy, and typically worsens during later gestation in women with pre-existing CKD. Testing contemporaneous serum samples taken during pregnancy would have better controlled for this effect, but there were not sufficient blood samples available for this purpose.

The study is limited by the relatively small sample size, meaning that it is likely to be underpowered to detect statistically significant differences in complement markers between groups. Pregnancy in women with CKD is relatively uncommon, so it was not pragmatic to extend the timeframe of recruitment any further. Subjects were recruited by convenience sampling, with blood testing taking place at routine clinic visits. Blood was drawn at differing timepoints during gestation for each subject, meaning that samples were not consistently available for all subjects for each specified trimester, and analysis of complement components across all 3 trimesters was only possible for a small proportion. This limitation was compounded by one of the properdin ELISA plates failing to read due to presumed substrate contamination. The plasma aliquots had already been thawed and

re-frozen so re-testing was not possible due to the likely confounding effect of complement activation. It is possible that those subjects with added pregnancy complications had more frequent antenatal blood testing and therefore more blood samples available for testing – thus a potential confounding effect. However, demographics and clinical outcome criteria very closely reflected those from the original CKD cohort (unpublished; described in a thesis by Dr Nadia Sarween, UoB (232)), with no evidence of a more severe phenotype in the current study.

Having more blood tests available for each subject across a range of gestations would have allowed a detailed longitudinal analysis of the change in complement biomarkers during pregnancy. Furthermore, analysis of placental histology would have enabled an evaluation of how circulating complement in the fluid phase relates to complement deposition in tissue in women with SPE.

Diagnosis of SPE in women with CKD is notoriously difficult, due to a blurring of disease phenotypes. Attempts were made to use a consistent definition of SPE, including the escalation of pre-existing hypertension or proteinuria, as employed by other research groups (79, 192). However, it is physiological for blood pressure to fall in mid-pregnancy, before returning to baseline later in pregnancy, requiring re-establishment of antihypertensives (177, 180, 193). Similarly, it is physiological for proteinuria to increase during healthy pregnancy (259). It is possible that these factors could have led to over-estimation of SPE in the CKD Cohort.

In the adverse pregnancy outcome analysis, there were gestational differences in the timing of first and third trimester blood draw, with samples being drawn earlier in subjects with an adverse pregnancy outcome. Ba concentration rises during pregnancy, particularly between the first and second trimester. Therefore, the results may have under-estimated the magnitude of the difference between Ba in adverse outcome and non-adverse outcome pregnancies.

The subjects in this study had relatively mild disease (median gestation at delivery 36.0 weeks in the SPE group). It would be interesting to compare those with severe, early-onset SPE with later-onset

disease, to assess whether there are differences in the complement profile between groups. Given the relatively small sample size, a subgroup analysis of this nature was not pragmatic.

4.4 Conclusions

This study did not confirm an association between complement activation and SPE in pregnant women with CKD. This is in contrast to the findings from Chapter 3, in which PE was associated with excessive complement activation in women with no pre-existing medical conditions.

Although there was excess Ba production in all 3 trimesters in women who later developed SPE, the differences between groups were not statistically significant. The diagnosis of SPE in women with CKD is difficult and may complicate analysis through misclassification of subjects. Furthermore, complement component tests have not been validated in pregnant subjects with pre-existing renal dysfunction. Impaired renal excretion in the SPE group may have confounded the results. Finally, the complement-mediated, placentally driven model of 'classical' PE in previously healthy women may differ from SPE pathogenesis in women with CKD, who have a predisposition for endothelial dysfunction. Further testing with a validation cohort of pregnant women with CKD would solidify the findings from this study.

The results from this study demonstrate excess production of circulating Ba in early, mid, and late pregnancy in women with CKD who developed adverse pregnancy outcomes. This potentially supports the theory of increased complement activation as a contributor to adverse pregnancy outcome; particularly excessive alternative pathway activity. Ba appears to provide excellent predictive accuracy of adverse pregnancy outcome in all three trimesters, raising the possibility of its utility as a biomarker for 'at-risk' pregnancy in women with CKD. Although third trimester Ba concentrations remained independently associated with adverse pregnancy outcome after controlling for pre-pregnancy renal function, significant differences in renal function between groups at the time of blood sampling may have confounded the results.

CHAPTER 5

INVESTIGATING THE ROLE OF COMPLEMENT IN THE PATHOGENESIS OF PRE-ECLAMPSIA IN WOMEN OF SUB-SAHARAN AFRICAN ETHNICITY

5 Investigating the role of complement in the pathogenesis of pre-eclampsia in women of sub-Saharan African ethnicity

5.1 Summary and overview

Maternal mortality and morbidity rates remain disproportionately high in the world's least developed regions. In 2017, approximately two-thirds of global maternal deaths occurred in SSA (84), often as a result of obstetric complications including PE and eclampsia (204). However, racial disparities in pregnancy outcomes exist even in more economically developed nations, including the UK (208, 209). Complex socioeconomic factors undoubtedly play a significant role in this, but it has been postulated that Black ethnicity itself confers an elevated risk of adverse pregnancy outcomes, including PE (175, 207, 253, 260). Population studies in the USA (174) and France (176) have reported significantly increased rates of PE in women of AA and SSA heritage, compared to women who identify as White American and White European, after controlling for maternal age and socioeconomic status. SSA ethnicity also appears to be a risk factor for early-onset PE (176) and PE with severe features (211). A genetic component may be responsible for conferring additional pregnancy risks in Black women. There is particular interest in high rates of KIR and HLA-C variants (216, 217), as well as high-risk alleles encoding the APOL-1 gene, which are only found in those with recent African ancestry (123, 124, 219).

There is very little published data on the role of complement in the pathogenesis of PE in women of SSA ethnicity. Existing evidence reports increased concentrations of alternative complement pathway activation fragment Bb in AA women with PE, both in early pregnancy (17), and at term (141). These studies featured relatively small cohort sizes and did not test any other complement components or biochemical parameters. A more comprehensive analysis of circulating complement markers in women of SSA is required, to assess for the potential contribution of complement activation to the high rates of PE seen in SSA women. Complement-modifying agents might present novel therapeutic opportunities for this group of women who would potentially have much to gain.

The specific aims of this arm of research were to:

- Investigate markers of complement activation in maternal circulation (Ba, properdin, C3, C4 and C5b-9) in a Ghanaian cohort of pregnant women, and to compare the relative changes in PE, gestational hypertension, and healthy pregnant controls.
- Analyse maternal blood samples for biochemical and immune markers of inflammation, to assess for any correlation with complement activation and pregnancy outcome group. Samples of maternal serum were tested for creatinine, cystatin C, urea, uric acid, B2M, LDH, HS-CRP, sFLCs, and immunoglobulins G, A and M.

5.2 Results

5.2.1 Cohort characteristics

Demographics and clinical outcome data are presented in ***Table 5.1.*** A total of 108 women were included in this study; 47 with a diagnosis of PE, 26 with gestational hypertension, and 35 healthy pregnant controls. All participants were of Ghanaian SSA ethnicity (from Ewe, Ga Adangbe, Akan, Dagbani, or Hausa background). There were no significant differences in maternal age, gravidity or parity between groups. A proportion of women in the PE group had pre-existing chronic hypertension (13%; versus none in the gestational hypertension or control groups, p=0.009). 11% of the PE group and 23% of the gestational hypertension group had had a previous diagnosis of hypertension in pregnancy, compared to none of the women in the control group (p=0.011). Antenatal clinic attendance was poorer in the PE group, compared to both other groups, but the overall difference between groups was not statistically significant (p=0.362).

5.2.1.1 Maternal and fetal outcomes

PE was associated with an increased risk of adverse pregnancy outcomes compared to the gestational hypertension and control groups. Median gestation in the PE group was 36.7 weeks, compared with 38.7 weeks for gestational hypertension, and 39.3 weeks for controls, p<0.001. Furthermore, there was a significantly higher proportion of preterm births among women with PE, as compared to gestational hypertension and controls (53%, vs. 4% and 17%, respectively, p<0.001). Women with PE were more likely to deliver by emergency Caesarean section (60%, versus 35% for gestational hypertension and 23% for controls, p=0.005). The mean peak recorded blood pressures, by definition, were significantly higher for both PE (168/108 mmHg) and gestational hypertension (156/102 mmHg), compared with controls (123/77 mmHg), p<0.001. Approximately 1 in 8 women in the PE group were diagnosed with eclampsia during their pregnancy, indicating severe or poorly controlled disease.

Adverse fetal outcomes were similarly commonplace among the PE group. The mean birth weight for pregnancies affected by PE was 2419 g, compared to 3232 g for gestational hypertension, and 3221 g for healthy controls, $p<0.001$. Almost half the babies born to mothers with PE were of low birth weight (less than 2500 g), compared with just 4% of the gestational hypertension group, and 11% of healthy controls ($p<0.001$). Likewise, SGA babies were seen more often in the PE group (51% of pregnancies) as compared to gestational hypertension and controls (16% and 20% respectively, $p=0.002$). There were 3 neonatal deaths among the PE group, but none in the other groups.

Table 5.1: Demographics and clinical outcome data: Ghana Cohort

	N	*Pre-eclampsia (n=47)*	*Gestational HTN (n=26)*	*Control (n=35)*	*p-value*
Maternal age at delivery (years)	99*	28.6 ± 6.7	30.1 ± 5.8	30.2 ± 6.4	0.487
Chronic HTN	108	6 (13%)	0 (0%)	0 (0%)	**0.009**
Pregnancy history:					
Parity	107*	1 (0-2)	2 (0-2)	1 (0-2)	0.756
Gravidity	95*	3 (2-4)	3 (2-4)	2 (1-3)	0.094
Previous HTN in pregnancy	108	5 (11%)	6 (23%)	0 (0%)	**0.011**
Regular antenatal clinic attendance	108	38 (81%)	23 (88%)	32 (91%)	0.362
Gestation (weeks)	107*	36.7 (34.0-39.4)	38.7 (38.0-40.7)	39.3 (38.0-40.9)	**<0.001**
Preterm birth (<37 weeks)	107*	25 (53%)	1 (4%)	6 (17%)	**<0.001**
Mode of delivery:	108				**0.005**
Vaginal		16 (34%)	14 (54%)	16 (46%)	
Emergency CS		28 (60%)	9 (35%)	8 (23%)	
Elective CS		2 (4%)	3 (12%)	7 (20%)	
Unknown		1 (2%)	0 (0%)	4 (11%)	
Peak recorded BP (mmHg):					
Systolic	107*	168 ± 15	156 ± 19	123 ± 9	**<0.001**
Diastolic		108 ± 11	102 ± 11	77 ± 10	**<0.001**
Eclampsia	47	6 (13%)	NA	NA	NA
Fetal outcomes:					
Twins	108	1 (2%)	2 (8%)	1 (3%)	0.455
*Birth weight (g)****	106*	2419 ± 879	3232 ± 528	3221 ± 608	**<0.001**
*Low birth weight (<2500 g)***	106*	22 (49%)	1 (4%)	4 (11%)	**<0.001**
*SGA (birth weight <10th centile)***	105*	23 (51%)	4 (16%)	6 (17%)	**0.001**
Neonatal death	108	3 (6%)	0 (0%)	0 (0%)	0.135

*Continuous variables are reported as mean ± standard deviation, with p-values derived from one-way ANOVA tests, or as median (interquartile range), with p-values from Kruskal-Wallis tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available from total of 108 pregnancies). **For twin pregnancies, outcomes were the same for both babies; hence these were combined for analysis. ***For twin pregnancies, the average weight of the two babies was assumed. NA=data were not available in the cohort for the stated variable. HTN = hypertension, CS = Caesarean section, BP = blood pressure, SGA = small-for-gestational-age.*

5.2.2 Complement results

Complement concentrations by group are presented in ***Table 5.2***. Data were available for 108 women (47 with PE, 26 with gestational hypertension, and 35 healthy controls) for Ba and C5b-9, and 107 women (46 with PE, 26 with gestational hypertension, and 35 controls) for C3 and C4 (1 participant had insufficient serum to test). For one plate of samples, the properdin ELISA test failed due to suspected substrate contamination. Properdin results are therefore only available for 88 subjects (35 with PE, 19 with gestational hypertension, and 34 healthy pregnant controls).

Post hoc analysis found that C5b-9 concentration was comparable between subjects with PE and controls but was significantly lower in the gestational hypertension group (720 ng/ml in PE vs. 544 ng/ml in gestational hypertension, p=0.040).

Post hoc analysis of Ba concentrations showed higher levels in subjects with PE than in gestational hypertension, with the difference between groups approaching but not reaching statistical significance (1222 ng/ml in PE vs. 993 ng/ml in gestational hypertension, p=0.069). Ba concentrations in women with PE were also generally higher than for controls, but the difference between groups was not statistically significant (1222 ng/ml, vs. 1074 ng/ml, respectively, p=0.543).

There were no significant differences in properdin, C3, or C4 between groups, with blood marker analysis returning very similar blood results across all 3 groups tested.

Table 5.2: Complement component results: Ghana Cohort

Blood test	*N*	*Pre-eclampsia (n=47)*	*Gestational HTN (n=26)*	*Control (n=35)*	*p-value*	*Post hoc test p-values*		
						PE vs control	*PE vs gest HTN*	*Gest HTN vs control*
Properdin (ng/ml)	88*	18802 ± 7208	17886 ± 4924	18229 ± 5320	0.855	0.919	0.857	0.979
Ba (ng/ml)	108	1222 (927-1708)	993 (770-1179)	1074 (851-1304)	0.067	0.543	0.069	0.960
C3 (g/L)	107*	1.6 ± 0.4	1.7 ± 0.3	1.6 ± 0.3	0.413	0.857	0.630	0.383
C4 (g/L)	107*	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.637	0.972	0.617	0.767
C5b-9 (ng/ml)	108	720 (550-922)	544 (407-724)	710 (563-890)	**0.025**	1.000	**0.040**	0.052

*Normally distributed variables are reported as mean ± standard deviation, with overall p-values derived from one-way ANOVA tests, or as median (interquartile range), with overall p-values for Kruskal-Wallis tests otherwise. Post hoc analyses were carried out using Tukey's test for multiple comparisons between groups for normally distributed variables, and Bonferroni-adjusted pairwise comparisons between groups for non-normally distributed variables. Bold p-values are significant at p<0.05. *= missing data (N denotes number of cases data available from a total of 108 pregnancies).*

5.2.2.1 Subgroup analysis: hypertensive disorders of pregnancy versus controls

Women with a background of chronic hypertension were not excluded from this study, which could potentially complicate the diagnosis of PE and lead to patients being misclassified. Furthermore, formal urine protein quantification was not available, thus proteinuria was detected using the more crude measure of urine dipstick testing to diagnose PE. This made it possible that PE was not always accurately diagnosed, and increased the complexity of the PE phenotype, with some potential crossover with the gestational hypertension group. The results analysis was therefore repeated by amalgamating subjects with PE and gestational hypertension into a single 'hypertensive disorder of pregnancy' group and comparing them to healthy control pregnancies.

Demographic and clinical outcome data are presented in ***Table 5.3***. The differences between groups were very similar to the overall analysis presented in ***Table 5.1***, with hypertensive disorders of pregnancy characterised by earlier delivery (median gestation 38.0 weeks, vs. 39.3 weeks for controls, p=0.003), higher rates of emergency Caesarean delivery (51%, vs. 23% of controls, p=0.003), lower mean birth weight (2717 g, vs. 3221 g in controls, p=0.002), and a larger proportion of SGA babies (39%, vs. 17% of controls, p=0.028). The differences in this new analysis related to gravidity, which was significantly higher in hypertensive disorders of pregnancy than for controls (median 3 vs. 2, p=0.030), and PTB rates which were no longer significantly different between groups (36% in hypertensive disorders of pregnancy, vs. 17% in controls, p=0.076).

Complement biomarker results comparing hypertensive disorders of pregnancy with healthy control pregnancies in the Ghana Cohort are shown in ***Table 5.4***. There were no statistically significant differences in any of the complement markers tested between groups, with largely comparable concentrations of all biomarkers between cases and controls.

Table 5.3: Demographic and clinical outcome data: hypertensive disorders of pregnancy versus controls

	N	***Hypertensive disorder of pregnancy (n=73)***	***Control (n=35)***	***p-value***
Maternal age at delivery (years)	99*	29.2 ± 6.4	30.2 ± 6.4	0.466
Chronic HTN	108	6 (8%)	0 (0%)	0.089
Pregnancy history:				
Parity	107*	1 (0-2)	1 (0-2)	0.455
Gravidity	95*	3 (2-4)	2 (1-3)	**0.030**
Previous HTN in pregnancy	108	11 (15%)	0 (0%)	**0.010**
Regular antenatal clinic attendance	108	61 (84%)	32 (91%)	0.377
Gestation (weeks)	107*	38.0 (35.1-39.6)	39.3 (38.0-40.9)	**0.003**
Preterm birth (<37 weeks)	107*	26 (36%)	6 (17%)	0.071
Mode of delivery:	108			**0.003**
Vaginal		30 (41%)	16 (46%)	
Emergency CS		37 (51%)	8 (23%)	
Elective CS		5 (7%)	7 (20%)	
Unknown		1 (1%)	4 (11%)	
Peak recorded BP (mmHg):				
Systolic	107*	164 ± 18	123 ± 9	**<0.001**
Diastolic		106 ± 11	77 ± 10	**<0.001**
Eclampsia	47**	6 (13%)	NA	NA
Fetal outcomes:				
Twins	108	3 (4%)	1 (3%)	1.000
*Birth weight (g)****	106*	2717 ± 861	3221 ± 608	**0.002**
*Low birth weight (<2500 g)***	106*	23 (32%)	4 (11%)	**0.031**
*SGA (birth weight <10th centile)***	105*	27 (39%)	6 (17%)	**0.028**
Neonatal death	108	3 (4%)	0 (0%)	0.549

*Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (IQR), with p-values for Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available for from total of 108 pregnancies). **For twin pregnancies, outcomes were the same for both babies; hence these were combined for analysis. ***For twin pregnancies, the average weight of the two babies was assumed. NA=data were not available in the cohort for the stated variable.*

Table 5.4: Complement biomarker results: hypertensive disorders of pregnancy versus controls

Blood test	*N*	*Hypertensive disorder of pregnancy*	*Control*	*p-value*
Properdin (ng/ml)	88	18480 ± 6462 (n=54)	18229 ± 5320 (n=34)	0.850
Ba (ng/ml)	108	1086 (864-1489) (n=73)	1074 (851-1304) (n=35)	0.625
C3 (g/L)	107	1.7 ± 0,4 (n=72)	1.6 ± 0.3 (n=35)	0.333
C4 (g/L)	107	0.3 ± 0.1 (n=72)	0.3 ± 0.1 (n=35)	0.875
C5b-9 (ng/ml)	108	675 (456-846) (n=73)	710 (563-890) (n=35)	0.272

Normally distributed variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values from Mann-Whitney U tests otherwise. Bold p-values are significant at p<0.05.

5.2.3 Biochemical and immunological markers

Biochemical and immunological markers were tested for a total of 107 women (46 with PE, 26 with gestational hypertension, and 35 healthy pregnant controls). 1 subject with PE from the overall Ghanaian cohort had insufficient serum available to perform testing. Results are presented in ***Table 5.5***.

Post hoc analysis found statistically significant differences between PE and control groups for several of the markers tested. Specifically, creatinine and cystatin C were both significantly raised in PE compared to healthy pregnant controls (median creatinine 66 μmol/L in PE, vs. 51 μmol/L in controls, p=0.015, and mean cystatin C 1.4 mg/L in PE, vs. 1.0 mg/L in controls, p=0.001). There were no statistically significant differences in other markers of excretory renal function (urea and uric acid) between groups, though.

B2M concentration was significantly raised in the PE group (3.0 mg/L, vs. 2.3 mg/L in controls, p=0.002). Conversely, HS-CRP was significantly lower in the PE group when compared to controls (4.8 mg/L, vs. 15.9 mg/L, respectively, p=0.013).

The only significant difference in biomarker comparisons between PE and gestational hypertension groups was serum creatinine, which was again significantly elevated in PE (66 μmol/L vs. 42 μmol/L, p=0.004).

There were no significant differences in biochemical or immunological markers between gestational hypertension and control groups. Similarly, there were no observed statistically significant differences in any of the immunoglobulin groups, serum free light chains, or LDH between any of the three study groups.

Table 5.5: Biochemical and immunological marker concentrations: Ghana Cohort

Blood test	Pre-eclampsia (n=46)	Gestational HTN (n=26)	Control (n=35)	p-value	Post hoc test p-values		
					PE vs control	PE vs gest HTN	Gest HTN vs control
B2M (mg/L)	3.0 (2.6-3.9)	2.5 (2.3-3.2)	2.3 (1.9-2.9)	**0.002**	**0.002**	0.064	0.671
Creatinine (μmol/L)	66.0 (51.0-81.0)	42.0 (39.0-55.5)	51.0 (39.0-60.0)	**0.001**	**0.015**	**0.004**	1.000
Urea (mmol/L)	2.4 (1.8-3.6)	1.8 (1.5-2.7)	2.1 (1.7-2.7)	0.234	1.000	0.269	1.000
Uric acid (mg/dL)	4.8 ± 1.4	4.1 ± 1.2	4.2 ± 1.2	0.065	0.144	0.105	0.952
Cystatin C (mg/L)	1.4 ± 0.5	1.3 ± 0.4	1.0 ± 0.4	**0.002**	**0.001**	0.385	0.157
IgG (g/L)	11.1 ± 3.4	12.1 ± 2.3	11.4 ± 2.7	0.373	0.826	0.339	0.688
IgA (g/L)	2.1 ± 0.9	1.9 ± 0.6	1.8 ± 0.7	0.118	0.119	0.370	0.901
IgM (g/L)	1.0 ± 0.6	1.2 ± 0.5	1.0 ± 0.5	0.313	0.902	0.465	0.298
Kappa (mg/L)	26.4 (22.1-33.3)	25.4 (21.0-31.9)	23.4 (18.6-28.0)	0.272	0.325	1.000	1.000
Lambda (mg/L)	25.7 (23.4-32.3)	26.6 (22.5-30.5)	26.5 (20.4-30.0)	0.612	0.974	1.000	1.000
HS-CRP (mg/L)	4.8 (1.7-15.0)	10.1 (2.0-20.7)	15.9 (5.3-33.5)	**0.015**	**0.013**	1.000	0.241
LDH (U/L)	601.9 (353.7-948.7)	647.5 (409.7-946.3)	594.2 (421.7-902.7)	0.941	1.000	1.000	1.000

Normally distributed variables are reported as mean ± standard deviation, with overall p-values derived from one-way ANOVA tests, or as median (interquartile range), with p-values for Kruskal-Wallis tests otherwise. Post hoc analyses were carried out using Tukey's test for multiple comparisons between groups for normally distributed variables, and Bonferroni-adjusted pairwise comparisons between groups otherwise. Bold p-values are significant at p<0.05. B2M = beta-2 microglobulin, HS-CRP = high-sensitivity C-reactive protein, LDH = lactate dehydrogenase.

5.2.3.1 Correlation between complement components and biochemical / immunological markers

The relationship between complement and biochemical / immunological markers was assessed in order to characterise the cohort in greater detail, to assess the relationship between complement and other markers of immunity and inflammation, and to be able to identify potential confounding factors. Correlations between complement components and biochemical / immunological markers are shown in ***Table 5.6***. Given that multiple comparisons were performed, a Bonferroni-corrected threshold for statistical significance of $p<0.004$ is also indicated.

Using the Bonferroni-corrected threshold, there were no significant correlations between properdin, C5b-9, or C3, and any of the biochemical and immunological markers tested.

In contrast, Ba was positively correlated with B2M (Spearman's rho 0.461, $p<0.001$), creatinine (rho 0.368, $p<0.001$), urea (rho 0.429, $p<0.001$), uric acid (rho 0.300, $p=0.002$), and HS-CRP (rho 0.334, $p<0.001$). There were no observed statistically significant correlations between Ba and any of the immunoglobulin classes, serum free light chains, cystatin or LDH, using the Bonferroni-corrected threshold.

C4 had a significant positive correlation with HS-CRP levels (rho 0.307, $p=0.001$).

Table 5.6: Correlation between complement components and biochemical / immunological markers: Ghana Cohort

	Complement component				
	Properdin	*Ba*	*C5b-9*	*C3*	*C4*
B2M	r: -0.073 p=0.504	**r: 0.461** **p<0.001***	r: 0.162 p=0.095	r: -0.077 p=0.430	r: 0.039 p=0.690
Creatinine	r: 0.112 p=0.339	**r: 0.368** **p<0.001***	r: 0.178 p=0.088	r: -0.067 p=0.524	r: 0.061 p=0.563
Urea	r: -0.076 p=0.482	**r: 0.429** **p<0.001***	**r: 0.244** **p=0.011**	r: -0.039 p=0.693	r: 0.070 p=0.477
Uric acid	**r: -0.227** **p=0.035**	**r: 0.300** **p=0.002***	r: 0.058 p=0.552	**r: -0.202** **p=0.037**	r: -0.109 p=0.266
Cystatin C	r: 0.040 p=0.710	**r: 0.198** **p=0.041**	r: -0.089 p=0.359	r: -0.162 p=0.095	r: -0.065 p=0.507
IgG	r: 0.033 p=0.759	r: -0.064 p=0.513	r: 0.004 p=0.967	r: 0.031 p=0.750	r: -0.223 p=0.021
IgA	r: 0.055 p=0.620	r: 0.047 p=0.637	**r: 0.237** **p=0.015**	r: 0.136 p=0.168	r: 0.083 p=0.398
IgM	r: 0.028 p=0.799	r: -0.115 p=0.242	r: 0.127 p=0.196	r: 0.108 p=0.272	r: 0.029 p=0.770
Kappa	r: 0.069 p=0.527	**r: 0.201** **p=0.039**	**r: 0.195** **p=0.045**	r: -0.039 p=0.689	r: -0.008 p=0.931
Lambda	r: 0.040 p=0.716	r: 0.159 p=0.103	**r: 0.206** **p=0.034**	r: -0.068 p=0.491	r: -0.032 p=0.748
HS-CRP	r: -0.034 p=0.755	**r: 0.334** **p<0.001***	**r: 0.232** **p=0.016**	r: 0.186 p=0.055	**r: 0.307** **p=0.001***
LDH	r: 0.048 p=0.659	r: 0.096 p=0.325	r: -0.082 p=0.401	r: 0.107 p=0.275	r: 0.023 P=0.817

*Analyses are based on a total of 107 blood results (N=68 for PE; NE=26 for gestational hypertension, and n=35 controls). Associations between variables are reported as Spearman's correlation coefficients, with associated p-values. Bold values are significant at p<0.05. r: Spearman's correlation coefficient. *p<0.004 (Bonferroni-corrected threshold for multiple comparisons).*

5.2.3.2 Logistic regression analysis

In this cohort, Ba concentrations closely correlated with biomarkers of excretory renal function. Furthermore, Ba concentrations were raised in women with PE (approaching a statistically significant difference when compared with the gestational hypertension group).

In Chapter 3, Ba was found *not* to be independently associated with PE after controlling for renal function. Contrary to this, in Chapter 4, third trimester Ba did remain independently associated with adverse pregnancy outcomes in women with CKD after controlling for pre-pregnancy renal function.

Logistic regression analysis was therefore conducted, using PE as the dependent variable, and controlling for serum creatinine. This found that, in the Ghana cohort, Ba was *not* independently associated with PE after controlling for renal function (OR for PE per 100 ng/ml increase in Ba: 1.04, 95% CI 0.96-1.14, p=0.337).

5.3 Discussion

5.3.1 Summary of findings

Existing literature highlights disproportionately high rates of hypertensive disorders of pregnancy, including PE, among women of SSA ethnicity (210, 211). This is also reflected in studies of more economically developed nations (176, 207, 215), and after controlling for demographic and socioeconomic factors (174). This is suggestive of an inherent risk of PE in this population of women. Women of SSA origin may also be at greater risk of severe disease (254, 261). A French study of 526 women reported rates of early-onset PE (diagnosis prior to 28 weeks' gestation) of 22% in SSA women versus 7% in white European women, and 7% in Maghrebian women ($p<0.01$) (176). Further evidence highlights disproportionately high rates of PE with severe features (211), and eclampsia (212), among women of SSA ethnicity, when compared to global rates.

A growing body of evidence, together with the results from Chapter 3 of this thesis, have linked excess complement activation with the pathogenesis of PE (4, 9-11). It was therefore postulated that women of SSA ethnicity – a population with already increased rates of PE, and with a high incidence of severe disease – would exhibit excessive complement activation in the setting of PE. The results from this study, however, do *not* confirm any significant association between complement dysregulation and PE in women of Ghanaian SSA ethnicity.

5.3.1.1 Complement biomarkers in women of SSA ethnicity

In this study, concentrations of properdin, C3 and C4 were comparable between women with PE, gestational hypertension, and controls. This is in direct contrast to the findings from Chapter 3, in blood samples taken from a UK cohort of women, which found properdin, C4 and C3 to be significantly reduced in women with PE when compared to healthy pregnant controls. Likewise, other publications have reported reduced C4 concentrations in women with PE (142), and high levels of split product C4d (34); indicative of C4 consumption via classical or lectin pathway activation. These findings are not reflected in this Ghanian cohort of women.

The present study found reduced concentrations of C5b-9 in Ghanaian women with gestational hypertension. However, when PE samples were compared with just healthy pregnant controls, there were no significant differences in C5b-9 between groups, and thus no evidence to confirm increased terminal pathway activation in Ghanaian women with PE. This is in contrast with some published research studies (of ethnically diverse groups), which report increased circulating C5b-9 concentrations in women with PE compared to healthy pregnant controls (34, 148, 150, 152), and associations with fetal growth restriction (34) and HELLP syndrome (80, 246). There were no cases of HELLP syndrome in the Ghana Cohort. No data were available about the timing of PE onset, but the median gestation at delivery was almost 37 weeks, with an interquartile range of 34.0 – 39.4 weeks, so it could be inferred that the majority of women in this cohort did not have early-onset disease. This may have contributed to the lack of difference in C5b-9 between PE and controls.

Although the present study did not report any significant difference in Ba concentration between groups, there was a trend towards elevated Ba levels in women with PE when compared with the gestational hypertension and healthy pregnant control groups. This is in keeping with the results from chapter 3, which found increased Ba in maternal and cord blood in subjects with PE, and Chapter 4, which found elevated Ba concentrations in all three trimesters in women with CKD with an adverse pregnancy outcome. Elevated Ba concentrations may be indicative of excessive alternative complement pathway activation in women with PE. The evidence base for alternative pathway activation in the maternal circulation in PE is conflicting. Elevated levels of complement Factor B and split product Bb have been reported during early pregnancy in prospective studies of women who later developed PE (17, 138). This is suggestive of early alternative pathway activation, as the placenta is developing. Further studies have reported high concentrations of Bb at term in cases of PE (141), and an association with PE severity (139), whereas other groups examining C3 levels reported no significant differences between cases of PE and healthy pregnant controls (142, 147).

Inclusion of women with chronic hypertension, and lack of formal protein quantification in this cohort Increased the complexity of the PE phenotype, with a potential blurring of diagnosis of PE and gestational hypertension. Thus PE and gestational hypertension patients were grouped together as 'hypertensive disorders of pregnancy' in subgroup analysis. However, there were no significant differences observed in any of the complement biomarkers between patients with a hypertensive disorder of pregnancy and healthy pregnant controls. Regrouping the patients in this way therefore did not alter the overall conclusions drawn.

5.3.1.2 Interpretation of findings

There is a scarcity of existing data on the role of complement in the pathogenesis of PE specifically in SSA populations, with publications limited to women of AA heritage living in the US. One prospective study by Lynch et al. examined maternal blood samples taken within the first 20 weeks of pregnancy for alternative pathway activation fragment Bb (17). From a total cohort of 701 women, 51 were of AA ethnicity. Overall, women with a Bb concentration measuring within the top decile were at a substantially increased risk of developing PE later in pregnancy (RR 3.3, 95% CI 1.6-7.0, p<0.001). Women of AA ethnicity were twice as likely as non-AA women to have a top decile Bb measurement (RR 2.2, 95% CI 1.2-4.0, p=0.008). The study did not report a significant increase in PE diagnoses in the AA group of women, but the numbers of affected women were very small and the study was underpowered to detect such a difference.

A later US-based study tested maternal and umbilical cord blood samples for Bb from a cohort of 291 women, of whom 78% were of AA ethnicity (141). From the overall cohort, maternal blood Bb concentrations were almost 30% higher in women with PE than in controls. Similarly, maternal blood Bb concentrations were significantly higher in AA women with PE, compared to AA women without PE (1.26 μg/ml vs. 0.96 μg/ml respectively, p=0.007). No significant differences in cord blood Bb concentrations between groups were found, and the study did not report a subgroup analysis of Bb concentrations in AA women with PE, versus women of non-AA ethnicity with PE.

Although these publications imply a potential role for alternative pathway activity in the maternal circulation in AA women with PE, the results are based on small cohorts of women and do not provide any analysis of biomarkers from other complement pathways.

Overall, there was a lack of evidence for excess complement activation in the setting of PE in this cohort of women of SSA ethnicity. The results may have been confounded by sampling from a resource-limited setting (with unavailability of formal urine protein quantification, angiogenic marker analysis, and uterine artery Doppler imaging), leading to potential misclassification of patients with PE. It may be the case that additional factors are at play and predispose women of SSA ethnicity to PE. Environmental and socioeconomic disparities – including differences in access to healthcare and higher rates of hypertension and obesity – undoubtedly play a role (175, 206, 253, 262).

Another hypothesis is that genetic differences in those with recent African ancestry confer a particular risk for developing PE during pregnancy. It has been proposed that the interaction between HLA-C expressed by trophoblasts, and KIRs expressed by dNK cells is important in successful trophoblast invasion, and therefore in healthy placental development (86, 98, 102, 103). Specific combinations of maternal KIR and fetal HLA-C alleles have been found more frequently in cases of PE (86, 98, 102). Interestingly, studies have shown high rates of these risk-conferring KIR and HLA-C variants in Ugandan (216) and Ghanaian (217) populations, which may contribute to the high rates of PE seen in women of SSA ethnicity.

Studies of the APOL-1 gene have also garnered much recent attention. ‘High risk’ variants of G1 and G2 alleles encoding the APOL-1 gene are only found in those with recent African ancestry and are known to confer an increased risk of kidney disease and hypertension (218, 263-266). In a Nigerian cohort study, approximately one in four people had two APOL-1 risk alleles, and among those with CKD, the prevalence rose to two-thirds (267). Although the presence of the maternal high-risk APOL-1 genotype has *not* been linked with the overall risk of developing PE, one study has reported an

association between the maternal APOL-1 G1 allele and the risk of developing early-onset PE (OR 2.2, p=0.03) (219). Instead, there appears to be a particular link between *fetal* high-risk APOL-1 genotypes and the risk of developing PE. One multicentre study of two US cohorts reported odds ratios for having a pregnancy affected by PE in the context of high-risk APOL-1 fetal genotype of 1.84 (95% CI 1.11, 2.93) and 1.92 (95% CI 1.05, 3.49), p<0.05 (123). This finding has been corroborated by later studies (124), and reinforces the theory that both maternal and fetal genetic factors are likely to play an important role in the pathogenesis of PE in women with recent African ancestry.

5.3.1.3 Relationship between complement and biochemical / immunological biomarkers

Biochemical analysis in this study found significantly elevated levels of B2M in Ghanaian women with PE. This association has previously been reported in a racially diverse cohort of women in the UK, where B2M was proposed to be a potentially useful biomarker for pregnancies at risk of PE (142). The authors also reported that B2M was strongly correlated with creatinine, meaning that elevated levels may be representative of impaired renal function rather than inflammation (142).

Women with PE in the present study had significantly higher levels of creatinine and cystatin C than healthy pregnant controls. These biomarkers are indicative of renal dysfunction and are expectedly elevated in the setting of PE. Ba concentrations were positively correlated with markers of excretory renal function, meaning that elevated Ba levels might be a product of poorer renal function, rather than necessarily being indicative of systemic complement activation. Ba is known to be renally excreted, and has been shown in previous research to be significantly elevated in patients with advanced chronic kidney disease compared to the assay normal range (249). However, the serum creatinine in the PE group (median 66.0 μmol/L) would still be considered to be within the normal range for pregnancy (250), thus the effect on renal excretion of small and middle-sized molecules would be expected to be minimal. Logistic regression analysis however confirmed that Ba was not significantly associated with PE after controlling for renal function. The trend towards elevated Ba

levels in SSA women with PE might therefore be interpreted as a surrogate marker of AKI, rather than excess complement activation.

In contrast to previous research (142), polyclonal serum free light chains were not significantly elevated in women with PE in this study when compared to controls. Interestingly, however, light chain concentrations for cases and controls in this Ghanaian cohort were almost double the reported levels in racially diverse UK cohorts in Chapter 3 of this thesis, and in published research (142). Furthermore, light chain concentrations were towards or above the upper limits of normal of stated laboratory reference ranges for non-pregnant populations (normal range for kappa 3.3-19.4 mg/L and lambda 5.7-26.3 mg/L (268)). Similarly, IgG concentrations for both PE cases and controls in the Ghana Cohort were significantly higher than levels reported during pregnancy in published research (142) and in Chapter 3 of this thesis. Previous studies have described higher IgG levels in Black populations than in White populations (269, 270), and higher proportions of Black patients with abnormal sFLC ratios than White patients, in the setting of monoclonal gammopathy (271). Despite this, there are no published reports to date on the link between inflammatory markers and ethnicity in pregnancy, except for a UK-based study of 88 women with PE and 107 healthy pregnant controls, in which Sarween et al. noted a trend towards higher levels of sFLCs and IgG in women of non-White ethnicity (142). This phenomenon could be contributory to the overall increased risk of inflammatory events in pregnancy, including PE, in women of SSA ethnicity.

5.3.2 Strengths and limitations

This study, for the first time, reports on a comprehensive panel of circulating complement biomarkers in a specific cohort of women of SSA ethnicity. This provides a valuable and unique insight into the potential contributors to PE pathogenesis in SSA women, who are known to be at a particularly high risk for developing PE during pregnancy. This cohort was recruited from a well-defined geographic area in Accra, Ghana, limiting the impact of geographical and socioeconomic

variations upon the results. Furthermore, there were no significant differences in antenatal clinic attendance between groups, which could be seen as an indicator of equality of access to healthcare between groups.

This study also provides a thorough review of biochemical and immunological markers in SSA women with PE, gestational hypertension, and healthy pregnancy. This has not previously been reported on and provides new potential mechanistic insights into the high rates of pregnancy complications seen in women of SSA ethnicity.

The results from this study are limited by the sample size: this study is likely to be underpowered to detect statistically significant differences in complement markers between groups. However, samples were tested retrospectively from an historically collected cohort, and all available samples were utilised.

Measurement of urine ACR was not possible within this healthcare setting, so the diagnosis of PE relied on more crude measures such as urine dipstick testing. Similarly, adjuncts to PE diagnosis used routinely in Western healthcare settings, including measurement of angiogenic markers (sFlt-1 and PlGF), and uterine artery Doppler ultrasonography were unavailable. Additionally, women with chronic hypertension were not excluded from this study, which could have further complicated the accurate diagnosis of PE and lead to potential misclassification. In an attempt to overcome this, a subgroup analysis was conducted, comparing all women with hypertensive disorders of pregnancy with healthy controls. This did not reveal any further significant differences between groups when compared to the overall results.

Another study limitation was the lack of availability of some demographic data. The gestation at PE onset and diagnosis was not recorded, so it was not possible to categorise the severity of PE. The gestation at blood draw was not recorded either, meaning that controlling for this effect in statistical analysis was not possible. However, the PE group delivered significantly earlier in pregnancy than gestational hypertension and control groups (albeit with a median gestation of 36.7 weeks in PE,

which would be considered almost ‘term’). It is therefore a possibility that gestational differences between groups could have impacted upon the recorded complement concentrations.

5.4 Conclusions

This study, for the first time, provides a comprehensive review of circulating biomarkers of complement activation during pregnancy in a cohort of women of SSA ethnicity. No significant differences were found in properdin, Ba, C3, C4, or C5b-9 concentrations between women with PE, women with gestational hypertension, or healthy pregnant controls. These results do not provide any conclusive evidence for complement activation being responsible for the excess rates of PE reported in women of SSA ethnicity.

Confounding factors were present which may have affected the conclusions drawn. The lack of availability of formal protein quantification and diagnostic adjuncts including uterine artery Doppler imaging and angiogenic marker analysis, and the inclusion of subjects with chronic hypertension, may have led to misclassification and a blurring of the PE phenotype. Future studies should aim to use standardised definitions of PE to enable accurate comparisons between groups. Furthermore, this study reports on a small population of women of Ghanaian ethnicity and is not representative of SSA as a whole.

Reasons for the increased rates of PE seen among women of SSA ethnicity are keenly debated. This study reported novel evidence of particularly high IgG and sFLC concentrations across all groups when compared to published data of racially diverse cohorts and normal laboratory reference ranges. This may contribute to an overall baseline systemic inflammation during pregnancy in SSA women, and high rates of pregnancy complications including PE.

This group of women have inherent differences to the racially diverse UK cohorts investigated in Chapters 3 and 4 of this thesis. The effect of complex environmental and socioeconomic factors cannot be overlooked and is likely to play a significant role in the high rates of PE seen in women of SSA ethnicity.

CHAPTER 6

A COMPARISON OF COMPLEMENT ACTIVATION IN PRE-ECLAMPSIA IN PREVIOUSLY HEALTHY WOMEN, AND IN HIGH-RISK GROUPS

6 A comparison of complement activation in pre-eclampsia in previously healthy women, and in high-risk groups

6.1 Summary and overview

PE is a heterogenous obstetric syndrome with hallmark features of hypertension and proteinuria, but wide variations in timing of onset and maternal and fetal clinical outcomes, including the development of HELLP syndrome and fetal growth restriction. This has led some commentators to question whether PE is in fact more than one disease with a shared clinical phenotype (107, 116), or if it has a single pathogenesis but manifests in different ways depending on individual sensitivities and risk factors (including age, obesity, ethnicity, and underlying health conditions including CKD) (118, 119).

The final chapter of this thesis aimed to compare complement markers within the maternal circulation across all three study groups (women with no pre-existing medical conditions in the Birmingham Cohort, women with renal disease in the CKD Cohort, and women of SSA ethnicity in the Ghana Cohort). Prior analysis in Chapter 3 (***section 3.2.9***) from a subset of patients from the Birmingham Cohort showed that complement assays conducted in Birmingham using commercially available ELISA kits returned the same statistically significant differences in complement biomarkers as MSD assays conducted in Newcastle. Therefore, the Birmingham ELISA test results from this subset of patients were compared with the CKD and Ghana cohort results.

The aim was to compare and contrast patterns of complement activation in PE and healthy pregnancy across a cohort of previously healthy women, women with CKD, and women of SSA ethnicity.

A summary discussion follows, providing a review of the principal novel findings arising from this thesis.

6.2 Results

6.2.1 Comparison of complement activation in PE: Birmingham vs. CKD vs. Ghana cohorts

A comparison of the demographic and clinical outcome data for Birmingham and Ghana Cohort PE groups, and the CKD SPE group are shown in ***Table 6.1***. It should be borne in mind that different criteria were used for the definition of PE or SPE across the three cohorts. In the Birmingham Cohort, PE was diagnosed according to international consensus criteria (see ***Table 1.3***), whereas for the Ghana Cohort, there was no availability of diagnostic adjuncts such as urine protein quantification with ACR or PCR, angiogenic marker analysis, or uterine artery Doppler. In the CKD Cohort, SPE was diagnosed according to the criteria listed in ***section 2.4.6***, which was adapted from previous research studies of SPE in women with CKD, in the absence of any standardised diagnostic criteria.

Data were available for a total of 78 women with PE: 16 from the Birmingham Cohort, 15 from the CKD cohort, and 47 from the Ghana Cohort. The only significant differences between PE cohorts related to ethnicity and parity. The Ghana Cohort was entirely comprised of women of Black ethnicity, whereas Birmingham and CKD cohorts were both more racially diverse; reflective of the population local to the West Midlands, UK. The Birmingham Cohort had a significantly higher proportion of nulliparous women (81%; vs. 47% of CKD and 30% of Ghana cohorts, p=0.001). Apart from this, cohorts were well matched for maternal age, gestation, mode of delivery, and fetal outcomes.

A comparison of complement biomarker concentrations in women with PE by cohort is displayed in ***Table 6.2***.

Post hoc analysis found that plasma properdin concentration was significantly lower in the Birmingham Cohort compared with the CKD Cohort (mean 12605 ng/ml, vs. 19065 ng/ml respectively, p=0.029), and with the Ghana Cohort (12605 ng/ml, vs. 18802 ng/ml, p=0.009).

C5b-9 concentrations were markedly raised in the Ghana Cohort when compared with the Birmingham Cohort (median 720 ng/ml, vs. 262 ng/ml respectively, $p<0.001$), and with the CKD Cohort (median 720 ng/ml, vs. 295 ng/ml, $p<0.001$).

There were no C3 or C4 results available for the CKD Cohort, but when comparing Birmingham with Ghana results, both C3 and C4 were significantly higher in the Ghana Cohort. Mean C3 concentration was 1.6 g/L for Ghana, vs. 1.3 g/L for Birmingham, $p<0.001$; and mean C4 concentration was 0.3 g/L for Ghana, vs. 0.2 g/L for Birmingham, $p=0.002$.

There were no statistically significant differences in plasma Ba concentrations across any of the three PE cohorts tested, although the median Ba concentration was notably higher in the CKD Cohort than in either of the other cohorts (1608 ng/ml for CKD, vs. 1286 ng/ml for Birmingham, and 1222 ng/ml for Ghana, $p=0.086$).

Table 6.1: Demographic and clinical outcome data for women with PE: Birmingham vs. CKD vs. Ghana Cohort

	N	***Birmingham (n=16)***	***CKD (n=15)***	***Ghana (n=47)***	***p-value***
Maternal age at delivery (years)	74*	28.5 ± 7.4	28.9 ± 4.7	28.6 ± 6.7	0.985
Nulliparous	78	13 (81%)	7 (47%)	14 (30%)	**0.001**
Ethnicity	78				**<0.001**
White		9 (56%)	8 (53%)	0 (0%)	
Asian		4 (25%)	5 (33%)	0 (0%)	
Black		3 (19%)	1 (7%)	47 (100%)	
Mixed/Other		0 (0%)	1 (7%)	0 (0%)	
Gestation (weeks)	78	36.9 (36.0-37.5)	37.1 (35.4-37.6)	36.7 (34.0-39.4)	0.826
Preterm birth (<37 weeks)	78	10 (63%)	7 (47%)	25 (53%)	0.697
Mode of delivery:	78				0.872
Vaginal		4 (25%)	4 (27%)	16 (34%)	
Caesarean		12 (75%)	11 (73%)	30 (64%)	
Unknown		0 (0%)	0 (12%)	1 (2%)	
Fetal outcomes:					
Birth weight (g)	75*	2311.3 ± 725.0	2659.9 ± 595.0	2418.7 ± 879.0	0.478
Low birth weight (<2500 g)	75*	10 (63%)	6 (43%)	22 (49%)	0.587
SGA (birth weight <10th centile)	75*	9 (56%)	3 (21%)	23 (51%)	0.103

*Continuous variables are reported as mean ± standard deviation, with p-values derived from one-way ANOVA tests, or as median (interquartile range), with p-values from Kruskal-Wallis tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available from total of 78 pregnancies).*

Table 6.2: Complement biomarker results in women with PE: Birmingham vs. CKD vs. Ghana Cohort

Blood test	*N*	*Birmingham*	*CKD*	*Ghana*	*p-value*	*Post hoc test p-values*		
						Bham vs CKD	*Bham vs Ghana*	*CKD vs Ghana*
Properdin (ng/ml)	65	12605 ± 3052	19065 ± 8341	18802 ± 7208	**0.008**	**0.029**	**0.009**	0.992
Ba (ng/ml)	72	1286 (977-1544)	1608 (1260-2581)	1222 (927-1708)	0.086	0.309	1.000	0.095
C3 (g/L)	62	1.3 ± 0.2	-	1.6 ± 0.4	**<0.001**	-	-	-
C4 (g/L)	62	0.2 ± 0.1	-	0.3 ± 0.1	**0.002**	-	-	-
C5b-9 (ng/ml)	72	262 (196-378)	295 (258-415)	720 (550-922)	**<0.001**	1.000	**<0.001**	**<0.001**

Normally distributed variables are reported as mean ± standard deviation, with overall p-values derived from independent samples t-tests, or one-way ANOVA tests. Non-normally distributed variables are reported as median (interquartile range), with overall p-values for Kruskal-Wallis tests. Post hoc analyses use Tukey's test for multiple comparisons between groups for normally distributed variables, and Bonferroni-adjusted pairwise comparisons between groups for non-normally distributed variables. Bold p-values are significant at p<0.05. Properdin data based on Birmingham N=16/ CKD N= 14/ Ghana N=35. Ba and C5b-9 data based on Birmingham N=10/ CKD N=15/ Ghana N=47. C3 and C4 data based on Birmingham N=16/ Ghana N=46.

6.2.2 Comparison of complement activation in pregnant controls: Birmingham vs. CKD vs. Ghana cohorts

Data were available for a total of 97 pregnant controls: 16 from the Birmingham Cohort, 46 from the CKD Cohort, and 35 from the Ghana Cohort. Baseline demographic and clinical outcome data are presented in ***Table 6.3***.

There was some heterogeneity in pregnant control group phenotypes across the three cohorts tested. Once again, there were inherent differences in the ethnic makeup of the groups tested. In addition, there were significant differences in mode of delivery, with the majority of the Birmingham Cohort controls delivering via Caesarean section, compared to a much more even split between vaginal and Caesarean deliveries in the CKD and Ghana controls. This is likely to be a result of many of the Birmingham Cohort controls being recruited from elective Caesarean section clinics. Note prior subgroup analysis in ***section 3.2.8.1*** which showed that mode of delivery did not appear to impact upon complement activation, however.

Control pregnancies in the CKD group had significantly earlier gestational age and smaller birth weights in the CKD cohort than in either the Birmingham or Ghana Cohort controls. Specifically, the CKD controls had a median gestational age of 37.9 weeks, vs. 39.3 weeks in both the Birmingham and Ghana cohorts, $p<0.001$. The mean birth weight for the CKD controls was 2824.3 g, vs. 3456.6 g for Birmingham, vs. 3221.1 g for Ghana, $p<0.001$. These differences are illustrative of the inherent challenges in managing pregnancy in women with renal disease and high rates of adverse outcomes even in the absence of PE (179, 180).

Complement biomarker concentrations for control groups are compared in ***Table 6.4***.

In contrast to PE groups, there were no significant differences in properdin concentrations between any of the study cohorts, or in C3 or C4 concentrations between Birmingham and Ghana cohorts.

Ba concentrations were significantly higher in CKD Cohort controls when compared to the Birmingham Cohort (1251 ng/ml, vs. 853 ng/ml respectively, p=0.006).

Once again, C5b-9 concentrations were in the order of 2.5 times greater in the Ghana Cohort than in either the Birmingham or CKD Cohort (710 ng/ml for Ghana, vs. 204 ng/ml for Birmingham, vs. 281 ng/ml for CKD; p<0.001).

Table 6.3: Demographic and clinical outcome data for pregnant control patients: Birmingham vs. CKD vs. Ghana Cohort

	N	***Birmingham (n=16)***	***CKD (n=46)***	***Ghana (n=35)***	***p-value***
Maternal age at delivery (years)	92*	30.1 ± 6.0	30.2 ± 5.8	30.2 ± 6.4	0.998
Nulliparous	96*	6 (38%)	16 (29%)	11 (32%)	0.959
Ethnicity	97				**<0.001**
White		9 (56%)	27 (59%)	0 (0%)	
Asian		4 (25%)	11(24%)	0 (0%)	
Black		2 (13%)	4 (9%)	35 (100%)	
Mixed/Other		1 (6%)	4 (9%)	0 (0%)	
Gestation (weeks)	96*	39.3 (39.0-40.6)	37.9 (37.0-38.9)	39.3 (38.0-40.9)	**<0.001**
Preterm birth (<37 weeks)	96*	0 (0%)	10 (22%)	6 (17%)	0.133
Mode of delivery:	97				**0.009**
Vaginal		3 (19%)	25 (54%)	16 (46%)	
Caesarean		13 (81%)	21 (46%)	15 (43%)	
Unknown		0 (0%)	0 (0%)	4 (11%)	
Fetal outcomes:					
Birth weight (g)	95*	3456.6 ± 344.0	2824.3 ± 471.2	3221.1 ± 608.3	**<0.001**
Low birth weight (<2500 g)	95*	0 (0%)	13 (30%)	4 (11%)	**0.011**
SGA (birth weight <10th centile)	95*	0 (0%)	12 (27%)	7 (20%)	0.053

*Continuous variables are reported as mean ± standard deviation, with p-values derived from one-way ANOVA tests, or as median (interquartile range), with p-values from Kruskal-Wallis tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *Missing data (N denotes number of cases data available from total of 97 pregnancies).*

Table 6.4: Complement biomarker concentrations in pregnant control patients: Birmingham vs. CKD vs. Ghana Cohort

Blood test	*N*	*Birmingham*	*CKD*	*Ghana*	*p-value*	*Post hoc test p-values*		
						Bham vs CKD	*Bham vs Ghana*	*CKD vs Ghana*
Properdin (ng/ml)	89	18669 ± 3915	17805 ± 5495	18229 ± 5320	0.844	0.841	0.958	0.935
Ba (ng/ml)	72	853 (626-1001)	1251 (891-2186)	1074 (851-1304)	**0.008**	**0.006**	0.148	0.271
C3 (g/L)	51	1.6 ± 0.2	-	1.6 ± 0.3	0.594	-	-	-
C4 (g/L)	51	0.3 ± 0.1	-	0.3 ± 0.1	0.859	-	-	-
C5b-9 (ng/ml)	72	204 (175-356)	281 (254-400)	710 (563-890)	**<0.001**	1.000	**<0.001**	**<0.001**

Normally distributed variables are reported as mean ± standard deviation, with overall p-values derived from independent samples t-tests, or one-way ANOVA tests. Non-normally distributed variables are reported as median (interquartile range), with overall p-values for Kruskal-Wallis tests. Post hoc analyses use Tukey's test for multiple comparisons between groups for normally distributed variables, and Bonferroni-adjusted pairwise comparisons between groups for non-normally distributed variables. Bold p-values are significant at p<0.05. Properdin data based on Birmingham N=16/ CKD N=39/ Ghana N=34. Ba and C5b-9 data based on Birmingham N=10/ CKD N=27/ Ghana N=35. C3 and C4 data based on Birmingham N=16/ Ghana N=35.

6.3 Discussion: comparison of complement biomarkers in pre-eclampsia in previously healthy women, and in high-risk groups

Analysis of complement biomarkers in both PE and healthy pregnancy across three distinct patient cohorts revealed different patterns of complement activity in each. Analysis of the Birmingham Cohort found significantly reduced concentrations of properdin in the maternal circulation of women with no pre-existing medical conditions in the setting of PE. This was not seen in the CKD or Ghana cohorts in women with PE, and in healthy control pregnancies, circulating properdin levels were comparable across all cohorts. The results are suggestive of excessive alternative complement pathway activity in women with PE in the Birmingham Cohort. Properdin is hypothesised to move from the maternal circulation and bind to activated complement in placental tissue – see ***section 3.3.2***) (224). This may in turn trigger further complement activation via the amplification loop.

Further differences between cohorts were seen in C3 and C4, which were both lower in the Birmingham PE Cohort than in the Ghana PE Cohort, and could be representative of complement consumption secondary to excessive activation of any of the 3 complement pathways.

Confounding factors may have contributed to the lack of properdin consumption seen among women with PE (or SPE) in the CKD and Ghana cohorts. One hypothesis is that impaired renal function in women with CKD could have caused reduced excretion of properdin (and other complement components), leading to relatively higher plasma concentrations in the CKD Cohort (256).

The raised levels of properdin, C3 and C4 seen in the Ghana PE Cohort could be ethnicity related. There are no published data relating to ethnic differences in complement components during pregnancy. Interestingly though, one recent study reported significantly elevated concentrations of properdin, C3 and C4 in a cohort of 89 Black African men, when compared to 96 White European

men (272). The authors suggest that this might predispose Black populations to systemic complement activation and inflammation, and could be contributory to the high rates of metabolic disease seen in this demographic.

C5b-9 concentrations were notably elevated in both PE and control pregnancies in the Ghana Cohort, compared to the Birmingham and CKD cohorts, with no overlap in the IQR between groups. Earlier subgroup analysis of the Birmingham Cohort also found a possible signal for raised C5b-9 levels in Black women with PE, versus non-Black women with PE (see ***sections 3.2.8.3 and 3.3.1.6***).

The evidence base for ethnicity-related differences in C5b-9 is limited and conflicting. Goff and colleagues did not report any significant difference in circulating levels of C5b-9 in their small cohort study of men of Black African heritage, versus White European men (272). Another small study found significantly reduced C5b-9 levels in 9 subjects of African origin, compared to 46 Caucasian subjects, $p<0.01$ (273). A final study compared the complement profile of 200 South Asians with 200 Caucasians, and found significantly elevated C5b-9 concentrations in South Asian participants (274). None of these studies included pregnant subjects, but they do highlight potential differences in normal ranges of complement components between ethnic groups. Having an elevated baseline C5b-9 level could theoretically predispose women of SSA ethnicity to inflammatory events during times of immune system stress, such as pregnancy. This may contribute to the high rates of PE with severe features and HELLP syndrome seen in the SSA population (80, 253).

Ba concentrations were higher in the CKD Cohort than in either the Birmingham or Ghana cohorts (although the differences between groups were only statistically significant for CKD vs. Birmingham pregnant controls). Note that the CKD control group had a significantly earlier gestation than Birmingham controls, so the reported difference between groups may be an under-estimate. Ba is known to be renally excreted, and has been shown to be significantly elevated in patients with advanced chronic kidney disease compared to the assay normal range (249). Previous analysis in Chapters 3 and 5 found Ba to be significantly correlated with biomarkers of renal function, although

third trimester Ba did remain independently associated with SPE after controlling for pre-pregnancy renal function in the CKD Cohort. Given that Ba concentrations are consistently elevated in CKD patients (even in control pregnancies not affected by SPE), and when compared to cohorts with normal kidney function, it seems likely that this is indicative of renal dysfunction rather than complement activation. Caution is needed when reviewing research studies reporting on Ba or Bb concentrations in PE, and study design should ideally control for or report on the renal function of participants.

It should be borne in mind that different diagnostic criteria were used for PE and SPE across the three cohorts tested, which may have affected interpretation of the results. Future studies should aim to use standardised and clearly defined disease descriptors, to enable reliable comparisons between groups.

6.4 Summary discussion

PE is a common hypertensive disorder of pregnancy, resulting in significant obstetric morbidity and mortality (74, 212, 237). Certain populations of women, including those with CKD and women of SSA ethnicity, have particularly high rates of PE (79, 190, 213, 262). The pathophysiology of PE remains poorly understood, and no definitive treatment is currently available, other than expedited delivery of baby and placenta (77, 86, 275). The identification of therapeutic targets could have substantial and much-needed benefits for at-risk women and help to reduce the extensive burden of disease (81).

There is increasing interest in the potential role of complement in the pathogenesis of PE. Animal models demonstrate placental complement activation inducing defective angiogenesis, abnormal placentation and fetal demise (15, 112, 114, 131), with complement inhibition rescuing pregnancy (15, 112). Small studies of human tissue report conflicting results but have demonstrated excessive placental complement deposition in women with PE, with potential links to disease severity (25, 31, 132). Other studies have reported raised concentrations of complement biomarkers in the maternal circulation in PE (17, 34, 148, 150). Data on umbilical cord blood complement is extremely limited. Despite the growing body of evidence, it remains unclear whether raised concentrations of circulating complement in human pregnancy affected by PE reflect a general heightened inflammatory state or are directly associated with placental complement-mediated injury.

This thesis examined the hypothesis that PE is associated with excessive complement deposition in placental tissue, with concurrent complement activation within the maternal and fetal circulation; and therefore, that abnormal levels of circulating complement are reflective of complement-mediated placental tissue damage. A secondary hypothesis was that groups with a high prevalence of PE, and of PE with severe features, (women with CKD and women of SSA ethnicity) would exhibit a significant degree of systemic complement activation.

There were three main arms of research; see ***Figure 2.1***. The first study examined placental tissue along with simultaneously collected samples of maternal and umbilical cord blood from a pregnant cohort of previously healthy women (PE cases and healthy controls), to explore how placental complement deposition might be related to levels of circulating complement in the fluid phase. The second and third arms of research examined maternal blood samples for complement biomarkers in cohorts at high risk of developing PE (pregnant women with CKD, and pregnant women of SSA ethnicity).

The aim was to provide further support for the role of complement activation in the pathogenesis of PE and also to provide mechanistic insight into the sequence of events leading to complement activation on a local and systemic level. This might in turn strengthen the evidence base towards future trials of complement-modifying agents in the treatment of PE.

6.4.1 Pre-eclampsia in previously healthy pregnant women is characterised by placental complement deposition and concurrent complement dysregulation in the maternal and fetal circulation

This study reports novel evidence of placental complement deposition associated with significant changes in complement biomarkers in the maternal and fetal circulation in women with PE. Specifically, women with PE had significant placental complement deposition (particularly C4d), with simultaneous significant reductions in properdin, C3 and C4 in the maternal circulation, and elevated Ba concentrations in both the maternal and fetal circulation.

Maternal plasma properdin emerged as being particularly strongly associated with PE. This is the first time a reduction in properdin in the context of PE has been reported. The findings were replicable in a separate validation cohort, and significant differences in properdin persisted after controlling for gestational age and excretory renal function. Furthermore, maternal plasma properdin concentration appears to provide excellent diagnostic accuracy in distinguishing cases of

PE from healthy pregnancy (AUROC 0.87 (SE 0.03), with properdin concentration < 5764 ng/ml suggestive of a PE diagnosis with sensitivity and specificity of 81%; reported in ***section 3.2.2*** and ***Figure 3.3***). Importantly, placental C4d deposition was negatively correlated with maternal properdin and C4, indicating that those women with the greatest complement consumption within the maternal circulation also had concurrent increased complement deposition within placental tissue.

This study also provided a novel review of complement biomarkers within the fetal circulation in PE and healthy pregnancy. Ba was the only complement component to significantly differ between PE and controls, but this may represent diffusion from the maternal circulation rather than fetal complement activation per se. This theory is reinforced by none of the fetal complement markers correlating with placental complement deposition, and all biomarkers except split products iC3b and Ba being lower in the fetal circulation than the maternal circulation.

Whilst this study cannot prove direct cause and effect, the results do provide new mechanistic insights into the role of complement in the pathogenesis of PE. The presence of significantly deranged circulating complement biomarkers with concurrent significant complement deposition support a role for complement activation being involved in the pathogenesis of PE rather than simply being present as a result of systemic inflammation. The proposed sequence of events using the new data from this thesis is that properdin binds to activated complement in placental tissue, resulting in depleted plasma concentrations. Classical pathway activation predominantly drives C4d deposition within the placenta (triggered by ischaemia-reperfusion injury) (31, 132), and the amplification loop exacerbates the cycle of activation via the alternative pathway.

Animal and in vitro laboratory studies have demonstrated links between aberrant complement activation and disordered angiogenesis, implicating them as crucial mechanisms of PE pathogenesis. Complement activation can stimulate the release and upregulation of antiangiogenic sFlt-1 (15, 137), leading to the formation of a poorly vascularised placenta, ischaemia-reperfusion injury, further

complement activation, and maternal endothelial dysfunction (133, 163). Conversely, complement blockade can reduce sFlt-1 production (15, 129), restoring placental perfusion (131), and rescuing pregnancy in mice (15, 112, 129).

In conclusion, this study, for the first time, demonstrates evidence of excessive placental complement deposition associated with significant concurrent changes in maternal and fetal circulating complement biomarkers in PE. Placental complement deposition is strongly correlated with complement activation within the maternal circulation, suggesting that those patients with the most excessive changes in circulating markers of complement activation also have the greatest extent of placental complement-mediated damage. Inhibition of complement activation might be a viable treatment option for women with PE, allowing improved pregnancy outcomes through blocking placental tissue injury and the endothelial dysfunction that results.

6.4.2 There is no evidence of excessive systemic complement activation in women with superimposed pre-eclampsia secondary to chronic kidney disease

Women with CKD have disproportionately high rates of SPE during pregnancy (180, 190). There is very little published literature on the role of complement in SPE in women with CKD. This study, for the first time, examined complement biomarkers in the maternal circulation in early, mid, and late pregnancy in a cohort of pregnant women with CKD; a proportion of whom developed SPE.

The data from this study did not reveal any significant differences in maternal circulating complement biomarkers between SPE and non-SPE pregnancies. Therefore, this study did *not* confirm any evidence for excessive complement activation being responsible for the high rates of SPE seen among women with renal disease.

Several confounding factors may have contributed to the lack of difference found between SPE and non-SPE groups. Impaired renal function may affect the excretion of complement components,

causing misleadingly raised biomarker concentrations in both groups. Secondly, the diagnosis of SPE in women with CKD is complicated by hypertension and proteinuria characterising both conditions, and may have led to some patients being misclassified. Misclassification of the SPE phenotype could be reduced in future studies by avoiding subjective measures, such as expert clinical review, and using objective diagnostic adjuncts instead, such as angiogenic marker analysis (sFlt-1 and PlGF). The caveat to this, however, is that angiogenic markers are less reliable in patients with more severe excretory renal impairment (192). Placental histology could also be examined to retrospectively classify patients according to the presence or absence of placental disease.

An alternative analysis found that maternal Ba concentrations were significantly elevated in women with CKD and an adverse pregnancy outcome, compared to those with no adverse outcomes, in all three trimesters of pregnancy. Third trimester Ba levels remained independently associated with adverse pregnancy outcome after controlling for pre-pregnancy renal function. Although adverse outcome does not equate to a diagnosis of SPE, the results are suggestive of excessive alternative pathway activation and raise the possibility of using Ba as a biomarker for 'at-risk' pregnancies. It is possible however that differences in renal function at the time of blood draw could have confounded the results, given that the adverse outcome group had significantly poorer pre-pregnancy renal function than the non-adverse outcome group.

Overall, the data from this study do not provide conclusive evidence of complement being a primary driving factor in the high rates of SPE seen in women with CKD. Impaired excretory renal function and the use of subjective measures in the diagnosis of SPE may have confounded the results. Irrespective of the potential confounding factors, it is possible that a different mechanism of disease predominates. One proposal is the 'sensitive vasculature' theory, whereby pre-existing endothelial dysfunction in women with CKD sensitises the maternal vasculature to circulating antiangiogenic factors, creating a lower threshold to develop angiogenic dysregulation and subsequent SPE, than for women without renal disease (79, 116, 201). Further research with a larger cohort of women

with renal disease is needed to validate the data from this study. The addition of placental tissue analysis would allow further mechanistic links to be made, to determine whether complement-mediated placental tissue injury is a feature of SPE in women with CKD.

6.4.3 There is no evidence of excessive systemic complement activation in women of sub-Saharan African ethnicity with pre-eclampsia

Unduly high rates of PE , and PE with severe features, complicate pregnancies in women of SSA ethnicity (175, 176, 210-212, 262), even after controlling for environmental and socioeconomic factors (174). This has led some commentators to question whether there is an inherent risk of PE conferred by Black ethnicity. There is a scarcity of published evidence on complement biomarker concentrations in SSA populations, either during or outside of pregnancy.

This study, for the first time, examined a broad panel of complement, biochemical and immunological biomarkers in a cohort of Ghanaian pregnant women. There was no evidence to support excessive complement activation in women with PE, or with any hypertensive disorder of pregnancy among women of SSA ethnicity, compared to healthy pregnant controls. A lack of access to formal urine protein quantification, uterine artery Doppler imaging, or angiogenic marker analysis may have led to misclassification of subjects, potentially confounding the results. Furthermore, this study reports on a small population of women of Ghanaian ethnicity and is not representative of SSA as a whole.

There were no patients in this study with HELLP syndrome and few PE patients with very early delivery. It would be interesting to conduct a sub-group analysis of these groups in women of SSA ethnicity, to assess for evidence of excessive complement activation in the setting of severe disease.

Interestingly, concentrations of C5b-9, IgG, and sFLCs were significantly higher than stated laboratory reference ranges in both PE and healthy control pregnancies from the Ghana Cohort. This

may be indicative of an overall state of heightened systemic inflammation in women of SSA ethnicity during pregnancy and might contribute to the risk of adverse pregnancy outcomes. Additional factors, such as high rates of risk-conferring APOL-1, KIR and HLA-C alleles, as well as socioeconomic barriers, may further compound the overall level of risk in women of African ancestry. Overall, however, the results from this study were not sufficient to support a hypothesis of excessive complement activation being a principal driver of excess rates of PE reported in women of SSA ethnicity.

6.4.4 There are different patterns of circulating complement expression in previously healthy women with PE compared to high-risk groups

Analysis of circulating complement in three distinct cohorts of pregnant women identified different patterns of complement biomarker expression in each.

The Birmingham Cohort study provided novel evidence linking abnormal maternal and fetal complement biomarker expression with excessive placental complement deposition. These data support a central role for excessive complement activation in the pathogenesis of PE. This led to a hypothesis of ischaemia-reperfusion injury during placentation resulting in complement deposition and placental tissue injury (predominantly mediated by classical pathway activation), potentiating further circulatory complement activation primarily within the maternal compartment, and mediated by excessive alternative pathway activity.

The evidence for excessive complement pathway activation in the CKD and Ghana cohorts was less clear. Impaired excretory renal function and possible misclassification of SPE in women with CKD may have confounded the results, masking any association between complement and pregnancy outcome in this cohort. Additional contributors in women of SSA ethnicity including genetic factors and a possible baseline heightened state of systemic inflammation may predispose these women to a heightened level of risk. Misclassification of PE may also take place in resource-limited settings

where there is a lack of routine availability to diagnostic adjuncts including urine protein quantification, uterine artery Doppler imaging, and angiogenic marker analysis.

Future studies should employ robust, objective diagnostic criteria for PE, to enable reliable comparisons between groups and to reduce the risk of misclassification of the phenotype. Furthermore, it would be pertinent to separate out early-onset cases of PE (where the disease is thought to be primarily driven by defective placentation from early in pregnancy (77, 98, 116)), from late-onset PE (which is thought to result from maternal factors including endothelial dysfunction (99, 118)), as the pathophysiology may differ considerably. Finally, it would appear to be important to control or match for risk factors including baseline renal function and ethnicity to allow accurate interpretation of results.

Overall, the evidence arising from this thesis supports the theory of PE being a heterogenous condition with different mechanisms of pathogenesis in specific groups but culminating in a common clinical syndrome (77, 86, 116). Inhibition of the complement system may ameliorate placental tissue injury and the resulting maternal endothelial dysfunction for some women with PE, allowing improved pregnancy outcomes for mother and child.

6.5 Therapeutic implications and future work

PE is a common disorder of pregnancy, carrying a significant global burden of disease (81). Surprisingly, there remains no definitive treatment except for iatrogenic delivery. The evidence from Chapter 3 of this thesis strongly supports a role for complement dysregulation in the pathogenesis of PE, and directly links derangement of circulating complement biomarkers with placental complement deposition. The results strengthen the evidence base towards potential future clinical trials of complement inhibiting therapies for use in PE. Inhibition of complement activation might enable improved pregnancy outcomes for women with PE through blocking complement-mediated placental tissue damage and the endothelial dysfunction that results. Those with severe, early-onset disease would potentially stand to gain the most from such treatment, through prolongation of pregnancy enabling improved maternal and fetal outcomes (168).

Eculizumab is a potent C5 inhibitor and already widely used in the treatment of aHUS and PNH, with a demonstrated safety profile in pregnancy (55, 58, 276). There are isolated case reports of its successful use in prolonging pregnancy in women with severe PE, with vital benefits to mother and baby through extending gestation (168-171). One small open-label phase 1 clinical trial investigating the use of eculizumab in HELLP syndrome is currently recruiting participants (277), but another similar trial investigating the use of eculizumab in severe PE has now been suspended (due to the principal investigator moving institutions) (278). There are inherent difficulties in recruiting patients for complement inhibition trials in the pregnant population, including obtaining ethical approval, and concerns about maternal and fetal immunosuppression (61) and potential long-term side effects. As the use of complement inhibition in the treatment of other diseases becomes more commonplace however, confidence may grow among clinicians and patients, leading to improved recruitment rates.

Numerous complement-modifying agents are currently undergoing development for use in other diseases and are in varying stages of clinical trials (164-167). Ravulizumab is a biosimilar drug to

eculizumab but has a longer half-life and therefore a reduced frequency of dosing is required. A phase 2 multicentre, international, randomised controlled trial for its use in patients with IgA nephropathy and lupus nephritis is currently recruiting participants (279). Another promising agent is the C5a receptor antagonist avacopan, which has been shown to be non-inferior to prednisolone in the treatment of ANCA-associated vasculitis (280). Avacopan has recently been approved by the US Food and Drug Administration (FDA) (281), and by NICE in the UK (282) for use as a steroid-sparing agent in the treatment of severe active cases of ANCA-associated vasculitis.

Agents that specifically target the alternative pathway have potential advantages over C5a or C5b-9 blockade by allowing other complement pathways to still function. Iptacopan is a highly-selective Factor B inhibitor and has shown favourable early results in a phase 2 clinical trial in patients with IgA nephropathy (283). Another promising therapeutic target could be properdin inhibition: particularly as significantly reduced properdin concentrations were found in the maternal circulation of previously healthy women with PE in this thesis, with strong associations with placental complement deposition. Interestingly, an anti-properdin antibody is currently in early development (224). In murine studies, properdin blockade has been shown to ameliorate renal ischaemia-reperfusion injury (284, 285). Furthermore, in a mouse model of aHUS, treatment with an anti-properdin mAb lead to significant improvements in thrombotic microangiopathy and overall survival when compared to a control treatment (286). The results from these studies indicate that properdin blockade can benefit diseases mediated by complement activation directed at self surfaces, by relieving or reducing host tissue injury (224). This could be of particular interest in PE, given that placental complement deposition was significantly associated with a reduction in circulating properdin in the research within this thesis. Thus properdin blockade could potentially ameliorate placental tissue injury, and further alternative pathway complement amplification. Furthermore, the benefits of properdin blockade in a mouse model of aHUS raises the question of whether this treatment could also benefit the phenotypically similar conditions of severe PE and HELLP syndrome.

There are some planned areas of future research to further investigate the pathogenesis of PE in high-risk groups. In the CKD arm of research, further analysis of maternal blood samples for properdin, Ba and C5b-9 is planned in a validation cohort of women with CKD from research collaborators in London. The additional numbers tested will strengthen the research conclusions and help to firmly establish whether there is evidence of systemic complement dysregulation in women with CKD and SPE, and in those with an adverse pregnancy outcome.

For the Ghana Cohort, genetic analysis for maternal APOL-1 variants is planned by research collaborators in Accra, Ghana. This will help to determine whether there is an association between maternal APOL-1 risk variants and hypertensive disorders of pregnancy (including PE and HELLP syndrome) within this specific population.

It is hoped that the data arising from this thesis will build the evidence base towards a future UK clinical trial of complement inhibition in the treatment of PE. Collaboration between research groups with an interest in hypertensive disorders of pregnancy would be necessary in order to recruit sufficient participants. A study of this nature should focus upon the general population first (those without CKD or other pre-existing health conditions), to avoid introducing potential confounding factors. Women with severe, early-onset disease could be offered complement inhibition treatment, with the aim of prolonging pregnancy. Maternal and fetal outcomes could be compared with women receiving the current standard of care (expectant management).

6.6 Final conclusions

The research conducted for this thesis has highlighted novel evidence of complement deposition in placental tissue, associated with concurrent changes in maternal and fetal circulating complement biomarkers, in previously healthy women with PE. This provides new mechanistic insights into the pathogenesis of PE, by demonstrating a link between systemic complement dysregulation and complement activation at a tissue level. Inhibition of complement activation might be a viable treatment option for women with PE and allow better pregnancy outcomes through blocking complement-mediated placental tissue damage and the endothelial dysfunction that results.

The results also suggest an association between alternative pathway activation and adverse pregnancy outcomes in women with CKD. Despite this, there was no definitive evidence for excessive complement activation in the maternal circulation of women with CKD and SPE, or in women of SSA ethnicity. The results may have been confounded by a lack of consensus criteria for the diagnosis of SPE in women with CKD, and by a lack of availability of diagnostic adjuncts in the Ghana Cohort, potentially complicating the diagnosis of (S)PE. Despite this, additional factors in specific patient groups – including pre-existing endothelial dysfunction in women with CKD, and genetic risk variants and socioeconomic factors in women of SSA ethnicity – may contribute to the disease pathogenesis.

Future studies should employ robust, objective diagnostic criteria for PE, to avoid misclassification of the phenotype. Further study of complement activation in healthy controls, and in normal pregnancy across a range of gestations is required, in order to better understand complement biomarker profiles in health and in disease states.

Overall, this body of research would support the hypothesis that PE is a heterogenous condition, with different mechanisms of pathogenesis in different patient groups culminating in a common clinical syndrome.

References

1. Walport MJ. Complement. First of two parts. N Engl J Med. 2001;344(14):1058-66.
2. Noris M, Remuzzi G. Overview of complement activation and regulation. Semin Nephrol. 2013;33(6):479-92.
3. Walport MJ. Complement. Second of two parts. N Engl J Med. 2001;344(15):1140-4.
4. Girardi G. Complement activation, a threat to pregnancy. Semin Immunopathol. 2018;40(1):103-11.
5. Medjeral-Thomas NR, Cook HT, Pickering MC. Complement activation in IgA nephropathy. Seminars in Immunopathology. 2021;43(5):679-90.
6. Smith RJH, Appel GB, Blom AM, Cook HT, D'Agati VD, Fakhouri F, et al. C3 glomerulopathy — understanding a rare complement-driven renal disease. Nature Reviews Nephrology. 2019;15(3):129-43.
7. Noris M, Remuzzi G. Atypical hemolytic-uremic syndrome. N Engl J Med. 2009;361:1676-87.
8. Regal JF, Gilbert JS, Burwick RM. The complement system and adverse pregnancy outcomes. Mol Immunol. 2015;67(1):56-70.
9. Regal JF, Burwick RM, Fleming SD. The Complement System and Preeclampsia. Curr Hypertens Rep. 2017;19(11):87.
10. Girardi G, Lingo JJ, Fleming SD, Regal JF. Essential Role of Complement in Pregnancy: From Implantation to Parturition and Beyond. Front Immunol. 2020;11:1681.
11. Pierik E, Prins JR, van Goor H, Dekker GA, Daha MR, Seelen MAJ, et al. Dysregulation of Complement Activation and Placental Dysfunction: A Potential Target to Treat Preeclampsia? Front Immunol. 2019;10:3098.
12. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res. 2004;114(5-6):397-407.
13. Griffiths SK, Campbell JP. Placental structure, function and drug transfer. Continuing Education in Anaesthesia Critical Care & Pain. 2015;15(2):84-9.
14. Jansen CHJR, Kastelein AW, Kleinrouweler CE, Van Leeuwen E, De Jong KH, Pajkrt E, et al. Development of placental abnormalities in location and anatomy. Acta Obstetricia et Gynecologica Scandinavica. 2020;99(8):983-93.
15. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. J Exp Med. 2006;203(9):2165-75.
16. Lynch AM, Gibbs RS, Murphy JR, Giclas PC, Salmon JE, Holers VM. Early elevations of the complement activation fragment C3a and adverse pregnancy outcomes. Obstet Gynecol. 2011;117(1):75-83.
17. Lynch AM, Murphy JR, Byers T, Gibbs RS, Neville MC, Giclas PC, et al. Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia. Am J Obstet Gynecol. 2008;198(4):385 e1-9.
18. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L, et al. Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. PLoS Med. 2011;8(3):e1001013.
19. Girardi G, Bulla R, Salmon JE, Tedesco F. The complement system in the pathophysiology of pregnancy. Mol Immunol. 2006;43(1-2):68-77.
20. Bulla R, Bossi F, Agostinis C, Radillo O, Colombo F, De Seta F, et al. Complement production by trophoblast cells at the feto-maternal interface. J Reprod Immunol. 2009;82(2):119-25.
21. Albieri A, Kipnis T, Bevilacqua E. A possible role for activated complement component 3 in phagocytic activity exhibited by the mouse trophoblast. American Journal of Reproductive Immunology. 1999;41:343-52.

22. Bulla R, Agostinis C, Bossi F, Rizzi L, Debeus A, Tripodo C, et al. Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. Mol Immunol. 2008;45(9):2629-40.
23. Agostinis C, Bulla R, Tripodo C, Gismondi A, Stabile H, Bossi F, et al. An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development. J Immunol. 2010;185(7):4420-9.
24. Singh J, Ahmed A, Girardi G. Role of complement component C1q in the onset of preeclampsia in mice. Hypertension. 2011;58(4):716-24.
25. Lokki AI, Heikkinen-Eloranta J, Jarva H, Saisto T, Lokki ML, Laivuori H, et al. Complement activation and regulation in preeclamptic placenta. Front Immunol. 2014;5:312.
26. Holmes CH, Simpson KL, Okada H, Okada N, Wainwright SD, Purcell DFJ, et al. Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). Eur J Immunol. 1992;22(6):1579-85.
27. Nishikori K, Noma J, Hirakawa S, Amano T, Kudo T. The change of membrane complement regulatory protein in chorion of early-pregnancy. Clinical Immunology and Immunopathology. 1993;69(2):167-74.
28. Faulk WP, Jarret R, Keane M, Johnson PM, Boackle RJ. Immunological studies of human placentae: complement components in immature and mature chorionic villi. Clinical and Experimental Immunology. 1980;40:299-305.
29. Wells M, Bennett J, Bulmer JN, Jackson P, Holgate CS. Complement component deposition in utero-placental (spiral) arteries in normal human pregnancy. J Reprod Immunol. 1987;12:125-35.
30. Tedesco F, Radillo O, Candussi G, Nazzaro A, Mollnes TE, Pecorari D. Immunohistochemical detection of terminal complement complex and S protein in normal and pre-eclamptic placentae. Clinical and Experimental Immunology. 1990;80:236-40.
31. Buurma A, Cohen D, Veraar K, Schonkeren D, Claas FH, Bruijn JA, et al. Preeclampsia is characterized by placental complement dysregulation. Hypertension. 2012;60(5):1332-7.
32. Goldberg M, Luknar-Gabor N, Keidar R, Katz Y. Synthesis of complement proteins in the human chorion is differentially regulated by cytokines. Mol Immunol. 2007;44(7):1737-42.
33. Richani K, Soto E, Romero R, Espinoza J, Chaiworapongsa T, Nien JK, et al. Normal pregnancy is characterized by systemic activation of the complement system. J Matern Fetal Neonatal Med. 2005;17(4):239-45.
34. Derzsy Z, Prohaszka Z, Rigo J, Fust G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. Molecular Immunology. 2010;47(7-8):1500-6.
35. Baines MG, Millar KG, Mills P. Studies of complement levels in normal human pregnancy. Obstetrics and Gynecology. 1974;43(6):806-10.
36. He YD, Xu BN, Song D, Wang YQ, Yu F, Chen Q, et al. Normal range of complement components during pregnancy: A prospective study. Am J Reprod Immunol. 2020;83(2):e13202.
37. Saleh M, Compagno M, Pihl S, Strevens H, Persson B, Wettero J, et al. Variation of Complement Protein Levels in Maternal Plasma and Umbilical Cord Blood during Normal Pregnancy: An Observational Study. J Clin Med. 2022;11(13).
38. Wolach B, Dolfin T, Regev R, Gilboa S, Schlesinger M. The development of the complement system after 28 weeks' gestation. Acta paediatrica (Oslo, Norway : 1992). 1997;86(5):523-7.
39. Grumach AS, Ceccon ME, Rutz R, Fertig A, Kirschfink M. Complement profile in neonates of different gestational ages. Scand J Immunol. 2014;79(4):276-81.
40. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, Molina H. A critical role for murine complement regulator crry in fetomaternal tolerance. Science. 2000;287:498-501.
41. Mao D, Wu X, Deppong C, Friend LD, Dolecki G, Nelson M, et al. Negligible Role of Antibodies and C5 in Pregnancy Loss Associated Exclusively with C3-Dependent Mechanisms through Complement Alternative Pathway. Immunity. 2003;19:813-22.

42. Kimura Y, Zhou L, Miwa T, Song W-C. Genetic and therapeutic targeting of properdin in mice prevents complement-mediated tissue injury. Journal of Clinical Investigation. 2010;120(10):3545-54.
43. Smyth A, Oliveira GHM, Lahr BD, Bailey KR, Norby SM, Garoyic VD. A Systematic Review and Meta-Analysis of Pregnancy Outcomes in Patients with Systemic Lupus Erythematosus and Lupus Nephritis. Clinical Journal of the American Society of Nephrology. 2010;5(11):2060-8.
44. Andreoli L, Bertsias GK, Agmon-Levin N, Brown S, Cervera R, Costedoat-Chalumeau N, et al. EULAR recommendations for women's health and the management of family planning, assisted reproduction, pregnancy and menopause in patients with systemic lupus erythematosus and/or antiphospholipid syndrome. Ann Rheum Dis. 2017;76(3):476-85.
45. Kim MY, Guerra MM, Kaplowitz E, Laskin CA, Petri M, Branch DW, et al. Complement activation predicts adverse pregnancy outcome in patients with systemic lupus erythematosus and/or antiphospholipid antibodies. Ann Rheum Dis. 2018;77(4):549-55.
46. Scambi C, Ugolini S, Tonello M, Bortolami O, De Franceschi L, Castagna A, et al. Complement activation in the plasma and placentas of women with different subsets of antiphospholipid syndrome. Am J Reprod Immunol. 2019:e13185.
47. Bramham K, Soh MC, Nelson-Piercy C. Pregnancy and renal outcomes in lupus nephritis: an update and guide to management. Lupus. 2012;21:1271-83.
48. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. Journal of Clinical Investigation. 2003;112(11):1644-54.
49. Minamiguchi S, Mikami Y, Nakajima N, Salah A, Kondoh E, Tatsumi K, et al. Complement split product C4d deposition in placenta in systemic lupus erythematosus and pregnancy-induced hypertension. Pathol Int. 2013;63(3):150-7.
50. Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. Am J Obstet Gynecol. 2007;196(2):167.e1.
51. Bais J, Pel M, von dem Borne A, van der Leile H. Pregancy and paroxysmal nocturnal hemoglobinuria. Eur J Obstet Gynecol Reprod Biol. 1994;53:211-4.
52. Fieni S, Bonfanti L, Gramellini D, Benassi L, Delsignore R. Clinical Management of Paroxysmal Nocturnal Hemoglobinuria in Pregnancy: A Case Report and Updated Review. Obstetrical and Gynecological Survey. 2006;61(9):593-601.
53. Hillmen P, Young NS, Schubert J, Brodsky RA, Socie G, Muus P, et al. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. N Engl J Med. 2006;355:1233-43.
54. Kelly RJ, Hill A, Arnold LM, Brooksbank GL, Richards SJ, Cullen M, et al. Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival. Blood. 2011;117(25):6786-92.
55. Kelly RJ, Hochsmann B, Szer J, Kulasekararaj A, de Guibert S, Roth A, et al. Eculizumab in Pregnant Patients with Paroxysmal Nocturnal Hemoglobinuria. N Engl J Med. 2015;373(11):1032-9.
56. Parikh SR, Borrow R, Ramsay ME, Ladhani SN. Lower risk of invasive meningococcal disease during pregnancy: national prospective surveillance in England, 2011-2014. Bjog. 2019;126(8):1052-7.
57. Fakhouri F, Roumenina L, Provot F, Sallee M, Caillard S, Couzi L, et al. Pregnancy-associated hemolytic uremic syndrome revisited in the era of complement gene mutations. J Am Soc Nephrol. 2010;21(5):859-67.
58. Gupta M, Govindappagari S, Burwick RM. Pregnancy-Associated Atypical Hemolytic Uremic Syndrome: A Systematic Review. Obstet Gynecol. 2020;135(1):46-58.
59. Palomo M, Blasco M, Molina P, Lozano M, Praga M, Torramade-Moix S, et al. Complement Activation and Thrombotic Microangiopathies. Clin J Am Soc Nephrol. 2019;14(12):1719-32.
60. Bruel A, Kavanagh D, Noris M, Delmas Y, Wong EKS, Bresin E, et al. Hemolytic Uremic Syndrome in Pregnancy and Postpartum. Clin J Am Soc Nephrol. 2017;12(8):1237-47.

61. Duineveld C, Wijnsma KL, Volokhina EB, van den Heuvel LPB, van de Kar N, Wetzels JFM. Placental passage of eculizumab and complement blockade in a newborn. Kidney Int. 2019;95(4):996.
62. Tichenor JR, Bledsoe LB, Opsahl MS, Cunningham DS. Activation of complement in humans with a first-trimester pregnancy loss. Gynecol Obstet Invest. 1995;39(2):79-82.
63. Banadakoppa M, Chauhan MS, Havemann D, Balakrishnan M, Dominic JS, Yallampalli C. Spontaneous abortion is associated with elevated systemic C5a and reduced mRNA of complement inhibitory proteins in placenta. Clin Exp Immunol. 2014;177(3):743-9.
64. Mohlin FC, Mercier E, Fremeaux-Bacchi V, Liszewski MK, Atkinson JP, Gris JC, et al. Analysis of genes coding for CD46, CD55, and C4b-binding protein in patients with idiopathic, recurrent, spontaneous pregnancy loss. Eur J Immunol. 2013;43(6):1617-29.
65. Abdi-Shayan S, Monfaredan A, Moradi Z, Oskouni MR, Kazemi T. Association of CD46 IVS1-1724 C>G Single Nucleotide Polymorphism in Iranian Women with Unexplained Recurrent Spontaneous Abortion (URSA). Iran J Allergy Asthma Immunol. 2016;15(4):303-8.
66. Lynch AM, Gibbs RS, Murphy JR, Byers T, Neville MC, Giclas PC, et al. Complement activation fragment Bb in early pregnancy and spontaneous preterm birth. Am J Obstet Gynecol. 2008;199(4):354 e1-8.
67. Lynch AM, Wagner BD, Deterding RR, Giclas PC, Gibbs RS, Janoff EN, et al. The relationship of circulating proteins in early pregnancy with preterm birth. Am J Obstet Gynecol. 2016;214(4):517 e1-e8.
68. Vaisbuch E, Romero R, Erez O, Mazaki-Tovi S, Kusanovic JP, Soto E, et al. Activation of the alternative pathway of complement is a feature of pre-term parturition but not of spontaneous labor at term. Am J Reprod Immunol. 2010;63(4):318-30.
69. Abalos E, Duley L, Steyn DW. Antihypertensive drug therapy for mild to moderate hypertension during pregnancy. Cochrane Database Syst Rev. 2014(2):CD002252.
70. Webster K, Fishburn S, Maresh M, Findlay SC, Chappell LC. Diagnosis and management of hypertension in pregnancy: summary of updated NICE guidance. BMJ : British Medical Journal (Online). 2019;366.
71. National Institute for Health and Care Excellence (NICE). Hypertension in pregnancy: diagnosis and management. London: National Institute for Health and Care Excellence (NICE); 2019 [NICE guideline (NG133)].
72. Magee LA, Brown MA, Hall DR, Gupte S, Hennessy A, Karumanchi SA, et al. The 2021 International Society for the Study of Hypertension in Pregnancy classification, diagnosis & management recommendations for international practice. Pregnancy Hypertension. 2022;27:148-69.
73. Bramham K, Parnell B, Nelson-Piercy C, Seed PT, Poston L, Chappell LC. Chronic hypertension and pregnancy outcomes: systematic review and meta-analysis. BMJ. 2014;348:g2301.
74. Shennan AH, Green M, Chappell LC. Maternal deaths in the UK: pre-eclampsia deaths are avoidable. Lancet. 2017;389(10069):582-4.
75. Knight M, Bunch K, Tuffnell D, Sheakespeare J, Kotnis R, Kenyon S, et al. Saving Lives, Improving Mothers' Care - Lessons learned to inform maternity care from the UK and Ireland Confidential Enquiries into Maternal Deaths and Morbidity 2015-17. Oxford: National Perinatal Epidemiology Unit, University of Oxford; 2019.
76. Hazelgrove JF, Price C, Pappachan VJ, Smith GB. Multicenter study of obstetric admissions to 14 intensive care units in southern England. Critical Care Medicine. 2001;29(4):770-5.
77. Chaiworapongsa T, Chaemsaithong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. Nat Rev Nephrol. 2014;10(8):466-80.
78. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. Obstetrics & Gynecology. 2020;135(6):e237-e60.
79. Wiles K, Chappell LC, Lightstone L, Bramham K. Updates in Diagnosis and Management of Preeclampsia in Women with CKD. Clin J Am Soc Nephrol. 2020;15(9):1371-80.

80. Burwick RM, Feinberg BB. Complement activation and regulation in preeclampsia and hemolysis, elevated liver enzymes, and low platelet count syndrome. Am J Obstet Gynecol. 2022;226(2S):S1059-S70.
81. Wang W, Xie X, Yuan T, Wang Y, Zhao F, Zhou Z, et al. Epidemiological trends of maternal hypertensive disorders of pregnancy at the global, regional, and national levels: a population-based study. BMC Pregnancy and Childbirth. 2021;21(1):364.
82. Abalos E, Cuesta C, Grosso AL, Chou D, Say L. Global and regional estimates of preeclampsia and eclampsia: a systematic review. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2013;170(1):1-7.
83. World Health Organisation (WHO). World health statistics 2015. Geneva: World Health Organisation (WHO); 2015 [
84. World Health Organisation (WHO). Trends in maternal mortality 2000 to 2017: estimates by WHO, UNICEF, UNFPA, World Bank Group and the United Nations Population Division. Geneva: World Health Organisation (WHO); 2019. [
85. Say L, Chou D, Gemmill A, Tunçalp Ö, Moller A-B, Daniels J, et al. Global causes of maternal death: a WHO systematic analysis. The Lancet Global Health. 2014;2(6):e323-e33.
86. Phipps EA, Thadhani R, Benzing T, Karumanchi SA. Pre-eclampsia: pathogenesis, novel diagnostics and therapies. Nat Rev Nephrol. 2019;15(5):275-89.
87. Brown MC, Best KE, Pearce MS, Waugh J, Robson SC, Bell R. Cardiovascular disease risk in women with pre-eclampsia: systematic review and meta-analysis. European Journal of Epidemiology. 2013;28(1):1-19.
88. Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. Bmj. 2007;335(7627):974.
89. Kristensen JH, Basit S, Wohlfahrt J, Damholt MB, Boyd HA. Pre-eclampsia and risk of later kidney disease: nationwide cohort study. BMJ. 2019;365:l1516.
90. Barrett PM, McCarthy FP, Kublickiene K, Cormican S, Judge C, Evans M, et al. Adverse Pregnancy Outcomes and Long-term Maternal Kidney Disease: A Systematic Review and Meta-analysis. JAMA Netw Open. 2020;3(2):e1920964.
91. McDonald SD, Han Z, Walsh MW, Gerstein HC, Devereaux PJ. Kidney disease after preeclampsia: a systematic review and meta-analysis. Am J Kidney Dis. 2010;55(6):1026-39.
92. Vikse BE, Irgens LM, Leivestad T, Skjaerven R, Iversen BM. Preeclampsia and the risk of end-stage renal disease. N Engl J Med. 2008;359(8):800-9.
93. Khashan AS, Evans M, Kublickas M, McCarthy FP, Kenny LC, Stenvinkel P, et al. Preeclampsia and risk of end stage kidney disease: A Swedish nationwide cohort study. PLoS Med. 2019;16(7):e1002875.
94. Wise J. Two new blood tests will help doctors rule out pre-eclampsia, says NICE. Bmj-British Medical Journal. 2016;353.
95. National Institute for Health and Care Excellence (NICE). Hypertension in pregnancy: diagnosis and management London: National Institute for Health and Care Excellence (NICE); 2011 [Clinical guideline (CG107)]. Available from: https://www.nice.org.uk/guidance/cg107/resources/hypertension-in-pregnancy-diagnosis-and-management-pdf-35109334011877.
96. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. The Lancet. 2008;371(9608):261-9.
97. Redman CW. Current topic: pre-eclampsia and the placenta. Placenta. 1991;12:301-8.
98. Redman CW, Sargent IL. Latest advances in understanding pre-eclampsia. Science. 2005;308(5728):1592-4.
99. Redman CWG, Sacks GP, Sargent IL. Preeclampsia: An excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol. 1999;180(2):499-506.
100. Deer E, Herrock O, Campbell N, Cornelius D, Fitzgerald S, Amaral LM, et al. The role of immune cells and mediators in preeclampsia. Nature Reviews Nephrology. 2023;19(4):257-70.

101. Cavalli RC, Cerdeira AS, Pernicone E, Korkes HA, Burke SD, Rajakumar A, et al. Induced Human Decidual NK-Like Cells Improve Utero-Placental Perfusion in Mice. PLoS One. 2016;11(10):e0164353.
102. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, et al. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. Journal of Clinical Investigation. 2010;120(11):4102-10.
103. Chazara O, Xiong S, Moffett A. Maternal KIR and fetal HLA-C: a fine balance. J Leukoc Biol. 2011;90(4):703-16.
104. Redman CW, Sargent IL. Immunology of pre-eclampsia. Am J Reprod Immunol. 2010;63(6):534-43.
105. Dekker G, Robillard PY, Roberts C. The etiology of preeclampsia: the role of the father. J Reprod Immunol. 2011;89(2):126-32.
106. Saito S, Sakai S. Th1/Th2 balance in preeclampsia. J Reprod Immunol. 2003;59:161-73.
107. Redman CW, Sargent IL, Staff AC. IFPA Senior Award Lecture: Making sense of pre-eclampsia – Two placental causes of preeclampsia? Placenta. 2014;35:S20-S5.
108. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. New England Journal of Medicine. 2004;350(7):672-83.
109. Maynard SE, Jiang-Yong M, Merchan J, Lim K-H, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. Journal of Clinical Investigation. 2003;111(5):649-58.
110. Zeisler H, Llurba E, Chantraine F, Vatish M, Staff AC, Sennstrom M, et al. Predictive Value of the sFlt-1:PlGF Ratio in Women with Suspected Preeclampsia. N Engl J Med. 2016;374(1):13-22.
111. Duhig KE, Myers J, Seed PT, Sparkes J, Lowe J, Hunter RM, et al. Placental growth factor testing to assess women with suspected pre-eclampsia: a multicentre, pragmatic, stepped-wedge cluster-randomised controlled trial. The Lancet. 2019;393(10183):1807-18.
112. Gelber SE, Brent E, Redecha P, Perino G, Tomlinson S, Davisson RL, et al. Prevention of Defective Placentation and Pregnancy Loss by Blocking Innate Immune Pathways in a Syngeneic Model of Placental Insufficiency. J Immunol. 2015;195(3):1129-38.
113. Ahmed A, Singh J, Khan Y, Seshan SV, Girardi G. A new mouse model to explore therapies for preeclampsia. PLoS One. 2010;5(10):e13663.
114. Sones JL, Merriam AA, Seffens A, Brown-Grant DA, Butler SD, Zhao AM, et al. Angiogenic factor imbalance precedes complement deposition in placentae of the BPH/5 model of preeclampsia. FASEB J. 2018;32(5):2574-86.
115. Lynch AM, Murphy JR, Gibbs RS, Levine RJ, Giclas PC, Salmon JE, et al. The interrelationship of complement-activation fragments and angiogenesis-related factors in early pregnancy and their association with pre-eclampsia. BJOG: An International Journal of Obstetrics & Gynaecology. 2010;117(4):456-62.
116. Ness RB, Roberts JM. Heterogeneous causes constituting the single syndrome of preeclampsia: A hypothesis and its implications. Am J Obstet Gynecol. 1996;175:1365-70.
117. Burton GJ, Redman CW, Roberts JM, Moffett A. Pre-eclampsia: pathophysiology and clinical implications. BMJ. 2019;366:l2381.
118. Roberts JM, Catov JM. Preeclampsia more than 1 disease: or is it? Hypertension. 2008;51(4):989-90.
119. Staff AC. The two-stage placental model of preeclampsia: An update. J Reprod Immunol. 2019;134-135:1-10.
120. Redman CW. Early and late onset preeclampsia: Two sides of the same coin. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health. 2017;7:58.
121. Gray KJ, Saxena R, Karumanchi SA. Genetic predisposition to preeclampsia is conferred by fetal DNA variants near FLT1, a gene involved in the regulation of angiogenesis. Am J Obstet Gynecol. 2018;218(2):211-8.

122. McGinnis R, Steinthorsdottir V, Williams NO, Thorleifsson G, Shooter S, Hjartardottir S, et al. Variants in the fetal genome near FLT1 are associated with risk of preeclampsia. Nat Genet. 2017;49(8):1255-60.
123. Reidy KJ, Hjorten RC, Simpson CL, Rosenberg AZ, Rosenblum SD, Kovesdy CP, et al. Fetal-Not Maternal-APOL1 Genotype Associated with Risk for Preeclampsia in Those with African Ancestry. Am J Hum Genet. 2018;103(3):367-76.
124. Miller AK, Azhibekov T, O'Toole JF, Sedor JR, Williams SM, Redline RW, et al. Association of preeclampsia with infant APOL1 genotype in African Americans. BMC Med Genet. 2020;21(1):110.
125. Chappell LC, Brocklehurst P, Green ME, Hunter R, Hardy P, Juszczak E, et al. Planned early delivery or expectant management for late preterm pre-eclampsia (PHOENIX): a randomised controlled trial. The Lancet. 2019;394(10204):1181-90.
126. Roberge S, Nicolaides K, Demers S, Hyett J, Chaillet N, Bujold E. The role of aspirin dose on the prevention of preeclampsia and fetal growth restriction: systematic review and meta-analysis. Am J Obstet Gynecol. 2017;216(2):110-20 e6.
127. Rolnik DL, Wright D, Poon LC, O'Gorman N, Syngelaki A, de Paco Matallana C, et al. Aspirin versus Placebo in Pregnancies at High Risk for Preterm Preeclampsia. N Engl J Med. 2017;377(7):613-22.
128. Chaiworapongsa T, Chaemsaithong P, Korzeniewski SJ, Yeo L, Romero R. Pre-eclampsia part 2: prediction, prevention and management. Nature Reviews Nephrology. 2014;10(9):531-40.
129. Qing X, Redecha PB, Burmeister MA, Tomlinson S, D'Agati VD, Davisson RL, et al. Targeted inhibition of complement activation prevents features of preeclampsia in mice. Kidney Int. 2011;79(3):331-9.
130. Petitbarat M, Durigutto P, Macor P, Bulla R, Palmioli A, Bernardi A, et al. Critical Role and Therapeutic Control of the Lectin Pathway of Complement Activation in an Abortion-Prone Mouse Mating. J Immunol. 2015;195(12):5602-7.
131. Lillegard KE, Johnson AC, Lojovich SJ, Bauer AJ, Marsh HC, Gilbert JS, et al. Complement activation is critical for placental ischemia-induced hypertension in the rat. Mol Immunol. 2013;56(1-2):91-7.
132. Kim EN, Yoon BH, Lee JY, Hwang D, Kim KC, Lee J, et al. Placental C4d deposition is a feature of defective placentation: observations in cases of preeclampsia and miscarriage. Virchows Arch. 2015;466(6):717-25.
133. Yonekura Collier AR, Zsengeller Z, Pernicone E, Salahuddin S, Khankin EV, Karumanchi SA. Placental sFLT1 is associated with complement activation and syncytiotrophoblast damage in preeclampsia. Hypertens Pregnancy. 2019:1-7.
134. Bos M, Buurma A, Veraar K, Nikkels P, Cohen D, Bloemenkamp K, et al. Placental complement activation and loss of placental thrombomodulin are associated with clinical characteristics in preeclampsia. Placenta. 2017;57.
135. Sinha D, Wells M, Faulk WP. Immunological studies of human placentae: complement components in pre-eclamptic chorionic villi. Clin Exp Immunol. 1984(56):175-84.
136. Wang W, Irani RA, Zhang Y, Ramin SM, Blackwell SC, Tao L, et al. Autoantibody-mediated complement C3a receptor activation contributes to the pathogenesis of preeclampsia. Hypertension. 2012;60(3):712-21.
137. Banadakoppa M, Balakrishnan M, Yallampalli C. Upregulation and release of soluble fms-like tyrosine kinase receptor 1 mediated by complement activation in human syncytiotrophoblast cells. Am J Reprod Immunol. 2018;80(5):e13033.
138. He YD, Xu BN, Wang ML, Wang YQ, Yu F, Chen Q, et al. Dysregulation of complement system during pregnancy in patients with preeclampsia: A prospective study. Mol Immunol. 2020;122:69-79.
139. Hoffman MC, Rumer KK, Kramer A, Lynch AM, Winn VD. Maternal and fetal alternative complement pathway activation in early severe preeclampsia. Am J Reprod Immunol. 2014;71(1):55-60.

140. Jia K, Ma L, Wu S, Yang W. Serum Levels of Complement Factors C1q, Bb, and H in Normal Pregnancy and Severe Pre-Eclampsia. Med Sci Monit. 2019;25:7087-93.
141. Velickovic I, Dalloul M, Wong KA, Bakare O, Schweis F, Garala M, et al. Complement factor B activation in patients with preeclampsia. J Reprod Immunol. 2015;109:94-100.
142. Sarween N, Drayson MT, Hodson J, Knox EM, Plant T, Day CJ, et al. Humoral immunity in late-onset Pre-eclampsia and linkage with angiogenic and inflammatory markers. Am J Reprod Immunol. 2018:e13041.
143. Kestlerová A, Feyereisl J, Frisová V, Měchurová A, Šůla K, Zima T, et al. Immunological and biochemical markers in preeclampsia. Journal of Reproductive Immunology. 2012;96(1):90-4.
144. Than NG, Romero R, Erez O, Kusanovic JP, Tarca AL, Edwin SS, et al. A role for mannose-binding lectin, a component of the innate immune system in pre-eclampsia. Am J Reprod Immunol. 2008;60(4):333-45.
145. Csuka D, Molvarec A, Derzsy Z, Varga L, Fust G, Rigo J, Jr., et al. Functional analysis of the mannose-binding lectin complement pathway in normal pregnancy and preeclampsia. J Reprod Immunol. 2010;87(1-2):90-6.
146. Larsen JB, Andersen AS, Hvas CL, Thiel S, Lassen MR, Hvas A-M, et al. Lectin pathway proteins of the complement system in normotensive pregnancy and pre-eclampsia. American Journal of Reproductive Immunology. 2019;81(4):e13092.
147. Boij R, Svensson J, Nilsson-Ekdahl K, Sandholm K, Lindahl TL, Palonek E, et al. Biomarkers of coagulation, inflammation, and angiogenesis are independently associated with preeclampsia. Am J Reprod Immunol. 2012;68(3):258-70.
148. He Y, Xu B, Song D, Yu F, Chen Q, Zhao M. Expression of the complement system's activation factors in plasma of patients with early/late-onset severe pre-eclampsia. Am J Reprod Immunol. 2016;76(3):205-11.
149. Denny KJ, Coulthard LG, Finnell RH, Callaway LK, Taylor SM, Woodruff TM. Elevated complement factor C5a in maternal and umbilical cord plasma in preeclampsia. J Reprod Immunol. 2013;97(2):211-6.
150. Burwick RM, Velasquez JA, Valencia CM, Gutierrez-Marin J, Edna-Estrada F, Silva JL, et al. Terminal Complement Activation in Preeclampsia. Obstet Gynecol. 2018;132(6):1477-85.
151. Penning M, Chua JS, van Kooten C, Zandbergen M, Buurma A, Schutte J, et al. Classical Complement Pathway Activation in the Kidneys of Women With Preeclampsia. Hypertension. 2015;66(1):117-25.
152. Burwick RM, Fichorova RN, Dawood HY, Yamamoto HS, Feinberg BB. Urinary Excretion of C5b-9 in Severe Preeclampsia Tipping the Balance of Complement Activation in Pregnancy. Hypertension. 2013;62(6):1040-5.
153. Guseh SH, Feinberg BB, Dawood HY, Yamamoto HS, Fichorova RN, Burwick RM. Urinary excretion of C5b-9 is associated with the anti-angiogenic state in severe preeclampsia. Am J Reprod Immunol. 2015;73(5):437-44.
154. Valencia CM, Hersh AR, Burwick RM, Velasquez JA, Gutierrez-Marin J, Edna F, et al. Soluble concentrations of the terminal complement complex C5b-9 correlate with end-organ injury in preeclampsia. Pregnancy Hypertens. 2022;29:92-7.
155. Lokki AI, Aalto-Viljakainen T, Meri S, Laivuori H. Genetic analysis of membrane cofactor protein (CD46) of the complement system in women with and without preeclamptic pregnancies. PLoS One. 2015;10(2):e0117840.
156. Banadakoppa M, Balakrishnan M, Yallampalli C. Common variants of fetal and maternal complement genes in preeclampsia: pregnancy specific complotype. Sci Rep. 2020;10(1):4811.
157. Wu W, Yang H, Feng Y, Zhang P, Li S, Wang X, et al. Polymorphisms in complement genes and risk of preeclampsia in Taiyuan, China. Inflammation Research. 2016;65(10):837-45.
158. Lokki AI, Kaartokallio T, Holmberg V, Onkamo P, Koskinen LLE, Saavalainen P, et al. Analysis of Complement C3 Gene Reveals Susceptibility to Severe Preeclampsia. Frontiers in Immunology. 2017;8.

159. Vaught AJ, Braunstein EM, Jasem J, Yuan X, Makhlin I, Eloundou S, et al. Germline mutations in the alternative pathway of complement predispose to HELLP syndrome. JCI Insight. 2018;3(6).
160. Lokki AI, Teirilä L, Triebwasser M, Daly E, Bhattacharjee A, Uotila L, et al. Dysfunction of complement receptors CR3 (CD11b/18) and CR4 (CD11c/18) in pre-eclampsia: a genetic and functional study. Bjog. 2021;128(8):1282-91.
161. Burwick R. Complement receptors in pre-eclampsia: cleaning up placental debris. BJOG: An International Journal of Obstetrics & Gynaecology. 2021;128(8):1292-.
162. Lokki AI, Ren Z, Triebwasser M, Daly E, FINNPEC, Perola M, et al. Identification of complement factor H variants that predispose to pre-eclampsia: A genetic and functional study. BJOG: An International Journal of Obstetrics & Gynaecology. 2023;130(12):1473-82.
163. Lynch AM, Salmon JE. Dysregulated complement activation as a common pathway of injury in preeclampsia and other pregnancy complications. Placenta. 2010;31(7):561-7.
164. Harris CL, Pouw RB, Kavanagh D, Sun R, Ricklin D. Developments in anti-complement therapy; from disease to clinical trial. Mol Immunol. 2018;102:89-119.
165. Ricklin D, Mastellos DC, Reis ES, Lambris JD. The renaissance of complement therapeutics. Nat Rev Nephrol. 2018;14(1):26-47.
166. Zipfel PF, Wiech T, Rudnick R, Afonso S, Person F, Skerka C. Complement Inhibitors in Clinical Trials for Glomerular Diseases. Front Immunol. 2019;10:2166.
167. Zelek WM, Xie L, Morgan BP, Harris CL. Compendium of current complement therapeutics. Mol Immunol. 2019;114:341-52.
168. Burwick RM, Feinberg BB. Eculizumab for the treatment of preeclampsia/HELLP syndrome. Placenta. 2013;34(2):201-3.
169. Lu AB, Lazarus B, Rolnik DL, Palmer KR. Pregnancy Prolongation After Eculizumab Use in Early-Onset Preeclampsia. Obstetrics & Gynecology. 2019;134(6):1215-8.
170. Lokki AI, Haapio M, Heikkinen-Eloranta J. Eculizumab Treatment for Postpartum HELLP Syndrome and aHUS—Case Report. Frontiers in Immunology. 2020;11.
171. Vaught AJ, Gavriilaki E, Hueppchen N, Blakemore K, Yuan X, Seifert SM, et al. Direct evidence of complement activation in HELLP syndrome: A link to atypical hemolytic uremic syndrome. Exp Hematol. 2016;44(5):390-8.
172. Stefanovic V. The Extended Use of Eculizumab in Pregnancy and Complement Activation–Associated Diseases Affecting Maternal, Fetal and Neonatal Kidneys—The Future Is Now? Journal of Clinical Medicine. 2019;8(3):407.
173. Zhang JJ, Ma XX, Hao L, Liu LJ, Lv JC, Zhang H. A Systematic Review and Meta-Analysis of Outcomes of Pregnancy in CKD and CKD Outcomes in Pregnancy. Clin J Am Soc Nephrol. 2015;10(11):1964-78.
174. Tanaka M, Jaamaa G, Kaiser M, Hills E, Soim A, Zhu M, et al. Racial disparity in hypertensive disorders of pregnancy in New York State: a 10-year longitudinal population-based study. Am J Public Health. 2007;97(1):163-70.
175. Nakimuli A, Chazara O, Byamugisha J, Elliott AM, Kaleebu P, Mirembe F, et al. Pregnancy, parturition and preeclampsia in women of African ancestry. Am J Obstet Gynecol. 2014;210(6):510-20 e1.
176. Anselem O, Girard G, Stepanian A, Azria E, Mandelbrot L. Influence of ethnicity on the clinical and biologic expression of pre-eclampsia in the ECLAXIR study. International Journal of Gynecology & Obstetrics. 2011;115(2):153-6.
177. Williams D, Davison J. Chronic kidney disease in pregnancy. British Medical Journal. 2008;336(7637):211-5.
178. Piccoli GB, Zakharova E, Attini R, Ibarra Hernandez M, Orozco Guillien A, Alrukhaimi M, et al. Pregnancy in Chronic Kidney Disease: Need for Higher Awareness. A Pragmatic Review Focused on What Could Be Improved in the Different CKD Stages and Phases. J Clin Med. 2018;7(11).
179. Wiles K, Webster P, Seed PT, Bennett-Richards K, Bramham K, Brunskill N, et al. The impact of chronic kidney disease Stages 3-5 on pregnancy outcomes. Nephrol Dial Transplant. 2020.

180. Wiles KS, Nelson-Piercy C, Bramham K. Reproductive health and pregnancy in women with chronic kidney disease. Nat Rev Nephrol. 2018;14(3):165-84.
181. Piccoli GB, Cabiddu G, Attini R, Vigotti FN, Maxia S, Lepori N, et al. Risk of Adverse Pregnancy Outcomes in Women with CKD. J Am Soc Nephrol. 2015;26(8):2011-22.
182. Piccoli GB, Attini R, Vasario E, Conijn A, Biolcati M, D'Amico F, et al. Pregnancy and Chronic Kidney Disease: A Challenge in All CKD Stages. Clinical Journal of the American Society of Nephrology. 2010;5(5):844-55.
183. Piccoli GB, Cabiddu G, Attini R, Gerbino M, Todeschini P, Perrino ML, et al. Outcomes of Pregnancies After Kidney Transplantation: Lessons Learned From CKD. A Comparison of Transplanted, Nontransplanted Chronic Kidney Disease Patients and Low-Risk Pregnancies: A Multicenter Nationwide Analysis. Transplantation. 2017;101(10):2536-44.
184. Shah S, Venkatesan RL, Gupta A, Sanghavi MK, Welge J, Johansen R, et al. Pregnancy outcomes in women with kidney transplant: Metaanalysis and systematic review. BMC Nephrol. 2019;20(1):24.
185. Lightstone L, Hladunewich M. Lupus nephritis and pregnancy: concerns and management. Semin Nephrol. 2017;37(4):347-53.
186. Wiles KS, Bramham K, Vais A, Harding KR, Chowdhury P, Taylor CJ, et al. Pre-pregnancy counselling for women with chronic kidney disease: a retrospective analysis of nine years' experience. BMC Nephrol. 2015;16:28.
187. Cabiddu G, Castellino S, Gernone G, Santoro D, Moroni G, Giannattasio M, et al. A best practice position statement on pregnancy in chronic kidney disease: the Italian Study Group on Kidney and Pregnancy. J Nephrol. 2016;29(3):277-303.
188. Bramham K, Seed PT, Lightstone L, Nelson-Piercy C, Gill C, Webster P, et al. Diagnostic and predictive biomarkers for pre-eclampsia in patients with established hypertension and chronic kidney disease. Kidney International. 2016;89(4):874-85.
189. Jones DC, Hayslett JP. Outcome of pregnancy in women with moderate or severe renal insufficiency. N Engl J Med. 1996;335:226-32.
190. Bramham K, Briley AL, Seed PT, Poston L, Shennan AH, Chappell LC. Pregnancy Outcome in Women with Chronic Kidney Disease: A Prospective Cohort Study. Reproductive Sciences. 2011;18(7):623-30.
191. Bramham K, Nelson-Piercy C, Gao H, Pierce M, Bush N, Spark P, et al. Pregnancy in renal transplant recipients: a UK national cohort study. Clin J Am Soc Nephrol. 2013;8(2):290-8.
192. Wiles K, Bramham K, Seed PT, Brockbank A, Nelson-Piercy C, Karumanchi SA, et al. Placental and endothelial biomarkers for the prediction of superimposed pre-eclampsia in chronic kidney disease. Pregnancy Hypertens. 2021;24:58-64.
193. Odutayo A, Hladunewich M. Obstetric nephrology: renal hemodynamic and metabolic physiology in normal pregnancy. Clin J Am Soc Nephrol. 2012;7(12):2073-80.
194. Morton A, Burke M, Jarvis E, Kumar S. Changes in proteinuria and diagnosing preeclampsia in CKD pregnancy. Pregnancy Hypertens. 2020;20:92-5.
195. Tomimatsu T, Mimura K, Endo M, Kumasawa K, Kimura T. Pathophysiology of preeclampsia: an angiogenic imbalance and long-lasting systemic vascular dysfunction. Hypertension Research. 2017;40(4):305-10.
196. Roumeliotis S, Mallamaci F, Zoccali C. Endothelial Dysfunction in Chronic Kidney Disease, from Biology to Clinical Outcomes: A 2020 Update. Journal of clinical medicine. 2020;9(8):2359.
197. van der Graaf AM, Toering TJ, Faas MM, Lely AT. From preeclampsia to renal disease: a role of angiogenic factors and the renin-angiotensin aldosterone system? Nephrol Dial Transplant. 2012;27 Suppl 3:iii51-7.
198. Fearn A, Sheerin NS. Complement activation in progressive renal disease. World J Nephrol. 2015;4(1):31-40.

199. Strevens H, Wide-Swensson D, Hansen A, Horn T, Ingemarsson I, Larsen S, et al. Glomerular endotheliosis in normal pregnancy and pre-eclampsia. BJOG: An International Journal of Obstetrics & Gynaecology. 2003;110(9):831-6.
200. Penning ME, Bloemenkamp KWM, van der Zon T, Zandbergen M, Schutte JM, Bruijn JA, et al. Association of Preeclampsia with Podocyte Turnover. Clinical Journal of the American Society of Nephrology. 2014;9(8):1377-85.
201. Tangren JS, Powe CE, Ankers E, Ecker J, Bramham K, Hladunewich MA, et al. Pregnancy Outcomes after Clinical Recovery from AKI. J Am Soc Nephrol. 2017;28(5):1566-74.
202. National Institute for Health and Care Excellence (NICE). PLGF-based testing to help diagnose suspected preterm pre-eclampsia 2022 [Diagnostics guidance [DG49]].
203. Wiles K, Bramham K, Seed PT, Kurlak LO, Mistry HD, Nelson-Piercy C, et al. Diagnostic Indicators of Superimposed Preeclampsia in Women With CKD. Kidney Int Rep. 2019;4(6):842-53.
204. Hounkpatin OI, Amidou SA, Houehanou YC, Lacroix P, Preux PM, Houinato DS, et al. Systematic review of observational studies of the impact of cardiovascular risk factors on preeclampsia in sub-saharan Africa. BMC Pregnancy Childbirth. 2021;21(1):97.
205. von Dadelszen P, Magee LA. Preventing deaths due to the hypertensive disorders of pregnancy. Best Pract Res Clin Obstet Gynaecol. 2016;36:83-102.
206. Maule SP, Ashworth DC, Blakey H, Osafo C, Moturi M, Chappell LC, et al. CKD and Pregnancy Outcomes in Africa: A Narrative Review. Kidney Int Rep. 2020;5(8):1342-9.
207. Sheikh J, Allotey J, Kew T, Fernandez-Felix BM, Zamora J, Khalil A, et al. Effects of race and ethnicity on perinatal outcomes in high-income and upper-middle-income countries: an individual participant data meta-analysis of 2 198 655 pregnancies. Lancet. 2022;400(10368):2049-62.
208. Knight M, Bunch K, Tuffnell D, Patel R, Shakespeare J, Kotnis R, et al. Saving Lives, Improving Mothers' Care: Lessons learned to inform maternity care from the UK and Ireland Confidential Enquiries into Maternal Deaths and Morbidity 2017-19. Oxford: National Perinatal Epidemiology Unit, University of Oxford; 2021.
209. Jardine J, Walker K, Gurol-Urganci I, Webster K, Muller P, Hawdon J, et al. Adverse pregnancy outcomes attributable to socioeconomic and ethnic inequalities in England: a national cohort study. The Lancet. 2021;398(10314):1905-12.
210. Gemechu KS, Assefa N, Mengistie B. Prevalence of hypertensive disorders of pregnancy and pregnancy outcomes in Sub-Saharan Africa: A systematic review and meta-analysis. Womens Health (Lond). 2020;16:1745506520973105.
211. Noubiap JJ, Bigna JJ, Nyaga UF, Jingi AM, Kaze AD, Nansseu JR, et al. The burden of hypertensive disorders of pregnancy in Africa: A systematic review and meta-analysis. J Clin Hypertens (Greenwich). 2019;21(4):479-88.
212. Abalos E, Cuesta C, Carroli G, Qureshi Z, Widmer M, Vogel JP, et al. Pre-eclampsia, eclampsia and adverse maternal and perinatal outcomes: a secondary analysis of the World Health Organization Multicountry Survey on Maternal and Newborn Health. BJOG. 2014;121 Suppl 1:14-24.
213. Fokom-Domgue J, Noubiap JJ. Diagnosis of hypertensive disorders of pregnancy in sub-Saharan Africa: a poorly assessed but increasingly important issue. J Clin Hypertens (Greenwich). 2015;17(1):70-3.
214. Ataklte F, Erqou S, Kaptoge S, Taye B, Echouffo-Tcheugui JB, Kengne AP. Burden of Undiagnosed Hypertension in Sub-Saharan Africa. Hypertension. 2015;65(2):291-8.
215. Boakye E, Kwapong YA, Obisesan O, Ogunwole SM, Hays AG, Nasir K, et al. Nativity-Related Disparities in Preeclampsia and Cardiovascular Disease Risk Among a Racially Diverse Cohort of US Women. JAMA Netw Open. 2021;4(12):e2139564.
216. Nakimuli A, Chazara O, Farrell L, Hiby SE, Tukwasibwe S, Knee O, et al. Killer cell immunoglobulin-like receptor (KIR) genes and their HLA-C ligands in a Ugandan population. Immunogenetics. 2013;65(11):765-75.
217. Norman PJ, Hollenbach JA, Nemat-Gorgani N, Guethlein LA, Hilton HG, Pando MJ, et al. Co-evolution of Human Leukocyte Antigen (HLA) Class I Ligands with Killer-Cell Immunoglobulin-Like

Receptors (KIR) in a Genetically Diverse Population of Sub-Saharan Africans. PLOS Genetics. 2013;9(10):e1003938.
218. Yusuf AA, Govender MA, Brandenburg J-T, Winkler CA. Kidney disease and APOL1. Human Molecular Genetics. 2021;30(R1):R129-R37.
219. Thakoordeen-Reddy S, Winkler C, Moodley J, David V, Binns-Roemer E, Ramsuran V, et al. Maternal variants within the apolipoprotein L1 gene are associated with preeclampsia in a South African cohort of African ancestry. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2020;246:129-33.
220. National Institute for Health and Care Research (NIHR). Good Clinical Practice (GCP) [Available from: https://www.nihr.ac.uk/health-and-care-professionals/learning-and-support/good-clinical-practice.htm.
221. Jia C, Tan Y, Zhao M. The complement system and autoimmune diseases. Chronic Diseases and Translational Medicine. 2022.
222. Chen M, Daha MR, Kallenberg CG. The complement system in systemic autoimmune disease. Journal of autoimmunity. 2010;34(3):J276-86.
223. Duckitt K, Qureshi A. Recurrent miscarriage. BMJ Clin Evid. 2011;2011:1409.
224. Michels M, Volokhina EB, van de Kar N, van den Heuvel L. The role of properdin in complement-mediated renal diseases: a new player in complement-inhibiting therapy? Pediatr Nephrol. 2019;34(8):1349-67.
225. Kolb WP, Morrow PR, Tamerius JD. Ba and Bb fragments of factor B activation: fragment production, biological activities, neoepitope expression and quantitation in clinical samples. Complement Inflamm. 1989;6(3):175-204.
226. Kim AHJ, Strand V, Sen DP, Fu Q, Mathis NL, Schmidt MJ, et al. Association of Blood Concentrations of Complement Split Product iC3b and Serum C3 With Systemic Lupus Erythematosus Disease Activity. Arthritis & rheumatology (Hoboken, NJ). 2019;71(3):420-30.
227. ThermoFisherScientific. User Guide: EZ-Link™Sulfo-NHS-Biotinylation Kit 2012 [Available from: https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0011559_EZ_Sulfo_NHS_Biotinylation_UG.pdf.
228. Roche Diagnostics. Roche Diagnostics eLabDoc 2022 [Available from: https://pim-eservices.roche.com/eLD/web/gb/en/home.
229. Than NG, Abdul Rahman O, Magenheim R, Nagy B, Fule T, Hargitai B, et al. Placental protein 13 (galectin-13) has decreased placental expression but increased shedding and maternal serum concentrations in patients presenting with preterm pre-eclampsia and HELLP syndrome. Virchows Arch. 2008;453(4):387-400.
230. Matrai CE, Rand JH, Baergen RN. Absence of Distinct Immunohistochemical Distribution of Annexin A5, C3b, C4d, and C5b-9 in Placentas From Patients With Antiphospholipid Antibodies, Preeclampsia, and Systemic Lupus Erythematosus. Pediatr Dev Pathol. 2019;22(5):431-9.
231. Perneger TV. What's wrong with Bonferroni adjustments. BMJ. 1998;316(7139):1236-8.
232. Sarween N. Biochemical and clinical factors which are associated with or predictive of pre-eclampsia in healthy women and those with chronic kidney disease. [PhD]: University of Birmingham; 2020.
233. National Institute for Health and Care Excellence (NICE). Chronic kidney disease: assessment and management 2021 [NICE guideline [NG 203]].
234. Hycult Biotech. Properdin human ELISA kit 2023 [Available from: https://www.hycultbiotech.com/product/properdin-human-elisa-kit/.
235. Quidel. MicroVue Complement Ba fragment EIA 2023 [Available from: https://www.quidel.com/research/elisa-kits/microvue-ba-eia-kit.
236. Quidel. MicroVue Complement SC5b-9 Plus EIA 2023 [Available from: https://www.quidel.com/research/elisa-kits/microvue-sc5b-9-plus-eia-kit.

237. Magee LA, Nicolaides KH, von Dadelszen P. Preeclampsia. N Engl J Med. 2022;386(19):1817-32.
238. Blakey H, Sun R, Xie L, Russell R, Sarween N, Hodson J, et al. Pre-eclampsia is associated with complement pathway activation in the maternal and fetal circulation, and placental tissue. Pregnancy Hypertens. 2023;32:43-9.
239. Drüeke TB, Massy ZA. Beta2-microglobulin. Seminars in dialysis. 2009;22(4):378-80.
240. Hutchison CA, Harding S, Hewins P, Mead GP, Townsend J, Bradwell AR, et al. Quantitative assessment of serum and urinary polyclonal free light chains in patients with chronic kidney disease. Clin J Am Soc Nephrol. 2008;3(6):1684-90.
241. Kemper C, Mitchell LM, Zhang L, Hourcade DE. The complement protein properdin binds apoptotic T cells and promotes complement activation and phagocytosis. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(26):9023-8.
242. Spitzer D, Mitchell LM, Atkinson JP, Hourcade DE. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. J Immunol. 2007;179(4):2600-8.
243. Zhang Y, Nester CM, Martin B, Skjoedt MO, Meyer NC, Shao D, et al. Defining the complement biomarker profile of C3 glomerulopathy. Clin J Am Soc Nephrol. 2014;9(11):1876-82.
244. Gou SJ, Yuan J, Chen M, Yu F, Zhao MH. Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis. Kidney Int. 2013;83(1):129-37.
245. Smith-Jackson K, Harrison RA. Alternative pathway activation in pregnancy, a measured amount "complements" a successful pregnancy, too much results in adverse events. Immunol Rev. 2023;313(1):298-319.
246. Haeger M, Unander M, Bengtsson A. Enhanced anaphylatoxin and terminal C5b-9 complement complex formation in patients with the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Obstet Gynecol. 1990;76(4):698-702.
247. Williams JP, Pechet TT, Weiser MR, Reid R, Kobzik L, Moore FD, Jr., et al. Intestinal reperfusion injury is mediated by IgM and complement. Journal of applied physiology (Bethesda, Md : 1985). 1999;86(3):938-42.
248. Rampersad R, Barton A, Sadovsky Y, Nelson DM. The C5b-9 membrane attack complex of complement activation localizes to villous trophoblast injury in vivo and modulates human trophoblast function in vitro. Placenta. 2008;29(10):855-61.
249. Oppermann M, Kurts C, Zierz R, Quentin E, Weber MH, Götze O. Elevated plasma levels of the immunosuppressive complement fragment Ba in renal failure. Kidney Int. 1991;40(5):939-47.
250. Wiles K, Bramham K, Seed PT, Nelson-Piercy C, Lightstone L, Chappell LC. Serum Creatinine in Pregnancy: A Systematic Review. Kidney Int Rep. 2019;4(3):408-19.
251. Sibikova M, Vitkova V, Jamrichova L, Haluzik M, Zivny J, Janota J. Spontaneous delivery is associated with increased endothelial activity in cord blood compared to elective cesarean section. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2020;251:229-34.
252. Ward PA, Murphy HS. Role of Complement in Endothelial Cell Activation. In: Serhan CN, Ward PA, editors. Molecular and Cellular Basis of Inflammation. Totowa, NJ: Humana Press; 1999. p. 1-27.
253. Johnson JD, Louis JM. Does race or ethnicity play a role in the origin, pathophysiology, and outcomes of preeclampsia? An expert review of the literature. Am J Obstet Gynecol. 2022;226(2S):S876-S85.
254. Shahul S, Tung A, Minhaj M, Nizamuddin J, Wenger J, Mahmood E, et al. Racial Disparities in Comorbidities, Complications, and Maternal and Fetal Outcomes in Women With Preeclampsia/eclampsia. Hypertension in Pregnancy. 2015;34(4):506-15.
255. Soto E, Romero R, Kusanovic JP, Ogge G, Hussein Y, Yeo L, et al. Late-onset preeclampsia is associated with an imbalance of angiogenic and anti-angiogenic factors in patients with and without placental lesions consistent with maternal underperfusion. J Matern Fetal Neonatal Med. 2012;25(5):498-507.

256. Onda K, Ohsawa I, Ohi H, Tamano M, Satoshi M, Wakabayashi M, et al. Excretion of complement proteins and its activation marker C5b-9 in IgA nephropathy in relation to renal function. BMC Nephrol. 2011;12:64.
257. Blatt AZ, Pathan S, Ferreira VP. Properdin: a tightly regulated critical inflammatory modulator. Immunol Rev. 2016;274(1):172-90.
258. Piccoli GB. What Is Superimposed Preeclampsia (and Does It Actually Exist)? Kidney Int Rep. 2019;4(6):759-62.
259. Cheung KL, Lafayette RA. Renal physiology of pregnancy. Adv Chronic Kidney Dis. 2013;20(3):209-14.
260. Sedor JR, Bruggeman LA, O'Toole JF. APOL1 and Preeclampsia: Intriguing Links, Uncertain Causality, Troubling Implications. Am J Kidney Dis. 2021;77(6):863-5.
261. Urquia M, Glazier R, Gagnon A, Mortensen L, Nybo Andersen A-M, Janevic T, et al. Disparities in pre-eclampsia and eclampsia among immigrant women giving birth in six industrialised countries. BJOG: An International Journal of Obstetrics & Gynaecology. 2014;121(12):1492-500.
262. Fasanya HO, Hsiao CJ, Armstrong-Sylvester KR, Beal SG. A Critical Review on the Use of Race in Understanding Racial Disparities in Preeclampsia. J Appl Lab Med. 2021;6(1):247-56.
263. Lipkowitz MS, Freedman BI, Langefeld CD, Comeau ME, Bowden DW, Linda Kao WH, et al. Apolipoprotein L1 gene variants associate with hypertension-attributed nephropathy and the rate of kidney function decline in African Americans. Kidney International. 2013;83(1):114-20.
264. Limou S, Nelson GW, Kopp JB, Winkler CA. APOL1 Kidney Risk Alleles: Population Genetics and Disease Associations. Advances in Chronic Kidney Disease. 2014;21(5):426-33.
265. Genovese G, Friedman DJ, Ross MD, Lecordier L, Uzureau P, Freedman BI, et al. Association of Trypanolytic ApoL1 Variants with Kidney Disease in African Americans. Science. 2010;329(5993):841-5.
266. Osafo C, Thomford NE, Coleman J, Carboo A, Guure C, Okyere P, et al. APOL1 genotype associated risk for preeclampsia in African populations: Rationale and protocol design for studies in women of African ancestry in resource limited settings. PLoS One. 2022;17(12):e0278115.
267. Ulasi II, Tzur S, Wasser WG, Shemer R, Kruzel E, Feigin E, et al. High Population Frequencies of APOL1 Risk Variants Are Associated with Increased Prevalence of Non-Diabetic Chronic Kidney Disease in the Igbo People from South-Eastern Nigeria. Nephron Clinical Practice. 2013;123(1-2):123-8.
268. Katzmann JA, Clark RJ, Abraham RS, Bryant S, Lymp JF, Bradwell AR, et al. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. Clinical chemistry. 2002;48(9):1437-44.
269. Tollerud DJ, Brown LM, Blattner WA, Weiss ST, Maloney EM, Kurman CC, et al. Racial differences in serum immunoglobulin levels: relationship to cigarette smoking, T-cell subsets, and soluble interleukin-2 receptors. Journal of clinical laboratory analysis. 1995;9(1):37-41.
270. Bunce CM, Drayson MT. Dissecting racial disparities in multiple myeloma-clues from differential immunoglobulin levels. Blood cancer journal. 2020;10(4):44.
271. Weiss BM, Minter A, Abadie J, Howard R, Ascencao J, Schechter GP, et al. Patterns of monoclonal immunoglobulins and serum free light chains are significantly different in black compared to white monoclonal gammopathy of undetermined significance (MGUS) patients. American journal of hematology. 2011;86(6):475-8.
272. Goff LM, Davies K, Zelek WM, Kodosaki E, Hakim O, Lockhart S, et al. Ethnic differences in complement system biomarkers and their association with metabolic health in men of Black African and White European ethnicity. Clin Exp Immunol. 2023.
273. Roumenina LT, Chadebech P, Bodivit G, Vieira-Martins P, Grunenwald A, Boudhabhay I, et al. Complement activation in sickle cell disease: Dependence on cell density, hemolysis and modulation by hydroxyurea therapy. American journal of hematology. 2020;95(5):456-64.

274. Siezenga MA, Chandie Shaw PK, van der Geest RN, Mollnes TE, Daha MR, Rabelink TJ, et al. Enhanced complement activation is part of the unfavourable cardiovascular risk profile in South Asians. Clin Exp Immunol. 2009;157(1):98-103.
275. Pennington KA, Schlitt JM, Jackson DL, Schulz LC, Schust DJ. Preeclampsia: multiple approaches for a multifactorial disease. Dis Model Mech. 2012;5(1):9-18.
276. Kelly R, Arnold L, Richards S, Hill A, Bomken C, Hanley J, et al. The management of pregnancy in paroxysmal nocturnal haemoglobinuria on long term eculizumab. Br J Haematol. 2010;149(3):446-50.
277. Johns Hopkins University, Eunice Kennedy Shriver National Institute of Child Health, Human Development. The Use of Eculizumab in HELLP Syndrome. 2022.
278. Cedars-Sinai Medical Center, Alexion Pharmaceuticals. Complement Regulation to Undo Systemic Harm in Preeclampsia. 2023.
279. Alexion Pharmaceuticals. Study of Ravulizumab in Proliferative Lupus Nephritis (LN) or Immunoglobulin A Nephropathy (IgAN). 2024.
280. Jayne DRW, Merkel PA, Schall TJ, Bekker P. Avacopan for the Treatment of ANCA-Associated Vasculitis. N Engl J Med. 2021;384(7):599-609.
281. Thorley J. FDA approves avacopan for ANCA-associated vasculitis. The Lancet Rheumatology. 2022;4(1):e21.
282. National Institute for Health and Care Excellence (NICE). Avacopan for treating severe active granulomatosis with polyangiitis or microscopic polyangiitis 2022 [Technology appraisal guidance [TA825]].
283. Barratt J, Rovin B, Zhang H, Kashihara N, Maes B, Rizk D, et al. POS-546 Efficacy and safety of iptacopan in IgA nephropathy: results of a randomized double-blind placebo-controlled phase 2 study at 6 months. Kidney International Reports. 2022;7(2):S236.
284. Miwa T, Sato S, Gullipalli D, Nangaku M, Song WC. Blocking properdin, the alternative pathway, and anaphylatoxin receptors ameliorates renal ischemia-reperfusion injury in decay-accelerating factor and CD59 double-knockout mice. J Immunol. 2013;190(7):3552-9.
285. Miao J, Lesher AM, Miwa T, Sato S, Gullipalli D, Song WC. Tissue-specific deletion of Crry from mouse proximal tubular epithelial cells increases susceptibility to renal ischemia-reperfusion injury. Kidney Int. 2014;86(4):726-37.
286. Ueda Y, Miwa T, Gullipalli D, Sato S, Ito D, Kim H, et al. Blocking Properdin Prevents Complement-Mediated Hemolytic Uremic Syndrome and Systemic Thrombophilia. J Am Soc Nephrol. 2018;29(7):1928-37.

Appendix 1: Publication and abstracts arising from thesis

Blakey H, Sun R, Xie L, Russell R, Sarween N, Hodson J, Hargitai B, Marton T, Neil D, Wong E, Sheerin N, Bramham K, Harris C, Knox E, Drayson M and Lipkin G . *Pre-eclampsia is associated with complement pathway activation in the maternal and fetal circulation, and placental tissue*. Pregnancy Hypertension. 2023; 32: 43-9. (See ***Appendix 8***).

Blakey H, Sun R, Xie L, Russell R, Sarween N, Hodson J, Hargitai B, Marton T, Neil D, Wong E, Sheerin N, Bramham K, Harris C, Knox E, Drayson M and Lipkin G. *Pre-eclampsia is associated with complement activation in maternal and fetal circulation, and placental tissue.*

Poster presentation at American Society of Nephrology Kidney Week 2022 conference – Orlando, Florida, USA.

Blakey H, Sarween N, Wong E, Sheerin N, Bramham K, Knox E, Drayson M, Lipkin G. *Chronic kidney disease and pregnancy: serial alternative complement pathway activity in women with superimposed pre-eclampsia.*

Poster presentation at UK Kidney Week 2021 conference.

Blakey H, Sun R, Xie L, Russell R, Wong E, Sheerin N, Bramham K, Knox E, Drayson M, Harris C, Lipkin G. *Alternative complement pathway dysregulation in women with pre-eclampsia.*

Poster presentation at UK Kidney Week 2020 conference.

Appendix 2: REC approval

NHS
Health Research Authority
National Research Ethics Service

NRES Committee North West - Haydock
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7827
Facsimile: 0161 625 7299

27 February 2015

Dr Jane C Steele
University of Birmingham
College of Medical and Dental Sciences
Edgbaston
Birmingham
B15 2TT

Dear Dr Steele

Title of the Research Tissue Bank:	**Human Biomaterials Resource Centre**
REC reference:	**15/NW/0079**
Designated Individual:	**Professor Jonathan Frampton**
IRAS project ID:	**171283**

Thank you for your submission 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 and the Second Reviewer.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Rachel Katzenellenbogen, nrescommittee.northwest-haydock@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

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.

Mental Capacity Act 2005

I confirm that the committee has approved this research project for the purposes of the Mental Capacity Act 2005. The committee is satisfied that the requirements of section 31 of the Act will be met in relation to research carried out as part of this project on, or in relation to, a person who lacks capacity to consent to taking part in the project.

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 standard conditions of ethical approval for Research Tissue Banks 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*
Human Tissue Authority licence [HTA research licence 12358]	N/A	19 December 2012
Other [Appendix 1. HTA licence 12358 structure]	1.0	12 January 2015
Other [Appendix 2. ATF governance structure]	1.0	12 January 2015
Other [Health Screening Questionnaire]	1.0	12 January 2015
Other [Appendix 3. ATF Management Committee. Membership and TOR]	1.0	12 January 2015
Other [January 2015. Annual report form]		21 January 2015
Other [January 2015. Summary of sample collection and release]		21 January 2015
Other [Annual Report: Applications for release of samples]		21 January 2015
Other [Appendix 1. HTA licence 12358 structure]	N/A	21 January 2015
Other [Appendix 2. ATF governance structure]	N/A	21 January 2015
Other [Health Screening Questionnaire]	N/A	21 January 2015
Other [Appendix 3. ATF Management Committee. Membership and TOR]	N/A	21 January 2015
Other [January 2015. Annual report form]	N/A	21 January 2015
Other [January 2015. Summary of sample collection and release]	N/A	21 January 2015

Participant consent form [UHBFT Consent For Investigation Or Treatment]	N/A	
Participant consent form [Adult Patient Consent Form with tracked changes]	2.0	20 February 2015
Participant consent form [Adult Patient Consent Form clean]	2.0	20 February 2015
Participant consent form [Healthy Volunteer Consent Form with tracked changes]	2.0	20 February 2015
Participant consent form [Healthy Volunteer Consent Form clean]	2.0	20 February 2015
Participant consent form [Parent/Child Consent Form with tracked changes]	2.0	20 February 2015
Participant consent form [Parent/Child Consent Form clean]	2.0	20 February 2015
Participant consent form [Consent Form for Patients with Neurodegenerative Disorders with tracked changes]	2.0	20 February 2015
Participant consent form [Consent Form for Patients with Neurodegenerative Disorders clean]	2.0	20 February 2015
Participant consent form [Personal Consultee Declaration with tracked changes]	2.0	20 February 2015
Participant consent form [Personal Consultee Declaration clean]	2.0	20 February 2015
Participant consent form [Nominated Consultee Declaration with tracked changes]	2.0	20 February 2015
Participant consent form [Nominated Consultee Declaration clean]	2.0	20 February 2015
Participant consent form [Consent Form for the Donation of Placenta, Umbilical Cord and Umbilical Cord Blood with tracked changes]	2.0	20 February 2015
Participant consent form [Consent Form for the Donation of Placenta, Umbilical Cord and Umbilical Cord Blood clean]	2.0	20 February 2015
Participant information sheet (PIS) [Adult Patient Information Sheet]	1.0	12 January 2015
Participant information sheet (PIS) [Healthy Volunteer Information Sheet]	1.0	12 January 2015
Participant information sheet (PIS) [Parent Information Sheet]	1.0	12 January 2015
Participant information sheet (PIS) [Information Sheet for Patients with Neurodegenerative Disorders]	1.0	12 January 2015
Participant information sheet (PIS) [Personal Consultee Information Sheet]	1.0	12 January 2015
Participant information sheet (PIS) [Nominated Consultee Information Sheet]	1.0	12 January 2015
Participant information sheet (PIS) [Child Information Sheet (under 8 years old) with tracked changes]	2.0	20 February 2015
Participant information sheet (PIS) [Child Information Sheet (under 8 years old) clean]	2.0	20 February 2015
Participant information sheet (PIS) [Child Information Sheet (8 - 12 years old) with tracked changes]	2.0	20 February 2015
Participant information sheet (PIS) [Child Information Sheet (8 - 12 years old) clean]	2.0	20 February 2015
Participant information sheet (PIS) [Child Information Sheet (over 13 years old) with tracked changes]	2.0	20 February 2015
Participant information sheet (PIS) [Child Information Sheet (over 13 years old) clean]	2.0	20 February 2015
Participant information sheet (PIS) [Information Sheet for the Donation of Placenta, Umbilical Cord and Umbilical Cord Blood with tracked changes]	2.0	20 February 2015

Participant information sheet (PIS) [Information Sheet for the Donation of Placenta, Umbilical Cord and Umbilical Cord Blood clean]	2.0	20 February 2015
Protocol for management of the tissue bank [HBRC Protocol]	1.0	12 January 2015
REC Application Form		13 January 2015
Relative information sheet [Donor Family Information Sheet]	1.0	12 January 2015

Licence from the Human Tissue Authority

Thank you for providing a copy of the above licence.

Research governance

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 permission is also not required by collaborators at tissue collection centres (TCCs) who provide tissue or data under the terms of a supply agreement between the organisation and the research tissue bank. TCCs are not research sites for the purposes of the RGF.

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.

Statement of compliance

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

After ethical review

Reporting requirements

The attached standard conditions give detailed guidance on reporting requirements for research tissue banks with a favourable opinion, including:

- Notifying substantial amendments
- Submitting Annual Progress reports

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

15/NW/0079	**Please quote this number on all correspondence**

Yours sincerely

On behalf of
Dr Tim S Sprosen
Chair

E-mail: nrescommittee.northwest-haydock@nhs.net

Enclosures: Standard approval conditions

Copy to: Professor Jonathan Frampton, University of Birmingham

Appendix 3: HBRC study approval

College of Medical and Dental Sciences
Research and Knowledge Transfer Office

8th May 2018

Dr Ellen Knox
Birmingham Women's Hospital
Mindelsohn Way
Edgbaston
Birmingham B15 2TG

Dear Dr Knox

Application Number:	**18-308**
Project Title:	**Exploring the role of the complement system in the pathogenesis of pre-eclampsia in healthy women and those with chronic kidney disease**
Application Received:	**6th March 2018**

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 return a signed copy of the letter covering the Terms of this approval (attached to the email) 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.

Yours sincerely

Dr Jane Steele
Director, Advanced Therapies Facility

Appendix 4: Patient Information Sheet

UNIVERSITY OF BIRMINGHAM

NHS Birmingham Women's and Children's 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.

UNIVERSITY OF BIRMINGHAM

NHS
Birmingham Women's and Children's
NHS Foundation Trust

- 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: 0121 627 2766

Appendix 5: Patient Consent Form

UNIVERSITY OF BIRMINGHAM

BWCH-171117-0055

NHS Birmingham Women's and Children's NHS Foundation Trust

DONATION OF SAMPLES TO THE HUMAN BIOMATERIALS RESOURCE CENTRE

Adult Patient Consent Form

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

1.	I have read the Information Sheet entitled 'DONATION OF SAMPLES TO THE HUMAN BIOMATERIALS RESOURCE CENTRE' dated 12-Jan-2015 (Version 1.0) and had the opportunity to ask questions.	***Please enter initials***
2.	I consent to the storage of my samples (may be tissue, blood or other body fluids) in the Human Biomaterials Resource Centre and for their use in ethically approved medical research projects, including genetic studies. I understand that occasionally samples may not be stored but may be released directly to approved projects.	***Please enter initials***
3.	I understand that donated 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 enter initials***
4.	I understand that giving my samples 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 or treatment being affected.	***Please enter initials***
5.	I understand that my health records may be accessed for research, both now and in the future, but that all extracted information will be anonymised.	***Please enter initials***
6.	I understand that my samples may be used by local researchers or by research institutions elsewhere in the UK and overseas, including private/commercial companies that do medical research. I understand that I will not receive any personal financial reward for donating my samples.	***Please enter initials***

Attach patient sticker here

PATIENT		PERSON TAKING CONSENT	
Name		Name	
Date		Role	
Signature		Date	
		Signature	

Version 2.0
20-Feb-2015

Pink copy to be given to the patient
Yellow copy to be retained by the HBRC
Top white copy to go in patient health record

Appendix 6: Ghana Cohort study ethics approval

In case of reply the number
And the date of this
Letter should be quoted
My Ref. No. KBTH/IRB/00056/2017
Your Ref. No.....

KORLE BU TEACHING HOSPITAL
P. O. BOX KB 77,
KORLE BU, ACCRA.

Tel: +233 302 667759/673034-6
Fax: +233 302 667759
Email: Info@kbth.gov.gh
pr@kbth.gov.gh
Website: www.kbth.gov.gh

25th October, 2017

DR. DWOMOA ADU
RENAL UNIT
KORLE BU

"PILOT STUDY OF GENETIC DETERMINANTS OF HYPERTENSIVE DISEASE IN PREGNANCY"

KBTH – IRB /00056/2017

Investigator: Dr. Dwomoa Adu

On 25th October, 2017, the Korle-Bu Teaching Hospital Institutional Review Board (KBTH IRB) reviewed and granted approval to the study entitled "Pilot Study of Genetic Determinants of Hypertensive Disease in Pregnancy"

Please note that the Board requires you to submit a final review report on completion of this study to the KBTH-IRB.

Kindly, note that, any modification/amendment to the approved study protocol without approval from KBTH-IRB renders this certificate invalid.

Please report all serious adverse events related to this study to KBTH-IRB within seven days verbally and fourteen days in writing.

This IRB approval is valid till 30th September, 2018. You are to submit annual report for continuing review.

Sincere regards,

[redacted]
OKYERE BOATENG (MR)
CHAIR (KBTH-IRB)

Cc: The Chief Executive Officer
Korle Bu Teaching Hospital

The Director of Medical Affairs
Korle Bu Teaching Hospital

In case of reply the number
And the date of this
Letter should be quoted
My Ref. No.... KBTH/MB/03617
Your Ref. No......

KORLE BU TEACHING HOSPITAL
P. O. BOX KB 77,
KORLE BU, ACCRA.

Tel: +233 302 667759/673034-6
Fax: +233 302 667759
Email: info@kbth.gov.gh
pr@kbth.gov.gh
Website: www.kbth.gov.gh

28th July, 2017

DR. DWOMOA ADU
RENAL UNIT
KORLE BU

SCIENTIFIC AND TECHNICAL COMMITTEE APPROVAL
PROTOCOL IDENTIFICATION NUMBER: **KBTH-STC 00056/2017**

The Korle Bu Teaching Hospital Scientific and Technical Committee (KBTH-STC), on 28th July, 2017 approved your submitted study protocol.

TITLE OF PROTOCOL: **"Pilot study of Genetic Determinants of Hypertensive Disease in pregnancy"**

PRINCIPAL INVESTIGATOR: Dr. Dwomoa Adu

This approval requires that you **forward your approved document to Korle Bu Teaching Hospital-Institutional Review Board (KBTH-IRB) for the ethical aspect of the proposal to be assessed before the project can be initiated.**

This STC approval is valid till 31st March, 2019
You may, however, request extension of the approval period, or renewal as the case may be, should the study extend beyond the stated period.

Upon completion, you are required to submit a final report on the study to the STC. This is to enable the STC ensure among others that, the project has been implemented as per the approved protocol. You are also required to inform the KBTH-STC and Research Directorate of any publications that may emanate from the research findings.

Kindly note that, should the need arise, the KBTH-STC or IRB may institute appropriate measures to satisfy itself that study is being conducted according to the highest scientific and ethical standards.

Please note that any modification to the study protocol without Scientific Technical Committee (STC) approval renders this approval invalid.

Sincerely,

Prof. G. Obeng Adjei
Chairman, KBTH-STC

Cc: The Chairman, KBTH-IRB

Appendix 7: Ghana Cohort study consent form

Consent Forms - Adults

KORLE-BU TEACHING HOSPITAL

TELEPHONE: 666987

Dept. of Medicine
P. O. Box 77,
Accra.

INFORMED CONSENT FORM

Participant ID Number:

Participant's initials:

Date:

Title: Pilot study of genetic determinants of hypertensive disease in pregnancy

You are being asked to take part in a research study looking at determinants of hypertension among pregnant women. Please read this form carefully and ask any questions you may have before agreeing to take part in the study. We will offer explanations or interpretation for those who cannot read.

What the study is about: The purpose of this study is to learn about the hereditary factors associated with hypertension during pregnancy. This study will also look at other non-hereditary determinants of hypertension in pregnancy.

What we will ask you to do: If you agree to be in this study, we will conduct an interview with you. The interview will include questions about your health and demographic information. We will also obtain some information such as your medical diagnosis, and medication from your clinical file. The interview will take about 30 minutes to complete.

Laboratory tests

Blood and saliva samples: During each study visit, we will take 20ml of blood. We may also ask you for a sample of your saliva. We will test DNA from your blood sample for genetic variation and sequence the DNA to read out all the genetic information in it. We will take a sample of urine. Blood samples will be drawn by experienced laboratory technicians or research personnel to avoid multiple needle

pricks. In addition about 5mls of blood from the umbilical cord of your baby will be drawn for test to look for hereditary predisposition to hypertension during pregnancy.

STORED SPECIMENS: Your blood and urine samples will be stored at a central laboratory (MDS-Lancet Laboratories, East Legon, Accra, Ghana) or a designated storage facility for future studies of kidney and heart diseases. This sample will not have your name and any other identifying information. It will be connected to your study results only by a unique study number assigned to each person in the study. This unique study number has no meaning outside the study. Your blood, urine and saliva specimens may be stored indefinitely. It may be used at any time during that period for more other studies not related to kidney disease. It is possible that new tests might be available in the future, which could be useful in understanding kidney disease. Stored blood and/or urine may be used later to find new risk factors for kidney heart and other diseases. These studies may be about your genetic makeup or to describe your genes or DNA. You will not be able to find out the results of these tests because your blood will no longer have your name attached. **Samples of all participants who withdraw from the study will not be analyzed and will be discarded. Your information on computer will be securely protected with password known to only the investigators.** Approval will be sought from the Korle Bu Ethical Review Committee for further use of the specimens.

Risks and benefits:

There is the risk that you may find some of the questions about your conditions to be sensitive. Potential risks of blood drawing include light-headedness, swelling, and inflammation at the site of the blood draw. Minor discomfort is associated with blood drawing. Participants are asked to spend about 30-40 minutes of your time for study intake and the one-time sample collection, which may be a minor inconvenience. Psychological risks are expected to be minimal. Although research-related injuries are not anticipated, any research-related injuries that occur will be treated by site staff, and appropriate referrals will be made for any services that the site cannot provide.

There are no benefits to you and you will not bear any cost associated with the study. Korle-Bu Teaching is the major referral centre for most medical conditions in Ghana and we hope to learn about this common condition to help clinicians in their practice.

Compensation: There will be no compensation

Your answers will be confidential. The records of this study will be kept private. In any sort of report we make public we will not include any information that will make it possible to identify you. Research records will be kept in a locked file; only the researchers will have access to the records.

Taking part is voluntary: Taking part in this study is completely voluntary. You may skip any questions that you do not want to answer. If you decide not to take part or to skip some of the questions, it will not affect your current or future relationship with Korle-Bu Teaching Hospital/School of Medicine and Dentistry, University of Ghana. If you decide to take part, you will be asked to sign a consent form.

If you have questions: The researchers conducting this study are Vincent Boima, Jerry Coleman, Dwomoa Adu and Dr. Charlotte Osafo. Please ask any questions you have now. If you have questions later, you may contact the above researchers via the contacts below;

Dr Vincent Boima –
Dr Jerry Coleman -
Dr Dwomoa Adu –
Dr Charlotte Osafo

If you want to speak with someone not directly involved in this research study please contact the Research Ethics Committee Administrator at the Korle Bu Teaching Hospital on **0302666766.**

You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Your Signature _____ Date _____

Your Name _____

Appendix 8: Publication (Chapter 3 study)

Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health 32 (2023) 43–49

Contents lists available at ScienceDirect

Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health

journal homepage: www.elsevier.com/locate/preghy

Pre-eclampsia is associated with complement pathway activation in the maternal and fetal circulation, and placental tissue

Hannah Blakey [a,b,*], Ruyue Sun [c], Long Xie [c], Rebecca Russell [c], Nadia Sarween [a], James Hodson [d], Beata Hargitai [e], Tamas Marton [e], Desley A H Neil [a], Edwin Wong [f], Neil S Sheerin [f], Kate Bramham [g], Claire L Harris [c], Ellen Knox [e], Mark Drayson [b], Graham Lipkin [a]

[a] Renal Medicine Department, Queen Elizabeth Hospital Birmingham, Birmingham, UK
[b] Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, UK
[c] Translational and Clinical Research Institute, Newcastle University, Newcastle, UK
[d] Research Development and Innovation, Institute of Translational Medicine, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK
[e] Birmingham Women's and Children's NHS Foundation Trust, Birmingham, UK
[f] National Renal Complement Therapeutics Centre, Newcastle, UK
[g] Department of Women and Children's Health, King's College London, London, UK

ARTICLE INFO

Keywords:
Pre-eclampsia
Complement system proteins
Pregnancy
Placenta
Biomarkers

ABSTRACT

Objectives: Pre-eclampsia (PE) is a leading cause of obstetric morbidity, with no definitive therapy other than delivery. We aimed to compare complement markers in maternal and fetal circulation, and placental tissue, between women with PE and healthy pregnant controls.

Study Design: Maternal and umbilical cord blood was tested for iC3b, C3, C4, properdin, Ba and C5b-9, and placental tissue for C3d, C4d, C9 and C1q, from women with PE (n = 34) and healthy pregnant controls (n = 33). Maternal properdin and Ba tests were repeated in a separate validation cohort (PE n = 35; healthy pregnant controls n = 35).

Main Outcome Measures: Complement concentrations in maternal and umbilical cord blood, and placental immunohistochemical complement deposition.

Results: Women with PE had significantly lower concentrations of properdin (mean: 4828 vs 6877 ng/ml, $p < 0.001$) and C4 (mean: 0.20 vs 0.31 g/l, $p < 0.001$), and higher Ba (median: 150 vs 113 ng/ml, $p = 0.012$), compared to controls. After controlling for gestational age at blood draw, average properdin concentration was 1945 ng/ml lower in PE vs controls (95 % CI: 1487–2402, $p < 0.001$). Of the cord blood markers assessed, only Ba differed significantly between PE and controls (median: 337 vs 233 ng/ml, $p = 0.004$). C4d staining of the syncytiotrophoblast membrane was increased in PE vs controls (median immunoreactivity score 3 vs 0, $p < 0.001$). Maternal properdin and C4 were significantly negatively correlated with placental C4d staining.

Conclusions: Our data confirm excessive placental complement deposition associated with significant concurrent changes in maternal and fetal circulating complement biomarkers in PE. Inhibition of complement activation is a potential therapeutic target.

1. Introduction

Pre-eclampsia (PE) is a multisystem disorder that affects around 5 % of pregnancies, and is a leading contributor to obstetric morbidity [1–3]. There remains no definitive treatment except for delivery of the baby and placenta, which is often necessitated preterm, due to declining maternal and/or fetal wellbeing.

There is increasing evidence implicating complement dysregulation in the pathogenesis of PE. Animal models demonstrate placental complement activation inducing defective angiogenesis, abnormal placentation, and fetal demise [4–6], with complement inhibition rescuing pregnancy [4,5]. Studies of human placental tissue from women with PE report excess C4d deposition compared to healthy pregnant controls, associated with preterm birth [7] and fetal growth restriction [8]. C3d and C5b-9 deposition may also be increased in PE [9–12], but other studies refute this [13]. Studies of maternal plasma drawn in early

* Corresponding author at: Renal Medicine, Queen Elizabeth Hospital Birmingham, Mindelsohn Way, Birmingham B15 2GW, UK.
E-mail address: hannahblakey@nhs.net (H. Blakey).

https://doi.org/10.1016/j.preghy.2023.04.001
Received 9 September 2022; Received in revised form 21 February 2023; Accepted 11 April 2023
Available online 21 April 2023

pregnancy report increased Bb concentrations in women who later developed PE [14,15], suggesting excessive alternative complement pathway activity during early placental development. Raised concentrations of C5b-9 in PE demonstrate terminal pathway activation [16–18] and may be associated with PE severity [19]. Data on umbilical cord blood (UCB) complement is extremely limited. UCB C5a appears to be raised in PE [20], suggesting that complement activation affects the fetal compartment, although data on UCB Bb are conflicting [21,22].

Complement-modifying agents are undergoing rapid development and may provide novel opportunities to treat complement-mediated diseases [23–27]. Despite the growing body of evidence, it remains unclear whether raised concentrations of circulating complement reflect a general heightened inflammatory state in PE or are directly associated with placental complement-mediated injury. We therefore aimed, for the first time, to simultaneously compare markers of local placental complement deposition with systemic complement activation in the maternal and fetal circulation between PE and healthy pregnancy. The results will potentially provide further support for the role of complement activation in the pathogenesis of PE, and may also provide insight into the sequence of events leading to complement activation on a local and systemic level.

2. Methods

2.1 Study population

The primary cohort comprised women recruited from Birmingham Women's Hospital between 2018 and 19 ("Birmingham Cohort"). The PE group included women with a confirmed diagnosis of PE, according to international consensus criteria [28,29]. Healthy pregnant controls were recruited from elective Caesarean section clinic and induction of labor suite. This only included subjects undergoing planned delivery for non-emergency reasons. Participants were recruited by convenience sampling. Exclusion criteria were a known history of: CKD, chronic hypertension, autoimmune disease, or recurrent miscarriage. Clinical outcome data were collated from obstetric records.

Health Research Authority approval was obtained through the University of Birmingham Human Biomaterials Resource Centre (Research Ethics Committee reference: 15/NW/0079). All study participants gave informed consent.

2.2 Sample collection

Samples of maternal plasma (EDTA) and serum were collected upon hospital admission, within seven days prior to delivery and before established labor. UCB samples and placental tissue were collected immediately following birth. Full thickness tissue sections were cut from a central portion of the placenta, then formalin-fixed and paraffin-embedded. Blood samples were centrifuged, separated into 0.5 mL aliquots, and frozen at −80 °C within four hours of collection to minimize ex vivo complement activation.

2.3 Laboratory analyses

Maternal and cord plasma samples were tested for properdin, Ba, iC3b, C3, and C5b-9. Complement biomarker concentrations were determined using in-house electrochemiluminescent immunoassays (MesoScale Diagnostics (MSD, Rockville, MD)) with either Gold 96-well Streptavidin QuickPlex plates or Small Spot Streptavidin plates (iC3b). Capture/detection antibodies were: C3: Clone 2898, Clone 3; iC3b: Ab807 anti iC3b/C3d, bH6; Properdin: Clone SIM 295, Clone 2.9; Ba: Clone D22/3, P21; C5b-9: aE11, E2 anti-C8. All antibodies were from Hycult Biotech (Netherlands), other than E2 anti-C8 (gift from BP Morgan, Cardiff University) and Ab807 (in-house anti iC3b/C3d HuCal antibody). Plates were read on a QuickPlex SQ 120 instrument (MSD). Analyte concentrations were determined by standard curve interpolation using MSD Workbench software v15.0. Samples were diluted to 1:2,000 (properdin), 1:5 (Ba), 1:200 (iC3b), 1:20,000 (C3) and 1:20 (C5b-9) and tested in duplicate, with the average value taken. Intra-assay coefficients of variation (CVs) were 0.9 % (properdin), 3.3 % (Ba), 2.3 % (iC3b), 2.0 % (C3), and 3.3 % (C5b-9). A minimum of two control plasma samples were used to measure inter-assay variance. Inter-assay CVs were 12 % (properdin and Ba), 7 % (iC3b), 8 % (C3), and 12.5 % (C5b-9). The C3 assay was designed to detect native, non-activated C3 which may differ from nephelometry assays that detect both native C3 and activation fragments.

Samples of maternal and cord serum were tested singly for C4 using an automated Hitachi Cobas 6000 Turbidimeter and commercially available assay kit (Roche diagnostics, West Sussex, UK). Intra-assay CV was 0.7–1.1 % and inter-assay CV 1.4–1.6 %.

2.4 Immunohistochemistry

Immunohistochemical analysis of placental tissue was performed for C3d, C4d, C9, and C1q. 4 μm placental tissue sections were stained using the Dako Autostainer Link 48 (Agilent, CA, USA). Placental sections were deparaffinized to enable antigen retrieval, endogenous peroxidase was blocked, and antibodies applied to C3d (DB Biotech; 1:200), C4d (Biomedica; 1:40), C9 (Abcam; 1:2000), and C1q (Dako Agilent; 1:60,000). Sections were also stained for hematoxylin and eosin, to provide a histological reference point. Prior to C1q staining, slides were protease-digested to enhance antigen retrieval; in line with established local protocol.

Immunostained slides were scanned to an online repository (Aperio eSlide Manager; Leica Biosystems, Nussloch, Germany). Placental sections were scored semi-quantitatively by a single examiner blinded to clinical data, using a modified placental immunoreactivity score [30,31] based upon staining intensity and distribution at the syncytiotrophoblast (STB) membrane. Intensity was scored as 0 = negative, 1 = weak (visible at 40x magnification), 2 = intermediate (10-20x magnification), or 3 = strong (4x magnification). Distribution was scored according to the proportion of cells staining positive, with 0 = negative, 1 = 1–10 % cells staining positive, 2 = 11–50 %, 3 = 51–80 %, and 4 = 81–100 %. Scores were multiplied to calculate a composite placental immunoreactivity score (range 0–12). A score of zero was classified as "absent" immunoreactivity, 1–8 as "focal" and 9–12 as "diffuse" [30]. Each slide was evaluated in ten random fields and scored on three separate occasions. The median score was determined as representative for that slide. A random sample of slides was verified independently by experienced histopathologists, to ensure consistency and accuracy of scoring.

2.5 Validation cohort

A separate cohort of patients was recruited from St Thomas' Hospital, London, between 2015 and 18 ("London Cohort"). Ethical approval was granted via the Integrated Research Application System (IRAS; study ID 83429, October 2014). Maternal blood samples were collected at the time of PE diagnosis (cases), and at routine antenatal hospital visits (healthy pregnant controls). The same inclusion and exclusion criteria were applied as for the Birmingham Cohort, with the additional exclusion of multifetal pregnancies. Maternal plasma samples were tested for properdin and Ba only, using the same methodology as above.

2.6 Statistical analysis

For comparisons between PE and control groups, nominal variables were assessed using Fisher's exact tests. Ordinal and continuous variables were reported as means ± standard deviation, with p-values derived from independent samples t-tests, when normally distributed. Medians, interquartile ranges and Mann-Whitney U tests were used otherwise. Blood markers found to differ significantly between groups were assessed using receiver operating characteristic (ROC) curves.

Maternal blood results were also assessed using linear regression to adjust for the effect of gestational age at blood draw. This initially included the group (PE vs control), gestational age at blood draw, and an interaction term as covariates. Where this interaction term was non-significant, it was removed from the model, and the analysis repeated. Goodness-of-fit of the resulting models was then assessed graphically. Where poor fit was detected, values were $\log_{10}$-transformed, and the analysis repeated. The resulting coefficients were then anti-logged and converted into percentage differences.

Correlations between variables were quantified using Spearman's correlation coefficients (rho). All analyses were performed using IBM SPSS 28 (IBM Corp. Armonk, NY), with $p < 0.05$ deemed indicative of statistical significance.

3. Results

3.1 Cohort characteristics

67 women were recruited in the Birmingham Cohort (34 PE cases and 33 healthy pregnant controls). The London cohort comprised a further 70 subjects (35 PE cases and 35 healthy pregnant controls). Baseline demographics and clinical outcome data are presented in Table 1. There were no significant differences in age, body mass index, ethnicity, parity, or delivery mode between PE and control groups for either cohort. In both cohorts, PE was characterized by significantly earlier delivery, lower birth weight, and increased rates of adverse pregnancy outcome.

3.2 Maternal blood complement

Maternal blood complement concentrations in the PE and control groups are reported in Table 2. In the Birmingham Cohort, women with PE had significantly lower concentrations of properdin (mean: 4828 vs 6877 ng/ml, $p < 0.001$), C4 (mean: 0.20 vs 0.31 g/l, $p < 0.001$), iC3b (mean: 489 vs 606 ng/ml, $p = 0.003$), and C3 (mean: 1.9 vs 2.4 g/l, $p < 0.001$), and significantly higher Ba (median: 150 vs 113 ng/ml, $p = 0.012$), compared to the control group. However, no significant differences in iC3b:C3 ratio ($p = 0.734$) or C5b-9 ($p = 0.753$) were detected between groups. Analysis of the London Cohort returned consistent results, with significantly reduced properdin concentrations in PE compared to controls (mean: 5282 vs 7021 ng/ml, $p < 0.001$). However, the difference in maternal Ba levels did not reach statistical significance (median: 165 in PE vs 151 ng/ml in controls, $p = 0.310$).

The analysis was repeated using a regression approach, to account for the observed differences in gestational age at blood sampling between groups (***Supplementary*** *Table S1*). For the pooled cohorts, the difference in maternal properdin remained significant, with concentrations being an average of 1945 ng/ml lower in the PE vs control groups (95 % CI: 1487–2402, $p < 0.001$, Fig. 1***a***). The differences in C3 and C4 in the Birmingham Cohort also persisted after adjustment for sample gestation, with concentrations being an average of 0.5 g/l (95 % CI: 0.2–0.8, $p < 0.001$) and 0.07 g/l (95 % CI: 0.02–0.13, $p = 0.014$) lower, respectively, in PE vs controls. However, after adjustment for gestation, the differences in iC3b ($p = 0.252$) and Ba ($p = 0.194$) were not statistically significant.

Further assessment using a ROC curve approach found maternal properdin to be the strongest predictor of PE diagnosis, with an area under the curve (AUROC) of 0.87 (SE: 0.03) when applied to the combined cohorts – this effect is visualized in Fig. 1***b***. This was followed by maternal C4 (AUROC 0.82; SE 0.05) and C3 (AUROC 0.80; SE: 0.06).

3.3 Umbilical cord blood complement

Data for UCB complement concentrations were available for 36

Table 1
Demographic and clinical outcome data by cohort.

	Birmingham Cohort				London Cohort			
	N	*Pre-eclampsia*	*Control*	*p-Value*	*N*	*Pre-eclampsia*	*Control*	*p-Value*
Maternal Age at Delivery (Years)	67	30.6 ± 6.6	29.6 ± 5.6	0.527	70	33.7 ± 6.5	33.0 ± 4.5	0.617
Body Mass Index (BMI) (kg/m^2)	67	28.5 ± 5.1	28.9 ± 5.1	0.741	70	28.8 ± 7.4	26.3 ± 4.5	0.094
Ethnicity	67			0.530	70			0.051
White		15 (44 %)	19 (58 %)			15 (43 %)	24 (69 %)	
Asian		12 (35 %)	9 (27 %)			1 (3 %)	0 (0 %)	
Black		6 (18 %)	3 (9 %)			12 (34 %)	4 (11 %)	
Mixed/Other		1 (3 %)	2 (6 %)			7 (20 %)	7 (20 %)	
Parity	67			0.082*	70			0.722*
0		25 (74 %)	16 (48 %)			22 (63 %)	22 (63 %)	
1		4 (12 %)	12 (36 %)			5 (14 %)	9 (26 %)	
2+		5 (15 %)	5 (15 %)			8 (23 %)	4 (11 %)	
Peak SBP (mmHg)	65	163 ± 14	128 ± 9	**<0.001**	70	173 ± 12	135 ± 13	**<0.001**
Peak DBP (mmHg)	65	105 ± 9	79 ± 7	**<0.001**	70	106 ± 8	85 ± 10	**<0.001**
Peak uPCR (mg/mmol)	34	259 (133–429)	*NA*	–	35	85 (54–200)	*NA*	–
Mode of Delivery	67			0.242	70			0.227
Caesarean		24 (71 %)	28 (85 %)			23 (66 %)	17 (49 %)	
Vaginal		10 (29 %)	5 (15 %)			12 (34 %)	18 (51 %)	
Gestation at (Weeks):								
Delivery	66	36.9 (36.0–37.7)	39.3 (39.0–40.6)	**<0.001**	70	36.1 (34.6–37.1)	39.6 (39.0–41.1)	**<0.001**
Blood Draw	65	36.6 (35.6–37.4)	39.0 (38.8–40.4)	**<0.001**	70	34.3 (32.7–35.9)	34.0 (32.1–35.9)	0.837
PE Diagnosis	34	35.9 (33.4–36.6)	*NA*	–	35	33.9 (32.6–35.3)	*NA*	–
Early-onset PE (<34 Weeks)	34	10 (29 %)	*NA*	–	35	19 (54 %)	*NA*	–
Twin Pregnancy	67	1 (3 %)	0 (0 %)	1.000	70	0 (0 %)	0 (0 %)	1.000
Preterm Birth (<37 Weeks)**	67	17 (50 %)	0 (0 %)	**<0.001**	70	24 (69 %)	2 (6 %)	**<0.001**
Birth Weight (g)***	67	2324 ± 670	3543 ± 491	**<0.001**	70	2397 ± 863	3592 ± 531	**<0.001**
Low Birth Weight (<2500 g)**	67	19 (56 %)	0 (0 %)	**<0.001**	70	20 (57 %)	0 (0 %)	**<0.001**
SGA (<10th Centile)**	67	22 (65 %)	5 (15 %)	**<0.001**	70	17 (49 %)	2 (6 %)	**<0.001**
Neonatal Unit Care**	67	13 (38 %)	4 (12 %)	**0.023**	70	22 (63 %)	1 (3 %)	**<0.001**

Continuous variables are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values for Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests, unless stated otherwise. Bold p-values are significant at $p < 0.05$. *p-Value from Mann-Whitney *U* test, as the factor is ordinal. **For the twin pregnancy, outcomes were the same for both babies; hence these were combined for analysis. ***For the twin pregnancy, the average weight of the two babies was assumed. NA = data were not available in the cohort for the stated variable.

Table 2
Maternal and umbilical cord blood complement concentration results.

Blood Marker	Birmingham Cohort				London Cohort			
	N	*Pre-eclampsia*	*Control*	*p-Value*	*N*	*Pre-eclampsia*	*Control*	*p-Value*
Maternal Blood								
iC3b (ng/ml)	66	489 ± 153	606 ± 157	**0.003**	*NA*	–	–	–
C3 (g/l)	66	1.90 ± 0.39	2.36 ± 0.39	**<0.001**	*NA*	–	–	–
iC3bC3 (x10^6)	66	245 (192–301)	251 (219–290)	0.734	*NA*	–	–	–
C4 (g/l)	66	0.20 ± 0.08	0.31 ± 0.08	**<0.001**	NA	–	–	–
Properdin (ng/ml)	66	4828 ± 806	6877 ± 1421	**<0.001**	70	5282 ± 1467	7021 ± 1317	**<0.001**
C5b-9 (ng/ml)	66	237 (198–335)	237 (185–334)	0.753	*NA*	–	–	–
Ba (ng/ml)	66	150 (119–223)	113 (89–148)	**0.012**	70	165 (117–268)	151 (113–198)	0.310
Umbilical Cord Blood								
iC3b (ng/ml)	36	740 (496–1179)	774 (631–1009)	0.788	*NA*	–	–	–
C3 (g/l)	36	0.81 ± 0.29	0.77 ± 0.20	0.610	*NA*	–	–	–
iC3bC3 (x10^6)	36	840 (502–1901)	1133 (734–1468)	0.546	*NA*	–	–	–
C4 (g/l)	31	0.12 ± 0.04	0.13 ± 0.05	0.551	*NA*	–	–	–
Properdin (ng/ml)	36	2943 ± 894	3321 ± 1170	0.333	*NA*	–	–	–
C5b-9 (ng/ml)	36	85.5 (58.2–111.8)	64.8 (41.2–97.5)	0.214	*NA*	–	–	–
Ba (ng/ml)	36	337 (273–370)	233 (155–261)	**0.004**	*NA*	–	–	–

Data are reported as mean ± standard deviation, with p-values from independent samples t-tests, or as median (interquartile range), with p-values for Mann-Whitney U tests. Bold p-values are significant at $p < 0.05$. For the Birmingham cohort, maternal blood concentrations were available for N = 33/N = 33 pre-eclampsia/control patients, whilst umbilical blood concentrations were available for N = 12/N = 24, with the exception of C4 (N = 10/N = 21). NA = data were not available in the cohort for the stated variable.

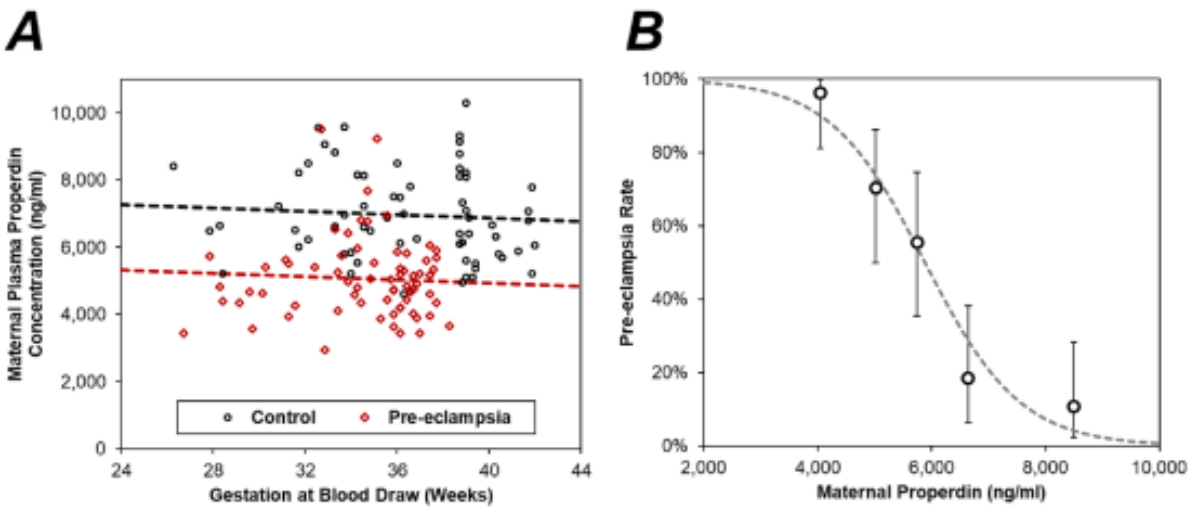


Fig. 1. Associations between pre-eclampsia and maternal properdin concentration Both figures include patients from both the Birmingham and London cohorts, with the exception of those with no properdin concentration recorded (N = 1); Figure A additionally excludes one patient for whom the gestation at blood draw was not recorded. In Figure A, points represent the data for individual patients, and broken lines are from a linear regression model, with gestation and pre-eclampsia status as covariates. In Figure B, points represent the observed pre-eclampsia rates within quintiles of the distribution, which are plotted at the mean of the interval, and whiskers represent 95 % confidence intervals. The broken line is from a binary logistic regression model on the patient-level data, with the maternal properdin concentration as a continuous covariate.

patients from the Birmingham Cohort (12 with PE, and 24 controls, Table 2). Ba was the only UCB measurement to differ significantly between PE and controls (median: 337 vs 233 ng/ml, p = 0.004; AUROC: 0.80, SE: 0.09).

Comparison of maternal and UCB complement found only C4 (rho: 0.39, p = 0.033) and Ba (rho: 0.44, p = 0.008) concentrations correlated significantly between maternal and fetal circulation (***Supplementary Table S2***). Absolute concentrations of all complement markers except activation fragments Ba and iC3b were lower in UCB samples.

3.4 Placental immunohistochemistry

Examples of placental immunohistochemistry are shown in Fig. 2. C4d staining was present on the STB membrane in almost all PE placentas (17/19, 89 %), compared to only 17 % (5/30) of controls; median immunoreactivity score 3 vs 0, respectively ($p < 0.001$, Fig. 3). When present, C4d was observed in a linear staining pattern on the STB surface and deposited circumferentially around placental villi (Fig. 2 ***image B and F***).

C3d staining was present at the STB membrane in all subjects. However, the PE group had significantly increased C3d deposition compared to controls (median immunoreactivity score 6 vs 4, p = 0.004). There was no apparent relationship between C3d and C4d staining, with co-localization observed infrequently. C9 was also detected more intensely at the STB membrane in PE compared to controls (median immunoreactivity score 4 vs 1, $p < 0.001$). C9 staining was observed most frequently in areas of villous injury, and did not show consistent co-localization with sites of C4d staining. C1q was not

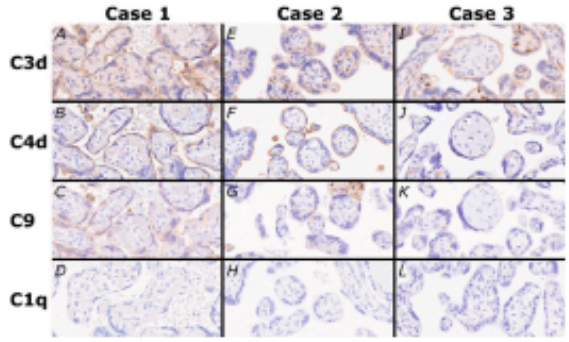


Fig. 2. Placental staining of C4d, C3d, C9 and C1q in preeclampsia and healthy pregnancy. Case 1 (Subject with PE): Diffuse C3d staining of the STB membrane [A]. Diffuse C4d staining localized to the STB membrane (strong, linear staining with syncytial cytoplasmic protrusions and membrane sheds) [B]. C9 staining shows weak membrane immunolocalization in STBs [C]. No immunostaining was detected for C1q [D]. **Case 2 (Subject with PE):** C3d: weak staining of STB membrane (co-localizes with C4d staining pattern) [E]. Strong C4d staining localized to the STB membrane in a circumferential, linear pattern around the villi [F]. C9 and C3d: non-specific background staining of stromal-mesenchymal cells and villous capillary plasma [G and E]. C1q: no immunostaining detected [H]. **Case 3 (Healthy control pregnancy):** C3d: weak, non-specific background staining [I]. C4d, C9 and C1q: absent immunostaining [J, K, L].

strongly detected in the placental tissue of PE patients or of controls, with the majority of subjects exhibiting absent or weak staining, and with no significant differences between groups ($p = 0.698$).

3.5 Relationship between circulating and placental complement

Associations between placental immunoreactivity scores and complement concentrations from maternal and UCB were assessed (Table 3). This identified a significant negative correlation between maternal properdin concentration and C4d deposition (rho: −0.524, $p < 0.001$). Specifically, mean maternal properdin concentrations declined from 6839 ± 1559 ng/ml in those with absent C4d deposition, to 5393 ± 1095 and 4367 ± 820 ng/ml in those with focal and diffuse C4d deposition, respectively. Maternal C4 concentration was also significantly negatively correlated with placental C4d deposition (rho: −0.337, $p = 0.019$). In addition, there was significant positive correlation between maternal Ba and placental C4d (rho: 0.337, $p = 0.019$).

There were no significant correlations between UCB complement concentration and placental complement deposition.

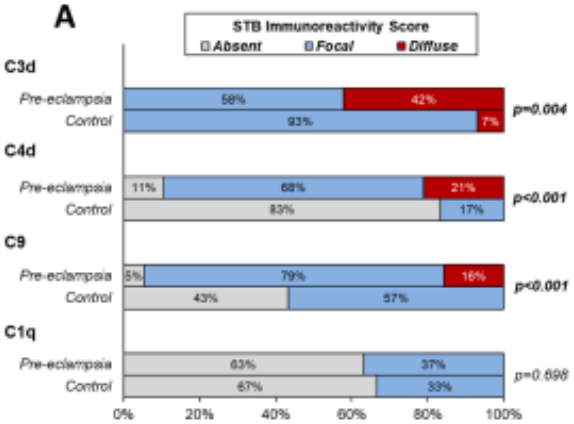
4. Discussion

There is accumulating evidence supporting the role of complement dysregulation in the pathogenesis of PE, but the precise mechanisms are debated [32,33], and the relationship between circulating complement and placental complement deposition is unclear. This study reports novel evidence of simultaneous placental complement deposition, associated with significant changes in complement biomarkers in the maternal and fetal circulation, in women with PE.

In this study, women with PE had significantly lower plasma properdin concentrations than healthy pregnant controls. This is the first time a reduction in properdin in the setting of PE has been reported. The findings were replicable in a separate validation cohort, and significant differences in properdin persisted after controlling for gestational age. Furthermore, maternal plasma properdin concentration appears to provide excellent diagnostic accuracy in distinguishing cases of PE from healthy pregnancy. Properdin acts as a positive regulator of the alternative complement pathway by stabilizing C3 convertase and prolonging its half-life. Reduced properdin levels are indicative of excess alternative pathway activity, through consumption from deposition in tissues.

Elevated concentrations of Ba in maternal and UCB provided further evidence of excessive alternative pathway activity in PE. However, the difference between groups was not statistically significant in the London Cohort, or after adjusting for gestational age at blood draw. Nevertheless, Factor B concentration has been shown to level off after 28 weeks in normal pregnancy [34]; thus gestational differences in the Birmingham Cohort may not be a significant confounding factor.

Although earlier research is conflicting [21,22], maternal and UCB Ba concentrations were significantly correlated in this study. This might indicate diffusion of this relatively small molecule across the maternal-fetal interface [35], rather than fetal circulatory complement activation per se; particularly given that no other UCB complement components significantly differed between PE and controls, together with the lack of



B

Stain	PE	Control	p-Value
C3d	6 (4-9)	4(3-6)	**0.004**
Absent	0 (0%)	0 (0%)	
Focal	11 (58%)	27 (93%)	
Diffuse	8 (42%)	2 (7%)	
C4d	3 (2-6)	0 (0-0)	**<0.001**
Absent	2 (11%)	25 (83%)	
Focal	13 (68%)	5 (17%)	
Diffuse	4 (21%)	0 (0%)	
C9	4 (2-6)	1 (0-3)	**<0.001**
Absent	1 (5%)	13 (43%)	
Focal	15 (79%)	17 (57%)	
Diffuse	3 (16%)	0 (0%)	
C1q	0 (0-1)	0 (0-1)	0.698
Absent	12 (63%)	20 (67%)	
Focal	7 (37%)	10 (33%)	
Diffuse	0 (0%)	0 (0%)	

Fig. 3. Placental immunoreactivity scores by complement stain and pre-eclampsia status *Results are based on N = 19 PE and N = 30 controls, with the exception of C3d, which only included N = 29 controls, due to missing data in one case. "Absent" is defined as a median immunoreactivity score at the STB membrane of 0, with "focal" and "diffuse" defined as scores of 1–8 and 9–12, respectively. Figure A shows the relative proportion of subjects with absent, focal and diffuse immunostaining for each complement marker, by PE status. In Figure B, data are reported as both median immunoreactivity score (interquartile range), and the N (%) within intervals of the score for each stain. p-Values are from Mann-Whitney U tests, and bold p-values are significant at p < 0.05. STB = Syncytiotrophoblast.*

Table 3
Correlation between maternal and cord plasma complement concentration, and placental immunoreactivity score.

	Maternal Plasma					Umbilical Cord Plasma				
	Properdin	*Ba*	*C5b-9*	*iC3b:C3*	*C4***	*Properdin*	*Ba*	*C5b-9*	*iC3b:C3*	*C4***
C3d – STB*	r: −0.130	r: 0.156	r: 0.240	**r: 0.291**	r: −0.217	r: −0.233	r: 0.257	r: 0.032	r: −0.115	r: −0.200
	p = 0.385	p = 0.296	p = 0.104	**p = 0.047**	p = 0.143	p = 0.216	p = 0.171	p = 0.867	p = 0.546	p = 0.326
C4d – STB	**r: −0.524**	**r: 0.337**	r: 0.216	r: −0.013	**r: −0.337**	r: −0.181	r: 0.308	r: 0.233	r: −0.238	r: 0.027
	p < 0.001	**p = 0.019**	p = 0.141	p = 0.931	**p = 0.019**	p = 0.329	p = 0.091	p = 0.208	p = 0.198	p = 0.893
C9 – STB	r: −0.184	r: 0.146	r: 0.264	r: 0.088	r: −0.267	r: −0.104	r: 0.337	r: 0.157	r: 0.054	r: −0.068
	p = 0.210	p = 0.322	p = 0.070	p = 0.550	p = 0.066	p = 0.578	p = 0.064	p = 0.399	p = 0.771	p = 0.738
C1q – STB	r: −0.038	r: 0.001	r: 0.070	r: 0.127	r: −0.106	r: 0.013	r: −0.158	r: −0.156	r: −0.346	r: 0.026
	p = 0.796	p = 0.995	p = 0.636	p = 0.388	p = 0.475	p = 0.947	p = 0.396	p = 0.401	p = 0.057	p = 0.897

Analyses are based on N = 48 for maternal plasma and N = 31 for umbilical cord plasma, unless stated otherwise. Associations between variables are reported as Spearman's correlation coefficients, with associated p-values. Bold values are significant at $p < 0.05$. *Correlations between C3d and maternal/umbilical cord plasma are based on N = 47/N = 30, due to missing data for one case. **Correlations with maternal/umbilical cord plasma are based on N = 48/N = 27. r: Spearman's correlation coefficient; STB: syncytiotrophoblast.

correlation between UCB complement and placental complement deposition.

Subjects with PE had reduced maternal blood concentrations of C4. This is in keeping with previous research [19,36], and is indicative of consumption through classical or lectin pathway activation. Maternal plasma C3 concentrations were also reduced in PE, which could indicate consumption. However, iC3b, a split product of C3b and biomarker of C3 activation, was not increased. This could be a gestational effect, as differences in iC3b between groups did not persist after adjusting for gestational age. Note that earlier research has not reported significant differences in C3 in PE, although assays measured both native and activated forms of the protein [36,37].

Although we did not find any significant differences between groups in plasma C5b-9 concentrations, previous studies have reported correlations between C5b-9 and PE outcomes, suggesting terminal pathway activity is a feature of severe disease [16–19]. There were no cases of HELLP syndrome in the Birmingham Cohort, and a relatively small proportion with early-onset PE, which may contribute to the lack of difference in C5b-9.

Immunohistochemical analysis demonstrated excessive deposition of C4d, C3d, and C9 in PE placentas, implying that placental complement activation extends as far as the terminal pathway. Prior studies have reported associations between placental C4d deposition and PE with adverse pregnancy outcomes [7,8]. It is postulated that poor placentation with associated oxidative stress and ischemia–reperfusion insults leads to excessive complement activation, primarily via the classical pathway [7,8]. Importantly, our data show that placental C4d deposition was negatively correlated with maternal properdin and C4, indicating that those with the greatest complement consumption within the maternal circulation also had concurrent high complement deposition within placental tissue.

We hypothesize that in PE, properdin binds to activated complement in placental tissue, resulting in depleted plasma concentrations. Classical pathway activation is thought to predominantly drive C4d deposition within the placenta and may be triggered by oxidative stress and repeated ischemia–reperfusion injury caused by defective placentation in PE [7,8]. The amplification loop exacerbates the cycle of activation via the alternative pathway. Interestingly, previous studies report alternative pathway dysregulation from early in pregnancy, suggesting that inappropriate complement activation begins early in PE as the placenta is developing, before clinical symptoms are detectable [14].

This study's major strength is the simultaneous analysis of complement across multiple tissue types, with findings being replicable in a separate validation cohort, and after controlling for gestational age. The primary limitation was the difference in gestation at blood draw between PE and control groups. If complement concentrations varied over the course of pregnancy, this difference would confound comparisons between groups. For maternal blood results, a regression analysis was used to account for any effect of gestation. However, the lack of crossover in gestational ages between the PE and control groups for the Birmingham cohort meant that these models extrapolated the trends for later gestations in PE, and earlier gestations in controls. Therefore, the accuracy of the adjustment cannot be confirmed, and over-adjustment or residual confounding may have been present in the final models.

5. Conclusions

This study, for the first time, demonstrates evidence of excessive placental complement deposition associated with significant concurrent changes in maternal and fetal circulating complement biomarkers in PE. Placental complement deposition is strongly correlated with complement activation within the maternal circulation, suggesting that those patients with the most excessive changes in circulating markers of complement activation also have the greatest extent of placental complement-mediated damage. Inhibition of complement activation might be a viable treatment option for women with PE, allowing improved pregnancy outcomes through blocking placental tissue damage and the endothelial dysfunction that results.

Funding

This study was supported by University Hospitals Birmingham Charities and Queen Elizabeth Hospital Kidney Patient Association. Prof Claire Harris receives funding from Kidney Research UK and Newcastle upon Tyne Hospitals NHS Charity.

CRediT authorship contribution statement

HB: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **RS:** Investigation, Methodology. **LX:** Investigation, Methodology. RR: Investigation, Methodology. **NS:** Conceptualization, Methodology, Supervision, Writing – review & editing. **JH:** Data curation, Formal analysis, writing – review & editing. **BH:** Investigation, Methodology, Writing – review & editing. **TM:** Investigation, Methodology, Writing – review & editing. **DAHN:** Investigation, Methodology, Writing – review & editing. **EW:** Methodology, Writing – review & editing. **NSS:** Methodology, Writing – review & editing. **KB:** Methodology, Writing – review & editing. **CLH:** Conceptualization, Methodology, Writing – review & editing. **EK:** Conceptualization, Methodology, Supervision, Writing – review & editing. **MD:** Conceptualization, Methodology, Supervision, Writing – review & editing. **GL:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful for the financial assistance provided by University Hospitals Birmingham Charities and the Queen Elizabeth Hospital Kidney Patient Association which allowed us to undertake this study. We gratefully acknowledge the contribution to this study made by the University of Birmingham's Human Biomaterials Resource Centre which has been supported through Birmingham Science City - Experimental Medicine Network of Excellence project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2023.04.001.

References

[1] E. Abalos, C. Cuesta, A.L. Grosso, D. Chou, L. Say, Global and regional estimates of preeclampsia and eclampsia: a systematic review, Europ. J. Obstet. Gynecol. Reproduct. Biol. 170 (1) (2013) 1–7.
[2] M.C. Brown, K.E. Best, M.S. Pearce, J. Waugh, S.C. Robson, R. Bell, Cardiovascular disease risk in women with pre-eclampsia: systematic review and meta-analysis, Eur. J. Epidemiol. 28 (1) (2013) 1–19.
[3] K. Wiles, L.C. Chappell, L. Lightstone, K. Bramham, Updates in Diagnosis and Management of Preeclampsia in Women with CKD, Clin. J. Am. Soc. Nephrol. 15 (9) (2020) 1371–1380.
[4] G. Girardi, D. Yarilin, J.M. Thurman, V.M. Holers, J.E. Salmon, Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction, J. Exp. Med. 203 (9) (2006) 2165–2175.
[5] Gelber SE, Brent E, Redecha P, Perino G, Tomlinson S, Davisson RL, et al. Prevention of Defective Placentation and Pregnancy Loss by Blocking Innate Immune Pathways in a Syngeneic Model of Placental Insufficiency. J Immunol. 2015;195(3):1129-38.
[6] J.L. Sones, A.A. Merriam, A. Seffens, D.-A. Brown-Grant, S.D. Butler, A.M. Zhao, X. Xu, C.J. Shawber, J.K. Grenier, N.C. Douglas, Angiogenic factor imbalance precedes complement deposition in placentae of the BPH/5 model of preeclampsia, FASEB J. 32 (5) (2018) 2574–2586.
[7] A. Buurma, D. Cohen, K. Veraar, D. Schonkeren, F.H. Claas, J.A. Bruijn, K. W. Bloemenkamp, H.J. Baelde, Preeclampsia is characterized by placental complement dysregulation, Hypertension 60 (5) (2012) 1332–1337.
[8] E.N. Kim, B.H. Yoon, J.Y. Lee, D. Hwang, K.C. Kim, JoonHo Lee, J.-Y. Shim, C. J. Kim, Placental C4d deposition is a feature of defective placentation: observations in cases of preeclampsia and miscarriage, Virchows Arch. 466 (6) (2015) 717–725.
[9] D. Sinha, M. Wells, W.P. Faulk, Immunological studies of human placentae: complement components in pre-eclamptic chorionic villi, Clin. Exp. Immunol. 56 (1984) 175–184.
[10] W. Wang, R.A. Imai, Y. Zhang, S.M. Ramin, S.C. Blackwell, L. Tao, R.E. Kellems, Y. Xia, Autoantibody-mediated complement C3a receptor activation contributes to the pathogenesis of preeclampsia, Hypertension 60 (3) (2012) 712–721.
[11] A.-R. Yonekura Collier, Z. Zsengeller, E. Pernicone, S. Salahuddin, E.V. Khankin, S. A. Karumanchi, Placental sFLT1 is associated with complement activation and syncytiotrophoblast damage in preeclampsia, Hypertens. Pregnancy 38 (3) (2019) 193–199.
[12] F. Tedesco, O. Radillo, G. Candussi, A. Nanzaro, T.E. Mollines, D. Pecorari, Immunohistochemical detection of terminal complement complex and S protein in normal and pre-eclamptic placentae, Clin. Exp. Immunol. 80 (1990) 236–240.
[13] A.I. Lokki, J. Heikkinen-Eloranta, H. Jarva, T. Saisto, M.L. Lokki, H. Laivuori, et al., Complement activation and regulation in preeclamptic placenta, Front. Immunol. 5 (2014) 312.
[14] A.M. Lynch, J.R. Murphy, T. Byers, R.S. Gibbs, M.C. Neville, P.C. Giclas, J. E. Salmon, V.M. Holers, Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia, Am. J. Obstet. Gynecol. 198 (4) (2008) 385.e1–385.e9.
[15] A.M. Lynch, B.D. Wagner, P.C. Giclas, N.A. West, R.S. Gibbs, V.M. Holers, The Relationship of Longitudinal Levels of Complement Bb During Pregnancy with Preeclampsia, Am. J. Reprod. Immunol. 75 (2) (2016) 104–111.
[16] R.M. Burwick, R.N. Fichorova, H.Y. Dawood, H.S. Yamamoto, B.B. Feinberg, Urinary Excretion of C5b-9 in Severe Preeclampsia Tipping the Balance of Complement Activation in Pregnancy, Hypertension 62 (6) (2013) 1040–1045.
[17] R.M. Burwick, J.A. Velásquez, C.M. Valencia, J. Gutiérrez-Marín, F. Edna-Estrada, J.L. Silva, J. Trujillo-Ossilvaro, J. Vargas-Rodríguez, Y. Bernal, A. Quintero, M. Rincón, J.E. Tolosa, Terminal Complement Activation in Preeclampsia, Obstet. Gynecol. 132 (6) (2018) 1477–1485.
[18] Y. He, B. Xu, D. Song, F. Yu, Q. Chen, M. Zhao, Expression of the complement system's activation factors in plasma of patients with early/late-onset severe pre-eclampsia, Am. J. Reprod. Immunol. 76 (3) (2016) 205–211.
[19] Z. Derzsy, Z. Prohaszka, J. Rigo, G. Fust, A. Molvarec, Activation of the complement system in normal pregnancy and preeclampsia, Mol. Immunol. 47 (7–8) (2010) 1500–1506.
[20] K.J. Denny, L.G. Coulthard, R.H. Finnell, L.K. Callaway, S.M. Taylor, T. M. Woodruff, Elevated complement factor C5a in maternal and umbilical cord plasma in preeclampsia, J. Reprod. Immunol. 97 (2) (2013) 211–216.
[21] M.C. Hoffman, K.K. Rumer, A. Kramer, A.M. Lynch, V.D. Winn, Maternal and fetal alternative complement pathway activation in early severe preeclampsia, Am. J. Reprod. Immunol. 71 (1) (2014) 55–60.
[22] I. Velickovic, M. Dalloul, K.A. Wong, O. Bakare, F. Schweis, M. Gamla, A. Alam, G. Medranda, J. Lekovic, W. Shuaib, A. Tedjasukmana, P. Little, D. Hanono, R. Wijetilaka, J. Weedon, J. Lin, R.D. Toledano, M. Zhang, Complement factor B activation in patients with preeclampsia, J. Reprod. Immunol. 109 (2015) 94–100.
[23] C.L. Harris, R.B. Pouw, D. Kavanagh, R. Sun, D. Ricklin, Developments in anti-complement therapy; from disease to clinical trial, Mol. Immunol. 102 (2018) 89–119.
[24] D. Ricklin, D.C. Mastellos, E.S. Reis, J.D. Lambris, The renaissance of complement therapeutics, Nat. Rev. Nephrol. 14 (1) (2018) 26–47.
[25] R.M. Burwick, B.B. Feinberg, Eculizumab for the treatment of preeclampsia/HELLP syndrome, Placenta 34 (2) (2013) 201–203.
[26] A.B. Lu, B. Lazarus, D.L. Rolnik, K.R. Palmer, Pregnancy Prolongation After Eculizumab Use in Early-Onset Preeclampsia, Obstet. Gynecol. 134 (6) (2019) 1215–1218.
[27] A.I. Lokki, M. Haapio, J. Heikkinen-Eloranta, Eculizumab Treatment for Postpartum HELLP Syndrome and aHUS—Case Report, Front. Immunol. 11 (2020).
[28] Gestational Hypertension and Preeclampsia, ACOG Practice Bulletin, Number 222, Obstet. Gynecol. 135 (6) (2020) e237–e260.
[29] National Institute for Health and Care Excellence (NICE). Hypertension in pregnancy: diagnosis and management. London: National Institute for Health and Care Excellence (NICE); 2019 [NICE guideline (NG133)].
[30] N.G. Than, O. Abdul Rahman, R. Magenheim, B. Nagy, T. Fule, B. Hargitai, M. Sammar, P. Hupuczi, A.L. Tarca, G. Szabo, I. Kovalszky, H. Meiri, I. Stiller, J. Rigo Jr., R. Romero, Z. Papp, Placental protein 13 (galectin-13) has decreased placental expression but increased shedding and maternal serum concentrations in patients presenting with preterm pre-eclampsia and HELLP syndrome, Virchows Arch. 453 (4) (2008) 387–400.
[31] C.E. Matrai, J.H. Rand, R.N. Baergen, Absence of Distinct Immunohistochemical Distribution of Annexin A5, C3b, C4d, and C5b-9 in Placentas From Patients With Antiphospholipid Antibodies, Preeclampsia, and Systemic Lupus Erythematosus, Pediatr. Dev. Pathol. 22 (5) (2019) 431–439.
[32] E. Pietik, J.R. Prins, H. van Goor, G.A. Dekker, M.R. Daha, M.A.J. Seelen, et al., Dysregulation of Complement Activation and Placental Dysfunction: A Potential Target to Treat Preeclampsia? Front. Immunol. 10 (2019) 3098.
[33] G. Girardi, Complement activation, a threat to pregnancy, Semin. Immunopathol. 40 (1) (2018) 103–111.
[34] Y.-D. He, B.-N. Xu, D.I. Song, Y.-Q. Wang, F. Yu, Q. Chen, M.-H. Zhao, Normal range of complement components during pregnancy: A prospective study, Am. J. Reprod. Immunol. 83 (2) (2020) e13202.
[35] W.P. Kolb, P.R. Morrow, J.D. Tamerius, Ba and Bb fragments of factor B activation: fragment production, biological activities, neoepitope expression and quantitation in clinical samples, Complement Inflamm. 6 (3) (1989) 175–204.
[36] N. Sarween, M.T. Drayson, J. Hodson, E.M. Knox, T. Plant, C.J. Day, G.W. Lipkin, Humoral immunity in late-onset Pre-eclampsia and linkage with angiogenic and inflammatory markers, Am. J. Reprod. Immunol. 80 (5) (2018) e13041.
[37] R. Boij, J. Svensson, K. Nilsson-Ekdahl, K. Sandholm, T.L. Lindahl, E. Palonek, M. Garle, G. Berg, J. Ernerudh, M. Jenmalm, L. Matthiesen, Biomarkers of coagulation, inflammation, and angiogenesis are independently associated with preeclampsia, Am. J. Reprod. Immunol. 68 (3) (2012) 258–270.