COLLEGE OF BASIC AND APPLIED SCIENCES

SCHOOL OF BIOLOGICAL SCIENCES

CIRCULATING ENDOTHELIAL PROGENITOR CELLS AND PREECLAMPSIA IN WOMEN WITH PLACENTAL MALARIA

THIS DISSERTATION IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

BY

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MARCH, 2019
DECLARATION

I hereby declare that this thesis is an original record of my research work carried out at the West African Centre for Cell Biology of Infectious Pathogens, the Noguchi Memorial Institute for Medical Research and the Korle-Bu Teaching Hospital. All resources and literature have been clearly specified and duly acknowledged. I further declare that this research has neither in whole nor in part been submitted for any previous degree or a degree elsewhere.

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DEDICATION

To the Almighty God who gave me the strength to sail through.

To my ‘sweetheart’ - John Addo Obiri and adorable kids - Nana Yaw, Nanaabena and Nanayaa. Thank you for the motivation and permission granted to work late and sometimes be away.

To my parents in-law Mr. & Mrs. Obiri Addo and my parents Mr. & Mrs. Osei for their immense support.

God bless you all.

$$$$

In loving memory of Helena Ivy Chinbuah
ACKNOWLEDGEMENTS

“Without ambition one starts nothing. Without work one finishes nothing. The prize will not be sent to you. You have to win it.”

Ralph Waldo Emerson

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACOG</td>
<td>American College of Obstetricians and Gynecologists</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanine</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted Reproductive Technology</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BP</td>
<td>Blood Pressure</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C Motif Chemokine Ligand 2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>cECs</td>
<td>Circulating Endothelial Cells</td>
</tr>
<tr>
<td>cEPCs</td>
<td>Circulating Endothelial Progenitor Cells</td>
</tr>
<tr>
<td>CRP</td>
<td>C - Reactive Protein</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin Sulphate A</td>
</tr>
<tr>
<td>CXCL10</td>
<td>C-X-C Motif Chemokine10</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic Blood Pressure</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESRI</td>
<td>Environmental Systems Research Institute</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocynate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HDP</td>
<td>Hypertensive disorders in pregnancy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HELLP</td>
<td>Haemolysis, Elevated Liver Enzymes and Low Platelet Count</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPF</td>
<td>High Powered Field</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>iEPCs</td>
<td>immature Endothelial Progenitor Cells</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPTp</td>
<td>Intermittent Preventive Treatment in Pregnancy</td>
</tr>
<tr>
<td>ISSHP</td>
<td>International Society for the Study of Hypertension in Pregnancy</td>
</tr>
<tr>
<td>KBTH</td>
<td>Korle-Bu Teaching Hospital</td>
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<tr>
<td>MDGs</td>
<td>Millennium Development Goals</td>
</tr>
<tr>
<td>mEPCs</td>
<td>matured Endothelial Progenitor Cells</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal Intensive Care Unit</td>
</tr>
<tr>
<td>NMIMR</td>
<td>Noguchi Memorial Institute for Medical Research</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PE</td>
<td>Preeclampsia/Preeclamptic</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein complex</td>
</tr>
<tr>
<td>PfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental Growth Factor</td>
</tr>
<tr>
<td>PM</td>
<td>Placental Malaria</td>
</tr>
<tr>
<td>PP13</td>
<td>Placental Protein 13</td>
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Thesis Structure

This thesis is presented in an article style format with three manuscripts in preparation for submission. Manuscripts are preceded with a general introduction section and succeeded by general discussion, conclusion and recommendation sections.
ABSTRACT

Background

Placental malaria and preeclampsia are major complications of pregnancy associated with high incidence of maternal/foetal morbidity and mortality particularly in sub-Saharan Africa. Similarities in pathophysiology such as systemic inflammation and generalized endothelial activation/dysfunction exist and are exacerbated in concurrent situations. The combined pathologic and immunologic effects of these pregnancy disorders, prevalent in malaria endemic regions, have not been tested. This study evaluated the pathologic risk associated with *Plasmodium falciparum* infection in the placenta and the outcome of preeclampsia. Immune mediators associated with placental malaria and the risk of preeclampsia were evaluated. In addition, the relationship between endothelial cell phenotypes implicated in vascular activation/injury/damage/repair and the factors that mediate their release were assessed.

Methods

A total of 140 pregnant women (18 - 42 years) diagnosed with and without preeclampsia were recruited into the study in Accra, Ghana. Peripheral blood samples were collected at delivery while cord blood, placental intervillous blood and placental biopsies were collected after delivery. Circulating endothelial cells (cECs) and circulating endothelial progenitor cell phenotypes (cEPCs, mEPCs and iEPCs) were quantified by flow cytometry. Immunological (inflammatory and angiogenic) factors were tested by multiplex ELISA using plasma separated from whole blood. Placental biopsies were histologically evaluated for unique placental lesions and placental malaria (classified as active and past infections).

Results

Of 133 placentas scored for placental malaria, women diagnosed with preeclampsia had 39 (29.3%) and 15 (11.3%) active and past infections respectively while the non-preeclamptic
women had 25 (18.8%) active infections and 6 (4.5%) past infections. Both active (adjusted odds ratio (AOR) 6.7, 95% CI 2.3 – 18.9; \( P < 0.0001 \)) and past infections (AOR 11.6, 95% CI 3.0 – 45.8; \( P < 0.0001 \)) increased the risk of preeclampsia. This association was enhanced in primigravidae (AOR 6.6, 95% CI 2.4 – 18.2; \( P < 0.0001 \)) and in women with pathological alterations in the placenta (AOR 3.0, 95% CI 1.2 – 7.5; \( P < 0.019 \)). The preeclamptic pregnancies with active placental parasites showed higher levels of proinflammatory and anti-angiogenic markers. In multivariate analysis, active parasite infection (AOR = 7.14, 95% CI = 1.1 – 44.7; \( P = 0.04 \)), past infection (AOR = 12.9, 95% CI = 1.1 – 155.5; \( P = 0.04 \)), primigravidity (AOR = 7.2, 95% CI = 1.1 – 48.0; \( P = 0.04 \)) and increased levels of the plasminogen activator inhibitor (PAI)-1 molecule (AOR = 7.1, 95% CI = 1.3 – 38.3; \( P = 0.02 \)) were all associated with placental malaria.

Furthermore, proportions of endothelial cell phenotypes were higher in the placenta and cord compared to peripheral blood. Alterations in their levels correlated strongly with angiogenic factors that stimulate their release mostly in non-preeclamptic compared to preeclamptic pregnancies.

**Conclusion**

Findings from this study have demonstrated that the pathophysiology of preeclampsia is pathologically and immunologically exacerbated in women exposed to placental malaria. In addition, endothelial cell phenotypes that contribute to vascular homeostasis and the factors that mediate their release into circulation are altered. Altogether, this study provides a new paradigm in assessing preeclampsia in malaria endemic regions.
CHAPTER ONE

1.0 Introduction

1.1 Trends in maternal mortality

Maternal mortality is referred to as death during pregnancy, childbirth or 42 days post-delivery (WHO, 2012). Global trends have necessitated the urgent need for pragmatic steps in reducing maternal mortality. This was encapsulated in the Millennium Development Goals (MDGs) and the current Sustainable Development Goals (SDGs). Global maternal mortality reduced by 44% between 1990 and 2015 (WHO, 2017b). However, the World Health Organization (WHO) reports a daily maternal death rate of approximately 830 from pregnancy and childbirth attributed to preventable causes (WHO, 2017b). About 99% of these deaths occur within the African region (Kassebaum et al., 2014, Alkema et al., 2016).

Several interventions put in place to improve maternal care facilitated Ghana’s significant advancement in reducing maternal mortality by 2.7% over a twenty five-year span. Notwithstanding, the MDGs in this area was not achieved. Between 1990 and 2015, maternal mortality reduced from 760 to 319 per 100,000 live births, (Figure 1.1) against a target of 190 deaths per 100,000 live births (UNDP, 2015, WHO, 2018c). Failure to achieve the set target may be attributed among others, to the high morbidity among pregnant women with either no known aetiology or inadequate intervention and treatment modalities. Building up from the MDGs’ progress requires that major stakeholders harness all efforts to meet SDG 3.1 target of reducing maternal mortality ratio to 70 per 100,000 live births by 2030.
Figure 1.1. Global trends in maternal mortality.

Maternal mortality ratio (per 100,000 live births), 2015. Arrow showing Ghana with mortality estimated at 319 per 100,000 live births. Adapted from Global Health Observatory: Maternal and reproductive health (WHO, 2018a).

1.2 Understanding the causes of maternal mortality

Direct maternal mortality is due to obstetric complications of pregnancy, intervention omissions, incorrect treatment or a combination of these events (Cross et al., 2010, WHO, 2012). On the other hand, indirect maternal mortality is caused by aggravated pregnancy conditions with no direct obstetric aetiology (WHO, 2012). This classification was well conceived, however, challenges with subjective classification that may mask the importance of indirect causes should not be ignored.
In a comprehensive WHO global report on the causes of maternal mortality, both direct and indirect obstetric complications contributed significantly to maternal mortality (Kassebaum et al., 2014, Say et al., 2014). This clearly showcased the importance of both direct and indirect obstetric complications to maternal mortality. Over the years, sub-Saharan Africa including Ghana, has seen a major proportion of maternal deaths attributed to direct causes such as haemorrhage, hypertensive disorders of pregnancy, abortions and some indirect causes such as HIV, malaria, anaemia and heart disease (Nour, 2008, Der et al., 2013, Say et al., 2014). However, there may also be a considerable increase in comorbidities due to both direct and indirect causes. For instance, high risk of gestational hypertension and preeclampsia were estimated in the United States of America among women with comorbid migraine and asthma (Czerwinski et al., 2012). Similarly in the United Kingdom, maternal deaths due to direct obstetric causes were significantly associated with comorbidities (Nair et al., 2015).

Evidence from low and middle-income countries suggest that hospital admission due to preeclampsia with comorbidity was associated with an increased risk of maternal death (Yego et al., 2014). On the contrary, a 5-year review of the causes of maternal mortality in Ghana independently reported on direct and indirect causes of mortality with no consideration for comorbidities (Der et al., 2013). However, complications that might have contributed to these deaths could have resulted from comorbidities as its effects were not measured. Furthermore, increasing incidence of comorbidities in relation to maternal mortality has been shown to vary significantly by race (Metcalf et al., 2018) possibly due to genetic differences or factors yet unknown. Thus, in addition to intensive management by health care practitioners, scientists need to decipher the pathophysiologic contributions of other obstetric complications that may contribute to maternal mortality.
1.3 Changing patterns of maternal morbidity and mortality

Maternal morbidity and mortality patterns are constantly changing. Increasing rates of maternal mortality due to indirect as well as comorbid causes are currently being reported (Say et al., 2014). This may be due to a realistic upward trend in incidence or inadequate reportage resulting from the unavailability of complete data in the past. Transition from maternal morbidity to mortality is often rapid and this requires critical monitoring for prevention and treatment. Postpartum haemorrhage, infections, hypertensive disorders (particularly preeclampsia and eclampsia), unsafe abortion and delivery complications account for over 75% of maternal deaths (Say et al., 2014, WHO, 2017b). Some of these conditions are either preventable or treatable, but there are others that require strict management due to unknown aetiology and lack of effective treatment modalities. In such cases, risk factor identification could aid in the prevention and management of pregnancy complications to reduce maternal and foetal mortality.

Hypertensive disorders in pregnancy (HDP) with reference to preeclampsia, have no definite aetiology and falls into the risk factor identification and management category for reduced maternal mortality. While increasing other obstetric risks, preeclampsia could also be a consequence of other indirect maternal complications. Areas with improved health systems and experts, have effectively managed obstetric haemorrhage and infections by conscious efforts. Hence, management of HDP has become critical. For example major tertiary hospitals in Ghana have observed HDP as the leading cause of maternal mortality ahead of postpartum haemorrhage (Lee et al., 2012, Adu-Bonsaffoh et al., 2013). Preeclampsia has been shown to increase the risk of postpartum haemorrhage while symptoms of preeclampsia and eclampsia have also been reported in women who have died from postpartum haemorrhage (auf Altenstadt et al., 2013, von Dadelszen and Magee, 2016). Therefore, postpartum haemorrhage should be
an important consideration in preeclampsia management.

1.4 The Impact of hypertensive disorders in pregnancy

Hypertensive disorders in pregnancy (HDP) which form a group of clinical syndromes with a hypertensive factor have been listed by the WHO as one of the major causes of maternal mortality (WHO, 2017b). Women who develop hypertensive disorders during pregnancy are at increased risk for long term cardiovascular disease and kidney dysfunc tion (Valdiviezo et al., 2012, Veerbeek et al., 2015, Schokker et al., 2015, Behrens et al., 2016, Thoullass et al., 2016, Tooher et al., 2017). The global prevalence ranges between 0.2 and 19.4% with women from developing countries being predominantly affected (Khan et al., 2006, Umesawa and Kobashi, 2017). Several regional and tertiary hospitals in Ghana have reported HDP as either the leading or one of the leading causes of maternal mortality at their facility within the last two decades (Lassey and Obed, 2004, Ganyaglo and Hill, 2012, Kwawununu et al., 2012, Lee et al., 2012, Adu-Bonsaffoh et al., 2013). Although the incidence of these disorders varies across countries with the greatest burden in developing countries, the long-term consequences on maternal and neonatal health may not be discriminatory.

Internationally, HDP is classified into four groups; these are gestational hypertension, preeclampsia, chronic hypertension and chronic hypertension with superimposed preeclampsia (ACOG, 2013, Tranquilli et al., 2014, Brown et al., 2018). Typically, these disorders are accompanied by elevated blood pressure, further differentiated by the onset of disease, associated organ damage or proteinuria. In addition, these disorders are associated with adverse maternal and perinatal morbidity. Preeclampsia accounts for the most devastating effects of HDP, particularly in low and middle income countries (Browne et al., 2015). Prediction of HDP and its adverse outcomes is crucial for optimal management (Ukah et al., 2018),
development and implementation of interventions particularly those geared toward preventing or minimizing the impact of preeclampsia. This, therefore, calls for more research into the understanding of underlying factor(s) promoting or aggravating preeclampsia.

1.4.1 Definition of preeclampsia

Clinical preeclampsia is characterised by an onset of hypertension (blood pressure $\geq 140/90$ mmHg) and proteinuria (Protein in urine $\geq 0.3$ g/24 hrs or 1+ dipstick) after 20 weeks of gestation in a woman who was previously healthy (ACOG, 2013, Tranquilli et al., 2014). It may also be diagnosed during systemic organ failure in the absence of proteinuria. Specifically, onset of high blood pressure together with low levels of blood platelets, impaired liver and kidney function, fluid retention in the lungs, cerebral symptoms or dysfunctional uterus and placenta can define preeclampsia (ACOG, 2013, Tranquilli et al., 2014, Brown et al., 2018). These additional symptoms characterise preeclampsia with severe features that may lead to the development of renal disease, haemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome and eclamptic seizures.

Early (<34 weeks gestation) or late (≥ 34 weeks gestation) onset of clinical presentation has been used to classify preeclampsia into two distinct phenotypes (Von Dadelszen et al., 2003, Roberts and Hubel, 2009). While early-onset preeclampsia has been linked with more placental and genetic factors, late onset preeclampsia has been associated with a more maternal and environmental factor with less debilitating and life threatening maternal and foetal effects (Boyd et al., 2013, Lisonkova and Joseph, 2013, Staff et al., 2013, Staff and Redman, 2018). Some groups have classified preeclampsia into mild or severe based on severity. However, this classification has not been encouraged as every presentation of preeclampsia can be life threatening (ACOG, 2013, Brown et al., 2018).
1.4.2 Epidemiology and risk factors of preeclampsia

Preeclampsia complicates about 2-10% of pregnancies worldwide (WHO, 2005, Steegers et al., 2010, Abalos et al., 2013, Stevens et al., 2017) with an annual mortality of > 500,000 foetal and neonatal deaths and > 70,000 maternal deaths (Brown et al., 2018). It is now one of the leading causes of maternal mortality in Ghana (Adu-Bonsaffoh et al., 2013, 2014). Described as a ‘disease of theories’, preeclampsia has no convincing evidence of a known aetiology. Several studies have proposed a multifactorial aetiology and a multifaceted presentation of disease (Pennington et al., 2012, Leavey et al., 2016). This presents a great challenge in the diagnosis despite standardized classification of the disorder. For instance, atypical presentation of preeclampsia suggests that the condition can be diagnosed with non-classical features such as gestational hypertension without proteinuria or gestational proteinuria without hypertension (Stella and Sibai, 2006, Sibai and Stella, 2009) generating overlap between other HDP.

Risk factor identification either at pre-conception counselling or early on in pregnancy have been paramount in identifying women at risk of preeclampsia. These factors have been used to predict women who are at possible risk of preeclampsia development after 20 weeks of gestation. Some of these risk factors have been highlighted (Table 1.1) from major systematic reviews and guidelines (Duckitt and Harrington, 2005, Milne et al., 2005, ACOG, 2013, Shamsi et al., 2013, Paré et al., 2014, Bartsch et al., 2016). These factors may either be independently associated with preeclampsia or are dependent on other risk factors for the development of this disorder.
<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Pattern of Risk</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>First Pregnancy</td>
<td>Risk of preeclampsia (PE) is higher in primigravids. This further predisposed</td>
<td>(Duckitt and Harrington, 2005), (Hernández-Díaz et al., 2009), (Boghossian et al., 2015)</td>
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<td></td>
<td>them to the condition in subsequent pregnancies.</td>
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<td>Low socioeconomic status</td>
<td>Low educational level, low monthly income, higher calorie intake/less protein</td>
<td>(Silva et al., 2008), (Bej et al., 2013), (Ramesh et al., 2014), (Kim et al., 2018)</td>
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<td></td>
<td>intake are some of the socioeconomic factors that increase the risk of PE.</td>
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<td></td>
<td>There are conflicting reports regarding the effects of access to health care on</td>
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<td></td>
<td>the risk of PE.</td>
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<td>Personal and Family History of PE</td>
<td>PE is linked to family history as well as previous history of the condition.</td>
<td>(Lie et al., 1998)</td>
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<td></td>
<td>Paternal genes in the developing foetus may also contribute to PE development.</td>
<td>(Mogren et al., 1999)</td>
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<td>These observations are pronounced in early onset PE.</td>
<td>(Boyd et al., 2013)</td>
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<td></td>
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<td>(English et al., 2015)</td>
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<td>Race</td>
<td>Racial disparities exist. Pregnant women of African-American decent or the</td>
<td>(Tanaka et al., 2007)</td>
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<td>black race are reported be at increased risk of PE with the tendency for more</td>
<td>(Lisonkova and Joseph, 2013)</td>
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<td></td>
<td>severe complications. Increased risk of placental vascular malperfusion is</td>
<td>(Shahul et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>associated with the black race.</td>
<td>(Assibey-Mensah et al., 2018)</td>
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<tr>
<td>Obesity</td>
<td>It has been consistently shown that as body mass index (BMI) increases, the</td>
<td>(O'Brien et al., 2003)</td>
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<td></td>
<td>risk of PE also increases.</td>
<td>(Canto-Cetina et al., 2018)</td>
</tr>
<tr>
<td>Maternal Age</td>
<td>Advanced maternal age in first time pregnancy; particularly those ≥ 35 years</td>
<td>(Jolly et al., 2000)</td>
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<td></td>
<td>have a higher risk of PE</td>
<td>(Jacobsson et al., 2004)</td>
</tr>
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<td></td>
<td></td>
<td>(Lamminpää et al., 2012)</td>
</tr>
<tr>
<td>Multiple Gestation</td>
<td>Twin gestation is shown to increase the risk of PE. Hydatidiform mole either</td>
<td>(Conde-Agudelo et al., 2000)</td>
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<td></td>
<td>independently or coexisting with a live foetus is also associated with PE.</td>
<td>(Coonrod et al., 1995)</td>
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<td></td>
<td></td>
<td>(Booth and Eskandar, 2018)</td>
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<tr>
<td>Pre-existing medical conditions</td>
<td>Preexisting diabetes mellitus, renal disease, chronic hypertension, sickle</td>
<td>(Skomsvoll et al., 2000)</td>
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<td>cell disease and autoimmune diseases such as rheumatic disease,</td>
<td>(Duckitt and Harrington, 2005)</td>
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<td>antiphospholipid syndrome and systemic lupus erythematosus have been shown to</td>
<td>(Oteng-Ntim et al., 2015)</td>
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<td></td>
<td>increase the risk of PE.</td>
<td>(Marchetti et al., 2016)</td>
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<td>(Saccon et al., 2017)</td>
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<td>Assisted reproductive technology</td>
<td>Patronage of ARTs have increased, but these have been independently associated</td>
<td>(Carbone et al., 2011)</td>
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<tr>
<td>(ART)</td>
<td>with PE. ARTs in combination with other risk factors such as advanced maternal</td>
<td>(Paré et al., 2014)</td>
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<td></td>
<td>age, obesity and multiple gestation further compound PE complications.</td>
<td>(Jie et al., 2015)</td>
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<tr>
<td></td>
<td></td>
<td>(Martin et al., 2016)</td>
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<td></td>
<td></td>
<td>(Omani-Samani et al., 2018)</td>
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<tr>
<td>Interval between pregnancies</td>
<td>Long interval between pregnancies have been associated with PE. It is also</td>
<td>(Skjærven et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>hypothesized that the risk is increased when a partner is changed.</td>
<td>(Cormick et al., 2016)</td>
</tr>
<tr>
<td>Infection</td>
<td>While most infections such as viral, bacterial and parasitic infections have</td>
<td>(Conde-Agudelo et al., 2008)</td>
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<tr>
<td></td>
<td>been indicated to increase the risk of PE, others have been reported to reduce</td>
<td>(Minassian et al., 2013)</td>
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<td></td>
<td>the risk.</td>
<td>(Huang et al., 2016)</td>
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Obstetric outcomes associated with complications from preeclamptic pregnancies include eclampsia, placental abruption, postpartum haemorrhage, stroke, HELLP syndrome, acute renal failure and mortality (Ghulmiyyah and Sibai, 2012, auf Altenstadt et al., 2013, Abalos et al., 2014). Foetal and neonatal effects include intrauterine growth restriction, premature birth, low birth weight, birth asphyxia, stillbirth and neonatal death (de Souza Rugolo et al., 2011, Lisonkova and Joseph, 2013).

1.5 Pregnancy, placenta and the pathophysiology of preeclampsia

Pregnancy is a normal physiological process for foetal development. This results in maternal anatomical and physiological adaptations that can either be normal or mimic symptoms of a medical disorder (Soma-Pillay et al., 2016). The placenta, which is a specialized and transitional organ in pregnancy, provides support for normal foetal development. Foetal membranes and maternal endometrium are conjoined into an intimate association (Reynolds and Redmer, 2001). Thus, foetal trophoblasts invade maternal uterine tissue and remodel the spiral arteries. This allows adequate supply of blood enriched with nutrients and required gases and simultaneous exportation of foetal metabolic waste (Gude et al., 2004, Mourad et al., 2016). Specialized anatomical features such as the placental villi (Figure 1.2) arise from this development. Open ended maternal blood vessels resulting from trophoblast invasion release blood into the intervillous spaces which interact with the outer trophoblast layer (syncytiotrophoblast). Continuous vascularisation becomes critical throughout gestation to support these foetal metabolic needs. In animals, endometrial capillaries shown from histochemical staining in pregnant and non-pregnant ewes suggest an appreciable increase in endometrial capillaries in pregnant compared to non-pregnant ewes (Reynolds and Redmer, 2001). Deficient vascularisation throughout gestation is associated with increased uterine vascular resistance and reduced uterine blood flow; the genesis of most placental related
pregnancy complications such as maternal preeclampsia and foetal intrauterine growth restriction (Khong et al., 1986, Reynolds and Redmer, 2001). This phenomenon reduces the ability of the placenta to perform its physiological functions and puts the pregnancy at risk.

**Figure 1.2: Placental anatomy in normal pregnancy.**

Adapted from Mourad et al. (2016). The placenta consists of a maternal decidual region while the foetal side forms the chorionic region. There is foetal and maternal blood vessel interaction that ensure exchange of nutrients and metabolic waste.

Preeclampsia is a well-known example of pregnancy and placental specific syndrome of dysfunction. It is hypothesized to resolve after the delivery of the placenta and not just the absence of foetus or molar pregnancy (Lam et al., 2005, Roberts and Escudero, 2012). It
remains a disease of theories with no definite aetiology but with multifactorial effects. The pathophysiology of preeclampsia is however linked to inadequate vascular remodelling and systemic maternal endothelial dysfunction that results from immunological and angiogenic imbalance during pregnancy (Pennington et al., 2012). These pathophysiologic events are the hallmarks of disease pathogenesis although nutritional, hormonal, genetic and other factors have been implicated (Conde-Aguadelo et al., 2008, Mutter and Karumanchi, 2008, Gupta and Chari, 2015, Scioscia et al., 2015).

The pathogenesis of preeclampsia is mainly defined by a two-stage model which involves the pre-clinical and clinical phases (Figure 1.3). Prior to 20 weeks of gestation, an asymptomatic phase of preeclampsia which is still a mystery in the pathophysiology is initiated. There is currently no test to predict this stage of preeclampsia. Characterised by abnormal placentation and dysregulation of placental molecules, the pre-clinical phase is responsible for the release of placental factors that diffuse into maternal circulation to initiate the second phase (Tannetta and Sargent, 2013). The second phase is the symptomatic maternal syndrome that results in changes such as hypertension, proteinuria and end organ damage or possibly death (Abalos et al., 2014). Roberts and Hubel (2009) in their seminal review, implied that the maternal syndrome is not initiated solely by the pre-clinical phase, however, genetic, behavioural, environmental and other maternal factors interact. More recently, this has been supported by placental gene expression profiling study which showed distinct pathophysiology and influence of maternal factors resulting in different aetiological clusters of preeclampsia (Leavey et al., 2016). Although these clusters may exist, the maternal constituent factors contributing to the pathophysiology of preeclampsia may overlap.
As earlier mentioned, several theories have been proposed for the aetiology of preeclampsia, classifying them as different phenotypes of the disease. There is no optimal consensus on these theories in literature. Pennington et al. (2012) suggest that a number of initial insults converge to emit the clinical syndrome of preeclampsia characterised by inadequate vascular remodelling and systemic anti-angiogenic response. However, the mechanisms responsible for these changes have not been elucidated but these abnormalities might interact.

Figure 1.3: The two-stage disease model of preeclampsia.

Proposed by Roberts and Hubel (2009). The first pre-clinical stage is asymptomatic and associated with placental malfunction which is later translated into a symptomatic stage of high blood pressure, proteinuria, end organ damage and death if management fails.
1.5.1 Abnormal placentation in preeclampsia

Abnormal placental development in preeclampsia (Figure 1.4) is a very popular phenomenon. Placental remodelling of the uterine vasculature is characterised by both morphological and functional changes as part of adaptations for foetal survival. In uncomplicated pregnancies, uterine artery diameter is approximately twice the pre-pregnancy size yielding a low resistance and high capacitance uterine circulation for increased blood flow (Palmer et al., 1992, Zhou et al., 1997, Konje et al., 2001). This has been reported to begin in the late first trimester and end between 18 to 20 weeks gestation in the second trimester (Lam et al., 2005). Similar changes have been reported in other mammals (Griendling et al., 1985, Moll, 2003). On the contrary, pathologic defects such as preeclampsia and intrauterine growth restriction result in placental malperfusion associated with a high resistance, low capacitance uterine circulation (Khong et al., 1986, Cerdeira and Karumanchi, 2012). This is largely due to reduced or deficient invasion by the foetal trophoblasts which replace maternal endothelial cells. The invasion of the trophoblast known as pseudovasculogenesis is required for increased surface area to maintain a hypoxic and ischaemic placental environment serving as a barrier between the maternal and foetal interface (Kaufmann et al., 2003). Although these changes are deficient in preeclamptic placentas, it has not been established if these abnormalities solely account for the development of preeclampsia, as other spectrum of pathologies have been associated with these defects (Papanikolaou et al., 2018).
Figure 1.4: The pathophysiology of preeclampsia.

Comparing foetal trophoblast invasion and remodelling of maternal spiral arteries in the placenta. Non-preeclamptic (non-PE) placenta showing adequate trophoblast invasion and a dilated spiral artery for enhanced foetal adaptation. Preeclamptic (PE) placenta showing inadequate invasion of foetal trophoblast and remodelling for a dilated spiral artery. Spiral artery appears constricted resulting in reduced blood flow which is inadequate for foetal support. Image adapted from Lam et al. (2005).
1.5.2 Angiogenic factors in preeclampsia

Functional roles of the anatomic structures of the placenta are tightly regulated. The angiogenic and vasculogenic networks ensure that the placenta is highly vascularised for adequate functionality, a process mostly regulated by angiogenic molecules. This explains why numerous studies have identified factors that compromise placental development as risk factors for preeclampsia (Reynolds et al., 2010).

Vasculogenesis is the de-novo formation of new blood vessels while angiogenesis is the sprouting of new blood vessels from preexisting ones. In their review, Cerdeira and Karumanchi (2012) indicated that endothelial tube segments formed by vasculogenesis are transformed into an organized network by angiogenesis. Also, blood vessels in the foetal compartment of the placenta are formed through vasculogenesis by the recruitment of the mesenchymal derived angioblasts while those in the maternal compartment, represented by decidualized endometrium, arise through angiogenesis (Lala and Nandi, 2016).

The theory of endothelial dysfunction points to preeclampsia being associated with impaired angiogenesis. Dysregulation of angiogenesis promoting and restraining factors have been identified as useful biomarkers of preeclampsia (Maynard et al., 2003, Ahmad and Ahmed, 2004, Maynard and Karumanchi, 2011). Overexpression of soluble vascular endothelial growth factor receptor one (sVEGFR-1), a vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) antagonist has been shown by several research groups in both animal models and humans to be abnormally increased in preeclampsia. This angiogenic antagonist is mainly secreted by the placenta into systemic circulation (Clark et al., 1998) resulting in symptomatic preeclampsia. Recently the usefulness of sVEGFR-1/placental growth factor
(PlGF) ratio in predicting preeclampsia in pregnancy was highlighted. A sensitivity of 0.80 (95% CI 0.68-0.88) and specificity of 0.92 (95% CI 0.87 – 0.97) sVEGFR-1/PIGF ratio in predicting preeclampsia (Agrawal et al., 2017) was reported. The angiopoietins (Ang) and receptor, fibroblast growth factor (FGF), stromal cell derived factor - 1 (SDF - 1), platelet derived growth factor (PDGF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), matrix metalloproteinases (MMPs), plasminogen activator inhibitor (PAI)-1, CCL2, CXCL10 and CD146 are among the numerous angiogenic molecules linked to the pathogenesis of preeclampsia (Hohlagschwandtner et al., 2002, Pasquier et al., 2005, Schanz et al., 2007, Wikström et al., 2009, Mosimann et al., 2013, Moreno-Eutimio et al., 2014). While some of these markers are expressed to maintain endothelial integrity, others have been shown to dysregulate angiogenesis and trigger activation of the endothelium which will subsequently lead to endothelial dysfunction characterised in preeclampsia.

1.5.3 Immune response in preeclampsia

The placenta also modulates the maternal immune response to avoid foetal rejection by producing and secreting cytokines, hormones and other signaling molecules required for pregnancy maintenance (Jansson and Powell, 2007, LaMarca et al., 2016). Aside the genetic, environmental and other factors, inflammation has a significant role in the pathogenesis of preeclampsia. According to Redman and Sargant (2010), the inflammatory contribution to overt preeclampsia is non-specific in contrast to specific maternal immune responses to foetal alloantigens in early pregnancy. Normal invasion of the trophoblast and foetal adaption require immune cells such as macrophages, dendritic cells, uterine natural killer cells on the innate arm and helper T cells, cytotoxic T cells and regulatory T cells on the adaptive arm of the immune system (Robertson et al., 2015, Shah et al., 2017). This is augmented by the release of cytokines and angiogenic molecules and other immune mediators (LaMarca et al., 2016). It is not certain
whether this immune adaption is due to tolerance or a suppression of the immune system. To verify this, a recent study has shown that immune cell phenotypes shift from an immune tolerance to an immune activation state from mid to late pregnancy (Shah et al., 2017). However, immune suppression occurs simultaneously by the upregulation of suppressive and regulatory molecules such as interleukin (IL)-10.

In preeclampsia, there is immune dysregulation where Th1 cells dominate with the release of proinflammatory and anti-angiogenic molecules to play a role in reduced trophoblast invasion, decreased vascular remodelling and endothelial dysfunction (Verlohren et al., 2009). The various bioactivities of these molecules further respond to a hypoxic and ischaemic environment perpetuated by preeclampsia pathology. Reactive oxygen species, vasoconstrictors and autoantibodies produced, precipitate and potentiate the symptomatic hypertension seen in preeclampsia (Sharma et al., 2007, Ferguson et al., 2017). This is proposed to be due to the release of excessive proinflammatory cytokines from the placenta into systemic circulation. Most studies have reported increase in the levels of proinflammatory and decrease in anti-inflammatory molecules in maternal sera of preeclamptic women (Sargent et al., 2006, Moreno-Eutimio et al., 2014, Cornelius, 2018). Experiments by Moreno-Eutimio et al. (2014), showed that regulatory T cells are fewer in patients with preeclampsia suggesting that systemic inflammation is associated with the pathogenesis of the disorder.

Understanding the comprehensive physiology of preeclampsia throughout gestation requires an ideal model. Reduced Uterine Perfusion Pressure (RUPP) rat model is by far the best characterised for the study of human preeclampsia (Li et al., 2012). This has led to the understanding of some characteristic features observed in preeclampsia such as hypertension, proteinuria, chronic immune activation, endothelial and renal dysfunction. Despite these
achievements, there are still limitations associated with mimicking the duration, severity and other pathophysiologic properties of preeclampsia.

The synergistic effect of poor placentation is associated with an ischaemic and hypoxic environment, the release of proinflammatory cytokines and anti-angiogenic factors into maternal circulation (Cerdeira and Karumanchi, 2012). The possible factors that trigger this process are numerous (as earlier outlined in associated risk factors), unspecific and may overlap. Also, there are several conflicting reports on these preeclampsia associated biomarkers. This could largely be attributed to differences in experimental procedures.

The intensity of preeclampsia research should continue to be robust, paying particular attention to the different risk factors reported as well as intrinsic and extrinsic contributions to the disease. As earlier mentioned, this pathophysiology is not uniquely ascribed to the aetiology and development of preeclampsia. The condition may interact with other pathogenic conditions that stimulate a proinflammatory state and may precipitate or exacerbate preeclampsia. Subsequently, the pathogenic basis of preeclampsia with reference to placental malaria due to several similarity indices will be outlined.

1.6 Protective effects, prevention and management of preeclampsia

The inverse of major risk factors of preeclampsia are expected to confer some protection to pregnant women. For instance, multigravid women are expected to have reduced risk of preeclampsia compared to primigravids (Hernández-Diaz et al., 2009). Other protective effects are described briefly.
Smoking during pregnancy has been shown to confer protection against preeclampsia but the exact mechanism is unknown (England and Zhang, 2007, Wei et al., 2015). Some reports show that the haeme-oxygenase-1/carbon monoxide pathway inhibits the release of anti-angiogenic molecules such as sVEGFR-1 and soluble endoglin (sEng) from endothelial cells (Cudmore et al., 2007). This has been supported by lower maternal concentrations of sVEGFR-1 among smokers with preeclampsia which is similar to what is observed in uncomplicated pregnancy (Jeyabalan et al., 2008). Potential targets in this direction could be explored to enhance treatment.

Prolonged exposure to paternal seminal fluid prior to conception reduces the risk of preeclampsia (Shiozaki and Saito, 2018). This means that contraception that prevents or decreases exposure to seminal fluid as well as assisted reproduction, increases the risk of preeclampsia (Jie et al., 2015). Saftlas et al. (2014), concluded that these findings are consistent with the immune maladaptation hypothesis of preeclampsia causation, indicating that paternal antigen exposure facilitates immune tolerance to paternal human leucocyte antigen.

There is no definite preventive formula for preeclampsia. Focus has been on risk factor identification before or during early pregnancy, life style modifications, nutritional adjustments and therapeutics (Bezerra Maia e Holanda Moura et al., 2012). These are primarily prognostic measures to aid in the early detection and management of preeclampsia in order to prevent adverse pregnancy outcomes. Prophylactic interventions such as diuretics, progesterone, nitric oxide, calcium supplementation and low dose aspirin have not been formalized for use but have been recommended for high risk populations (Sibai et al., 1993, Henderson et al., 2014, Achamrah and Ditisheim, 2018, ACOG, 2018).
Due to the lack of understanding of preeclampsia aetiology over several decades of research, management of this disorder has been carefully guided to reduce mortality. Prevention, early detection and treatment (English et al., 2015) are the three main modalities used by clinicians in accordance with international guidelines. Guidelines from the American College of Obstetricians and Gynecologists (ACOG) and the International Society for the Study of Hypertension in Pregnancy (ISSHP) are commonly used worldwide. Hospital-based management is centred on case observation and delivery in severe complications. Magnesium Sulphate (MgSO₄) has consistently been used as the drug of choice in preventing progression from preeclampsia to life threatening eclampsia resulting in the reduction of seizures (WHO, 2017a). Anti-hypertensive therapies have been recommended for use in the prevention of severe hypertension and stroke. Labetalol, hydralazine, nifedipine, and alpha methylldopa are the recommended first line drugs (English et al., 2015, WHO, 2017a). Delivery is the last option if blood pressure and complications are not controlled and threatens the life of the pregnant woman. The current research trend is focused on targeted therapy for the treatment of preeclampsia. Meiri et al. (2017) proposed placental protein 13 (PP13) produced predominantly in the placenta as a therapeutic target. RNA interference modulation of placental sVEGFR-1 in pregnant mice has also shown reduced sVEGFR-1 levels implicated in the pathology of preeclampsia (Turanov et al., 2018).

1.7 Preeclampsia and infections

Among the numerous risk factors associated with preeclampsia are infections. This is particularly important for developing countries where the burden of infectious diseases are generally high (Bhatta et al., 2014). Pregnant women are among the most vulnerable groups susceptible to infections and several observational studies have associated infections with the risk of preeclampsia. Considering the high prevalence of preeclampsia and the higher risk of
death in developing regions (Abalos et al., 2013, Abalos et al., 2014), fundamental biological investigations are required in order to draw definite conclusions. In a systematic review of maternal infections and the risk of preeclampsia, urinary tract infection and periodontal disease showed some association whilst malaria, HIV and other infections were not associated (Conde-Agudelo et al., 2008). However this review asserts that, these infections that were not significant are linked to the indirect effect of enhanced inflammation during an infection (Conde-Agudelo et al., 2008). Direct mechanisms include pathogen interaction with the trophoblast and endothelium leading to injury and dysfunction as seen in malaria infections (Gueneuc et al., 2017). Interestingly, of all the studies that assessed the risk of infections to preeclampsia, none was done in a developing or malaria endemic region except studies that assessed malaria as a risk factor (Conde-Agudelo et al., 2008). This suggests that although several risk factors might be involved in the development of preeclampsia, prevalent infectious diseases might open up pathophysiological mechanisms for better understanding of preeclampsia. Among pregnant women, placental malaria seems a unique model for trophoblast and endothelial interaction leading to the development of preeclampsia in malaria endemic regions.

1.8 Placental malaria

*Plasmodium falciparum* accounts for the greater burden of pregnancy associated malaria worldwide, often resulting in severe placental malaria. This is prevalent in areas of stable malaria transmission especially within sub-Saharan Africa where more than 50 million pregnant women are exposed to infections yearly (Dellicour et al., 2010, Conroy et al., 2012). Estimated risk of placental malaria due to *P. falciparum* in Africa is about 12.4 million in women without pregnancy specific protection (Walker et al., 2014). In Ghana, the prevalence of placental malaria in a rural setting was shown to be 19.7% (Ofori et al., 2009). Quite
recently, a study in an urban tertiary hospital showed a prevalence of 57.3% by polymerase chain reaction in placental tissues against 6.3% in peripheral blood specimen (Blay et al., 2015). This suggests that focus on placental malaria prevention even with considerable coverage of intermittent preventive treatment in pregnancy (IPTp) which has shown probable recrudescence in Ghana and elsewhere (Duah et al., 2012, Moussiliou et al., 2013, Okell et al., 2017) should be paramount.

Several maternal and foetal adverse effects have been associated with placental malaria. Congenital malaria, low birth weight, preterm delivery, intrauterine growth restriction, maternal/foetal anaemia, abortion, small for gestational age infants, perinatal mortality and effects on foetal anthropometric parameters are some of the associated effects (Uneke, 2007, Ofori et al., 2009, Kapisi et al., 2017, Lufele et al., 2017). It is interesting to note that even in subclinical manifestations these effects occur and primarily in primigravid women (Cohee et al., 2014, Cottrell et al., 2015). Although IPTp reduces parasite load, it is taken after the first trimester of pregnancy and does not prevent re-infection of parasites hence questioning its protective effects.

Placental malaria occurs as part of immune evasion mechanisms of the malaria parasite. Hence, susceptibility of primigravids who have no prior exposure to the *P. falciparum* placental phenotype is high. Infection is characterised by the sequestration of infected erythrocytes into the maternal intervillous spaces of the placenta. This is mediated by the *P. falciparum* erythrocyte membrane protein 1(PfEMP-1) which is encoded by a diverse family of *var* genes (Fried and Duffy, 1996). Specifically, parasites expressing VAR2CSA have been shown to preferentially bind to chondroitin sulphate A (CSA) of the placental syncytiotrophoblast (Fried and Duffy, 1996, Miller et al., 2002, Turner et al., 2013) and sometimes to hyaluronic acid.
(Rasti et al., 2006) and the endothelium (Miller et al., 2002) leading to reduced blood flow and other effects. This is a unique feature that discriminate placental malaria pathogenesis from other forms of malaria. Several factors however interplay in disease severity. Women develop immunity against CSA binding in subsequent pregnancies reducing the effects of severe disease. According to a review by Miller and colleagues (2002), numerous parasite, host, and environmental factors converge as determinants of asymptomatic to severe disease during an infection however, maternal preexisting immunity and placental integrity are fundamental.

In normal pregnancy, a predominant T helper (Th) 2 response is induced to suppress inflammation for foetal adaptation. This predisposes pregnant women to opportunistic infections such as *P. falciparum* with the tendency of enhancing the Th1 response, a proinflammatory state which might be damaging to the foetus. Several cellular mechanisms interact to accommodate the developing foetus however this complex milieu is compromised during placental malaria. For instance, elevated levels of Th1 cytokines such as Tumour Necrosis Factor alpha (TNF-α), Interferon gamma (IFN-γ), IL-1β, IL-2 is evidenced in women with placental malaria than uninfected women (Fried et al., 1998, Suguitan et al., 2003). Histological alterations, infiltration of immune cells and accumulation of parasites in the intervillous spaces also affect placental integrity. Agudelo et al. (2014) have earlier reported on a compromised placenta during submicroscopic infections indicating the elevated expression of inflammatory, hypoxic and apoptotic markers. These factors are required for parasite clearance but become deleterious to the pregnancy. Identifying biomarkers to placental malaria is associated with several complexities due to their simultaneous detection in peripheral blood. Ruizendaal et al. (2015) suggest that several markers of placental malaria have not shown individual specificity but, targeting multiple markers from different pathophysiological pathways could ensure accurate prediction of placental malaria.
There is currently no vaccine to prevent placental malaria however, VAR2CSA *P. falciparum* antigen holds some promise for the development of a vaccine. This is because VAR2CSA of the *PfEMP1* protein family, is a unique and currently known ligand involved with infected erythrocyte binding to CSA in the placenta (Fried and Duffy, 1996, Viebig *et al.*, 2005, Viebig *et al.*, 2007). It is a conserved 350-kD protein organised into six Duffy-binding (DBL) domains, interdomain regions and an intracellular domain (Clausen *et al.*, 2012). The current vaccine strategy is to block the binding interaction between this antigen and the host receptor CSA by inducing the production of anti-VAR2CSA antibodies. This means that such vaccine should be administered to women of childbearing age so that anti-VAR2CSA antibodies would be present in maternal circulation during their first pregnancy (Pehrson *et al.*, 2017). Two vaccine candidates PAMVAC (ClinicalTrials.gov Identifier: NCT02647489) (Mordmuller *et al.*, 2019) and PRIMVAC (ClinicalTrials.gov Identifier: NCT02658253) (Chêne *et al.*, 2019) have been listed at varying stages of clinical trials.

1.9 Placental malaria and preeclampsia: The association

Placental malaria and preeclampsia share significant similarities such as high risk in primigravidas, adverse maternal and foetal outcomes, placental pathophysiology and higher risk of death in developing countries (Brabin and Johnson, 2005, Muehlenbachs *et al.*, 2006, Conde-Agudelo *et al.*, 2008, Gueneuc *et al.*, 2017).

The longstanding challenge with preeclampsia is the disillusioned aetiology and lack of effective treatment. Preeclampsia and placental malaria would be expected to occur frequently and sometimes simultaneously in malarious regions such as Ghana, but whether or not they interact is unclear. Some studies have found no link between both conditions (Shulman *et al.*, 2001, Dorman *et al.*, 2002), however, many other studies have established an association.
between them (Sartelet et al., 1996, Muehlenbachs et al., 2006, Ndao et al., 2009, Adam et al., 2011). Findings from these studies suggest that seasonal variations, malaria transmission intensity, gravidity and parity play a role in this interaction. In an earlier study in Tanzania, investigators indicated that an immunological pathway is responsible for a maternal and foetal conflict in placental malaria which is further selective for hypertension, particularly, in primigravidas (Muehlenbachs et al., 2006). An update of an earlier review is necessary in the advent of further studies which have found an association between placental malaria and preeclampsia. With the multifactorial aetiology and several risk factors attributed to preeclampsia, the influence of co-existing factors on the pathophysiology cannot be underestimated. Also, further probe into understanding the pathogenesis of these two conditions is warranted.

As earlier highlighted in their pathophysiology, both preeclampsia and placental malaria are characterised by placental insufficiency (Pennington et al., 2012, Agudelo et al., 2014), enhanced inflammation, endothelial activation/dysfunction, hypoxia and ischaemia. While preeclampsia translates into a maternal phenotype of hypertension and proteinuria with the associated organ dysfunction, placental malaria may remain asymptomatic but can directly or indirectly contribute to the episode of preeclampsia.

Several studies have reported weak regulation of the cytokine balance in the pathogenesis of preeclampsia. Predominant levels of proinflammatory cytokines such as TNF-α, IL1, IL12 (Arriaga-Pizano et al., 2005, Sharma et al., 2007, Szarka et al., 2010, Kalinderis et al., 2011) have been found to be associated with this disorder. Several markers of dysregulated angiogenesis, a consequence of the proinflammatory state such as intercellular adhesion molecule 1 (ICAM1), VEGF, PlGF, Ang1 and 2 and their receptors have also been implicated
in preeclampsia (Molvarec et al., 2010, Szarka et al., 2010). Placental malaria promotes a proinflammatory state, an important factor to consider in circulatory disorders such as preeclampsia. Excessive production of these placental factors, contributed either directly or indirectly from *P. falciparum* infections, results in further interaction with other organs leading to systemic inflammation. Consequently, the endothelium may lose its anti-inflammatory function due to upregulation of adhesion molecules and down regulation of factors that maintain the integrity of the endothelium (angiogenic factors). The resultant effect is endothelial activation/damage. TNF-α has been shown to cause widespread endothelial activation during infection from malaria parasites (Fried et al., 1998, Miller et al., 2002).

1.10 Vascular regeneration and repair

The body has its own mechanism of repair when damage occurs. Circulating endothelial cells (cECs) resulting from injury are thought to be associated with endothelial damage (Prater et al., 2007, Smadja et al., 2009). This further aggravates the inflammatory pathway leading to a state of chronic inflammation and endothelial activation. Conventionally known mediators of blood vessel integrity include but not limited to the VEGF family of molecules and angiopoietins (Muehlenbachs et al., 2006, Cerdeira and Karumanchi, 2012). Additionally, microvascular damage induces the release and mobilization of bone marrow derived circulating endothelial progenitor cells (cEPCs) involved in the development, regulation and repair of the vasculature (Luppi et al., 2010). These cEPCs are incorporated into damaged sites in the microvasculature and augment the local response, which may be insufficient to repair extensive or chronic injury.

This phenomenon was initially thought to be solely due to angiogenesis. In a seminal work by Asahara et al. (1997), they established that damage can also be repaired by circulating
endothelial progenitor cells (cEPCs) which was initially thought to be absent in adults. These bone marrow-derived stem cells, migrate, proliferate and differentiate into functional endothelial cells at sites of damage. Extensive studies in hypertensive disorders of pregnancy such as preeclampsia and cardiovascular disorders have shown low levels of cEPCs in peripheral blood (Robb et al., 2007, Heimrath et al., 2014). This has been associated with microvascular imbalance, a situation that results in persistent inflammation and aggravates endothelial dysfunction. Mobilization and migration of these cEPCs to damaged sites are augmented by the expression and activation of certain growth factors, chemokines and proteases including stromal cell derived growth factor 1 (SDF-1) and MMP-9. This involves differentiation of stem cells towards endothelial lineage followed by cEPCs proliferation, mobilization, and homing to sites of endothelial damage (Lapid et al., 2012).

Vascular requirements increase as gestational age increases. Endothelial cells and their progenitors of both maternal and foetal origin have also been identified in the vascularisation process. There is increasing evidence that changes in circulating levels of angiogenic molecules are promising targets for preeclampsia prediction and therapeutics. This is because numerous molecules have been implicated in the regulation of vascular growth in the placenta and other parts of the body. A unified understanding of cEPCs in underdeveloped populations is expected to correlate with previous studies especially in the paradigm of an infectious disease like malaria. The role and function of cEPCs have not been studied in detail in placental malaria although this may mirror association between low levels of cEPCs in children with cerebral malaria, a condition connected to damaged cerebral microvasculature (Gyan et al., 2009). In this study, they indicated that cerebral malaria was associated with insufficient mobilization of cEPCs to the cerebral microvasculature and highlighted the role of cEPCs in microvascular disorders. These cells were defined by the co-expression of CD34+/VEGFR2+ (matured
progenitor cells) and CD34+/CD133+ (immature progenitor cells) in peripheral blood of children with cerebral malaria by flow cytometry (Gyan et al., 2009). In line with these findings, and in addition to the reduced cEPCs in patients with preeclampsia as earlier reported, scientific interests might be ignited in the area of aetiology, diagnostics and therapeutics.

1.11 Problem statement

Although Ghana significantly reduced its maternal mortality between 1990 and 2015, the MDG in this area was not achieved (190 deaths per 100,000) (UNDP, 2015). Currently, the SDG 3.1 requires maternal mortality to be reduced to 70 deaths per 100,000 live births. Strong efforts must therefore be put in place to significantly reduce maternal deaths from the current levels of 319 per 100,000 live births (UNDP, 2015, WHO, 2018c).

Preeclampsia is almost the leading cause of maternal mortality particularly in tertiary hospitals in Ghana (Adu-Bonsaffoh et al., 2013, Adu-Bonsaffoh et al., 2014). Aetiology is still an illusion but multifactorial theories have been proposed, with several risk factors linked to the condition (Wang et al., 2009, Chappell and Morgan, 2006, Veerbeek et al., 2015). The rise in this condition against the background of the burden of infectious diseases such as malaria which affect pregnant women in malaria endemic regions, is alarming.

Placental malaria is one of the severe phenotypes of malaria resulting from parasite sequestration in the placenta as well as chronic inflammation and endothelial damage (associated with malaria in general) (Moxon et al., 2014). Although the WHO has recommended the use of IPTp to reduce adverse effects associated with malaria in pregnancy (WHO, 2018b), the policy suffers many constraints. While the availability of this therapy particularly in rural settings may be limited or absent, others may suffer the constraint of
unsupervised administration or low/late antenatal attendance by pregnant women (Azizi et al., 2018).

Current risk factors identified to increase the risk of preeclampsia are hinged on studies carried out mostly in non-malarious and high-income regions giving less disparity on how this condition may be managed or treated. Other studies in malarious regions have been observational with little emphasis on pathophysiology. The placental malfunction, inflammation and endothelial dysfunction during placental malaria and preeclampsia which this study sought to look at through circulating mediators may influence disease management and treatment of preeclampsia in malaria endemic regions. There could be a paradigm shift in the interaction between malaria and hypertensive disorders in general which might help reduce maternal deaths.

1.12 Rationale

Women in malaria endemic areas like Ghana are often infected with parasites but may remain asymptomatic. This leads to chronic untreated placental malaria with insidious consequences (Fried et al., 2012). It is important to know whether these insidious consequences precipitate preeclampsia development or exacerbate the condition. While some studies have found no link between placental malaria and preeclampsia (Shulman et al., 2001, Dorman et al., 2002), a significant number have established an association between these two conditions in Africa (Sartelet et al., 1996, Muehlenbachs et al., 2006, Ndao et al., 2009, Adam et al., 2011). Understanding the pathophysiologic link between placental sequestration of *P. falciparum*, inflammation and angiogenesis will provide further insights into similar pathogenic pathways and aid in the management of preeclampsia.
According to Etyang et al. (2016), pathogen elimination is the focus of most treatment for infectious diseases. The consequences associated with dysregulated inflammation and angiogenesis are not targeted. This study sought to provide a new concept in understanding further, the pathogenesis of placental malaria in the context of inflammation and endothelial integrity. Intensifying research on already identified marker for preeclampsia and placental malaria as well as new targets will be imperative in decreasing disease burden and move towards its elimination.

1.13 Hypothesis

Based on the background above, the study hypothesized that;

1. Mechanical damage and inflammation due to sequestration of parasites in the placenta result in endothelial activation/damage and a predisposition to preeclampsia.

2. Alterations in endothelial cell phenotypes and the angiogenic molecules that stimulate their function will differ between preeclamptic and non-preeclamptic pregnancies exposed to placental malaria.

1.14 Aim and objectives

Aim

The overall aim of this study was to determine the association between placental malaria and preeclampsia among pregnant women delivering at term in relation to placental infection state, inflammatory and angiogenic mediators. The contribution of endothelial cells and their progenitors were also assessed by comparing their levels in women with and without preeclampsia.
Specific objectives:

Objective 1: To assess placental pathology and the risk of preeclampsia in women exposed to *Plasmodium falciparum* infections in the placenta.

Sub Objectives

1. To identify placental histological changes among women diagnosed with preeclampsia
2. To identify *Plasmodium falciparum* placental exposure and the risk of preeclampsia
3. To determine the relationship between placental pathology and *Plasmodium falciparum* exposure in the placenta of women diagnosed with preeclampsia.

Objective 2: To determine the Inflammatory and angiogenic network in preeclampsia and the risk associated with *Plasmodium falciparum* exposure.

Sub Objectives

1. To compare levels of disease mediators in peripheral and placental blood.
2. To compare levels of disease mediators in non-preeclamptic and preeclamptic pregnancies.
3. To determine the relationship between *Plasmodium falciparum* exposure, disease mediators in non-preeclamptic and preeclamptic pregnancies.

Objective 3: To determine the endothelial cell phenotypes in pregnancy, preeclampsia and pregnancy exposed to *Plasmodium falciparum* infections in the placenta.

Sub Objectives

1. To compare levels of endothelial cell phenotypes in peripheral, cord and placental blood in non-preeclamptic and preeclamptic pregnancies.
2. To identify the relationship between endothelial cell phenotypes in peripheral, and placental blood in non-preeclamptic and preeclamptic pregnancies.

3. To identify the relationship between *Plasmodium falciparum* exposure and endothelial cells in non-preeclamptic and preeclamptic pregnancies.
CHAPTER TWO

2.0 Paper 1: Placental pathology and the risk of preeclampsia in women exposed to *Plasmodium falciparum* Infections in the placenta.

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2.1 Abstract

**Background:** Preeclampsia (PE) is a placental disorder with multifactorial aetiologies that present with different phenotypes. In malaria endemic regions, high incidence of PE is reported, with debilitating maternal and foetal effects particularly among first time pregnant women. However, the relationship between placental pathology, *Plasmodium falciparum* infection in the placenta and PE is underexplored.

**Methods:** A total of 134 placentas were sampled at delivery from 69 women without PE (non-PE group) and 65 women diagnosed with PE (PE group) at a tertiary hospital in Ghana. Demographic details were documented and placentas examined histologically for pathological lesions and placental malaria (PM).

**Results:** Placental pathology was increased in the PE group compared to the non-PE group, with syncytial knots being the specific pathology associated with PE ($P = 0.003$). Pathology was also found to be dependent on first time pregnancy and blood pressure to induce PE using a regression model (Adjusted odds ratio (AOR) 2.8, 95% CI = 1.2 – 6.4). Of 133 placentas scored for placental parasite exposure, 64 (48.1%) and 21 (15.8%) represented active and past infections respectively. Placental parasite exposure was significantly higher in the PE group [39 (29.3%) and 15 (11.3%) active and past infections respectively] compared to the non-PE group [25 (18.8%) active infections and 6 (4.5%) past infections, $P < 0.0001$]. Absence of placental exposure was 10 (7.5%) in the PE and 38 (28.6%) in the non-PE group. Multivariate analysis showed placental pathology (AOR 3.0, 95% CI = 1.2 – 7.5), exposure to PM [(active infection: AOR 6.7, 95% CI = 2.3 – 19.1); (past infection: AOR 12.4, 95% CI = 3.0 – 51.0)], first pregnancy (AOR 6.6, 95% CI = 2.4 – 18.2) but not blood pressure to be significantly associated with PE.

**Conclusion:** Placental histological changes and PM are risk factors for PE particularly in primigravids. Mechanisms associated with this finding should further be investigated.
2.2 Introduction

Maternal syndrome of preeclampsia (PE) complicates 2 – 10% of pregnancies worldwide (Stevens et al., 2017) and contributes significantly to maternal and foetal morbidity and mortality (Brown et al., 2018). It is clinically characterised by hypertension and proteinuria on or after 20 weeks of gestation in previously normotensive women (ACOG, 2013, Tranquilli et al., 2014). Although little is known about the pathophysiology, the placenta has evolved as the main organ responsible for the initiation of the disorder. Over the course of the 20th century, investigators succeeded in understanding placental changes associated with PE pathology.

Examination of placental biopsies from women diagnosed with PE and foetal growth retardation revealed inadequate trophoblast invasion and impaired remodelling of maternal spiral arteries (Gerretsen et al., 1981, Khong et al., 1986). Further on, the two stage model of PE by Roberts and Hubel (2009) suggests that these placental insufficiencies (stage 1) are responsible for the clinical symptoms of the disorder (stage 2). Poor placental perfusion has been associated with placental histopathological changes such as infarcts, increase in syncytiot knots, fibrin deposits, atherosclerosis of the arterial wall, accelerated villous maturation and calcification (Staff et al., 2010, Souza et al., 2011, Vinnars et al., 2011, Stevens et al., 2013, Ezeigwe et al., 2018, Wallingford et al., 2018). These changes result from ischaemic and hypoxic mechanisms secondary to poor perfusion (Roberts and Escudero, 2012). Additionally, variable effects on the clinical severity of PE and foetal outcomes have been shown (Ezeigwe et al., 2018) suggesting the contribution of not only a single but several placental factors.

Women in malaria endemic regions exposed to placental parasites could be at an increased risk of PE compared to women in non malarious regions. Although there are conflicting reports, most studies have confirmed this theory (Etard et al., 2003, Muehlenbachs et al., 2006, Ndao
Sequestration of parasites in the placenta is the hallmark of disease pathogenesis with associated links to PE pathogenesis as previously reviewed (Brabin and Johnson, 2005, Etyang et al., 2016). Similar to PE, placental malaria (PM) is associated with placental pathological alterations leading to adverse maternal and foetal effects such as low birth weight and foetal death (Dorman et al., 2002, Ofori et al., 2009, Kapisi et al., 2017, Lufele et al., 2017). In Gabon, a study observed excessive fibrinoid deposits associated with syncytiotrophoblast damage, and ultra-structural damage leading to basal lamina thickening in placentas affected by PM (Walter et al., 1982). According to the authors, immunopathologic processes were related to these placental changes. Additionally in an animal model, Plasmodium berghei ANKA-infected mice showed pathological alterations associated with inflammation, tissue disorganisation and reduced vascular spaces leading to reduced blood supply in placental tissues (Neres et al., 2008). Hypoxic markers have also shown higher expression in PM compared to non-PM placentas (Agudelo et al., 2014).

Over the years, the effects of placental changes during PM have focused mainly on foetal outcome, maternal anaemia and death. Maternal death due to PM is believed to be indirect. However, most women with placental parasite infections are asymptomatic possibly due to some degree of acquired immunity which may be associated with insidious consequences. The relationship between pathology, P. falciparum infection and PE is underexplored. This study identified placental alterations, parasite infections and other risk factors associated with the presence or absence of PE by testing the hypothesis that placental histological changes and PM are associated with the PE syndrome.
2.3 Methods

2.3.1 Study site

The study was conducted at the Department of Obstetrics & Gynaecology of the Korle-Bu Teaching Hospital (KBTH) in Accra, the capital city of Ghana. KBTH is a tertiary hospital and a leading national referral centre that also incorporates the University of Ghana School of Medicine and Dentistry. The Department of Obstetrics and Gynaecology is the largest department in the hospital with a 275-bed capacity for obstetric care (~10,000 deliveries/year) (KBTH, 2016). It has the capacity and medical expertise to manage major obstetric complications hence serves as a pivot for obstetric referrals from the southern half of the country (Figure 2.1). Due to this, it is involved with local, national and international collaborative research to identify causes of maternal morbidity and mortality and the way forward. Previous studies have linked hypertensive disorders of pregnancy, particularly PE, as one of the major causes of mortality in this facility (Adu-Bonsaffoh et al., 2013, 2014).

Most of the participants recruited into the study came from various suburbs of Accra while others came from the surrounding regions. Regular attendants of the KBTH antenatal clinic were about 34% while 66% were referrals from other health centres. The hospital is about 11 miles from the Noguchi Memorial Institute for Medical Research (NMIMR) where most of the experimental procedures were conducted.
2.3.2 Study design and eligibility

This study was designed to obtain both demographic and clinical information using case report forms (questionnaire). This was carried out between January and December 2017. Pregnant women presenting for delivery at the hospital and diagnosed with PE were screened for inclusion into the study. Another group of women presenting with healthy pregnancies (non-
PE) were also screened for inclusion from the same hospital. The study was explained to each participant and those who voluntarily consented were recruited. Case report forms were appropriately completed. Each participant was assigned a unique identification code prior to sampling. Laboratory data were collected at delivery and transported to the designated laboratory. Both primigravidae and multigravidae within the age range of 18 – 45 years were enrolled into the study.

2.3.3. Definition of preeclampsia

Clinical PE was defined as pregnant woman who presented with a history of sustained hypertension (blood pressure ≥ 140mmHg systolic or ≥ 90mmHg diastolic at least 4 hours apart) with proteinuria (two readings of 1+ or higher on urinalysis) ≥ 20 weeks of gestation as diagnosed by an obstetrician. This definition is in accordance with the guidelines used by the American College of Obstetricians and Gynaecologists (ACOG) as well as the International Society for the Study of Hypertension in Pregnancy (ISSHP) for the diagnosis of clinical PE (ACOG, 2013, Tranquilli et al., 2014).

2.3.4 Early and late onset preeclampsia

This was based on the time of disease occurrence. Definition as classified by Lisonkova and Joseph (2013) was used.

1. Early onset PE was defined as the occurrence of PE before 34 weeks of gestation.

2. Late onset PE was defined as the occurrence of PE on or after 34 weeks of gestation.

2.3.5 Preeclampsia with mild or severe features

1. The PE with mild features was defined as history of sustained hypertension
(blood pressure ≥ 140mmHg systolic or ≥ 90mmHg diastolic at least 4 hours apart) with proteinuria (two readings of 1+ or higher on urinalysis) ≥ 20 weeks of gestation as diagnosed by an obstetrician with no evidence of organ damage.

2. The PE with severe features was defined as

History of sustained hypertension (blood pressure ≥ 160mmHg systolic or ≥ 110mmHg diastolic at least 4 hours apart) with proteinuria (two readings of 1+ or higher on urinalysis) ≥ 20 weeks of gestation or

1. Platelet count of < 100,000/µl
2. Major organ dysfunction
3. Renal insufficiency
4. Pulmonary oedema
5. Visual disturbance such as blurred vision or temporary loss of vision.

Participants selected for definition 2 occurred in the presence of hypertension as earlier defined for PE. Definition adapted from ACOG (2013).

2.3.6. Normal, healthy or non-preeclamptic pregnancy

This was defined as pregnant woman at ≥ 20 weeks of gestation with a history of sustained blood pressure < 140mmHg systolic or < 90mmHg diastolic) without proteinuria at time of enrolment as confirmed by a clinician/midwife and medical records.

2.3.7 Measure of exposure to placental malaria

Placental Malaria (PM) was defined as the presence of parasites in the placental blood and/or parasite pigment deposition in placental biopsies after delivery as previously described (Bulmer
et al., 1993, Muehlenbachs et al., 2010).

1. No infection: Absence of parasites and pigments in the intervillous spaces
2. Acute infection: Parasites in the intervillous spaces.
3. Chronic infection: Parasites in maternal red blood cells and pigment in fibrin or cells within fibrin and or chorionic villous syncytiotrophoblast of stroma.
4. Past infection: No parasites but pigment confined within fibrin or cells within fibrin.

Acute and chronic infections were subsequently combined and classified as active infection to be compared against past infection and absence of infection.

2.3.8 Exclusion criteria

1. Participants who were unable to give informed consent or unwilling to comply with the requirements of the protocol were excluded from the study.
2. Patients with history of chronic hypertension, underlying renal disease, diabetes mellitus, and cardiovascular diseases were excluded from the study.
3. Pregnant women with obvious clinical evidence of urinary tract infections and other concomitant infections at time of enrolment were excluded from the study.
4. Pregnant women, who met inclusion criteria but were critically ill based on obstetrician’s recommendation, were excluded from the study.

2.3.9 Sample size determination

Sample size was determined based on prior studies. The expected frequency of exposure to PM in women with non-PE pregnancies (controls) was 27% while the frequency of exposure expected among PE pregnancies (cases) was 53% with an estimated odds ratio of 3.0 (Sartelet
et al., 1996). Based on these assumptions and given a ratio of 1:1 between cases and controls, we estimated a minimum sample size of 128 at 95% CI and a power of 80% to study the effect of PM as a risk factor for PE (openEpi version 3). Anticipating a 10% refusal rate, inadequate sampling and incomplete data of participants, a total sample size of 140 pregnant women (70 normal pregnancies and 70 pregnancies complicated by PE) was deemed adequate for the study.

2.3.10 Ethical considerations

Approval to conduct this study was obtained from the Institutional Review Board of the NMIMR (IRB# 00001276)) registered with OHRP (FWA 00001824) and the Ethical and Protocol Review Committee of the KBTH. Demographic data and biological specimen were obtained following informed consent from selected participants. Participation was strictly voluntary with no restrictions if participant decided to opt out of study.

2.3.11 Placental tissue sampling and processing

To investigate the association between placental pathology and *P. falciparum* infection to the risk of PE, placental tissues were sampled from PE and non-PE women after delivery. A portion of incised tissue was washed in physiological saline to remove excess blood and immediately placed in a well labelled container with 100 ml of 10% neutral buffered formalin for tissue fixation (Appendix I). This was to maintain tissue integrity and minimize the formation of formalin pigment in the tissue. Biopsy was immediately transported to the Department of Pathology, KBTH, where tissue processing was carried out. Fixative was changed from time to time until a clear fixative was obtained that ensured that the tissue was well fixed before processing. Processing of fixed tissue was carried out within 72 hours of collection.
2.3.12 Placental tissue processing

Placental tissues were processed using Leica TP1020 (Leica Biosystems, Germany) tissue processor. Briefly, a 3 cm³ (3x1x1cm) portion of the placental tissue was grossed and placed into a well labelled tissue processing cassette and fixed in 10% neutral buffered formalin for about an hour. The tissue portion in the cassette was dehydrated in ascending grades of ethanol (60%, 70%, 80% and 90%) for an hour and 30 minutes. This was then placed in three changes of absolute ethanol to achieve full dehydration. After dehydration, tissue was cleared in two changes of xylene and transferred into molten paraffin wax at a melting temperature of 56 °C. Infiltration was carried out for 2 hours. For paraffin embedding, Leica EG1150, (Leica Biosystems, Germany) modular tissue embedder was used to form tissue blocks. (Appendix II).

2.3.13 Placental tissue sectioning and staining

Placental tissue block was trimmed at 10 microns and placed on ice to cool, after which 3 microns thick of tissue was sectioned into ribbons using a rotary microtome (Leica RM2235, Leica Biosystems, Germany). Ribbons were floated onto a protein-free water bath (Boekel Scientific 14793, USA) at a temperature of 50 ºC. Two floating ribbons from each placental tissue were mounted onto well labelled grease-free frosted end glass slides. Subsequently, the slides were air dried and heat fixed on a hot plate. Placental tissue sections mounted on each slide were stained using standard haematoxylin and eosin (H&E) stains and Giemsa respectively. The H & E staining was carried out to differentiate cellular components of placental tissues using Mayer’s staining protocol. Briefly, sections were deparaffinized in xylene, rehydrated in absolute alcohol, conditioned in varying gradients of alcohol and stained with haematoxylin. Sections were counter stained in eosin, dehydrated, cleared and mounted.
with a xylene based mounting medium (DPX 44581, Sigma) (Appendix III). Similar procedure was carried out for Geimsa staining (Appendix IV).

2.3.14. Microscopic examination of histologically stained placental tissues

Slides were examined using a standard light microscope and a histological grading system adapted from Muehlenbachs et al. (2010) (Appendix V). Presence or absence of parasites, haemzoin, inflammation and unique placental changes were recorded under 10x, 40x or 100x [high powered field (HPF)]. Infection state was classified into four groups (described in section 2.3.7). Unique pathologies observed during the examination of the placenta were also reported. Pathologist was unaware of the disease states or any characteristics of the study participants.

2.3.15 Statistical analysis

Data were analysed using GraphPad prism v6 and R v3.5.1 (R Development Core Team). Means and proportions were compared between two continuous variables using Student’s t-test for normally distributed data. Mann-Whitney U test was used for data that were not normally distributed. One way ANOVA (parametric) or Kruskal-Wallis (non-parametric) test were used to compare means and medians respectively between three or more continuous variables. Pair-wise post-hoc comparisons were done using Dunn’s multiple comparison test. Categorical data was analysed using Chi-square test or Fisher’s exact test for association. Univariate and multivariate regression analyses were carried out to predict the risk of PE. For all analyses, \( P < 0.05 \) was considered statistically significant.
2.4 Results

2.4.1 Demographic and clinical characteristics of study participants

The study sampled 140 pregnant women comprising 71 diagnosed with PE (PE group) and 69 without PE (non-PE group) delivering at the KBTH. Demographic and clinical details of participants have been summarized (Table 2.1). The age of the participants ranged between 18-42 years with a mean age of 29 ± 6 years for non-PE pregnant women and 28 ± 6 years for women diagnosed with PE. This was not statistically different. Mean maternal age, body mass index (BMI) and intermittent preventive treatment in pregnancy (IPTp) use were not different between the PE and the non-PE group ($P > 0.05$ in all cases). Women who have had multiple pregnancies were the majority within the study population ($P = 0.004$). However, there were more primigravids in the PE than the non-PE group ($P = 0.004$). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were significantly higher in the PE (mean SBP = 150.7 ± 17.9, mean DBP = 95.2 ± 14.4) compared to non-PE group (mean SBP 122.2 ± 14.2, mean DBP = 75.5 ± 8.8; $P < 0.0001$ respectively). Proteinuria was present in the PE group only (69 (97.2; $P < 0.0001$). Management of PE with magnesium sulphate and anti-hypertensive drugs are reported in the PE group. Most women were administered with at least one dose of IPTp during the course of gestation (91.3%). Differences were not observed between the PE and non-PE groups in terms of IPTp administration.
Table 2.1: Demographic and clinical characteristics among women diagnosed with or without preeclampsia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-PE n= 69</th>
<th>PE n= 71</th>
<th>Total n= 140</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean maternal age (years)</td>
<td>29±6</td>
<td>28±6</td>
<td>28±6</td>
<td>0.43</td>
</tr>
<tr>
<td>Primigravid (%)</td>
<td>16 (23.2)</td>
<td>34 (47.9)</td>
<td>50 (35.7)</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>Multigravid (%)</td>
<td>53 (76.8)</td>
<td>37 (52.1)</td>
<td>90 (64.3)</td>
<td></td>
</tr>
<tr>
<td>Mean BMI (kg/m^2)</td>
<td>26±4.9</td>
<td>28.1±6.3</td>
<td>27.0±5.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>122.2±14.2</td>
<td>150.7±17.9</td>
<td>136.4±21.5</td>
<td><strong>&lt;0.0001</strong></td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.5±8.8</td>
<td>95.2±14.4</td>
<td>85.3±15.5</td>
<td><strong>&lt;0.0001</strong></td>
</tr>
</tbody>
</table>

Proteinuria

<table>
<thead>
<tr>
<th></th>
<th>Non-PE</th>
<th>PE</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>69 (100)</td>
<td>2 (2.8)</td>
<td>71 (50.7)</td>
<td><strong>&lt;0.0001</strong></td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>69 (97.2)</td>
<td>69 (49.3)</td>
<td></td>
</tr>
</tbody>
</table>

Treatment

<table>
<thead>
<tr>
<th></th>
<th>Non-PE</th>
<th>PE</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>25 (36.2)</td>
<td>25 (18.1)</td>
<td><strong>&lt;0.0001</strong></td>
</tr>
<tr>
<td>No</td>
<td>69 (100)</td>
<td>44 (63.8)</td>
<td>113 (81.9)</td>
<td></td>
</tr>
</tbody>
</table>

Anti-hypertensive drugsa

<table>
<thead>
<tr>
<th></th>
<th>Non-PE</th>
<th>PE</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>0</td>
<td>60 (88.2)</td>
<td>60 (43.8)</td>
<td><strong>&lt;0.0001</strong></td>
</tr>
<tr>
<td>No</td>
<td>69 (100)</td>
<td>8 (11.8)</td>
<td>77 (56.2)</td>
<td></td>
</tr>
</tbody>
</table>

IPTp use (%)

<table>
<thead>
<tr>
<th></th>
<th>Non-PE</th>
<th>PE</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose 4</td>
<td>1 (1.5)</td>
<td>3 (4.8)</td>
<td>4 (3.1)</td>
<td>0.50</td>
</tr>
<tr>
<td>Dose 3</td>
<td>36 (55.4)</td>
<td>27 (42.9)</td>
<td>63 (49.2)</td>
<td></td>
</tr>
<tr>
<td>Dose 2</td>
<td>10 (15.4)</td>
<td>15 (23.8)</td>
<td>25 (19.5)</td>
<td></td>
</tr>
<tr>
<td>Dose 1</td>
<td>12 (18.5)</td>
<td>13 (20.6)</td>
<td>25 (19.5)</td>
<td></td>
</tr>
<tr>
<td>Dose 0</td>
<td>6 (9.2)</td>
<td>5 (7.9)</td>
<td>11 (8.6)</td>
<td></td>
</tr>
<tr>
<td>No record</td>
<td>8</td>
<td>4</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean and standard deviations for continuous variables and percentage for categorical variables. P-values were generated using Student’s t-test for continuous data and fisher’s exact test for categorical data. BMI = Body mass index, IPTp= intermittent preventive treatment in pregnancy. Differences were considered statistically significant if P < 0.05. ‘*’ data for these variables has missing observations and do not add up to the total number of samples.
2.4.2 Maternal birth outcome of study participants

Details of birth outcome are described in Table 2.2. The mean gestational age at delivery in the non-PE group (39 ± 2) was significantly higher than that of the PE group (36 ± 4, \( p < 0.0001 \)). All the women in the non-PE group had vaginal delivery, while 78.9% of women in the PE group had delivery by C-section (\( P < 0.0001 \)). Preterm delivery in the non-PE (4.3%) differed significantly from the PE group (49.3% \( p < 0.0001 \)). Low Apgar score and high number of neonatal intensive care unit (NICU) admissions were reported for births from women diagnosed with PE than the non-PE group.

Table 2.2: Birth Outcome among pregnant women diagnosed with or without preeclampsia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-PE N= 69</th>
<th>PE N= 71</th>
<th>Total N= 140</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery age (weeks)</td>
<td>39.5 ± 2</td>
<td>36.0 ± 4</td>
<td>37.7 ± 4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>69 (100)</td>
<td>15 (21.1)</td>
<td>84 (60)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C section</td>
<td>0</td>
<td>56 (78.9)</td>
<td>56 (40)</td>
<td></td>
</tr>
<tr>
<td>Preterm delivery</td>
<td>3 (4.3)</td>
<td>35 (49.3)</td>
<td>38 (27.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.2 ±0.5</td>
<td>2.4±1.0</td>
<td>2.8±0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apgar at 1 min</td>
<td>7.1 (1.1)</td>
<td>6.3 (1.3)</td>
<td>6.7 (1.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apgar at 5 min</td>
<td>8.3 (0.9)</td>
<td>7.6 (1.2)</td>
<td>8.0 (1.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex of baby(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (47.8)</td>
<td>31 (44.3)</td>
<td>64 (46.0)</td>
<td>0.80</td>
</tr>
<tr>
<td>Female</td>
<td>36 (52.2)</td>
<td>39 (55.7)</td>
<td>75 (54.0)</td>
<td></td>
</tr>
<tr>
<td>NICU admission(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (10.3)</td>
<td>40 (58.8)</td>
<td>47 (34.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>No</td>
<td>61 (89.7)</td>
<td>28 (41.2)</td>
<td>89 (65.4)</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean and standard deviations for continuous variables and percentage for categorical variables. \( P \)-values were generated using Student’s t-test for continuous data and fisher’s exact test for categorical data. Differences were considered statistically significant at \( P < 0.05 \).\(^a\) data for these variables have missing observations and do not add up to the total number of samples.
2.4.3 Placental histological findings in women diagnosed with and without preeclampsia

Placentas from 134 pregnant women were retrieved at delivery. Of this, 69 were from non-PE women and 65 from PE pregnant women. Histological changes were observed in both non-PE (29% of 69) and PE (47.7% of 65) placentas (Table 2.3). However, there were generally more non-pathologic (61.9% of 134) placentas than pathologic (38.1% of 134) ones in the study. From the photomicrographs (Figure 2.2), some unique pathological features were reported from the placentas. While most of the placentas (61.9% of 134) showed no pathological features, the percentage of placentas that showed pathological features were significantly higher ($P = 0.03$) in the PE group (47.7% of 65) than the non-PE (29% of 69).

Table 2.3: Observed histological findings in non-preeclamptic and preeclamptic placentas

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Non-PE (%)</th>
<th>PE (%)</th>
<th>Total (%)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>49 (71)</td>
<td>34 (52.3)</td>
<td>83 (61.9)</td>
<td>0.03</td>
</tr>
<tr>
<td>Yes</td>
<td>20 (29)</td>
<td>31 (47.7)</td>
<td>51 (38.1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>69 (100)</td>
<td>65 (100)</td>
<td>134 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as proportions between non-preeclamptic group (non-PE) and preeclamptic group (PE).

$P$-value obtained by Chi-square test.

Calcification, syncytial knot, infarction, atherosis, accelerated maturation of villi and a combination of these pathologies (mixed) were the major changes observed in the sampled placentas. The distribution of these histological changes are shown (Figure 2.3). A higher proportion of PE placentas had syncytial knots (21.5% of 65) compared to the proportion in the non-PE group (2.9% of 69, $P = 0.002$). On the other hand, the proportions with calcification
were not significantly different between the non-PE group (23.2% of 69) and the PE group (11.3% of 65, \( P = 0.06 \)). Mixed pathologies, atherosclerosis, accelerated maturation and infarction were also not significantly different between the PE and non-PE groups (Figure 2.3).
Figure 2.2: Photomicrographs of placental changes observed among pregnant women from the study.

a) A non-PE placenta showing normal villi (black arrow) and intervillous spaces (red arrow) b) Calcifications (black arrow) in a 33-week old PE placenta. (x400) c) Infarction (coagulative necrosis) of large area of the placenta from ischaemia (black arrow) H&E x400 29-week placenta with intrauterine foetal death) d) Accelerated villous maturation (thin finger-like or slender villi with reduced branching) (H&E x100); e) Atherosis showing accumulation of lipid laden macrophages within sub-endothelial area of arterial wall (H&E x400), f) Increased syncytial knots showing densely stained and closely packed nuclei (H&E x400).
Figure 2.3: Distribution of unique histological findings in normal and pathologic placentas in the study groups.

Proportions of specific pathological features between PE and non-PE placentas. *P*-value obtained by Chi-square test.

### 2.4.4 Pathological features and maternal factors among participants

Histological observations in the placenta were assessed in terms of maternal and pregnancy outcomes (gravidity, delivery age and birth weight) in PE and non-PE pregnancies (Figure 2.4). Placental pathology was prevalent in primigravids in the PE group compared to the non-PE group. The proportion of primigravids with no pathology was not different between groups. Also, multigravids with pathologic placentas were less prevalent in the PE group compared to the non-PE group. In non-PE women with normal placentas, mean delivery age was significantly higher (40 weeks) than the mean delivery age for PE women with a normal placenta (36 weeks *P* < 0.0001). Similarly, non-PE women with pathologic placentas had
higher delivery age (39 weeks) than the PE women (37 weeks; \( P = 0.03 \)). Mean birth weight of babies delivered to non-PE women was higher (3.2 kg) than those from PE women (2.6 kg; \( P = 0.0006 \)) in the non-pathologic placenta group. Again, the pathologic placentas also showed the non-PE women delivering babies with higher birth weight (3.1 kg) as compared to birth weight from PE women (2.5 kg; \( P = 0.01 \)).
Figure 2.4: Association between pregnancy outcome and placental histological observations in women with and without preeclampsia.

Pathologic effects on (A) Gravidity (B) delivery age and (C) birth weight in PE and non-PE women. Student’s t-test was used to compare means between two variables and Chi-square test for proportions.
2.4.5 Risk factors associated with pathology in non-preeclamptic and preeclamptic pregnancies

Generally, placental pathology was significantly associated with PE. Presence of placental pathology increased the odds of PE (OR 2.2, 95% CI 1.1 – 4.6; \( P = 0.027 \)). Syncytial knots were the only specific pathology that increased the risk of PE (OR 10.1, 95% CI 2.2 – 47.3; \( P = 0.003 \)). Calcification, mixed pathology, infarction, accelerated villous maturation and atherosis were not significantly associated with PE. Systolic and diastolic blood pressure as well as primigravidity in univariate analysis predicted PE [odds ratio: 1.0, 95% CI (1.0 – 1.1), \( P = 0.010 \); 1.0, 95% CI (1.0 – 1.1); \( P = 0.042 \) and 3.0, 95% CI (1.5 – 6.3), \( P = 0.003 \) respectively). After adjusting for specific maternal factors that independently predicted PE, presence of pathology, primigravidity and systolic blood pressure significantly predicted PE (Table 2.4).

Table 2.4: Placental pathology, maternal factors and the risk for preeclampsia

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95 % CI)</th>
<th>P-value</th>
<th>AOR (95 %CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental Pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>2.2 (1.1 – 4.6)</td>
<td>Ref</td>
<td>0.027</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td>Ref</td>
<td>2.8 (1.2 – 6.4)</td>
</tr>
<tr>
<td>Specific Pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed Pathology</td>
<td></td>
<td>7.21 (0.8 – 64.5)</td>
<td>0.077</td>
<td>-</td>
</tr>
<tr>
<td>Infarction</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calcification</td>
<td></td>
<td>0.63 (0.2 – 1.7)</td>
<td>0.361</td>
<td>-</td>
</tr>
<tr>
<td>Atherosis</td>
<td></td>
<td>2.8 (0.3 – 33.1)</td>
<td>0.395</td>
<td>-</td>
</tr>
<tr>
<td>Accelerated maturation</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Syncytial Knots</td>
<td></td>
<td>10.1 (2.2 -47.3)</td>
<td>0.003</td>
<td>-</td>
</tr>
<tr>
<td>1(^{st}) visit SBP (mmHg)</td>
<td></td>
<td>1.0 (1.0 – 1.1)</td>
<td>0.010</td>
<td>1.0 (1.0 - 1.1)</td>
</tr>
<tr>
<td>1(^{st}) visit DBP (mmHg)</td>
<td></td>
<td>1.0 (1.0 – 1.1)</td>
<td>0.042</td>
<td>1.0 (0.9 – 1.1)</td>
</tr>
<tr>
<td>Primigravid</td>
<td></td>
<td>3.0 (1.5 – 6.3)</td>
<td>0.003</td>
<td>4.5 (2.0 – 10.7)</td>
</tr>
</tbody>
</table>

Univariate and multivariate analysis showing factors associated with placental pathology, maternal and pregnancy outcome and the risk of PE. Data presented as odds ratio (OR), adjusted odds ratio (AOR) and confidence interval (CI). Specific placental pathology was not adjusted for the risk of PE. SBP and DBP = systolic and diastolic blood pressure respectively.
2.4.6 *Plasmodium falciparum* placental exposure and the risk of preeclampsia

From the 140 women recruited, six placentas could not be obtained from the PE group while one was heavily pigmented and impossible to evaluate. Placental malaria classification guide by Bulmer *et al.* (1993) was used to score the absence of infection, acute, chronic and past infections as illustrated in the photomicrographs (Figure 2.5) for all the 133 placentas. The distribution of this scoring is described below (Table 2.5). Some of the placentas examined were infected with active parasites (n=64, 48.1%). Of the 69 placentas that were not actively infected, 15.8% presented with evidence of past infections while 36.1% showed no level of exposure or infection. Most of the women in the non-PE group were not exposed to placental infections (55.1% of 69) while active parasite infection was detected in the placentas of most PE diagnosed women (60.9% of 64) (Figure 2.6).

<table>
<thead>
<tr>
<th>Bulmer score</th>
<th>Non-PE (%)</th>
<th>PE (%)</th>
<th>Total (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Infection</td>
<td>38 (55.1)</td>
<td>10 (15.6)</td>
<td>48 (36.1)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Acute</td>
<td>5 (7.2)</td>
<td>11 (17.2)</td>
<td>16 (12.0)</td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>20 (29.0)</td>
<td>28 (43.8)</td>
<td>48 (36.1)</td>
<td></td>
</tr>
<tr>
<td>Past Infection</td>
<td>6 (8.7)</td>
<td>15 (23.4)</td>
<td>21 (15.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>69</strong></td>
<td><strong>64</strong></td>
<td><strong>133</strong></td>
<td></td>
</tr>
</tbody>
</table>

Data presented as proportions between non-preeclamptic group (non-PE) and preeclamptic group (PE).

*P*-value obtained by Chi-square test.
Figure 2.5: Placental infections observed among pregnant women from the study.

a) Normal placental architecture with uninfected RBCs in the intervillous spaces (H & E x100). b) Acute malaria infection X600: Only parasitized red cells. No parasite pigments c) Chronic malaria infection X600: Parasites and parasite pigments are present in red cells, 38-week old placenta d) Past malaria infection X600: Showing pigments in fibrin.
Figure 2.6: Distribution of placental infections in women diagnosed with or without preeclampsia.

Data presented as proportions between active, past and absence of infections in the non-PE and PE groups by Chi square test.

2.4.7 Placental exposure, maternal and pregnancy outcomes in preeclampsia

Classification of the parasite infection state was by active, past and absence of infections. Distribution of the parasite infection state by gravidity between the study groups was determined as described (Table 2.6). Majority of the primigravids in the PE group had active and past infections (51% of 47) compared to the non-PE group (13% of 47). Absence of infection was 15% and 21% of 47; \( P = 0.03 \) in the PE and non-PE group respectively. In the 86 multigravidae, 33% of the non-PE group and 4% of the PE group had no infections. Exposure to parasites (active and past infections) was 29% of 86 in the non-PE group and of 35% of 86 in the PE group \( (P < 0.0001) \). Mean delivery age was higher (39 weeks) in women with active infection in the non-PE group compared to the PE group (36 weeks; \( P = 0.0002 \)).
Mean age in women with past infection or absence of infections did not differ from each other. Mean birth weight of babies was not significantly different amongst groups with active, past or no infection in both non-PE (3.2 ± 0.5 kg, 3.3 ± 0.3 kg and 3.1 ± 0.5 kg respectively; \( P = 0.8 \)) and PE women (2.4 ± 1.0 kg, 2.4 ± 0.9 kg and 3.1 ± 1.1 kg respectively; \( P = 0.07 \)). However, in the active and past infection group, mean birth weight of babies were significantly higher in the non-PE group (3.2 ± 0.5 kg, 3.3 ± 0.3 kg respectively) compared to the PE group (2.4 ± 1.0 kg, 2.4 ± 0.9 kg respectively; \( P = 0.0003 \) and 0.03 respectively) (Figure 2.7).

### Table 2.6: Distribution of parasite infection status by gravidity between study groups

<table>
<thead>
<tr>
<th></th>
<th>No Infection (%)</th>
<th>Active Infection (%)</th>
<th>Past Infection (%)</th>
<th>Total (%)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primigravid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-PE</td>
<td>10 (58.8)</td>
<td>4 (17.4)</td>
<td>2 (28.6)</td>
<td>16 (32.0)</td>
<td>0.03</td>
</tr>
<tr>
<td>PE</td>
<td>7 (41.2)</td>
<td>19 (82.6)</td>
<td>5 (71.4)</td>
<td>31 (68.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Multigravid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-PE</td>
<td>28 (90.3)</td>
<td>21 (51.2)</td>
<td>4 (28.6)</td>
<td>53 (58.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PE</td>
<td>3 (9.7)</td>
<td>20 (48.8)</td>
<td>10 (71.4)</td>
<td>33 (41.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>48</td>
<td>64</td>
<td>21</td>
<td>133</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as proportions between non-PE and PE groups in primigravid and multigravid women.

\( P \)-value obtained by Chi-square test.
Figure 2.7: Association between pregnancy outcome and histological observation in non-PE and PE diagnosed women.

Delivery age and birth weight were compared between PE and non-PE women with active past and no placental infection. Centred bar in data distribution represents the mean within groups by ANOVA and between groups by unpaired Student’s t-test. * \((P < 0.05)\), ** \((P < 0.01)\), *** \((P < 0.001)\).
2.4.8 Placental malaria, pathology and pregnancy factors as predictors of preeclampsia

The study compared placental infections, pathology and other significant maternal risk factors for PE development using a logistic regression model (Table 2.7). Presence of past and active placental infections increased the chance of developing PE compared to women with no placental infections [odds ratio: 5.9 (2.6 – 14.0), \( P < 0.0001 \) and 9.5 (2.9 – 30.8), \( P < 0.0001 \) respectively]. Placental pathology, blood pressure at first antenatal visit and primigravidity were also associated with PE as earlier described (Section 2.4.5). A multivariate analysis was used to determine further predictors of PE. Presence of active or past placental infections further increased the risk of PE [adjusted odds ratio: 6.7 (2.3 – 19.1), \( P < 0.0001 \); 12.4 (3.0 – 51.0), \( P < 0.0001 \) respectively] in the presence of placental pathology [adjusted odds ratio: 3.0 (1.2 – 7.5), \( P = 0.019 \)] and among primigravids [adjusted odds ratio: 6.6 (2.4 – 18.2), \( P < 0.0001 \)]. Blood pressure was not a predictor of PE in the presence of these factors.
Table 2. 7: Crude and adjusted odds ratios for factors associated with placental malaria and the risk of preeclampsia.

<table>
<thead>
<tr>
<th></th>
<th>Non-PE</th>
<th>PE</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>AOR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Infection</td>
<td>38</td>
<td>10</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Active Infection</td>
<td>25</td>
<td>39</td>
<td>5.9 (2.6 – 14.0)</td>
<td>&lt;0.0001</td>
<td>6.7 (2.3 – 19.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Past Infection</td>
<td>6</td>
<td>15</td>
<td>9.5 (2.9 – 30.8)</td>
<td>&lt;0.0001</td>
<td>12.4 (3.0 – 51.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Pathology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>49</td>
<td>34</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>31</td>
<td>2.2 (1.1 – 4.6)</td>
<td>0.027</td>
<td>3.0 (1.2 – 7.5)</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>1st visit SBP (mmHg)</strong></td>
<td>69</td>
<td>64</td>
<td>1.0 (1.0 - 1.1)</td>
<td>0.010</td>
<td>1.04 (1.0 – 1.1)</td>
<td>0.083</td>
</tr>
<tr>
<td><strong>1st visit DBP (mmHg)</strong></td>
<td>69</td>
<td>64</td>
<td>1.0 (1.0 – 1.1)</td>
<td>0.042</td>
<td>1.0 (0.9 – 1.1)</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Primigravidity</strong></td>
<td>16</td>
<td>31</td>
<td>3.0 (1.5 -6.3)</td>
<td>0.003</td>
<td>6.6 (2.4 – 18.2)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

OR (odds ratio); AOR (adjusted OR). Logistic regression model used. P value is significant if < 0.05.

PM represents placental malaria, PE = preeclampsia, SBP and DBP = systolic and diastolic blood pressure respectively.
2.5 Discussion

Placental examination is an important, non-invasive means to continuously decipher pathological conditions in order to improve maternal and foetal health. Therefore, histological examination of placentas after delivery from PE and non-PE diagnosed women in a malaria endemic region will provide further insight into the pathophysiology of the disorder. Histological alterations occurred more frequently in PE placentas than non-PE placentas in this study. The placenta is the source of PE pathogenesis (Roberts and Escudero, 2012) hence this was expected. Placental alterations are reported to be non-specific to PE, however it has been an observation in several PE associated studies (Falco et al., 2017). This suggests that, these alterations may contribute either directly or indirectly to PE pathogenesis.

Of the specific alterations associated with PE, syncytial knots were identified as the main contributor to placental pathology in women diagnosed with PE. It was about ten-fold more frequent in PE than non-PE placentas. These aggregates of syncytial nuclei are associated with conditions of uteroplacental malperfusion (Loukeris et al., 2010) and hypoxia (Heazell et al., 2007) such as PE. The syncytiotrophoblasts mainly serve as protective barrier against harmful effects to the foetus as well as a foeto-maternal exchange portal for nutrients and waste products. Functional deficits of the syncytiotrophoblasts result in the formation of syncytial knots which support the ‘Tenney-Parker’ changes of the placenta used as an index of well-being (Tenney Jr and Parker Jr, 1940, Benirschke et al., 2012, Fogarty et al., 2013). Our data also confirm findings from a previous report of excessive syncytiotrophoblasts in PE placentas compared to normotensive women (Devisme et al., 2013). Increased syncytial knots have also been associated with PE severity (Devisme et al., 2013, Weel et al., 2016). The biological inference here is that increased syncytial knots may result in sporadic release of factors into the localized placental environment that may cause further damage to the placenta.
Subsequently, these factors such as sVEGFR1, are exported into maternal circulation to cause the clinical symptoms of preeclampsia. These bioactive factors could be used as surrogate markers to enhance the management of PE.

Accelerated villous maturations and infarctions were reported in the PE group only; hence statistical significance could not be established. Calcifications, atherosis and presence of mixed pathologies were not different between the groups. Although these are biologically significant pathologic alterations in PE pregnancies, literature reports a discordance in their pathophysiologic ramifications (Staff et al., 2010, Wallingford et al., 2018). In addition, the overall association of placental pathology with PE shown in this study suggests that these placental lesions may have a contributory effect either directly or indirectly to the PE syndrome.

Many independent risk factors for PE highlighted earlier were excluded at recruitment in order to limit confounders. Other risk factors such as high BMI were not different between the PE and non-PE groups as shown in the demographic characteristics. Primigravidity and blood pressure were independent risk factors for PE in the univariate analysis. Presence of this association was still evident in a multivariate analysis that included the presence of placental pathology with the exception of diastolic blood pressure. Increased blood pressure has been linked to the development of hypertensive disorders in pregnancy (HDP) (Boghossian et al., 2015). Krielessi correlated the extent of placental lesions with the level of hypertensive disorders and found that extensive placental lesions were associated with a higher level of hypertension (Krielessi et al., 2012). However high blood pressure alone, is indicative of gestational hypertension which is a different phenotype from PE and may not present with placental pathology as reported elsewhere (Melamed et al., 2014, Stanek, 2017). Primigravidity
is also a known risk factor for PE (Duckitt and Harrington, 2005, Hernández-Díaz et al., 2009). Our study has shown a combined effect of pathology, primigravidity and blood pressure as contributors to PE that discriminates from other HDP. Our data also support some of the adverse pregnancy and foetal outcomes associated with PE.

Malaria endemicity may be an added risk factor for PE pathogenesis as demonstrated in earlier studies (Sartelet et al., 1996, Etard et al., 2003, Muehlenbachs et al., 2006, Adam et al., 2011). The histological grading system for PM showed a high exposure rate in PE than non-PE women. Consistent with earlier studies, PM is an independent risk factor for PE with adverse outcomes such as low birth weight. Approximately nine- and six-fold increase in the risk for PE were observed for past and active infections respectively, with absence of infections as a reference. This study has linked placental infections and not peripheral infections to the risk of PE. In a similar study in Sudan, PM was associated with PE mostly by past malaria infection in placental histological grading (Adam et al., 2011). In Senegal, histological diagnosis was made but the study did not present data based on our histological grading system and as done in Sudan (Sartelet et al., 1996). Therefore, conclusion cannot be drawn on whether the association was based on only active infections, past infections or both. Other studies generally associated PM with HDP and not PE only (Muehlenbachs et al., 2006, Ndao et al., 2009) as this present study has shown. Placental pathology was not specifically related to PM and other factors in these studies. Muehlenbachs et al. (2006) however showed elevated levels of sVEGFR-1, a PE biomarker, in primigravids with PM, hypertension or both. They concluded that this marker, associated with endothelial or placental dysfunction, may be under selective pressure during first pregnancies in malaria endemic regions. Our study has brought to bear the link between placental pathology, PM (active or past infections) and PE. In the presence of PM, blood pressure was not significant in the multiple comparison model. This is because
malaria is known to decrease blood pressure. In Muehlenbachs study, parasite density was inversely associated with blood pressure however, chronic placental infections by histology were associated with high blood pressure (Muehlenbachs et al., 2006). Although our investigation of PM was retrospective and subclinical, the increased risk of preeclampsia in primigravid women with placental pathology and PM highlights the devastating effects of these factors on PE pathogenesis.

2.6 Conclusion

In conclusion, this study has demonstrated an increase in placental alteration in PE placentas specifically linking increased syncytial knots to the PE outcome. In addition, PM is independently associated with PE or may be dependent on factors such as first pregnancy and placental pathology to induce PE. Mechanisms that induce this phenotype of PE should be further investigated.
CHAPTER THREE

3.0 Paper 2: Placental malaria is a risk factor associated with inflammatory and angiogenic network in women diagnosed with preeclampsia.

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6. Department of Pathology, Korle-Bu Teaching Hospital, Accra, Ghana
3.1 Abstract

**Background:** Preeclampsia (PE) and placental malaria (PM) are associated with inflammatory and angiogenic alterations in the placenta and maternal circulation resulting in poor pregnancy outcomes. However, it is unknown if the inflammatory and angiogenic pathways during PM precipitate or aggravate the syndrome of PE.

**Methods:** Exposure to PM was determined among 74 pregnant women diagnosed with (n = 44) and without (n = 30) PE at term by histology. Peripheral and placental plasma were obtained at delivery to measure levels of 26 inflammatory and angiogenic analytes using a multiplex bead-based ELISA assay.

**Results:** There were 42 active placental infections with a significantly higher proportion in the PE group (n = 27) compared to the non-PE group (n = 15, \( P = 0.04 \)). The placenta generally showed a higher concentration of markers compared to maternal circulation in both women diagnosed with and without PE. Levels of proinflammatory markers and anti-angiogenic markers were elevated in PE compared to non-PE pregnancies. The PE pregnancies with active placental parasites also showed higher levels of proinflammatory and anti-angiogenic markers compared to the non-PE pregnancies. Factors that significantly increased the risk of PE in a multivariate analysis included active parasite infection (Adjusted odds ratio (AOR) = 7.14, 95% CI = 1.1 – 44.7; \( P = 0.04 \)), past infections (AOR = 12.9, 95% CI = 1.1 – 155.5; \( P = 0.04 \)), primigravidity (AOR = 7.2, 95% CI = 1.1 – 48.0; \( P = 0.04 \)) and increased levels of plasminogen activator inhibitor (PAI)-1 molecule AOR = 7.1, 95% CI = 1.3 – 38.3; \( P = 0.02 \)).

**Conclusion:** This study has generally shown that PE is associated with dysregulated inflammation and angiogenesis which is enhanced in women with PM. Also, PAI-1 showed a predictive potential and should further be investigated to ascertain its diagnostic and therapeutic potential in PM and PE pathogenesis.
3.2 Introduction

Inflammation and angiogenesis are closely related and tightly regulated immune mechanisms. Their role in pathological conditions are well documented especially in vascular pathologies such as atherosclerosis, diabetes, and hypertension (Laitinen et al., 2013, Nazir et al., 2014, Quarck et al., 2015). Angiogenesis involves the sprouting of new blood vessels from preexisting ones and may occur during physiological processes such as organ development, tissue repair and inflammation. This process is mediated or inhibited by cytokines, chemokines, growth factors, adhesion molecules and proteases through the migration of immune cells (Szekanecz and Koch, 2007). Although inflammation triggers angiogenesis, this process further enhances the inflammatory pathway that could precipitate a pathologic condition. An activated endothelium ensures this vicious cycle of immune cell infiltration and the propagation of inflammation and angiogenesis (Szekanecz and Koch, 2007).

Preeclampsia (PE) is a complex obstetric vascular disorder that is defined mainly by clinical symptoms due to unknown aetiology. Altered inflammation and angiogenesis are proposed aetiological theories associated with vascular dysfunction in this disorder (Roberts et al., 1989, Verlohren et al., 2009, Redman and Sargent, 2010). Although a mild systemic inflammatory state is triggered during the third trimester of pregnancy to induce uterine contractions, this is further enhanced during PE (Redman and Sargent, 2003, Roberts and Escudero, 2012, Gueneuc et al., 2017). The systemic observations in PE are triggered mainly by placental factors. Abnormal placentation during PE triggers a local inflammatory response with altered angiogenesis that leaks deranged placental factors into systemic circulation to cause the clinical syndrome (Roberts et al., 1989, Maynard and Karumanchi, 2011). Factors that exaggerate the inflammatory and angiogenic network of PE are important as the search for aetiological, diagnostic and therapeutic tools continue.
Pregnancy is generally skewed towards an anti-inflammatory T helper (Th) 2 response for foetal adaptation but predisposes pregnant women to infections (Gueneuc et al., 2017). This explains why placental malaria (PM) is frequent, particularly in first time mothers in malaria endemic regions. Sequestration of parasites in the intervillous spaces of the placenta releases proinflammatory cytokines that initiate the cascade of dysregulated angiogenesis, enhanced inflammation and endothelial dysfunction (Gueneuc et al., 2017). This is detrimental for foetal adaptation and growth. Placental dysregulation due to PM has mainly been linked to maternal anaemia, foetal growth restrictions and low birth weight infants (Ofori et al., 2009, Lufele et al., 2017). However, these placental events might be initiators or promoters of local and systemic pathophysiologic events that may lead to PE.

Several markers that promote or inhibit inflammation, angiogenesis and endothelial or placental dysfunction are similar in both PM and PE but may lack specificity to a single disease. For instance, the vascular endothelial growth factor (VEGF) and angiopoietin (Ang) family of markers which play a role in angiogenesis have been reported as factors in both PM and PE pathophysiology (Muehlenbachs et al., 2006, Leinonen et al., 2010, Ataide et al., 2015, Adu-Bonsaffoh et al., 2017). The non-specificity of these markers hinders their predictive, diagnostic or therapeutic potential. Assessing a cocktail of markers with different physiologic pathways may provide a diagnostic lead to identifying women at risk of PM and PE.

Although PE and PM are placental disorders releasing placental factors into maternal circulation, measuring these factors in the periphery may not always reflect placental assessment. Inflammatory and angiogenic markers have been shown to differ between the placental and the peripheral plasma with some showing either high or low expression in the placenta (Bouyou-Akotet et al., 2004, Keelan and Mitchell, 2007, Djontu et al., 2016,
Okamgba et al., 2018). How these differences translate to disease is a question of interest. This study proposed that PM perpetuates the inflammatory and angiogenic milieu in the placenta, leading to the precipitation or exacerbation of PE. Inflammatory and angiogenic molecules were measured in maternal circulation and the placenta of women diagnosed with and without PE. This was to determine the presentation pattern of these molecules at these sites and how they relate to PE. Additionally, *P. falciparum* exposure in the placenta was determined to assess alterations in inflammatory and angiogenic molecules in women diagnosed with and without PE.
3.3 Methods

3.3.1 Ethical considerations

The study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR) (IRB# 00001276)) registered with OHRP (FWA 00001824) and the Ethical Review Committee of the Korle-Bu Teaching Hospital (KBTH) in Ghana. Consenting pregnant women between 18 and 45 years were recruited from the Obstetrics and Gynaecology Unit of the KBTH in Ghana.

3.3.2 Recruitment of participants

One hundred and forty pregnant women were recruited into the study for a broader overview of assessing the risk of PE in women exposed to PM (Chapter 2). Preeclampsia (PE) was defined according to international guidelines of blood pressure ≥ 140mmHg systolic or ≥ 90mmHg diastolic at least 4 hours apart with proteinuria (ACOG, 2013, Tranquilli et al., 2014). Exposure to PM was defined by histological grading as described elsewhere (Bulmer et al., 1993, Muehlenbachs et al., 2006). In this present study peripheral and placental blood were obtained at delivery and after delivery respectively from 44 women diagnosed with PE and 30 without PE.

3.3.3 Peripheral blood sampling, processing and storage

At delivery, maternal peripheral blood was collected into anticoagulant ethylenediaminetetraacetic acid (EDTA) tubes, spun down at 2000rpm for 10 minutes at 4°C, and plasma stored at -80°C for subsequent immunoassays.
3.3.4 Placental blood sampling and processing

Expelled placenta was immediately sampled after delivery. Two to three incisions were made (about 2 mm deep) from the maternal decidua in an off-centre position, one quarter distance from the edge of the placenta as described by Bulmer et al. (1993). Pooled blood from the area of incision was collected using Pasteur pipettes into labelled EDTA treated tubes. Samples were transported and processed immediately and their products stored at -80 °C for subsequent immunoassays.

3.3.5 Histological evaluation of placental malaria

The placental tissues sampled were evaluated histologically as earlier described (Bulmer et al., 1993) for the presence of placental parasites. Briefly, placental tissues were incised, fixed in 10% neutral buffered formalin, processed and subsequently embedded in paraffin wax. Tissues were sectioned onto well-labelled glass slides, stained with H & E and microscopically graded as active, past or absence of Plasmodium infections.

3.3.6 Measurement of inflammatory and angiogenic markers by multiplex ELISA

To investigate inflammation, angiogenesis and the risk of PE, a panel of markers functionally linked to alteration or regulation of these factors were analysed using the magnetic multiplex bead assay with premixed multi-analyte kit (R & D Systems, USA). Twenty six markers associated with either PE or PM pathophysiology were measured on placental and peripheral plasma samples from women diagnosed with and without PE. Assay principle was based on the use of analyte-specific antibodies pre-coated onto colour-coded magnetic microparticles. The manufacturer’s protocol was followed in all reagent preparations and assay procedures. Briefly, microparticles pre-coated with analyte-specific capture antibodies were incubated with
placental and peripheral plasma samples with specified dilutions and detection range (Table 3.1). This was followed by the addition of biotin antibody cocktail diluted in assay buffer. After incubation, streptavidin-phycoerythrin was added and read on a Luminex 200 analyser (Luminex Xmap Technology, USA). Median fluorescence intensity was measured using analyser software (xPONENT 3.1).
Table 3.1: Summary of analytes in the multiplex ELISA assay

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Plasma Dilution</th>
<th>Detection Range (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Panel 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG -2</td>
<td>1:2</td>
<td>90.5 - 22,000</td>
</tr>
<tr>
<td>CCL2/Monocyte chemoattractant protein (MCP) 1</td>
<td>1:2</td>
<td>31.9 - 7,740</td>
</tr>
<tr>
<td>CXCL10/Interferon gamma-induced protein (IP) 10</td>
<td>1:2</td>
<td>2.8 – 690</td>
</tr>
<tr>
<td>CXCL12/Stromal derived factor (SDF)-1 alpha</td>
<td>1:2</td>
<td>151 - 36,720</td>
</tr>
<tr>
<td>CXCL8/Interleukin (IL)-8</td>
<td>1:2</td>
<td>4.7 - 1,140</td>
</tr>
<tr>
<td>Fibroblast growth factor basic (bFGF)</td>
<td>1:2</td>
<td>1.89 – 460</td>
</tr>
<tr>
<td>Granulocyte-macrophage colony-stimulating factor (GM-CSF)</td>
<td>1:2</td>
<td>12.5 - 3,030</td>
</tr>
<tr>
<td>Interferon-gamma (IFN-γ)</td>
<td>1:2</td>
<td>299 - 24,200</td>
</tr>
<tr>
<td>Interleukin (IL)-1 beta</td>
<td>1:2</td>
<td>16.3 - 3,960</td>
</tr>
<tr>
<td>IL-10</td>
<td>1:2</td>
<td>3.7 – 890</td>
</tr>
<tr>
<td>IL-17A</td>
<td>1:2</td>
<td>9.26 - 2,250</td>
</tr>
<tr>
<td>IL-21</td>
<td>1:2</td>
<td>29.4 - 7,140</td>
</tr>
<tr>
<td>IL-6</td>
<td>1:2</td>
<td>4.9 - 1,180</td>
</tr>
<tr>
<td>CD146</td>
<td>1:2</td>
<td>215 - 52,240</td>
</tr>
<tr>
<td>PlGF</td>
<td>1:2</td>
<td>1.9 – 470</td>
</tr>
<tr>
<td>Tie-2</td>
<td>1:2</td>
<td>614 - 149,166</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1:2</td>
<td>8.9 - 2,160</td>
</tr>
<tr>
<td>VEGF R1/Flt-1</td>
<td>1:2</td>
<td>72.1 - 17,530</td>
</tr>
<tr>
<td><strong>Panel 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF)-A</td>
<td>1:2</td>
<td>8.5 - 2,070</td>
</tr>
<tr>
<td>Intercellular adhesion molecule (ICAM)-1</td>
<td>1:2</td>
<td>6,615 - 1,607,350</td>
</tr>
<tr>
<td>VEGF R2</td>
<td>1:2</td>
<td>154 - 37,400</td>
</tr>
<tr>
<td><strong>Panel 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG-1</td>
<td>1:50</td>
<td>114 – 27,610</td>
</tr>
<tr>
<td>Matrix metalloproteinase (MMP )-9</td>
<td>1:50</td>
<td>133.6 – 32,460</td>
</tr>
<tr>
<td>Platelet derived growth factor (PDGF) -BB</td>
<td>1:50</td>
<td>13.6 – 3,310</td>
</tr>
<tr>
<td><strong>Panel 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-reactive protein (CRP)</td>
<td>1:200</td>
<td>104 - 25,200</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor (PAI)-1</td>
<td>1:200</td>
<td>19.5 - 4,730</td>
</tr>
</tbody>
</table>

Four multiplex ELISA kits (panel 1 – 4) each with different dilution factor and detection range were used for the experiment.
3.3.7. Statistical analysis

Statistical analyses were carried out using R package software version 3.5.1 (R Development Core Team). Demographic and clinical details of study participants were summarised as mean and standard deviations for data that passed the normality test using the Student’s t-test for continuous variables and Chi-square test for categorical variables. Non-parametric data were analysed and reported as median and range. Mann-Whitney rank sum test was used as a comparator between two variables while Kruskal-Wallis test (non-parametric) was used with Dunn’s multiple comparison test as comparator for more than two variables. Logistic regression analysis was used to establish association between the analytes, maternal parameters, PM and the risk of PE in a univariate and multivariate model. Level of significance was set at $P < 0.05$. 


3.4 Results

3.4.1 Demographic and clinical characteristics of study participants

Maternal and birth outcomes were compared between PE and non-PE groups (Table 3.2). In all, plasma samples were obtained from 74 pregnant women consisting of 30 without PE and 44 diagnosed with PE. Maternal age, haemoglobin levels and platelet count of the PE and non-PE groups were not different. There were 31 primigravids (41.9% of 74) and 43 multigravids (58.1% of 74) within the study population however, there were more primigravids (77% of 31) in the PE compared to the control group (23% of 31 \(P = 0.015\)). Birth outcome showed differences in the delivery age of (39.7 ± 1.7 weeks), birth weight (3.2 ± 0.4 kg) in the non-PE compared to the PE women (36.1 ± 3.9 weeks, \(P < 0.0001\) and 2.3 ± 0.8 kg, \(P < 0.0001\) respectively). Mode of delivery showed a higher proportion of vaginal deliveries in the non-PE group (77% of 39) than the PE group (23% of 39 \(P < 0.0001\)).
Table 3.2: Maternal and birth outcome among study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-PE</th>
<th>PE</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n= 30</td>
<td>n= 44</td>
<td>n= 74</td>
<td></td>
</tr>
<tr>
<td>Mean maternal age (years)</td>
<td>29 ± 6</td>
<td>27 ± 6</td>
<td>28 ± 6</td>
<td>0.20</td>
</tr>
<tr>
<td>Primigravid (%)</td>
<td>7 (23.3)</td>
<td>24 (54.5)</td>
<td>31 (41.9)</td>
<td>0.015</td>
</tr>
<tr>
<td>Multigravid (%)</td>
<td>23 (76.7)</td>
<td>20 (45.5)</td>
<td>43 (58.1)</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin levels (g/dL)</td>
<td>11.6 ± 1.4</td>
<td>12.3 ± 2.4</td>
<td>12.0 ± 2.1</td>
<td>0.18</td>
</tr>
<tr>
<td>Platelet count (x10⁹/L)</td>
<td>194.9 ± 75.0</td>
<td>175.4 ± 80.9</td>
<td>183.5 ± 78.6</td>
<td>0.31</td>
</tr>
<tr>
<td>Placental parasites</td>
<td>27 (65.9)</td>
<td>15 (50.0)</td>
<td>42 (59.2)</td>
<td>0.04</td>
</tr>
<tr>
<td>Delivery age (weeks)</td>
<td>39.7 ± 1.7</td>
<td>36.1 ± 3.9</td>
<td>37.6 ± 3.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>30 (100)</td>
<td>9 (20.5)</td>
<td>39 (52.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C section</td>
<td>0</td>
<td>35 (79.5)</td>
<td>35 (47.3)</td>
<td></td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.2 ± 0.4</td>
<td>2.3 ± 0.8</td>
<td>2.7 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apgar at 1 min</td>
<td>7.1 ± 0.8</td>
<td>6.4 ± 1.2</td>
<td>6.7 ± 1.1</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Apgar at 5 min</td>
<td>8.4 ± 0.6</td>
<td>7.8 ± 0.9</td>
<td>8.0 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are represented as mean and standard deviation or proportions between groups. Values highlighted represent comparison with significant P values at < 0.05. PE represents women diagnosed with preeclampsia and non-PE are women without preeclampsia.
3.4.2 Inflammatory profile in peripheral and placental blood

The concentration of proinflammatory (CRP, IFN-γ, IL1, IL6, IL8, IL17 and TNF-α) and anti-inflammatory (IL10 and IL21) molecules were measured in peripheral and placental blood of PE and non-PE women (Figure 3.1). Concentrations of proinflammatory molecules IFN-γ, IL1, IL8, and TNF-α were higher in the placenta \((P < 0.05)\) compared to peripheral plasma in both groups of pregnant women. The CRP on the other hand showed higher concentrations in the periphery compared to the placenta in all pregnant women \([\text{non-PE} (P < 0.001) \text{ and PE} (P < 0.0001)]\). Interestingly, both IL6 and IL17 concentrations were found to be higher in the placenta as compared to the periphery plasma samples among the PE diagnosed women and the differences were statistically significant \((P < 0.0001 \text{ and } P < 0.05 \text{ for } \text{IL6 and IL17 respectively})\). This observation was not seen among the non-PE group. For the anti-inflammatory molecules, IL10 showed higher levels in placenta compared to the periphery \((P = 0.025)\) among the non-PE group with no significant difference among the PE group. On the other hand, the concentration levels of IL21 did not show any differences between the placenta and the periphery in both non-PE and PE diagnosed women.
Figure 3.1: Peripheral and placental blood levels of inflammatory molecules in women diagnosed with and without preeclampsia.

Comparisons are for differences between markers in peripheral and placental blood. Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. \( P \) values represented as *\( <0.05 \), **\( <0.01 \), ***\( <0.001 \), ****\( <0.0001 \).
3.4.3 Profile of anti-angiogenic molecules in peripheral and placental blood

The concentrations of molecules associated with dysregulated angiogenesis (PAI-1, MMP9, ICAM1, Ang2, CCL2, CXCL10, CD146 and sVEGFR1) were measured in peripheral and placental blood. Anti-angiogenic molecules PAI-1, Ang2, CCL2 and sVEGFR1 levels were found to be higher in the placenta as compared to the periphery ($P < 0.01$) of both non-PE and PE women (Figure 3.2). Also, ICAM1 levels were found to be higher in the peripheral samples as compared to the placental plasma samples ($P = 0.042$) among the non-PE women only. An opposite was observed for CD146 where higher levels were seen in the placenta as compared to the periphery plasma samples ($P = 0.043$). There were no statistically significant differences in the concentration levels of MMP9 and CXCL10 in the placental and peripheral plasma samples between the non-PE and PE women.
Figure 3.2: Peripheral and placental blood levels of anti-angiogenic molecules in women diagnosed with and without preeclampsia.

Comparisons are for differences between markers in peripheral and placental blood. Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
3.4.4 Profile of pro-angiogenic molecules in peripheral and placental blood

Pro-angiogenic molecules (Ang1, PDGF, VEGF, VEGFR2, SDF1, bFGF, GM-CSF, PI GF and Tie 2) which mediate angiogenesis were measured in peripheral and placental plasma in non-PE and PE women. Levels of bFGF and Tie2 were higher in placenta compared to peripheral plasma \( (P < 0.0001) \) in both Non-PE and PE women (Figure 3.3). High concentrations of PDGF were observed in the placental plasma as compared to the peripheral plasma \( (P < 0.001) \) of non-PE but this was not observed among the women with PE. The Ang1, VEGFR2 and GM-CSF levels were all found to be higher in the placental plasma of the women with PE but not among the non-PE women. The concentration levels of the last three pro-angiogenic molecules (VEGF, PI GF and SDF1) were not different between the groups.
Figure 3.3: Peripheral and placental blood levels of pro-angiogenic markers in women diagnosed with and without preeclampsia.

Comparisons are for differences between markers in peripheral and placental blood. Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. P values represented as *<0.05, **<0.01, ****<0.0001.
3.4.5 Inflammatory mediators in non-preeclamptic and preeclamptic pregnancies

Proinflammatory (CRP, IFN-γ, IL1, IL6, IL8, IL17 and TNF-α) and anti-inflammatory (IL10 and IL21) molecules were measured in the localized placenta and maternal systemic circulation. This was to find out differences in their levels between PE and non-PE women. Inflammatory mediators measured within the localized placenta did not show any differences between PE and non-PE women. In the maternal circulation, pro-inflammatory molecules CRP and IL8 showed high levels in the PE compared to non-PE women. The expression of all the other markers were not statistically different (Figure 3.4).
Figure 3.4: Comparison of inflammatory molecules between preeclamptic and non-preeclamptic pregnancies in the placenta and maternal circulation.

Comparing differences between women diagnosed with (PE) and without (non-PE) preeclampsia in these sites. Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. $P$ values represented as ** ($P < 0.01$).
3.4.6 Anti-angiogenic molecules in non-preeclamptic and preeclamptic pregnancies

Eight markers believed to be associated with dysregulated angiogenesis (PAI-1, MMP9, ICAM1, Ang2, CCL2, CXCL10, CD146 and VEGFR1) were measured among PE and non-PE women. No statistically significant differences were observed in the levels of these molecules between the PE and non-PE women in the placenta. However, in the maternal circulation, PAI-1, CCL2, CXCL10 and VEGFR1 showed higher expression levels in the maternal circulation between the PE and non-PE women ($P < 0.0001$, $P = 0.005$, $P = 0.008$ and $P < 0.0001$ respectively). Although, a similar trend was seen in the placenta the differences were not statistically significant (Figure 3.5).
Figure 3.5: Anti-angiogenic molecules between preeclamptic and non-preeclamptic pregnancies in the placenta and maternal circulation.

Comparing differences between women diagnosed with (PE) and without (non-PE) preeclampsia in these sites. Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. ** \((P < 0.01)\), **** \((P < 0.0001)\).
3.4.7 Pro-angiogenic molecules in non-preeclamptic and preeclamptic pregnancies

Pro-angiogenic molecules (Ang1, PDGF, VEGF, VEGFR2, SDF1, bFGF, GM-CSF, PlGF and Tie 2) mediate angiogenesis. In this study, we measured levels of nine pro-angiogenic molecules in the placenta and maternal circulation and compared between the non-PE and PE women (Figure 3.6). Levels of these molecules were not different between the non-PE and PE women in the placenta. The levels of PlGF was higher in the maternal circulation of non-PE women compared to the PE group ($P = 0.04$) while, the concentration of PDGF and GM-CSF were elevated in PE diagnosed women but not the non-PE women ($P = 0.02$ and 0.03 respectively. Levels of Ang1, VEGF, VEGFR2, SDF1, and Tie 2 were not statistically different.
Figure 3.6: Pro-angiogenic molecules between preeclamptic and non-preeclamptic pregnancies in the placenta and maternal circulation.

Comparing differences between women diagnosed with (PE) and without (non-PE) preeclampsia in these sites. Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. * ($P < 0.05$).
3.4.8 Placental malaria and levels of inflammatory mediators in circulation

Study participants were further categorised based on the histological grading of PM infection status (active, past and no infection) as earlier described. Level of exposure to PM was determined by active and past infections. Absence of infection is attributed to “no exposure”.

The concentration of inflammatory, anti-angiogenic, and pro-angiogenic molecules were compared between the placenta and maternal circulation and also between non-PE and PE diagnosed women.

Proinflammatory (CRP, IFN-γ, IL1, IL6, IL8, IL17 and TNF-α) and anti-inflammatory (IL10 and IL21) molecules were compared in maternal circulation to find the association between PM exposure in non-PE and PE diagnosed women (Figure 3.7). Levels of CRP and IL8 were higher in PE women with active parasite infection in the placenta compared to non-PE women ($P$ was each <0.01). Past and no infection did not show differences between the groups. However, concentrations of IL6 and anti-inflammatory IL10 ($P < 0.05$ each) were higher in the maternal circulation of non-PE women with past placental infections. Levels of IFN-γ, IL1, IL17, TNF-α and IL21 were not different between the groups.
Figure 3.7: Relationship between inflammatory markers and placental malaria exposure in the maternal circulation of women diagnosed with or without preeclampsia.

Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. * ($P < 0.05$), ** ($P < 0.01$).
3.4.9 Placental malaria and levels of inflammatory mediators in the placenta

In the placenta, proinflammatory (CRP, IFN-γ, IL1, IL6, IL8, IL17 and TNF-α) and anti-inflammatory (IL10 and IL21) molecules were compared to find the association between PM malaria exposure in non-PE and PE diagnosed women (Figure 3.8). IL1, IL6, IL8 and IL17 were significantly increased in PE diagnosed women with active infection ($P < 0.01$ for IL6 and IL17 and $P < 0.05$ for IL1 and IL8) compared to the non-PE women. IL10 levels were higher in non-PE women with no placental infections ($P < 0.05$) while CRP, IFN-γ, IL21 and TNF-α were not different between the groups. Also, there were no differences in level of these mediators in non-PE and PE diagnosed women with past infections.
Figure 3.8: Relationship between inflammatory markers and placental malaria exposure in the placenta of women diagnosed with or without preeclampsia.

Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. * (P < 0.05), ** (P < 0.01).
3.4.10 Placental malaria and levels of anti-angiogenic molecules in circulation

Anti-angiogenic factors (PAI-1, MMP9, ICAM1, Ang2, CCL2, CXCL10, CD146 and sVEGFR1) were assessed in maternal circulation of non-PE and PE women based on PM exposure (Figure 3.9). PAI-1, CCL2, CXCL10, CD146 and sVEGFR1 were found to be significantly elevated in PE diagnosed women with active parasite infections ($P < 0.0001$ for PAI-1 and VEGFR2, $P < 0.05$ for CCL2, CXCL10 and CD146) compared to non-PE women. Other anti-angiogenic factors measured were not different between the groups.
Figure 3.9: Relationship between anti-angiogenic markers and placental malaria exposure in the maternal circulation of women diagnosed with or without preeclampsia.

Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. * ($P < 0.05$), **** ($P < 0.0001$).
3.4.11 Placental malaria and levels of anti-angiogenic molecules in the placenta

In the placenta, anti-angiogenic factors (PAI-1, MMP9, ICAM1, Ang2, CCL2, CXCL10, CD146 and sVEGFR1) were measured and compared between non-PE and PE women based on PM exposure (Figure 3.10). PE diagnosed women with active parasite infections had elevated levels of PAI-1 ($P < 0.01$), ANG2 ($P < 0.05$) and sVEGFR1 ($P < 0.05$) compared to the non-PE group. Levels of CXCL10 were higher in non-PE women with no infection ($P < 0.05$) compared to PE diagnosed women. The other markers did not show any differences between the groups.
Figure 3.10: Relationship between anti-angiogenic markers and placental malaria exposure in the placenta of women diagnosed with or without preeclampsia.

Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. * \((P < 0.05)\), ** \((P < 0.01)\).
3.4.12 Placental malaria and levels of pro-angiogenic molecules in circulation

Pro-angiogenic molecules (Ang1, PDGF, VEGF, VEGFR2, SDF1, bFGF, GM-CSF, PI GF and Tie 2) were measured in the peripheral blood of women in the study population (Figure 3.11). Levels of ANG1 and GM-CSF were elevated in non-PE women with past infections ($P < 0.05$ and 0.01 respectively) compared to non-PE women. There were no differences in the levels of the pro-angiogenic markers measured in women who had no infection between the non-PE and the PE women. Active infection showed elevated level of VEGFR2 in non-PE women ($P < 0.01$) while FGF and SDF1 levels were reduced in these women ($P < 0.05$ each) compared to the PE diagnosed group.
Figure 3.11: Relationship between pro-angiogenic markers and placental malaria exposure in the maternal circulation of women diagnosed with or without preeclampsia. Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. * \( P < 0.05 \), ** \( P < 0.01 \).
3.4.13 Placental malaria and levels of pro-angiogenic molecules in the placenta

The pro-angiogenic molecules (Ang1, PDGF, VEGF, VEGFR2, SDF1, bFGF, GM-CSF, PlGF and Tie 2) were compared between groups (Figure 3.12). PlGF levels were higher ($P < 0.05$) in non-PE diagnosed women with no parasite infections however in women with active parasite infection, the expression was lower in the same group ($P < 0.05$). Tie2 levels were elevated in the PE group with no infection while FGF levels were lower in the same group ($P < 0.05$ each). GM-CSF was the only marker that significantly showed a reduced expression in the PE group with past infection ($P < 0.05$). Additionally, women with active infections, had higher concentration of VEGF and SDF1 in the PE ($P < 0.05$) compared to non-PE diagnosed women.
Figure 3.12: Relationship between pro-angiogenic markers and placental malaria exposure in the placenta of women diagnosed with or without preeclampsia.

Black dots represent the individual measured levels while the blue dots represent the median level for each set of measurements using the Mann-Whitney U test. * ($P < 0.05$)
3.4.14 Predictors of preeclampsia during placental malaria

In a univariate model, five markers (PAI-1, CCL2, CXCL10, sVEGFR1, and PDGF) were predictive of PE in peripheral blood between non-PE and PE women. However, in a multivariate analysis that adjusted for placental parasite exposure and gravidity, PAI-1 was the only marker that was predictive of PE (AOR 3.30 (1.03 – 10.54; \( P < 0.05 \)) while active, past infections and primigravidity were still associated with PE (Table 3.3).

Table 3.3: Factors predicting preeclampsia during exposure to placental malaria

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95 % CI)</th>
<th>( P )-value</th>
<th>AOR (95 % CI)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Infection</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Active Infection</td>
<td>5.9 (2.6 – 14.0)</td>
<td>&lt;0.0001</td>
<td>7.14 (1.1 – 44.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>Past Infection</td>
<td>9.5 (2.9 -30.8)</td>
<td>&lt;0.0001</td>
<td>12.9 (1.1 – 155.5)</td>
<td>0.04</td>
</tr>
<tr>
<td>Primigravid</td>
<td>3.0 (1.5 – 6.3)</td>
<td>0.003</td>
<td>7.2 (1.1 – 48.0)</td>
<td>0.04</td>
</tr>
<tr>
<td>Multigravid</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>10.3 (3.4 – 31.8)</td>
<td>&lt;0.0001</td>
<td>7.1 (1.3 – 38.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>CCL2</td>
<td>3.0 (1.2 - 7.3)</td>
<td>0.016</td>
<td>1.9 (0.4 – 7.9)</td>
<td>0.41</td>
</tr>
<tr>
<td>CXCL10</td>
<td>2.1 (1.2 – 3.8)</td>
<td>0.011</td>
<td>1.7 (0.6 – 4.6)</td>
<td>0.29</td>
</tr>
<tr>
<td>sVEGFR1</td>
<td>6.1 (2.4 – 15.6)</td>
<td>&lt;0.0001</td>
<td>1.6 (0.5 – 2.1)</td>
<td>0.85</td>
</tr>
<tr>
<td>PDGF</td>
<td>1.7 (1.0 – 2.7)</td>
<td>0.04</td>
<td>1.1 (0.5 – 2.1)</td>
<td>0.85</td>
</tr>
</tbody>
</table>

OR (odds ratio); AOR (adjusted OR) in logistic regression model. \( P \) is significant if <0.05. ‘Ref’ represents the reference category.
3.5 Discussion

Placental malaria (PM) and preeclampsia (PE) are placental conditions that are frequent in first time pregnant women (Muehlenbachs et al., 2006, Ndao et al., 2009, Adam et al., 2011). Pathophysiology is marked by placental inflammatory response that cascades into endothelial and placental dysfunction (Muehlenbachs et al., 2006, Ndao et al., 2009). Increasing evidence points to PM as a contributing factor to PE due to infiltration of immune cells and release of cytokines that infringe the placenta. In PE, factors in maternal circulation are expected to mimic placental factors as indicated in the pathophysiology. Therefore, profile of inflammatory and angiogenic mediators and inhibitors in the placenta and maternal circulation will allow further probe into their role in pregnancy or obstetric pathologies. In addition, variations in these markers, will further enhance understanding of the pathophysiologic basis of PE in the context of PM.

Generally, the study showed higher placental levels of proinflammatory (IFN-γ, IL1, IL8, and TNF-α), anti-angiogenic (PAI-1, Ang2, CCL2 and sVEGFR1) and pro-angiogenic (bFGF and Tie2) markers than those in maternal circulation for both pregnancy groups. These findings therefore support the notion that placental trophoblasts, decidual and infiltrating immune cells are major sources of these products as reported elsewhere (Keelan and Mitchell, 2007). Normal pregnancy is divided into 3 distinct immunological phases (implantation, foetal growth/development and parturition). The implantation and parturition phases are characterised by an increased proinflammatory state while foetal growth and development is more anti-inflammatory (Mor and Cardenas, 2010). Various immune mediators such as those measured in this study are known to be produced to modulate the various stages of pregnancy (Keelan and Mitchell, 2007). Data from non-PE pregnancies were obtained mostly from women with spontaneous deliveries which relate to a proinflammatory state similar to PE. For
instance, IL8 concentrations have been shown to be higher in amniotic than maternal plasma at delivery (Laham et al., 1993, Oláh et al., 1996). However Oláh et al. (1996), further showed no association between the IL6, IL8 and IFN-γ levels in placental and peripheral plasma. These differences may be due to a relatively small sample size, sampling, experimental procedures and the absence of infections in their study. Notwithstanding, the results suggest that the placenta which predicts pregnancy outcome is biased towards a proinflammatory state compared to maternal circulation during PE or parturition in normal pregnancy.

The levels of CRP in this study reiterated the placenta as the possible site of production of these immune markers. As an acute phase protein, CRP which is produced mainly by hepatocytes in response to inflammation, (Thompson et al., 1999, Salazar et al., 2014) was the only molecule with significantly low expression in the placenta compared to peripheral plasma. Malek and colleagues (2006) however demonstrated that CRP may be produced by placental tissues but are exported to maternal circulation. Their presence in the placenta suggest that they have a role in both normal and pathologic pregnancies. Regardless of this, our findings support the hypothesis that placentation leads to the release of placental factors that may be exported into maternal circulation to aid parturition or provoke a pathological condition such as PE.

Interestingly, IL6 and IL17 levels were also elevated in the placenta of women with PE. Both markers have been shown in some studies to be elevated in PE pregnancies (Udenze et al., 2015, Poordast et al., 2017) and in the placenta of other obstetric vascular disorders like diabetes (Hara et al., 2016). It has also been shown that placental decidua has a higher expression of IL17 compared to maternal circulation during normal pregnancy (Nakashima et al., 2010). These findings support the hyper inflammatory state during PE.
Also, IL10, an anti-inflammatory mediator was elevated in the placenta of non-PE pregnancies only. This finding is consistent with other reports that found IL10 to suppress the production and function of other cytokines during pregnancy and have been suggested to play a role in normal and term deliveries (Denney et al., 2011). High levels of other pro and anti-angiogenic cytokines were also shown in the placenta than peripheral plasma in all pregnancy types. This supports the hypothesis that the placenta is an active site for angiogenesis particularly during the third trimester of increased vascularisation and foetal growth (Chen and Zheng, 2014). Bioavailability of these molecules may be important in maintaining a healthy balance during pregnancy and their alterations are determinants of pathologies such as PE.

The high expression of placental inflammatory factors did not correlate with disease states. Levels of all analytes were comparable in placental plasma of both non-PE and PE pregnancies. However, peripheral plasma showed higher levels of proinflammatory CRP and IL8 in women diagnosed with PE compared to non-PE women. The angiogenic molecules PAI-1, CCL2, CXCL10, PDGF and sVEGFR1 (sFLT1) were also elevated in PE pregnancies. However, GM-CSF and PlGF were found to be higher in non-PE pregnancies. The increase in the levels of proinflammatory and anti-angiogenic markers in maternal circulation of PE pregnancies, confirms the characteristic pathophysiologic features of PE (Roberts et al., 1989, Verlohren et al., 2009, Szarka et al., 2010, Cemgil Arikan et al., 2012, Adu-Bonsaffoh et al., 2017). Other inflammatory and angiogenic cytokines such as IFN-γ, TNF-α, IL1, IL6, CCL2 and CXCL10 did not show any differences between the non-PE and PE pregnancies. This might be due to paracrine effects and short half-life of some of these cytokines in maternal plasma (Szarka et al., 2010, Cemgil Arikan et al., 2012).
This study further demonstrated variations in inflammatory and angiogenic cytokines in PM (exposed and non-exposed) women with or without PE in both placenta and peripheral circulation. Placental malaria (PM) is associated with \textit{P. falciparum} sequestration and infiltration of immune cells in the intervillous spaces of the placenta leading to poor obstetric outcomes (Fried and Duffy, 1996). Ofori and colleagues (2018) reported that PM aetiology that is often overlooked, may arise from pre-existing parasites prior to conception. These parasites are able to switch to a VAR2CSA PfEMP1 variant that invade the placenta in addition to the acquisition of new infections. Also, malaria drugs mainly target parasite clearance but not the immune alterations which may linger even after parasite clearance (Etyang \textit{et al.}, 2016). Pregnant women with subclinical infections based on environmental and immune factors may be asymptomatic with associated insidious consequences such as chronic, recurrent infections and a prolonged inflammatory state (Fried and Duffy, 1996, Duffy, 2007, Agudelo \textit{et al.}, 2014, Ofori \textit{et al.}, 2018). These features may be characteristic initiators of PE according to several reports (Brabin and Johnson, 2005, Muehlenbachs \textit{et al.}, 2006, Etyang \textit{et al.}, 2016).

Data from this study revealed high expression of inflammatory and angiogenic molecules in the placenta, (IL1, IL6, IL8, IL17, PAI-1, ANG2, sVEGFR1, VEGF, PIGF and SDF1) of PE compared to non-PE women with active placental infections. This trend was similar in maternal circulation (CRP, IL8, PAI-1, CCL2, CXCL10, CD46, sVEGFR1, FGF and SDF1) although the expression of the markers was different. This high level of markers in active placental infections at delivery mirrors the extent of immune dysregulation when pregnant women are infected earlier on in pregnancy. This finding supports a current review of literature which revealed that maternal infections particularly those that are transmissible \textit{in utero}, results in an immunological shift that enhances the development of PE (Shiadeh \textit{et al.}, 2017). Consistency
is also shown in findings from Muehlenbachs et al. (2006) who showed high levels of VEGR1 in primigravid women with hypertension and PM.

The histological grading system used in this study was able to detect pregnant women who have had past Plasmodium infections in both PE and non-PE placentas. Although the study could not trace the pattern of inflammation at the time of infection, regulatory IL10 and pro-angiogenic ANG1 levels were low in these women. No study has specifically looked at this interaction but high levels of IL10 and ANG1 have independently been associated with pregnancy. Kappou and group demonstrated increased expression of ANG2 and reduced expression of ANG1 in pregnancies complicated by PE and intrauterine growth restriction (Kappou et al., 2014). Low levels of IL10 have also been reported in PE pregnancies at term (Markova et al., 2016). This is in part supported by the higher expression of IL10 in the placental of non-PE pregnancies with no parasite infection in this study. Assessing all markers, PM exposure and other maternal factors in a univariate analysis, women with past and active infections, primigravids and higher level of anti-angiogenic markers PAI-1, CCL2, CXCL10 and sVEGFR1 had a higher risk of predicting PE. Past and active infections and primigravidity continued to show increased risk of PE in a multivariate model however, PAI-1 was the only marker which showed an increased risk. PAI-1 is a thrombotic factor associated with increased fibrin deposition during PM (Avery et al., 2012) and a risk factor for thrombotic disorders such as atherosclerosis (Hunt et al., 2015). PAI-1 is also elevated in PE pregnancies following exposure to inflammatory and angiogenic molecules in hypoxic conditions (Anteby et al., 2004). Increased fibrin deposition and infarction associated with PAI-1 is a sign of placental damage occurring in both PM and PE. Findings from this study should further be strengthened by adapting this model in malaria naïve pregnant women. Longitudinal and genetic studies
should be carried out to model the levels of these markers at various stages of pregnancy in order to draw vivid conclusions.

3.6 Conclusion

In conclusion, variations have been shown in the concentration of inflammatory and angiogenic markers in the placenta and maternal circulation of both PE and non-PE placentas. The study further demonstrated that PE is associated with dysregulated inflammation and angiogenesis which is further enhanced by *P. falciparum* placental infections particularly in primigravid women. PAI-1 levels have been shown for the first time to increase the risk of PE in primigravid women with placental pathology and placental malaria. This should further be investigated to ascertain its diagnostic and therapeutic potential in PM and PE pathogenesis.
CHAPTER FOUR

4.0 Paper 3: Circulating endothelial cells and endothelial progenitor cells are altered in preeclampsia and placental malaria.

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4.1 Abstract

**Background:** Preeclampsia (PE) and placental malaria (PM) are pregnancy conditions associated with endothelial dysfunction that significantly contribute to disease severity. Circulating endothelial cells (cECs) and circulating endothelial progenitor cells (cEPCs) are identified markers of endothelial damage and repair respectively. Based on this knowledge, the current study explored the profile of endothelial cell phenotypes [(cECs, cEPCs, immature cEPCs (iEPCs) and matured cEPCs (mEPCs)] in PE and non-PE pregnancies and how these cells discriminate against angiogenic factors and PM.

**Methods:** Levels of cECs (CD11b+/CD34+/CD31bright/CD133+), cEPCs (CD45dim/CD309+/CD34+/CD133+), iEPCs (CD34+/CD133+) and mEPCs (CD34+/CD309+) cells were quantified from peripheral, placental intervillous and cord blood in 71 PE and 69 non-PE women in Accra, Ghana. Peripheral and placental intervillous plasma concentrations of angiogenic markers were also measured by ELISA.

**Results:** Cord blood cEPCs were higher in PE compared to non-PE pregnancies ($P = 0.009$) while the levels cECs, iEPCs and mEPCs were statistically similar between groups. The mEPCs showed a strong correlation between mEPCs and fibroblast growth factor ($r = 0.69, P < 0.0001$) and a moderate one with CD146 ($r = 0.46, P < 0.01$) in the peripheral blood of the non-PE group. Observations in the PE group were generally weak or non-existent. The mEPCs were higher in the peripheral blood of PE women compared to non-PE women with past PM ($P = 0.03$). In the placenta, cECs were lower in PE women compared to non-PE women with active PM ($P = 0.03$). The expression pattern of all endothelial cell phenotypes was generally high in the placenta compared to peripheral blood in either PE or non-PE women with active or past placental infections. This observation was also seen in the placenta of non-PE women with no placental infections but not that of the PE group.
**Conclusion:** Findings from the study show higher levels of endothelial cell phenotypes in the uteroplacental region compared to maternal circulation of both non-P and PE pregnancies. Circulating endothelial progenitor cells in PE pregnancies correlated weakly with growth factors that stimulate their release and function. Finally, placental malaria altered the levels of circulating endothelial cell phenotypes in the peripheral and placental blood of both non-PE and PE women instigating the need for further characterisation and functional assessment.
4.2 Introduction

The endothelium is made up of monolayer of endothelial cells that serve as inner lining of blood vessels and maintain vascular integrity. Pathophysiologic function of these endothelial cells are maintained by the synthesis of several active molecules such as cytokines, chemokines, angiogenic factors, proteases as well as endothelial relaxing and constricting factors (reviewed in (Vane et al., 1990, Aird, 2012, Vestweber, 2015)). During pregnancy, the placental vasculature is remodelled to sustain the developing foetus. The vasculogenic and angiogenic processes of blood vessel formation which are not common in adults, have both been shown to be utilized during pregnancy (Poole and Coffin, 1989, Zygmunt et al., 2003, Tal et al., 2019). Abnormalities in the maternal vasculature have been associated with obstetric vascular pathologies such as preeclampsia (PE) and intrauterine growth restriction (Roberts et al., 1989, Arroyo and Winn, 2008, Roberts and Escudero, 2012).

Hypertensive disorders of pregnancy, particularly PE and the infectious disease PM, are common obstetric presentations of pregnancy in the tropics (Muehlenbachs et al., 2006, Ndao et al., 2009, Adam et al., 2011). Endothelial dysfunction underpins the pathophysiology of both conditions (Gueneuc et al., 2017). Abnormal interaction between Plasmodium falciparum infected erythrocytes and the vascular wall in the placenta, systemic circulation and other organs such as the brain is linked to disease severity. For instance, investigations have shown that parasite sequestration in the brain results in microvascular congestion leading to coma in patients who died from cerebral malaria (Ponsford et al., 2012, Castillo et al., 2013). Sequestration in the placenta and other major organs also cause debilitating effects but may not lead to severe disease (Castillo et al., 2013). In PE, endothelial dysfunction is a consequence of reduced uteroplacental perfusion, exaggerated inflammation and impaired angiogenesis.
(reviewed in (Roberts, 1998, Redman and Sargent, 2005, LaMarca, 2012, Pennington et al., 2012)).

Changes in the levels of immunologically defined circulating factors have gained considerable interest in the evaluation of endothelial function. Some of these include circulating endothelial cells (cECs) and endothelial progenitor cells (cEPCs) which have been measured as markers of vascular damage and regeneration respectively. The cECs are characterised as matured endothelial cells that are displaced from the endothelium due to vascular injury (Goon et al., 2005) and some studies have shown high numbers of these markers in pathological conditions such as PE (Grundmann et al., 2008, Heimrath et al., 2014, Tuzcu et al., 2015). They have also been associated with endothelial damage in infectious diseases such as cerebral malaria (Oduro, 2015, Yoo et al., 2015).

The identification of cEPCs in peripheral circulation ignited a paradigm shift in adult vascular biology. Asahara et al. (1997) indicated from their seminal findings that adult vascular regeneration is not limited to local repair (angiogenesis) instead, recruitment of bone marrow derived cEPCs to localized sites of injury augment angiogenesis. The regenerative capacity of cEPCs is stimulated by various angiogenic factors such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF), fibroblast growth factor (FGF), and stromal cell derived factor -1 (SDF-1) (Asahara et al., 1999, Gyan et al., 2009, Qi et al., 2013). Flow cytometric analyses have utilized combinations of cell surface antigens to characterise cEPCs in order to delineate them from other haematopoietic stem cells. Typically CD34⁺, CD309 (KDR)⁺ and CD133⁺ have been used singly or in combination to identify cEPCs in several studies to understand disease pathologies (Asahara et al., 1997, Gammill et al., 2007, Gyan et al., 2009, Qi et al., 2013, Sakashita et al., 2014).
Indeed, changes in the circulating levels of cECs and cEPCs in normal and PE pregnancies have been fairly investigated (Robb et al., 2007, Heimrath et al., 2014, Sakashita et al., 2014). However, these changes in combination with PM have not been considered. Gyan et al. (2009) showed low levels of cEPCs in Ghanaian children with cerebral malaria while Oduro (2015) showed low cEPCs and high cECs in peripheral circulation of similar children using different marker combinations. Additionally, alternative sources of cEPCs exist aside those isolated from the peripheral blood and bone marrow. The placenta (Sölder et al., 2012, Rapp et al., 2012) and cord blood (Xia et al., 2007, Gumina et al., 2017) undergo continuous vascularization during pregnancy and are also expected to be sites for circulating cECs and cEPCs. In view of these, levels of endothelial phenotypes were compared within peripheral, maternal and foeto-placental circulation between PE and non-PE pregnancies. The relationship between the endothelial cell phenotypes and the angiogenic molecules that stimulate their function was assessed. Further comparison based on PM exposure was also evaluated.
4.3 Methods

4.3.1 Study population and enrollment
The study was carried out at the Obstetrics and Gynaecology Department of the Korle-Bu Teaching Hospital (KBTH) in Accra, the Capital City of Ghana, which mainly attend to women with obstetric complications. Pregnant women (140) diagnosed with PE (71) and without PE (69) were recruited at delivery after obtaining written informed consent. Study protocols were approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR) and the Ethical Review Committee of the KBTH. Preeclampsia (PE) was diagnosed as a history of sustained hypertension (blood pressure $\geq 140$mmHg systolic or $\geq 90$mmHg diastolic at least 4 hours apart) with proteinuria (two readings of $1^{+}$ or higher on urinalysis) $\geq 20$ weeks of gestation as diagnosed by an obstetrician (Tranquilli et al., 2014). Presence of PM was tested in placental biopsies after delivery.

4.3.2 Sample collection and processing
At delivery, 5 ml of maternal peripheral, cord and placental blood were collected into EDTA tubes. Peripheral blood was initially used for the determination of haematological parameters (Sysmex Automated Haematology Analyser Model KX-21N, USA). Placental blood was sampled by the incision method. Briefly, two to three incisions were made (about 2 mm deep) from the maternal decidua in an off-centre position, one quarter distance from the edge of the placenta as described by Bulmer et al. (1993). Pooled blood from the area of incision was collected using Pasteur pipettes into labelled EDTA treated tubes. Samples were immediately transported on ice to the NMIMR for flow cytometric analysis and processing of plasma for storage at -80 °C for subsequent ELISA assays. Placental biopsies were sampled and tested for PM as previously described (Bulmer et al., 1993).
4.3.3. Phenotypic characterisation of endothelial cell phenotypes by flow cytometry

Phenotypic characterisation involved the staining of cells based on the expression of cell surface markers on endothelial cell phenotypes. These cells were quantified based on size and internal complexity using single cell analysis by flow cytometry (BD FACSCalibur, San Jose, California, USA). In this experiment, blood leucocytes were processed by staining prior to flow cytometry analysis.

4.3.4 Immunostaining of blood and quantification of endothelial cell phenotypes

Based on the expression of specific cell surface markers, endothelial cell phenotypes were quantified using a lyse and wash whole blood protocol in previous methods with slight modification (Duda et al., 2007, Oduro, 2015). EDTA treated blood sampled from periphery, cord and placenta was processed within two hours of sample collection and analysed. To prevent non-specific binding, diluted blocking reagent IgG chrome (Jackson ImmunoResearch Labs, USA) was aliquoted into the polystyrene tube (Table 4.1) prior to the addition of blood and antibody cocktail. This was done for each blood type (cord, placenta and periphery) and incubated at 4 °C for 10 minutes. Immunostaining was carried out with fluorescently labelled monoclonal antibody combinations (Table 4.1). Monoclonal antibodies against the various endothelial cell receptors considered in the study and their isotypes were added to the panel. Approximately 3μl each of CD45 (FITC; BD, USA), CD11b (FITC; Miltenyi Biotech, USA), CD34 (PE; BD, USA), CD133 (PerCP; Miltenyi Biotech, USA) and CD31 (APC; Miltenyi Biotech, USA) was pipetted into their respective polystyrene tube (BD, Belgium) and incubated for 30 minutes at 4 °C in the dark. After incubation, 2 ml of diluted FACS lysing solution (BD, USA) was used for the lysis of the red blood cells, incubated at room temperature.
in the dark for 10 minutes and washed to get rid of debris and any unbound antibodies. Cells were washed 2x and re-suspended in 200 µl of FACSFlow (BD, USA) for flow cytometry quantification.

Table 4.1: Panel showing monoclonal antibodies and fluorochromes used for the phenotypic characterisation of endothelial cell phenotypes

<table>
<thead>
<tr>
<th>Tube</th>
<th>Blood Volume (µl)</th>
<th>Cell</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>Unstained</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>Positive control</td>
<td>CD15</td>
<td>CD14</td>
<td>CD4</td>
<td>CD8</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>cECs</td>
<td>CD11b</td>
<td>CD133</td>
<td>CD34</td>
<td>CD31</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>cEPCs</td>
<td>CD45</td>
<td>CD133</td>
<td>CD34</td>
<td>CD309</td>
</tr>
</tbody>
</table>

Manual compensation was carried out using calibrate beads in addition to single cell analysis before cells from participants were analysed by flow cytometry. The circulating endothelial cells (cECs) were gated as cells co-expressing CD11b/CD34+/CD31bright/CD133+ markers (Figure 4.1). Similarly, circulating endothelial progenitor cells (cEPCs) co-expressed CD45dim/CD309+/CD34+/CD133+ (Figure 4.2). Both cECs and cEPCs were expressed as percentage of the initial leucocyte gating (G1) of 250,000 events. CD34+/CD133+ cEPCs and CD34+/CD309+ cEPCs representing matured (mEPCs) and immature (iEPCs) phenotypes respectively were also analysed.
Figure 4.1: Flow cytometric quantification of circulating endothelial cells.
A represents total leucocyte population, followed by B which is indicated by CD11b/CD34+ population from the initial leucocyte population. The C represents final gating for inclusion of CD11b'/CD34+/CD31[^bright]/CD133+ cells for the enumeration of cECs. The “-” population represent no expression of associated marker, “+” represents high expression of associated marker and the “bright” population represent very high expression of associated marker.

Figure 4.2: Flow cytometric quantification of circulating endothelial progenitor cells.
A represents initial leucocyte gating; followed by B which is gating for CD45[^dim] population; C represents CD45[^dim]/CD309+ population; while D represents final gating for CD45[^dim]/CD309+/CD34+/CD133+ cells for the enumeration of cEPCs population. The “[^dim]” population represent minimal expression of associated marker, “-” represents no expression of associated marker and “[^+^]” represents high expression of associated marker.
4.3.5. Measurement of angiogenic factors by ELISA

Peripheral and placental intervillous plasma stored at -80 °C were thawed and concentrations of VEGF, PI GF, FGF, Ang 1 & 2, SDF-1, MMP9 and sVEGFR-1 measured by multiplex ELISA. Samples were analysed in duplicate with inter and intra assay variability of less than 20%.

4.3.6. Statistical analysis

Data were analysed using GraphPad Prism (v 6) and R statistical software (v 3.5.1). Demographic data were compared between PE and non-PE groups. Categorical variables were expressed as percentages using the Chi-square test. Endothelial cells were expressed as percentages. Continuous variables are expressed as mean and standard deviation and compared using the Student’s t-test. Median comparison between two groups used the Mann-Whitney U test and Kruskal-Wallis test for three groups. Spearman’s rank correlation test was used to determine the relationship between endothelial cell phenotypes and angiogenic molecules.
4.4 Results

4.4.1 Demographic and clinical characteristics of Study Participants

The maternal and infant characteristics are similar to those described in chapter 2. Briefly, 71 pregnant women diagnosed with PE and 69 women without PE were recruited into the study. Mean maternal age was not different between the women diagnosed with PE and those without PE (28 ± 6 and 29 ± 6 years respectively, \( P = 0.43 \)). There was a higher number of primigravids (68% of 50 primigravids) and less multigravids (41% of 90) in the PE group compared to non-PE pregnancies (\( P = 0.004 \)). Mean systolic and diastolic blood pressures were significantly higher in PE women (mean systolic blood pressure: 150.7 ± 17.9 mmHg; diastolic blood pressure: 95.2 ± 14.4 mmHg) than non-PE women (systolic blood pressure: 122.2 ± 14.2 mmHg; diastolic blood pressure: 75.5 ± 8.8 mmHg) (\( P < 0.0001 \) in both cases). All PE women had received some form of therapy for management of PE before sampling was carried out (anti-hypertensive therapy or Magnesium Sulphate). Mean delivery age was 36.0 ± 4 years in the PE compared to 39.5 ± 2 years in the non-PE group (\( P < 0.0001 \)). There was a higher rate of caesarean deliveries, preterm delivery and neonatal intensive care admissions in the PE group compared to non-PE group. Also, mean birth weight (2.4 ± 1.0 kg) and Apgar scores were lower in PE women (1 minute = 6.3 ± 1.3, 5 minutes = 7.6 ± 1.2) than in non-PE women (mean birth weight 3.2 ± 0.5 kg, Apgar at 1 minute = 7.1 ± 1.1, at 5 minutes = 8.3 ± 0.9; \( P < 0.001 \)). No differences were observed in the haematological parameters, but \( P. falciparum \) infection status showed difference between the groups (Table 4.1). Women with active parasite infection were the majority in the PE diagnosed women (61% of 64) while most of the non-PE women were not infected (55.1% of 69 \( P < 0.0001 \)).
Table 4.2: Haematological and *Plasmodium falciparum* infection status among PE and non-PE pregnancies

<table>
<thead>
<tr>
<th>Clinical Characteristic</th>
<th>Non-PE (N= 69)</th>
<th>PE (N = 71)</th>
<th>Total (N = 140)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematological Parameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cells- WBC (x 10⁹/L)</td>
<td>11.6 ± 5.4</td>
<td>11.6 ± 5.4</td>
<td>10.8 ± 4.9</td>
<td>0.06</td>
</tr>
<tr>
<td>Platelets (x10⁹/L)</td>
<td>196.4 ± 78.0</td>
<td>181.6 ± 91.1</td>
<td>189.1 ± 84.7</td>
<td>0.33</td>
</tr>
<tr>
<td>Red blood cells- RBC (x10¹²/L)</td>
<td>4.1 ± 0.6</td>
<td>4.1 ± 0.7</td>
<td>4.1 ± 0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>11.4 ± 1.7</td>
<td>11.8 ± 2.0</td>
<td>11.6 ± 2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Parasite Infection</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Active Infection</td>
<td>25 (36.2)</td>
<td>39 (61.0)</td>
<td>64 (48.1)</td>
<td></td>
</tr>
<tr>
<td>Past Infection</td>
<td>6 (8.7)</td>
<td>15 (23.4)</td>
<td>21 (15.8)</td>
<td></td>
</tr>
<tr>
<td>No Infection</td>
<td>38 (55.1)</td>
<td>10 (15.6)</td>
<td>48 (36.1)</td>
<td></td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD (Student’s t-test) for continuous variables and % for categorical variables ($\chi^2$ test). Active infection represents parasite + pigment in placenta, past infection represents pigment only in the placenta and no infection represents absence of both parasite and parasite pigment in the placenta. PE = Preeclampsia. *P* is significant if < 0.05.
4.4.2 Levels of endothelial cell phenotypes

Four endothelial cell phenotypes were assessed based on co-expression of cell surface markers in the peripheral, placental and cord blood between PE and non-PE pregnancies (Figure 4.3). The cECs (CD11b\textsuperscript{−}/CD34\textsuperscript{+}/CD31\textsuperscript{bright}/CD133\textsuperscript{+}) had a higher expression in the placental ($P < 0.0001$) and cord ($P = 0.01$) blood compared to the periphery in non-PE pregnancies. Higher expression was only shown in the placenta compared to periphery in PE pregnancies ($P = 0.02$). There was however no difference in expression between PE and non-PE groups for this endothelial cell phenotype (Figure 4.3 A). On the other hand, cEPC (CD45\textsuperscript{dim}/CD309\textsuperscript{−}/CD34\textsuperscript{+}/CD133\textsuperscript{−}) showed differences between peripheral and placental blood ($P < 0.0001$) and between placental and cord blood ($P = 0.006$) in non-PE pregnancies. In PE pregnancies, cEPCs expression was elevated in the placenta ($P < 0.0001$) and cord ($P = 0.0004$) compared to maternal circulation. Cord blood cEPCs were higher in the PE ($P = 0.009$) compared to the non-PE group (Figure 4.3 B). The expression levels of mEPCs (CD34\textsuperscript{−}CD309\textsuperscript{−}) were different only between placenta and cord blood ($P = 0.003$) in the non-PE group. Similar to the cEPCs, cord blood expression of mEPCs in PE pregnancies were higher ($P = 0.0008$) compared to non-PE pregnancies (Figure 4.3 C). Finally, iEPCs (CD34\textsuperscript{−}CD133\textsuperscript{−}) were significantly elevated in the placental ($P < 0.0001$) and cord ($P = 0.008$) blood in comparison to peripheral blood of non-PE women. This trend was similar in the PE group with elevated iEPCs in the placental ($P < 0.0001$) and cord ($P = 0.02$) blood compared to peripheral blood iEPCs. Differences were not observed between the expression of iEPCs in PE and non-PE pregnancies (Figure 4.3 D).
Figure 4.3: Differences between endothelial cell phenotypes in peripheral, cord and placenta blood of women diagnosed with and without preeclampsia.

“A” represents circulating endothelial cells (cECs) shed during damage and co-expressed as % CD11b-CD34-CD31brightCD133+; “B” represents circulating endothelial progenitor cells (cEPCs) co-expressed as % CD45dim/CD309+/CD34+/CD133+. “C” represents mature endothelial progenitor cells (mEPCs) co-expressed as % CD34+CD309+, “D” represents immature endothelial progenitor cell (iEPCs) co-expressed as % CD34+CD309+. “B, C and D represent mediators of endothelial repair. PE = preeclamptic group, Non-PE = non-preeclamptic group. Comparison between non-PE and PE group (Mann-Whitney U test) and comparison between peripheral, placental and cord blood (Kruskal-Wallis test). P is significant if < 0.05.
4.4.3 Correlation between endothelial cell phenotypes and angiogenic molecules.

Endothelial cell phenotypes measured in the study (cECs, cEPCs, mEPCs and iEPCs) as well as the angiogenic factors (VEGF, PI GF, FGF, Ang 1 & 2, SDF-1, MMP9 and sVEGFR-1) that aid in endothelial cell proliferation, migration and homing into sites of injury were assessed. This relationship was analysed on cells in maternal circulation (Figure 4.4) and the placental intervillous blood (Figure 4.5) of PE and non-PE pregnancies respectively. In the peripheral blood of the non-PE group, the frequency of mEPCs correlated strongly with that of iEPCs and fibroblast growth factor (FGF) \( (r = 0.76, P < 0.0001 \text{ and } r = 0.69, P < 0.0001, \text{ Figure 4.4 A}) \). A moderate positive correlation was shown between iEPCs and FGF as well as CD146 \( (r = 0.53, P < 0.001 \text{ and } r = 0.46, P < 0.01 \text{ respectively}) \). Also, a weak correlation was shown between mEPCs and cEPCs \( (r = 0.36, P < 0.001) \). In the placenta of the same group of women (Figure 4.5 C), mEPCs correlated strongly with cEPCs \( (r = 0.71, P < 0.0001) \) and weakly with iEPCs \( (r = 0.36, P < 0.001) \). Angiogenic factors Tie2 and vascular endothelial growth factor (VEGF) also correlated weakly with cEPCs and iEPCs respectively \( (; r = 0.39, P < 0.01; r = 0.39, P < 0.01, \text{ Figure 4.5 A}) \).

For women diagnosed with PE (Figure 4.4 B), cEPCs correlated strongly with mEPCs \( (r = 0.70, P < 0.0001) \) and moderately with iEPCs \( (r = 0.55, P < 0.0001) \) in peripheral blood. Also, mEPCs had a strong positive correlation with iEPCs \( (r = 0.65, P < 0.0001) \) and a weak correlation with CD146 \( (r = 0.39, P < 0.01) \). In addition, cECs showed weak correlation with cEPCs, mEPCs and CD146 \( (r = 0.28, P < 0.01; r = 0.43, P < 0.001 \text{ and } r = 0.33, P < 0.01 \text{ respectively}) \). In the placenta of women diagnosed with PE (Figure 4.5 B), cEPCs had moderate interaction with mEPCs \( (r = 0.54, P < 0.0001) \) and iEPC \( (r = 0.51, P < 0.0001) \). Also, mEPCs correlated moderately with iEPCs \( (r = 0.64, P < 0.0001) \). Other interactions between the endothelial cell phenotypes and angiogenic factors were weak. Levels of Ang 2 correlated
strongly with FGF, placental growth factor (PIGF) and moderately with stromal cell derived factor 1 (SDF1) ($r = 0.69, P < 0.0001; r = 0.7, P < 0.0001; r = 0.57, P < 0.0001$ respectively).

Other associations shown with other markers were moderate to weak (Figure 4.5 B).

**Figure 4.4: Correlation coefficients ($r$) between endothelial cell phenotypes and angiogenic factors in peripheral blood.**  
A and B represent non-PE and PE pregnancies respectively. cECs = circulating endothelial cells (CD11b/CD34+/CD31bright/CD133+), cEPCs = circulating endothelial progenitor cells (CD45dim/CD34+/CD309+/CD133+), mEPCs = matured endothelial progenitor cells (CD34+/CD309+), iEPCs = immature endothelial progenitor cells (CD34+/CD133+). *<0.01, **<0.001, ***<0.0001 are represented as $P$ values.
**Figure 4.5: Correlation coefficients \((r)\) between endothelial cell phenotypes and angiogenic factors in the placenta**

A and B represent non-PE and PE pregnancies respectively. cECs = circulating endothelial cells (CD11b^CD34^CD31^{bright}CD133^), cEPCs = circulating endothelial progenitor cells (CD45^{dim}CD34^CD309^CD133^), mEPCs = matured endothelial progenitor cells (CD34^CD309^), iEPCs = immature endothelial progenitor cells (CD34^CD133^). *<0.01, **<0.001, ***<0.0001 are represented as \(P\) values.
4.4.4 Endothelial cell phenotypes in pregnancies exposed to placental malaria

Placental malaria (PM) during the course of pregnancy was assessed by histological analysis and graded into active or past infections, or an absence of *Plasmodium falciparum* placental infections as earlier described. A sub-analysis was carried out to further assess endothelial phenotypes based on infection status between PE and non-PE pregnancies. In peripheral blood, expression of mEPCs was higher in PE women with past infections compared to non-PE women (\( P = 0.03 \); Figure 4.6 C). The expression of cECs, cEPCs and iEPCs were not different between the groups (Figure 4.6 A, B and D respectively).

In the intervillous blood of the placenta, cECs were lower in PE pregnancies with active infections (\( P = 0.03 \)) compared to the non-PE group (Figure 4.7A). These cells were also higher in past infections compared to active infections (\( P = 0.01 \)) in PE pregnancies only. The levels of iEPCs were higher in non-PE pregnancies with past infection compared to active and no infections (\( P = 0.02 \) and 0.01 respectively; Figure 4.7 D). Levels of cEPCs and mEPCs were not different within and between groups (Figure 4.7 B and C respectively).
Figure 4.6: Endothelial cell phenotypes circulating in peripheral blood from non-preeclamptic and preeclamptic pregnancies with or without placental malaria.

Group A shows cECs (CD11b−CD34−CD31 bright CD133+), B = eEPCs (CD45 dim CD34+CD309+CD133+), C = mEPCs (CD34+CD309+) and D = iEPC (CD34+CD133+). Horizontal bar across dotplots represent the median within the distribution and significance is at $P < 0.05$. PE = preeclamptic group, Non-PE = non-preeclamptic group. Comparison between non-PE and PE group (Mann-Whitney U test) and comparison between active, past and no infections (Kruskal-Wallis test).
Figure 4.7: Endothelial cell phenotypes circulating in placental blood from non-preeclamptic and preeclamptic pregnancies with or without placental malaria.

Group A shows cECs (CD11bCD34⁺CD31^{bright}CD133⁺), B = cEPCs (CD45^{dim}CD34⁺CD309⁺CD133⁺), C = mEPCs (CD34⁺CD309⁺) and D = iEPC (CD34⁺CD133⁺). Bar represents the median within the distribution and significance is at $P < 0.05$. PE = preeclamptic group, Non-PE = non-preeclamptic group. Comparison between non-PE and PE group (Mann-Whitney U test) and comparison between active, past and no infections (Kruskal-Wallis test).
4.4.5 Placental malaria and endothelial phenotypes in preeclamptic and non-preeclamptic pregnancies

The pattern of endothelial cell expression in the peripheral and placental circulation was measured in both groups. In the PE group (Figure 4.8), women with active and past parasite infections had higher cEPCs and iEPCs levels in the placenta compared to the periphery ($P < 0.05$). In addition, cECs were higher in the placenta of women with past infections only ($P < 0.05$). Women with past parasite infections did not show any differences in endothelial markers between the sampling sites. In the non-PE group, cEPCs and iEPCs levels were higher in the placenta compared to periphery for women with active infections ($P < 0.05$, Figure 4.9). Women with no infection had higher placental levels of cECs, cEPCs and iEPCs ($P < 0.05$) while those with past infection had higher placental levels of cEPCs only.
Figure 4.8: Pattern of endothelial cell expression and *Plasmodium falciparum* exposure in the peripheral blood and placenta of women diagnosed with preeclampsia.

The violin density plot shows the distribution of endothelial cell phenotypes with a median blue dot. $P$ is significant at < 0.05. Median differences between the peripheral blood and placental blood analysed by the Mann-Whitney U test.
Figure 4.9 Pattern of endothelial cell expression and *Plasmodium falciparum* exposure in the maternal circulation and placenta of non-preeclamptic women.

The violin density plot shows the distribution of endothelial cell phenotypes with a median blue dot. Significance is at *P* < 0.05. Median differences between the peripheral blood and placental blood analysed by the Mann-Whitney U test.
4.5 Discussion

Vascular perturbations affect the anti-inflammatory, and anti-thrombotic functions of the vascular endothelium. This dysfunction to endothelial cells precedes organ damage and is known to occur in both acute and chronic disease states (Bermejo-Martin et al., 2018). In such conditions, a reparative mechanism is employed such as the recruitment of circulating endothelial progenitor cells (cEPCs) which have been established as markers of endothelial repair (Asahara et al., 1997, Gyan et al., 2009, Qi et al., 2013). However under pathologic circumstances such as in cardiovascular diseases, cEPCs have impaired proliferative ability and function (Vasa et al., 2001).

In the present study, co-expression of endothelial cell markers at different levels of maturation was shown. Phenotypic expression of these markers was quantified in periphery, cord and placenta of PE and non-PE pregnancies in addition to PM exposure. Several antigen combinations were used as earlier described (Oduro, 2015). Comparison between circulating endothelial cell phenotypes have become increasingly difficult due to lack of consensus on the specific markers that define them. Differences in study design and methods of cell enumeration are also a competing challenge. The current study quantified endothelial cells using cell surface staining by flow cytometry while other studies have explored the analysis of colony forming units in culture, magnetic bead sorting and or flow cytometry (Asahara et al., 1997, Gammill et al., 2007, Nolan et al., 2007, Gumina et al., 2017). Molecular analyses of endothelial cell phenotypes have also been investigated (Shi et al., 1998, Nolan et al., 2013). Endothelial cell phenotypes were enumerated by excluding other cells of hematopoietic stem cell origin expressing CD11b or CD45 antigens (Duda et al., 2007, Nolan et al., 2007, Oduro, 2015) for cECs and cEPC respectively. A CD31 bright population (endothelial and monocytic marker) was further used in combination with the well-studied CD34 and CD133 markers to characterise
cECs (Oduro, 2015). A combination of commonly used antibodies CD45/CD309/CD34/CD133, CD34/CD133 and CD34/CD309 represented total, immature and matured progenitor cells phenotypes (Gyan et al., 2009, Oduro, 2015).

Generally, high levels of these markers were observed in the cord and placental regions compared to peripheral circulation in both PE and non-PE women. Similarity among the endothelial progenitor cell phenotypes was enhanced in the PE group. The strength of cell mobilization was also observed to be greater in the non-PE compared to PE pregnancies. The significance of this observation is supported by reports of shedding and re-endothelialization during pregnancy either by angiogenesis or vasculogenesis which may be enhanced in pathologic conditions (Robb et al., 2007). Several endogenous and exogenous factors stimulate this process (Asahara et al., 1999, Takahashi et al., 1999). The strong correlation shown by mEPCs (CD34+/CD309+) and iEPCs (CD34+/CD133+) to FGF in the peripheral blood of non-PE women suggests its role in endothelial progenitor cell mobilization for vascular repair. Sai’s group, previously showed that basic FGF is involved in the proliferation and maintenance of cEPCs while deficiency leads to cEPC differentiation to smooth muscle cells (Sai et al., 2014).

In the placenta of PE pregnant women, moderate associations were shown between iEPCs and angiogenic factors Ang 2, FGF and SDF-1. Although expression of these angiogenic factors have been implicated in endothelial cell mobilization (Kim et al., 2014, Sai et al., 2014, Szmigielska-Kaplon et al., 2015), these circulating cells in PE may be attenuated and dysfunctional. In addition, the use of anti-hypertensive therapies such as metoprolol, methyldopa and nifedipine in pregnancy have been shown to boost the numbers of endothelial cell phenotypes in circulation (Wang et al., 2019). Hence, further evaluation might be needed to test the functionality of these progenitors and the factors that mediate vascular repair under drug exposure in PE.
Endothelial cell phenotypes cECs, mEPCs and iEPCs were comparable in the peripheral, cord and placental blood of PE and non-PE pregnancies. However, cEPCs were increased in the cord blood of PE pregnancies compared to non-PE pregnancies but not in the placenta or peripheral blood. On the contrary, most studies have observed decreased cord blood cEPCs in PE pregnancies (Xia et al., 2007, Hwang et al., 2008, Muñoz-Hernandez et al., 2014, Gumina et al., 2017). In addition, Hwang et al. (2008) associated these cells with increased senescence and reduced differentiation ability. Similarities in the formation of new vascular networks in both PE and non-PE pregnancies which are independent of other obstetric characteristics have also been demonstrated (Muñoz-Hernandez et al., 2014). This suggests that the comparability of endothelial cell phenotypes in PE and non-PE pregnancies observed in this study may be dependent on other obstetric characteristics. Anti-hypertensive drugs as earlier described and other therapies used in the management of PE may alter the levels of circulating endothelial cell phenotype subsets (Wang et al., 2019). In addition, race and geographical settings of pregnant women induce changes that are not comparable to previous studies (Monga et al., 2012).

Despite the pathophysiologic link and related birth outcome between PE and PM, endothelial cell phenotypes as vascular mediators in PM or in combination with PE have not been considered. In view of this, a subgroup analysis was carried out to evaluate effect of *P. falciparum* placental infections on endothelial cell phenotypes in PE and non-PE women. Both PE and PM represent a state of placental dysfunction and high levels of circulating cECs in PE but not PM have been reported (Canbakan et al., 2007, Grundmann et al., 2008). Other markers of endothelial dysfunction similar to those in PE have also been evaluated in PM (Gueneuc et al., 2017). High percentage cECs similar to markers evaluated in this study have been shown in cerebral malaria patients (Oduro, 2015). This was however different from findings by
Heimrath et al. (2014) who observed low cECs in women with pregnancy induced hypertension.

Contrary to what has been observed in cerebral malaria and hypertensive disorders in pregnancy, particularly PE, our findings showed similarity in the levels of all endothelial phenotypes (cECs, cEPCs, mEPCs and iEPCs) in the placenta of pregnant women with active or past infections as well as in those with no PM in both PE and non-PE women. It could be explained that, heterogeneity among study populations, differences in antigen combinations and site of blood sampling exist. This, in addition to other biological, environmental and socioeconomic factors may account for variations in outcome from other studies with similar pathologies.

In the peripheral blood, PE women with past *Plasmodium* infection had a matured phenotype of progenitors expressing CD34+/CD309+ cells. These cells identify as a combined stem cell and endothelial cell marker with the capacity to differentiate into endothelial cells (Gammill et al., 2007) and thus signify the host’s attempt at repair. Indeed, this finding is consistent with the studies that identified low cEPC levels in children with cerebral malaria and subsequent increase in cEPC levels at recovery (Gyan et al., 2009, Oduro, 2015) which may suggest a past infection state. However, high levels of cEPCs were also found in children with severe malaria anaemia (Gyan et al., 2009). These variations call for the urgent need for cEPC characterisation in all malaria phenotypes. In combined conditions like PM and PE, endothelial cell phenotypes should be further characterised with emphasis on effects of drugs and other concomitant obstetric effects.
Finally, the levels of all endothelial cell phenotypes with the exception of mEPCs was higher in the placenta compared to the periphery of both PE and non-PE women with active and past PM. Percentage of cECs was however similar between the placenta and periphery in the non-PE but not PE group with past infection. In non-PE women with no infections, endothelial cell phenotypes were elevated in the placenta in contrast to periphery. The expression levels in PE women with no infection was not different. Although differences were not seen between non-PE and PE women in terms of parasite exposure, the pattern of expression was different in the peripheral and placental circulation of both non-PE and PE pregnancies. These alterations suggest a possible effect of PM on vascular stress and the subsequent development or exacerbation of PE. Recently, a group isolated blood outgrowth endothelial cells from paediatric malaria patients for the study of *P. falciparum* infected red blood cell adhesion (Ecklu-Mensah *et al.*, 2018). This experiment could be replicated to extensively study the functional characterisation of endothelial cells in PE pregnancies with or without PM. This will enhance pathophysiologic knowledge and lead to the identification of novel diagnostic and therapeutic targets.

4.6 Conclusion

This study has demonstrated high levels of endothelial cell phenotypes in the uteroplacental region compared to maternal circulation of non-PE and PE pregnancies. Circulating endothelial progenitor cells in PE pregnancies did not correlate strongly with growth factors that stimulate their release and function. Further alterations in the levels of endothelial cell phenotypes have been shown in peripheral and placental blood of non-PE and PE pregnancies exposed to PM.
CHAPTER FIVE

5.0 General Discussion and Conclusion

5.1 Discussion

Preeclampsia is a complex and pregnancy-specific syndrome and up until now, delivery of the placenta is the optimum means of treatment. The variability in clinical presentation clearly supports the multifactorial aetiology of preeclampsia. In an unsupervised placental gene expression profiling, Leavey et al. (2016) identified probable distinct subclasses of preeclampsia with divergent aetiologies. These phenotypes include “maternal preeclampsia” with healthy placentas at delivery, “canonical preeclampsia” with placental histopathological features and “immunologic preeclampsia” associated with severe foetal growth restriction (Leavey et al., 2016). Although the current study did not consider this phenotypic clustering, it may be a future direction in understanding the molecular basis of preeclampsia in the context of placental malaria.

Both intrinsic and extrinsic factors could influence the phenotypic differences in preeclampsia. For instance, in malaria endemic regions, foetal mechanisms that promote sVEGFR-1 (a preeclampsia marker) expression, have been postulated to be under selective pressure during pregnancy (Muehlenbachs et al., 2006). This is coupled with data from observational studies that have found increased risk of preeclampsia within the African sub-region (Sartelet et al., 1996, Ndao et al., 2009, Adam et al., 2011). However, the synergy between preeclampsia and placenta malaria is unclear and this necessitates further testing to expand pathophysiologic knowledge particularly in the context of placental malaria exposure. Therefore, this study sought to address this problem by linking placental infections to the risk of preeclampsia together with the associated inflammatory and angiogenic alterations. Endothelial integrity was
further assessed based on proportions of endothelial cell phenotypes (cECs, cEPCs, mEPCs and iEPCs).

Overall, the study identified placental pathology particularly syncytial knots and the presence of past and active placental parasites to increase the risk of preeclampsia development or exacerbate the condition. In the preeclampsia group, inflammation and angiogenesis were dysregulated based on elevation of pro-inflammatory and anti-angiogenic molecules in both the placenta and maternal circulation. In addition, patterns of endothelial cell phenotypes that mediate endothelial integrity were altered particularly in preeclampsia diagnosed women exposed to placental parasites. Angiogenic molecules that mediate the release and mobilization of these endothelial cells were not strongly correlated in women diagnosed with preeclampsia. This suggests that although these cells might be in circulation, their function may be impaired leading to the inability of vascular repair.

Indeed, the placenta is a hub for preeclampsia and placental malaria to thrive; necessitating the need for histological examination as carried out in this study. This method has been employed severally in the identification of placental lesions/injury such as syncytial knots associated with both preeclampsia and placental malaria (Ismail et al., 2000, Devisme et al., 2013, Ezeigwe et al., 2018). In addition, histological examination has been shown to be the gold standard for diagnosing sequestered parasites and their pigment during placental malaria due to variability in the sensitivity of other methods (Anchang-Kimbi et al., 2009, Souza et al., 2011, Fried et al., 2012). Despite recent advances in sensitive diagnostic technology such as quantitative PCR, histology is the most reliable tool for the diagnosis of past Plasmodium infections based on parasite pigment deposition in placental biopsies (Fried et al., 2012). This study explored histology to identify past parasite infections in placental biopsies in consonance with the timing
of preclinical preeclampsia (< 20 weeks of gestation). The assumption is that, placental malaria early on in pregnancy could precipitate the onset of preeclampsia. It is evidenced that most infections occur even before pregnant women seek obstetric/prenatal care (Kalilani-Phiri et al., 2013, Ofori et al., 2018). On the other hand, infections that occur during clinical preeclampsia could exacerbate the condition.

The retrospective diagnosis of placental malaria which is mostly asymptomatic, complicates its clinical significance. This is because, placental parasitaemia does not always reflect in peripheral circulation making it difficult for clinicians to detect and manage effects of damaged placenta and poor pregnancy outcomes. Specific biomarkers and tools for diagnosing both parasite and immune factors associated with placental malaria or in combination with preeclampsia in the peripheral blood during pregnancy should be identified.

Activation of the placental microvasculature during *P. falciparum* infections is mediated either directly by parasite interaction or indirectly through the release of inflammatory factors (Mantel et al., 2016). Parasite ligands on infected erythrocytes interact with both syncytiotrophoblast and endothelial receptors of the placenta although via different mechanisms (Duffy and Fried, 2003). The immunological cascade associated with malaria is expected to be elicited even in asymptomatic *Plasmodium* infections. It has been found that submicroscopic *Plasmodium* infections of the placenta elicit an immunological imbalance and hypoxic conditions (Agudelo et al., 2014). Also, Moxon and colleagues (2014) showed in a group of Malawian children that endothelial activation and inflammation could persist even after the clearance of parasites. They suggested that drugs that reduce the risk of cardiovascular diseases such as statins be administered to reduce the effects of endothelial activation during malaria. In resonance with this study, both active and past placental infections increased the
risk of preeclampsia in addition to increased pathological and inflammatory consequences. This suggests that, although parasite density may be important in clinical manifestation of malaria, asymptomatic and submicroscopic infections may have subtle consequences. Host immune response to these infections is also an equally important determinant of disease.

Alterations in inflammatory and angiogenic mediators shown in this study have been independently measured in placental malaria and preeclampsia (Muehlenbachs et al., 2006, Leinonen et al., 2010, Ataide et al., 2015, Mantel et al., 2016). This is expected to be enhanced when both conditions occur concurrently as confirmed from this study. Collectively, proinflammatory and anti-angiogenic markers were elevated in preeclamptic compared to non-preeclamptic pregnancies. This was further highlighted in preeclampsia diagnosed women with active parasite infections. Dysregulated inflammation and angiogenesis alter placental vascular development resulting in blood vessel constriction, impaired blood flow and adverse foetal outcomes (Conroy et al., 2013, Mantel et al., 2016). The high expression of proinflammatory and anti-inflammatory factors in both preeclampsia and placental malaria suggests potential damage to the placenta. Although most of these factors may not be specific to each condition or their synergistic occurrence, a few may show diagnostic and therapeutic promise. For instance, Muehlenbachs’ study found correlation between placental malaria and hypertension with increased levels of sVEGFR-1 (Muehlenbachs et al., 2006). This study has confirmed and identified additional pro-angiogenic molecules (sVEGFR1, PDGF, PAI-1, Ang2 and CCL2) to be associated with increased risk of preeclampsia during placental malaria exposure. This was independent of other obstetric factors and might not show specificity. Therefore, in a multivariate analysis, PAI-1 was found to increase seven-fold in the peripheral blood of preeclampsia diagnosed primigravid women with active or past placental infections. This
unlocks a new prospect in predicting placental damage specific to both preeclampsia and placental malaria in peripheral blood during pregnancy.

The anti-angiogenic factor PAI-1, a plasminogen activator inhibitor, acts on the fibrinolytic pathway by breaking down fibrin in blood clots during endothelial cell injury (Bhattacharjee and Bhattacharyya, 2014, Ye et al., 2017). High expression of this factor results in fibrin deposits and impaired placentation implicated in several reproductive pathologies such as preeclampsia, intrauterine growth restriction, recurrent pregnancy loss and gestational diabetes (Ye et al., 2017). This is so because, the state of hyper-coagulation in normal pregnancy with high levels of PAI-1 (the main inhibitor of the fibrinolysis) is intensified in these pathologic pregnancies such as that shown in preeclampsia (Halligan et al., 1994, Coolman et al., 2006, Elzein et al., 2016). In terms of placental malaria, little is known about their expression. A study among Sudanese pregnant women did not find significant differences between PAI-1 levels in women with placental malaria and that of normal pregnant women (Mostafa et al., 2015). This suggests that PAI-1 is underexplored hence, further studies should validate the potential of this marker as a promising diagnostic and therapeutic target for preeclamptic pregnancies exposed to placental malaria.

In addition to PAI-1, the levels of cECs which are matured endothelial cells displaced from blood vessel walls were measured as markers of endothelial injury. Levels of different phenotypes of endothelial progenitor cells with capacity for maintaining blood vessel integrity by regeneration and repair were also measured. All endothelial cells were quantified based on flow cytometric analysis of endothelial cell specific marker combinations. The challenge in this area is the lack of standardized phenotypic characterisation and methodological contradictions in the quantification and isolation of endothelial cell phenotypes (Chopra et al., 2018).
Different markers of endothelial cell origin have been used discordantly by different groups to represent endothelial cells and their phenotypes (Gyan et al., 2009, Heimrath et al., 2014, Gumina et al., 2017, Chopra et al., 2018, Zahran et al., 2019). This complexity is further expounded by the different sources of endothelial progenitor cells such as bone marrow, peripheral, umbilical cord blood, cardiac and adipose tissues (Chopra et al., 2018). In spite of these irregularities, circulating endothelial cell phenotypes are being harnessed in maintaining endothelial health and alleviating vascular pathologies. The clinical use of anti-hypertensive therapy for the management of preeclampsia has also been strengthened. This is because Wang et al. (2019), demonstrated that drugs such as metoprolol, methyldopa and nifedipine were associated with increased levels and function of cEPCs. Their mechanism of action should further be investigation to aid in preventive therapy. Various clinical trials are also underway in testing therapeutic effects of circulating endothelial cell phenotypes for various cardiovascular disorders and other systemic conditions (Chopra et al., 2018).

This study was exploratory and the assessment of endothelial cell variability in pregnancy exposed to placental malaria and in combination with preeclampsia is a novel contribution to understanding pathophysiology of both conditions. The study was limited by small number of subjects particularly in the sub-analysis group with placental malaria exposure due to rigorous exclusion criteria to exclude multiple confounding factors. Drugs used in the management of preeclampsia were not accounted for. Despite these limitations, new grounds for the study of preeclampsia in malaria endemic regions will further expound potential phenotypic differences in the aetiology of preeclampsia and open up diagnostic and therapeutic avenues.
5.2 Conclusions

The study demonstrated associated risk for preeclampsia in women exposed to placental malaria by showing that;

1. Placental pathology and *P. falciparum* placental infections dependently or independently increased the risk of preeclampsia particularly in first time pregnant women resulting in poor birth outcomes.

2. The placenta is a site for production of most inflammatory and angiogenic molecules for pregnancy maintenance. Proinflammatory and anti-angiogenic were enhanced in both the placenta and maternal circulation of preeclampsia diagnosed women exposed to placental malaria.

3. Levels of proinflammatory markers and anti-angiogenic markers associated with endothelial dysfunction were elevated in preeclamptic compared to non-preeclamptic pregnancies.

4. Preeclamptic pregnancies with active placental parasites also showed higher levels of proinflammatory and anti-angiogenic markers.

5. High levels of plasminogen activator inhibitor 1 (PAI-1) was associated with increased risk of preeclampsia in first time pregnancy with active or past placental malaria.

6. Levels of endothelial progenitor cell phenotypes were elevated in the placental circulation compared to peripheral circulation in both preeclamptic and non-preeclamptic pregnancies.

7. Circulating endothelial progenitor cells (cEPCs) correlated with angiogenic molecules that mediate their release in non-preeclamptic but not in preeclamptic pregnancies.

8. The expression pattern of all endothelial cell phenotypes were generally high in the placenta compared to peripheral blood in both preeclampsia diagnosed and undiagnosed women with active or past placental infections.
9. Higher placental expression of endothelial cell phenotypes was seen in non-preeclamptic pregnancies in all placental malaria exposure group (No infection, active infection and past infection) than in peripheral circulation. However, in preeclamptic pregnancies, the placental expression was higher only in women with active and past infection compared to peripheral circulation.

5.3 Recommendations

Outcome of this study may be important for the following policy-related reasons;

1. Systems that encourage early antenatal attendance such as free maternal care and conscious sensitization should be put in place to ensure adequate monitoring of pregnancy and early detection of pregnancy complications such as preeclampsia.

2. Free pre-conception counselling centres should be set up to help minimize risk associated with preeclampsia. If women are sensitized to attend these centres, high risk women will be identified even before pregnancy leading to early prenatal management and improved outcome.

3. The current preventive treatment in pregnancy using IPTp should be revisited to ensure its effectiveness and impact in clearing placental parasites. Currently, dosage has been increased from 3 to 5 but not all women take the appropriate dose during pregnancy. It is recommended that home visits by health personnel are incorporated into the antenatal programme to ensure higher coverage of IPTp to late or non-attendant pregnant women.

4. The current management guideline for preeclampsia in Ghana is laudable. However, a multidisciplinary approach involving experts such as infectious disease specialists and cardiologists in the management of preeclampsia will be an added advantage for improving pregnancy outcomes.
5. Continuous efforts targeted at pregnancy-based malaria vaccines should be intensified as the best preventive means to placental malaria and its associated outcomes such as preeclampsia.

For further research, the following are recommended;

1. The placenta should further be explored in understanding the relationship between preeclampsia and placental malaria. Placental tissue localization of these study markers should be evaluated and compared to their expression in the peripheral circulation and assess how this can improve diagnosis and identify therapy. The placenta (although a temporal organ) is a rich source of information on maternal and foetal health. It is a hub for understanding the pathophysiology of most pregnancy related conditions and targets for diagnostic and therapeutic markers. In a larger context, a placental bio-bank to serve as repository for placental research in Ghana will be a great initiative to compact most of the maternal and health related complications.

2. Further experiments to test the functionality of endothelial cell phenotypes in relation to placental malaria and preeclampsia should be explored.

3. Larger longitudinal studies are also needed to further confirm the observations in this study. This will involve different time point assessment during pregnancy and their relation to placental and birth outcomes. Comparison should also be made with malaria naïve pregnant women in order to strengthen findings.

4. The contribution of other major infectious diseases prevalent in the tropics to the risk of preeclampsia should be explored.

Finally, and to the research community, the complexity of preeclampsia requires a multidisciplinary approach towards addressing preeclampsia and placental malaria related
complications. Research design must incorporate the expertise of researchers from other disciplines such as molecular biologists, nutritionists, epidemiologists etc. for a holistic intervention.
REFERENCES


microvascular endothelial cell heterogeneity in organ maintenance and regeneration. 

*Developmental Cell*, 26, 204-219.


human placenta have greater vasculogenic potential than circulating endothelial progenitor cells from umbilical cord blood. *Cell Medicine*, 2, 85-96.


progenitor cells inversely correlate with risk factors for coronary artery disease. 


APPENDICES

Appendix I: Preparation of 10% Neutral Buffered Formalin

Materials
An Erlenmeyer’s flask (about 1.5L volume)
Stock formalin fixative (40%)
Measuring cylinder
Funnel
900 ml Distilled water
Sodium Phosphate, dibasic (Na$_2$HPO$_4$)
Sodium Phosphate, monobasic (NaH$_2$PO$_4$)

To prepare 1L of 10% neutral buffered formalin,

- Measure 100 ml of the 37% stock formaldehyde (formalin) using the measuring cylinder, into the Erlenmeyer’s flask with the help of a funnel.
- Weigh 6.5 g of the sodium Phosphate-dibasic, and 4 g of the sodium Phosphate-monobasic into a beaker.
- Dissolve these salts with some amount of the 900 ml distilled water, and stir gently with the stirring rod until all salts get dissolved.
- Gently transfer the solution of the sodium Phosphate salts (monobasic and dibasic) into the Erlenmeyer’s flask with the help of the funnel.
- Rinse the beaker with a little of the 900 ml distilled water remaining, and pour it into the Erlenmeyer’s flask again.
- Gently pour the remainder of the distilled water into the Erlenmeyer’s flask with the help of the funnel.
- Cover the flask and mix well the preparation.
- Then store at room temperature.

NB:
Right proportions of the sodium Phosphate salts and the stock formaldehyde (formalin) should be calculated for use, depending on the concentration and/or volume needed to be prepared.
Appendix II: Tissue processing protocol (automated processing machine-Carousel).

<table>
<thead>
<tr>
<th>Position</th>
<th>Reagent Name</th>
<th>Time of Incubation</th>
<th>Operating Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% Buffered Neutral formalin</td>
<td>1 hr 30 minutes</td>
<td>Agitation</td>
</tr>
<tr>
<td>2</td>
<td>60% alcohol (ethanol)</td>
<td>1 hr 30 minutes</td>
<td>Agitation</td>
</tr>
<tr>
<td>3</td>
<td>70% alcohol</td>
<td>1 hr</td>
<td>Agitation</td>
</tr>
<tr>
<td>4</td>
<td>80% alcohol</td>
<td>1 hr</td>
<td>Agitation</td>
</tr>
<tr>
<td>5</td>
<td>90% alcohol</td>
<td>1 hr</td>
<td>Agitation</td>
</tr>
<tr>
<td>6</td>
<td>100% alcohol</td>
<td>1 hr</td>
<td>Agitation</td>
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<tr>
<td>7</td>
<td>100% alcohol</td>
<td>1 hr</td>
<td>Agitation</td>
</tr>
<tr>
<td>8</td>
<td>100% alcohol</td>
<td>1 hr</td>
<td>Agitation</td>
</tr>
<tr>
<td>9</td>
<td>Xylene</td>
<td>1 hr 30 minutes</td>
<td>Agitation</td>
</tr>
<tr>
<td>10</td>
<td>Xylene</td>
<td>1 hr</td>
<td>Agitation</td>
</tr>
<tr>
<td>11</td>
<td>Paraffin wax (molten)</td>
<td>1 hr 30 minutes</td>
<td>Heat / Agitation</td>
</tr>
<tr>
<td>12</td>
<td>Paraffin wax (molten)</td>
<td>1 hr</td>
<td>Heat / Agitation</td>
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Appendix III: Haematoxylin and Eosin Staining Protocol for Placental Tissue

<table>
<thead>
<tr>
<th>Procedure/solution</th>
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<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
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<td>Xylene</td>
<td>5 minutes</td>
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<tr>
<td>Xylene</td>
<td>5 minutes</td>
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<tr>
<td>Absolute ethanol</td>
<td>5 minutes</td>
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<tr>
<td>Absolute ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Rinse in tap water</td>
<td>1 minute</td>
</tr>
<tr>
<td>Mayer’s haematoxylin</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Blue in tap water (control nuclei staining microscopically)</td>
<td></td>
</tr>
<tr>
<td>90% ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>45 seconds</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>45 seconds</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>45 seconds</td>
</tr>
<tr>
<td>Xylene</td>
<td>1 minute</td>
</tr>
<tr>
<td>Xylene</td>
<td>1 minute</td>
</tr>
</tbody>
</table>

Mount with DPX and coverslip.

Appendix IV: Giemsa Staining Protocol for Placental Tissue

<table>
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<th>Procedure/solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
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<td>Xylene</td>
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<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
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<tr>
<td>Absolute ethanol</td>
<td>5 minutes</td>
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<tr>
<td>Absolute ethanol</td>
<td>4 minutes</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>2 minutes</td>
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<tr>
<td>70% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Rinse in running tap water</td>
<td></td>
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</tbody>
</table>
Stain with Diluted Giemsa (made fresh 1/10 dilution) 10 minutes
Distilled water Few dips (rinse in water)
Air dry stained slides
Xylene 1 minute
Xylene 1 minute

Mount with DPX and coverslip.
## Appendix V: Case Summary Table for Histological Diagnosis of Malaria Parasites

<table>
<thead>
<tr>
<th>ID</th>
<th>Formalin Pigment Y/N</th>
<th>Parasites Y/N</th>
<th>Parasitaemia</th>
<th>Parasite Pigment (% hpf)</th>
<th>Pigment Score</th>
<th>Pigment Y/N</th>
<th>Inflammation Y/N</th>
<th>Inflammation Score</th>
<th>Bulmer score</th>
<th>Unique non-malaria placental pathology/injury</th>
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<tbody>
<tr>
<td>P17001 H&amp;E</td>
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<td>P17001 G</td>
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