UNIVERSITY OF CAPE COAST

CYCLOOXYGENASE (COX) AND LIPOXYGENASE (LOX) GENE EXPRESSION IN CO-MORBIDITY OF PRE-ECLAMPSIA AND PLACENTAL MALARIA: A CASE-CONTROL STUDY IN KORLE-BU TEACHING HOSPITAL, GHANA

RICHARD DARKO

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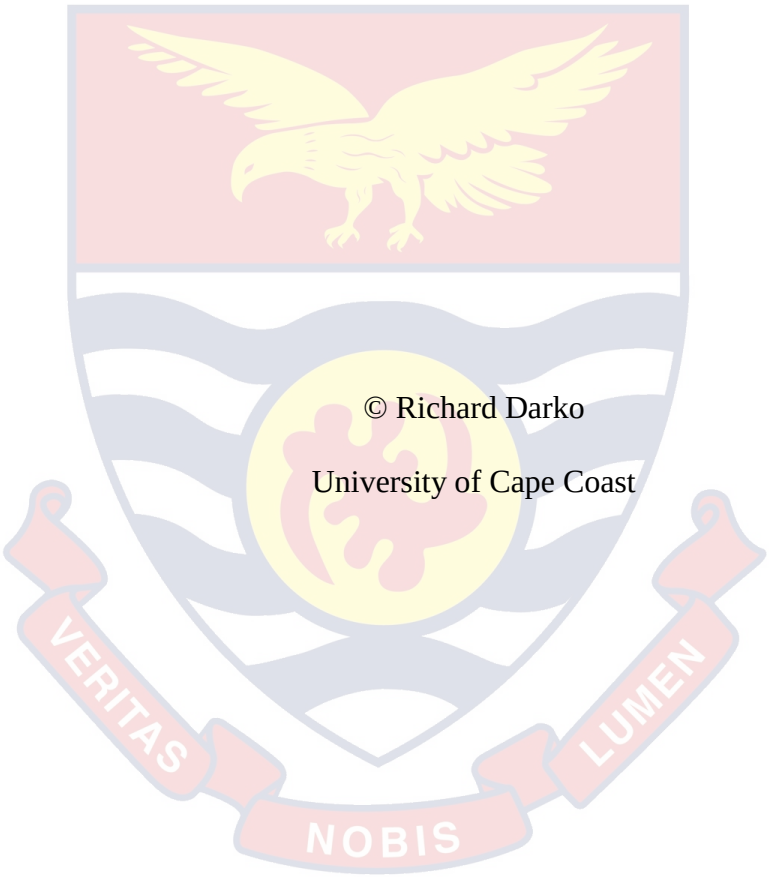

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BY

RICHARD DARKO

Thesis submitted to the Department of Microbiology and Immunology of the College of Health and Allied Sciences, School of Medical Sciences, University of Cape Coast, in partial fulfillment of the requirements for the award of Master of Philosophy degree in Infection and Immunity.

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ABSTRACT

Over the years there has been an increased incidence of maternal health problems including high maternal death. Though pregnancy-related complication such as sepsis, severe bleeding, unsafe abortions, and unskilled health personnel have been implicated to this high maternal mortality rate, also the effect of hypertensive diseases such as pre-eclampsia and infectious diseases like placental malaria are major complications of pregnancy. Studies have not specifically outlined the mechanisms of pre-eclampsia aetiology and therefore it remains poorly understood. However, a multifactorial aetiology has been proposed with several risk factors. Significant numbers of studies have established an association between pre-eclampsia and placental malaria in Africa. Also, the rise in pre-eclampsia is occurring against the background of malaria infection in malaria endemic regions though malaria prevalence has decreased in recent times. Expressions of cyclooxygenase (COX) and lipoxygenase (LOX) are reported to be involved in pregnancy-related pathology such as pre-eclampsia and placental malaria. This study sought to assess whether the association between pre-eclampsia and placental malaria is through induction of either COX-1, COX-2, or 15-LOX by malaria infection. Study participants were pregnant women reporting for routine antenatal care. Using RT-PCR, we quantified the expression levels of the mRNA genes of COX-1, COX-2, and 15-LOX from placental tissue of the participants. The study established that the odds of developing pre-eclampsia are 2.09 greater for women with placental malaria compared to women without exposure, confirming the association between placental malaria and pre-eclampsia development. The study also demonstrated a significant increase in mRNA levels for COX-2 in pre-eclampsia women with placental malaria. Placental malaria could play a role in pre-eclampsia by inducing production of COX-2 leading to inflammation and consequently pre-eclampsia.

KEYWORDS

Arachidonic acid

Cyclooxygenase (COX)

Lipoxygenase (LOX)

Placental malaria

Pre-eclampsia

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DEDICATION

To my Parents,

Mr. Emmanuel Akwesi Sakyi and Mrs. Janet Aquah.

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LIST OF ACRONYMS

COX	Cycloxygenase
LOX	Lipoxygenase
15-LOX	15-Lipoxygenase
SDGs	Sustainable Development Goals
FFPE	Formalin Fixed Paraffin Embedded
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid or Micro Ribonucleic Acid
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
PPIA	Peptidyl Propyl Isomerase A
NMIMR	Noguchi Memorial Institute for Medical Research
NMIMR-IRB	Noguchi Memorial Institute for Medical Research Institutional Review Board

CHAPTER ONE

INTRODUCTION

Pregnant women are considered to be a special demographic group due to their susceptibility to some infections. Increasing rate of pregnancy has been associated with an increased incidence of maternal health problems including high maternal death in developing countries. A report by World Health Organization (WHO) in 2017 indicated that an expected 303,000 maternal deaths occurred globally, yielding a general Maternal Mortality magnitude relation of 216 maternal deaths for every 100,000 live births from 183 countries. They also emphasized that developing regions accounted for nearly 99% of the evaluated worldwide maternal passings in 2015, with sub-Saharan Africa alone representing approximately 66%, trailed by Southern Asia.

Although Ghana has made significant progress in improving maternal health, it still fell short of reaching the Millenium Development Goal (MDG) 5 target at the end of 2015 (NDPC, 2015). Maternal mortality over the years has decreased in Ghana. However, the figure is above 75 deaths per 100,000 live births in spite of several efforts and interventions by government and development partners. Though unskilled health personnel and factors of pregnancy-related complications; including sepsis, severe bleeding, unsafe abortions have been implicated in this high maternal mortality rate, also the effect of hypertensive diseases such as pre-eclampsia and infectious diseases like placental malaria are major complications of pregnancy associated with high incidence of maternal/fetal death. Several researchers have established an association between placental malaria and pre-eclampsia development in

Africa (Sartelet *et al.*, 1996, Muehlenbachs *et al.*, 2006, Ndao *et al.*, 2009, Adam, *et al.*, 2011). This study, therefore, seeks to determine whether the association between pre-eclampsia and placental malaria is through induction of either COX-1, COX-2, or 15-LOX by malaria infection as this will provide a firsthand concept in understanding the interactions between pre-eclampsia and placental malaria.

1.0 Background to the Study

Pre-eclampsia, presently one of the main sources of maternal mortality in Ghana (Adu-Bonsaffoh, Obed, and Seffah, 2014), is a placenta-dependent condition with both resident and systemic abnormalities. Studies have shown that the commencement of pre-eclampsia syndrome is during the initial phases of gestation (English, Kenny, and McCarthy, 2015; Tanaka *et al.*, 2015). However, it manifests after twenty weeks of pregnancy and is characterized by the onset of hypertension and proteinuria (Zhang *et al.*, 2016). Evidence suggests that alteration in circulating levels of controllers of angiogenesis cause several clinically significant symptoms of pre-eclampsia (Pennington *et al.*, 2012). Trophoblast invasion has also been suggested to contribute to the commencement of pre-eclampsia (Nadeem *et al.*, 2011). Furthermore, the insufficient transformation of the spiral arteries, as well as endothelial dysfunction and inflammation (Craici *et al*, 2008), are reported to be pathogenic factors of pre-eclampsia making the development of pre-eclampsia multifactorial.

Placental malaria infection is one of the most important protozoan infections that occurs during pregnancy and has been broadly utilized as a standard marker to describe protozoan infection in epidemiological

investigations (Uneke, 2007). The condition is common in first-time pregnancies and has adverse pathophysiologic effects. Malaria infection throughout gestation could be an important public health issue with substantial risks for the pregnant mother, and also the newborn kid. Malaria-associated maternal ill health and low birth weight is usually the results of *Plasmodium falciparum* infection and happens preponderantly in regions of stable transmission in Africa (WHO, 2017).

Malaria infections generally have been difficult to manage because the body is awful at developing long-lasting immunity to the parasite hindering vaccine development for decades. Research has shown that the sturdy provocative reaction that accompanies and drives clinical protozoan infection is additionally to blame for silencing the key immune cells required for long-run protection against the parasite. This indicates that during infection vital inflammatory molecules really arrest the event of helper T cells and, therefore, the B cells do not get the mandatory directions to create antibodies (Ryg-Cornejo et al., 2016) and downstream immune response. Though several molecules have been characterized as contributing factors to pre-eclampsia and placental malaria, some enzyme complex in the metabolism of arachidonic acid has also been reported to be involved in pathology during pregnancy as they are expressed in the placenta.

Arachidonic acid is a key unsaturated carboxylic acid that is the precursor for a good type of lipid mediators that are involved with several physiological and pathophysiological forms in human (Powell and Rokach, 2015). It is derived from a phospholipid molecule by the enzyme phospholipase A2 but can also be made from diacylglycerol (DAG) by

diacylglycerol lipase (Baynes and Marek, 2005). This arachidonic acid is metabolized to eicosanoids using several pathways depending on enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 monooxygenase and epoxygenase.

Cyclooxygenase (COX) work in one division of the arachidonic acid metabolic pathway to yield prostaglandins (PGs) and thromboxanes (TXs), while the activities of lipoxygenase and its related super molecules add the opposite arm of the metabolic pathway to create leukotrienes (Lutz and Cornett, 2013; Sakai *et al*., 2012). However, there has been conflicting reports on the expression levels of these enzymes during pre-eclampsia. For instance, Akarasereenont *et al.* (1999) reported COX-2 overexpression during pre-eclampsia, whereas Khan *et al.* (1999) described a reduction of COX-2 in preeclamptic-placentas. Interestingly, another study reported that COX-2 mRNA expression in placental malaria decreases with parity (Sarr *et al*., 2010). Sarr *et al*., (2010) further indicated that increase in COX-2 is associated with anaemia in pregnant women while the increase in COX-1 and 15-LOX transcripts is associated with low birth weight in placental malaria similar to what is observed in pre-eclampsia.

Ordi *et al*., (2001) previously reported that COX-2 expression increases after a lengthy period of active malaria infection of the placenta. This agrees with findings by Sarr *et al* (2010) that COX-2 and IL-10 are highly expressed in active placental malaria. Hence, COX-1, COX-2 and 15-LOX were considered as parameters in this study to show if there is any association through inflammatory mediator induction between placental malaria and pre-eclampsia development.

1.2 Statement of the Problem

The incidence of pre-eclampsia and placental malaria is common among women living in malaria transmission settings. Placental malaria is one of the severe phenotypes of protozoan infections resulting in parasite sequestration in the placenta as well as chronic inflammation (Moxon *et al*., 2014). While some studies have found no link between placental malaria and pre-eclampsia (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).

COX-2 over-expression has been linked to pre-eclampsia pathogenesis (Akarasereenont *et al.,* 1999) and has also been associated with anaemia in pregnant women during malaria infection. COX-1 and 15-LOX over-expression have also been described during pregnancy in placental malaria (Sarr *et al*., 2010) with complications similar to pre-eclampsia.

Currently, the sustainable development goals (SDGs) have set a target for maternal mortality to be decreased below 70 deaths per 100,000 live births by the year 2030. Efforts must, therefore, be put in place to significantly reduce maternal deaths. Factors that contribute to morbidities and mortalities, in an effort to attain goal 3 of the Sustainable development goals (SDGs) should, therefore, be identified. Among the leading causes of maternal mortality in Ghana are hypertensive disorders of pregnancy with pre-eclampsia in the lead (Adu-Bonsaffoh et al., 2014, 2013). Decades of studies have not specifically outlined the mechanisms of pre-eclampsia aetiology and therefore it remains poorly understood. A multifactorial aetiology has been

proposed with several risk factors associated with the condition (Wang *et al*., 2010, Chappell and Morgan, 2006, Pennington *et al*., 2012, Veerbeek *et al*., 2015).

It is therefore important to know whether these metabolic enzymes of arachidonic acid participate in co-morbidity of placental malaria and pre-eclampsia development to enhance early detection and management for a successful pregnancy. Therefore, this study seeks to assess whether the association between pre-eclampsia and placental malaria is through induction of either COX-1, COX-2, or 15-LOX by malaria infection.

1.3 Significance of the Study

This study seeks to assess whether there is an association between pre-eclampsia and placental malaria through the induction of COX-1, COX-2, and 15-LOX by malaria infection. The study will, therefore, provide a firsthand concept in understanding the interactions between pre-eclampsia and placental malaria. This will consequently contribute to the management of malaria related maternal health issues, reducing maternal and fetal mortality, and attaining the SDG three.

1.4 Hypothesis

Placental Malaria infection increases cyclooxygenase and lipoxygenase pathways of converting arachidonic acid into prostaglandins which lead to increased inflammation and subsequently pre-eclampsia.

1.5 Purpose of the Study

To assess whether the association between pre-eclampsia and placental malaria is modulated by the induction of either COX-1, COX-2, or 15-LOX by malaria infection.

1.6 Objectives

1) To determine the relationship between placental malaria and pre-eclampsia development.
2) To measure and compare the levels of COX-1, COX-2, and 15-LOX mRNA gene expressions among pregnant women with pre-eclampsia and placental malaria, women with placental malaria only and women with no pre-eclampsia or placental malaria.

1.7 Delimitations

COX and LOX are believed to increase inflammatory molecules such as cytokines during their metabolic activity on arachidonic acid. Also placental malaria influences the levels of haemoglobin in pregnancy. Thus, it would be great to measure cytokine profiles and some haematological indices and access how they interact with variables of interest in this study. However, this study did not measure these variables and thus inferences drawn are without recourse to cytokine profiles and haematological indices.

1.8 Limitation

Placenta tissues obtained were formalin-fixed and paraffin embedded (FFPE). The method of fixing the tissue sections and inserting them in paraffin usually results to some level of RNA degradation and chemical alteration. Also, the nucleic acid is cross-linked with a macromolecule throughout the

formalin fixation method, and most of the RNA secluded from FFPE tissues is extremely degraded and decreased to a far lower yield than that of RNA secluded from a similar quantity of contemporary tissues (Guo *et al*., 2016). Hence, the struggle of estimating gene expression levels in FFPE samples.

1.9 Organisation of the Study

This study seeks to assess whether the association between pre-eclampsia and placental malaria is through induction of COX-1, COX-2, and 15-LOX by malaria infection. The thesis is organized into five chapters.

Chapter one introduces the study with respect to the subject, statement of the problem and the significance of the study. The hypothesis, purpose of the study and objectives were also mentioned.

Chapter two focused on reviewing other related literature where existing knowledge specifically on this study was elaborated. Chapter 3 provides brief descriptions of research methods geared towards this study where research design, sampling processes, data collection techniques and data processing and analysis were highlighted. Chapter four presents results obtained and their interpretations, and discussions as well where findings from the study were compared to other related studies. Finally, the study concluded and some recommendations were made in chapter five.

CHAPTER TWO

LITERATURE REVIEW

This study seeks to assess whether the association between pre-eclampsia and placental malaria is through stimulation of either COX-1, COX-2, or 15-LOX by malaria infection. The levels of COX-1, COX-2, and 15-LOX mRNA gene expressions will be evaluated and compared in women diagnosed with or without pre-eclampsia that are infected or uninfected by *P. falciparum.* This chapter provides knowledge available specifically to the study where pre-eclampsia disease, pathogenesis of pre-eclampsia (abnormal development of placenta in pre-eclampsia, immunologic factors, systemic endothelial dysfunction, inflammation, genetic factors and diet), biomarkers of pre-eclampsia, malaria disease, pathogenesis of malaria, placental malaria, and co-morbidity of pre-eclampsia and placental malaria were elaborated in chronology.

2.1 Pre-eclampsia

Pre-eclampsia is a pregnancy-related condition characterized by hypertension and excess protein in the urine. Also, in the absence of substantial proteinuria, hypertension in the presence of any end-organ impairment, for example, thrombocytopenia, impaired liver function, renal disorder, pulmonic edema, or cerebral instabilities is adequate to establish a diagnosis (Monica, Ravi, and Karumanchi, 2015). The condition can be differentiated into mild and severe forms. Mild pre-eclampsia occurs when the systolic circulatory strain of >140 mmHg or a diastolic pulse >90 mmHg together with 300 mg of proteinuria more than 24 hours, and serious pre-

eclampsia is described by extreme heights of circulatory strain or proof of alternative end-organ pathology (Pennington *et al*., 2012).

The order of events in pre-eclampsia is suggested to occur in two phases; a placental phase of insufficient development of maternal spiral arteries leading to decreased maternal blood supply to the placenta, achieving placental ischemia and hypoxia, and a peripheral phase where the exemplary appearances of broad epithelium pathology, high blood pressure, proteinuria, and edema occurs (Sargent *et al*., 2006). Pre-eclampsia, presently one of the main sources of maternal mortality in Ghana (Adu-Bonsaffoh *et al*., 2014), is a placenta-dependent condition with both resident and systemic abnormalities. Thus, the condition of the placenta is essential to the advancement of pre-eclampsia since in most circumstances eliminating the placenta generally resolves the condition and continues to be the only effective treatment. Pre-eclampsia may be one among the foremost precarious complication of gestation as a result of its impact on maternal and newborn child outcomes (Leeman and Fontaine, 2008).

2.2 Pathogenesis of Pre-eclampsia

Pre-eclampsia is a multi-systemic syndrome, involving several factors in its pathogenesis and pathophysiology and the lone known treatment is delivery of the fetus and placenta (Figure 1).

It is generally divided into early and late-onset. In the early-onset type, the clinical signs appear before 33 gestational weeks, while the late-onset type occurs from 34 weeks (Gathiram and Moodley, 2016). The chief pathological feature of early-onset pre-eclampsia is the partial transformation of the spiral

arteries, resulting in hypoperfusion of the placenta and reduced nutrient supply to the fetus.

Despite decades of research, the pathogenesis and pathophysiology of pre-eclampsia are still poorly understood. However, abnormal development of the placenta, diet, genetic factors, increased sensitivity to angiotensin II, and immunologic factors have been proposed (Karumanchi *et al.*; 2011) to be major contributing factors.

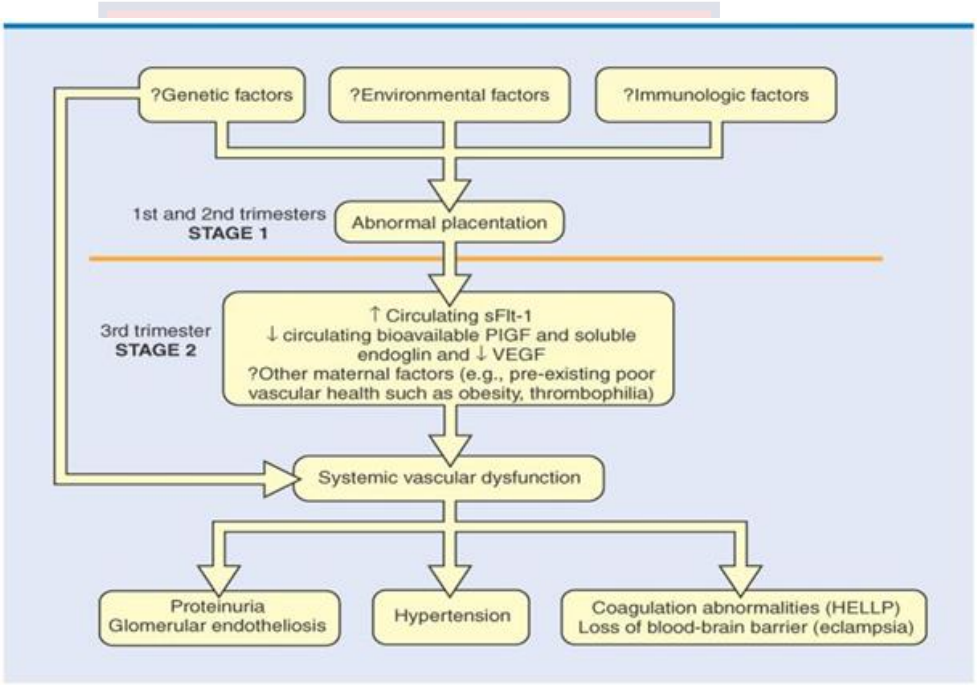

Figure 1: Schematic Representation of the Pathophysiology of Pre-eclampsia Picture credit (Gallery of 54 New Pathophysiology of Pre-eclampsia in Flow Chart,http://dailyrevshare.com/pathophysiology-of-pre-eclampsia-in-flow-chart/Obtained on 01/10/2018).

2.2.1 Abnormal Development of Placenta in Pre-eclampsia

Pre-eclampsia is a disorder that is described by the inability of the trophoblast to invade the decidual supply routes prompting modifications in placental improvement, placental perfusion and an inadequate transport of supplements. Placentation and trophoblast invasion of the maternal tissue involves two processes; firstly vascularisation to establish a feto-placental vascular network, and secondly, the incursion of the maternal spiral arteries by the cytotrophoblasts or endovascular trophoblasts (Gathiram and Moodley, 2016).

Trophoblast invasion has been suggested to contribute to the inception of pre-eclampsia (Nadeem *et al*., 2011) and insufficient transformation of the spiral arteries (Craici, Wagner, and Garovic, 2008) is also reported to be a pathogenic factor of pre-eclampsia. In pathologies with an anomalous placentation, the trophoblast invasion is poor and restricted solely to spiral arteries present in shallow decidua. Amid early ordinary placental advancement, extravillous cytotrophoblasts of fetal birthplace invade the uterine artery corridors of the decidua and myometrium. These obtrusive cytotrophoblasts supplant the endothelial layer of the maternal spiral corridors, changing them from little, high-obstruction vessels into extensive bore capacitance vessels equipped for giving sufficient placental perfusion to feed the fetus (Young, Levine, and Karumanchi, 2010). In pre-eclampsia, however, this transformation is incomplete.

The deformity of placental vascularization causes a reduction in blood flow in the intervillous spaces, prompting gradual chronic hypoxia (Gueneuc, Deloron, and Bertin, 2017).

Valenzuela *et al*., (2012) noted that the events that prompt the improvement of pre-eclampsia could also be clarified by the principal phase of defective trophoblastic intrusion, which happens from the get-go in gestation, with uteroplacental circulation remaining in an exceeding state of high resistance throughout physiological pregnancy, which may be detected by high resistance of the female internal reproductive organ arteries. The perseverance of the condition under perfusion produces placental hypoxia and native oxidative pressure leading to a general inflammatory reaction and endothelial pathology, prompting the beginning of the clinical symptoms of pre-eclampsia. Placental ischemia and hypoxia enhance release of trophoblast microparticles into the maternal circulation which stimulates increased induction of proinflammatory cytokines and the activation of maternal endothelial cells (Raghupathy, 2013).

In summary, the pathophysiological process of pre-eclampsia starts with insufficient trophoblast intrusion right off the bat in pregnancy, which delivers an expansion in oxidative pressure adding to the improvement of general endothelial pathology in the later periods of the condition, prompting the trademark clinical appearance of pre-eclampsia, with hypertension, proteinuria, and edema.

2.2.2 Role of Immune Receptors

Maternal immunological response during pregnancy is essential. Anti-inflammatory cytokines that aid to control the immune reaction, such as IL-10 and IL-4, play crucial roles in a successful pregnancy by giving equilibrium to the immune system. This indicates tolerance to the semi-allogeneic fetus, which has half maternal genes as well as half paternal genes (Gleicher, 2007).

There is increasing evidence suggesting that both innate and adaptive immune processes take part in the pathogenesis of pre-eclampsia. Harmon *et al*., (2016) indicated that the alteration in immune balance is believed to contribute to the overall pathophysiology associated with pre-eclampsia, which includes the production of reactive oxygen species (ROS), increased endothelin-1 expression, and B cell assembly of autoantibodies to the Angiotensin II (AngII) type 1 receptor (AT1- AA), which all culminating in the development of hypertension during pregnancy. Immunologic irregularities, like those seen in organ rejection graft versus host disease, have been demonstrated in preeclamptic women (Gleicher, 2007).

Sargent, Borzychowski, and Redman, (2006) proposed that pre-eclampsia syndrome is a condition which involves two stages. According to them, the first stage is a poor incursion by trophoblasts as a result of an insufficient manifestation of HLA-G resulting to a diminished stimulation of decidual NK cells which partly is involved in the assembly of immunoregulatory cytokines and angiogenic factors.

Trophoblast cells express an uncommon collection of histocompatibility antigens, including human leucocyte C, E and G class antigens (HLA-C, HLA-E, HLA-G), of which just HLA-C shows marked polymorphism (Chelbi and Vaiman, 2008). Interaction between natural killer (NK) cells and extravillous trophoblast (EVT) cells have been proposed to regulate placental implantation where NK cells that express an assortment of receptors such as CD94, KIR, and ILT known to perceive class I molecules penetrate the maternal decidua in close association with EVT cells (Loke and King, 2000).

Generally, NK cells, macrophages, and dendritic cells are mediators of innate immunity during which macrophages and dendritic cells are the foremost antigen-presenting cells within the uterus that enhance the adaptation of the immunologic response to the void of denial of the growing embryo (Young *et al*., 2010). However, alteration in killer immunoglobulin receptors (KIR) interaction on uterine natural killer (uNK) cells with HLA-C on interstitial trophoblast modifies the decidual immune reaction, leading to damaged extravillous trophoblast invasion and inadequate spiral artery remodeling, related to pre-eclampsia (Williams and Broughton, 2011). Patients with pre-eclampsia have high levels of agonistic antibodies to the angiotensin AT-1 receptor which can prepare intracellular free calcium and may represent augmented plasminogen activator 1 assembly and shallow trophoblast intrusion found in pre-eclampsia (Dechend et al., 2004; Thway *et al*., 2004). Laresgoiti-Servitje, Gomez-Lopez, and Olson, (2010) indicated that the role of immune system in developing pre-eclampsia involves more than NK cells and HLA-G, as dendritic cells (DCs), T $CD4^+$ subpopulations, $CD8^+$ T cells, adhesion molecules and cytokines additionally are involved.

Endothelial dysfunction

Endothelial dysfunction is likewise an immunological factor that shows the improved arrangement of variables, for example, endothelin, reactive oxygen species (ROS) and expanded vascular sensitivity to angiotensin II (LaMarca, 2012).

Studies have shown that renal blood vessel during pregnancy increases in diameter, and this vasodilatory reaction leads to improved renal plasma drift and glomerular filtration rate (GFR) indicating that maternal systemic

circulation undergoes significant changes during pregnancy (Monica, Ravi, and Karumanchi, 2015), and maternal vascular endothelium seems to be a crucial target of factors stimulated throughout pre-eclampsia (LaMarca, 2012). According to Hod, Cerdeira and Karumanchi, (2015) data from experimental, epidemiological, and restorative investigations from a few research centers have given convincing confirmation that proposes that modifications in circulating angiogenic factors assume a pathogenic role in pre-eclampsia development.

Platelet-derived growth factor, cellular fibronectin, endothelin, von Willebrand antigen, soluble tissue factor, soluble E-selectin, soluble vascular adhesion molecule, endothelial leukocyte adhesion molecule-1, and cytokines like TNF-α, IL-8, IL-6 are particular serum indicators of endothelial stimulation and endothelial dysfunction that have been noted to disturb routine activities in women with pre-eclampsia. Host molecules like the soluble VEGF receptor-1, soluble endoglin, the angiotensin II type-1 receptor autoantibody, play a part in the impairment of the maternal vascular endothelium. Hypoxia stimulated catechol-O-methyltransferase (COMT) or 2-methoxyoestradiol (2-ME) and/or HO-1 insufficiency and high immune cells and molecules have also been described in endothelial dysfunction (LaMarca, 2012; Young *et al*., 2010).

Role of inflammatory cytokines

The maternal disorder in pre-eclampsia is fundamentally that of generalized dysfunction of the maternal endothelium, and this summed up endothelial dysfunction seems to be a part of an excessive systemic inflammatory reaction that includes maternal leukocytes and pro-inflammatory

cytokines (Raghupathy, 2013). The second stage of pre-eclampsia is described by a systemic inflammatory reaction comprising leukocytes and endothelium which seems to begin in the placenta (Sargent, Borzychowski, and Redman, 2006). Harmon *et al*., (2016) noted that pre-eclampsia is related to chronic immune stimulation that yields to a high assembly of inflammatory cytokines by pro-inflammatory T cells, and a lower regulatory and anti-inflammatory cytokines, which additionally advances to the inflammatory state during pre-eclampsia.

Extreme discharge of tumour necrosis factor alpha (TNFα) has been embroiled attributable to its commitment to endothelial activation, which thus could play a part in maternal symptoms (LaMarca, *et al*., 2007). IL-10 has additionally been ensnared in the pathologic process of pre-eclampsia by enhancing the inflammatory reaction towards trophoblast cells leading to lessened invasion and redesigning of the spiral arteries (Williams and Broughton, 2011).

Williams and Broughton, (2011) further indicated that two other inflammatory genes, IL-1α and the interleukin 1 receptor antagonist (IL1Ra) have been identified to have an association with pre-eclampsia development. According to Gathiram and Moodley, (2016), it was shown in a study that serum levels of circulating cytokines, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-18, INF-γ, TNF-α and chemokine interferon-γ-inducible protein (IP-10), monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) were raised in pre-eclampsia compared to controls. TNF-α, IL-6, and IL-17 which are pro-inflammatory in nature and are usually secreted from activated Th1

and Th17 cells during immunological challenges promote cytotoxic and inflammatory responses (Harmon *et al*., 2016).

It is believed that in pre-eclampsia syndrome compared to a usual pregnancy, there is a swing to Th-1 type from the Th-2 type of immunity. Thus, the generalized inflammation in pre-eclampsia is the preponderance of Th1-type immunity (Ramma and Ahmed, 2011). The predominance of Th1 immunity is not only related to poor placentation but also to the exaggerated inflammatory response and endothelial dysfunction seen in pre-eclampsia (Saito and Sakai, 2003).

High level of chemokines and molecules equipped for engaging macrophages and dendritic cells; have been demonstrated in preeclamptic placentas. The increase in chemokines, macrophages, and dendritic cells in placentas influenced by pre-eclampsia underpins the thought that a provocative milieu present both in the principal trimester and at the period of clinical presentation of pre-eclampsia may enhance immune maladaptation, resulting to damaged trophoblast incursion at the level of the spiral arteries (Young et al., 2010). T regs and regulatory cytokines ensure the proper control and function of pro-inflammatory cells and their actions to ensure proper invasion. However, placental ischemia, resulting from the insufficient trophoblast invasion seen in pre-eclampsia, has been shown to produce an imbalance in immune function that leads to chronic inflammation and presents similarly to an autoimmune disease.

2.2.3 Genetic Factors

Genetic factors are believed to play a part in pre-eclampsia development (Skjaerven *et al*., 2005). Young *et al*., (2010) indicated that most

cases of pre-eclampsia ensue in nulliparous women devoid of a family history of the condition. It has likewise been accounted for that ladies with first degree relations with pre-eclampsia have five times more danger of developing the condition, while those with second-degree relations have their risk doubled (Valenzuela *et al*., 2012). It is believed that paternal genes additionally assume a significant role in the advancement of pre-eclampsia, following evidence suggesting an increased danger of pre-eclampsia development in ladies with pregnancies to men who have already been associated with pregnancies entangled with pre-eclampsia (Haig, 1996). Change in paternity from a past pregnancy, prolonged interpregnancy interim, utilization of boundary contraception, and conception by intracytoplasmic sperm infusion, involve the restricted introduction of paternal antigen as an inclining factor to pre-eclampsia (Camille, Levine and Karumanchi, 2011). Thus, while pre-eclampsia is often considered to be a disease of the first pregnancy, the risk increases when the women had limited sperm exposure with their partner, suggesting a maternal adaptation to immunological sperm parameters (Chelbi and Vaiman, 2008).

The contention among maternal and paternal genes is likewise thought to initiate the irregular placental implantation through expanded NK cell actions. Six genes with significant maternal-fetal genotype interaction including insulin-like growth factor 1 (IGF1), interleukin 4 receptor (IL4R), insulin-like growth factor 2 receptor (IGF2R), G protein subunit beta 3 (GNB3), colony stimulating factor 1 (CSF1), and thrombospondin 4 (THBS4) have been indicated to be related to pre-eclampsia (Valenzuela *et al*., 2012).

Storkhead box 1 (STOX1), a new transcription factor gene, has demonstrated conflicting relationship with pathophysiological cascade leading to the onset of pre-eclampsia (Chelbi and Vaiman, 2008). In a study of sisters with pre-eclampsia, it was demonstrated that the mother developed pre-eclampsia only when the fetus/placenta inherited a maternal STOX1 missense mutation on 10q22; when the fetus/placenta carried the imprinted paternal homolog, the pre-eclampsia phenotype was not expressed (Karumanchi *et al*., 2011). Genetic studies looking at polymorphisms in killer immunoglobulin receptors on maternal NK cells and the fetal HLA-C haplotype propose that ladies with KIR– AA genotype and fetal HLA-C2 genotype were enormously at risk of pre-eclampsia condition (Sargent, Borzychowski, and Redman, 2006a).

2.2.4 Diet

Though the etiology of pre-eclampsia remains vague, evidence suggests that diet may serve as a contributing factor since hypertensive disorders of pregnancy are characterized by metabolic instabilities like those seen in cardiovascular diseases and type 2 diabetes mellitus as well as endothelial dysfunction, inflammation, oxidative stress, insulin resistance and dyslipidemia (Schoenaker, Soedamah-Muthu, and Mishra, 2014). Several interventions have been demonstrated and assessed in the prevention of pre-eclampsia with respect to diet.

Brantsaeter *et al*., (2011) showed that many epidemiologic investigations have revealed that, eating of vegetables, fruits and dietary fiber is associated with lower pre-eclampsia development. In their observational examination, they showed an autonomous protective relationship between consumption of probiotic milk items and pre-eclampsia, particularly serious

pre-eclampsia, suggesting that probiotics may particularly target and adjust the kind of inflammation underpinning extreme pre-eclampsia.

An earlier study indicated that ladies who develop pre-eclampsia have been found to have lower biochemical markers of n-3 unsaturated fat consumption (Williams, Zingheim, King, and Zebelman, 1995). Another study also suggested that eating high doses of n-3 long-chain polyunsaturated fatty acids (LCPUFA) in early pregnancy, or other food nutrients seen in cod-liver oil could increase the risk of developing hypertensive conditions in pregnancy (Olafsdottir *et al*., 2006).

The usage of antiplatelet agents and calcium supplementation has been connected to a 17% and 55% decline in the risk of pre-eclampsia respectively (Duley, Henderson-Smart, Meher, and King, 2007; Hofmeyr, Lawrie, Atallah, and Duley, 2010). However, a study conducted by Oken *et al*., (2007) did not support the idea that maternal consumption of calcium or vitamin C and vitamin E throughout gestation can avert hypertensive disorders of gestation, in spite of the fact that they do suggest a potential advantage for extended n-3 fatty acids in averting pre-eclampsia. Dodd, Brien, and Grivell, (2014) in their review also showed that ladies with lower dietary calcium consumption were more probable to be diagnosed with gestational hypertension. It was further demonstrated that there was no critical relationship between the dietary element of vitamins C and E, vitamin D and n-3 polyunsaturated fats with pre-eclampsia. Meanwhile, Achkar *et al*., (2015) indicated that maternal vitamin D deficiency early in gestation may be an independent cause for pre-eclampsia.

In a prospective study, Schoenaker *et al*., (2014) also demonstrated no evidence that consumption of calcium, folate, or antioxidant vitamins

decreases the risk for pre-eclampsia or gestational hypertension, or that consumption of n-6 or trans fatty acids increases risk.

2.3 Biomarkers of Pre-eclampsia

Huppertz, (2015) noted that depending on the causative routes resulting to the progress of pre-eclampsia syndrome, markers may or may not have a prognostic value and mother or child may show long-term effects later in life. Evidence suggests that alterations in circulating levels of regulators of angiogenesis cause several clinically significant symptoms of pre-eclampsia (Pennington *et al*., 2012).

Angiogenesis, the development of a new blood vessel from existing vessels, is controlled by angiogenic factors. These angiogenic elements are vital for the maintenance of the typical vessel. However, studies show that modifications in circulating angiogenic elements contribute to pre-eclampsia development (Hod, Cerdeira, and Karumanchi, 2015). Hod, Cerdeira, and Karumanchi, (2015) further indicated that high levels of the antiangiogenic elements, soluble forms-like tyrosine kinase 1 (sFlt-1) and soluble Endoglin (sEng) trap circulating VEGF, placental growth factor (PlGF) and transforming growth factor b (TGFb) correspondingly, reducing their free levels, resulting to endothelial dysfunction and the clinical manifestations of the condition.

Levine *et al*., (2006) demonstrated a significant mean sFlt-1 value of 3 times higher in ladies with pre-eclampsia condition than in usual gestation. Ladies having a subsequent pre-eclampsia had 2 times higher concentrations of sFlt-1 at sixteen to twenty weeks than controls (Wathén *et al*., 2006). Hence, quantifying angiogenic elements utilizing automated assays of sFlt-1

and PlGF has shown to be clinically important for the routine diagnosis of pre-eclampsia syndrome (Knudsen *et al*., 2012).

Five proteins have been identified to be hub molecules, including ubiquitin B (UBB), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1), microtubule-associated protein family member 1 (MAPRE1), VEGFA, and integrin subunit beta 1 (ITGB1) of which PIK3R1, VEGFA, and ITGB1 are identified to be linked with pre-eclampsia syndrome (Jiang *et al*., 2015).

Placental ischemia and hypoxia also cause discharge of trophoblast microparticles into the maternal circulation which stimulates high proinflammatory cytokines and the activation of maternal endothelial cells (Raghupathy, 2013). The impairment of placental vascularization leads a reduction of blood flow in the intervillous spaces, which gradually results into the chronic hypoxia (Gueneuc, Deloron, and Bertin, 2017).

Fetal hemoglobin has now been characterized as a new prognostic biomarker for pre-eclampsia and has also been hypothesized to be a causal element (Huppertz, 2015).

Excretion of lysosomal enzymes is well-thought-out to be a probable diagnostic marker in pre-eclampsia as pre-eclampsia patients have proximal tubule epithelial injury which leads to the release of lysosomal enzymes (Jiang *et al*., 2015). The multifaceted interaction between factors released from the placenta and the reaction of the maternal vascular system lastly defines whether or not a pregnant mother advances into pre-eclampsia.

2.4 Malaria Disease

Malaria remains one amongst the foremost serious infectious diseases that threaten almost half the world's population and led to many thousands of deaths in 2015, preponderantly among youngsters in African continent (Phillips *et al*., 2017). Pregnant mothers and non-immune individuals mostly die as victims of cerebral manifestations and anemia and is responsible for a huge quantity of spontaneous abortions, premature deliveries, and low birth weight (Asma, Taufiq, and Khan, 2014).

Malaria occurs in humans by 5 Apicomplexans of single-celled eukaryotic Plasmodium parasites (Phillips *et al*., 2017). These include *P. falciparum, P. malariae, P. vivax, P. ovale and P. knowlesi* that is spread by the bite of Anopheles spp. *Plasmodium falciparum* is responsible for the most fatal form of malaria in humans with it virulence triggered by many elements like parasite proteins on the surface of infected red blood cells (Wassmer and Grau, 2017) with serious life-threatening problems like cerebral malaria, severe anemia, respiratory distress, jaundice, acute renal failure, etc. (Das, 2008). Serious falciparum malaria involves a broad spectrum of diseases including complications that involve particular organs like the brain in cerebral malaria and the placenta during malaria in pregnancy.

Storm and Craig, (2014) noted that severe malaria is defined by WHO as severe anemia, respiratory distress (or acidosis) or coma, or a combination of these. Mohanty *et al*., (2015) indicated that the occurrence of falciparum malaria is influenced by age, exposure and immune status. Fever is the basic symptom, though no set of symptoms reliably differentiate malaria from other causes of fever (Basu and Sahi, 2017). Along with high fever; a headache,

nausea, fatigue, general body aches and weakness, chills, and flu-like symptoms can also be seen. The use of long-lasting insecticidal bed nets, anti-malaria drugs use during pregnancy; and treatment using anti-malaria drugs such as the artemisinin combination therapy (ACT) are some of the interventions included in malaria control programmes (K'Oyugi, 2015; Sharma and Dutta, 2011).

2.5 Pathogenesis of Malaria

Plasmodium spp. are worldwide pathogens with a mind-boggling life cycle interchanging between female *Anopheles* mosquitoes and vertebrate that require the development of distinctive zoite structures to attack diverse cell types at particular stages (Cowman, Healer, Marapana, and Marsh, 2016). *Plasmodium* sporozoites are injected into host dermis during blood meal by *Anopheles* mosquitoes. While in the host, they infect hepatocytes, which are followed by the asexual cycle in the blood.

During the asexual phase, the ring stages advance into replicative schizont that releases multiple aggressive daughter merozoites. In each replication cycle, a small portion of asexual parasites progresses into male and female gametocytes which are the lone stages that are transmissible (Meibalan and Marti, 2017) by a feeding *Anopheles* mosquito, finalizing the cycle. The pathology of malaria and associated clinical manifestations are primarily ascribed to the erythrocytic phase (Meibalan and Marti, 2017). The ultimate features of *P. falciparum* infection that account for the pathogenesis of serious illness and death are exponential parasite growth, stimulation of inflammatory reaction, and vascular obstruction owing to adherence of parasites to vessels (Cowman *et al.*, 2016). The intracellular parasites transform the red blood

cells in many ways leading to hypoglycemia by changing glucose into lactic acid through anaerobic glycolysis allowing cell membranes to become less deformable, resulting in hemolysis and anemia. (Asma *et al*., 2014; Brattig *et al*., 2008).

Acute renal failure is frequently seen in *P. falciparum* infection, however, *P. vivax* and *P. malariae* can also cause renal damage (Das, 2008). According to Zaki *et al*., (2013) many hypotheses such as mechanical obstruction caused by cytoadherence and sequestration of infected red blood cells, immune-mediated glomerular pathology, induction of cytokines, and reactive oxygen intermediates by activated mononuclear cells, and alterations in the renal and systemic hemodynamics have been proposed as the mechanisms for renal failure in *falciparum* malaria. Acute respiratory distress (ARD) arises when parasites obtain energy from anaerobic glycolysis producing lactic acid, contributing to clinical manifestations of hypoglycemia and lactic acidosis. This leads to respiratory distress in a few severely infected individuals (Asma *et al*., 2014).

The principal pathophysiological processes in the development of cerebral malaria comprise of an imbalance of pro- and anti-inflammatory reaction to *Plasmodium* infection, endothelial cell activation, as well as damage to or loss of blood-brain barrier integrity (Dunst, Kamena, and Matuschewski, 2017). Lennartz *et al*., (2017) noted that the idea of vascular obstruction that leads to cerebral malaria is based on the fact that the ability of mature infected red blood cells to sequester in the vasculature by binding of *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) on the erythrocyte

surface to endothelial cell surface proteins like ICAM-1, VCAM-1, cluster of differentiation 36 (CD36), or endothelial protein C receptor (EPCR).

Malaria neurological disorder demonstrates as impaired consciousness and convulsions which occasionally results into a coma (Idro, Marsh, John, and Newton, 2010). Jaundice, one of the severe life-threatening complications of malaria appears to be the result of the collective effects of hemolysis, hyperbilirubinemia, and liver impairment (Zaki *et al*., 2013).

2.6 Placental Malaria

Placental malaria infection is one of the most important options of protozoan infection throughout gestation and has been broadly utilized as a standard marker to describe protozoan infection in epidemiological investigations (Uneke, 2007). The condition is associated with the placenta, mostly occurring in first-time pregnancies and has adverse pathophysiologic effects resulting in poor pregnancy outcomes.

Generally, sequestration of infected red blood cells in the intervillous space leads to secretion of chemokines resulting in inflammatory cells recruitment and cytokine production which is associated with poor pregnancy outcomes (Rogerson *et al*., 2003; Suguitan *et al*., 2003). Malaria associated maternal ailment and low birth weight is usually the result of *P. falciparum* infection which occurs mostly in regions of stable transmission in Africa (WHO, 2017). During placental malaria, parasites collect in the placenta with an increased risk of low birth weight (Sarr *et al*., 2010). Anemia, preterm delivery, and fetal growth restriction are also the major contributing factors of malaria associated low birth weight babies. Therefore, malaria throughout

gestation is an essential public health problem with significant risks for the pregnant mother and the newborn.

Placental malaria can be categorized as acute, chronic, or previous infection. While chronic placental malaria has been linked a high risk of fetal development restriction, acute placental malaria has been associated with preterm delivery in malaria-endemic areas (Menendez *et al*., 2000). Placental malaria changes the environment in the intervillous space of the placenta. This occurs as a result of *P. falciparum*-infected erythrocytes binding to syncytiotrophoblast, a continuous, multinucleated, specialized epithelial layer that covers the interior of the villous of the placenta. *P. falciparum*-infected erythrocytes specifically bind on syncytiotrophoblast receptors known as chondroitin sulphate A (CSA) and hyaluronic acid (Beeson, Rogerson, and Brown, 2002; Gamain *et al*., 2005). Sequestration of infected red blood cells in the intervillous spaces of the placenta is as a result of binding of infected red blood cells to syncytiotrophoblast. The ability of infected erythrocytes to bind on CSA is conferred by the parasite's variant surface antigen belonging to var. gene subfamily VAR2 that is manifested on the surface of infected red blood cells called VAR2CSA (Kidima, 2015).

Studies have also shown high levels of monocytes and macrophages in the intervillous space of the placenta during active placental malaria (Rogerson *et al*., 2003). Earlier studies by Fried, Muga, Misore, and Duffy (1998) reported elevated levels of T-helper-1 cytokines in the placental plasma of placental malaria-positive women such as TNF-α and IFN- γ).

Pregnancy-associated malaria shares several characteristics with pre-eclampsia, both being more frequent in primigravidae and several biomarkers

linked with placental dysfunction have also been recognized as associated with pre-eclampsia (Gueneuc *et al*., 2017). Many studies suggest that inflammatory response and an unusual secretion of secondary cytokines throughout placental malaria might be origins of pre-eclampsia and eclampsia (Muehlenbachs, *et al*., 2006; Ndao *et al*., 2009).

Clinically, reduction in placental perfusion is a shared association between these pathologies that are linked with fetal development restriction (Mol *et al*., 2016). Placental protein 13 (PP13) is high in pre-eclampsia syndrome (Burger et al., 2004), whiles pregnancy-associated plasma protein A (PAPPA) produced by the placenta is reduced.

2.7 Co-Morbidity of Pre-eclampsia and Placenta Malaria

Similarities between pregnancy-associated malaria and pre-eclampsia syndrome are remarkable and may lead to crucial biomarkers in the framework of pregnancy-associated malaria as pre-eclampsia probable markers (Gueneuc *et al*., 2017). Certainly, many markers are high in both pathologies, such as TNF, IFN-γ, ICAM-1, IL10, and IL6. Other elements like nitric oxide synthase, s-endoglin, or TGF-ß soluble receptor are linked to pre-eclampsia, as well as severe malaria in youngsters, not leaving infected primigravidae (Silver *et al.*, 2011). Inhibin A, complicated in gonadotropin repression, is also high in pre-eclampsia. The level of visfatin, a placental element engaged in glucose homeostasis, is reduced in maternal blood (Kar, 2014). Some enzymes engaged in the metabolism of arachidonic acid have also been reported to be involved in pathology during pregnancy as they are expressed in the placenta.

Arachidonic acid is a key unsaturated carboxylic acid that is the precursor for a good type of lipid mediators that are engaged with several physiological and pathophysiological forms in human (Powell and Rokach, 2015). It is derived from a phospholipid molecule by the enzyme phospholipase A2 but can also be made from diacylglycerol (DAG) by diacylglycerol lipase (Baynes and Marek, 2005). This arachidonic acid is metabolized to eicosanoids using several pathways depending on enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 monooxygenase and epoxygenase (Figure 2).

Cyclooxygenase (COX) work in one division of the arachidonic acid metabolic pathway to yield prostaglandins (PGs) and thromboxanes (TXs), while the activities of lipoxygenase and its related super molecules add the opposite arm of the metabolic pathway to create leukotrienes (Lutz and Cornett, 2013; Sakai *et al*., 2012).

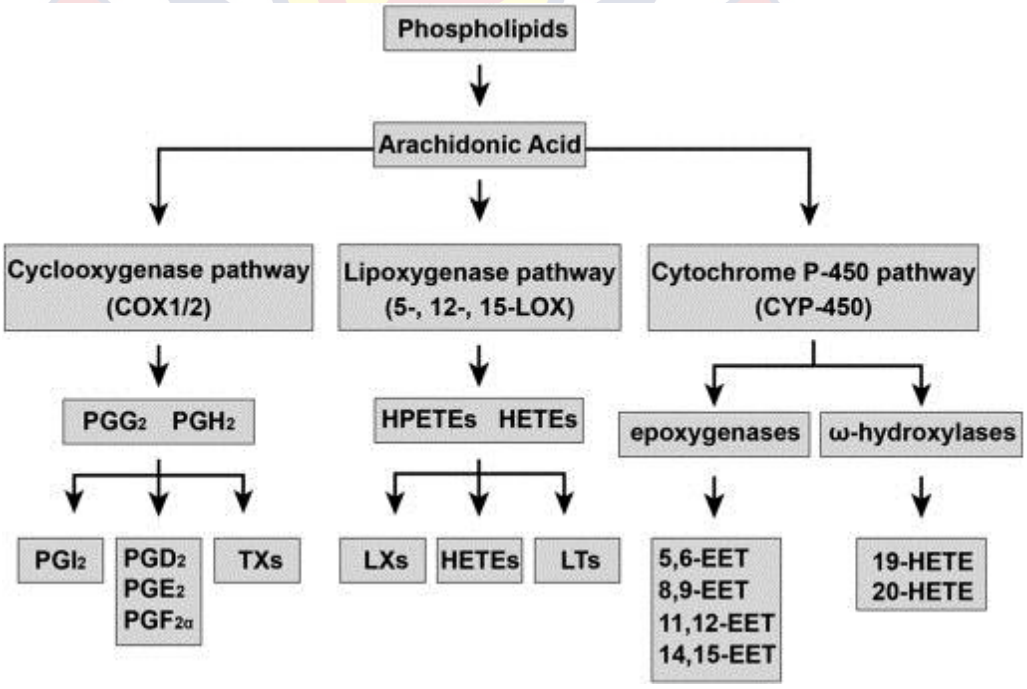

Figure 2: Schematic Pathway of Arachidonic Acid Metabolism (Yuan et al., 2014)

Akarasereenont *et al.* (1999) reported COX 2 over-expression during pre-eclampsia in 1999. However, Khan *et al.* (1999) described a reduction of COX 2 in preeclamptic placentas in the same year. Interestingly, a recent study (Sarr *et al*., 2010) has shown that COX 2 mRNA expression in placenta malaria decreases with parity. Pre-eclampsia is also noted to occur mostly in first-time pregnancies. Sarr *et al*., (2010) further indicated that increase in COX 2 is associated with anaemia in pregnant women while the increase in COX 1 and 15 LOX transcripts is associated with low birth weight in placental malaria. Ordi *et al*., (2001) has previously reported that long-term active malaria infection of the placenta has seen COX 2 over-expression.

CHAPTER THREE

RESEARCH METHODS

This study sought to assess whether the association between pre-eclampsia and placental malaria is through induction of COX-1, COX-2, and 15-LOX by malaria infection. The levels of COX-1, COX-2, and 15-LOX mRNA gene expressions were evaluated and compared in women diagnosed with or without pre-eclampsia that are infected or uninfected by *P. falciparum*. This chapter describes the study area and population followed by study design, a method of sampling, ethical consideration, procedure and the statistical analysis.

3.0 Study Design

This study is a case-control study conducted at the Department of Obstetrics and Gynaecology of the KBTH and NMIMR where pregnant mothers diagnosed with or without pre-eclampsia condition (cases and controls respectively) were identified, consented and enrolled based on a set of inclusion and exclusion criteria.

3.1 Study Area and Population

The study was conducted at the Department of Obstetrics and Gynaecology of the Korle-Bu Teaching Hospital (KBTH) and NMIMR. All samples were collected at KBTH and transported to NMIMR for laboratory work. The Department of Obstetrics and Gynaecology is the largest department in the facility and referral centre for obstetric complications with a total number of deliveries of about 12 000 per year.

KBTH is the first medical centre in Ghana that was established on 9th October 1923. To the southern part of Ghana, it is the only tertiary hospital and is presently the third biggest health facility in Africa and the foremost nationwide referral center in Ghana. KBTH is affiliated with the School of Medical Sciences, the University of Ghana with three centers of excellence; the National Cardiothoracic Centre, the National Plastic and Reconstructive Surgery and the Radiotherapy Centers. With infrastructural development, the teaching hospital has got new buildings like the Maternity, Surgical, Children Blocks and State of the Art laboratories. KBTH is precisely situated in the Accra Metropolitan District of Greater Accra Region, Ghana.

Currently, Accra Metropolitan District is among one of the 10 districts that make up the Accra Metropolitan Area (Ghana Statistical Service, 2014). The Accra Metropolitan district is bounded to the north by the Ayawaso East and Okaikoi Municipal districts, to the west by the Ablekuma East Municipal district, to the east by La Dade Kotopon Municipal district, and to the south by the Gulf of Guinea (AMA, 2017). Also, the report from AMA (2017) indicated that there were about one million, six hundred and sixty five thousand and eighty-six persons leaving in the district during the 2010 census. As of March 2017, it spanned an area of approximately 6.56 km^2 (2.53 sq mi) and encompassed the Ashiedu Keteke and Osu Klottey sub-metropolitan district councils with 72 communities and 76 Electoral Areas. 38.4% of the population was under the age of 19, 12.4% between 20 and 24, 11.5% between 25 and 29, 21.5% between 30 and 44, 12.2% between 45 and 64, and 4% aged 65 and over (AMA, 2017).

The total number of families in the locality remained at 501,903 family units, out of which 450,794 families lived in the 149,789 houses inside the area. The average family unit measure in the area is 3.7 people for each family unit and the populace per house is evaluated at 11.1, demonstrating that compound houses are the most widely recognized sort of dwelling place (67.7%) in the district (Ghana Statistical Service, 2014).

3.2 Sampling

Purposive and convenience sampling was used in the data collection. Considering 95% confidence level, with an estimated odds ratio of 3 for malaria risk among patients with pre-eclampsia, power of 80% to study the effect of placental malaria in the development of pre-eclampsia, 1:1 ratio of controls to cases, hypothetical quantity of controls with exposure of forty and a hypothetical quantity of cases with exposure of about sixty-seven, a minimum sample size of 110 was estimated using the Kelsey method of OpenEpi Software. A total of 141 pregnant women between 18 – 45 years, of gestational age ≥ 20 weeks visiting the study site for medical services were enrolled.

Case Definition

Clinical Pre-eclampsia:

Pregnant woman with history of sustained hypertension (blood pressure ≥140mmHg systolic or ≥ 90mmHg diastolic at least 4 hours apart), with proteinuria (≥300 mg of total protein measured in a 24-h urine collection or two readings of 1+ or higher on urinalysis) ≥ 20 weeks of gestation (Tranquilli *et al*., 2014) as diagnosed by an obstetrician.

Control Definition

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

Exposure

Placental Malaria:

Defined as the presence of parasites in the placental blood and/or parasite pigment deposition in tissue after delivery according to (Bulmer *et al*., 1993).

3.3 Inclusion and Exclusion Criteria

Pregnant women who fit into either the case or control definition were included if they clearly understood the study and voluntarily decided to join after signing an informed consent form. Patients with a history of chronic hypertension, pre-gestational kidney disease, diabetes, cardiovascular disease, thyroid dysfunction, and evidence of concomitant infection at the time of enrolment as confirmed by a clinician/midwife and medical records were excluded from the study. Patients who were unable to give informed consent or unwilling to comply with the requirements of the protocol were also excluded from the study.

3.4 Ethical Consideration

Ethical approval was sought from the NMIMR Institutional Review Board (IRB). Approval was also sought from the KBTH-IRB (a collaborating institution). Placental tissues obtained were given unique identities. This made

patients anonymous so that apart from the investigator the identities could not be traced to the patients concerned.

3.5 Placental Tissue Collection and Nucleic Acid (RNA and DNA) Extraction

Placental tissues were collected at delivery between January and December 2017 after explaining the nature and purpose, potential benefits, and possible risks associated with participating in the research and agreed by participants. This began just after the research was reviewed and approved by the NMIMR-IRB and KBTH-IRB.

The tissues were formalin fixed and paraffin embedded (FFPE) immediately after collection. RNAs were extracted and purified from sections of FFPE tissues following strictly the protocol (Protocol II and III of Appendix A), using RNeasy FFPE kit (Qiagen Sample and Assay Technologies). RNA concentrations were assessed using the Nanodrop One (Thermo Fisher Scientific).

DNAs were also extracted from the same FFPE tissues after sectioning following strictly the protocol (Protocol I of Appendix A), using QIAamp DNA FFPE Tissue Kit (Qiagen Sample and Assay Technologies). DNA concentrations were also assessed using the Nanodrop One (Thermo Fisher Scientific).

3.6 RT-PCR and mRNA Quantification

A volume of 10µL RNAs extracted was reversed transcribed using High-Capacity cDNA transcription kit and the RT2 PreAMP cDNA synthesis kit from Applied Biosystems and Qiagen Sample and Assay Technologies

respectively following strictly the protocol (Protocol IV and VI of Appendix A). Amplification was performed in a Quantstudio 5 system (Thermo Fisher Scientific) using Luna universal probe qPCR master mix (New England Biolabs) containing primers and fluorogenic probes following a detailed protocol (Protocol VII of Appendix A). The sequence of primers and probes used for this study was obtained from a study conducted by Sarr *et al*., (2010). However, slight modifications were made at the 3' end of the sequence of the probes to increase their wave lengh (Table 7 and 8 of Appendix B).

All measurements were performed in duplicate together with Peptidyl Propyl Isomerase A (PPIA) and Beta-Actin (ACTB) on the same plate. PPIA was selected for standardization.

Quantification of the gene was carried out according to (Livak and Schmittgen, 2001). For every measurement of the gene, the mean Ct-values of the duplicates from results were recorded. The results were adjusted using the level of PPIA in the same sample. For each gene, the relative quantity (RQ) of mRNA obtained was expressed in arbitrary units as a fold increase in comparison with the control group.

The method of fixing the tissue sections and inserting them in paraffin usually results to some level of RNA degradation and chemical alteration. Also, the nucleic acid is cross-linked with a macromolecule throughout the formalin fixation method, and most of the RNA secluded from FFPE tissues is extremely degraded and decreased to a far lower yield than that of RNA secluded from a similar quantity of contemporary tissues (Guo *et al*., 2016). Hence, there was the struggle of estimating gene expression levels in FFPE samples.

3.7 Molecular Detection of Plasmodium Falciparum (Nested PCR)

Molecular detection (Nested PCR) of *P. falciparum* was conducted after DNA was extracted from the FFPE tissue samples. Amplification was performed in a Master cycler nexus gradient (Eppendorf) using OneTaq Universal PCR Master Mix (New England Biolabs). Nest 1 and Nest 2 reaction reagents and primers with their concentrations indicating the cycling conditions of the reactions are highlighted (Table 5 and 6 of Appendix A). Specifications and sequence of primers for *P. falciparum* are also shown (Table 9 of Appendix B). The integrity of *P. falciparum* DNA obtained was assessed on an ethidium bromide stained denaturing agarose gel. Gel images were obtained indicating *P. falciparum* positives and negatives (Figure 6 and 7 of Appendix C).

3.8 Statistical Analysis

Data generated from the laboratory were presented in summary tables and subjected to statistical analyses. Descriptive statistics were performed and tables generated using SPSS 21(SPSS Inc, Chicago, IL, USA). Graphs were generated using GraphPad Prism 6.01. Difference between cases and control groups were compared using the Mann-Whitney U test. The difference between preeclamptic women with placental malaria was also compared to women without placental malaria and pre-eclampsia using Mann-Whitney U test in using GraphPad prism. The alpha value was set at 0.05.

CHAPTER FOUR

RESULTS AND DISCUSSION

This study sought to assess whether the association between pre-eclampsia and placental malaria is through induction of either COX-1, COX-2, or 15-LOX by malaria infection. The levels of COX-1, COX-2, and 15-LOX mRNA gene expressions were evaluated and compared in women diagnosed with or without pre-eclampsia that are infected or uninfected by *P. falciparum.* Results obtained from this study are shown clearly followed by discussions in this chapter.

4.0 Results

The odds of developing pre-eclampsia during *P. falciparum* infection

During the study, a total of 141 pregnant women were recruited. Their demographic data are summarized accordingly (Table 1). However, due to incomplete data from some of the participants, 134 pregnant women were examined from which the odds of developing pre-eclampsia were determined. All *P. falciparum* positive results obtained from the placenta tissue after performing PCR were considered as women with placental malaria.

Using a two by two cross tabulation between placental malaria and pre-eclampsia the number and percentages of women with placental malaria developing pre-eclampsia were determined. Women without placental malaria developing pre-eclampsia was also indicated (Table 2).

To ascertain whether there is an association between placental malaria and pre-eclampsia development for this case-control study, the odds of developing pre-eclampsia was determined (Table 2).

Table 1: Demographic data of study participants

Variable	Pre-eclampsia	Without Pre-eclampsia	p-value
Gravidity			0.002
No. of Women	71	70	
Primigravidae n(%)	34(47.9%)	16(22.9%)	
Multigravidae n(%)	37 (52.1%)	54(77.1%)	
Parity			0.076
Primiparous n(%)	39(54.9%)	28(40.0%)	
Multiparous n(%)	32(45.1%)	42(60.0%)	
Sickling status			0.976
Positive n(%)	7(10.6%)	7(10.8%)	
Negative n(%)	59(89.4%)	58(89.2%)	
Placental malaria			0.0392
No. of Women	69	65	
Positive n(%)	38(61.3%)	24(38.7%)	
Negative n(%)	31(43.1%)	41(56.9%)	
Age (yrs)	27(18-42)	28.5(18-41)	0.391
Gestational age at first visit (weeks)	16.5(5-36)	17 (7-41)	0.552

All analysis was done at alpha value of 0.5. Continuous variables were presented as median (range) and compared using Mann-Whitney U test. Categorical variables were presented as counts (%) and compared using chi-square analysis.

Summary of the risk estimation of developing pre-eclampsia to ascertain whether there is indeed an association between placental malaria and pre-eclampsia development. The odds of developing pre-eclampsia was 2.094 (P = 0.0392), which is significantly greater in women with placental malaria (P. *falciparum* positive) compared to women without placental malaria (*P. falciparum* negative) (Table 2).

Table 2: Placental Malaria * Pre-eclampsia Crosstabulation

Placental Malaria	Pre-eclampsia n (%)		Odds Ratio (CI at 95%)	P-Value
	Pre-eclampsia	Without Pre-eclampsia		
P. falciparum positive	38 (61.3%)	24 (38.7%)	2.094 (1.048-4.183)	0.0392
P. falciparum negative	31 (43.1%)	41 (56.9%)	1.424 (1.022-1.982)	0.0675
Total	69 (51.5%)	65 (48.5%)		

All P. falciparum positives in placenta tissue represent women with placental malaria while those without placental malaria were P. falciparum negatives.

Level of expression of COX-1, COX-2 and 15-LOX genes among the study population

A summary of the clinical and laboratory features of the study population used for the quantification of gene expression is outlined (Table 3). For the purpose of the gene expression study, 50 pregnant women were randomly selected from the 134; 25 cases (Pre-eclampsia) and 25 controls (without pre-eclampsia) from which their placental malaria status was also determined. Out of the 25 cases 8, 14, and 10 were positive for COX-1, COX-2 and 15-LOX representing 32%, 56% and 40% respectively, and 8, 13, and 16 were positive for COX-1, COX-2 and 15-LOX representing 32%, 52% and 64% respectively for the controls (Table 4).

Expression levels of COX-1, COX-2, and 15-LOX were compared between pre-eclampsia women with placental malaria and women without pre-eclampsia but with placental malaria.

The expression levels of COX-1, COX-2 and 15-LOX again were compared between women with placental malaria without pre-eclampsia and women without placental malaria and pre-eclampsia (healthy women).

Finally, pre-eclampsia women with placental malaria were compared to women without pre-eclampsia and placental malaria.

Table 3: Clinical and laboratory characteristics of the study population used for the gene expression study

Parameter	COX-1	COX-2	15-LOX
No. of women	16(26.7%)	26(43.3%)	18(30.0%)
PE	4(25.0%)	7(26.9%)	5(27.8%)
nPE	4(25.0%)	7(26.9%)	5(27.8%)
PM	4(25.0%)	6(23.1%)	4(22.2%)
nPM	4(25.0%)	6(23.1%)	4(22.2%)
Ages (Yrs)	27(18-42)	27(18-42)	29(20-43)

All categorical data were presented in count and percentages, and age is presented in median (range). PE= Pre-eclampsia, nPE= without pre-eclampsia, PM= Placental malaria, nPM= Without Placental malaria.

Table 4: Number and percentage of women that were positive and negative to COX-1, COX-2 and 15-LOX

Parameter	COX-1	COX-2	15-LOX
PM			
No. of Women	11(23.4%)	21(44.7%)	15(31.9%)
PE	7(63.6%)	14(66.7%)	9(60.0%)
nPE	4(36.4%)	7(33.3%)	6(40.0%)
PE			
No. of Women	25(33.3%)	25(33.3%)	25(33.3%)
Positive	8(32.0%)	14(56.0%)	10(40.0%)
Negative	17(68.0%)	11(44.0%)	15(60.0%)
nPE			
No. of Women	25(33.3%)	25(33.3%)	25(33.3%)
Positive	8(32.0%)	13(52.0%)	16(64.0%)
Negative	17(68.0%)	12(48.0%)	9(36.0%)

PE= Pre-eclampsia, nPE= without pre-eclampsia, PM= Placental malaria, nPM= Without Placental malaria

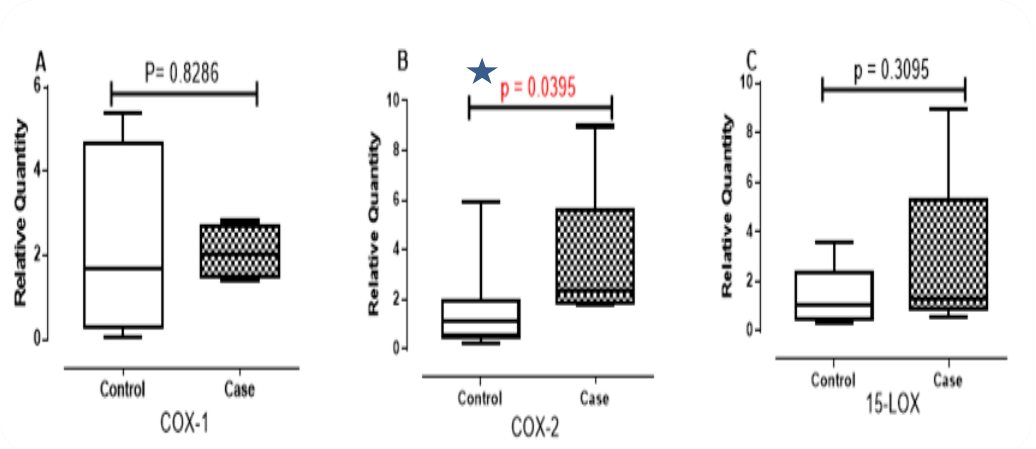

Figure 3: Expression of COX-1 (A), COX-2 (B) and 15-LOX (C) mRNA in the placenta of women with placental malaria diagnosed with and without preeclampsia

*The expression levels of COX-1, COX-2 and 15-LOX where PPIA was used as a reference gene to calibrate expression levels for each gene. Delta Ct (ΔCt) was obtained from the equation [**ΔCt = Ct gene – Ct PPIA**] and normalized using control (women without pre-eclampsia). Delta delta Ct (ΔΔCt) was obtained from the equation [**ΔΔCt = ΔCt - mean ΔCt control**]. Relative Quantity was evaluated using the equation [**RQ = 2^- (ΔΔCt**]. Results obtained were plotted according to control (women without pre-eclampsia) and cases (women diagnosed with pre-eclampsia). P value indicated with star shows a significant test ($p < 0.05$) for an increase in COX-2 gene expression only.*

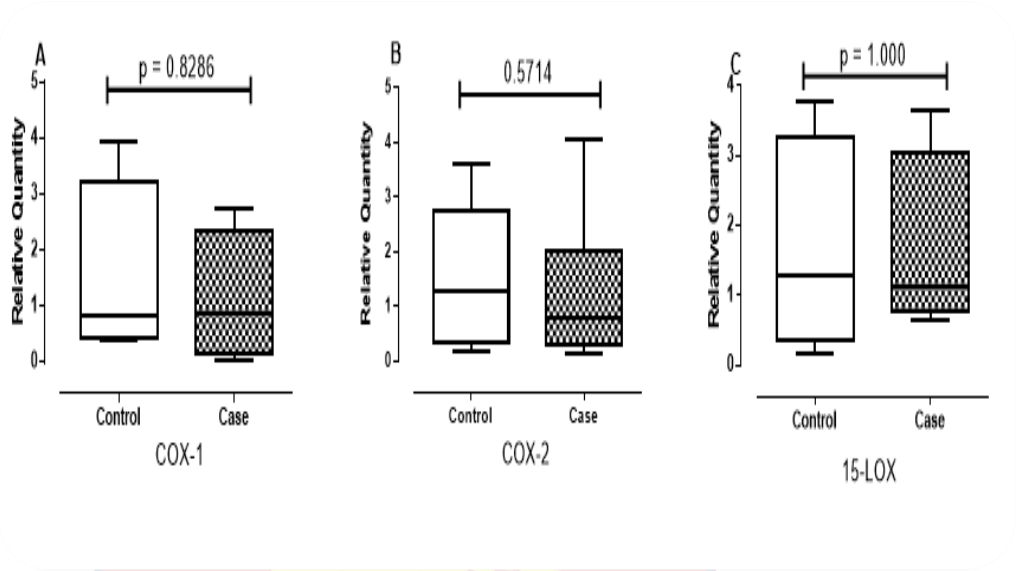

Figure 4: Expression of COX-1 (A), COX-2 (B) and 15-LOX (C) mRNA in the placenta of placental malaria women without preeclampsia.

*The expression levels of COX-1, COX-2 and 15-LOX where PPIA was used as a reference gene to calibrate expression levels for each gene. Delta Ct (**ΔCt**) was obtained from the equation [**ΔCt = Ct gene – Ct PPIA**] and normalized using control (women without pre-eclampsia and placental malaria). Delta delta Ct (ΔΔCt) was obtained from the equation [**ΔΔCt = ΔCt - mean ΔCt control**]. Relative Quantity was evaluated using the equation [**RQ = 2^-(ΔΔCt**]. Results obtained were plotted according to control (women without pre-eclampsia) and cases (women diagnosed with pre-eclampsia).*

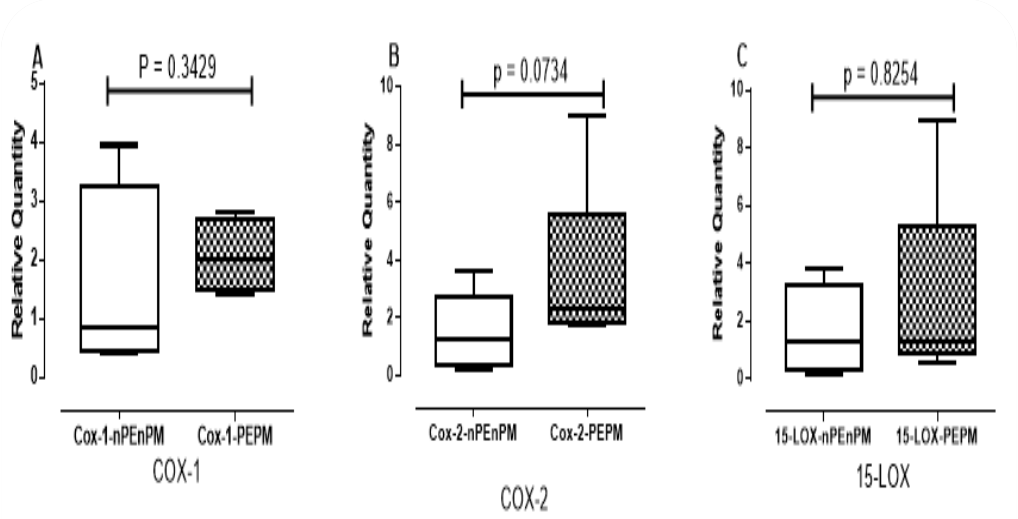

Figure 5: Comparing expression levels of COX-1 (A), COX-2 (B) and 15-LOX (C) mRNA in the placenta of PE women with PM (PEPM) and women without PE and PM (nPEnPM)

This compares the expression levels COX-1, COX-2 and 15-LOX mRNA in the placenta of pre-eclampsia women with placental malaria (PEPM) and women without pre-eclampsia and placental malaria (nPEnPM) from Figures I and II using Mann-Whitney U test.

4.1 Discussion

The study observed that women with placental malaria were twice as likely to develop pre-eclampsia relative to those without placental malaria. While some studies have found no link between placental malaria and pre-eclampsia (Shulman *et al*., 2001, Dorman *et al*., 2002), this study confirms previously reported association between placental malaria and pre-eclampsia development in Africa (Sartelet et al., 1996, Muehlenbachs *et al*., 2006, Ndao *et al*., 2009, Adam *et al*., 2011). This therefore suggests that placental malaria could play a role in pre-eclampsia development.

To assess whether the association between pre-eclampsia and placental malaria is through induction of either COX-1, COX-2, or 15-LOX by malaria

infection, the expression levels of COX-1, COX-2, and 15-LOX were compared between pre-eclampsia women with placental malaria and women without pre-eclampsia but with placental malaria. The mRNA levels of COX-1, COX-2 and 15-LOX increased by about 2, 3 and 2 folds respectively in pre-eclampsia women with placental malaria from the control groups (without pre-eclampsia but with the exposure). However, only COX-2 increased significantly (P = 0.0395, Figure 3). This supports the study of Akarasereenont *et al.* (1999) who reported an increased expression levels of COX-2 in pre-eclampsia women though they did not compare their study to placental malaria. Sarr *et al*., (2010) also observed increased levels of COX-2 in women with placental malaria. They also did not relate their study to preeclamptic women.

To indicate the expression levels in women with placental malaria only to ascertain whether there will be an increase levels in the enzymes especially COX-2 as reported previously, the expression levels of COX-1, COX-2 and 15-LOX were compared between women with placental malaria without pre-eclampsia and women without placental malaria and pre-eclampsia (healthy women) (Figure 4). However, there was no significant difference between these levels. Suggesting that the lack of significant difference between these levels may denote that the support is weak and the data are inconclusive. Sarr *et al*., (2010) reported increased levels of COX-2 in placental malaria. They further indicated that expression of COX-1 tends to increase during past infection but remained low in all their placentae without significant differences between their positive and negative control group, agreeing with this study to some extent though this study did not report on past, and acute

infections. They also reported a lower level of 15-LOX in their case group but not significantly lower as observed in this study.

Comparing pre-eclampsia women with placental malaria to women without pre-eclampsia and placental malaria, there was no significant difference in the expression levels of these genes though we observed increase level of COX-2 in pre-eclampsia women with placental malaria near significance. Again, this suggests that the lack of significant difference between these levels may denote that the support is weak and the data are inconclusive.

It would have been fair to compare expression levels of COX-1, COX-2 and 15-LOX between pre-eclampsia women with placental malaria to pre-eclampsia women without placental malaria. However, all pre-eclampsia cases that were positive for COX-1 COX-2 and 15-LOX turned to be positive for placental malaria except one pre-eclampsia case that was negative for placental malaria for 15-LOX gene.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

5.0 Summary

The study is a hospital-based case-control study conducted at the Department of Obstetrics & Gynaecology of the Korle-Bu Teaching Hospital (KBTH) and Noguchi Memorial Institute of Medical Research (NMIMR) where pregnant women diagnosed with or without pre-eclampsia (cases and controls respectively) were identified, consented and enrolled based on set inclusion and exclusion criteria.

The study sought to assess whether the association between pre-eclampsia and placental malaria is through induction of either COX-1, COX-2, or 15-LOX by placental malaria. This will provide a novel factor in understanding the interactions between pre-eclampsia and placental malaria which will consequently contribute to the management of maternal health issues, reducing maternal and fetal mortality; and attaining the SDG three.

The study hypothesized that Placental Malaria infection increases COX-1, COX-2 and 15-LOX pathways of converting arachidonic acid into prostaglandins which leads to increased inflammation and subsequently pre-eclampsia.

Molecular detection (Nested PCR) of *P. falciparum* was conducted on DNA extracted from placenta tissues obtained to ascertain whether subjects were exposed or not exposed to *P. falciparum* infection. All *P. falciparum* positive results obtained from the placenta tissue after performing PCR were considered as women with placental malaria.

To ascertain whether there is an association between placental malaria and pre-eclampsia development for this case-control study, the odds of developing pre-eclampsia was determined, where the odds of developing pre-eclampsia was 2.094 which is significantly greater in women with placental malaria (P. *falciparum* positive) compared to women without placental malaria (*P. falciparum* negative).

To assess whether the association between pre-eclampsia and placental malaria is through induction of either COX-1, COX-2, or 15-LOX by malaria infection, expression levels of COX-1, COX-2, and 15-LOX were compared between pre-eclampsia women with placental malaria and women without pre-eclampsia but with placental malaria (Figure 3). The mRNA levels of COX-1, COX-2 and 15-LOX increased in pre-eclampsia women with placental malaria from the control groups (without pre-eclampsia). However, only COX-2 increased significantly (P = 0.0395) supporting the study of Akarasereenont *et al.* (1999) who reported an increased expression levels of COX-2 in pre-eclampsia women though they did not relate their study to placental malaria.

The expression levels of COX-1, COX-2 and 15-LOX again were compared between women with placental malaria without pre-eclampsia and women without placental malaria and pre-eclampsia (healthy women). However, the data were not significant suggesting that the lack of significant difference between these levels may denote that the support is weak and the data are inconclusive. Comparing pre-eclampsia women with placental malaria to women without pre-eclampsia and placental malaria, there was no significant difference in the expression levels of these genes.

5.1 Conclusion

Indeed this study established that the odds of developing pre-eclampsia are 2.09 greater for women with placental malaria (*P. falciparum*) compared to women without placental malaria (*P. falciparum* negative) (P = 0.0392) confirming the association between pre-eclampsia development and placental malaria. This study has also shown a significant increase in mRNA levels of COX- 2 in pre-eclampsia women with placental malaria (P = 0.0395). In addition, all pre-eclampsia cases that were positive for COX-1 COX-2 and 15-LOX turned to be positive for placental malaria except one pre-eclampsia case that was negative for placental malaria for 15-LOX gene. The data suggest that transcripts levels of COX-2 may play a role in the development of pre-eclampsia in women with placental malaria infection. Hence, COX-2 could potentially be used as a diagnostic marker for women with placental malaria and pre-eclampsia.

5.3 Recommendation

There is the need to replicate this study in other malaria-endemic regions as well as regions where there is an increased incidence of pre-eclampsia. A large sample size of expression levels of COX-1, COX-2 and 15-LOX should also be used to demonstrate whether there is an association between pre-eclampsia and placental malaria through induction of these enzymes.

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APPENDICES

APPENDIX A: PROTOCOLS

I. Isolation of Genomic DNA from FFPE Tissue Sections using QIAamp DNA FFPE Tissue Kit

1. Clean working environment, equipment and hands with RNase and DNase free cleaner.
2. Using a scalpel, trim excess paraffin off the sample block.
3. Cut 4 tissue sections of 10 µm thick each. ***If the sample surface has been exposed to air, discard the first 2–3 sections.***
4. Immediately place the sections in a 1.5 or 2 ml microcentrifuge tube, and add 1 ml xylene to the sample. Close the lid and vortex vigorously for 10 s.
5. Centrifuge at full speed for 2 min at room temperature (15–25°C).
6. Remove the supernatant by pipetting. Do not remove any of the pellets.
7. Add 1 ml ethanol (96–100%) to the pellet, and mix by vortexing.
8. Centrifuge at full speed for 2 min at room temperature.
9. Remove the supernatant by pipetting. Do not remove any of the pellets. ***Carefully remove any residual ethanol using a fine pipet tip.***
10. Open the tube and incubate at room temperature or up to 37°C. Incubate for 10 min or until all residual ethanol has evaporated.
11. Resuspend the pellet in 180µl Buffer ATL. Add 20µl proteinase K, and mix by vortexing.
12. Incubate at 56°C for 1 h (or until the sample has been completely lysed).

13. Incubate at 90°C for 1 h.

If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 90°C.

14. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid. ***If RNA-free genomic DNA is required, add 2 µl RNase A (100 mg/ml) and incubate for 2 min at room temperature before continuing with step 14. Allow the sample to cool to room temperature before adding RNase A.***
15. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200µl ethanol (96–100%), and mix again thoroughly by vortexing.

It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the QIAamp procedure.

16. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
17. Carefully transfer the entire lysate to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.

18. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
19. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

20. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely. ***This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.***
21. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the flow-through.

Carefully open the lid of the QIAamp MinElute column and apply 20–100 µl Buffer ATE to the center of the membrane. Important*: Ensure that Buffer ATE is equilibrated to room temperature. If using small elution volumes (<50 µl), dispense Buffer ATE onto the center of the membrane to ensure complete elution of bound DNA. QIAamp MinElute columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. The volume of eluate will be up to 5 µl less than the volume of elution solution applied to the column.*

22. Close the lid and incubate at room temperature for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min.

Incubating the QIAamp MinElute column loaded with Buffer ATE for 5 min at room temperature before centrifugation generally increases DNA yield.

Nested Polymerase Chain Reaction (PCR)

Table 5: Nest 1 Reaction

Reagent	Concentration	Vol. 1X	X100	X50	Cycling condition
ddH_2O		5.99	599	299.5	94 ^{0}C– 4mins
5X PCR Buffer	1X	3	300	150	94 ^{0}C –30secs 55^{0}C – 45secs } 35 cycles
dNTP mix	167nmol/L	0.25	25	12.5	
$MgCl_2$	2.5uM	0.42	42	21	68^{0}C – 45secs
PLU 5	80nmol/L	0.12	12	6	68^{0}C – 5mins
PLU 6	80nmol/L	0.12	12	6	4 ^{0}C - ∞
OneTaq	1uL	0.1	10	5	
Template		5/15			

Table 6: Nest 2 Reaction

Reagent	Concentration	Vol. 1X	X100	X50	Cycling condition
ddH_2O		10.49	1049	524.5	94 ^{0}C– 4mins
5X PCR Buffer	1X	3	300	150	94 ^{0}C –30secs
dNTP mix	167nmol/L	0.25	25	12.5	58^{0}C–45secs (35 cycles)
$MgCl_2$	2.5uM	0.42	42	21	
FAL 1	80nmol/L	0.12	12	6	68^{0}C – 1min
FAL 2	80nmol/L	0.12	12	6	68^{0}C – 5mins
OneTaq	1uL	0.1	10	5	4 ^{0}C - ∞
Template		0.5/15			

II. Extraction and purification of total RNA from formalin fixed paraffin embedded (FFPE) tissue using RNeasy FFPE kit (QIAGEN)

1. Clean working environment, equipment and hands with RNase and DNase free cleaner

2. Using a scalpel, trim excess paraffin off the sample block.

3. Cut 3-4 tissue sections of 10 µm thick each. *If the sample surface has been exposed to air, cut and discard the first 2 sections.*

4. Immediately place the sections in a 2 ml microcentrifuge tube and close the lid.

5. Add 160 µl Deparaffinization Solution, vortex vigorously for 10s, and centrifuge briefly to bring the sample to the bottom of the tube.

6. Incubate at 56°C for 3 min, and then allow cooling at room temperature.

7. Add 150 µl Buffer PKD, and mix by vortexing.

8. Centrifuge for 1 min at 10,000 rpm.

9. Add 10µl proteinase K to the lower, clear phase. Mix gently by pipetting up and down.

10. Incubate at 56°C for 15 min, then at 80°C for 15 min. If a heating block without a shaking function is used, briefly mix by vortexing every 3 min. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

11. Transfer the lower, uncolored phase into a new 2 ml microcentrifuge tube.

12. Incubate on ice for 3 min. Then, centrifuge for 15 min at 13,500 rpm.

13. Transfer the supernatant to a new microcentrifuge tube taking care not to disturb the pellet. 14. Add DNase Booster Buffer equivalent to a tenth of the

total sample volume (approximately 15 µl) and 10 µl DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

16. Incubate at room temperature for 15 min.

17. Add 320 µl Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.

III. RNA Purification

1. Add 720µl ethanol (100%) to the sample and mix well by pipetting.

2. Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15s at ≥10,000 rpm.

3. Discard the flow-through and reuse the collection tube in step 4.

4. Repeat step 2 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 3.

5. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥10,000 rpm. Discard the flow-through.

6. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥10,000 rpm to wash the spin column membrane. Discard the collection tube with the flow-through.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

7. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through. *To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise). It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.*

8. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 30µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

IV. First-Strand cDNA Synthesis **(**QIAGEN)

Important points before starting.

RNA samples are very sensitive to RNase digestion; therefore, wear gloves and maintain an RNase-free work area while performing this protocol. The RT2 PreAMP cDNA Synthesis Kit is not compatible with the chemicals in DNA-free™ kits from Ambion. If your RNA sample has been treated with DNA-free reagents, contact QIAGEN Technical Services.

Procedure

1. Prepare the genomic DNA elimination mix for each RNA sample in a sterile PCR tube according to Table 1. Mix gently by pipetting up and down and then centrifuge briefly.

Genomic DNA Elimination Mix

Component	Amount for one sample (RNA from fresh/frozen sample)	Amount for one sample (RNA from FFPE sample)
RNA	1 ng–100 ng	100 ng–1 µg
Buffer GE	2 µl	2 µl
RNase-free water	Variable	Variable
Total volume	**10 µl**	**10 µl**

2. Incubate the genomic DNA elimination mix at 42°C for 5 min, then place immediately on ice for at least 1 min.

3. Prepare the reverse-transcription mix according to the table below.

For multiple reactions scale up the volumes shown in the table below accordingly.

Reverse-Transcription Mix

Component	Volume for 1 reaction
5x Buffer BC3	4 µl
Control P2	1 µl
cDNA Synthesis Enzyme Mix	1 µl
RNase Inhibitor	1 µl
RNase-free water	3 µl
Total volume	**10 µl**

4. Add 10 µl reverse-transcription mix to each tube containing 10 µl genomic DNA elimination mix. Mix gently by pipetting up and down. Centrifuge briefly to remove any air bubbles and collect all the liquid to the bottom of the tube.

5. Incubate at 42°C for exactly 30 min. Then immediately stop the reaction by incubating at 95°C for 5 min.

6. Place the reactions on ice and proceed with the preamplification protocol.

If you wish to store the reactions overnight prior to real-time PCR, transfer to a –20°C freezer. Longer storage times are not recommended.

V. Preamplification of cDNA Target Templates

Important point before starting

Each RT2 PreAMP Pathway Primer Mix is specific for a particular pathway or disease RT2 Profiler PCR Array. Check the label to verify that the correct pathway-specific RT2 PreAMP Pathway Primer Mix is used for the RT2 Profiler PCR Array. Verify that the lot number of the RT2 PreAMP Pathway Primer Mix is compatible with that of the RT2 Profiler PCR Array to be used. If the RT2 PreAMP Pathway Primer Mix and the RT2 Profiler PCR Array have been purchased at different times, check with QIAGEN Technical Services to ensure their compatibility.

Procedure

1. Thaw the RT2 PreAMP PCR Mastermix and RT2 PreAMP Pathway Primer Mix at room temperature. If precipitates are visible, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.

2. Prepare the preamplification mix according to Table 3. For multiple reactions, scale up the volumes shown in Table 3 accordingly.

Preamplification Mix

Component	Amount for one sample
RT^2 PreAMP PCR Mastermix	12.5 µl
RT^2 PreAMP Pathway Primer Mix	7.5 µl
Total volume	**20 µl**

3. Pipet 5 µl cDNA synthesis reaction (from step 6, page 18) into a 0.2 ml PCR tube. Then add 20 µl preamplification mix.

4. Mix gently by pipetting up and down. Spin briefly to remove any air bubbles and collect all the liquid to the bottom of the tube.

5. Program the real-time cycler according to Table 4 or Table 5. Place the tubes in the real-time cycler and start the program.

Cycling Conditions for Preamplification of cDNA from FFPE Samples

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	95°C	60 seconds	1
Denaturation	95°C	15 seconds	40–45
Extension	60°C	30 seconds (+plate read)	

6. When cycling is finished, take the tubes from the real-time cycler and place on ice.

7. Add 2µl Side Reaction Reducer to each preamplified reaction. Mix gently by pipetting up and down. Spin the tubes briefly to remove any air bubbles and collect all the liquid at the bottom of the tube.

8. Incubate at 37°C for 15 min followed by heat inactivation at 95°C for 5 min.

9. Immediately add 84µl nuclease-free water. Mix well.

10. Place on ice prior to real-time PCR, or store overnight at –20°C.

VI. cDNA Synthesis (Applied Biosystems)

Prepare the 2X RT master mix

1. Allow the kit components to thaw on ice.

2. Calculate the volume of components needed to prepare the required number of reactions. Note: Prepare the RT master mix on ice.

2X RT Master Mix

Component	Volume	
	With RNase Inhibitor	Without RNase Inhibitor
10X RT Buffer	2.0 µL	2.0 µL
25X dNTP Mix (100 mM)	0.8 µL	0.8 µL
10X RT Random Primers	2.0 µL	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL	1.0 µL
RNase Inhibitor	1.0 µL	—
Nuclease-free H_2O	3.2 µL	4.2 µL
Total per reaction	10.0 µL	10.0 µL

3. Place the 2X RT master mix on ice and mix gently.

Prepare the reverse transcription reactions

1. Pipette 10µL of 2X RT master mix into each well of a 96-well reaction plate or individual tube.

2. Pipette 10 µL of RNA sample into each well, Pipette up and down two times to mix.

3. Seal the plates or tubes.

4. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.

5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

Thermal cycling conditions for 2X RT Master Mix

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	∞

VII. Quantitative Polymerase Chain Reaction (qPCR)- Luna Universal Probe qPCR (New England Biosystem)

Before Use:

• Prepare DNA or cDNA of interest using desired DNA extraction and purifcation method. • Make dilutions of DNA or cDNA to be used for the standard curve. These should be prepared fresh before each experiment and can be diluted in either water or TE.

Reaction Setup: For best results, we recommend running each DNA standard and sample in triplicate.

Luna Universal Probe qPCR Master Mix

COMPONENT	20 µl REACTION	FINAL CONCENTRATION
Luna Universal Probe qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	0.8 µl	0.4 µM
Reverse primer (10 µM)	0.8 µl	0.4 µM
Probe (10 µM)	0.4 µl	0.2 µM
Template DNA	variable	< 100 ng
Nuclease-free Water	to 20 µl	

1. Thaw Luna Universal Probe qPCR Master Mix and other reaction components at room temperature, then place on ice. After thawing completely, briefly mix each component by inversion, pipetting, or gentle vortexing.

2. Determine the total volume for the appropriate number of reactions, plus 10% overage and prepare assay mix of all components except DNA template accordingly. Mix thoroughly but gently by pipetting or vortexing. Collect liquid to the bottom of the tube by brief centrifugation.

3. Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.

4. Add DNA templates to qPCR tubes or plate. Seal tubes with flat, optically transparent caps; seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.

5. Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 2,500–3,000 rpm).

6. Program real-time instrument with indicated thermocycling protocol (see table below). Ensure a plate read is included at the end of the extension step. Confrm the appropriate detection channel is selected for the fluorophore used in the assay.

We recommend using the "Fast" cycling profle where applicable (e.g., Applied Biosystems StepOnePlus®, QuantStudio®, 7500 Fast instruments).

Cycling conditions for Luna Universal Probe qPCR Master Mix

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	95°C	60 seconds	1
Denaturation	95°C	15 seconds	40–45
Extension	60°C	30 seconds (+plate read)	

APPENDIX B: TEMPLATES, PRIMER AND PROBE SEQUENCES, AND RESULTS

PCR plate template for *P. falciparum* detection showing sample IDs

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	2	3	4	5	6	7	9	10	11	12	13
B	14	15	16	17	18	19	20	21	22	23	24	25
C	26	28	29	30	31	32	33	34	35	36	37	38
D	39	40	41	42	43	44	45	46	47	48	49	50
E	51	52	53	54	55	56	57	58	59	60	61	62
F	63	64	65	66	67	68	69	70	71	72	73	74
G	75	76	77	78	79	80	81	82	83	84	85	86
H	87	88	89	90	91	92	93	94	92	**+ c**	**+ c**	**- c**

RT qPCR plate template for COX-1, COX-2 and 15-LOX with their IDs

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Cox-1 108	Cox-1 108	Cox-1 108	Cox-2 108	Cox-2 108	Cox-2 108	15-Lox 108	15-Lox 108	15-Lox 108	PPIA 108	PPIA 108	PPIA 108
B	ACTB 108	ACTB 108	ACTB 108	Cox-1 1	Cox-1 1	Cox-1 1	Cox-2 1	Cox-2 1	Cox-2 1	15-Lox 1	15-Lox 1	15-Lox 1
C	PPIA 1	PPIA 1	PPIA 1	ACTB 1	ACTB 1	ACTB 1	Cox-1 114	Cox-1 114	Cox-1 114	Cox-2 114	Cox-2 114	Cox-2 114
D	15-Lox 114	15-Lox 114	15-Lox 114	PPIA 114	PPIA 114	PPIA 114	ACTB 114	ACTB 114	ACTB 114	Cox-1 113	Cox-1 113	Cox-1 113
E	Cox-2 113	Cox-2 113	Cox-2 113	15-Lox 113	15-Lox 113	15-Lox 113	PPIA 113	PPIA 113	PPIA 113	ACTB 113	ACTB 113	ACTB 113
F	Cox-1 64	Cox-1 64	Cox-1 64	Cox-2 64	Cox-2 64	Cox-2 64	15-Lox 64	15-Lox 64	15-Lox 64	PPIA 64	PPIA 64	PPIA 64
G	ACTB 64	ACTB 64	ACTB 64	Cox-1 NTC	Cox-1 NTC	Cox-1 NTC	Cox-2 NTC	Cox-2 NTC	Cox-2 NTC	15-Lox NTC	15-Lox NTC	15-Lox NTC

Table 7: Specifications and sequence of primers for COX-1, COX-2, 15-LOX and PPIA

Name	Scale	Purification	5' Modification	Sequence	3' Modification
COX-1 F	0.01 (umole)	Desalting	None	ATGATGGGCCTGCTGTGGA	None
COX-1 R	0.01 (umole)	Desalting	None	CCAACACTCACCATGCCAAAC	None
COX-2 F	0.01 (umole)	Desalting	None	GCTCAAACATGATGTTTGCATTC	None
COX-2 R	0.01 (umole)	Desalting	None	GCTGGCCCTCGCTTATGA	None
15-LOX F	0.01 (umole)	Desalting	None	GGAGACAGTGATGGCGACACT	None
15-LOXR	0.01 (umole)	Desalting	None	TCTGCCCAGCTGCCAAGT	None
PPIA F	0.01 (umole)	Desalting	None	GTCAACCCCACCGTGTTCTT	None
PPIA R	0.01 (umole)	Desalting	None	CGTCTGTCTTTGGGACCTTGT	None

Table 8: Specifications and sequence of probes for COX-1, COX-2, 15-LOX and PPIA

Name	Scale	Purification	5' Modification	Sequence	3' Modification
COX-1	0.2 (umole)	PAGE	5' 6-FAM (0.2)	CTGGCCTCAGCACTCTGGAATGAC	3' BHQ-1™ (0.2)
COX-2	0.2 (umole)	PAGE	5' 6-FAM (0.2)	TGCCCAGCACTTCACGCATCAGTT	3' BHQ-1™ (0.2)
15-Lox	0.2 (umole)	PAGE	5' 6-FAM (0.2)	TCCACCAGGCTTCTCTCCAGATGTCC	3' BHQ-1™ (0.2)
PPIA	0.2 (umole)	PAGE	5' 6-FAM (0.2)	AGCTCAAAGGAGACGCGGCCCA	3' BHQ-1™ (0.2)

Table 9: Specifications and sequence of primers for *P.falciparum*

Name	Scale	Purification	5' Modification	Sequence	3' Modification
rPLU6	0.01 (umole)	Desalting	None	TTAAAATTGTTGCAGTTAAAACG	None
rPLU5	0.01 (umole)	Desalting	None	CCTGTTGTTGCCTTAAACTTC	None
rFAL1	0.01 (umole)	RP-Cartridge	None	TTAAACTGGTTTGGGAAAACCAAATATATT	None
rFAL2	0.01 (umole)	RP-Cartridge	None	ACACAATGAACTCAATCATGACTACCCGTC	None

Mean Ct, ΔCt, ΔΔCt, 2^-ΔΔCt (fold change values) together with the reference gene (PPIA) for COX-1 among women with pre-eclampsia and placental malaria and women with placental malaria without pre-eclampsia

	PCR ID	SAMPLE ID	Ct 1	Ct 2	COX-1 Mean Ct	St. Dev 1	PPIA Ct 1	PPIA Ct 2	PPIA Mean Ct	St. Dev 2	ΔCt	St. Dev 3	ΔΔCt	2^-ΔΔCt
PfPE	116	**P17011**	36.9	36.959	36.9	0.026	35.7	35.8	35.8	0.057	1.2	0.063	-1.5	2.82
	107	**P17008**	35.742	37.077	36.4	0.944	34.507	33.964	34.2	0.383	2.2	1.019	-0.5	1.41
	101	**P17016**	35.593	35.556	35.6	0.026	34.393	33.809	34.1	0.413	1.5	0.414	-1.2	2.30
	104	**P17023**	37.2	36.6	36.9	0.416	36.1	33.9	35.0	1.587	1.9	1.641	-0.8	1.73
PfnPE(control)	36	**C17025**	34.4	35.325	34.9	0.658	35.1	34.1	34.6	0.725	0.25	0.979	-2.4	5.38
	64	**C17017**	38.1	39.341	38.7	0.912	35.9	35.993	35.9	0.072	2.75	0.915	0.1	0.95
	60	**C17007**	37.6	37.530	37.5	0.014	31.608	30.863	31.2	0.526	6.30	0.526	3.6	0.08
*	50	**C17065**	38.879	38.959	38.919	0.056569	37.512	37.5512	37.5	0.028	1.39	0.064	-1.3	2.44
										Avg.	2.67			

Mean Ct, ΔCt, ΔΔCt, 2^-ΔΔCt (fold change values) together with the reference gene (PPIA) for COX-1 among women with placental malaria without pre-eclampsia

PCR ID	SAMPLE ID			COX-1 Mean Ct	St. Dev 1	PPIA Ct 1	PPIA Ct 2	C Mean Ct	St. Dev 2	ΔCt	St. Dev 3	ΔΔCt	2^-ΔΔCt
36	**C17025**	34.4	35.325	34.9	0.658	35.1	34.1	34.6	0.725	0.25	0.979	-1.45	2.74
64	**C17017**	38.1	39.341	38.7	0.912	35.9	35.993	35.9	0.072	2.75	0.915	1.05	0.48
60	**C17007**	37.6	37.530	37.5	0.014	31.608	30.863	31.2	0.526	6.30	0.526	4.60	0.04
50	**C17065**	38.879	38.959	38.919	0.056569	37.512	37.5512	37.5	0.028	1.39	0.064	-0.31	1.24
63	**C17004**	37.0	36.728	36.9	0.177	34.2	33.4	33.8	0.553	3.03	0.581	1.3	0.40
58	**C17002**	38.4	38.571	38.5	0.141	36.4	37.4	36.9	0.747	1.57	0.760	-0.1	1.10
51	**C17014**	37.4	38.066	37.7	0.495	34.529	35.925	35.2	0.987	2.49	1.104	0.8	0.58
41	**C17018**	37.0	37.6	37.3	0.475	38.377	36.787	37.582	1.125	-0.28	1.221	-2.0	3.95
									Avg.	1.70			

Mean Ct, ΔCt, ΔΔCt, 2^-ΔΔCt (fold change values) together with the reference gene (PPIA) for COX-2 among women with pre-eclampsia and placental malaria and women with placental malaria without pre-eclampsia

	PCR ID	SAMPLE ID	Ct 1	Ct 2	COX-2 Mean Ct	St. Dev 1	PPIA Ct 1	PPIA Ct 2	PPIA Mean Ct	St. Dev 2	ΔCt	St. Dev 3	ΔΔCt	2^-ΔΔCt
	93	**P17028**	36.3	36.425	36.380	0.064	36.4	37.433	36.934	0.705	-0.554	0.709	-2.489	5.61
PfPE	116	**P17011**	35.9	34.937	35.437	0.707	35.7	35.8	35.763	0.057	-0.326	0.709	-2.261	4.79
	109	**P17015**	36.1	36.181	36.151	0.042	37.4	37.383	37.384	0.002	-1.233	0.042	-3.168	8.99
	78	**P17001**	35.1	35.135	35.137	0.003	33.4	35.422	34.422	1.414	0.715	1.414	-1.220	2.33
	84	**P17029**	35.126	35.773	35.450	0.458	34.092	34.692	34.392	0.424	1.058	0.624	-0.877	1.84
	114	P17030	36.3	36.320	36.321	0.001	35.90784	34.51959	35.21372	1.0	1.107	1.000	-0.828	1.77
	101	P17016	35.86081	34.10374	34.98228	1.242	34.39256	33.8088	34.10068	0.412777	0.882	1.309	-1.053	2.08
	35	**C17024**	35.6	35.465	35.515	0.070	31.233	33.999	32.616	1.956	2.899	1.957	0.965	0.51
PfnPE(control)	36	**C17025**	36.8	35.9	36.359	0.632	35.1	34.1	34.614	0.725	1.745	0.962	-0.189	1.14
	13	**C17026**	35.760	37.087	36.424	0.938	32.9	31.9	32.352	0.707	4.072	1.175	2.137	0.23
	54	**C17005**	36.178	35.494	35.836	0.484	33.362	34.900	34.131	1.088	1.705	1.191	-0.230	1.17
	19	**C17021**	35.2	32.169	33.669	2.121	35.4	33.2	34.301	1.539	-0.633	2.621	-2.567	5.93
	60	**C17007**	34.426	33.657	34.042	0.544	31.608	30.863	31.235	0.526	2.807	0.757	0.872	0.55
	64	**C17017**	35.9	37.890	36.890	1.414	35.9	35.993	35.942	0.072	0.948	1.416	-0.987	1.98
										Avg.	1.935			

Mean Ct, ΔCt, ΔΔCt, 2^-ΔΔCt (fold change values) together with the reference gene (PPIA) for COX-2 among women with placental malaria without pre-eclampsia

PCR ID	SAMPLE ID			COX-2 Mean Ct	St. Dev 1	PPIA Ct 1	PPIA Ct 2	PPIA Mean Ct	St. Dev 2	ΔCt	St. Dev 3	ΔΔCt	2^-ΔΔCt
35	**C17024**	35.6	35.465	35.515	0.070	33.9	31.5	32.700	1.660	2.815	1.661	1.433	0.37
36	**C17025**	36.8	35.9	36.359	0.632	35.1	34.1	34.614	0.725	1.745	0.962	0.363	0.78
13	**C17026**	35.760	37.087	36.424	0.938	32.9	31.9	32.352	0.707	4.072	1.175	2.690	0.16
54	**C17005**	36.178	35.494	35.836	0.484	33.362	34.900	34.131	1.088	1.705	1.191	0.323	0.80
64	**C17017**	35.9	37.890	36.890	1.414	35.9	35.993	35.942	0.072	0.948	1.416	-0.434	1.35
19	**C17021**	35.2	32.169	33.669	2.121	35.4	33.2	34.301	1.539	-0.633	2.621	-2.015	4.04
62	**C17008**	35.1	35.143	35.138	0.007	31.5	33.280	32.369	1.288	2.769	1.288	1.387	0.38
34	**C17023**	33.018	34.244	33.631	0.866	33.9	31.5	32.700	1.660	0.931	1.872	-0.451	1.37
55	**C17010**	36.6	36.290	36.433	0.202	35.4	37.331	36.341	1.400	0.092	1.414	-1.290	2.45
12	**C17027**	35.5	34.521	35.021	0.707	33.470	34.275	33.873	0.570	1.149	0.908	-0.233	1.18
52	**C17013**	37.8	37.867	37.817	0.071	33.925	34.082	34.004	0.111	3.813	0.132	2.431	0.19
41	**C17018**	36.5	37.731	37.121	0.863	38.377	36.787	37.582	1.125	-0.461	1.418	-1.843	3.59
									Avg.	1.382			

Mean Ct, ΔCt, ΔΔCt, 2^-ΔΔCt (fold change values) together with the reference gene (PPIA) for 15-LOX among women with pre-eclampsia and placental malaria and women with placental malaria without pre-eclampsia

	PCR ID	SAMPLE ID	Ct 1	Ct 2	15-LOX Mean Ct	St. Dev 1	PPIA Ct 1	PPIA Ct 2	PPIA Mean Ct	St. Dev 2	ΔCt	St. Dev 3	ΔΔCt	2^-ΔΔCt
PfPE	93	**P17028**	35.9	38.588	37.3	1.884	36.4	37.433	36.9	0.705	0.3	2.012	-0.4	1.28
	116	**P17011**	36.8	37.600	37.2	0.563	35.7	35.8	35.8	0.057	1.4	0.566	0.8	0.59
	78	**P17001**	25.831	38.041	31.9	8.634	33.4	35.422	34.4	1.414	-2.5	8.749	-3.2	8.94
	84	**P17029**	34.092	34.692	34.4	0.424	34.092	34.692	34.4	0.424	0.0	0.6	-0.7	1.60
	107	**P17008**	34.6	34.677	34.7	0.021	34.507	33.964	34.2	0.383	0.4	0.384	-0.2	1.19
PfnPE(control)	28	**C17019**	37.9	38.783	38.3	0.636	36.843	35.427	36.135	1.002	2.2	1.187	1.5	0.35
	1	**C17022**	35.9	37.813	36.9	1.342	37.270	35.261	36.266	1.421	0.6	1.955	-0.1	1.05
	129	**C17009**	36.5	37.447	37.0	0.700	35.796	35.517	35.656	0.197	1.3	0.727	0.6	0.65
	19	**C17021**	34.8	34.692	34.8	0.106	35.4	33.2	34.301	1.539	0.5	1.543	-0.2	1.16
	35	**C17024**	31.5	31.365	31.4	0.099	31.233	33.999	32.616	1.956	-1.2	1.959	-1.9	3.62

Mean Ct, ΔCt, ΔΔCt, 2^-ΔΔCt (fold change values) together with the reference gene (PPIA) for 15-LOX among women with placental malaria without pre-eclampsia

PCR ID	SAMPLE ID			15-LOX Mean Ct	St. Dev 1	PPIA Ct 1	PPIA Ct 2	PPIA Mean Ct	St. Dev 2	ΔCt	St. Dev 3	ΔΔCt	2^-ΔΔCt
1	**C17022**	35.9	37.813	36.86	1.342	37.270	35.261	36.27	1.421	0.60	1.955	-0.09	1.07
129	**C17009**	36.5	37.447	36.95	0.700	35.796	35.517	35.66	0.197	1.30	0.727	0.60	0.66
19	**C17021**	34.8	34.692	34.77	0.106	35.4	33.2	34.30	1.539	0.47	1.543	-0.23	1.17
35	**C17024**	31.5	31.365	31.43	0.099	31.233	33.999	32.62	1.956	-1.18	1.959	-1.87	3.66
55	**C17010**	35.7	36.775	36.22	0.792	35.4	37.331	36.34	1.400	-0.13	1.608	-0.82	1.76
34	**C17023**	34.216	37.538	35.88	2.349	33.9	31.5	32.70	1.660	3.18	2.876	2.49	0.18
63	**C17004**	32.6	32.592	32.60	0.005	34.2	33.4	33.82	0.553	-1.23	0.553	-1.92	3.78
64	**C17017**	37.7	36.1	36.88	1.128	35.9	35.993	35.94	0.072	0.94	1.130	0.25	0.84

APPENDIX C: IMAGES

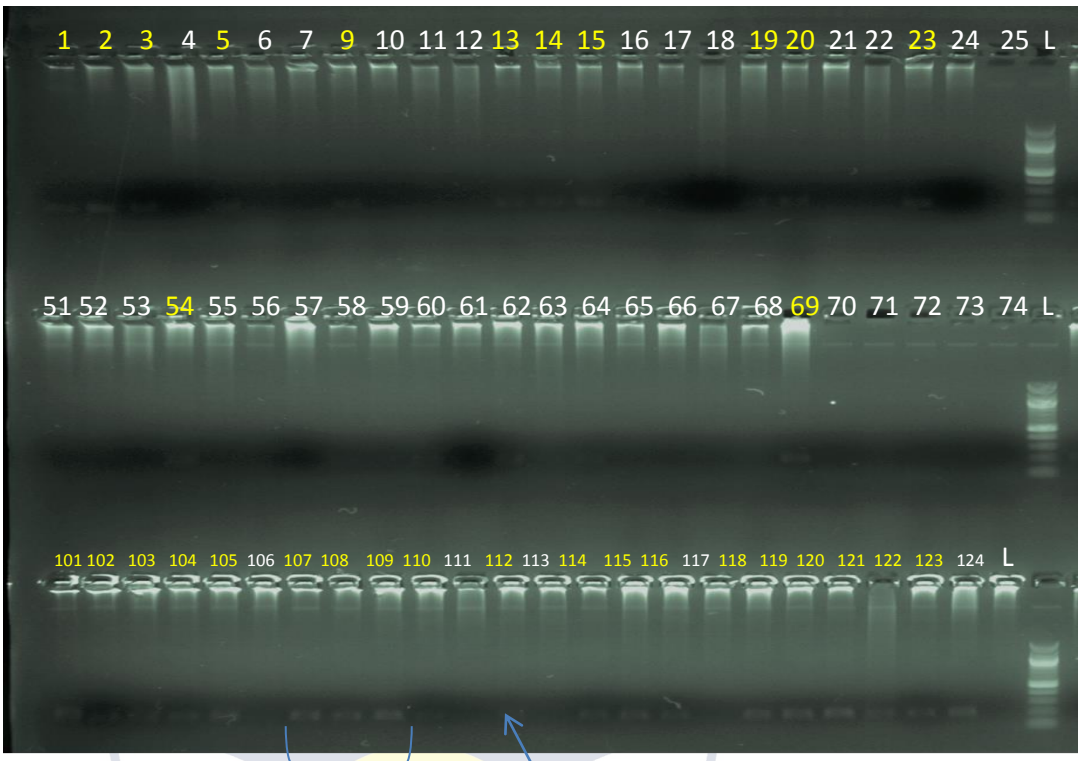

Figure 6: Gel Image showing DNA positive samples for P. falciparum with their sample IDs

Yellow numbered colour code represents *P. falciparum* positive samples and white represents negative samples.

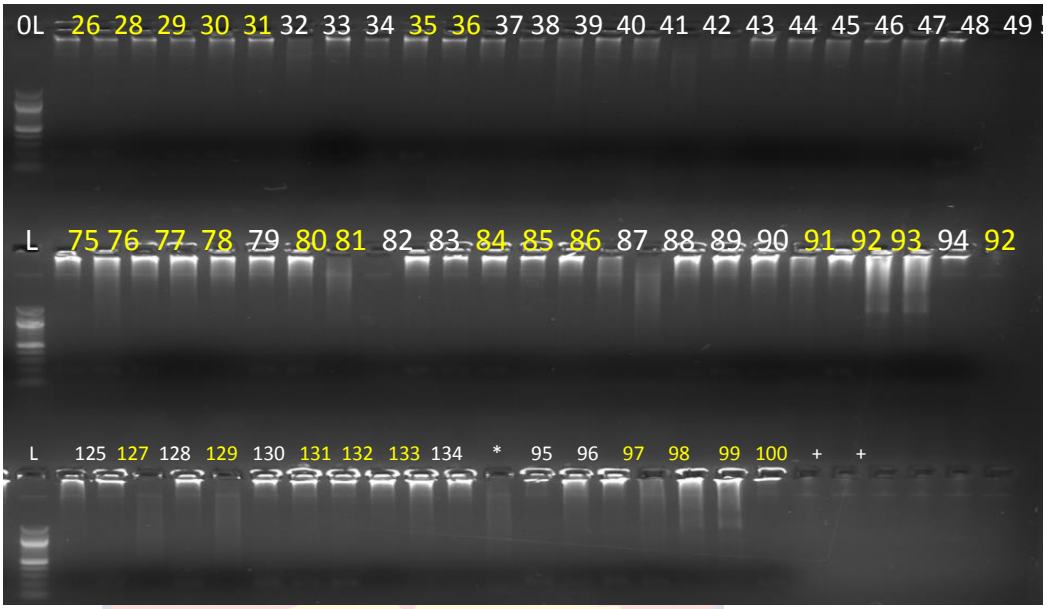

Figure 7: Gel Image showing DNA positive samples for P. falciparum with their sample IDs

Yellow numbered colour code represents *P. falciparum* positive samples and white represents negative samples.

Graphical Representation of Research Procedure