MALARIA-ASSOCIATED CHANGES IN FETAL SYNCYTIOTROPHOBLAST FUNCTION

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DEDICATION

I dedicate this thesis to my lovely father, the late Mr. Benedict John Kidima. Indeed you left me too early to see the fruit of the "seed" you sowed in me. I also dedicate this to my mom Margareth Lacha Kidima. To you both my parents, thank you for the unconditional love and guidance, and the gift of education you have offered me.

I am also dedicating this piece of work to my beautiful sons Leka and Kidima and to my husband Vincent Leyaro: I love you all so much.

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ABSTRACT

The fetus is entirely dependent on syncytiotrophoblasts (ST) that line the intervillous space (IVS) of the placenta as ST play a critical role in fetal development. During placental malaria (PM), *Plasmodium falciparum* infected erythrocytes (IE) bind to ST causing inflammation; leading to low birth weight babies due to fetal growth restriction (FGR) and preterm delivery (PTD). We hypothesize that the microenvironment that prevails during PM alters ST functions that are important for fetus growth.

We conducted genome-wide expression studies of fetal cells from PM-infected placentas to investigate whether changes occur in fetal cells during to PM. We then developed a protocol and established an *in vitro* system to investigate if the changes identified *in vivo* were occurring in ST. The BeWo cell line with characteristics of ST was co-cultured with CS2 IE, schizogonic products, cytokines and chemokines prepared *in vitro* by co-culturing THP-1IE.

Our microarray data showed that insulin like growth factor 1(IGF-1), insulin, mammalian target of ramphamycin (mTOR), vascular endothelia growth factor (VEGF), transforming growth factor 1 (TGFβ1), epidermal growth factor (EGF) and prostaglandin synthesis and regulation signaling pathways were significantly ($p \leq 0.05$) dysregulated in fetal cells from chronically PM-infected placentas. Several genes (leptin, system A amino acid transporters and placental 11beta-hydroxysteroud dehydorogenase-2-gene) known to be important in fetal growth) were also downregulated. *In vitro* results demonstrated that optimal response of BeWo cell line to CS2 IE occurred when BeWo were treated with 10µM forskolin for 72 hrs and cultured with 10 IE: 1 BeWo for 48 hrs. Using the same protocol, we showed inflammatory cytokines and chemokines, but not IE or IE with schizogonic products, significantly altered genes linked with vasculogenesis, blood

vessel formation and negative regulation of vasoconstriction. Interestingly, cytokines and chemokines significantly dysregulated growth pathways similar to those that were altered in chronically infected placentas, including IGF1, insulin, mTOR. TGFβ1, EGF, platelet derived growth factor and prostaglandin synthesis. We concluded that perturbation of ST functions by the cytokines and chemokines secreted by maternal cells upon exposure to IE explains the biological mechanisms that lead to increased risk of FGR during chronic PM.

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LIST OF ABBREVIATIONS AND SYMBOLS

IE	infected erythrocytes
ST	syncytiotrophoblast/trophoblasts
FGR	fetal growth restriction
LBW	low birth weight
IGF	insulin-like growth factor
IGFBP	IGF binding protein
IUGR	Intrauterine growth restriction
mTOR	mammalian target for ramphamycin
IVS	intervillous space
P.	Plasmodium
VEGF	vascular endothelial growth factor
PIGF	placental growth factor
PTD	pre-term delivery
TNF	Tumor Necrosis Factor
PfEMP 1	*Plasmodium falciparum* erythrocyte membrane protein 1
CSA	chondroitin sulphate A
VAR2CSA	variant PfEMP antigen exposed on CSA-binding erythrocytes
ATP	adenosine triphosphate
HSD	hydroxysteroid dehydrogenase
ANGPTL	angiopoetin-like
PAMP	pathogen associate molecular patterns
PRR	pattern recognition receptors

TLR	toll-like receptors
DNA	deoxyribonucleic acid
IL	interleukin
MIP	macrophage inhibitory protein
cAMP	cyclic adenomonophosphate
CS2	strain of *Plasmodium falciparum* that binds to CSA

CHAPTER 1

Introduction

1. 1 Placental malaria

Plasmodium falciparum is known to cause the most severe form of malaria; a disease that claims the lives of about one million people annually[1]. The disease is severe in children under the age of five and in pregnant women. In pregnant women, malaria is associated with an increased risk of poor pregnancy outcomes, including maternal anemia , preterm delivery (PTD) (i.e., delivery before 37 weeks of gestation) and fetal growth restriction (FGR) which is defined as inability of the fetus to reach genetically predetermined size and weight at term [2]. Anemia, preterm delivery, and FGR are major causes of malaria-associated low birth weight (LBW) babies. The prevalence of LBW babies as a result of placental malaria has been well documented in malaria endemic areas including; Thailand, Papua New Guinea and Sub-Saharan Africa countries and overall prevalence of malaria-associated LBW from studies from year 1985 to 2000 has been estimated to be 20% of live births. Estimates indicate that about 8% of malaria associated LBW babies are due to PTD; whereas, LBW caused by FGR ranges between 8-15% and those caused by maternal malaria-anemia are 20% in malaria endemic areas (reviewed in Steketee R. et al., 2001) [3]. Infants born with LBW not only have increased risks of dying in the first year of life, but have potential health problems in adulthood [4,5]. Studies have shown that the prevalence of infant mortality due to maternal malaria ranges from 50-160 per 1000 of live infants [100]. Efforts to understand the mechanism of disease pathology that leads to poor pregnant outcomes are important for finding targets for future intervention(s) and providing approaches that could lead to disease prevention.

Placental malaria occurs as a result of *P. falciparum* infected erythrocytes (IE) binding to syncytiotrophoblast (ST) receptors, mostly chondroitin sulphate A (CSA) and hyaluronic acid. Binding of IE to ST leads to sequestration of IE in the intervillous space (IVS) of the placenta. The ability of IE to bind on ST is conferred by the parasite's protein VAR2 chondroitin sulphate A (VAR2CSA) expressed on the surface of IE, a variant of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). Generally, sequestration of IE in the IVS leads to secretion of chemokines that recruit inflammatory cells that produce cytokines that have been reported to be associated with poor pregnancy outcomes [6,7,8].

Although placental malaria is largely asymptomatic, there is a correlation between peripheral and placental parasitemia at delivery. Placental malaria is categorized as acute, chronic, and past infection using specific pathological criteria [9] While chronic placental malaria has been associated with an increased risk of FGR, acute placental malaria has been associated with PTD in malaria endemic areas[10]. However, the major question to date is the mechanisms on how chronic placental malaria induces FGR and PTD.

1.2 Prevalence of placental malaria

Placental malaria is prevalent where *P. falciparum* is endemic. This includes most regions of sub-Saharan Africa, South America, and Asia. Of all endemic areas, Sub-Saharan Africa experiences the highest level of *P. falciparum* transmission, thus the burden of placental malaria-associated with poor pregnancy outcomes is high on the African continent. In Africa, both symptomatic and asymptomatic cases of placental malaria are common, with symptomatic infection with fever increase risk of PTD.

In high malaria transmission areas, placental malaria is more prevalent in women in their first pregnancy, i.e., primigravidae, as opposed to low transmission areas where susceptibility to placental malaria is similar in women of all gravidities. The reason for this observation is explained by exposure-related immunity. That is, subpopulations of parasites expressing VAR2CSA are selected for during pregnancy and therefore are only present in pregnant women. Consequently, primigravidae, lack immunity towards VAR2CSA, making them highly susceptible to placental malaria. This phenomenon explains the difference observed in the burden of placental malaria among women of different gravidities between low versus high malaria transmission areas. That is, while the burden of placental malaria is more among the primigravidae who lack immunity to placental malaria in high transmission areas, in low transmission area the burden is across all gravidity groups since they all lack protection from placenta malaria . Apart from gravidity, maternal age has also been shown to be an independent risk factor for malaria in pregnancy, indicating malaria-age-specific immunity is also important in placental malaria [11,12] Infection by Human immunodeficiency virus (HIV) have also been shown to increase prevalence of placental malaria in pregnant women[13].

Cross-sectional studies have shown that peak prevalence of malaria measured by peripheral parasitemia is around the second trimester [14]. Studies by Huynh et al.,(2011)[15] and Contrell et al., (2007) [16] have shown that the prevalence of parasitemia in the first trimester is a contributing factor for poor pregnancy outcomes. In addition, peripheral parasitemia in the third trimester has been positively associated with FGR[17]. Together, these observations suggest malaria may affect pregnancy outcome anytime across the gestational period.

1.3 Pathogenesis of placental malaria

Maternal responses to placental malaria

Although pathogenesis of placental malaria is not completely understood, IE bind to fetal ST and induce chemokines that recruit maternal monocytes into the IVS, resulting into elevated levels of pro-inflammatory cytokines. Chemokines that aid in recruitment and adhesion of monocytes and macrophage to the IVS include MCP-1, MIP-1α, MIP-1β, IP-10, 1-309 and IL- 8 [18]. In addition, studies in pregnant women from malaria endemic areas reported that levels of Th1 cytokines are high in the placenta plasma of placenta malaria-positive women, including TNFα, IFNγ [8, 6] and IL-2, [8]. Other cytokines associated with placental malaria are IL -10 and macrophage inhibition factor (MFI) [19,20]. Elevated secretion of cytokines by maternal monocytes has been associated with LBW babies [8,20,] with women in their first pregnancy being at the greatest risk.

Evidence fetal trophoblasts respond to malaria

The role of fetal tissue in the pathogenesis of placental malaria is only beginning to be unveiled. Studies have shown that ST respond to IE and natural hemozoin (a pigmented by-product of *P. falciparum* digestion of hemoglobin) by secreting cytokines and chemokines commonly found in placental malaria. In *in vitro* studies using primary trophoblasts and BeWo cell line, Lucchi et al., [21,22] showed that the binding of CSA-binding parasites to BeWo stimulated BeWo to secrete MIF, MIP-1α/CCL3 (detected at protein level) and TGFβ (at transcription level), as well as, induced phosphorylation of trophoblasts proteins [22,23]. Additional data from the same group indicated that hemozoin pigment could activate the ERK1/2 pathway and induce secretion of the chemokines CXCL8, CCL3 and CCL4 as well as the cytokine TNFα and soluble intracellular adhesion molecule-1 (ICAM-1) [21]. These results therefore suggest that ST respond to the binding of IE hemozoin pigment and malarial bi-products. Moreover, the

phosphorylation of trophoblast proteins following IE binding implies that changes in the regulation of protein functions are induced. However, during schizogony, IE may produce other soluble and insoluble bioactive molecules to which ST might respond. Studies identifying the response of ST to IE, hemozoin, and other IE bioactive molecules are needed.

1.4 Placental malaria pathology and pregnancy outcome

Studies on biopsies of placental malaria-infected placentas have characterized histological and immunochemical changes associated with placental malaria. Early histological studies by Bulmer et al.,1984 characterized placental malaria into four main groups or categories, namely i) *acute infection* defined as presence of parasites in maternal erythrocytes and malaria pigment in erythrocytes at IVS without inflammation, ii) *chronic malaria* as presence of parasites in maternal erythrocytes in IVS and pigment in maternal erythrocytes, monocytes, fibrin or villous ST, iii) *past infection* characterized as no IE and placental tissue having pigment confined to fibrin or cell within fibrin, and iv) placental malaria negative; no evidence of infection [9]. Generally, chronic placental malaria has been associated with FGR babies; whereas, acute placental malaria has been linked to PTD [24]. Other pathological variables like severe mononuclear intervillositis and severe pigment deposition in placental villi have also been shown to be associated with LBW babies[10, 9].

Occurrence of localized destruction of placental villi, ST degradation, increased syncytial knotting, trophoblast basement membrane thickening, and fibrinoid deposition on placental villi in malaria-infected placentas has also been reported [25, 26]. Currently, a study has reported ST denudation (thinning or loss of the ST layer) in chronic infected placenta, indicating ST integrity may be compromised during placental malaria [27].

1.5 Consequences of placenta malaria to neonates

Congenital malaria

Congenital malaria is defined as malaria acquired *in utero*, leading to the presence of parasites in the infant during the first week of life. *P. falciparum* IE have been reported in cord blood of newborns in malaria endemic areas [28]. Although congenital malaria is rare with an estimated prevalence of about 4-6% [29], its impact on a child's health is may be significant. The impact of malaria during the first year of life leads to an increased risk of anemia, infant morbidity and mortality [30].

Susceptibility of malaria during the first year of life

During placental malaria, the fetus can be exposed to soluble and insoluble malarial antigens *in utero*. Exposure to malaria antigens may influence the fetal immune responses [31, 32]. While activation of malaria-specific immune responses may be beneficial to the development of a child's antimalarial immunity, exposure without priming of fetal immune cells might result into tolerance [33] ; a phenomenon that may partially explain the increased susceptibility of children to malaria in the first year of life.

Anemia-induced low birth weight

Studies show that placental malaria-positive mothers are likely to be anemic (ref). Anemia may result from increased RBC destruction during malaria through rupture, phagocytosis of IE, phagocytosis of non-IE in the spleen, and hyper-splenomegaly (reviewed in Clark et. al., 2006)[34]. Other patho-physiological mechanisms include inflammation-induced erythroid hypoplasia, suppression of erythropoietin synthesis, and dyserythropoiesis[35]. It has been proposed that anemia may affect fetus growth by affecting oxygen transport to the fetus. Positive correlation between placental malaria-induced maternal anemia and LBW has been reported, with women having hemoglobin level below 7g/L having a higher risk of LBW babies [8].

Low birth weight related to premature delivery

Low birth weight, as a result of placental malaria associated-PTD, is common in both seasonal and intense perennial malaria transmission areas. Suguitan et al., 2003 showed that cytokine imbalance during placental malaria due to acute infection increases a woman's chances of having a PTD [6]. Other studies in endemic areas from women with placental malaria have reported that cord blood parasitaemia [36] and placental parasitemia [6,36] correlated with PTD. Suguitan and colleagues, also reported a correlation between anemia and PTD,[6] as well as placental parasitaemia of >1% and over expression of placental IL-10. Generally, it has been suggested that placental inflammation and dysfunction due to acute infection are important factors leading to PTD.

Low birth weight related to fetal growth restriction

Theories explaining mechanism of LBW due to FGR during placental malaria are still being debated. The most prevailing theory is that placental insufficiency associated with parasite sequestration and inflammation in the IVS leads to FGR. The placental insufficiency theory attributes malaria as having the ability to reduce placental capacity to transport oxygen and nutrients to the fetus by impairing formation and remodeling of placental vasculature. Early studies in Kenya by Dorman et al.,(2001), demonstrated the effect of maternal malaria, as measured by peripheral parasitemia, on uterine blood flow[37]. In this study, parasitemia during the third trimester of gestation was associated with abnormal uterine blood flow as measured by Doppler ultrasound, suggestive of impairment of blood flow after normal placental development. However, a longitudinal study on umbilical artery Doppler flow velocity waveforms in women attending antenatal clinic in Malawi, suggested a potential influence of malaria early during placenta development [38]. These two studies indicate that malaria during pregnancy may either

affect placental development early or dysregulate hemodynamics though vasoconstriction or vascular damage later in gestation[37].

Dysregulation of ST functions have also been proposed to be responsible for FGR in placental malaria. Several in *vivo* and *in vitro studies* have implicated dysregulation of placental angiogenesis [39,40] and transporting activities [41] during placental malaria. Since placental malaria exposes the fetal ST to *P. falciparum*-IE, hemozoin, parasites bi-products, cytokines, and maternal cells, impairment of their normal functioning is likely. It is important that the nature and extent of placental functions that are hampered by placental malaria be investigated.

1.6 The placenta and its response to malaria

1.6.1 Overview of the placenta

The placenta plays a vital role in regulating the exchange of nutrient and gases, water, ions and waste products between the mother and developing fetus [42]. It consist of ST, cytotrophoblast, the connective tissue villous tree, and the endothelia cells of the fetus capillaries (Fig 1.1). Cytotrophobasts differentiate into ST during villous formation and development [43]. Of all the cells, ST are the dominant cell type for controlling fetal development, and create a thin multinucleated surface that covers the villous trees. Syncytiotrophoblast maintains fetal homoeostasis by conducting physiological functions that, after delivery, are carried out by the kidneys, gastrointestinal tract, lungs and endocrine glands of the new born [44, 45,46].

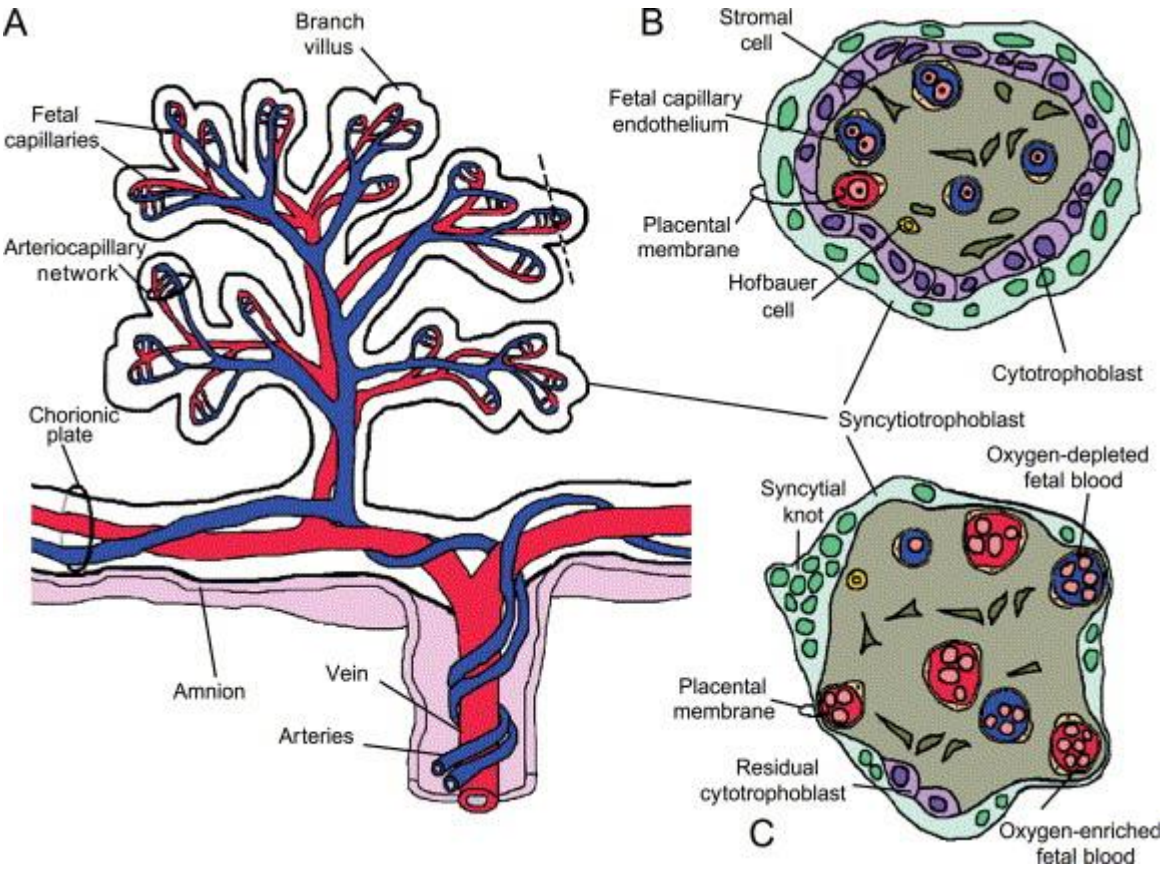

Figure 1.1 Fetal placental circulation A, chorionic villous B section chorionic villous at approximately 10 weeks C section through the chorionic villous (At full termhttp://origin-ars.els-cdn.com.eres.library.manoa.hawaii.edu/content/image/1-s2.0-S0049384804003421-gr1.jpg)

At the end of the first trimester, the maternal blood supply becomes established and blood enters the placenta via spiral arteries into the IVS. On the fetal side, fetal blood enters the placenta through two umbilical arteries, which branch and form a capillary network in the terminal villi of the villous tree. The terminal villous trees freely float in the maternal blood of the IVS.

The ST layer has an intimate relationship with fetal vessels [47] (Fig 1.1). Thus, the perturbations at the maternal-fetal interface can potentially alter the histology and physiological functions of the ST and therefore growth and development of the embryo.

1.6.2 Functions of the placental trophoblasts

Physical barrier protects the fetus from pathogens

The ST form a physical barrier, separating maternal and fetal blood. Under normal circumstances, ST also prevents hematogenous transmission of infection from mother to the fetus. The barrier has additional antimicrobial activities: synthesizing high levels of nitric oxide synthase, producing microvillus-associated glycosaminoglycans, and secreting interferon's which are antiviral. Thus, ST perform an important role in the first line of defense.

Immunological protection

The fetal trophoblastic cells, unlike other nucleated cells, do not express classical HLA A and B, but expresses HLA E and G which are ligands for inhibitory receptors found on natural killer cells, the CD94/NKG2/A/B/C [48]. Interaction between HLA G and E expressed on fetal trophoblasts and natural killer inhibitory receptors, prevent killing of ST by maternal NK cells, cytotoxic T cells, and macrophages.

Innate active barrier to pathogens

Fetal trophoblasts are immunological active cells that help prevent pathogens, such as bacteria, parasites and viruses, from crossing the maternal fetal interface. Like immune cells, trophoblast express pattern recognition receptors (PRR), including toll-like receptors (TLR 1-10) [49,50,51] and nucleotide-binding oligomerization domain (NOD) receptors. While TLR recognize and respond to both extracellular and intracellular pathogen-associated molecular patterns (PAMPS), NOD receptors are cytosolic

sensors. Studies on human trophoblasts have shown that TLR expression on trophoblasts differ with gestation age with certain types of TLR being differentially expressed in the first, second or third trimesters (reviewed in Abraham M (2008) [52]. This temporal expression of TLR across gestation suggests that different responses are produced by trophoblasts to various TLR ligands at different points in gestation. For example, TLR2 forms heterodimers with either TLR1 or 6 upon stimulation. Since TLR6 is only expressed by third trimester trophoblasts, TLR2 ligands may not induce a response earlier in gestation. Furthermore, TLR2/TLR1 stimulation leads to apoptotic death in the first trimester trophoblast; whereas, ligation of the same ligand through TLR2/TLR6 heterodimers prevents cell death in human third trimester primary trophoblast [53].

P. falciparum has a number of well-characterized PAMPs. For instance, most malaria parasite proteins are anchored to glycosylphosphatidylinositol (GPI), at the C-terminus of the protein. The parasite GPI is known to activate TLR2/TLR1 and TLR2/TLR6 heterodimers. [54] Furthermore hemozoin, a malarial pigment, activates the NOD-like receptor called NLRP3 inflammasome [55] and parasite DNA is known to stimulate TLR9. Generally, PRR-expression by the fetal trophoblasts aids in combating pathogens in the IVS, including parasites, viruses and bacteria, but the resulting immune response they induce may contribute to poor neonatal outcomes associated with malaria IE and other infections at the maternal fetal interface.

Transport and metabolism

Syncytiotrophoblast, the major transporting epithelium in the placenta, are polarized with the ability for uptake and transfer of nutrients, such as amino acids, glucose, fatty acids, minerals, cholesterol, and some xenobiotics. The apical surface of ST is perfused by

maternal blood; whereas, the basal membrane is in intimate contact with fetal capillary networks. The ST regulates maternal-fetal transport of nutrients, including glucose, amino acids, lipids, gases, and ions. Owing to its syncytial nature, transplacental transfer of nutrients, e.g., amino acid and glucose, is made possible by carrier proteins that are expressed on both apical and basal membranes of the ST. Generally, the driving force of the transport of most nutrients through ST depends on concentration and an electrochemical gradient (e.g., glucose and ions). However, another form of transport operates against concentration gradient of solutes and thus requires energy in terms of hydrolysis of Adenosine-5'-triphosphate (ATP). Amino acid transporters require energy in the form of ATP to transfer amino acids from the apical surface to the basal membrane of ST.

Fetal growth is dependent upon the ST transporting activities of various transporters. Accordingly, fetuses with restricted growth often have low plasma concentrations of amino acids and are hypoglycemic. This association has been reported by Glazier et al., 1997 and Jasson et al., 2002 [56], who have shown that the activities of several transporters are dysregulated in pregnancies associated with idiopathic FGR, including transporters for system A amino acids (SLC38A), leucine, sodium-dependent and -independent taurine, Na+/K+-ATPase, Ca2+ and Na+/H+ exchanger. Uptake of nutrients by the fetal ST influences metabolism and subsequent nutrient delivery to the fetus. However, little is known about how nutrients modify placental malaria pathogenesis, particularly the activities and expression of the transporters and therefore nutrient uptake during disease processes.

Transfer of nutrients across ST is influenced by the uteroplacental and umbilical blood flow as well as the activity or expression of transporters on the ST which may be

regulated by various fetal, maternal, and placental signals [57]. Several placental genes, placental signaling molecules, and placental hormones synthesized and secreted by the placenta facilitate transport of nutrients across ST (reviewed in Lager & Powel 2012) [57], influencing fetus growth.

1.6.3 Endocrine functions

Syncytiotrophoblasts are the main coordinators of the biomolecular interactions that occur between the fetus and the mother [80]. Generally, ST produce growth factors and hormones that regulate and support fetal growth, as well as, integrating various maternal and fetal signals. The hormones, growth factors, signalling pathways and proteins produced by ST can be categorized in the following functional groups.

Regulation of vasculogenesis and angiogenesis

Vasculogenesis and angiogenesis are processes essential for creating and maintaining utero-placental blood flow, and therefore, influence the exchange of nutrients between mother and fetus. While angiogenesis refers to as the formation of new blood vessels from pre-existing ones, vasculogenesis is the formation of blood vessels from endothelia cells. The main factors produced by ST that regulate vasculogenesis and angiogenesis are vascular endothelial growth factors (VEGF) and placental growth factor (PIGF). The VEGF induces its action via two specific receptors; VEGFR 1 (FLT) and VEGFR 2, by inducing the production of nitric oxide (NO) from trophoblasts. Literature suggests that VEGF, PIGF and their respective receptors are important for regulating trophoblast survival and angiogenesis. VEGF and EGF induce angiogenesis and regulate vasculogenesis, and therefore placental vasculature. Dysregulation of angiogenesis has been implicated in FGR [58,37].

Potentiating angiogenesis are placental angiopoietins 1 (ANGPT 1 and 2). The ANGPT are expressed by the trophoblasts and induce their effect via receptor kinases, *Tunica* internal endothelial cell kinase-2 (Tie-2) receptor expressed on endothelial cells. Ang-Tie forms a vascular-specific ligand/receptor system that controls endothelia cell survival and vascular maturation, as well as plays a role of in the development of placental maturation and therefore growth.

Placental vasculature is also maintained by factors called vasodilators and vasoconstrictors that are produced locally by the trophoblasts. The common placental trophoblast vasodilators and vasoconstrictors include the renin-angiotensin system, arachinoid metabolites (thromboxane and prostacyclin), endothelin and its receptors, as well as nitric oxide. The balance between vasodilators and constrictors is important for homeostatic balance of placental vasculature [80].

Overall, angiogenesis is essential for facilitating *utero*-placental blood flow; therefore, exchange of nutrients and gas between the mother and the fetus. Alteration of angiogenic factors during pregnancy is associated with bad pregnancy outcomes particularly FGR-induced LBW.

Trans placental transfer of nutrients, cells survival and proliferation

IGF-1 axis

The insulin-like growth factor (IGF) axis consists of two polypeptide hormones, IGF-1 and IGF 2; cell surface receptors IGF-1R and IGF-2R; soluble IGF-binding proteins; and an IGF-binding protease, which all control growth of many organs, including the placenta. During pregnancy, the insulin growth factors are mainly secreted by the placenta and the growing fetus; thereby, regulating fetus and placental growth

throughout gestation in both autocrine and paracrine fashions. [59,60] Gene-disruption studies show that mice carrying the mutant allele of IGF-1 have a 40% reduced birth weight; whereas, mutation in the IGF-1 gene in one human severely reduced fetus growth by 60%, as well as subsequent postnatal growth [61]. On the other hand, IGF-R2 gene targeting in mice resulted in placental and fetus over-growth [62]. In addition, Laviola et al*.*, *(*2005) found a decrease in expression of IGF-1R and signaling molecules in human intrauterine FGR restricted-placentas.[63] These observations indicate that impairment of both IGF-I action through absence of IGFs expression and IGF signaling molecules may lead to abnormal fetus and placental growth.

Dysregulation of the IGF axis has been studied in LBW babies and pathological pregnancies due to various causes. Chiesa et al. (2008) reported an association of reduced maternal IGF-1 with intrauterine growth restriction (IUGR), with IGF-1 concentration being lower in pregnancies with IUGR than in normal birth weight babies[64]. In addition, studies in preeclamptic patients showed alteration of IGFBP-1 and IGF-2 at the fetus: maternal interface [65]. More recently, it has been shown that IGF-1 concentrations in the cord blood of neonates born to placental malaria-positive mothers is lower than those born to mothers without placental malaria and these positively correlated with birth weight [66]. In this study, maternal IGF-1 concentration was positively correlated with chronic placental malaria.

IGF-1 signaling

IGFs are structurally similar to insulin [67] and so are their receptors. The activity of IGF-1 is induced via tyrosine kinase [60]. IGF-1 and IGF-2 can initiate their mitogenic and metabolic effects by binding to IGF-R1, whereas, IGF-2 can only bind to IGF-2R. IGF-1 signaling involves two main pathways, namely the MAPK/ERK and PI3K/AKT pathways

[68]. Signaling through these two pathways leads to cells proliferation and survival by enhancing protein synthesis, amino and glucose uptake, prevention of apoptosis, and angiogenesis.

Local bioavailability of IGF-I and 2 is controlled by soluble IGFBPs [69,70, 71] which are secreted by many cell types, including cells at the maternal : fetal interface [65]. Of all the regulators, IGFBP-I has been shown to be the most important regulator of IGF functions. IGFBP-1 binds IGF-I and 2 with higher affinity than their respective IGF receptors, thereby inhibiting their mitogenic effect.

Leptin

Originally leptin was considered as adipose-derived signaling molecule that was important in central metabolism, but it also has an influence on placental cells during pregnancy [72,73] as well as having some immune-modulating properties. In the placenta both leptin protein and receptors are expressed on ST [74,75,76] and studies suggested that leptin acts in both a paracrine and autocrine fashion. [77] *In vitro* studies have indicated that leptin induces proliferation of trophoblastic cells, [78] has a positive impact on angiogenesis, and provides an anti-apoptotic effect [79] on ST survival. Using human trophoblastic cell lines and isolated human villous explants, Pérez-Pérez et al. [80] showed that leptin stimulated protein synthesis [81] and influenced placental amino acid transport. Data from Von Versen-Höynck and colleague [82] showed an increase in system A amino acid transporter activities after incubation of placental villous tissue with leptin. These data demonstrate that leptin is important hormone for the growth of the fetus.

1.6.4 Proteins and pathways important for fetus growth

Placental mammalian target for ramphamycin (mTOR) pathway

The placental mTOR protein, expressed in the cytosol of ST [83], is a protein kinase that facilitates and promotes translation of proteins important for cellular growth. The activities of placental mTOR are regulated by growth factors, hormones, nutrients, such as amino acids, glucose [43,83–85] energy, and stress [43]. Studies using human trophoblastic cells by Ross et al. 2009 reported that activation of amino acid transporting activities by the growth factors, IGF-1 and insulin, occurred through the mTOR signaling pathway[86]. Consequently, mTOR activities were reported to be reduced in IUGR placentas. [87] These studies suggest a possible role for the placental mTOR signaling pathway in regulating fetal growth by influencing transplacental transport of nutrients, predominantly amino acids.

Placental enzymes 11β-hydroxysteroid dehydrogenase (11β-HSD-2 and 1)

The 11β-HSD isoforms are essential enzymes that maintain the placental cortisol concentration. They are expressed by all trophoblasts. Although cortisol is essential for regulation of fetus development, excess cortisol or prolonged exposure of the fetus to cortisol has detrimental effects on fetus growth. Maternal cortisol concentrations are higher than those of the fetus [88]. Owing to its lipophilic nature, cortisol readily crosses the placental barrier to the fetus. Placental 11β-HSD-2 protects the fetus from endogenous maternal cortisol by converting active cortisol into inactive form, cortisone. Reduced activities of 11β-HSD-2 have been reported to associate with FGR. [88] In addition, the 11β-HSD-2 has been implicated in fetal programming in which inactivity of this enzyme during pregnancy increases the risk of newborn predisposition to later diseases in life [89,90].

Xenobiotic metabolizing enzymes

Xenobiotics are defined as any chemical that is found in an organism, but which is not normally produced or expected to be present in it. Syncytiotrophoblasts express a wide variety of enzymes that metabolize, bio-transform, and detoxify xenobiotics including drugs and some environmental toxins; thereby, protecting the fetus from their potential toxic effects. The metabolizing enzymes include phase I metabolizing enzymes, some cytochrome P450 isoforms [45] and other enzymes involved in phase II metabolism. The placental phase II enzymes are comprised of glutathione S-transferases, UDP-glucoronosyltransferases, epoxide hydrolyses, and sulfotransfarase enzymes among others (reviewed in [91]). While phase I metabolizing enzymes are mainly involved in oxidative, reductive and hydrolyzing activities, phase II enzymes detoxify xenobiotics by conjugating them with various functional groups, thereby enhancing their excretion. However, several reactive intermediates generated by the phase II enzymes can be toxic. Thus, if generated endogenously in ST, exposure of the fetus to toxins can potentially cause adverse outcome as reported in some studies (reviewed in).[92]

1.6.5. Possible effect of placental malaria on fetal growth

As noted above, ST play significant roles in the growth of the baby *in utero*. The binding of IE to ST may cause changes in ST function. Still, much to be learned on what the changes are*. In vivo* studies have measured growth factor levels in maternal peripheral and intervillous blood. Studies have found decrease in factors for angiogenesis (e.g., VEGF/Fltl, endoglin, ANG-1), growth (IGF-1, leptin), and but it remains unclear if these factors are dysregulated in fetal cells and whether the changes are brought about as a result of parasite binding to ST or infiltration of maternal monocytes at IVS and associated inflammatory cytokines.

1.7 BeWo cell line

Origin and characteristic

The BeWo cell line was derived about 1959 from a human gestational choriocarcinoma and established by serial transplantation in the hamster cheek pouch. [93] Patillo and Gey (1968) [94] adapted BeWo to continuous *in vitro* culture and reported they had similar endocrine characteristics as normal trophoblasts cells. BeWo cell line secretes functional marker of trophoblasts, e.g., human chorionic gonadotrophin (hCG) and other hormones characteristic of pregnancy demonstrating BeWo retain many normal placental functions.

Phenotypically, BeWo cell lines are similar to the cytotrophoblablast. Treating BeWo with a fusogenic compound that activate cAMP (forskolin), stimulates BeWo to acquire characteristics of ST, including formation of multinucleated giant cells and secretion of hCG [95]. Studies by Kudo et al., (2004) have demonstrated that treatment of BeWo with forskolin induces BeWo to express genes that are important for placental hormone synthesis and trans-epithelial transport. [95] In addition, gene-expression studies have documented expression of genes that code for various placental hormones, such as placental growth hormones, placental growth factor, chorionic somatotrophin hormone 1, and some glycoprotein in BeWo cell line treated with forskolin [96]. Others genes induced are enzymes important for metabolism, such as cytochrome p450 family, Glutathione S transferase the hydroxysteroid 17 beta dehydrogenase. Together these observations suggest close similarities between BeWo cell line and placental syncytial trophoblasts.

BeWo cell line and placental binding parasites

Monolayer of BeWo cells forms a structural surface that binds to the *P. falciparum*-IE expressing VAR2CSA, showing that BeWo do express the CSA ligand. This characteristic has made BeWo suitable for selecting *in vitro P. falciparum* IE that selectively binds to the placenta during malaria via CSA [97].

1.8 CS2 Parasites

CS2 parasite culture

Historically, placental-binding parasites were derived from parasite strain FAF-EA8CHO5, D7CHO6 and E10CHO6 originally derived from *P. falciparum*-Brazilian isolate, ITG2F6. The placental-binding parasite phenotype from these strains was derived by panning of infected erythrocyte on Chinese hamster ovarian cells 5 to 6 times. The specificity of binding of these placental binding parasites collectively known as CS2 *P. falciparum* strain occurs via binding of VAR2CSA to a proteoglycan, CSA. Evidently, the FAF-EA8CHO5, D7CHO6 and E10CHO6 parasite strain bind CSA and HA[98,99] expressed on ST.

1.9 Summary and goals of the study

The contribution of fetal cells in the pathogenesis of placental malaria is currently unknown; this impedes the understanding of biology of placenta in response to malaria. Maintaining placental function throughout gestation is essential for the growth of the fetus. Unfortunately, placental malaria changes the micro-environment in the IVS of the placenta, increasing the risk of poor neonatal outcomes. Recent studies of placental plasma from placental malaria-positive women provided evidence for the dysregulation of ST functions, including IGF, leptin, VEG, ANG1/ANG2, soluble fms-like tyrosine kinase 1 (sFLT) and soluble endoglin [39, 66,100,40]. In these studies, important factors for fetus growth were dysregulated in placental malaria-positive mothers who delivered LBW babies. However, it is hypothesized that placental inflammation not IE, is the cause of development of LBW babies due to FGR. It is still not understood how inflammation might increase the risk of FGR. It is important to understand the pathophysiology induced by placental malaria and identify mechanisms that lead to poor pregnancy outcomes. Establishing the role ST in the pathogenesis of placental malaria may aid in understanding the pathophysiology induced by placental malaria and will have implications in finding focus that could lead to prevention of poor neonatal outcome.

Evidence are already showing that binding of infected erythrocytes to syncytiotrophoblasts increases the tyrosine phosphorylation of proteins in ST cells and that ST respond to infected erythrocyte, hemozoin and parasite DNA by secreting chemokines and cytokines [22,101,102]. However, direct influence of IE on ST functions has not been characterized. Placental malaria exposes ST to multiple parasite factors, including hemozoin, parasite DNA -hemozoin complexes, parasite GPI-anchored protein and other soluble and insoluble proteins. For example glycosylphosphatidylinisitol (GPI) anchor is a TLR-2/TLR1 and TLR2/TLR6 heterodimer ligand, hemozoin is an NALP3

inflammasome activator, and TLR9 is activated by parasite's DNA-hemozoin complex. It is possible that activation of these ST's PRR by parasite components alter the physiology and therefore dysregulate ST functions during placental malaria Fig.1.2.

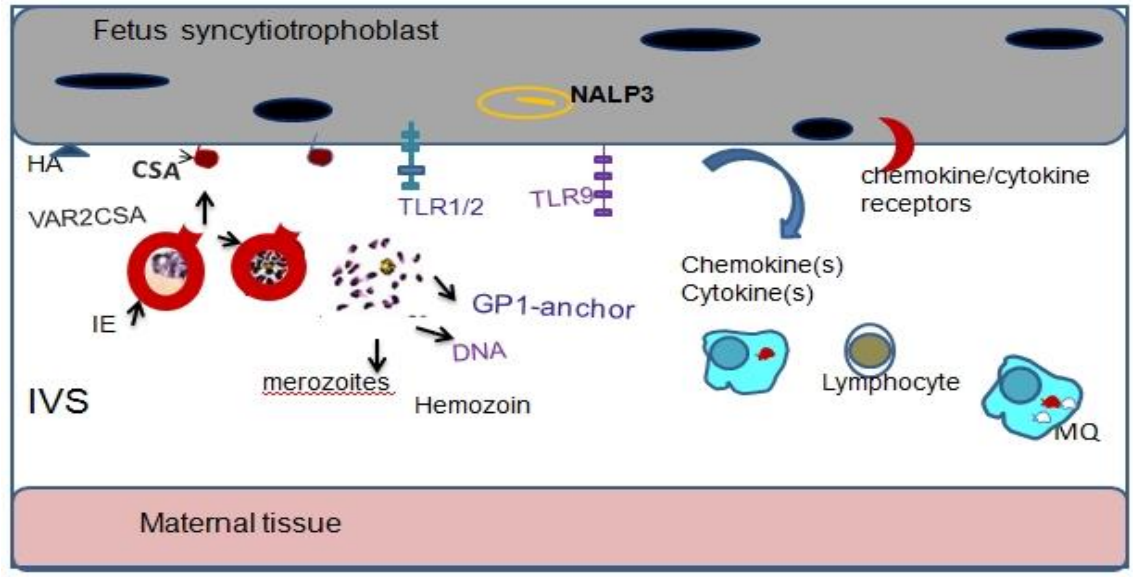

Figure 1.2 Microenvironment in the intervillous space during placental malaria, interaction of IE, parasites bioactive of schizogony with ST receptors

Consequently, if IE induces changes in ST that is associated with increased risk of FGR, a vaccine that will generate antibodies to prevent binding of infected erythrocyte to ST and therefore sequestration, would be vital. However if inflammation is responsible for the changes of ST function during placental malaria, then prompt and better diagnosis remains to be important.

Overall Aims

Overall aim of this study is to investigate placental malaria-associated changes in syncytiotrophoblast functions and determine if binding of IE, parasite products or cytokines induce the changes.

Hypotheses

- *Placental malaria will dysregulate metabolic, cellular and regulatory pathways that are important for placental and fetal growth.*
- *The binding of IE and parasite molecules released during schizogony will induce downregulation of pathways involved in placental and fetal growth.*

Aim 1

To identify changes in fetal cells from placental malaria infected placentas using genome-wide microarray analysis.

Hypothesis

Placental malaria will dysregulate metabolic, cellular and regulatory pathways that are important for placental and fetal growth.

Aim 2

Establish an *in vitro* model using the human trophoblastic BeWo cell line to determine -

i. If changes occur in ST functions.
ii. If factors contributing to fetal growth are altered by *P. falciparum*-infected erythrocytes.

Aim 3

Determine if infected erythrocytes, infected erythrocytes plus schizogony products, and a mixture of proinflammatory cytokines induce changes in gene expression or pathways in ST that are important for fetal growth

Hypothesis

BeWo cell lines exposed to both bound parasites and bi-products will dysregulate more placental pathways than those induced by binding of intact malaria parasites alone and cytokines.

CHAPTER 2

Malaria Associated Changes in Syncytiotrophoblast Function

To be submitted to *Placenta*

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Abstract

During placental malaria, infected erythrocytes adhere to fetal syncytiotrophoblasts (ST) and sequester in the intervillous space (IVS), creating pathology that leads to poor pregnancy outcome including low birth weight (LBW) babies. The biological mechanisms that occur in the placenta leading to LBW babies, especially fetal growth restriction, are unknown. Syncytiotrophoblasts line the IVS and play a critical role in fetal development. Information on the contribution of ST in malaria pathogenesis will aid in understanding the biology of placental malaria and may help identify preventive measures.

We carried out genome wide expression analysis of fetal cells from biopsies of 3 malaria-positive placentas and compared the results with a pool of 6 placental malaria-negative-placentas. The biopsies contained fetal ST, stromal cells, macrophages, and blood vessel endothelial cells. We assessed the nature of biological relationship between genes that were differentially-expressed using microarrays and pathway analysis with Pathway Miner Version 1.1. Metabolic, cellular and regulatory pathways were significantly altered during placental malaria. These included dysregulation of the Insulin / IGF-1, mammalian target for ramphamycin (mTOR) and vascular endothelial growth factor (VEGF) signaling pathways. In addition, the transforming growth factor β (TGF β) and epidermal growth factor (EGF) signaling pathways were altered. Several genes encoding proteins known to be important in birth weight were down-regulated; leptin, system A amino acid transporter, placental inhibin A, and insulin-regulated glucose transporter gene. Placental 11 beta-hydroxysteroid dehydrogenase-2 enzyme, which prevents maternal cortisol from reaching fetus compartment, was also down-regulated, indicating the placental barrier might be compromised.

Placental malaria perturbs normal functioning of fetal cells, including changes that are potential risk factors for reduced fetal growth. The pathways that are altered can be used as targets for intervention against placental-malaria related outcomes.

2.1 Introduction

The accumulation of *Plasmodium falciparum* infected erythrocytes (IE) in the placenta changes the microenvironment in the intervillous space (IVS). Initially the IE bind to syncytiotrophoblast (ST) resulting in the accumulation of IE in the IVS, initiating an inflammatory response, including infiltration of maternal inflammatory cells that produce type 1 cytokines. Although inflammatory responses have been associated with low birth weight (LBW) babies, the pathogenic mechanism(s) responsible for poor neonatal outcome are still unclear. In addition, the contribution of fetal cells to disease pathogenesis is not well studied. Understanding the contribution of fetal cells to placental malaria pathology is important as it may lead to the identification of targets for potential intervention.

The objective of this study was to investigate changes in fetal tissues lining the IVS induced by placental malaria. Fetal microvilli are covered by fetal ST that are in direct contact with maternal blood, where they play an important role in fetal growth. ST also synthesize and secrete hormones for placental and fetal growth, as well as, regulating transport and synthesis of nutrients [103,104,103.] In addition, ST metabolize toxins and drugs acquired from maternal circulation [45,46,105]. All of these processes are tightly regulated. Impairment of any of these physiological functions can compromise fetal growth.

Studies show that in pregnant women who have placental malaria, the level of some hormones synthesized by the placenta that are important for fetal and placenta development are dysregulated. [39,40,106,66] Furthermore, studies have documented that the binding of IE induces biochemical changes in ST [22,107]. These observations suggest the potential involvement of fetal-derived cells in disease pathogenesis. However, to date, the nature and extent of the contribution of fetal-derived cells to disease

pathogenesis remains unclear. Thus, it is important to identify changes in ST that contribute to malaria-associated placental pathology that increase the risk of poor pregnancy outcomes. Based on the literature, ***we*** **hypothesize that** ***placental malaria changes ST function, resulting in dysregulation of pathways in ST that are important for fetal growth*** **and** ***modification of signals that facilitate growth and transport capabilities of ST***. We carried out genome-wide genes expression analysis of fetal tissue from malaria-infected placentas compared to non-infected placentas from women in living in a malaria endemic area using human DNA microarray. Differentially-expressed genes were subjected to pathway analysis.

2.2 Materials and methods

Placenta tissue Thirty placentas from consenting women were obtained within 15 minutes of delivery from women living in Yaoundé, Cameroon. Biopsies were collected, decidual tissue was dissected away, maternal IVS blood was removed by extensive washing, and the residual fetal tissue (containing ST, stromal cells, Hofbaeur macrophages, and fetal blood vessels) was placed in RNAlater and kept at -80ºC.

Histological sections of placental tissue Placental tissues were analyzed for presence or absence of IE in IVS, hemozoin in the IVS or monocytes or ST, and lymphocytes in the IVS. The placenta malaria status of the tissue was classified using Bulmer classification[108].

DNA microarrays Human DNA microarrays were produced in the molecular genomic core facility of Drexel University of Medicine using the Opero Human Oligo v2.0 set containing ≈ 21,000 Oligo nucleotides (70mer). The Oligonucleotide representing well characterized human genes from UniGene Database.

RNA extraction and RNA quality assessment Total RNA from placenta malaria positive and negative fetal tissues were extracted using an RNeasy RNA isolation kit (Qiagen, Inc.). The negative placental malaria control consisted of a pool of six fetal tissues collected from placental malaria-negative women. The pool used as control ensured an effective assessment of differentially-expressed genes induced by malaria without bias. The quality of RNA was assessed by measuring O.D. with a Nanodrop spectrophotometer (Thermo scientific). RNA at 260/280 and ratio of >2.00 was considered pure enough for further study.

cDNA synthesis Complimentary DNA (cDNA) synthesis from isolated RNA was conducted using Amino Ally Message Amp II aRNA kit (Ambion, Inc., Austin, TX)

following the manufacturer's protocol. Briefly, reverse transcription master mix containing 1µl Array Script, 2µl 10x first strand buffer, 1µl T7 Oligo (dT) primer, 4µl of dNTP mix, 1µl RNase inhibitor in 1µl nuclease-free water was transferred to a microtube (nuclease-free) containing 5µg of the sample RNA for synthesis of first strand cDNA. Cycling parameters for first strand cDNA synthesis were 42°C (50°C lid) 2 hrs for 1 cycle and 4°C hold.

For second strand cDNA synthesis, 80µl of a master mix, containing 10µl of10x second strand buffer, 4µl dNTP mix, 2µl DNA polymerase and 1µl RNase H in 63µl nuclease free water was added to each synthesized sample of first strand cDNA. Cycling parameter for second strand cDNA synthesis included 16°C (heat-disabled lid, or no lid) for 2 hrs for 1 cycle and a 4°C hold.

cDNA purification For purification of all sample cDNA synthesized, 250 µl of cDNA binding buffer was added to each sample cDNA synthesized, and mixed by pipetting up and down 2–3 times, then flicking the tube 3–4 times. Following a brief centrifugation, the reaction was collected at the bottom of the tube. The mixture was then passed through a cDNA filter cartridge, centrifuged, and washed using washing buffer. Purified cDNA was eluted using preheated nuclease-free water at 55°C. Sample cDNA was stored on -20ºC for further analysis.

In vitro transcription to synthesize amino allyl-modified aRNA A master mix containing aaUTP (50 mM), 12 µl of ATP, CTP, GTP Mix, 6ul of UTP solution (50 mM), T7 reaction buffer (10x) and T7 enzyme mix was added to cDNA sample, incubated at 37°C (default lid; 100–105°C) for 14 hrs; for 1 cycle and kept at 4°C on hold in thermal cycler.

aRNA purification and quality assessment To remove unincorporated aaUTP and Tris from the *in vitro* transcription reactions (that would otherwise compete with the aRNA for dye coupling, enzymes, salts, and other unincorporated nucleotides), 350 µl of aRNA binding buffer was added to each sample followed by 250 µl of 100% ethanol. The mixture was thoroughly mixed by pipetting. Samples were then passed through an aRNA filter cartridge, washed with washing buffer, and eluted with pre-heated nuclease-free water and quality assessed by a Nanodrop spectrophotometer.

Dye preparation, coupling to aRNA and labeled aRNA cleanup Mono-reactive NHS esters of Cy3 (550nm maximum absorbance) and Cy5 (650nm maximum absorbance) dye (Amino Ally MessageAmp II Kit) were used for labeling aRNA. The Cy5 dye was used to label the aRNA of pooled placental malaria-negative tissue, whereas Cy3 was used for aRNA from malaria positive samples.

For the preparation of the dye, 11 µL of DMSO was added to one tube of Cy3 or Cy5 reactive dye and vortexed to mix thoroughly, and kept in the dark at room temperature (20-25ºC) for a maximum of 1 hr. Then, ~ 5µg of amino allyl-modified aRNA were placed in a nuclease-free microfuge tube and vacuum dried until no liquid remained. Then, dried aRNA was re-suspended in 9 µl of coupling buffer, 11µl of prepared dyes were added to the aRNA, and mixed well by gently vortexing. This step was followed by 30 min incubation at room temperature in the dark. To stop the reaction, 4.5 µl of 4M hydroxylamine were added and mix well by gently vortexing, and the reaction incubated at room temperature in the dark room for 15min. The solution was then brought up to 30µl by adding nuclease-free water.

Dye labeled aRNA purification and quantification To remove excess dye from aRNA, 105 µl of aRNA binding buffer was added to each aRNA sample, followed by 75 µl of

100% ethanol and mixed by pipetting the mixture up and down for 3 times. The samples were then added to filter cartridges. Cartridges containing the labeled aRNA were then centrifuged for 1 min, and washed once with 500µl of wash buffer for 1min at 1000g x g. The flow-through was discarded and the aRNA eluted with 10µl pre-heated nuclease free water. Elution with 10µl preheated nuclease free water was repeated two time. The purified aRNA was stored at -20ºC in the dark until further use.

Preparation of labeled aRNA for hybridization Labeled aRNA of each sample was concentrated to 10µl by vacuum drying and then fragmented for hybridization to oligonucleotide microarrays of 60-200 nucleotide using 10x fragmentation reagent (Ambion P/N AM8740). Then, components of hybridization mixture was added one by one to the fragmented aRNA; i. e.; 1.2µl 1M HEPES (pH 7.0), 9µl 20x SSC, 10µl 1mg/mL of polydA, 10µl human cot DNA^{R} (used to block nonspecific hybridization in microarray screening) (Invitrogen), and 1.2µl 10% SDS. The mixture was boiled for 2 minutes, and then cooled to room temperature 25ºC for 10min in the dark.

Preparation of the arrays and washing hybridized slides *First,* 6ul of 3x SSC was placed at each end of the human oligonucleotide array and then 60ul of each sample for hybridization was added. Hybridization was done for 16 hrs at 65ºC. Several washing steps with SSC and SDS, followed by drying of the microarray.

Scanning slides, initial analysis, and data acquisition After drying, microarray slides were scanned using GenePixPro 5.1 Software (Axon). Data was then acquired, and initial analysis was performed Initial analysis involved flagging irregular and missing features on scanned microarray slides (GenePixPro 5.1 Software Axon). Flagging criteria included empty SSC, spots with diameters ≤ 60µm, and a signal/noise ratio (SNR) of < 2.In conducting the microarrays, RNA from each of the 3 placental malaria

positive samples (individually in triplicate) was compared with a pool of RNA from 6 placental-malaria negative (control samples).

Functional profiling *Differentially* up- and down-regulated genes were assigned to various functional categories using National Center for Biotechnology Information (NCBI) and other public databases.

Pathway analysis Pathway analysis for differentially-expressed genes was done using pathway miner Version 1.1 1(http://www.biorag.org/pathway.php) that utilizes databases from three independent sources: GeNMAP, Biocarta and KEGG. Input required for analysis included accession numbers and gene expression values in terms of Log fold change. Gene expression profiles were analyzed for significant pathway representation based on Fisher exact statistical test. The statistical test was done for pathways from all three independent resources. The pathway includes both the cellular and regulatory pathways.

Statistical analysis Analysis of microarray data was conducted using the Limma package in Bioconductor and R statistical analysis software. The preprocessing of gene expression intensities on log2 scale included background correction and normalization. Background correction was made using Norm Exp offset 16 and data normalization within the array was conducted using Print Tip Group Loess. The normalized microarray data were fitted using the linear model with least squares estimators to quantify the differential expression of genes between groups. Differentially expressed genes were then selected based on an adjusted P value of <0.01 with false discovery rate controlled at 1% and log fold change of ≥ 1. Significant changes in gene expression profiles of a variety of biochemical pathways were identified based on Fisher's exact test with multiple testing error rate controlled at 5%. These changes included both up- and down-

regulate genes for each placenta. Pathways with adjusted P values ≤ 0.05 and ≥ 2 fold changes was considered significantly altered.

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2.3 Results

Pathological findings Among 30 placentas collected, *P. falcip*arum IE were found in only 3 placentas. Table 2.1 provides information on the women from whom the 3 placental malaria-positive placentas were obtained. The 3 placentas (Fig 2.1) were from A) a primigravid woman with intervillositis due to a chronic infection with massive infiltration of monocytes, macrophages and lymphocytes, but a low placental parasitemia B) a secundigravid women who also had a chronic infection but with mild accumulation of inflammatory cells in the IVS; whereas, C) was a multigravid woman with a mild infection, few parasites, and no evidence of inflammation in the IVS.

Differential gene expression between infected versus non-infected placentas

Genes with adjusted p values of <0.01 and a log fold change of ≥1 in each of the three infected placentas relative to the negative pool were considered to be significant up- or down-regulated. The number of differentially-expressed genes is shown in (Table 2.1). Significant changes were detected in gene expression between the normal placenta pool and the 2 chronically-infected placentas.

Functional profiling

Overall, genes that code for proteins that perform various immunological, placental metabolism, growth and transporting functions were altered; with some being up-regulated and others down-regulated. Figure 2.2 and Table 2.2 depict functions of genes that were differentially up- and down-regulated. For the placenta A with intervillositis, a high percentage of down-regulated genes were involved in metabolism (34%) and transport (34%). Others down-regulated were pregnancy related (25%). In placenta B, 31% down-regulated genes involved in metabolism, 21% in transport and 33% were pregnancy associated genes.

The majority of genes (65%) up-regulated in placenta A (intervillositis a severe chronic placenta malaria) were genes that code for the immune response genes, including i) pattern recognition receptors (TLR-1, LY6, TLR-2, TLR-4, MD2, TLR8, formyl peptide receptor 1), ii) chemokines and their receptors (IL-8, CCL5, CXCL9, CCR5, CCR1, MIP-1α), iii) both pro- and anti-inflammatory cytokines (IL-1β, IL-10RA, IL-2R2, CCL-18, IL-18, Tumor necrosis factor ligand superfamily member 13 TACI), and iv) adhesion molecules (ITG2, CD83, CD58, CD11A, ITGA6). Others up-regulated genes were complement components (C_1QA, C3, C_1QB), genes that codes proteins for antigen processing and presentation (HLA 2: HLA-A, HLA DRB1, HLA DRA, CD74; and HLA 1: HLA-F, HLA-A). Up-regulated immune genes in placenta B included TLR1, MD2, LY6, CD36, CD64, CD58, P2RX5, CD32 and Fc fragment low affinity IIB and complement 7.

Interestingly, about 220 differentially expressed genes were common in placenta A and B, with 99% of change being in the same direction. In a mildly-infected placenta C, however, only 5 genes were differentially expressed, but all were related to pregnancy and fetal development (Table 2.0).

In placentas A and B, a number of genes were identified that code for proteins known to be important in fetal growth, including leptin (LEP); system A amino acid transporter (solute carrier family 38, member 1& 2, [SLC38A1& 2]); insulin-like growth factor binding protein 1, 5 and 7 (IGFBPs); Inhibin A; placenta enzyme 11 β hydroxysteroid dehydrogenase 2 (11βHSB 2); placenta growth factor (VEGF), angiopoietin I and II (ANGPT-I & 2); and insulin-regulated glucose transporter gene.

Cellular metabolic and regulatory pathway altered in chronic infected placenta

To gain a better understanding about the relationships between genes that were differentially expressed, microarray data were subjected to pathway analysis. Important

pathways that may have significant impact on fetal ST, and therefore fetal growth, were selected using data from the two placentas with chronic inflammation (Placentas A+B), since chronic inflammation is known to increase the risk of LBW babies (5, 15, 29, 35).

The major pathways that were dysregulated ($p \leq 0.05$, fold change ≥ 2) were: 1) insulin-like growth factor 1, 2) insulin, and 3) mTOR signaling pathways, 4) pathways that influence angiogenesis and vascular permeability (e.g., VEGF), 5) TGFβ and 6) pathways required for maintaining cellular integrity, cell-cell communication, and focal adhesion.

2.4 Discussion

We have identified changes in gene expression and associated biochemical pathways that could alter fetal growth in fetal cells from malaria-infected placentas. We found that several growth pathways important for growth are dysregulated in chronic placental malaria-infected fetal tissues. However, we did not observe significant changes in terms of biochemical pathways in the acute, mildly infected placenta (C) without inflammation. Biochemical changes observed suggest placental malaria can induce pathology by changing important growth pathways. This might occur as a result of prolonged interactions between fetal tissue with IE and parasite's bioactive molecules or inflammatory cytokines or maternal inflammatory cells or both in the IVS.

The results are in line with earlier studies that hypothesized changes in ST function during placental malaria [39, 40, 66]. We observed dysregulation of pathways that are essential for fetus and placental growth in chronic infected placentas. These include the insulin/IGF-1 signaling pathway that is important for in utero growth throughout gestation. Our results indicate IGF-1 signaling components are down-regulated (Table 2.5). Studies have shown that abnormalities in IGF-1 signaling do occur in pregnancies complicated with FGR compared to normal pregnancies with or without placental malaria. IGF-1 signaling involves the two main pathway namely; the MAPK/ERK and PI3K/AKT pathways. Signaling through these two pathways leads to cells proliferation and survival; enhanced protein synthesis, amino and glucose uptake, prevention of apoptosis and angiogenesis. In our study components of both pathways are dysregulated. That is, insulin/IGF-I receptor and its post-receptor signaling components (IR, IRS-1, and the threonine kinase AKT-3, RAS p21 protein activator and v-fos FBJ indicate that genes that code for potent regulators of IGF-1 activities, e.g., IGFBP-1, 5 and 7 are up-regulated (Table 1). IGFBPs decrease the bioavailability of IGF-1 and

therefore its mitogenic effect on the placental tissues. Although high protein levels of IGFBP-1 have been reported in placental malaria-positive mothers, the role of IGFBP-5 and 7 has not been established in placental malaria.

Down-regulation of the mTOR pathway, which is important for fetal growth, was observed in chronically-infected placentas. It is well established that the mTOR pathway is a downstream from a number of growth factors signaling pathways, including the insulin and IGF-1 pathways (reviewed in [109,110]). In addition, leptin which was down-regulated in our study has been shown to activate the mTOR pathway. Specifically, the mTOR pathway regulates amino acid transporters at the apical membrane of ST. Consequently, system A amino acid transporter (SLC38A1 and 2) are down-regulated (Table 2.5) in chronic placentas. Our data therefore indicates that the dysregulation of the insulin/IGF-1, mTOR, and insulin signaling pathways may be involved in the pathogenesis of placental malaria that leads to LBW. These pathways work in concert with each other. Thus, it is possible that LBW observed in infants from placental malaria-positive mothers might result from impairment of transplacental transport of amino acid mediated through dysregulation of these growth pathways. Additional studies are warranted to investigate the role of these genes and corresponding proteins in the pathogenesis of placental malaria.

Dysregulation of angiogenesis has also been implicated in placental malaria associated pathology. Placental inhibin A has been implicated in the impairment of angiogenesis and therefore uteroplacental blood flow [111]. In our study, the gene encoding placental INHBA was up-regulated. Moreover, the VEGF signaling pathway was also dysregulated (Table 2.4). The VEGF signaling is important in vasculogenesis. Impairment of vasculogenesis during placental malaria may affect *utero*-placental blood flow and

therefore maternal fetal exchange of nutrients, wastes and gases, thereby affecting *in utero* growth.

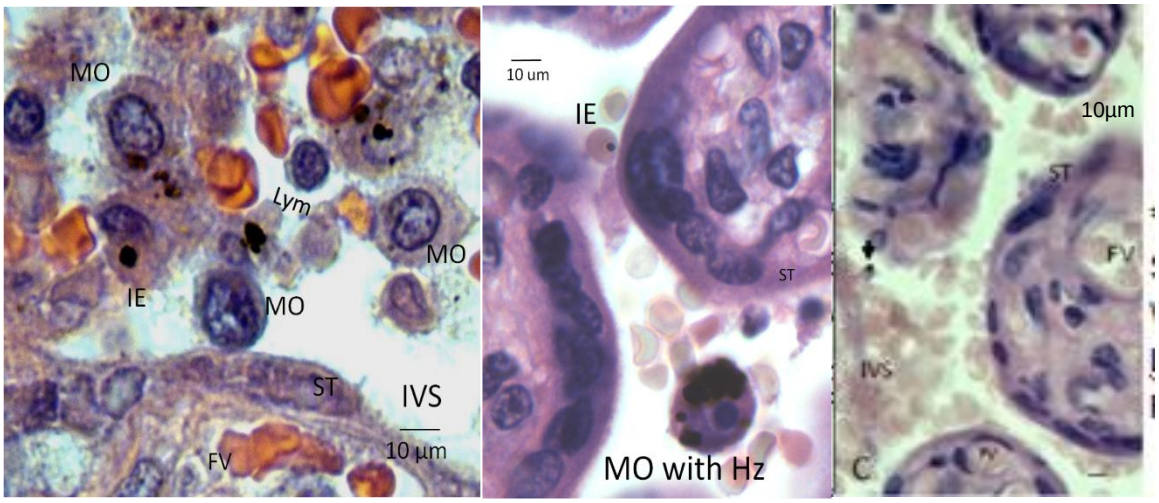
Regulation of cortisol levels at the fetal unit in the placenta is kept in check by the hydroxysteroid 11β dehydrogenase 2 enzyme expressed in ST. The gene encoding this enzyme is down-regulated, implying potential compromised placental metabolic barrier during chronic placental malaria. Low activity of this enzyme leads to fetal exposure to cortisol which has been associated with LBW in idiopathic FGR [88] . Cortisol has been shown to affect fetal programming, exposing the fetus to health problems in the adulthood [112].

Although our results are based on the analysis of only two chronically-infected placentas, they support the observation of increased risk of having LBW babies when mothers have chronic placental malaria. Furthermore, the degree of congruency in terms of pattern of gene expression between the two chronic infected placentas is credible. The commonality implies that fetal tissues responded to malaria in a same way, that is interactions between the parasite components and fetal tissue during chronic infection results in more or less similar responses. However, since the fetal tissues analyzed are composed of five cell types (i.e., ST, Hofbauer macrophages, fetal endothelial cells, and fetal stroma) the changes described cannot be attributed to ST alone. However, notwithstanding this limitation, the study describes the potential biological mechanisms on how chronic placental malaria can lead to increased risk of having FGR. Figure 2.3 depicts a model showing relationship between changes in fetal cells (in terms of altered pathways) due placental malaria can lead to FGR. Additional studies are needed to investigate the potential utilization of the proteins encoded by these genes dysregulated

in the pathways (Fig. 2.3) for improvement of diagnosis of placental malaria in endemic areas.

2.5 Conclusion

ST functions that are essential during *in utero* growth are perturbed during chronic placental malaria. While waiting for a vaccine for placental malaria to come to fruition, the interventional approach targeting these pathways to alleviate placental malaria-associated LBW in pregnant women at risk of placental malaria areas may be possible by designing therapy that will target proteins in pathways that are severely dysregulated. However, more studies on chronically infected placentas with larger samples sizes are warranted to investigate expression of genes and proteins belonging to specific pathways.



Placenta A **Placenta B** **Placenta C**

Figure 2.1 Hematoxylin & Eosin staining of placental histological sections. A) massive intervillositis with a few IE but many macrophages, monocytes, and lymphocytes infected erythrocytes, B) both IE and macrophages containing hemozoin pigment (Hz) present, and C) mild acute infection with only a few IE and no evidence of inflammation.MO= macrophage, ST = syncytiotrophoblast, Lym = lymphocyte, and FV = fetal vessels

Table 2.1 Placental parasitological and histological results

Placenta	Age (yrs)	Gravidity	Placental Parasitemia	Placental findings	Number of Differentially -expressed genes
A	18	Primigravidae	0.02%	Chronic	409
B	29	Secundigravidae	0.03%	Chronic	1104
C	29	Multigravidae	0.01%	Mild	5

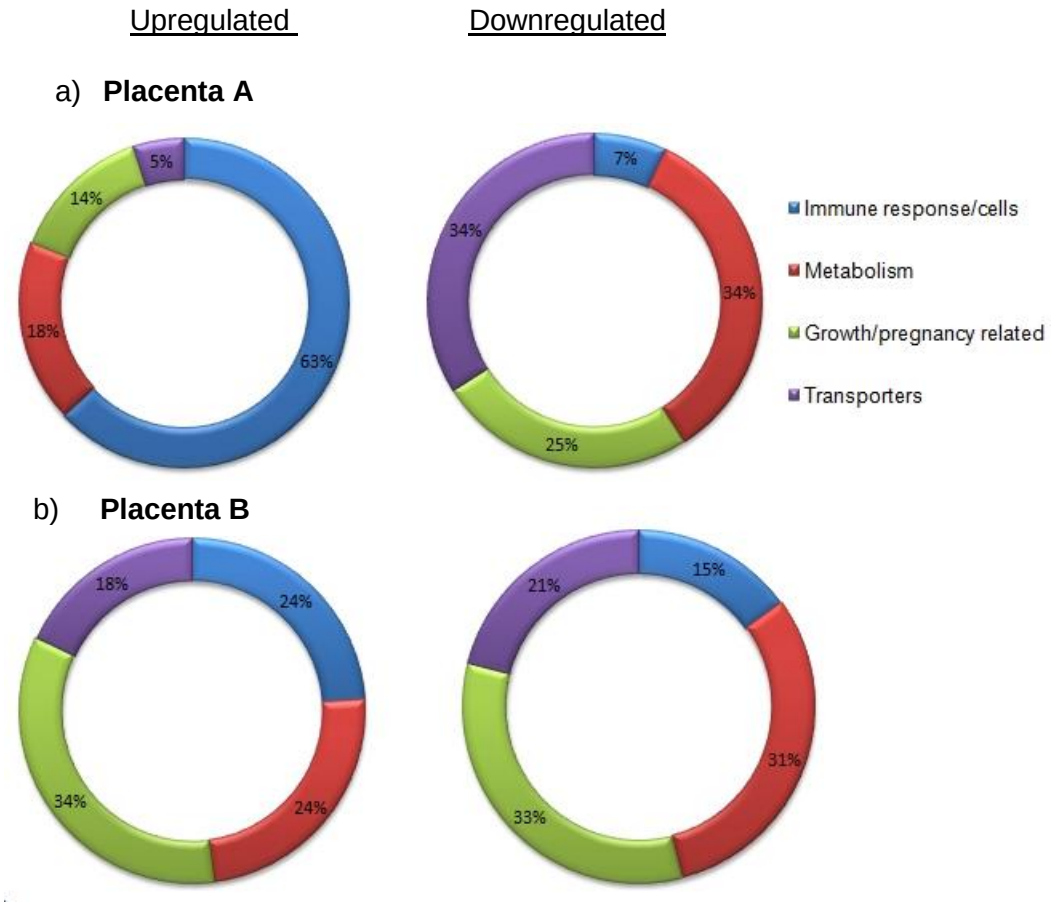


Figure 2.2 Function profiling of differentially-expressed genes in fetal cells from placentas with chronic placental malaria a) **Placenta A**. percent upregulated genes (left panel) and down-regulated genes (right side) b) **Placenta B** percentage upregulated (left panel) and down-regulated (right panel) genes. Functional categories grouped in percentages

Table 2.2 Functions of differentially expressed genes placenta C: mild infection

Gene ID	Name	Function
Upregulated genes		
NM_002571	PAEP(Progestagen-associated endometrial protein)	Immunosuppressive neovascularization
NM_000948	PRL(Prolactin)	Growth regulators for many tissue/immunomodulator
NM_012242	DKK1 dickkopf homolog 1	Embryonic development through its inhibition of the WNT signaling pathway
Downregulated genes		
NM_005252	v-fos FBJ murine osteosarcoma viral oncogene homolog	Regulation of transcription
NM_001008	RPS4Y1(ribosomal protein s4, y-linked)	translation

Table 2.3 Number of pathways found to be differentially-expressed in three malaria-infected placentas

Source	Placenta A	Placenta B	Placenta C
Biocarta	150	195	29
GenMAPP	25	38	4
KEGG	28	131	9

Table 2.4 Pathway important for growth significantly altered in chronically-infected placentas

Pathway Name	P-value < 0.05
Prostaglandin synthesis regulation	0.00002
Insulin signaling pathway	0.00003
TGF-beta signaling pathway	0.00044
VEGF signaling pathway	0.00044
mTOR signaling pathway	0.00583
IGF-1 Signaling Pathway	0.00195
Focal adhesion	0.00001
Cell-cell communication	0.00001

Table 2.5 Differentially-expressed genes in placental A that are important in fetal growth

Gene Name	Gene symbol	Fold change	adj. P val
Insulin-like growth factor 1	IGF-1	3.88	7.92^{-4}
Angiopoietin-like 1	ANGPTL1	3.14	7.13^{-3}
Angiopoietin 1	ANGP1	2.06	1.33^{-3}
Insulin-like growth factor binding protein 7	IGFBP-7	2.02	5.64^{-3}
Insulin-like growth factor binding protein 1	IGFBP-1	4.94	1.22^{-3}
Inhibin, beta A	IHBA	4.1	1.80^{-3}
Hydroxysteroid (11-beta) dehydrogenase 2	HSD11B2	-2.5	4.44^{-3}
Related RAS viral oncogene homolog 2	R-RAS	-2	2.64^{-3}
Solute carrier family 38, member 2	SLC38A2	-2.18	1.68^{-3}
Insulin receptor substrate 1	IRS-1	-2.34	1.65^{-3}
Placental growth factor	PGF	-2.96	1.27^{-3}
FK506 binding protein 12-rapamycin associated protein 1	mTOR	-3.08	1.35^{-3}
Angiopoietin 2	ANGP-2	-3.34	1.20^{-3}
V-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	-3.92	7.92^{-4}
Insulin receptor	IR	-4.06	1.02^{-3}
Leptin	LEP	-4.82	9.88^{-4}
Angiopoietin-like 4	ANGPTL4	-4.98	7.92^{-4}

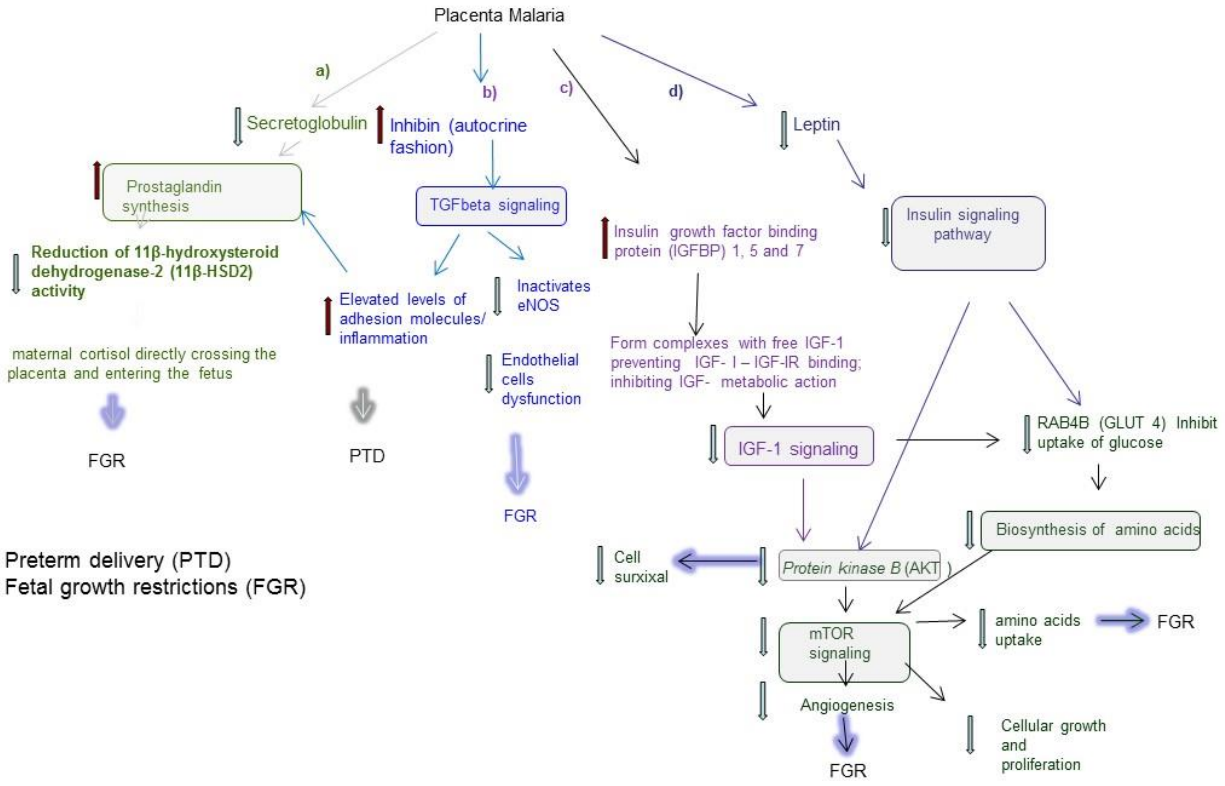


Figure 2.3 Mechanisms (a, b, c & d) that may lead to fetal growth restriction (FGR) based on response of fetal cells to placental malaria. Placental malaria may impair the activity placenta 11β-HSD2 enzyme thereby exposing fetus to excess cortisol leading to FGR. Also placental malaria may cause FGR by decreasing transplacental transfer of amino acids, protein synthesis and angiogenesis via dys-regulation of IGF-1 and insulin signaling pathways as well as impairing leptin hormone secretions. Effects of these pathways on fetal growth may be mediated via placental mTOR signaling pathway. Placental malaria may also cause endothelia cell dysfunction by down-regulating TGFβ signaling pathway by up-regulating secretion of the hormone inhibin A. The later may also lead to elevation of adhesion molecules and therefore inflammation leading to risk of preterm delivery early during pregnancy.

CHAPTER 3

Optimization of the BeWo Model to Investigate Responses of Syncytiotrophoblast to *Plasmodium falciparum*-Infected Erythrocytes

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Abstract

Establishment of an *in vitro* model to study placental malaria is essential for understanding the biology and pathogenesis of placental malaria. We defined experimental variables for obtaining optimal responses of BeWo cells to placental-binding *Plasmodium falciparum*-infected erythrocytes (CS2 parasites). This included i) optimal concentration of forskolin, a c*yclic adenosine monophosphate inducer* important in the induction of syncytialization of BeWo, ii) suitable period of incubating BeWo with forskolin, iii) ratio of *P. falciparum*-infected erythrocytes to BeWo to induce responses in BeWo cells, and iv) length of BeWo-infected erythrocyte co-culture for BeWo cells to be stimulated by infected erythrocytes. We used human chorionic gonadotrophin hormone as a marker to measure the efficiency of different concentration of forskolin and incubation times for syncytialization. Several markers were measured by ELISA and RT-PCR to evaluate responses of BeWo to infected erythrocytes. Results showed that 72 hrs incubation of BeWo with 10µM forskolin resulted into higher levels of hCG compared with 25µM and 50µM. The optimal response of forskolin-treated BeWo to CS2 -infected erythrocytes occurs at 48 hrs incubation of BeWo cells with infected erythrocytes at a ratio of 10 infected erythrocytes to 1 BeWo.

An extract of the parasite at a concentration of 1 parasite equivalent to 1 BeWo, induced gene expression changes (inhibin and progesterone associated endometrial protein) at 24 hrs of incubation of BeWo with the parasite extract. Overall, data showed that stimulating BeWo cells treated with 10µM forskolin for 72 hrs followed by incubation with a 10:1 ratio of infected erythrocytes for 48 hrs was the best protocol for testing the influence of *P. falciparum* infected erythrocytes on placental trophoblasts.

3.1 Introduction

Pathogenesis of placental malaria may result from infected erythrocytes (IE) binding to syncytiotrophoblasts (ST), resulting in sequestration of IE at the intervillous space (IVS) that stimulate infiltration of maternal monocytes and secretion of proinflammatory cytokines in the IVS. The binding of IE to ST is mediated by the parasite protein, variable surface antigen that is expressed on the surface of IE, called VAR2CSA and ST receptor called chondroitin sulfate A (CSA). Therefore, during placental malaria, fetal ST interact with IE, maternal monocytes that infiltrated during inflammation and inflammatory cytokines that are secreted by maternal monocytes and ST. The major outcome of this interaction of ST with IE in malaria-endemic areas, is low birth weight (LBW) babies [36] as a result of preterm delivery (PTD) and fetal growth (FGR). Little is known about the pathogenesis of placental malaria that leads to low birth weight babies.

One approach to investigate the pathogenesis of PM would be *in vivo* studies using an animal model. However, the human ST are in many aspects different from rodent ST and rodents are a poor model for human placental malaria [113]. In addition, the parasite variable surface antigens (VSA) that mediate binding to ST leading to sequestration in the IVS are different in human and rodent placental malarial parasites [114]. While human perfusion model and trophoblasts-primary cells are an alternative for ex-*vivo* and *in vitro* studies of placental-malaria pathogenesis, human ST from term placentas have a very short life-span in culture and are in a senescent state at delivery. BeWo cells are a stable cytotrophoblast cell line capable of acquiring the ST phenotypic and endocrine characteristics upon treatment with forskolin, an inducer of cyclic adenosine monophosphate (cAMP). The BeWo system has recently been used as an *in vitro* model to select *Plasmodium falciparum* placental-binding parasites [23] as well as in studies on placental malaria pathogenesis [21,22,41]. However the study of placental malaria

pathogenesis using the BeWo cell lines system has not been optimized and therefore standardized protocols have not been established. The optimization of BeWo model and establishment of *in vitro* protocol will allow for the study of pathophysiology of ST associated with placental malaria that lead to bad pregnancy outcome.

During placental malaria, ST respond to a wide range of placental parasitemias, inflammatory cytokines, and parasite bioactive molecules that increase the risk of a range of poor pregnancy outcomes in malaria endemic areas. Thus, ST may be exposed to a number of different conditions *in vivo*, over an extended period of time. In this study, we sought to optimize various experimental variables and establish an *in vitro* system that mimicked different aspects of the malaria-induced microenvironment in which the parasite helps mediate placental malaria pathology. Gene expression and protein assays were used to study the dynamics of BeWo responses to various concentrations of intact IE at different time points. Several genes whose proteins are reported to be dysregulated in pregnant women during placental malaria [20,40, 39] and factors important for placental and fetal growth were selected for study. The genes included vascular endothelia growth factor (VEGFA) A; which is important in inducing angiogenesis, vasculogenesis and as well mediate vascular permeability, endoglin (END); a transforming growth factor coreceptor which is also involved in regulation of angiogenesis, inhibin A (INHA); is an anti-angiogenic molecule, mammalian target of ramphamycin (mTOR); implicated in the regulation of growth in utero involves and angiopoietin-2 (ANGPT2); a key regulator of angiogenesis. In addition, we measured IL-8 and Macrophage Inhibitory Factor (MIF), which are elevated during placental malaria [19,20], to assess the optimal number of intact IE required to induce responses to BeWo at a particular incubation period. Furthermore, significant intervillous- monocyte infiltration occurs during placental malaria [115], and association between monocytes infiltrates and

inflammatory cytokines in the IVS have been reported [116].Therefore we also assessed levels of chemokines (MCP-1, MIP-1, IL-8) and cytokines (TNFα, IFNɣ, IL 6) secreted by monocyte cell line (THP-1) upon exposure to CS2-IE.

3.2 Material and Methods

CS2 parasite culture The CS2 strain of *P. falciparum* was grown in type O+ blood at 5% hematocrit in RPMI-1640 media (Invitrogen) supplemented with 50 µg/mL hypoxanthine (Sigma), 0.1 mg/ml gentamicin sulfate (Sigma), 5% albumax, and 5% human AB serum at 37^{o}C in the incubator with 5% CO_2, 90% N_2 and 5% O2.

Culturing the BeWo cell line BeWo cells, obtained from ATCC, were grown in Ham's F12 medium (source) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% Penicillin/Streptomycin and incubated in humidified atmosphere at 37°C in the presence of 5% CO_2. For all of the experimental purposes, BeWo were seeded on 24-well plates at 2.0 x 10^5 cells/well and incubated until 90% confluent, before being treated with 10µM forskolin. For parallel experiments for determination of viability in various treatments, 96 well (1 x 10^4 cells/well) format was used to seed BeWo.

Determining optimal concentration of forskolin and length of incubation with BeWo cells To determine the effective concentration and timing of forskolin treatment required for BeWo to acquire phenotypic and endocrine characteristics of ST, the concentration of β-human chorionic gonadotrophin hormone (hCG) secreted by the cells into culture supernatants was measured using a commercial ELISA kit (Alpha diagnostic). BeWo that were treated with different concentration of forskolin including, 10µM, 25µM and 50µM forskolin (Calbiochem) using DMSO as a vehicle in Hams F12 medium. Forskolin-treated BeWo were then incubated at different time points: 24, 48, 72 and 96 hrs. Viability of BeWo was determined using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay.

Preparation of parasites extract To investigated whether malaria parasite extracts induces responses to forskolin treated BeWo, previous freeze-thawed 3D7 parasites-

infected erythrocytes (~50% parasitemia stored at -20ºC) and their corresponding normal red blood cell controls collected at the same time, were thawed for about 6 minutes at 37ºC, mixed thoroughly by vortexing, and sonicated for 2 minutes. Volumes containing known numbers of parasite equivalent per BeWo were added to each well. For example, at 50% parasitemia, there are 5 x 10^8 IE /100ul of packed cells. Therefore we added 0.04µl of parasite extract (PE) to one well with BeWo cell at 2 x 10^5 cells per well for 1 PE: 1 BeWo treatment. Same number of red blood cells extract were prepared and added to BeWo as a control. Different concentrations of PE-treat BeWo cells were cultured for different periods of time.

Magnetic separation purification technique To concentrate the late stage (trophozoite stage which express proteins required for binding on ST of the placenta) CS2 parasites from the *in vitro* culture for the experiment, the magnetic column (LS Columns Miltenyi Biotec) with Quadro MACS separator was used. The columns positively separated trophozoite-stage parasites. CS2 culture parasites from synchronized cultures were washed with PBS. The parasite pellet was then suspended in sterile filtered MACS buffer (PBS, pH 7.2., 0.5% BSA, 2Mm EDTA). Then MACS buffer was added drop-wise to pre-wet the columns. Then MACS-parasite suspension was added to the columns attached to MACS separator, and flow-through containing ring-stage parasites were collected and discarded. Before eluting the trophozoites, the columns were detached from the Quadro MACS separator. The trophozoites stage parasites were then eluted with 5ml MACS buffer, the trophozoites collected in a 15ml tube. The eluted parasites were then centrifuged at 2500rpm for 5 min, supernatant removed and parasitemia determined under the microscope. The eluate consisted of trophozoites, schizonts, free hemozoin and parasite debris (hemozoin-DNA complexes, parasite DNA).

Determination of timing of BeWo response towards intact infected erythrocyte

To understand the dynamics of responses and define the critical time point at which IE induces detectable responses in forskolin-treated BeWo, three different concentrations of intact IE were used to stimulate forskolin-treated BeWo and four different incubation times were utilized. Briefly, cells were seeded on 24 well plate at 2 x 10^5 cells/ well, treated with forskolin, at 90% confluence were treated with forskolin and incubated for 72 hrs at 37ºC. Then the forskolin-treated BeWo were incubated with intact IE at the ratios of 10, 1, and 0.1 IE to 1 BeWo for 6, 12, 24 and 48 hrs. Normal culture-red blood cells that were used to culture CS2 parasite were used as treatment control. Both protein assay (ELISA) and gene expression (RT-PCR) were used to measure expression of selected markers; i.e., measure gene transcription and amount of protein secreted.

Selection of markers for analysis **of BeWo responses to IE** Selected markers include factors thought to be dysregulated during placental malaria, expressed by ST and are important in placental malaria pathogenesis including MIF [20,117] which is involved in recruiting and retaining maternal monocytes in the IVS, IL-8; a chemokine that correlates with maternal monocytes during placental malaria, ANGPT- 2, END, VEGF-A and INHA; genes that code for proteins essential in angiogenesis, and mTOR; involved in regulation of in *utero growth*.

ELISA assay for detection MIF in the supernatant The amount of MIF, a cytokine that is elevated in placental malaria-positive placentas, was measured in the supernatant of forskolin treated-BeWo cells incubated with IE for different periods of time using an ELISA kit (R& D, Duoset Cat No DY289). Briefly, 2µg/µl of mouse anti-human MIF capture antibody was used for coating ELISA plates (Maxisorp) and incubated at 4˚C overnight. The capture antibody was then aspirated and the wells washed three times

with 0.05% Tween in PBS. Then, 300µl of blocking solution 1% BSA in PBS (filtered with 0.2µM) was added and incubated for 2 hrs at 37ºC. The blocking solution was aspirated and wells washed three times with 0.05% Tween in PBS. Samples (pooled supernatants from triplicate wells) and standards were then added and incubated for 2 hrs at 37ºC. After aspirating and washing, 100µl of 100ng/mL of biotinylated goat ant-human MIF detection antibody were added and incubated for 2 hrs. Then, 100µl of streptavidin-HRP at a 1:200 dilution (in diluent) was added to each well and incubated for 30min. The intensity of the color change was then recorded at 450/630nm.

BeWo RNA extraction Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's protocol. Quality and yield of RNA was assessed using a nano-drop UV spectrophotometer. RNA preparations were considered pure when the A260/A280 absorbance ratio was equal to 1.9-2.0. Assessment of gene expression on selected genes from stimulated forskolin-treated BeWo was determined using real time PCR (RT-PCR).

cDNA synthesis and RT-PCR for selected markers High quality RNA (A260/A280 ratio = 1.9-2.0) was used for cDNA synthesis that was carried out according to the manufacturer's protocol (Quanta Bioscience). cDNA was diluted at 1:5 with nuclease-free water for RT-PCR assays. Three independent amplification assays was carried out in duplicate for each gene using a total reaction volume of 20 µl. The reaction contained 1.5µl of sample cDNA, 10µL PerfeCta SYBR Green SuperMix (Quanta Bioscience 59), 250nM (0.5µl) of gene-specific forward and reverse primers (Bio-Rad) in nuclease free water. A master mix containing forward and reverse primers for *Beta actin* was prepared for amplification as an internal control (reference gene). The RNA was reverse transcribed using SYBR Green qPCR under cycling conditions recommended by the

manufacturer. Data were acquired using iCycler (Bio Rad). Gene expression levels were normalized using a single internal control, the *beta actin* gene. Mean relative RNA expression levels in reference to the internal control were determined and compared among the infected erythrocyte and normal red blood cell treatment groups.

Assess chemokine's and cytokine secreted by monocyte cell line (THP-1 cells) upon exposure of infected erythrocytes inflammatory cytokines and chemokines are elevated in the IVS of women with placental malaria. Also correlation of maternal monocytes and inflammatory cytokines in the IVS exists. We therefore assessed cytokines and chemokines secreted by human monocytic cell line (THP-1 cells passage 8) were seeded at 4 x 10^5 cells/ ml in T-75 flask and treated with purified CS2-infected erythrocytes at a ratio of 10 IE : THP- 1 in THP-1 media (11mL), and incubated at 37ºC in humidified atmosphere in 5% CO_2 for 24 hrs. A T-75 flask containing same number of THP-1 cells was treated with same volume of THP-1 media and used as control. At 24 hrs, supernatants from both flasks were collected by centrifugation at 2500rpm, at 30ºC for 7 minutes and then stored -80ºC. The concentrations of cytokines and chemokines in the condition and spent media were measured using a Luminex assay (R& D).

Luminex assay for detection of chemokines and cytokines

Supernatant from BeWo cultures

Using microparticles (VersaMap) that were coated with IL-8-specific antibodies, IL-8 in BeWo culture supernatant were measured for the various treatments. Briefly, 50µl of IL-8 pre-coated microparticles were pipetted into wells 96 well plate (previously pre-wet wells with washing buffer). Then, 200µL of samples and 50µl of standards were added into wells containing microparticles and incubated for 2 hrs at room temperature on a

horizontal orbital microplate shaker set at 500 ± 50 rpm. The liquid was then removed by vacuum aspiration and three washing steps with washing buffer was performed. For detection, 50µl of IL8 -specific biotinylated antibody was added and incubated for one hour room temperature on a shaker. After three washing, 50µl of streptavidin-phycoerythrin conjugate antibody was added and incubated for 30min. After washing, 100µl of washing buffer was added and MIF read using a MicroChip M100.

Supernatant from THP-1 cultures

Supernatants collected fromTHP-1-IE co-cultures and THP-1 cells treated with medium alone for 24 hrs, were screened for TNFα, IFNγ, MIP1α, IL-6, CXCL1, and MCP1α using procedures described above with slight modification. That is, 50µL of sample supernatants were incubated the microspheres.

3.3 Results

Levels of human chorionic gonadotropin hormone secreted by BeWo treated with different concentrations of forskolin

To assess efficiency of different concentration of forskolin in inducing syncytialization to BeWo we measured levels of hCG secreted by BeWo in the supernant of BeWo that were treated with10µM, 25 µM and 50µM of forskolin. The results shows that BeWo without forskolin secreted less hCG compared to BeWo treated by 10µM, 25 µM and 50µM of forskolin (Fig.3.1A). Lower doses of forskolin (10µM) forskolin induced about 23% more hCG in BeWo at 72 hrs of incubation compared to BeWo treated with 25µM for the same time period. That is the amount of hCG seemed to increase with time, with higher concentrations at time 72 hrs at lower doses of forskolin. Compared to BeWo treated with 50µM for 24 and 48 hrs, the 10µM treatment increased hCG secretion by 50% (Fig.3.1A). Figure 3.2 shows BeWo monolayer without and with 10µM forskolin.

Viability of BeWo incubated with different concentrations of forskolin

To investigate whether different concentration of forskolin (in DMSO) had effect on viability of BeWo cells was also assessed to viability of BeWo using an MTT assay. There was no different between viability of BeWo treated with different concentrations of forskolin. However, as expected, there was a clear difference in O.D. of the MTT assay between BeWo treated with forskolin and untreated controls, because BeWo cells in the untreated controls continued to proliferate and increase in number, whereas, forskolin-treated BeWo formed syncytia and their numbers remained constant (Fig.3.2). Viability of BeWo treated with forskolin at 10µM concentration was stable until 96 hrs of incubation (Fig.3.1B). Thus, treating BeWo with 10 µM forskolin for 72 hrs was used in subsequent experiments.

Protein and gene expression in BeWo cells co-cultured with different numbers of IE for 12 and 48 hrs

To assess the influence the different number of IE in the induction of response in forskolin-treated BeWo, BeWo cell line were treated with 10µM forskolin for 72 hrs and exposed to different number of purified IE overnight and at 48 hrs using same numbers of normal red blood cells cultured *in vitro* for 48 hrs (nRBC) as controls. The protein level of IL 8, RNA expression of inhibin, ANG2, mTOR, endoglin were measured in IE-treated BeWos at two time points; overnight and 48 hrs of incubation. The results indicated no change of amount of IL-8 secreted by BeWo treated overnight with 10, 1, 0.1 intact IE. However, at 48 hrs, a ratio of 10 IE: 1 BeWo resulted in the induction of a 4.5 fold increase in IL-8 compared to treatment with 1 IE: 1 BeWo, and a 27 fold increase compared with treatment at 0.1 IE: 1 BeWo (Fig. 3.3). Treating BeWo with 10:1 IE induced BeWo to secrete about 87-fold more IL 8 compared to the nRBC controls.

Likewise at 48 hrs of forskolin- treated BeWo with 10 IE resulted in 5-fold increase in VEGFA RNA expression compared to forskolin-treated-BeWo treated with equivalent number of nRBC (10:1 IE) at 48 hrs of incubation and overnight (3.4). Minimal differences were observed in the RNA expression of mTOR at 48 hrs of incubation, with expression being less than 2 fold between the 10 IE and the 10:1 nRBC treatment groups. Thus, overall, higher expression of selected genes and protein was observes at 48 hrs with 10 IE: 1 BeWo treatment group compared to overnight treatment.

Relative RNA expression of inhibin, ANG2, mTOR and endoglin in BeWo cells cultured with a 10:1 IE to BeWo ratio for 6, 12, 24 and 48 hrs

Since 10:1 IE ratio induced significant changes in gene expression at 48 hrs, we analyzed the expression of other genes (markers) in forskolin treated BeWo at different time points at a 10IE:1 BeWo ratio using same number of nRBC as control treatment. RNA expression levels of END in all treatment groups were higher at 48hr regardless of the treatment, with 10:1 IE inducing about 1.5 folds more END than 10:1 nRBC. With IHBA, optimal RNA expression was achieved at 48 hrs, with 10:1 IE treatment inducing at least 2 folds more RNA compared to nRBC control.

RNA expression levels and pattern of ANGPT -2 were similar at 12 and 24 hrs, with about a two-fold decrease in RNA expression in the IE treatment group compared to nRBC controls. At 48 hrs, ANGPT-2 expression pattern had a 1.5 fold increase in IE treatment group compared to nRBC controls (Fig 3.5).

BeWo secreted a fair amount of MIF at all-time points in all treatments plus control, with a slight increase in MIF concentration found by the IE treatment group at 12 hrs (Fig 3.6).

Relative RNA expression of inhibin A and PAEP in response to the parasite extract

To investigate whether the *P. falciparum* infected IE-extract induces changes in gene expression in BeWo, an extract of 3D7 IE was used, i.e., a strain of *P. falciparum* that does not express VAR2CSA. When used at a 1:1 ratio of parasite extract: 1BeWo, IHBA RNA expression was observed starting at 24 hrs and at PAEP RNA was observed, but a similar increase was observed in all treatment groups, including BeWo treated with medium alone (control) (Fig 3.8).

Cytokines secreted by THP1 cells on exposure to infected erythrocytes

Since correlation of maternal monocytes and inflammatory cytokines in the IVS during placental malaria exists, we measured types and levels of cytokines and chemokines in the supernatant from THP-1 cells upon exposure of IE (conditioned media) at 24 hrs of incubation and supernatant from THP-1 cells with media alone (spent media). There were higher levels of cytokines and chemokines: TNFα, MIP1α, IL-6, CXCL1, MCP1α in conditioned media compared to levels found in the spent medium (Fig 3.9).

3.4 Discussion

In this study we sought to identify culture conditions for measuring parasite-induced changes in BeWo with characteristics of ST. We found that incubation of BeWo with 10µM of forskolin at 72 hrs stimulated the cells to secrete higher levels of hCG. Also to induce a measurable response, a ratio of intact IE to BeWo required for optimal responses was 10 IE to 1 BeWo, with most of responses (in terms of RNA expression) starting at 24 hrs to 48 hrs of incubation. Detectable differences in expression between treatment and control became more apparent at 48 hrs. These results indicate that response of BeWo to intact IE (based on the selected markers used in our study) do not occur immediately after treatment.

The results are consistent with earlier studies by Chaisavaneeyakorn et al, (2005)[118], who showed that IE induce significant higher MIF in BeWo. However, in our study higher level of MIF in BeWo treated with IE were observed in the first 12 hrs of treatment. After 12 hrs, levels of MIF increased in both normal red blood cells and medium treated-BeWos; further validating earlier observations by Chaisavaneeyakorn and colleagues (2002)[117], that cytotorophoblasts and ST intrinsically express MIF and that MIF elevated in the IVS of women with placental malaria [20], may be secreted by other cells types in addition to ST.

In vivo studies by Moormann et al., (1999) indicated that maternal macrophage from placental malaria -infected tissues express IL-8 [119] and increased IL-8 RNA expression correlated with maternal monocytes in placental malaria infected placentas [18]. We show an increase in IL-8 secretion in forskolin-treated BeWo treated with IE at 10 IE: 1 BeWo after 48 hrs of incubation as observed by Lucchi et al., (2008) [101]. Difference in the amount of parasites, concentration of forskolin used as well cell types could contribute in timing of increased secretion of IL-8 in response to IE. Increase in IL-8 secretion by

BeWo treated with 10:1IE emphasize the immunological role of ST; recognizing and responding to infection (in this case *P. falciparum* IE and its bioactive molecules). IL-8 is an important chemokine that recruit maternal monocytes at IVS and it is elevated in the IVS during placental malaria. Induction of IL-8 secretion by BeWo in response to IE, therefore demonstrates that ST are partly responsible to elevated levels of IL-8 *in vivo*.

While *in vivo* studies have shown dysregulation of growth factor in intervillous space of placental malaria-positive women, we show that BeWo model (10µM forskolin –treated BeWo, incubated for 72 hrs and treated at a ratio of 10 IE: 1 BeWo for 48 hrs) do result in changes in RNA expression of selected markers important for placental and fetal growth: mTOR, INHA, VEGFA and ANG-2 at 48 hrs. However this study is based on screening of few selected markers and can therefore not generalized for all genes.

Overall our experimental study shows that 80% of the selected markers screened for RNA and protein expression are positively induces by 10 IE: 1BeWo treatment. That is, IE induces expression VEGFA, and, ANGPT-2 relative higher compared to the nRBC control indicating potential involvement of parasite-derived ST responses during placental malaria. Increase in expression of an angiogenic molecule i.e. VEGFA and regulator of angiogenesis ANGTP-2 in BeWo (forskolin-treated) that were treated with IE, demonstrate potential modulation of angiogenic processes by ST in response to placental malaria. However it impossible to know how these responses compare with the *in vivo* condition. Although evidence indicate that forskolin-treated BeWo do form syncytial [120], there is no data to date that indicate forskolin- treated BeWo forms single intact layer of cells similar to ST *in vivo*. However to ensure that we have a monolayer of cells in our BeWo system, we observed the BeWo monolayer under microscope until we saw a situation similar to that seen *in vivo* and treat them with fusogenic molecule forskolin to form monolayer of cells to mimic ST.

In the future, we wish to study malaria-induced changes in BeWo as a model for events that occur in ST *in vivo*. Overall the results from this study have helped identify culture conditions for use in these future studies.

Figures and Tables

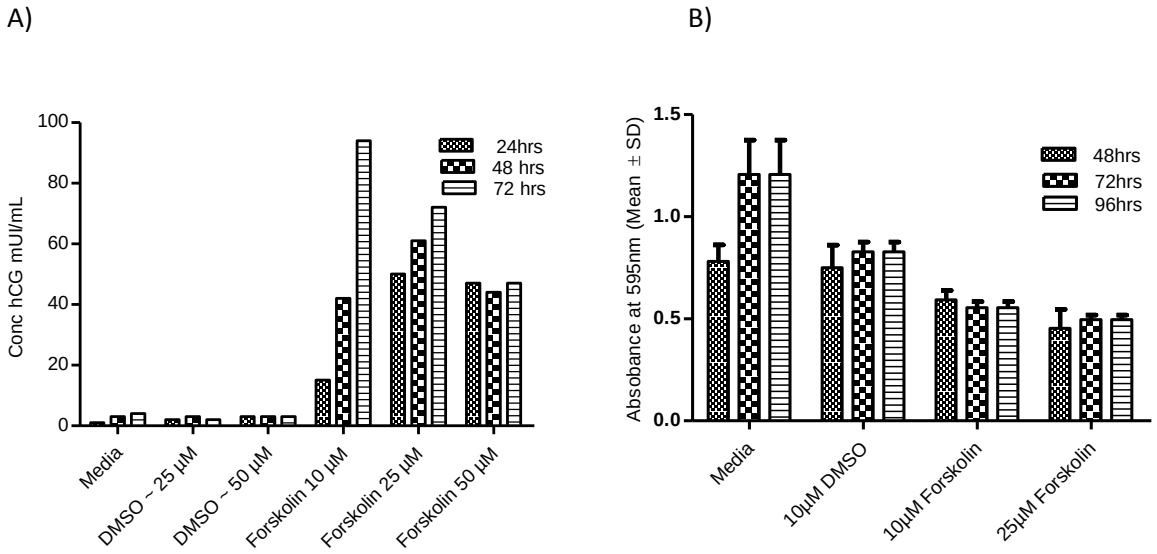

Figure 3.1 Effect of forskolin on BeWo hCG secretion and viability. A) Concentration of hCG secreted by BeWo treated with 10, 25 and 50µM of forskolin in DMSO. B) Viability of BeWo treated with different concentrations of forskolin. 1 x 10^{4} BeWo were seeded in 96 wells and treated with different concentrations of forskolin in DMSO or DMSO alone for different periods of time.

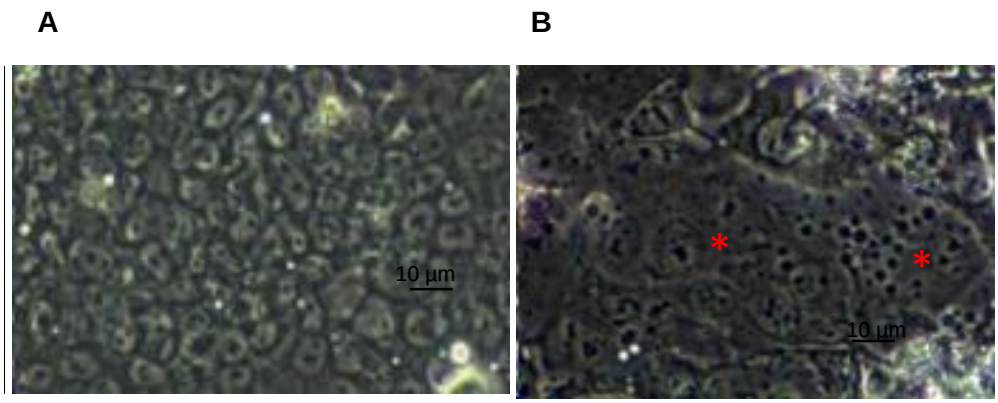

Figure 3.2 BeWo cell monolayers A) before and after B) treatment with 10µM forskolin, incubated for 72 hrs. 2 x 10^5 BeWo cells seeded on 24-well plate.* monolayer of syncytialized BeWo.10x magnification

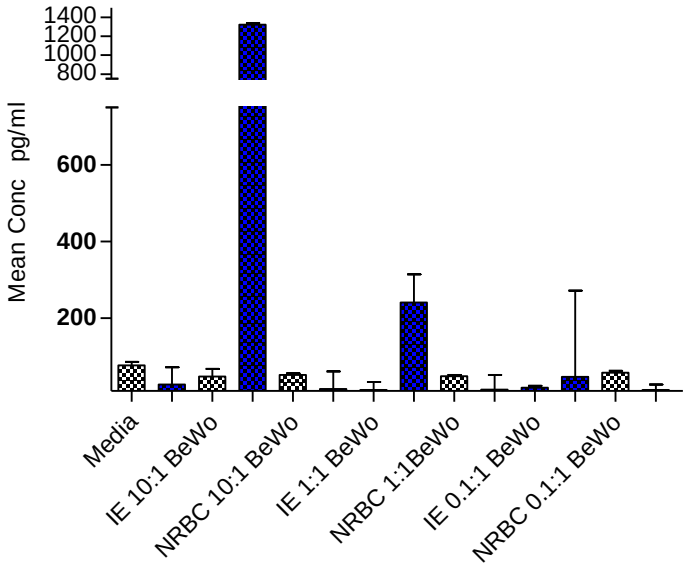

Figure 3.3. Protein expression of IL-8 by BeWo treated with different concentrations of intact CS2 infected erythrocytes using normal red blood cells as controls. Forskolin-treated BeWo (1= 2 x 10^5) cells were seeded in a 24 well format and treated in triplicates with different concentrations of IE and incubated overnight dotted-white bars and for 48 hrs blue bars. Data presented in mean ± SD

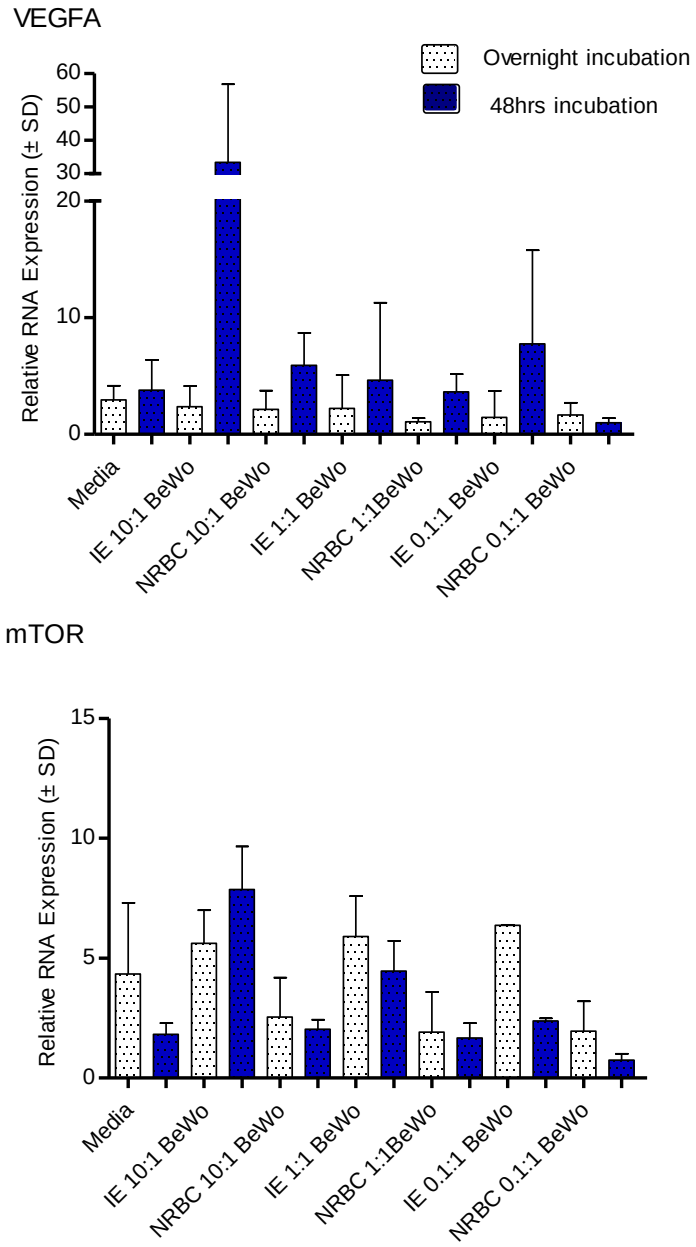

Figure 3.4 Relative RNA expression of selected markers by BeWo treated with different concentrations of intact CS2 infected erythrocytes (mean RNA expression relative to the internal control, *β-actin* gene). 10µM forskolin-treated BeWo (1 = 2 x 10^5) cells were seeded in a 24 well format and treated with different concentrations of IE incubated overnight [dotted-white bars] and for 48 hrs [blue bars. Figures shows expression of selected markers (Mean ± SD)

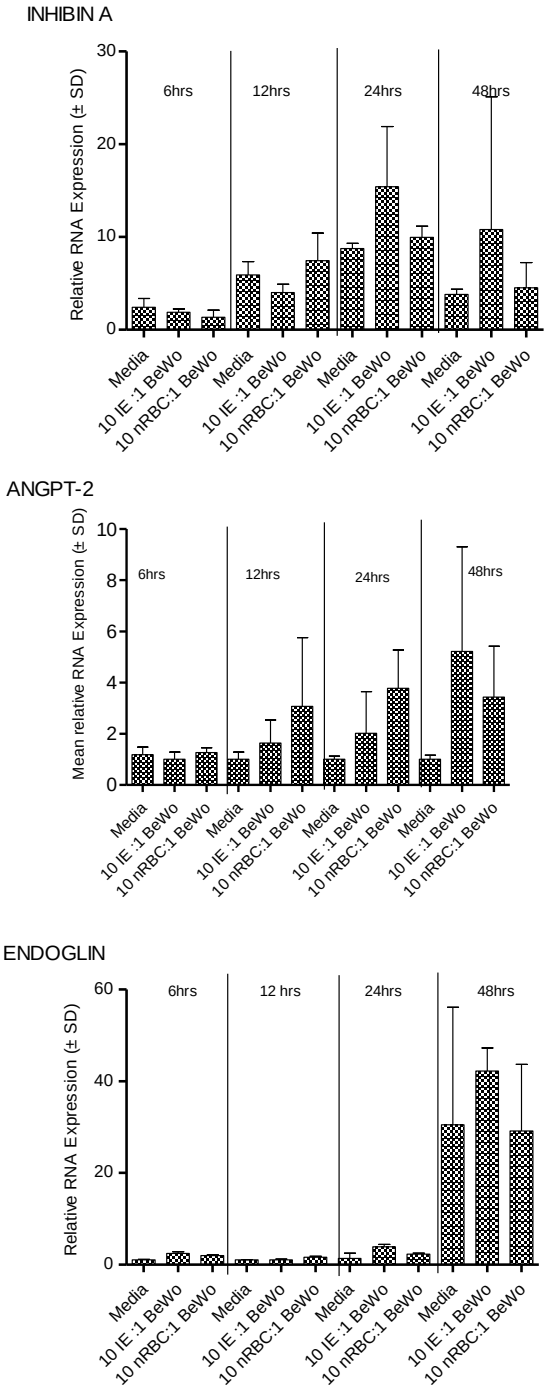

Figure 3.5 Time course expression of selected markers in BeWo cells. 2 x 10^5 forskolin-treated BeWo were incubated with 2 x10^6 CS2 IE using normal RBC as control and incubated for 6, 12, 24, and 48 hrs. Results show mean relative RNA expression levels of A) endoglin, B) Inhibin A, and C) ANG 2 RNA expression in BeWo cells relative to β-actin. Data presented in mean ± SD. Data presented in mean ± SD in triplicate.

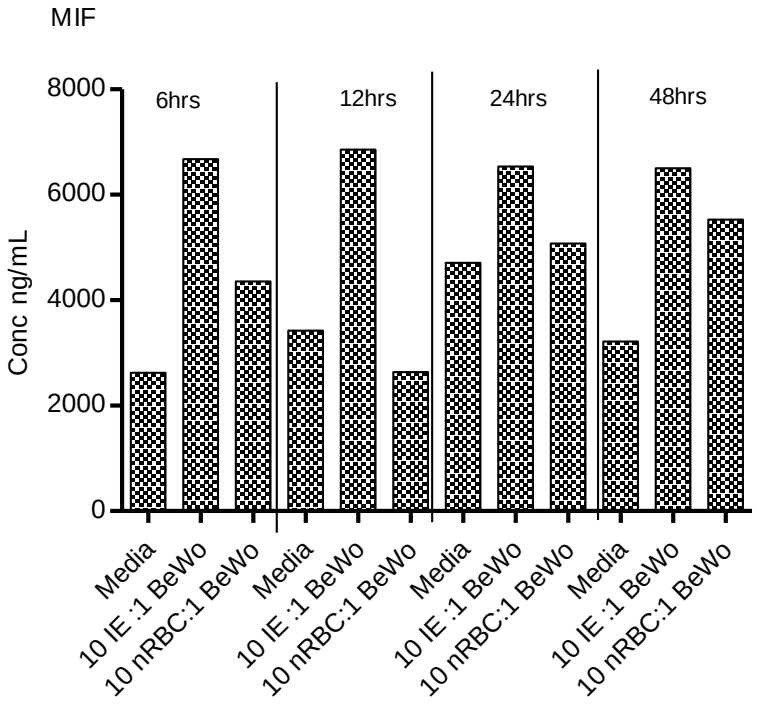

Figure 3.6 Time course expression of MIF in BeWo cells. 2 x 10^5 forskolin-treated BeWo were incubated with 2 x10^6 CS2 IE using normal RBC as control and incubated for 6, 12, 24, and 48 hrs. Supernatant were pooled from triplicates only one sample analyzed. Results show concentration of MIF (ng/ml) secreted by BeWo at different times of incubation.

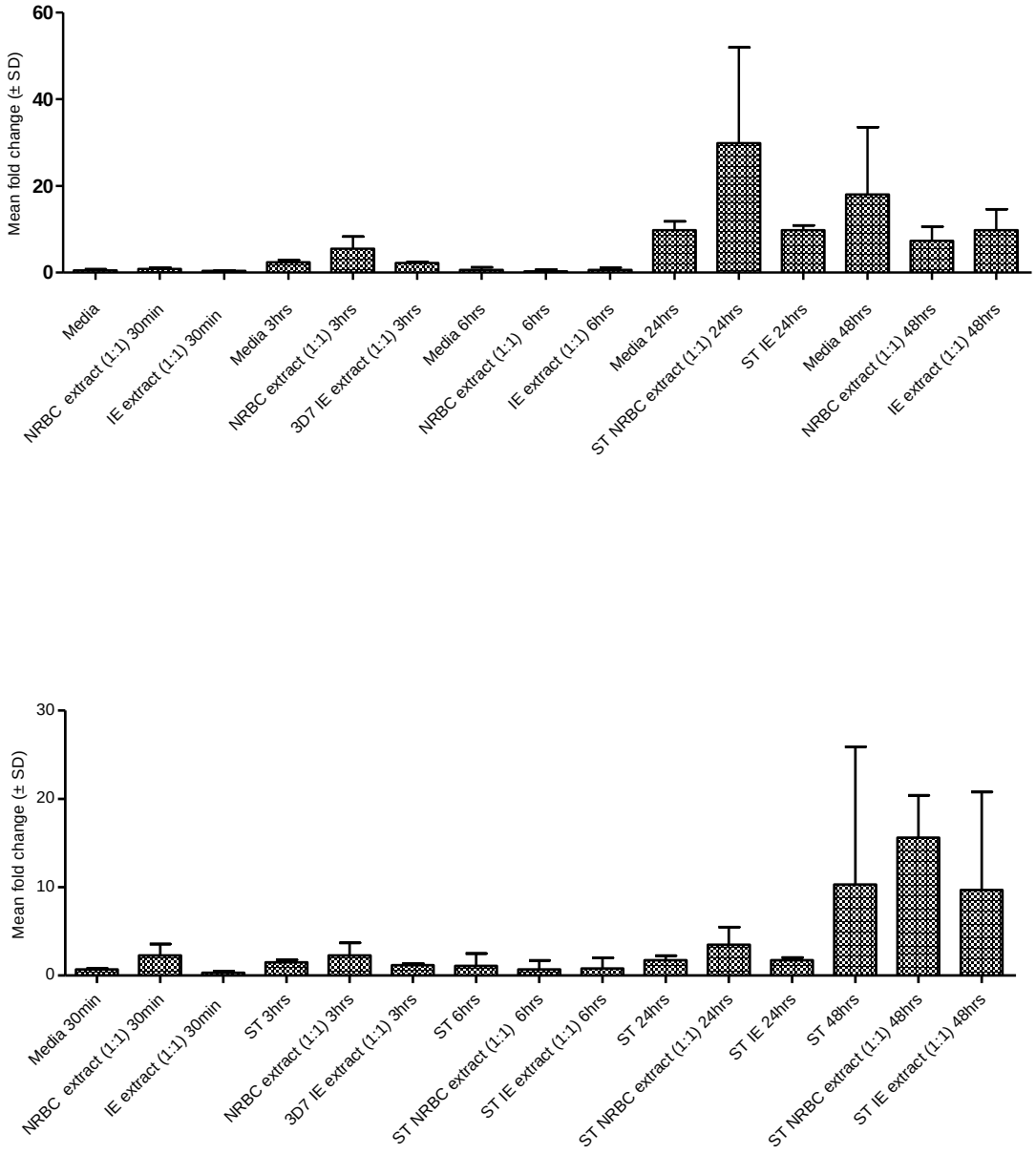

Figure 3.8. Time course expression of inhibin beta A and PAEP from BeWo cells. 2 x 10^5 forskolin-treated BeWo were incubated with 2 x10^5 3D7 IE extract using normal RBC as control and incubated for 30 min, 3, 6, 24, 12, 24, and 48 hrs. Results show mean relative RNA expression levels relative to β-actin. Data presented in mean ± SD in triplicate.

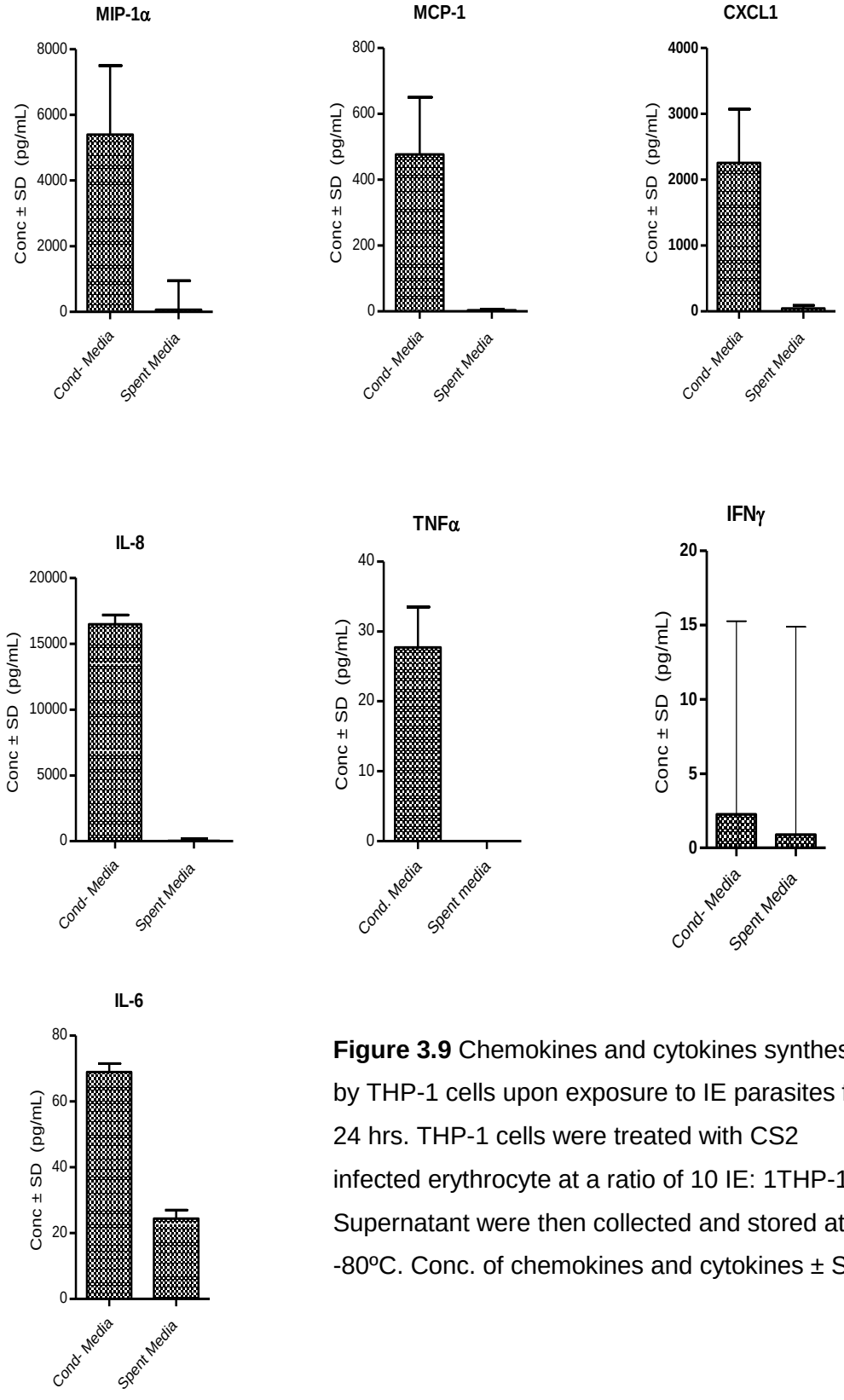

Figure 3.9 Chemokines and cytokines synthesis by THP-1 cells upon exposure to IE parasites for 24 hrs. THP-1 cells were treated with CS2 infected erythrocyte at a ratio of 10 IE: 1THP-1. Supernatant were then collected and stored at -80ºC. Conc. of chemokines and cytokines ± SD

CHAPTER 4

Gemone-Wide Analysis of Changes in BeWo Cells Induced by CS2 *Plasmodium falciparum*-Infected Erythrocytes, Schizogonic Products, and Cytokines

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Abstract

Placental malaria (PM) changes the microenvironment at the intervillous space (IVS) of the placenta which is essential for fetus growth by exposing the syncytiotrophoblast (ST) to P*. falciparum* infected erythrocytes (IE), *P. falciparum* schizogonic products, and inflammatory cytokines leading to low birth weight (LBW) babies. Changes of ST functions occurs during PM, however it is not known what contribute to these changes. In order to understand the influence of PM on ST function, we analyzed gene expression changes in the BeWo cell line produced by the binding of IE, IE and parasite schizogonic products, and cytokines produced by THP-1 monocytes co-cultured with IE.

BeWo cells were syncytialized for 72 hours using 10 µM forskolin in triplicate cultures and exposed to intact CS2 IE, intact CS2 IE plus schizogonic products, and conditioned medium produced by THP-1cells upon exposure to IE for 24 hrs) for 48 hrs. Gene expression changes were then analyzed by the microarray. Functional annotation of differentially expressed genes (fold change ≥ 1.5 and adjusted P value ≤ 0.01) was determined using GO-term context with DAVID version 6.7v and altered pathways were identified using Pathway Miner (BioRag).

Conditioned medium containing inflammatory cytokines and chemokines, but not IE alone or IE with schizogonic products, significantly altered genes associated with biological processes and signaling pathways in BeWo cells that are important for fetus and placental growth. The categories of genes enriched (P ≥ 0.05) among down-regulated genes in BeWo cells treated with conditioned medium included, genes linked with vasculogenesis, blood vessel formation and negative regulation of vasoconstriction, as well as, for “female pregnancy”. Growth pathways significantly altered (P ≤ 0.05) included insulin like growth 1, insulin, mammalian target of ramphamycin, and

transforming growth factor beta 1. Other important growth pathways significantly altered included the platelet derived growth factor, epidermal growth factor signaling and prostaglandin synthesis and regulation.

These results suggest that cytokines and chemokine dysregulate pathways and biological processes in BeWo cells that are involved in placental vascular development and remodeling. The changes in these pathways may explain why placental insufficiency occurs as a result of malaria infection leading to FGR during placental malaria.

4.1 Introduction

During placental malaria, infected erythrocyte (IE) bind to syncytiotrophoblasts (ST) and accumulate in the intervillous space (IVS) of the placenta. If not quickly eliminated, parasite antigens, metabolites, and toxins are released into the IVS during schizogony that can induce an inflammatory response and pathology. The trophozoite-stage IE expresses an antigen called VAR2CSA that binds to ST via chondroitin sulfate A (CSA). Although Moore et al., have suggested that biochemical changes occur in ST functions upon the binding of IE to ST, little is known the changes that occur. Once IE bind to ST, the parasites mature into schizont-segmenters, and subsequently release bio-products during schizogony. The parasite molecules released are well characterized Pathogen Associated Molecular Patterns (PAMPs), including hemozoin, hemozoin-DNA complex, GP1-achor protein, and parasite DNA. *In vitro* studies using primary trophoblasts have shown that ST respond to purified hemozoin [102]. In chronic placental malaria, malaria pigment may be found in chorionic villous ST[108]. In addition, studies have shown that malarial GPI that anchors proteins to parasite membranes activates TLR2/TLR1 and TLR2/TLR6 heterodimers [54] hemozoin pigment activates the NALP3 inflammasome[55]; and parasite DNA-hemozoin complexes stimulates TLR9 [121]. These receptors are expressed on ST. Since ST express receptor for these PAMPs, it is likely they play a role in dysregulation of ST functions during placental malaria. Additional information on the influence of IE and parasite products on induction of pathology is central in understanding the biology of placental malaria. In addition, information regarding the contribution of IE to placental malaria pathogenesis is important in the design and development of future placental malaria vaccine.

Studies in pregnant women residing in malaria endemic areas show that levels of Th1 cytokines are elevated in IVS plasma of malaria-positive women, including TNFα, IFNγ [86] and IL-2, [8] and there is a positive association between elevated cytokine levels and number of maternal monocytes in the IVS [119]. Elevated cytokines and maternal monocytes levels in the IVS of placental malaria-positive women have been associated with LBW babies [8,20]. However to date, the mechanism(s) by which inflammatory cytokines and maternal monocytes contribute to LBW, particularly fetal growth restriction (FGR), have not been elucidated. Data by Boeuf and colleague (2013) showed that system A amino acid transporters are reduced in ST of women who develop malaria-related intervillositis and deliver FGR babies [41]. These data suggest that impaired amino transport may be one of the mechanisms leading to LBW infants. However, studies have shown that chronic placental malaria without intervillositis are also associated with an increased risk of FGR related LBW babies [122]. Clearly, additional mechanisms are possible. Thus, the changes in ST associated with the increased risk of LBW as a result of placental *P. falciparum* infections and associated inflammation have not been identified.

The objective of this study, therefore, is to identify changes in ST induced by intact CS2 IE, parasite bioactive molecules released during schizogony, and cytokines in an *in vitro* model using the BeWo cell line. We hypothesize that the *binding of IE and parasite molecules released during schizogony will induce down-regulation of pathways involved in placental and fetal growth.* Genome-wide expression studies will be used to identify dysregulated genes in BeWo cells exposed to intact CSA expressing IE, IE plus parasite bioactive molecules, and a milieu of cytokines similar to those found in the IVS of women with chronic placental malaria. The results will not only contribute to our/the general understanding of the pathogenesis of placental malaria, but also will answer the major

question as to whether the IE and/or parasite products have a direct influence on ST functions.

4.0 Materials and Methods

CS2 parasite culture The CS2 strain of *P. falciparum* was grown in type O+ blood at a 5% hematocrit in RPMI-1640 media (Invitrogen) supplemented with 50 µg/mL hypoxanthine (Sigma), 0.1 mg/ml gentamicin sulfate (Sigma), 5% albumax, and 5% human AB serum. IE were incubated at 37^{o}C in an incubator in the presence of 5% CO_2, 90% N_2 and 5% O2.

BeWo cell culture and syncytialization BeWo cells, obtained from ATCC, were grown in Ham's F12 medium (source) supplemented with10% Fetal Bovine Serum (Hyclone), 1% L-glutamine, and 1% Penicillin/Streptomycin (Sigma) at 37°C in the presence of 5% CO_2. For experimental purposes, BeWo cells were seeded on 6-well plates at 5.0 x 10^5 cells/well. When the cells reached 90% confluence, syncytialization was induced by adding 10uM of forskolin (Calbiochem) in a vehicle DMSO (dimethyl sulfoxide). Syncytialization was confirmed microscopically and by increased secretion of β-HCG after forskolin treatment using a commercial ELISA kit (Alpha Diagnostic, Int Inc).

Separation and maintenance of knobby parasites The gelatin floatation method was used to isolate late-stage IE (knobby-stage). Briefly, the IE pellet was suspended in sterile gelatin medium (sterile-filtered 0.7% gelatin in RPMI at 37°C) and allowed to sediment for 30-45 minutes. Then, 1ml of the top, turbid, fraction containing trophozoites was removed, IE were washed twice with complete medium, and the percent parasitemia was determined by light microscopy.

Panning of CS2 parasites To select for IE that bind to CSA, the *P. falciparum* CS2 line was panned on monolayers of BeWo cells previously treated with 10 µM of forskolin for 72 to 96 hrs. Briefly, gelatin-floated parasites were washed three times with RPMI and

centrifuged at 2,500 rpm for 5 minutes. The pellet containing late-stage IE (trophozoite) was resuspended in 20ml of RPMI. The 20ml of suspended IE were added to a monolayer of BeWo in T75 flask and incubated for 1 hour at 37 ºC. After an hour, the unbound parasites were removed by washing three times with PBS, leaving only bound parasites on the BeWo monolayer (Figure 4.2). To elute the bound IE, 2mg/mL of soluble CSA was added to BeWo cell cultures and incubated for 30 minutes at 37ºC. The CSA-binding parasites were then washed by RPMI three times and returned to *in vitro* culture.

Purification of Trophozoite-stage IE using magnetic column purification IE were purified using magnetic columns and a Quadro MACS separator (LS Columns Miltenyi Biotec). The technique purifies trophozoite stage parasites from parasite culture. CS2 IE from synchronized cultures were first purified by gelatin floatation, washed with PBS, and then suspended in sterile filtered MACS buffer (PBS, pH 7.2., 0.5% BSA, 2mM EDTA). The columns were re-wet with 5 ml of MACS buffer added drop-wise. Then, the suspension of IE was added to the columns attached to MACS separator. The flow-through containing ring-stage parasites were collected and returned to culture or discarded. Before eluting the trophozoites, the columns were detached from the Quadro MACS separator. The trophozoite stage IE were then eluted with 15ml MACS buffer and collected in a 15 ml tube. The last 2ml of MAC buffer were then plunged against magnetic column to allow for break-up of intact schizonts and trophozoites. The eluted parasites were then centrifuged at 2,500 rpm for 5 min, supernatant was removed, and the parasitemia was determined using microscope. The eluate consisted of trophozoites, schizonts, free hemozoin, hemozoin-DNA complexes, parasite DNA and host and parasite membranes.

THP-1 culture THP- 1 cells are a human monocyte cell line. The cells were obtained from ATCC and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, and 0.05mM 2-β mercaptoethanol (Sigma). Cells were cultured in the presence of 5% CO_2 and the medium was change every 2-3 days.

THP-1- and IE co-culture *Mononuclear* cells accumulate in the IVS during placental malaria, where they phagocytize IE and secrete a complex mixture of cytokines. THP-1 and IE were co-cultured to obtain a milieu of cytokines similar to that produced during placental malaria. THP-1 cells (passage 8) were seeded at 4 x 10^5 cells/ ml in T-75 flask and co-cultured with purified CS2-IE at a ratio of 10 IE to 1 THP- 1 in THP-1 media (11mL). The cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 24 hrs to produce cytokine-containing medium. A parallel T-75 flask containing same number of THP-1 cells was cultured identically and supernatant as used as a negative control. At 24 hrs, supernatant from both flasks were collected by centrifugation at 2,500rpm and 30ºC for 7 minutes and then stored -80ºC. Supernatant was assayed for chemokines: (IL-8 (CXCL8), monocyte chemo tactic protein-1 (MCP-1), the chemokine MIP1α (CCL3) and CXCL1, cytokine: macrophage inhibitory factor (MIF) and tumor necrosis factor alpha (TNFα) by Luminex assay (R& D). Levels of cytokines and chemokines levels presented in Table 4.7

BeWo co-culture experiments **BeWo** cells were plated in 6-well plates at 5 x 10^5 cells/ well (3ml/ well). At 90% confluence, BeWo cells were treated with 10µM forskolin (Calbiochem) for 72 hrs. After 72 hrs of incubation, BeWo cultures were washed and then the following treatments were added: i) intact CS2 IE purified by gelatin flotation at a ratio of 10 IE: 1BeWo and normal RBC cultured *in vitro* from 48 hours (nRBC), equal to

the number of RBC present in the IE cultures, as a negative control, ii) *intact CS2 IE plus schizogony bioproducts purified by magnetic column* separation at a ratio of 10 IE: 1BeWo and parallel culture containing nRBC equivalent to nRBC present in the IE treatment as control, and iii) THP-1 cytokine-containing–medium (from THP-1: IE coculture) and negative-control THP-1 culture medium (Table 1). After 48 hrs of incubation, supernatants were collected, cells were washed by PBS (37ºC) and lysed using RLT buffer (Qiagen, Inc.). The resulting cell lysates were transferred onto RNAase-free microtubes and kept at -80ºC until used. A parallel experiment was carried out in 96-well format to determine viability of cells.

DNA microarrays Human DNA microarrays were produced in the molecular genomic core facility of Drexel University of Medicine using the Opero Human Oligo v2.0 set containing ≈ 26,000 (70mer) oligonucleotides. The oligonucleotide included well characterized human genes from the UniGene Database. Microarray analysis was conducted in triplicate arrays for each individual sample. That is, each co-culture combination (e.g., BeWo + IE) was conducted in triplicate and triplicate microarray analysis of each culture was made, such that each data point is based on the data for 9 microarrays.

RNA extraction and RNA quality assessment Total RNA from triplicate treatment and control cultures were extracted using an RNeasy RNA isolation kit (Qiagen, Inc.). The quality of RNA was assessed by measuring O.D. with a Nanodrop 2000 spectrophotometer (Thermo Scientific). Samples with ratios of > 2.00 when measured at 260/280 were considered pure enough for further analysis.

Complementary DNA synthesis (cDNA) Complimentary DNA synthesis for each purified RNA sample was conducted using Amino Ally Message Amp II aRNA kit (Ambion, Inc., Austin, TX) following the manufacturer's protocol. Briefly, 10µ of reverse transcription master mix (containing 1µl Array Script, 2µl 10x first strand buffer, 1µl T7 Oligo (dT) primer, 4µl of dNTP mix, 1µl RNase inhibitor in 1µl nuclease-free water) was added to nuclease-free microtubes and 10µl (5µg) of sample RNA was added. Cycling parameters for first strand cDNA synthesis were 42°C (50°C lid) 2 hrs for 1cycle and 4°C hold.

For second strand cDNA synthesis, 80µl of a master mix, containing 10µl of10x second strand buffer, 4µl dNTP mix, 2µl DNA polymerase and 1µl RNase H in 63µl nuclease free water, was added to each synthesized sample of first strand cDNA (~20µl). Cycling parameter for second strand cDNA synthesis included 16°C (heat-disabled lid, or no lid) for 2 hrs for 1 cycle and a 4°C hold.

Complementary DNA synthesis purification For purification of all sample cDNA synthesized, 250 µl of cDNA binding buffer was added to each cDNA sample and mixed by pipetting up and down 2–3 times, then flicking the tube 3–4 times. Following a brief centrifugation, the solution was collected at the bottom of the tube. The mixture was then passed through a cDNA filter cartridge, centrifuged, and washed using washing buffer. Purified cDNA was eluted from the column using preheated nuclease-free water at 55°C. cDNA was stored on -20ºC for further analysis.

In vitro transcription to synthesize amino allyl-modified RNA (aRNA) First, 26µl of a master mix consisting of 3µl aaUTP (50 mM), 12 µl of the ATP-CTP-GTP- Mix, 3µl of UTP solution (50 mM), 4 µl T7 reaction buffer (10x) and 4µl T7 enzyme mix was added

to each cDNA sample, incubated at 37°C (default lid; 100–105°C) for 14 hrs; for 1 cycle and kept at 4°C on hold in thermal cycler

aRNA purification and quality assessment To remove unincorporated aaUTP and Tris from the *in vitro* transcription reactions (that would otherwise compete with the aRNA for dye coupling, enzymes, salts, and other unincorporated nucleotides), 350 µl of aRNA binding buffer was added to each sample followed by 250 µl of 100% ethanol. The mixture was thoroughly mixed by pipetting. Samples were then passed through an aRNA filter cartridge, washed with washing buffer, and eluted with pre-heated nuclease-free water and quality assessed was made using a Nanodrop spectrophotometer.

Dye preparation, coupling to aRNA and labeled aRNA cleanup Mono-reactive NHS esters of Cy3 (550nm maximum absorbance) and Cy5 (650nm maximum absorbance) dye (Amino Ally MessageAmp II Kit) were used for labeling aRNA. The Cy5 dye was used to label the aRNA of pooled placental malaria-negative tissue; whereas, Cy3 was used for labeling aRNA from malaria-positive samples.

For the preparation of the dye, 11 µL of DMSO was added to one tube of Cy3 or Cy5 reactive dye(Life technologies) and vortexed to mix thoroughly, and allowed to dissolve in the dark at room temperature (20-25ºC) for an of 1 hr. At the same time, ~ 5µg of amino allyl-modified aRNA was added to a nuclease-free microfuge tube and vacuum dried until liquid no longer remained. Then, the dried aRNA was re-suspended in 9 µl of aRNA-dye-coupling buffer and 11µl of the dye was added and mixed well by gently vortexing. The aRNA and dye mixture was incubated for 30 min at room temperature in the dark. To stop the reaction, 4.5 µl of 4M hydroxylamine was added and mix well by gently vortexing, and the reaction mixture was incubated at room temperature in the dark

room for 15min. The solution was then brought up to 30µl by adding nuclease-free water.

Dye labeled aRNA purification and quantification To remove excess dye from aRNA, 105 µl of aRNA binding buffer was added to each aRNA sample, followed by 75 µl of 100% ethanol and mixed by pipetting up and down for 3 times. The samples were then added to filter cartridges. Cartridges containing the labeled aRNA were then centrifuged for 1 min, and washed once with 500µl of wash buffer for 1 min at 1000g x g. The flow-through was discarded and the aRNA eluted with 10µl pre-heated nuclease-free water. Elution with 10µl preheated nuclease-free water was repeated two times. The purified aRNA was stored at -20ºC in the dark until further used.

Preparation of labeled aRNA for hybridization Labeled aRNA for each sample was concentrated to 10µl by vacuum drying and then fragmented to 60-200 nucleotide using 10x fragmentation reagent (Life technologies P/N AM8740). Then, components of hybridization mixture was added one by one to the fragmented aRNA; i. e.; 1.2µl 1M HEPES (pH 7.0), 9µl 20x Saline-sodium citrate (SSC) buffer, 10µl 1mg/mL of polydA, 10µl human cot DNA^{R} (used to block nonspecific hybridization in microarray screening) (Invitrogen), and 1.2µl 10% Sodium dodecyl sulfate (SDS). The mixture was boiled for 2 minutes and then cooled to room temperature 25ºC for 10min in the dark.

Preparation of the arrays and washing hybridized slides First, 6ul of 3x SSC was placed at each end of the human oligonucleotide array and then 60µl of each sample for hybridization was added. Hybridization was done for 16 hrs at 65ºC. Several washing steps with SSC and SDS was performed followed by drying of the microarray.

Scanning slides, initial analysis, data acquisition and detection of differentially expressed genes After drying, microarray slides were scanned and data was then

acquired and initial analysis was performed. Initial analysis involved flagging irregular and missing features on scanned microarray slides (GenePixPro 5.1 Axon). Flagging criteria included empty SSC, spots with diameters ≤ 60μm, and a signal/noise ratio (SNR) of < 2. Analysis of microarray data was conducted using Limma GUI in R package [123]. Background correction was made using Norm Exp offset 16 and data normalization within the array using Print Tip Group Loess. Fitting of normalized microarray data involved the linear model using least of square method. Differentially-expressed genes were then determined using adjusted p value of <0.01 and fold change of ≥ 1.5

Functional annotation To facilitate biological interpretation of genes that were differentially expressed into functional categories, Database for Annotation, Visualization and Integrated discovery (David Bioinformatics Resources Version 6.7v) [124] in the context of gene ontology terms GO-terms were used to assign biological functions to dysregulated genes. Each category is assigned enrichment score (p value) a modified Fischer exact test adopted to measure the gene-enrichment of biological processes using GO from public databases.

Pathway analysis Pathway analysis for differentially-expressed genes was conducted using pathway miner Version 1.1 1(http://www.biorag.org/pathway.php) that utilizes databases from three independent sources: GeNMAP, Biocarta and KEGG. Input required for analysis includes accession numbers and gene expression values in terms of log-fold change. Gene expression profiles were analyzed for significant pathway representation based on Fisher exact statistical test. The statistical tests for pathways from all three different databases were conducted. The pathways include both the cellular and regulatory pathways.

Validation of microarray RT PCR experiment The cut-off used in the analysis as 1.5 fold change. To verify this cut-off was appropriate, 5 genes with ~1.5-fold changes were selected for follow-up studies using real time semiquantative PCR (RT-PCR). High quality RNA (A260/A280 ratio = 1.9-2.0) was used for cDNA synthesis that was carried out according to the manufacturer's protocol (Quanta Bioscience). cDNA was diluted at 1:5 with nuclease-free water for RT-PCR assays. Amplification assays were carried out in triplicate for each gene using a total reaction volume of 20 µl. The reaction contained 4µl of sample cDNA, 10µL PerfeCta SYBR Green SuperMix (Quanta Bioscience), 250nM (0.5µl) of gene-specific forward and reverse primers (fibroblast growth factor 17 (FGR17), solute carrier family 3 member 1 (SLC3A1), solute carrier family 38, member 2 (SLC38A2), transforming growth factor, beta 1 (TGFβ1), Inhibin, beta A (INHA) (Biorad) Bio-Rad) in nuclease-free water. A master mix containing forward (0.5µl) and reverse primers (0.5µl) for *Beta actin* was prepared for amplification as an internal control (reference gene). The RNA was reverse transcribed using SYBR Green qPCR under cycling conditions recommended by the manufacturer. Changes in gene expression in treatment sample relative to controls were analyzed using comparative CT method. Two sample t-test was used to investigate the difference between normalized CTs.

Statistical analysis. Fisher's exact test was adopted to measure the gene-enrichment whereas p value ≤ 0.05 was considered strongly enriched in the annotation categories. Significant changes in gene expression profiles of a variety of biochemical pathways were also identified based on Fisher's exact test with multiple testing error rate controlled at 5%. These changes included both up- and down-regulate genes for each placenta. Pathways with p values ≤ 0.05 and ≥ 2 fold changes was considered significantly altered.

4.4 Results

Changes in gene expression

Using a cutoff of a ≥1.5-fold change in gene expression and adjusted p values of 0.01, 170 genes were found to be differentially expressed in BeWo cells co-cultured with intact CS2 IE; among which 145 genes were upregulated and 26 genes were down regulated Whereas, a larger number of genes were differentially expressed in BeWo cells cultured with intact CS2 IE and schizogonic products. Overall, 330 genes were differentially expressed, with; 185 genes being upregulated and 145 genes downregulated. One the other hand, a total of 516 genes were differentially expressed in BeWo cells cultured with conditioned cytokine-containing medium compared to spent medium. Among the 516 differentially expressed 195 genes were upregulated and 321 genes were downregulated. Thus, significant gene expression occurred in BeWo cells co-cultured for 48 hours with CS2 IE (n=170 genes), IE plus parasite schizogonic products (n=330 genes), and culture medium containing cytokines (n=516 genes).

Biological processes significantly altered in differentially expressed genes

The biological characteristics of the differentially-expressed genes was explored using DAVID bioinformatics tool 6.7v [124]. The program analyzes the biological properties of each gene product (protein) and groups of proteins that perform related biological processes, and then calculates the likelihood the biological process being altered. In the primary analysis, different biological processes were categorized into Gene Ontology (GO) terms. GO terms with an EASE score of ≥ 0.1and p values of ≤ 0.05 (Fisher exact test) were considered significantly enriched (i.e., altered). Biological processes likely to contribute to fetal and placental growth and development, which are of special interest in this study, were selected and are listed in Tables 4.1 for upregulated genes and Table 4.2 for downregulated genes. Results of the analysis identified only three GO terms the

up- and down-regulated genes when BeWo cells were co-cultured with intact CS2 IE. None of the biological processes were related to growth and fetus development (Table 4.2 and Table 2). However, when BeWo cells were cultured with intact IE plus schizogonic products, 18 GO terms were identified; 5 for up-regulated genes and 13 for downregulated genes. Those of highest interest among the up-regulated processes were innate immune responses, metal ion transport, and basic amino acid transport. A number of important processes were identified among downregulated genes related to fetal and placental development (Table 4.2), including vasculogenesis (vascular development, pregnancy, and cellular responses. The vasculogenesis category included the following genes; Cbp/p300-interacting transactivator; (CITE 2), caveolin 1; CAV1, hairy/enhancer-of-split related with YRPW motif 1(HEY). Table 4.3 provides a description of the biological functions of the above genes. The female pregnancy category consisted of genes that code for proteins involved in physiological processes that allow fetal development within the body of a female: including, 26 serine protease, CITE2, adrenomedullin (ADM) a potent vasodilator, and v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) Others of potential importance included the “cellular response to stress” and ‘lipid storage”. Amine acid transport, cellular amino acid biosynthesis, and carbohydrate transport were not significantly enriched.

BeWo exposed to THP-1-conditioned medium demonstrated a distinct pattern of GO terms for both upregulated and downregulated genes (Table 4.1 & 4.2). Most of the categories important for cells survival were enriched in upregulated genes as well as processes involved in transport, proteolysis and some metabolic processes. Among the downregulated genes, processes involved in placental vascular activities, female pregnancy and response hormones, hormone activity, response to stress, cellular signaling and metabolic processes were identified. Closely related to the etiology of

placental malaria were GO terms for vasculogenesis, blood vessel morphogenesis, female pregnancy, and vasculogenesis category consisted of genes that facilitate embryonic vascularization and angiogenesis (Table 4.4). Related to vasculogenesis are genes involved in blood vessel morphogenesis. There were 8 genes in the blood vessel morphogenesis category and all of them play various regulation of blood vessel formation (See Table 4:4). The "female pregnancy" GO term category consisted of the following genes: ADM a vasodilator, insulin like 4 (insl4) associated with viability of trophoblasts; TGFβ1 a vital regulator of placental development and function; urocortic a neuropeptide which is known to stimulate corticotropin-releasing hormone (CRH); 26 protease serine which is important in placental function and development, and FOS which is implicated in growth factor signaling.

Oxidative stress may also play a role in the etiology of placental malaria with inflammation [125,126]. Interestingly, genes related to stress were enriched in BeWo exposed to cytokines. These included genes that code for cytochrome P450, family 11, subfamily A, polypeptide 1, epidermal growth factor receptor, erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian), ion peptidase 1, mitochondrial protein phosphatase 1, regulatory (inhibitor) subunit 15B , superoxide dismutase 2, mitochondrial syndecan 1, urocortin, v-fos FBJ murine osteosarcoma viral oncogene homolog.

Pathway associated with differentially expressed genes

BeWo exposed to intact CS2 IE *P*athways altered by the 170 genes that were differentially expressed (fold change ≥1.5, Adj. p ≤ 0.01) were identified by pathway miner. The results indicated that five main pathways were significantly altered (Table 4.5). Co-culture of BeWo cells with IE upregulated the immune pathway which is involved in adhesion and diapedesis of granulocytes (adhesive molecules p= 0.003) and

a multifunctional pathway the MAPK signaling pathway (p= 0.001) and electron transport chain (p=0.003).

BeWo cells treated with intact CS2 parasites with schizogonic product A number of pathways in BeWo cells cultured with intact IE and schizogonic products were dysregulated (Table 4.5). In addition to the MAPK signaling pathway ($p \leq 0.001$) and electron transport chain altered by IE, important pathway for fetal growth were altered, included the TGFβ signaling pathway (p=0.02), and the focal adhesion signaling pathway ($p \leq 0.001$). In addition, pathways essential in signal transduction, focal adhesion signaling were also dysregulated. Pathways involved in translation of proteins, i.e., ribosome pathway and sucrose metabolism, were also influenced. Finally, immune pathways for cytokine-cytokine receptor interaction and the complement and coagulation pathways were up-regulated. Thus, schizogonic products stimulated a number of growth and immune regulator pathways.

BeWo cells treated with conditioned medium In contrast to the limited number of changes observed above, a large number of important pathways for fetal growth were altered in BeWo cells treated with conditioned medium (Table 4.5). Changes included significantly altered pathways involved in cellular survival, angiogenesis, proliferation, differentiation, cellular amino acid and glucose transport, insulin-like growth factor 1 (IGF-1), insulin, and the mammalian target for ramphamycin (mTOR) pathways. Other important growth pathways important in angiogenesis were significantly altered i.e., the transforming growth factor beta (TGFβ1), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) pathways. The adipocytokine signaling pathway, a pathway that is involved modulating metabolism and focal adhesion signaling was also downregulated.

Immune response pathways were also stimulated, including the cytokine-cytokine receptor interaction signaling pathway, as well as, pathways reported to be induced by cytokines and other environmental stimuli, including the MAPK signaling pathways: the classical MAPK kinase: p38 MAPK Signaling Pathway, Erk1/Erk2 MAPK signaling pathway, as well as the Jak-STAT signaling pathway. Thus, exposure of BeWo cells to cytokines and chemokines significantly dysregulate the inflammatory pathways including complement and coagulation cascades and prostaglandin synthesis regulation.

Confirmation of interpretation of microarray results by RT-PCR. The above dysregulated genes and pathways were identified using a cut-off of ≥1.5-fold change and adjusted p values of ≥0.01. To confirm the appropriateness of this cut-off, 5 genes were selected with ~1.5-fold change and evaluated by RT-PCR. Results in Table 4.6 confirm the microarray results.

Summary of Results A summary of biological processes and metabolic pathways that were altered by IE, IE plus schizogonic products and cytokines is shown in Fig. 1. Clearly, the binding of IE to BeWo cells stimulated few responses, the binding of schizogonic products to trophoblasts induced upregulation of vasculogenesis, response to stress and the MARK pathway; however, numerous processes and pathways with the potential for altering placental and fetal growth were suppressed.

4.5 Discussion

The effect of IE in the IVS and subsequent inflammatory response on ST function is unknown. In the current study, the BeWo cell line syncytialized by treatment with 10µM forskolin was co-cultured with placental binding CS2 IE; placental-binding CS2 IE plus schizogonic products (e.g., hemozoin, parasite DNA, GPI-anchored proteins); and cytokines and chemokines produced by THP-1 monocytes upon exposure to IE and changes in gene expression was studied using microarray analysis. Results showed that CS2 IE alone, CS2 IE plus schizogonic products, and cytokine induced different pattern of gene expression in BeWo cells. *In vitro*-produced supernatants containing a mixture of cytokines commonly found in the IVS of women with placental malaria significantly induced changes in BeWo cell processes and pathways that could influence fetal growth in utero as the result of placental malaria associated pathology and LBW babies.

According to the *in vitro* results, neither biological processes nor pathways associated with fetal growth were altered in BeWo cells cultured with intact CS2 IE alone, suggesting that binding of the IE alone to ST does not have an impact on fetal growth. . However, as shown in Tables 4.1, 4.2, 4.3, and Fig.1, BeWo cells incubated with intact IE plus schizogonic products downregulated genes that code for proteins that inhibit vasculogenesis, namely, CITE 2, HEY and CAV 1 suggesting potential local adaptive response to increase local placental blood flow in response to exposure to IE plus parasite products released during schizogony. Conversely in BeWo cells cultured with cytokines expressed genes that code for proteins that both facilitate and inhibit vasculogenesis; creating seemingly counteracting effects that could increase the risk of dysregulated vasculogenesis, that is, increasing the risk of poor blood vessel formation, *in vivo.* Placenta-adaptive changes in response to malaria have been suggested in several *in vivo* studies. Leke and colleagues (2002) showed an increase in both the

number and diameter of fetal blood vessels in fetal villi in placental malaria-positive women who delivered normal birth weight babies[145]. Recently, a study in Malawi by Griffin et al., (2012) showed that malaria infection early pregnancy women who were placental malaria-positive at delivery irrespective of gravidity, resulted to decreased umbilical artery resistance late in the third trimester[127].The present *in vitro* study suggests that ST response to malaria in the absence of inflammation (i.e., intact IE plus schizogonic products respond by downregulating genes that code for proteins that inhibit vasculogenesis (in this case CITE 2, HEY and CAV 1) as a compensatory mechanism to increase local *utero*- placental flow.

Pathophysiology of placental malaria is correlated with hypertensive disorder observed in preeclampsia [128,129] particularly in first time mothers and women who have preterm deliveries. In the current *in vitro* study, a trend towards downregulation of key vasodilators and pro-angiogenic molecules was observed in BeWo cells treated with both intact IE plus schizogonic products and cytokines, with especially high numbers of downregulated genes found in BeWo cells exposed to cytokines (Table 4.2 and 4.5). The genes downregulated included the vasodilators; adrenomodullin and urocortin [130,131]; angiogogenic molecules; TGFβ1 , CITE2 [132], RASA1, C1GALT1 , APA, [133–135], the ID 1 gene; NUS1P3 that all participate in angiogenesis and vascular remodeling; and the growth regulatory factor ZFP36L11. Since the placental is not innervated [136], local expression of vasodilators and vasoconstrictors are essential in maintaining vascular tone during pregnancy for optimal transplacental transport of nutrient and gases that are essential for placental and fetal growth. Results from our *in vitro* study therefore have identified possible mechanisms for ST responses to cytokines during placental malaria and suggests how these responses might increase the risk of FGR, PTD, as well as hypertension. The results emphasize the role of cytokines in the placental pathology

observed in women with placental malaria when inflammation occurs in the IVS. These observations therefore suggest that inflammation due to (chronic placental malaria, and not IE themselves increase risk of poor/bad pregnancy outcomes.

Syncytiotrophoblasts are the coordinators of the bimolecular interactions that occur between the fetus and the mothers; integrating maternal and fetal signals [137] that facilitate fetal growth *in utero*. Both autocrine and paracrine factors signals are released and received by ST, thereby regulating vasculogenesis, angiogenesis, transport of nutrients, cell survival, and proliferation through various developmental and growth pathways. Abnormalities in ST growth factor pathways have been linked to reduced fetal growth *in utero* [68]. In our *in vitro* study, important growth pathways were significantly altered in BeWo cells exposed to cytokines compared to the other treatments: including IGF-1, EGF, PDGF, insulin, and the mTOR signaling pathways. Others pathways included TGFβ1, prostaglandin synthesis and regulatory signaling pathways (Table 4.5). Our *in vitro* data are in agreement with our earlier genome-wide studies reported in Chapter 2 where *in vivo*-derived fetal tissue collected from the placentas of women with chronic inflammatory placental malaria were assessed. Major pathway ways related to fetal grown identified both *in vivo* and *in vitro* included the mTOR, IGF-1, TGFβ1, insulin, prostaglandin synthesis, and regulation signaling pathways. The current findings are supported by studies reported previously, For example Umber et al (2011) reported that reduced levels of IGF-1 were present in maternal plasma of women who had placental malaria inflammation at delivery [66]. Our study using the BeWo cell model found that inflammatory cytokines dysregulated the IGF-1 signaling pathway when BeWo cells are co-cultured with cytokines, supporting the role of cytokines in placental pathogenesis. Abnormality in IGF-1 signaling has also been reported to be associated with reduced fetal growth *in utero*, suggesting that IGF-1 post-receptor signaling molecules, e.g.,

IRS2, are important in mediating the growth action of IGF-1[68,63]. Accordingly, we found IRS2 gene as one of several genes downregulated in IGF-1 signaling pathway in BeWo treated with cytokines. These data provide insight into the role altered growth and developmental pathways in ST that increase the risk of FGR in placental malaria where inflammation occurs.

The identification of other altered growth pathways other than IGF-1 (Table 4.5) in BeWo cells exposed to cytokines bring attention to the potential involvement of other growth pathways in the pathogenesis of placental malaria-associated inflammation. For instance, the mTOR pathway in ST (which was significantly altered in our study) is downstream from several important growth factors pathways, such as IGF-1, insulin and leptin [109, 110, 86]. The mTOR pathway regulates amino acid uptakes via sodium-dependent amino acid transporter 1 and 2 (SNAT1 & 2), mediates protein translation and facilitates angiogenesis [85], which makes the mTOR pathway important in FGR . Particularly prominent in our study was the downregulation of system A amino acid transporter gene (SNAT2 i.e., SLC38A2) that codes for neutral amino acid transporter in addition to mTOR signaling pathway. Downregulation of SNAT2 in BeWo cells treated with cytokines is in agreement with data by Boeuf et al., (2013) that showed downregulation of transcription of SLC38A2 in ST from malaria-infected placentas with inflammation [41]. Other signaling pathways that are important in angiogenesis and embryonic development include the TGFβ1, EGF, and PDGF signaling pathways that were also dysregulated in BeWo cells treated with cytokines. These observations indicate that placental malaria with inflammation may alters ST function in multiple ways. Further studies on the role of mTOR, TGFβ1, EGF and PDGF in the pathogenesis of placental malaria are needed.

Our study is based on direct influence of IE, schizogonic products and cytokines on BeWo cells for only 48 hours. Results from the current may be comparable to what happens in non-immune women who become infected and lack neutralizing antibodies against the parasite protein VAR2CSA. *In vivo*, ST may be exposed to the binding of intact IE, schizogonic products, and cytokines over an extended period of time. The current study allowed us to identify the effect of each of these factors individual on ST, however, *in vivo* ST are exposed to all three conditions when women develop chronic infections. Therefore, further *in vitro* studies are warranted that investigate the combined effect of IE, schizogonic products and cytokines on BeWo cell gene expression.

4.6 Conclusion

Inflammation is important in the pathophysiology of placental malaria and is associated with an increased risk of FGR and PTD. Cytokines significantly induced changes in both biological processes and metabolic pathways that are important for placental and fetal development and growth *in utero*. Downregulation of genes that are important in vasculogenesis, vasodilation and vasoconstriction, as well as angiogenesis provide possible mechanisms by which cytokines might induce placental insufficiency during placental malaria. Furthermore, intact placental binding IE with or without schizogonic product do not seen to significantly dysregulate growth pathway in BeWo cells, suggesting that directly parasite binding of IE or parasite bioproducts via PRR is not likely to be the primary cause of pathologies associated with bad pregnancy outcomes. In contrast, the secretion of chemokines and cytokines that recruit and retain maternal macrophages at IVS appear to be primary factors that are responsible for increasing the risk of poor pregnancy outcomes. Our study therefore has helped elucidate the mechanisms by which inflammation in the IVS increases the risk of reduced fetus growth.

Table 4.1

Functional annotation chart of biological processes associated with differentially upregulated genes

Biological processes	Genes	P value
BeWo treated with intact IE		
GO:0000041~transition metal ion transport	NM_003459, NM_000578, NM_017964	0.05
BeWo treated with intact IE and schizogonic products		
GO:0051252~regulation of RNA metabolic process	NM_005710, AB032976, NM_020657, AK000580, NM_006442, AL137483, NM_030756, NM_004083, AK055592, NM_003900, AF055019, AK056903, AL110236, NM_000578, NM_006735, NM_014862, NM_002700, NM_014650, NM_030763, NM_020646, NM_005120, AK025812, BC016334, AB021641, NM_003436, NM_052860, NM_003433, BC007307	0.04
GO:0045087~innate immune response	NM_015077, NM_001710, NM_000578, NM_020393, NM_014726, D17525, NM_014314	≤ .001
GO:0030001~metal ion transport	NM_006539, U07139, AB032970, NM_017964, NM_000718, AK055913, NM_024076, AK054816, NM_003459, NM_001038, NM_006749, NM_000578, NM_014227, AB033091	≤ .001
GO:0006355~regulation of transcription, DNA-dependent	NM_005710, AB032976, NM_020657, AK000580, NM_006442, AL137483, NM_030756, NM_004083, AK055592, NM_003900, AF055019, AK056903, AL110236, NM_000578, NM_006735, NM_014862, NM_002700, NM_014650, NM_030763, NM_020646, NM_005120, AK025812, BC016334, AB021641, NM_003436, NM_052860, NM_003433, BC007307	0.03
GO:0015802~basic amino acid transport	NM_000341, NM_000578	0.06

BeWo treated with cytokines		
GO:0007049~cell cycle	NM_001255, NM_018410, NM_018101, NM_016359, NM_018944, NM_022346, NM_018492, D38553, NM_001809, D42045, NM_005192, NM_004159, NM_002266, NM_001237, NM_012177, NM_006845, NM_006101, NM_001070, NM_001168, NM_016343, NM_001786, BC008589, NM_004701, NM_006185, NM_007019, NM_001790, NM_003600, NM_004856, NM_003318, NM_012112, NM_002358, NM_004217, NM_031299	≤ .001
GO:0000226~microtubule cytoskeleton organization	NM_007019, NM_012177, NM_006845, NM_006101, NM_001070, NM_001809, NM_003600, NM_004856, NM_006082, NM_003318, NM_016359	≤ .001
GO:0051439~ ubiquitin-protein ligase activity during mitotic cell cycle	NM_007019, NM_001255, NM_012177, NM_001786, NM_002358, NM_004159	≤ .001
GO:0007093~mitotic cell cycle checkpoint	NM_003318, NM_001786, NM_002358, NM_016343, NM_001237	≤ .001
GO:0051438~regulation of ubiquitin-protein ligase activity	NM_007019, NM_001255, NM_012177, NM_001786, NM_002358, NM_004159	≤ .001
GO:0051248~negative regulation of protein metabolic process	NM_007019, NM_001255, NM_012177, NM_002358, NM_004159, D17525, NM_004095	≤ .001
GO:0034622~cellular macromolecular complex assembly	NM_018154, NM_012177, NM_001070, NM_001809, NM_006082, NM_018410, AK001696, NM_016343, NM_002129	0.01
GO:0010498~proteasomal protein catabolic process	NM_007019, NM_001255, NM_001786, NM_002358, NM_004159	0.01
GO:0030001~metal ion transport	AK055913, NM_006539, NM_005714, NM_003459, NM_001038, NM_006749, NM_000578, U07139, NM_006598, NM_017964	0.01
GO:0002253~activation of immune response	NM_001710, U07139, NM_001831, D17525	0.04

Table 4.2

Functional annotation of enriched biological processes associated with differentially downregulated genes

Term	Genes	P value
BeWo treated with intact IE		
GO:0006986~response to unfolded protein	NM_005345, NM_006295, NM_006597, NM_006929, NM_005346	≤ .001
GO:0022613~ribonucleoprotein complex biogenesis	AF177341, AK024391, NM_006170	0.04
BeWo treated with intact IE and schizogonic products		
GO:0006916~anti-apoptosis	AF155827, NM_000700, AK057120, AF109161, NM_000095, NM_006595, NM_014350, BC002538	≤ .001
GO:0031668~cellular response to extracellular stimulus	NM_002350, NM_005566, NM_000210, NM_001753, NM_005252	≤ .001
GO:0033554~cellular response to stress	NM_002350, NM_002875, NM_001124, NM_005591, AL137691, AK057120, NM_001753, NM_005252, NM_000251, AL049709, NM_005066, NM_012415	≤ .001
GO:0019915~lipid storage	L21934, NM_001753, NM_003489	0.01
GO:0001570~vasculogenesis	NM_012258, AF109161, NM_001753	0.04
GO:0007565~female pregnancy	NM_001124, NM_006025, AF109161, NM_005252	0.05
GO:0009628~response to abiotic stimulus	NM_002350, NM_020169, NM_001124, NM_000140, NM_006516, NM_001753, NM_005252, NM_000251, NM_012415	0.01
GO:0051258~protein polymerization	NM_005718, NM_001233, NM_001753	0.05
GO:0044271~nitrogen compound biosynthetic process	NM_001124, AL390172, NM_005956, NM_000140, NM_001033, AK025615, NM_000251, NM_000791, NM_000097	0.01
GO:0006974~response to DNA damage stimulus	NM_002350, NM_002875, NM_005591, AK057120, NM_000251, AL049709, NM_005066, NM_012415	0.02
GO:0009967~positive regulation of signal transduction	NM_002350, NM_003810, NM_003270, NM_020648, NM_001233, AF109161, NM_001753	0.03
GO:0015837~amine transport	NM_002350, NM_003982, NM_014270, AK025102	0.06
GO:0008643~carbohydrate transport	NM_012243, NM_006931, NM_006516	0.08

BeWo treated with cytokines		
GO:0006468~protein amino acid phosphorylation	NM_002350, NM_001258, NM_004850, NM_005627, NM_005713, NM_005762, NM_001892, NM_012290, NM_000660, BC012761, AF092132, NM_004333, NM_003565, AY032950, NM_006255, NM_005228, AF212224, NM_003618, AK023426, AK025306	0.02
GO:0006633~fatty acid biosynthetic process	AK023717, NM_021727, AL137506, NM_032823, AK001887, AF043897	0.04
GO:0031328~positive regulation of cellular biosynthetic process	AB033061, AF346509, NM_003069, NM_003353, NM_005762, NM_002467, NM_021961, NM_000660, NM_006941, NM_000636, NM_003405, NM_005977, NM_005252, NM_005935, AF073310, NM_005228, AL133574, AF109161, NM_003489	0.05
GO:0007565~female pregnancy	NM_002195, NM_001124, NM_003353, NM_006025, NM_000660, AF109161, NM_005252	0.01
GO:0001570~vasculogenesis	NM_012258, NM_004926, AF109161, NM_002890	0.03
GO:0045906~negative regulation of vasoconstriction	NM_001124, AK023426	0.03
GO:0048514~blood vessel morphogenesis	NM_020156, NM_001977, NM_012258, NM_004926, NM_002165, AF109161, NM_002890, U82319	0.05
GO:0009725~response to hormone stimulus	AL049246, NM_000781, NM_002350, NM_003225, NM_001124, NM_003353, BC001854, AF073310, NM_003256, NM_000660, NM_005252, NM_002997	0.03
GO:0048545~response to steroid hormone stimulus	AL049246, NM_000781, NM_003225, NM_001124, NM_003353, NM_000660, NM_005252, NM_002997	0.03
GO:0033554~cellular response to stress	AK055175, NM_002350, NM_005713, NM_004793, NM_012290, NM_002912, NM_003589, NM_000636, NM_005252, NM_001124, NM_015251, AL137691, NM_003618, AK023426, NM_032833, NM_013451, NM_005395	0.02
GO:0006979~response to oxidative stress	NM_000781, NM_003353, NM_004793, NM_005228, NM_000636, NM_005252, NM_002997, NM_032833	0.02
GO:0000302~response to reactive oxygen species	NM_000781, NM_000636, NM_005252, NM_002997, NM_032833	0.03
GO:0001666~response to hypoxia	NM_019058, NM_001124, NM_004793, NM_000660, NM_000636, AF109161	0.06

Table 4.3

Functions of important genes from selected enriched GO terms from BeWo with intact IE and schizogonic products

VASCULOGENESIS			
ID	Gene symbol	Name	Function
AF109161	CITE2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	interferes with the binding of transcription factors HIF-1a induced genes and therefore suppress embryonic vascularization
NM_012258	HEY1	hairy/enhancer-of-split related with YRPW motif 1	inhibit vessel promoting effect of VEGF in angiogenesis
NM_001753	CAV1	caveolin 1, caveolae protein, 22kDa	regulate placental angiogenesis (negative regulator eNOs

Table 4.4

Functions of important genes from selected enriched GO terms from BeWo with cytokines

VASCULOGENESIS			
category			
ID	Gene symbol	Name	Function
AF109161	CITE2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	interferes with the binding of transcription factors HIF-1a- induced genes and therefore suppress embryonic vascularization
NM_012258	HEY1	hairy/enhancer-of-split related with YRPW motif 1	inhibit vessel promoting effect of VEGF in angiogenesis
NM_002890	RASA1	RAS p21 protein activator 1	enhance angiogenesis by inhibiting Ras- cyclic AMP pathway
NM_004926	ZFP36L1	zinc finger protein 36, C3H type-like 1	
BLOOD VESSEL MORPHOGENESIS			
AF109161	CITE2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	interferes with the binding of transcription factors HIF-1a- induced genes and therefore suppress embryonic vascularization
NM_002890	RASA1	RAS p21 protein activator 1	enhancing angiogenesis
NM_020156	C1GALT1	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	facilitate angiogenesis
NM_001977	APA	aminopeptidase A	regulator of blood vessel formation

NM_012258	HEY1	hairy/enhancer-of-split related with YRPW motif 1	inhibit vessel promoting effect of VEGF in angiogenesis
NM_002165	ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	facilitate angiogenesis
U82319	NUS1P3	nuclear undecaprenyl pyrophosphate synthase 1	important in vascular remodeling and angiogenesis
NM_004926	ZFP36L1	zinc finger protein 36, C3H type-like 1	regulating the response to growth factors
NEGATIVE REGULATION OF VASOCONTRICTION			
NM_001124	ADM	adrenomedullin	vasodilator
AK023426	BMPRII	bone morphogenetic protein receptor, type II	embryonic vasculogenesis

Table 4.5

Important pathways associated with differentially expressed genes

Pathway Name	P-value
BeWo exposed to intact CS2 IE	
Adhesion and Diapedesis of Granulocytes	0.009
Electron Transport Chain	0.003
MAPK signaling pathway	0.001
Regulation of actin cytoskeleton	0.003
BeWo exposed to intact CS2 parasites plus schizogonic product	
Electron Transport Chain	0.020
MAPK signaling pathway	0.000
Regulation of actin cytoskeleton	0.001
TGF Beta Signaling Pathway	0.020
Calcium signaling pathway	0.011
Cytokine-cytokine receptor interaction	0.002
Focal adhesion	0.001
Complement and coagulation cascades	0.050
Starch and sucrose metabolism	0.011
Ribosome	0.011
BeWo exposed to cytokines	
Insulin signaling pathway	0.002
mTOR signaling pathway	0.002
EGF Signaling Pathway	0.002
IGF-1 Signaling Pathway	0.015
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)	0.015
PDGF Signaling Pathway	0.015
TPO Signaling Pathway	0.015
TGF-beta signaling pathway	0.000
Wnt signaling pathway	0.008
MAPK signaling pathway	0.000
p38 MAPK Signaling Pathway	0.015
Jak-STAT signaling pathway	0.040
Adipocytokine signaling pathway	0.008
Phosphatidylinositol signaling system	0.008
Activation of Src by Protein-tyrosine phosphatase alpha	0.015
Erk1/Erk2 Mapk Signaling pathway	0.015
Focal adhesion	0.000

Cytokine-cytokine receptor interaction	0.000
Complement and coagulation cascades	0.040
Prostaglandin synthesis regulation	0.013
Cell cycle	0.000

Table 4.6
Comparison of fold-change in gene expression by RT-PCR and microarray results for selected genes

Gene ID	Name	Microarray		RT-PCR results		
		Fold change	Adj. P value	Mean fold change	P value	BeWo treatment
NM_003867	FGF17	1.6	0.00	5.2	0.000	IE
NM_003867	FGF17	1.6	0.00	21.9	0.002	IE +Schiz
NM_000341	SLC3A1	2.6	0.01	17.0	0.005	IE +Schiz
NM_000660	TGFβ 1	-1.5	0.00	-1.6	0.091	Condtn M
NM_018976	SLC38A2	-1.6	0.00	-1.6	0.005	Condtn M
NM_002192	IHBA	1.5	0.01	2.9	0.003	Condtn M

Abbreviations: fibroblast growth factor 17 (FGR17), solute carrier family 3 member 1 (SLC3A1), solute carrier family 38, member 2 (SLC38A2), transforming growth factor, beta 1 (TGFβ1), Inhibin, beta A (INHA), Schiz (schizogony), Condtn M (condition medium)

Table 4.7 Concentration of chemokine*'s and cytokines concentration (pg/ml) secreted by THP-1 upon exposure to P. falciparum* infected erythrocytes

Cytokines		
Treatment	TNFα	IL 6
Conditioned medium	28 (± 5)	69 (± 2)
Spent medium	0	24.3 (±2)

Chemokines				
Treatment	IL 8	CXCL1	MIP 1 α	MCP-1
Conditioned medium	16,468 (± 714)	2,259 (± 813)	5,402 (± 714)	476 (± 173)
Spent medium	16 (± 19)	46.1 (± 46)	63 (± 228)	3.3 (± 4)

Figure 4.1 Summary of the influence of malaria on metabolic pathways in BeWo cells related to placental and fetal growth

	Response of BeWo to IE alone	Response of BeWo to IE and schizogony product	Response of BeWo to cytokines
Biological Processes		↑ Vasculogenesis ↑ Cellular response to stress	↓ Vasculogenesis ↓ Blood vessel morphogenesis ↓ Amino acid phosphorylation ↑ Vasoconstriction ↑ Cellular response to stress
pathways altered	↑ MAPK signaling	↑ MAPK ↓ TGFβ1	↓ Insulin / IGF1/mTOR ↓ EGF / PDGF ↓ TGFβ1 ↓ MAPK ↑ Prostaglandin synthesis and regulation ↑ Complement activation ↑ Apoptotic DNA fragmentation and tissue homeostasis

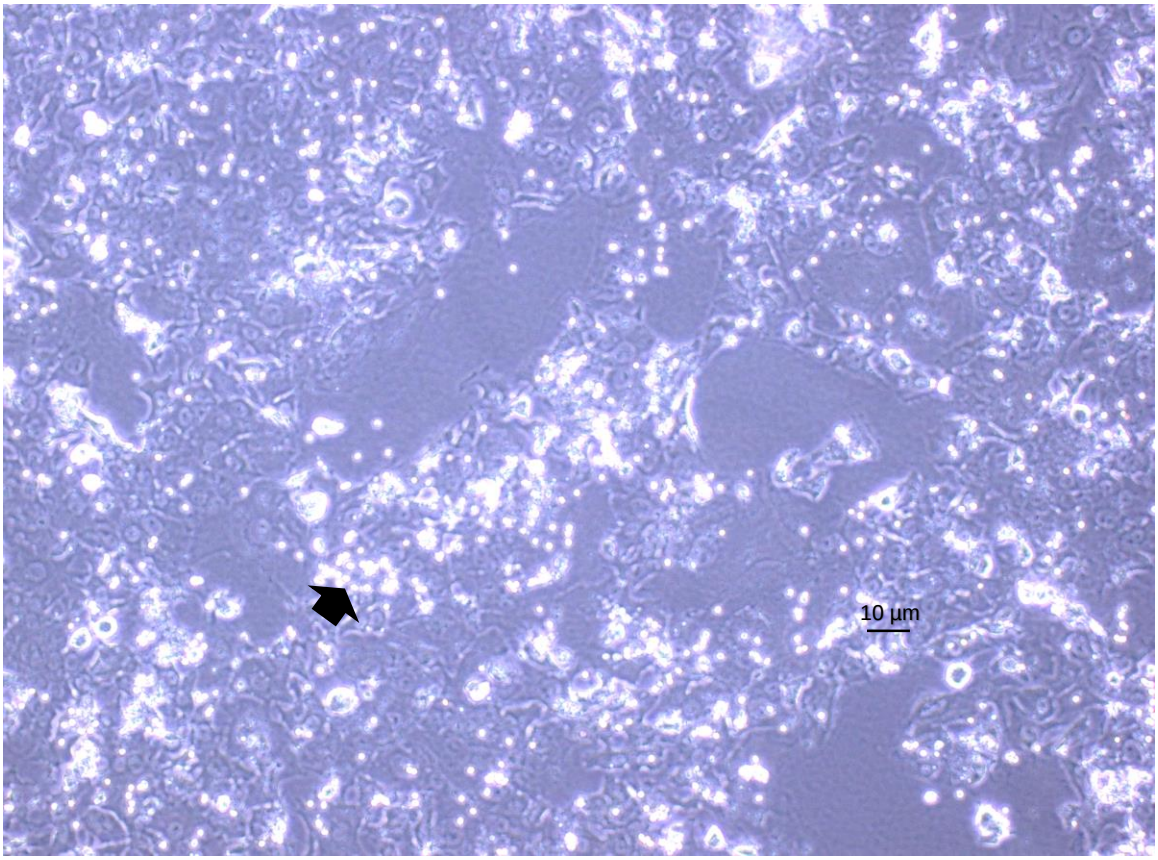

Figure 4.2 Selected CS2 *P falciparum* after 1 hour incubation with forskolin treated BeWo cells and washed by PBS. A black arrow show CSA-binding IE. 10x magnification.

CHAPTER 5

Summary, Discussion, Significance and Possible Future Studies

Maintenance of placental function throughout gestation is essential for the growth of the fetus in utero. Placental malaria changes the microenvironment in the intervillous space (IVS) due to the binding of the parasite protein VAR2CSA on the surface of infected erythrocytes (IE) to the syncytiotrophoblast receptor, chondroitin sulfate A CSA. As a result, IE accumulate in the IVS, i.e., sequestration of IE occurs, which often stimulates an inflammatory response leading to poor pregnancy outcomes. One important consequence of placental malaria is an increased risk of women having low birth weight (LBW) babies, due either to fetal growth restriction (FGR) or preterm delivery (PTD). The major mechanism responsible for PTD appears to be early induction of labor by pro-inflammatory cytokines, especially TNFα, produced by maternal macrophages in the IVS during placental malaria [6]. However, the biological mechanism(s) leading to LBW babies due to FGR remains unclear.

Several studies have shown that chronic placental malaria, defined as the presence of IE, maternal monocytes, hemozoin pigment in maternal monocytes, as well as in fibrin and syncytiotrophoblasts (ST), is associated with an increased risk of LBW due to FGR [26]. Furthermore, in malaria endemic areas, data show that high levels of inflammatory cytokines, including TNF alpha and maternal anemia are positively associated with LBW babies [8, 6]. The question remains: how does placental malaria influence fetal development that results in LBW babies due to FGR?

In vitro studies by Lucchi et al., (2005, 2006) demonstrated that the binding of *P. falciparum* IE to trophoblasts induced tyrosine phosphorylation of ST proteins and

showed that ST secreted cytokines and chemokines capable of recruiting and maintaining maternal monocytes in the IVS [138,102, 23] during placental malaria. These were the first studies to suggest that ST can respond to malaria with physiological changes. Moreover, during placental malaria, deposition of schizogonic bioactive molecules, including hemozoin, parasites antigens, hemoglobin, GPI-anchor, parasite DNA as well hemozoin-parasite DNA complexes, may come in contact with ST in the IVS [139]. *P. falciparum* schizogonic products have well-characterized pathogen associated molecular patterns (PAMPs); that is, ligands for specific pattern recognition receptor (PRR) expressed on ST. PRR on ST can directly bind hemozoin (activate inflammasome), parasite DNA (TLR 9), GPI-anchor (TLR2/TLR1), as well as hemozoin and DNA hemozoin complexes (TLR9). Generally, no data exist to elucidate the influence of IE binding on ST function and thus on the pathogenesis placental malaria. Although TLRs are essential innate receptors at the maternal fetal interface, the immediate response of ST to *P. falciparum*-derived PAMPs cannot be studied *in vivo*. However, the role of TLRs in pathological pregnancies has been reported [140]. Therefore the question is: Does the binding of IE alone to ST induce pathophysiological changes that lead to FGR? Do *P. falciparum*-associated PAMPs influence ST function?

Since ST play important roles during pregnancy in placental and fetal growth throughout gestation, and *in utero* growth is impaired during placental malaria, we hypothesized that placental malaria alters ST functions by dysregulating major growth pathways important for both placenta and fetus growth. To test this hypothesis we carried out gene expression studies in fetal cells (ST, fetal endothelia cells, fetal stromal cells and fetal macrophages) from placental malaria-infected placentas to assess whether changes in ST function occurs during placental malaria. Then, we asked the following questions: Are the changes occurring in ST? Are intact *P. falciparum* infected erythrocytes

responsible for the changes? Do both intact IE and the schizogonic products released during schizogony induce these changes? Alternatively, are the changes induced by the cytokines and chemokines released by the maternal monocytes at the IVS as a result of malaria? Results obtained from these major research questions have great impact on the understanding the biology of placental malaria. In addition, the information obtained is important for vaccine development, identification of potential new drug targets, and creating new diagnostic tools for detection of placental malaria.

We showed in our genome-wide gene expression analysis of fetal cells using malaria-infected placentas with chronic infections that placental malaria significantly dysregulated a number of growth pathways, including IGF-1, insulin, mTOR, TGFβ1, prostaglandin synthesis and regulation pathways, as well as, significantly downregulated individual genes, such as, inhibin A, leptin, amino acid transporters, and placental 11βHSD2, that are known to be important for fetus growth.

In order to investigate whether the changes observed in our *in vi*vo study occurred in ST, we established an *in vitro* model and develop a protocol to study the response of ST to malaria. First, we demonstrated that the BeWo cell line formed syncytia after treatment with 10μM forskolin for 72 hours, responded optimally when co-cultured with a 10 IE: 1 BeWo cells for 48 hours (Chapter 3), and underwent gene expression of various proteins that are important for growth *in vivo.* Then, we used the BeWo model to address three major questions: Are the changes observed in ST *in vivo* using fetal cells from placentas chronically infected with malaria induced by i) direct binding of intact IE, ii) interaction of intact IE plus schizogonic product binding to PRR, or iii) cytokines and chemokines released by maternal monocytes in response to IE in at the IVS? We conducted gene expression studies of BeWo treated with a) intact placental binding-IE alone, b) intact

placental binding-IE plus schizogonic products, and c) conditioned medium containing cytokines and chemokines released by the THP-1 monocytic cell line upon exposure to IE, using microarray analysis. Using differentially expressed genes (fold change ≥ 1.5, p value ≤0.01), we analyzed biological processes of genes enriched as well as pathways that were significantly altered.

Results from our *in vitro* study demonstrated that BeWo cells exposed to conditioned medium, containing a mixture of cytokines and chemokine like those found in the IVS during placental malaria, portrayed pathophysiology changes similar to those reported in the literature for cases related to poor pregnancy outcome. That is, cytokines and chemokines significantly induced changes in genes that coded for proteins important in vasculogenesis, blood vessel morphogenesis, and negative regulation of vasoconstriction, i.e., genes for these processes were significantly enriched in the list of downregulated genes. Downregulated genes, such as adrenomodulin, amino peptidase A, TGFB 1, RASA1, C1GALT1, APA, the ID 1gene urocortin, and NUS1P3, have been implicated in placental vascular development and vascular remodeling[133–135]. Indeed, downregulation of these genes clearly shows that it is possible that cytokines and chemokine produced during placental malaria are associated with an increased risk of progression to poor placental blood flow reported in women [37] during placental malaria.

From their studies, Dorman et al., (2001) and Griffin et al., (2012) hypothesized that placental malaria might affect placental development and remodeling by dysregulating hemodynamics through vasoconstriction or vascular damage leading to placental insufficiency [37,141]. Other studies have shown that elevated levels of anti-angiogenic molecules, that are known to be produced by ST for regulating placental functions, are elevated by placental malaria, such as fms-like tyrosine kinase-1 (sFLT1), a soluble

vascular endothelia growth factor receptor that sequesters the vascular endothelia growth factor 1 (VEGF1). Other anti-angiogenic molecules elevated during placental malaria include soluble endoglin (sEND)[142, 39] and angiopoietin-2,[40] which are synthesized by the ST to regulate placental development and fetal growth. Alterations in the production of these factors by ST could lead to poor vascularization of the placenta, and hence fetal growth. Our *in vitro* data suggest that impairment of blood flow observed *in vivo* may be a result of dysregulation of angiogenic and vasculogenesis factors of ST origin by the inflammatory cytokines secreted by maternal monocytes in the IVS. Our *in vitro* study further corroborates the theory that placental insufficiency occurs during placental malaria. Fig. 5.1 illustrates our model on how placental malaria with chronic inflammation might lead to FGR. We also propose that dysregulation of the genes that are essential for placental vascular development may lead to placental vascular dysfunction that occurs in placental malaria associated-PTD as well as placental malaria-associated hypertension reported in malaria endemic areas [128,129]. The results therefore support a role for cytokines in the pathogenesis of placental malaria.

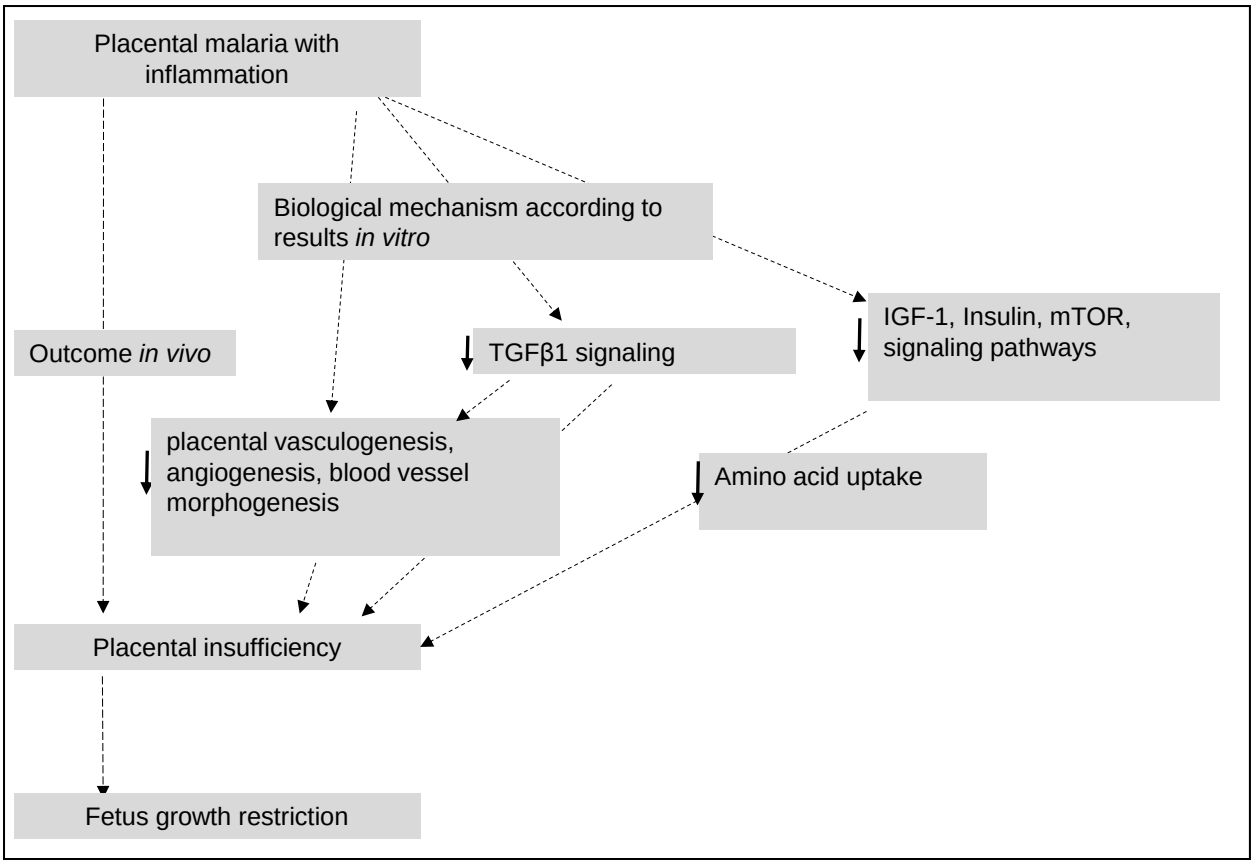

Figure 5.1 Model explaining biological mechanisms that explain development of placental insufficiency as a result of placental malaria with inflammation.

Components of the insulin growth factor 1 (IGF-1) axis have been reported to be altered during placental malaria with inflammation *in vivo* [66]. We found several growth pathways that are important for fetal growth in addition to IGF-1 that were altered in BeWo cells treated with cytokines and beta chemokines. IGF-1 and insulin pathways are essential for survival of the placenta as well as for the fetus (reviewed in [68]). Studies have demonstrated that IGF-1, IGF1R and its post-receptor signaling molecules are important in mediating growth effect. Consequently, any abnormality in the IGF-1 signaling pathway in the fetal-maternal unit will be associated with FGR [63]. Moreover IGF-1 is deregulated in other causes of FGR, including preeclampsia. The proliferative effect of IGF-1 is mediated via mammalian target of ramphamycin (mTOR), an important

regulator of fetal growth. The placental mTOR pathway has multiple roles in mediating translation of cellular proteins, cell survival, proliferation, angiogenesis, and amino acid uptake. Some *in vitro* studies have shown that amino acid transporters are downstream of mTOR [143, 86]. Consequently, in normal pregnancies system A amino acid transporters are upregulated by IGF-1, insulin and leptin via the mTOR-dependent pathway[85]. Rogerson et al., (2011) have shown that the inflammatory cytokine milieu in the IVS interferes with the fetal IGF-1 axis leading to LBW [66] babies. However, in their study, the ST-IGF-1 post-receptor signaling molecules were not studied and downstream pathways to IGF-1 that are affected by malaria have not been explored. In our study, we showed that IGF-1, insulin, as well as the mTOR signaling pathway, and the system A amino acid transporter 2 (SNAT2) are also dowregulated in the trophoblastic BeWo cell line. Our results support the observation by Boeuf et al., (2013) that inflammation downregulates amino acid transporter expression in ST [41]. Lower expression of amino acid transporters as a result of placental malaria may lead to reduced uptake of amino acids and contribute to FGR, and ultimately LBW babies.

To conclude, the effect of inflammation in the IVS in response to placental malaria, and not the IE themselves, are the major cause of the pathophysiology associated with placental malaria and bad pregnancy outcomes. IE plus its schizogonic products cause only minimal pathology to ST cells. However binding of IE and schizogony products may contribute to secretion of chemokines and cytokines that recruit maternal macrophages at maternal fetal interface triggering local inflammatory response. It appears that the interaction of cytokine receptors on ST is the main driving force of placental malaria pathophysiology, and that much of the pathology occurs through downregulation of angiogenic and vasodilators that are essential in placental vascular development and maintenance of placental blood flow. Our results add insight into how placental

insufficiency arises during placental malaria when inflammation is present in the IVS. The results advocate detecting the level of markers of inflammation, placental angiogenesis, and placental vasoconstriction in the peripheral blood of women to diagnoses the risk of bad pregnancy outcome in women with placenta malaria who are exposed to malaria.

These results also imply that severe peripheral inflammation during pregnancy, regardless of origin, for example peripheral malaria other infectious diseases, and non-infectious conditions may also potentiate changes in ST functions during pregnancy and may increase the risk of FGR and preterm delivery. Thus, for malaria, controlling of parasitemia is essential throughout pregnancy to prevent infected erythrocyte from sequestrating and therefore causing inflammation. For other inflammatory diseases, safe anti-inflammatory drugs may be needed.

Clearly, our data provide a foundation for many future studies. For example, one might evaluate different biomarkers for detecting placental malaria-related inflammation. Longitudinal studies investigating different vasodilators and anti-angiogenic molecules as correlates of chronic placental malaria might also be investigated for the diagnosis of placental malaria with inflammation. In addition, animal models might be used to investigate the role of mTOR, insulin, and TGFB1 in placental malaria. Current studies are demonstrating that maternal nutrition plays an important role in placenta malaria pathogenesis [127], and pathology may occur via activation of the mTOR pathway since nutrients are activators of the mTOR pathway [144]. High levels of nutrients in the IVS may be able to over-ride the effects of inflammatory cytokines and diminish the effects of cytokines on fetus growth. So, future research should also consider that intermittently

supplement high risk women who have placental malaria with low levels of essential amino acids and other micronutrients in malaria endemic areas.

We support the importance of developing a vaccine that will induce antibodies that prevent the sequestration of IE in the placenta. Antibodies that inhibit binding of IE on ST may reduce the prevalence of LBW babies in malaria endemic areas. Neutralization of binding of IE will halt secretion of chemokines and cytokines by ST that recruit and retain macrophages in the IVS and consequently prevent inflammation. Reduction of the burden of LBW babies also relies on reduction of the parasite burden in the IVS after infection. Intermittent prevent treatment during pregnancy (IPTp) has been instrumental in reducing the burden of placental malaria in areas where low parasite resistance to prophylactic drugs exist. Use of insecticide treated bed nets is also essential in minimizing infection. However, these malaria preventive measures must be sustainable in endemic areas. Lack of sustainability of preventive measures may result to ‘‘malaria rebound effect” to previously placental-malaria immune women, a situation that will unfortunately increase the burden of placental malaria in pregnant women around the world.

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