The Effects of Malaria in Pregnancy on Utero- and Fetoplacental Blood Flow and Fetal
Growth: a longitudinal Doppler ultrasound study from
Kinshasa, Democratic Republic of Congo

Jennifer Beth Griffin

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in

partial fulfillment of the requirements for the degree of Doctor of Philosophy in the

Department of Epidemiology, Gillings School of Global Public Health

Chapel Hill

2011

Approved by:

Steven R. Meshnick, MD, PhD

Julie L. Daniels, MPH, PhD

Amy H. Herring, ScD

Steven J. Rogerson, PhD

John M. Thorp, Jr., MD

ABSTRACT

JENNIFER BETH GRIFFIN: The Effects of Malaria in Pregnancy on Utero- and
Fetoplacental Blood Flow and Fetal Growth: a longitudinal Doppler ultrasound study
from Kinshasa, Democratic Republic of Congo
(Under the direction of Steven R. Meshnick)

Malaria during pregnancy is thought to affect both uterine and umbilical artery blood

flow, leading to decreased oxygen and nutrient exchange between the mother and fetus,

and ultimately to intrauterine growth restriction.

We examined the effect of concurrent malaria on changes in uterine artery and umbilical

artery resistance indices over gestational age. We used data from 177 pregnant women

enrolled in a longitudinal Doppler ultrasound study. Women with high uterine artery and

umbilical artery resistance had neonates that weighed less and were smaller. Compared

to multigravidae with no malaria, primigravidae with concurrent malaria had an 11-13%

increase in uterine artery resistance. Compared to women with no concurrent malaria

and a male fetus, concurrent malaria caused an acute 10-14% increase in umbilical

artery resistance among women female fetus, while women with a male fetus and

concurrent malaria had a 2-3% increase in umbilical artery resistance.

We also examined the effect of early pregnancy malaria parasitemia (≤20 weeks'

gestation) on subsequent changes in uteroplacental blood flow and fetal growth among a

subset of 128 women with early pregnancy malaria exposure data. Early pregnancy

malaria infection affected placentation, reflected by changes in uterine artery blood flow.

Among nourished women, early pregnancy malaria decreased uterine artery resistance

(-0.036; 95% CI: -0.065, -0.0058), but among undernourished women, early pregnancy malaria increased uterine artery resistance (+0.022; 95% CI: -0.031, 0.074). Among primigravidae, early pregnancy malaria decreased umbilical artery resistance, reflecting adaptive villous angiogenesis with early pregnancy malaria. Primigravidae with early pregnancy malaria had 3.6 times the risk of subsequent IUGR (95% CI: 2.1, 6.2) compared to multigravidae with no early pregnancy malaria.

Our findings point to both the acute effects of concurrent parasitemia and the effects of early pregnancy malaria on placental development. The acute effects of malaria on placental blood flow indicate the need for management and control strategies during pregnancy. Early pregnancy malaria infection leads to changes in placentation, villous angiogenesis, and intrauterine growth restriction. Our findings support the initiation of malaria prevention and control efforts earlier in pregnancy.

For Ayla and Vivian

ACKNOWLEDGEMENTS

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AC	Abdominal circumference
ANC	Antenatal care
BPD	Biparietal diameter
BMI	Body mass index
CI	Confidence interval
CSA	Chondroitin sulphate A
D	Diastole
DAG	Directed acyclic graph
EFW	Estimated fetal weight
EMM	Effect measure modifier
FL	Femur length
GA	Gestational age
HC	Head circumference
HIV	Human immunodeficiency virus
IE	Infected erythrocyte
IL	Interleukin
INF	Interferon
ITN	Insecticide treated bed net
IUGR	Intrauterine growth restriction
LBW	Low birth weight
LMP	Last menstrual period
MUAC	Mid upper arm circumference
PTD	Preterm delivery
qPCR	Real-time quantitative polymerase chain reaction

RI	Resistance index
RR	Risk ratio
S	Systole
SES	Socioeconomic status
SGA	Small for gestational age
SD	Standard deviation
SP	Sulfadoxine Pyrimethamine
TNF	Tumor necrosis factor
UA	Umbilical artery
UtA	Uterine artery
WHO	World Health Organization

CHAPTER 1 - SPECIFIC AIMS

Plasmodium (P.) falciparum malaria in pregnancy is associated with adverse

pregnancy outcomes for the mother and fetus, including maternal anemia and low birth

weight (LBW) (<2500 gms) due to preterm and intrauterine growth restriction (IUGR)

(Brabin and Rogerson 2001; Steketee, Nahlen et al. 2001). LBW infants have an

increased risk of acute morbidity and mortality, as well as having an increased risk of

chronic disease later in life (Williams, Creasy et al. 1982; Ashworth 1998; Kramer 2003).

While there are likely multiple mechanisms by which malaria in pregnancy leads to LBW

(Rogerson, Hviid et al. 2007), one is thought to be the reduction of utero- and

fetoplacental blood flow (Arbeille, Carles et al. 1998; Dorman, Shulman et al. 2002).

The placenta develops with the primary function of providing oxygen and

nutrients to the growing fetus. The uterine artery brings blood enriched with oxygen and

nutrients from the mother to the placenta, while the umbilical artery brings deoxygenated

blood with waste products from the fetus to the placenta. In the placenta, oxygen and

nutrient exchange occurs. Pathophysiological events that alter the placental environment

can decrease blood flow in the uterine or umbilical arteries, leading to a reduced

capacity to transport oxygen and nutrients and, ultimately, to restricted fetal growth.

Malaria in pregnancy has previously been found to be associated with abnormal utero-

and fetoplacental circulation in the late second and early third trimesters (Arbeille, Carles

et al. 1998; Dorman, Shulman et al. 2002).

Longitudinal ultrasound studies can be used to investigate the effects of malaria

infection on utero- and fetoplacental circulation and fetal growth throughout pregnancy.

Doppler ultrasound of the uterine and umbilical arteries directly evaluates blood perfusion of the maternal and fetal compartments of the placental circulation, respectively (Abramowicz and Sheiner 2008). In addition, fetal biometry measurements can be used to estimate fetal weight and to identify IUGR fetuses by comparing estimated fetal weights to population growth charts.

The primary objective of this dissertation is to longitudinally examine the effect of malaria on changes in placental blood flow and on intrauterine growth restriction. Several definitions of malaria parasitemia will be explored in the analyses, as these definitions may help elucidate pathological processes. In our analyses, will use quantitative real-time polymerase chain reaction (qPCR) detected *P. falciparum* parasitemia; using qPCR we have shown many misclassifications of malaria diagnosis by peripheral blood microscopy in this cohort (Taylor, Juliano et al.).

The data are from Project ECHO, a prospective, longitudinal study of 182 pregnant women conducted at Binza Maternity Hospital in Kinshasa, Democratic Republic of Congo between May 2005 and May 2006 (Landis, Lokomba et al. 2009). Women participated in monthly follow-up visits during which malaria, maternal anthropometrics, fetal biometrics, and Doppler ultrasound data were measured. All enrolled women received iron supplementation and intermittent preventive treatment (IPTp) for malaria at two time points as a part of routine antenatal care. Women received additional treatments for malaria at monthly visits in which women were found to be peripheral blood smear-positive for malaria infection.

Project ECHO data uniquely allow us to examine the relationship between malaria, blood flow and fetal size for several reasons: 1) accurate pregnancy dating; 2) longitudinal measures of utero- and fetoplacental blood flow and fetal biometrics; and 3) concurrent serial measures of malaria parasitemia, based on qPCR.

Specific Aim 1

To examine the effect of malaria parasitemia on changes in uterine artery (UtA) and umbilical artery (UA) resistance indices (RI) over time.

Justification

Decreased uteroplacental blood flow is a hypothesized mechanism by which malaria in pregnancy is thought to lead to LBW. Previous studies have shown acute effects of malaria during the late second and early third trimesters on uterine artery resistance among primigravidae and on umbilical artery resistance in all gravidae. However, the effect of malaria on uteroplacental blood flow over the course of pregnancy has not been examined in a longitudinal setting. We will use linear mixed effect models to estimate the effect of malaria on UtA RI and UA RI from approximately 18 weeks' gestation to term. The main binary, time-dependent malaria exposure will be defined as whether a woman was positive for *P. falciparum* malaria by qPCR of peripheral blood at the visit in which the ultrasound measure of uteroplacental blood flow were taken. We will determine if the effect of malaria varies over gestational age. We will also examine effect measure modification by gravidity, fetal sex, and maternal nutritional status.

Hypotheses to be tested

1. Is the effect of malaria on UtA RI and UA RI different than the null?
2. Does the effect of malaria on UtA RI and UA RI change over time?
3. Is the effect of malaria on UtA RI and UA RI modified by gravidity, fetal sex, or maternal nutritional status?

Specific Aim 2

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A. Estimate the effect of malaria in early pregnancy on subsequent uterine artery and umbilical artery resistance indices over time

B. Estimate the effect of malaria in early pregnancy on subsequent intrauterine growth restriction

Justification

It has been hypothesized that malaria in early pregnancy disrupts the process of placentation, particularly extravillous trophoblast invasion and transformation of the maternal spiral arteries (as in preeclampsia). Should an early pregnancy malaria insult disrupt placentation, it could lead to problems in later pregnancy, including intrauterine growth restriction. To date, the effect of early pregnancy malaria on subsequent utero- and fetoplacental blood flow (providing information on placentation and villous angiogenesis, respectively) and fetal growth over the course of pregnancy has not been investigated. We will use linear mixed effect models to estimate the effect of malaria on the continuous, time-dependent outcomes, including uterine artery resistance index and umbilical artery resistance index, from 21 weeks' gestation to term. We will use log-binomial general estimating equation regression models with an exchangeable working correlation matrix to estimate the effect of early pregnancy malaria on the time-varying, binary outcome intrauterine growth restriction from 21 weeks' gestation to term. The main binary, time-independent malaria exposure will be defined as whether a woman was positive for *P. falciparum* malaria by qPCR of peripheral blood prior to 21 weeks' gestation. We will determine if the effect of early pregnancy malaria varies over gestational age. We will also examine effect measure modification by gravidity, fetal sex, and maternal nutritional status.

Hypotheses to be tested

1. Is the effect of early pregnancy malaria on subsequent UtA RI, UA RI or IUGR different than the null?

2. Does the effect of early pregnancy malaria on UtA RI, UA RI or IUGR change over time?

3. Is the effect of early pregnancy malaria on UtA RI, UA RI or IUGR modified by gravidity, fetal sex, or maternal nutritional status?

CHAPTER 2 – BACKGROUND AND SIGNIFICANCE

Epidemiology of malaria in pregnancy

Pregnant women are more vulnerable to malaria infection than non-pregnant adults, with more frequent parasitemia, higher parasite densities, and more severe disease (Brabin 1983; McGregor 1984; Brabin and Rogerson 2001). In sub-Saharan Africa, there are 30 million pregnancies occurring annually in areas with *Plasmodium falciparum* transmission, with the vast majority (98.7%) living in areas of stable transmission (WHO 2003; Dellicour, Tatem et al. 2010). Approximately one-quarter of pregnant women in African malaria-endemic settings have evidence of either peripheral or placental parasitemia at delivery (McGregor, Wilson et al. 1983; Bulmer, Rasheed et al. 1993; Steketee, Nahlen et al. 2001; Guyatt and Snow 2004). The peak prevalence of malaria infection in pregnancy occurs from approximately 13 to 20 weeks' gestation and decreases throughout pregnancy (Brabin 1983).

The risk of malaria in pregnancy varies based on factors such as gravidity, maternal age, and the existence of co-morbidities, particularly human immunodeficiency virus (HIV) infection. Malaria in pregnancy is more frequent and severe in first and second pregnancies, with approximately twice the prevalence of malaria among primigravidae compared to multigravidae (Brabin 1983; McGregor, Wilson et al. 1983; Brabin and Rogerson 2001). Age is an independent risk factor for malaria in pregnancy, with younger pregnant women being particularly susceptible to malaria, regardless of gravidity (Rogerson, Van den Broek et al. 2000; Walker-Abbey, Djokam et al. 2005). HIV-infection has been shown to shift the burden of malaria from primigravidae to all

gravidae, causing more frequent peripheral and placental malaria, higher parasite densities, more severe illness, and worse pregnancy outcomes (Steketee, Nahlen et al. 2001; ter Kuile, Parise et al. 2004).

The epidemiology of malaria in pregnancy is also highly dependent on the intensity of transmission. In stable transmission settings, adults have high levels of naturally acquired immunity and most malaria infections are asymptomatic. In these areas, malaria in pregnancy primarily causes asymptomatic infection; however, malaria in pregnancy may lead to maternal anemia and placental parasitemia, both of which contribute to low birth weight (Nosten, Rogerson et al. 2004). In low transmission settings, adults have no significant levels of acquired immunity and typically have malarial disease when parasitemic. In these areas, malaria in pregnancy may cause episodes of severe or cerebral malaria, which can lead to stillbirths, spontaneous abortion, or premature delivery, as well as maternal mortality (Nosten, Rogerson et al. 2004).

Malaria in pregnancy is known to have significant direct health effects on the mother, the fetus and the neonate including: severe maternal anemia and related maternal mortality; low birth weight from both preterm delivery and intrauterine growth restriction; and, infant mortality (Steketee, Nahlen et al. 2001). Malaria in pregnancy is estimated to contribute 2-15% of maternal anemia in endemic settings (Steketee, Nahlen et al. 2001), which may lead to severe maternal anemia. Severe maternal anemia is a known risk factor for maternal mortality (Brabin, Hakimi et al. 2001), and is an independent cause of low birth weight (Rasmussen 2001; Levy, Fraser et al. 2005). In endemic areas, *P. falciparum* malaria during pregnancy has been estimated to contribute approximately 13 - 70% of low birth weight due to intrauterine growth restriction and 8 - 36% of low birth weight due to preterm (Steketee, Nahlen et al. 2001). Considering that infant mortality varies across malaria-endemic settings (from 50-160

deaths per 1,000 live births), it has been estimated that 75,000 to 200,000 infant deaths are attributable globally to malaria in pregnancy each year (Steketee, Nahlen et al. 2001).

Epidemiology of intrauterine growth restriction

Low birth weight (<2500 g or 5 lb, 8 oz) is caused by both preterm delivery (<37 weeks' gestation) (PTD) and IUGR. While LBW remains a frequently used designation of fetal growth, particularly in the developing world, Lubchenco first demonstrated that small fetuses relative to those of the same gestational age (i.e. "small for gestational age" or "SGA") best identified small neonates at risk of neonatal morbidity and mortality (Lubchenco, Hansman et al. 1963). SGA fetuses include both constitutionally small fetuses, which are meeting their genetic growth potential, and IUGR fetuses, which are small due to underlying pathology and have an increased risk of morbidity and mortality (Ott 1997; Baschat and Weiner 2000). SGA and IUGR are frequently used interchangeably in the medical literature, leading to confusion in terminology.

In developing countries, where the prevalence of LBW is very high, most LBW is due to IUGR rather than preterm (Villar and Belizan 1982; de Onis, Blossner et al. 1998; Steketee, Nahlen et al. 2001; Kramer 2003). A recent review of LBW estimated that 24% of all neonates in developing countries were IUGR, or approximately 30 million infants per year (de Onis, Blossner et al. 1998). One in five infants in sub-Saharan Africa is estimated to suffer from IUGR (de Onis, Blossner et al. 1998; Kramer 2003). In malaria-endemic regions, maternal undernutrition (characterized by low gestational weight gain, low pre-pregnancy BMI, and short stature) and malaria are the primary risk factors for IUGR (Kramer 2003). Other important determinants of IUGR in the developing world include: smoking; primiparity; pregnancy-induced hypertension; congenital anomalies; and, other genetic factors (Kramer 2003).

Intrauterine growth restricted fetuses have an increased risk of mortality, acute morbidity and an increased risk of chronic disease later in life (Williams, Creasy et al. 1982; Ashworth 1998; Kramer 2003). Generally, the risk of morbidity and mortality increases at lower weights. IUGR fetuses have an increased risk of stillbirth and IUGR infants have an increased risk of neonatal death (Kramer 2003). Acute morbidity includes hypoglycemia, hypocalcemia and polycythemia; chronic effects include small, permanent deficits in growth and neurocognitive development (Kramer 2003). Additionally, recent research indicates that several important chronic diseases of middle-age including coronary heart disease, hypertension and type-2 diabetes may originate in impaired intrauterine growth and development (Godfrey and Barker 2000; Barker 2006).

Intrauterine growth restriction is sometimes sub-classified as symmetrical or asymmetrical, with approximately 70% of IUGR fetuses presenting with asymmetrical growth (Pollack and Divon 1992; Regnault, Galan et al. 2002). In symmetrical IUGR, there is an overall reduction in fetal size, including head circumference and skeletal size; in asymmetrical IUGR, the skeletal dimensions and head circumference are spared, while the abdominal circumference is decreased due to decreased liver size and a scarcity of fat (Resnik 2002). However the utility of these sub-categories is questionable as the association is confounded by the severity of the growth restriction, with asymmetric IUGR infants tending to be more severely growth restricted (Kramer 2003)

An overview: the placenta and fetal growth

From early pregnancy through the second trimester, there are two waves of trophoblast invasion, in which the maternal spiral arterioles are transformed into larger, less rigid vascular channels that are maximally dilated (Guzman 2005; Abramowicz and Sheiner 2008). Concurrently, there is extensive angiogenesis in the placental tissue and development of tertiary villi in the placenta, leading to a sharp increase in umbilical blood

flow. Both the transformation of the uterine vasculature and placental angiogenesis are critical for the conversion of the previously high resistance system into a low resistance system that ensures adequate blood flow to the placenta and fetus during pregnancy (Guzman 2005; Abramowicz and Sheiner 2008; Abuhamad 2008).

Normal fetal growth is primarily determined by nutrient transport across the placenta from the maternal to the fetal circulation. In the placenta, the maternal and fetal circulations come into close proximity to one another for respiratory gas and nutrient exchange, but remain separated by the villous trophoblast (Gude, Roberts et al. 2004). Maternal uterine arteries feed the spiral arteries which supply maternal blood to the intervillous spaces of the placenta via pulsatile blood flow where it circulates around the chorionic villi. The chorionic villi are supplied with deoxygenated fetal blood by terminal branches of the umbilical arteries. Gas exchange occurs in the villi and the blood is returned to the fetal circulation by branches of the umbilical vein (Gude, Roberts et al. 2004; Abramowicz and Sheiner 2008). After gas exchange, the deoxygenated maternal blood is drained from the placenta by the endometrial veins (Gude, Roberts et al. 2004). In addition to respiratory gas and nutrient exchange, the placenta releases metabolic products; protects the fetus from infection; and, releases hormones affecting pregnancy, metabolism, and fetal growth (Gude, Roberts et al. 2004).

The placenta develops with the primary function of providing oxygen and nutrients to the growing fetus; however, pathophysiological events may impede this function. The capacity of the placenta to meet the nutritional needs of the fetus is dependent on several factors including adequate uterine and umbilical artery blood flows; the concentration gradient between maternal and fetal blood; and the expression and activity of transporters located both on the microvillous and the basal plasma membrane of the syncytiotrophoblast (Jones, Powell et al. 2007). Pathophysiological events that impede either the transformation of the uterine vasculature or placental

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angiogenesis may lead to increased impedance in the uterine and/or umbilical arteries, a reduced capacity to transport oxygen and nutrients (i.e. placental insufficiency) and, ultimately, restricted fetal growth (Abramowicz and Sheiner 2008).

Doppler ultrasonography of blood flow velocity waveforms

Doppler ultrasound is a powerful clinical tool that to assess hemodynamic information and placental performance in pregnancy via blood flow velocity waveforms (Maulik 2005; Abramowicz and Sheiner 2008). As the velocity of flow in a particular vascular bed is inversely proportional to downstream impedance to flow, evaluation of the change in maximal Doppler shifts over time provides information on the downstream impedance to flow (Guzman 2005; Abuhamad 2008). When sampled from the arterial circulation, a flow velocity waveform depicts one cardiac cycle. The left limit of the wave corresponds with the onset of systole, the zenith of the wave with peak systole, and the right limit with end diastole (Maulik 2005). The frequency shift is highest during systole, when the blood flow is at its fastest, and lowest during end-diastole, when the blood flow is at its slowest in the peripheral circulation (Abuhamad 2008). Flow velocity waveforms with high diastolic flow are seen when downstream resistance is low, and those with low (or reverse) diastolic flow are found when downstream resistance is high (Guzman 2005).

Techniques for analyzing the flow velocity waveform involve deriving Doppler indices or ratios from various combinations of the peak systolic, end-diastolic, and temporal mean values of the maximum frequency shift envelope from a single cardiac cycle (Maulik 2005). There are three semi-quantitative techniques for the analysis of Doppler waveforms that are widely used in obstetric practice, including the resistance index (RI) of Pourcelot [(S-D)/S]; the pulsatility index (PI) [(S-D)/ the temporal average frequency over one cycle]; and the SD ratio (Figure 2.1). Some have argued that no

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particular index has proven to offer a clinical advantage over others (Guzman 2005), while others have argued in favor of a particular index (Maulik 2005). In one prospective study, the RI was found to have the best and the PI the worst discriminatory performance for adverse pregnancy outcomes (Maulik 2005).

Doppler assessment of the uterine and umbilical arteries

The left and right uterine arteries are the main blood supply to the uterus via the radial, basal and spiral arteries (Abramowicz and Sheiner 2008). Although the uterine arteries do not directly perfuse the placenta, several conditions ultimately affecting the placenta and the fetus may be predicted through Doppler analysis of the uterine arteries (Abramowicz and Sheiner 2008). The assessment of uterine blood flow is considered the best clinical method to evaluate the uterine circulation and the maternal compartment of the placental circulation (Abramowicz and Sheiner 2008; Abuhamad 2008).

In early pregnancy, normal uterine artery waveforms are characterized by a high systolic component and low end-diastolic pattern, with a prominent early diastolic notch (Coppens, Loquet et al. 1996; Abramowicz and Sheiner 2008). Following extravillous trophoblast conversion of the spiral arteries around 15-17 weeks' gestation, the uteroplacental circulation is converted to a high-flow, low resistance system. As a result, Doppler indices decrease and the early diastolic notch disappears. While the early diastolic notch can sometimes persist until 20-26 weeks' gestational age in a normal pregnancy, persistent notching after 17-22 weeks' gestation is thought to be indicative of abnormal uterine artery circulation (Guzman 2005; Abuhamad 2008). An early diastolic notch that persists after 17 weeks' gestation is associated with an increased risk of IUGR and hypertensive disorders of pregnancy (Abramowicz and Sheiner 2008). The various waveform indices of the uterine artery show a progressive decrease until

approximately 26 weeks' gestational age, after which there are few changes with gestational age (Guzman 2005; Abramowicz and Sheiner 2008).

Defective trophoblast invasion and failure of physiologic transformation of the spiral arteries result in persistent notching and/or elevation of the uterine artery Doppler index (Miller, Turan et al. 2008). Several criteria define an abnormal uterine artery waveform: 1) elevated Doppler indices; 2) presence of a diastolic notch after 22 weeks; and, 3) a lack of difference in indices obtained from the uterine arteries on the placental and non-placental side (Abramowicz and Sheiner 2008; Miller, Turan et al. 2008). Increased uterine artery resistance and notching after 20 weeks' gestation is associated with preeclampsia, IUGR and adverse pregnancy outcomes (Trudinger, Giles et al. 1985).

The assessment of umbilical blood flow is the best clinical method to evaluate blood perfusion of the fetal compartment of the placental circulation (Baschat 2005; Abramowicz and Sheiner 2008). The typical flow waveform in the umbilical cord consists of a saw-tooth arterial tracing above the baseline (Abramowicz and Sheiner 2008). Normal umbilical artery flow waveforms are characterized by an absent end-diastolic component prior to 12 weeks' gestation. Diastolic velocities emerge between 12 and 14 weeks' gestation, with a progressive increase in end-diastolic flow seen with advancing gestation due to the proliferation of tertiary stem villi and small arterial channels that occurs with placental maturation (Coppens, Loquet et al. 1996; Abramowicz and Sheiner 2008; Abuhamad 2008). This increase in end-diastolic flow is reflected in a decrease in the resistance indices with advancing gestational age. In humans, umbilical blood flow increases in direct proportion to the increase in fetal body weight (Abramowicz and Sheiner 2008).

Diseases that reduce the proliferation of tertiary stem villi and small arterial channels in the placenta result in increased vascular resistance, which is reflected by a

decrease in the end-diastolic flow of the umbilical artery (Abramowicz and Sheiner 2008). Umbilical artery Doppler indices become elevated when 30% of villous vasculature is abnormal (Miller, Turan et al. 2008). With even greater vascular resistance, absent or revered end-diastolic velocity may be seen (Baschat, Gembruch et al. 2001; Abuhamad 2008). The presence of absent or revered end-diastolic velocity in the umbilical arteries represents advanced fetal compromise, associated with greater than 70% of placental arterial obliteration (Abuhamad 2008). However, placental vascular dysfunction with fetal hypoxemia may exist in the absence of any Doppler findings (Miller, Turan et al. 2008). Doppler changes precede clinical manifestations for adverse changes in the fetus (Baschat, Gembruch et al. 2001; Miller, Turan et al. 2008; Turan, Turan et al. 2008). Increased umbilical artery resistance is associated with fetal distress and IUGR (Giles, Trudinger et al. 1985; Trudinger, Giles et al. 1985).

Doppler ultrasonography for identification of the IUGR fetus

The ability to accurately identify a small fetus is dependent on three things, namely: 1) estimated gestational age; 2) estimated fetal weight; and, 3) a weight percentile calculated from an appropriate fetal growth curve. Standardized Doppler fetal biometric parameters are used to estimate gestational age and fetal weight, including the biparietal diameter (BPD), the head circumference (HC), the abdominal circumference (AC) and the femur length (FL).

Estimating gestational age

There are two primary methods to estimating *in utero* gestational age (GA): 1) the first day of the last menstrual period (LMP); and, 2) ultrasound dating via fetal biometry parameters. While straightforward and inexpensive, the validity of LMP is decreased by the uncertain recall of LMP dates; digit preference; and, biologic variation

in the timing of ovulation, the timing of fertilization, and non-menstrual bleeding (Lynch and Zhang 2007). Ultrasound dating has been shown to provide a superior estimate of gestational age (Savitz and Terry 2002), even when LMP dates are considered certain (Mongelli, Wilcox et al. 1996). In the ultrasound estimation of gestational age, multiple fetal biometric parameters are measured and compared to a fetal size nomogram that has been correlated with gestational age. The main limitation of this method is that estimates of gestational age will be biased if the fetus is either large (upward bias) or small (downward bias). However, dating formulae that rely on multiple fetal parameters are thought to reduce the magnitude of this bias; nearly all estimates of gestational age from published formulae fall within one-week of the actual gestational age (Chervenak, Skupski et al. 1998).

Doppler assessment of estimated fetal weight

There are numerous regression formulae utilizing Doppler-assessed fetal biometric parameters for the *in utero* estimation of fetal weight (EFW), reviewed in (Kurmanavicius, Burkhardt et al. 2004; Dudley 2005; Anderson, Jolley et al. 2007; Scioscia, Vimercati et al. 2008). The abdominal circumference alone is a good marker for estimating fetal weight, with the accuracy of the measurement increasing with the addition of BPD, HC, and FL parameters (Hadlock, Harrist et al. 1985; Dudley 2005; Scioscia, Vimercati et al. 2008). The addition of parameters beyond AD, BPD, HC and FL does not improve accuracy in the estimation of fetal weight due to increased measurement error.

While sonographically estimated fetal weight is commonly used in clinical settings, the various formulas for the estimation of fetal weight have various methodological and/or analytical faults. Errors in fetal weight estimation may originate from several sources: 1) systematic and random errors in measurement, reflected in

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inter-and intra-observer variability; 2) combining 2-dimensional measures to approximate 3-dimensional fetal volume; and 3) the use of fetal volume to estimate fetal weight, a measure of mass (i.e. volume*density) (Zhang, Merialdi et al.). Most formulae have an overall mean absolute percentage error of less than 10% and no formula has been consistently shown to be superior (Dudley 2005; Scioscia, Paine et al. 2008), with estimated fetal weight subject to poorer precision in very low and very high birth weight babies (Kurmanavicius, Burkhardt et al. 2004; Anderson, Jolley et al. 2007; Scioscia, Vimercati et al. 2008).

Doppler assessment of IUGR

The most frequently used technique for diagnosing abnormal growth in a fetus is to calculate the EFW using ultrasound-derived fetal biometric data and to compare the EFW with a fetal growth curve in order to identify fetuses falling under a certain percentile. Generally, an EFW below the 10th percentile for gestational age is used to identify the potentially growth restricted fetus. However, there is no "gold standard" for the identification of IUGR based on EFW and other cutoffs have been suggested, including EFW less than the 25th, 5th and 3rd percentiles and an EFW 2 standard deviations below the mean (corresponding to approximately the 2nd or 3rd percentile) (Pollack and Divon 1992).

There are many published reference curves for fetal growth; interpretations of the estimated fetal weight (or AC) percentile are dependent on the birth weight reference or standard used. A birth weight reference represents an entire population, while a standard excludes high-risk pregnancies assesses fetal size in comparison to normally grown fetuses and is likely of superior clinical utility (Zhang, Merialdi et al.). Furthermore, it is known that fetal size is influenced by factors such as race, gender, SES and altitude and these confounding factors may explain much of the variation among standard fetal

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growth curves (Zhang, Merialdi et al.). Finally, while many reference curves have been constructed from cross-sectional studies, longitudinal studies are preferable due to improved data quality and the ability to study fetal growth, versus fetal size (Zhang, Merialdi et al.). Alternatives to population or standard growth curves have been suggested to deal with these shortcomings, including mathematical models (Rossavik and Deter 1984); customized fetal growth charts (Gardosi 1998); and, regression methods (Bukowski, Smith et al. 2007). However, the clinical utility of these methods in identifying abnormal fetal size has not been demonstrated (Hutcheon, Zhang et al. ; Zhang, Merialdi et al. ; Hutcheon and Platt 2008; Hutcheon, Zhang et al. 2008).

The pathology of malaria in pregnancy

Susceptibility to malaria in pregnancy

Pregnant women, and particularly primigravidae, are more susceptible to *P. falciparum* malaria infection than non-pregnant adults due to a unique phenotypic subset of parasites that sequester in the placenta. In malaria-endemic regions, people acquire immunity to malaria with continued exposure to the parasites, including acquiring anti-adhesion antibodies that inhibit parasite adherence to various host receptors (e.g. CD36). During malaria in pregnancy, parasite-infected erythrocytes (IEs) sequester in the placenta via the adhesion to chondroitin sulfate A (CSA) receptors, located on the syncytiotrophoblast lining of the villous surface of the placenta and in the intervillous space (Duffy and Fried 1999; Muthusamy, Achur et al. 2004). Anti-adhesion antibodies to the CSA-binding parasite appear in multigravidae, but primigravidae lack anti-adhesion antibodies against this parasite phenotype (Fried, Nosten et al. 1998). Natural immunity to CSA-binding parasites builds over the progression of pregnancy and

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through subsequent pregnancies, likely leading to the differential effects of malaria infection on birth outcomes based on gravidity.

Modification of the immune response during pregnancy (Raghupathy 1997) and pregnancy hormones (Roberts, Walker et al. 2001) are also believed to play a role in susceptibility to malaria during pregnancy. Immune system modulation during pregnancy is characterized by a shift away from inflammatory, cell-mediated immunity (Th1-type), with decreases in Th1-type cytokines (e.g. interleukin [IL]-2, interferon [INF]γ and tumor necrosis factor [TNF]-α), toward humoral, anti-inflammatory (Th2-type) immunity, with increases in Th2-type cytokines (e.g. IL-4, IL-6 and IL-10) (Raghupathy 1997). Th1-type immune responses are known to be incompatible with a successful pregnancy and may result in fetal rejection (Raghupathy 1997). The bias toward a Th2-type immunity is thought to leave the pregnant woman more susceptible to Th1-dependent infections, such as malaria (Fievet, Moussa et al. 2001).

Pathophysiology of malaria in pregnancy

Plasmodium falciparum placental malaria is characterized by several changes in the placenta (Figure 2.2). First, IEs adhere to and sequester in the intervillous space of the placenta (Fried and Duffy 1996; Rogerson, Mwapasa et al. 2007). IEs can achieve high densities in the placenta (Brabin, Romagosa et al. 2004); and may be retained there even after apparently adequate therapy (Procop, Jessen et al. 2001). Second, IEs may be accompanied by intervillous infiltrates of monocytes and macrophages, with or without malaria pigment (i.e. hemozoin) (Rogerson, Hviid et al. 2007). Intervillous infiltrates of inflammatory cells are associated with low birth weight (Leopardi, Naughten et al. 1996; Menendez, Ordi et al. 2000; Rogerson, Pollina et al. 2003). Finally, malaria pigment may be found in fibrin deposits in the intervillous spaces without active parasites, representing past chronic infection (Bulmer, Rasheed et al. 1993). Several

studies have found that hemozoin is associated with lower birth weight and lower hemoglobin concentrations (Rogerson, Mkundika et al. 2003); however, others have not found an association between hemozoin and poor birth outcomes (Sullivan, Nyirenda et al. 2000). Several systems have been developed to characterize the pathological changes of malaria in pregnancy (Bulmer, Rasheed et al. 1993; Leopardi, Naughten et al. 1996; Ismail, Ordi et al. 2000; Rogerson, Pollina et al. 2003).

The identification of malaria in pregnancy

The antenatal diagnosis of placental malaria is constrained by the unavailability of information regarding placental infection prior to delivery. In pregnant populations, the microscopy of peripheral blood smear underestimates the prevalence of placental malaria infection using placental blood smear microscopy as referent (Leke, Djokam et al. 1999; Rogerson, Mkundika et al. 2003; Akum, Kuoh et al. 2005; Tako, Zhou et al. 2005), although these findings have not been universal (Kasumba, Nalunkuma et al. 2000). Generally, microscopy of peripheral thick blood smears during pregnancy is considered a poor proxy for placental malaria, as malaria parasites sequestered in the placenta may not be in the peripheral blood or may be submicroscopic in the peripheral blood (Mockenhaupt, Ulmen et al. 2002).

PCR of peripheral blood has been shown to have nearly 100% sensitivity with placental blood smear as the referent; however, specificity is lower (75%) (Mockenhaupt, Ulmen et al. 2002). "False positives" using PCR of peripheral blood may be due to the low sensitivity of placental blood smear microscopy (76-78% compared to the gold standard referent of placental histology) (Rogerson, Mkundika et al. 2003); peripheral parasitemia in the absence of placental infection; and/or remnant DNA in the peripheral blood from resolved placental infection. To date, PCR of peripheral blood at delivery and the gold standard of placental histology have not been compared. While qPCR is highly

sensitive and specific, the clinical relevance of submicroscopic infections is under debate (Mankhambo, Kanjala et al. 2002; Malhotra, Dent et al. 2005; Mockenhaupt, Bedu-Addo et al. 2006).

Malaria in pregnancy, placental function and fetal growth

Malaria in pregnancy is thought to affect fetal growth primarily through placental insufficiency, although the exact mechanisms are unclear and are likely to be multifactorial (Figure 2.3). Chronic malaria infection is particularly associated with the risk of low birth weight and asymmetrical growth, while preterm is associated with acute infection and high parasite densities (Brabin, Romagosa et al. 2004; Rogerson, Hviid et al. 2007; Rogerson, Mwapasa et al. 2007). There are several hypothesized pathological mechanisms of malaria in pregnancy believed to affect placental function and fetal growth including: 1) disrupted trophoblast invasion and inadequate spiral artery remodeling, resulting in decreased placental blood flow; 2) placental inflammatory infiltrates physically decreasing blood flow in the placenta or releasing Th1-type cytokines; 3) morphologic changes in the placenta and placental lesions; and 4) maternal anemia.

A potential mechanism by which malaria in pregnancy may impact fetal growth is via the disruption of trophoblast invasion and the process of spiral artery remodeling from malaria infection during early pregnancy (Dorman, Shulman et al. 2002; Rogerson and Boeuf 2007). The peak prevalence of infection in primi- and multigravidae occurs from approximately 13 to 20 weeks' gestation; coinciding with the second wave of trophoblast invasion of the maternal spiral arteries (Brabin 1983; Dorman, Shulman et al. 2002). If the process of spiral artery remodeling is impaired, it may lead to inadequate blood flow later in pregnancy, similar to preeclampsia.

The most significant association with active malaria infection is the intervillous infiltration by inflammatory cells, particularly in primigravidae, who lack a significant Th2-type anti-inflammatory response (Brabin, Romagosa et al. 2004). *P. falciparum* infection favors the Th1-type, inflammatory immune response, with increased levels of TNF-α; IFNγ; and IL-12 (Fievet, Moussa et al. 2001). TNF-α and IFNγ have direct cytotoxic effects toward both intracellular organisms and the villous trophoblast (Brabin, Romagosa et al. 2004). Both placental levels of Th1-type cytokines and inflammatory infiltrates in the intervillous space have been consistently associated with low birth weight (Leopardi, Naughten et al. 1996; Menendez, Ordi et al. 2000; Rogerson, Pollina et al. 2003). Birth weight reduction is particularly dramatic in the case of massive chronic intervillositis (Ordi, Ismail et al. 1998; Menendez, Ordi et al. 2000). Placental infiltration of inflammatory cells, infected erythrocytes and fibrin can be massive, with more than three-quarters of the intervillous space involved with infiltration (Ordi, Ismail et al. 1998). These inflammatory infiltrates may decrease placental blood flow mechanically. Intervillous infiltrates may also contribute to the effect of malaria in pregnancy on fetal growth via damage to the villous trophoblast or the release of Th1-type cytokines.

There are several morphologic and vascular changes that have been consistently found to characterize active infection in malaria in pregnancy, including perivillous fibrinoid deposits, increased syncytial knotting; and trophoblastic basement membrane thickening (Bulmer, Rasheed et al. 1993; Ismail, Ordi et al. 2000; Brabin, Romagosa et al. 2004). These changes are associated with damage to the syncytiotrophoblast and to cytotrophoblast proliferation (Bulmer, Rasheed et al. 1993; Brabin, Romagosa et al. 2004). [It is worth noting that past infections show minor histological abnormalities, indicating that resolved placental malaria infections leave few to no residual changes in the placenta (Brabin, Romagosa et al. 2004)]. Trophoblastic basement membrane thickening is seen in both acute and chronic infections (Bulmer, Rasheed et al. 1993);

however, most of these changes are typically seen in chronic infection or severe parasitemia (Brabin, Romagosa et al. 2004; Rogerson, Hviid et al. 2007). These placental lesions, particularly the thickening of the trophoblastic basement membrane (Bulmer, Rasheed et al. 1993), may alter nutrient transport and could contribute to the effect of malaria in pregnancy on fetal growth.

Malaria induced maternal anemia is an additional mechanism through which maternal malaria may impact fetal growth. Maternal iron-deficiency anemia often accompanies maternal undernutrition, which may confound the association between anemia and fetal growth. Anemia might contribute to changes in placental growth and vascular structure, leading to hypoxia and reduced nutrient exchange (Mayhew, Charnock-Jones et al.). In malaria in pregnancy, increased TNF-α and monocyte infiltrates are associated with maternal anemia (Fried, Muga et al. 1998; Rogerson, Pollina et al. 2003). Moderate to severe anemia is a recognized cause of low birth weight (Rasmussen 2001; Levy, Fraser et al. 2005). The relationship between maternal malaria, maternal anemia and low birth weight is complicated by the finding that iron deficiency anemia may confer protection from placental malaria, particularly in primigravidae (Kabyemela, Fried et al. 2008).

Previous studies, gaps in knowledge and summary

Two cross-sectional studies have previously reported an association between malaria and abnormal utero- and fetoplacental blood flow. In Kenya, peripheral malaria infection during the third-trimester was associated with concurrent bilateral uterine artery notching, with 18.3% of women with peripheral parasitemia having bilateral notching, compared to 8.2% of women who were not parasitemic (RR=2.24; 95% CI: 1.31, 3.83) (Dorman, Shulman et al. 2002). Among pregnant women experiencing acute malaria crises in French Guinea, Doppler indices were abnormal, with umbilical artery

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resistance increasing by 5 to 20%; uterine artery resistive indices were also increased in approximately half of the pregnancies (Arbeille, Carles et al. 1998). These studies provide evidence that at least some malaria-associated IUGR is explained by decreased utero- and fetoplacental blood flow. However, the effects of malaria parasitemia have yet to be explored longitudinally over the course of pregnancy.

It has also been hypothesized that malaria infection during early pregnancy affects the process of trophoblast invasion and remodeling of the maternal spiral arteries, as in preeclampsia (Rogerson and Boeuf 2007). Should malaria in early pregnancy affect trophoblast invasion, it could lead to altered uterine artery blood flow, reduced oxygen and nutrient exchange, and, ultimately, intrauterine growth restriction. To date, this hypothesis has not been tested. The potential for malaria parasitemia to alter fetoplacental blood flow has not been discussed in the literature. However, malaria in early pregnancy could also affect villous angiogenesis in the placenta. To date, the effect of malaria during early pregnancy on uterine and umbilical artery blood flow over the course of pregnancy has not been explored.

There is a considerable literature linking malaria in pregnancy to low birth weight at delivery (Cot, Le Hesran et al. 1995; Steketee, Wirima et al. 1996; Verhoeff, Brabin et al. 1998; Menendez, Ordi et al. 2000); and, due to the difficulties of accurate pregnancy dating, a smaller number of studies showing a small, significant increased risk of SGA at delivery (Steketee, Wirima et al. 1996; Sullivan, Nyirenda et al. 1999; Verhoeff, Brabin et al. 2001). Placental malaria has also been found to be associated with an increased risk of SGA at delivery (Steketee, Wirima et al. 1996; Menendez, Ordi et al. 2000; Verhoeff, Brabin et al. 2001). An important limitation of these studies is that they have described the association of antenatal and/or placental malaria infection and fetal weight cross-sectionally at delivery.

Evidence is mounting that early pregnancy malaria infection is associated with decreased fetal weight at delivery. To our knowledge, three previous studies have examined various definitions of early pregnancy malaria exposure with birth weight or low birth weight outcomes (Table 2.1). These studies appear to demonstrate a j- or u-shaped relationship between the timing of malaria exposure and both decreased birth weight and risk of low birth weight (Taha, Gray et al. 1993; Cottrell, Mary et al. 2007; Huynh BT 2011). While studies describing effect of early pregnancy malaria infection on fetal growth are of interest, inaccurate pregnancy dating and a lack of longitudinal fetal growth data have prevented such analyses, to date.

In summary, there are important gaps in knowledge regarding the relationship between acute and early pregnancy malaria on utero- and fetoplacental blood flow and fetal growth. The relationship between malaria and utero- and fetoplacental blood flow has not been explored longitudinally. Additionally, the effect of malaria during early pregnancy on utero- and fetoplacental blood flow and fetal growth has not been examined. Thus, questions remain regarding when and how malaria in pregnancy leads to increased uterine and umbilical artery resistance and reduced fetal growth, for example: Is it an acute process? Does parasitemia in early pregnancy affect trophoblast invasion and lead to impaired uteroplacental circulation, as in preeclampsia? Does the effect of malaria on blood flow and fetal growth change over pregnancy? Answers to questions such as these can lead to a better understanding of the pathogenesis of malaria in pregnancy; a better understanding of disease processes can be used to inform the type and timing of public health interventions, leading to improved health outcomes for mothers and their infants.

Figure 2.1 Typical umbilical artery Doppler with Doppler Index equations. S is peak

systole; D is end-diastole; A is the temporal average frequency; RI is resistance index;

PI is pulsatility index.
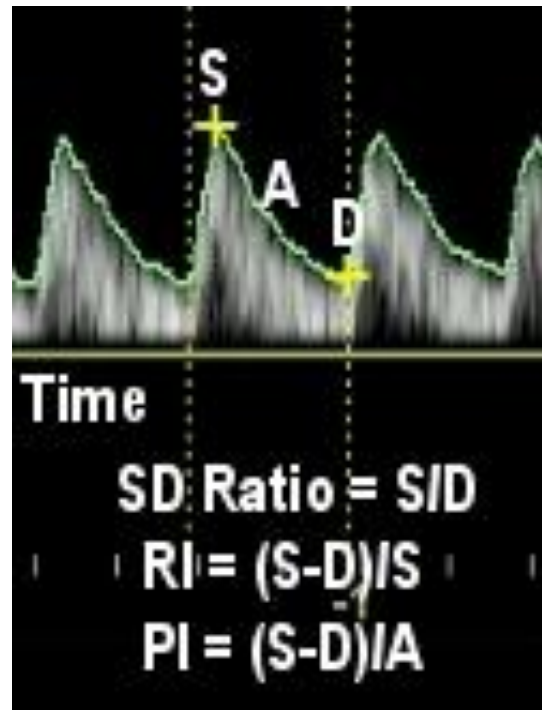
Figure 2.2 Histologic features of placental malaria. IVS=intervillous space; V=placental villi; F=fibrin; arrowheads=infected erythrocytes; asterisk = inflammatory cells; circle = pigment (hemozoin versus formalin artifact). Giemsa stain, 400x. (Image courtesy of Atis Muehlenbachs)
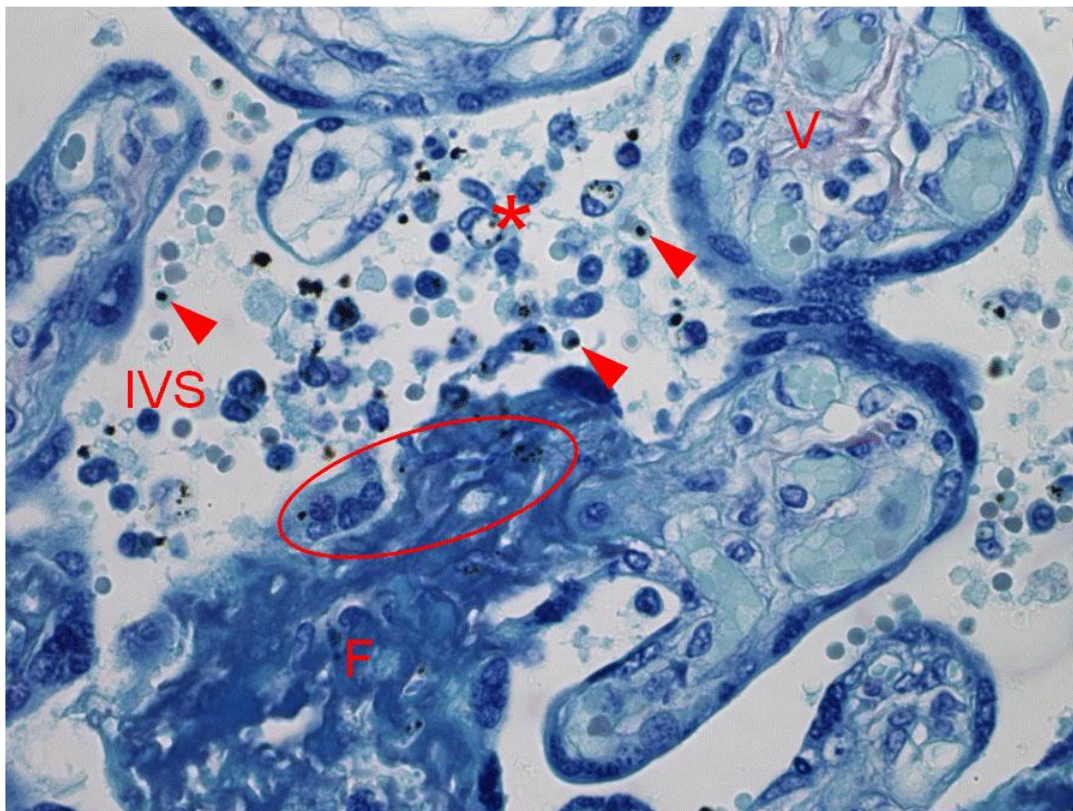
Figure 2.3 Possible pathways by which placental sequestration of infected erythrocytes could activate monocytes and other cells to cause changesin placental function that result in growth retardation. Infected erythrocytes or their products can activate both syncytiotrophoblasts and monocytes to release chemokines and cytokines. The former contribute to monocyte accumulation, whereas the latter could have direct effects of placental growth (through angiogenesis). Cytokines and cell accumulations can lead to placental hypoxia. Cytokines could directly or indirectly affect nutrient transport mechanisms. Decreased placental growth, or decreased nutrient transport are probable final common pathways by which malaria leads to fetal growth restriction. Glut1=glucose transporter 1. (Rogerson, Hviid et al. 2007).
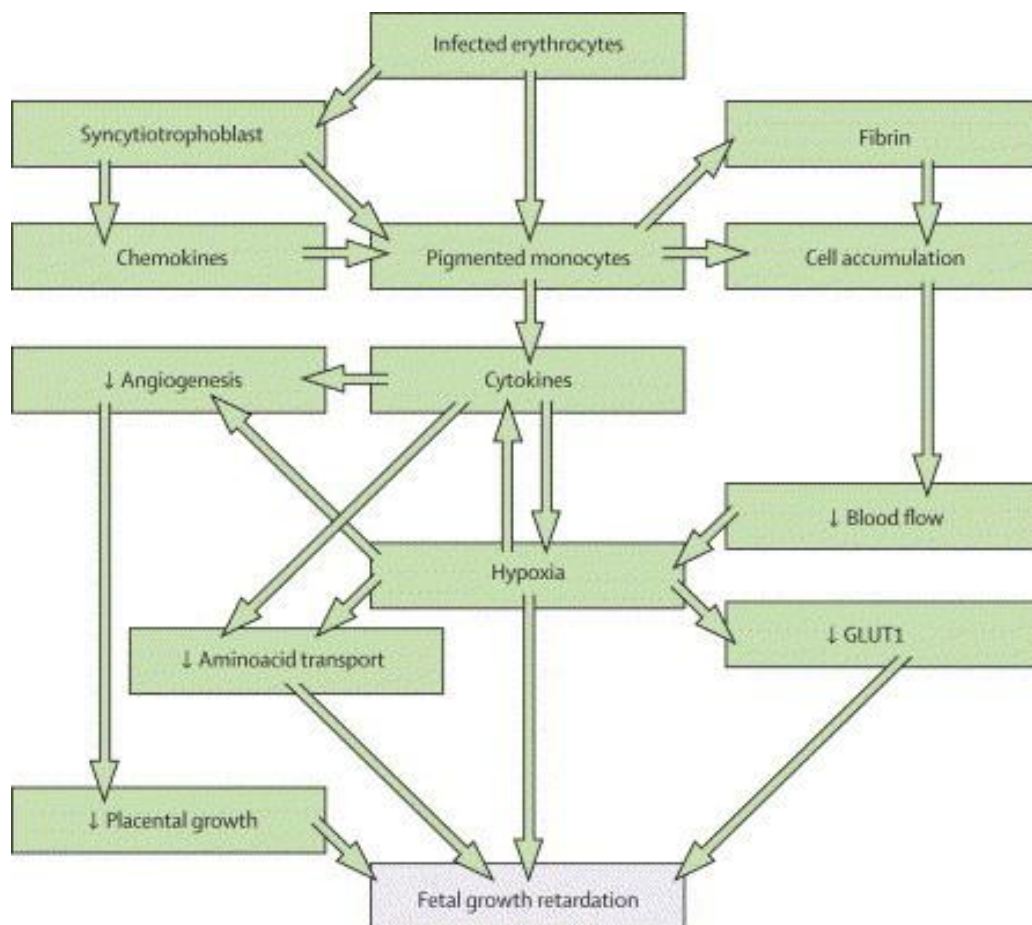
Table 2.1 Association between early pregnancy malaria infection and pregnancy outcomes.

Location	Design	N	Outcome	Timing	Effect type	Effect (95% CI)	Citation
Benin	Cohort	1037	Birth weight	<4 months	Mean difference	-98.5 g[a] (-189, -9)	(Huynh BT 2011)
				5-6 months	Mean difference	35.4 g (-42, 113)	
				>6 months	Mean difference	-22.0 g (-82, 39)	
			LBW	<4 months	Odds Ratio	1.2 (0.6, 2.6)	
				5-6 months	Odds Ratio	1.0 (0.5, 2.0)	
				>6 months	Odds Ratio	1.4 (0.8, 2.5)	
Burkina Faso	Cohort	1190	Birth weight	<4 months	Mean difference	-68 g[b] (-145, 10)	(Cottrell, Mary et al. 2007)
				4-6 months	Mean difference	18 g (-68, 101)	
				>6 months	Mean difference	-105 g (-190, -22)	
			LBW	<4 months	Odds ratio	1.3 (0.7, 2.4)	
				4-6 months	Odds ratio	1.1 (0.6, 1.8)	
				>6 months	Odds ratio	1.8 (1.1, 3.1)	
Central Sudan	Case-control	215/117	LBW	Trimester 1	Odds ratio	1.9[c] (1.2, 2.7)	(Taha, Gray et al. 1993)
				Trimester 2	Odds ratio	1.6 (1.0, 2.6)	
				Trimester 3	Odds ratio	1.3 (0.4, 4.1)	

LBW = Low birth weight; LBW was defined as <2500 grams in all studies
a. Models adjusted for parity, newborn sex, rainy season at delivery, maternal BMI, education, duration of gestation, number of SP intakes, number of consultations, bednet use
b. Models adjusted for parity; infant sex; transmission period; maternal BMI; duration of gestation; prophylaxis before and after 6 months of pregnancy
c. Models adjusted for malaria treatment, use of insecticides, parity, other illnesses, infant sex, antenatal care, maternal weight

CHAPTER 3 - METHODS

Study Design

In order to investigate the specific aims of this dissertation, we conducted secondary analyses using data from the "Project ECHO" study. Project ECHO was a longitudinal Doppler ultrasound cohort study conducted in Kinshasa, Democratic Republic of Congo. Project ECHO data uniquely provide the ability to examine the relationship between malaria and fetal size for several reasons: 1) ultrasound dating of gestational age; 2) prospective serial measures of Doppler fetal biometrics and blood flow velocity waveforms; and 3) prospective serial measures of incident malaria parasitemia. The Project ECHO study has continuing UNC Institutional Review Board (IRB) approval.

Study Setting and Population

Project ECHO was conducted in the Binza Maternity Hospital in Kinshasa, Democratic Republic of Congo and was a collaboration of University of North Carolina-Chapel Hill School of Public Health and the Kinshasa School of Public Health. Binza Maternity Hospital has been in operation for over 30 years and is Kinshasa's second largest urban maternity hospital, with between 6,000 and 10,000 births per year.

Pregnant women who presented for first antenatal care at Binza Maternity Hospital in Kinshasa, Democratic Republic of Congo during two recruitment stages between May 2005 and May 2006 were considered for enrollment in the study. Women aged 18 or older, with a singleton pregnancy with no known fetal congenital

malformations and an ultrasound-derived gestational age of 22 weeks, 0 days or less were considered eligible for enrollment.

Selection Criteria

Inclusion criteria: In order to be eligible for the study, women had an ultrasound confirmed singleton pregnancy with an ultrasound-derived gestational age of 22 weeks, 0 days or less; were 18 years of age or older; agreed to HIV testing; and consented to participate in the study.

Exclusion criteria: Women were excluded who were younger than 18 years of age; had a fetus with detectable congenital malformations; or, were hypertensive (systolic blood pressure >140 mmHg and/or diastolic > 90 mmHg) at baseline. Women with evidence of placenta previa, fetal abnormalities and multiple gestations during ultrasound were referred to the Department of Obstetrics and Gynecology at the Clinique Universitarie in Kinshasa for high-risk pregnancy follow-up care.

Data Collection

Recruitment

During routine antenatal registration and clinical evaluation at Binza Maternity Hospital, all pregnant women presenting for their first antenatal care visit were screened for study pre-eligibility. The three pre-eligibility criteria were: 1) aged 18 or older; 2) systolic blood pressure less than 140 mmHg and diastolic blood pressure less than 90 mmHg; and, 3) less than 23 weeks gestation by patient reported last menstrual period. Women who met pre-eligibility criteria were asked to consent to have a baseline ultrasound examination to estimate gestational age (Hadlock, Deter et al. 1984; Hadlock, Shah et al. 1992) and fetal weight (Hadlock, Harrist et al. 1985) within three to five days

of pre-eligibility screening. Amniotic fluid volume index (four quadrant method), gender of the fetus and placental location were also recorded. Pre-eligible women having an ultrasound-confirmed singleton pregnancy less than 22 weeks, 0 days were considered eligible for the Project ECHO study. Eligible women met with a Project ECHO recruiter who explained the objectives and procedures of the longitudinal study and administered written informed consent for enrollment. Results from recruitment are reported in Figure 3.1.

<u>Baseline visit</u>

After providing written consent, enrolled women participated in the baseline interview to assess sociodemographic characteristics; medical and obstetric history; alcohol and tobacco use; and, current malaria status. All enrolled women participated in HIV voluntary counseling and testing at baseline via an ongoing Glaser Foundation Preventing Mother to Child Transmission program conducted at Binza Maternity Hospital. Woman identified as HIV-positive, and their infants, received Nevirapine treatment as per the Glaser Foundation program protocol.

A baseline medical examination collected maternal anthropometric measurements (weight, height and mid-upper arm circumference); blood pressure; pulse; temperature; and pallor. The onsite Binza Maternity Hospital laboratory conducted a urine test for albumin and a hematocrit test. The laboratory collected malaria thick and thin smears and filter paper samples from fingerprick blood samples. Malaria thick smears were read onsite via microscopy for a gross determination of parasitemia. The Ecole Sante Publique de Kinshasa provided quality control; assessment of parasite density; and, parasite speciation via microscopy.

In accordance with Congolese National Policy, all enrolled women received a dose of intermittent presumptive therapy in pregnancy (IPTp) with sulfadoxine-

pyrimethamine (SP: 1500 mg sulfadoxine + 75 mg pyrimethamine) between 16 and 27 weeks' gestation, regardless of malaria status. If women had their baseline visit prior 16 weeks' gestation, they received IPTp at their first follow-up visit. In accordance with Congolese National Policy, women received iron supplementation as part of routine ANC at Binza Maternity Hospital. At the end of the baseline visit women were provided with an insecticide treated bed net.

Follow-up visits

Participants returned to Binza Maternity Hospital for monthly follow-up visits until delivery. At each follow-up visit, a medical examination assessed maternal weight and mid-upper arm circumference; blood pressure; pulse; temperature; and symptoms of anemia. The Binza Maternity Hospital laboratory conducted a urine test for albumin at each follow-up visit and a hematocrit test at every other visit. A follow-up interview assessed recent malaria symptoms, use of anti-malarial medications, and bed net use.

At each follow-up visit, an ultrasound examination was used to estimate gestational age and fetal weight from fetal biometric measurements; amniotic fluid volume index; and, placental location. Doppler ultrasound additionally interrogated left and right uterine and umbilical artery blood flow waveforms; arterial notching of the uterine arteries; and end diastolic flow of the umbilical artery.

The onsite laboratory collected malaria thick and thin smears and filter paper samples from finger-prick blood samples. Malaria thick smears were read onsite via microscopy for a gross determination of parasitemia, with quality control from Ecole Sante Publique de Kinshasa. At all visits, women found to have a positive malaria slide were provided with free anti-malarial treatment by Project ECHO, with treatment determination by the attending physician at Binza Maternity Hospital. In accordance with Congolese National Policy, all enrolled women received a second dose of intermittent

presumptive therapy (IPT) with SP between 28 and 32 gestational weeks, regardless of malaria status.

Interim visits

Women were instructed to return to the clinic any time they felt ill or had malaria symptoms. At interim visits, the maternity provided regular medical care. A Project ECHO staff member recorded all medical procedures performed/medications prescribed and prepared a thick smear and filter paper sample from finger prick blood samples. All medications and laboratory tests were provided free of charge by Project ECHO.

Labor and Delivery

Project ECHO participants were encouraged to deliver at Binza Maternity Hospital and were reimbursed for one-third of delivery-related fees. If a study participant did not delivery at Binza Maternity Hospital, a Project ECHO staff member traveled to the hospital or clinic where the woman delivered as soon after delivery as possible to collect the necessary samples and infant information.

Prior to delivery, the onsite laboratory collected malaria thick and thin smears and filter paper samples from finger-prick blood samples and conducted a hematocrit test. If biospecimen samples were not collected prior to delivery, attempts were made to collect them in the 24 hours following delivery. Delivery date, method, abnormal events and outcome were recorded. After delivery, a placental thick smear and a placental biopsy were taken to assess placental malaria infection. The infant was weighed, gender and congenital malformations were noted, and infant anthropometrics (crown to heel, crown to rump, head circumference and abdominal circumference) were taken within 24 hours of delivery.

Enrolled women participated in 979 regular appointments that included an ultrasound scan, with 11 in the first trimester, 423 in the second trimester and 545 in the third trimester. There were 172 interim visits which did not include an ultrasound scan. Women participated in 4.5 follow-up visits (SD = 0.9 visits) and were enrolled for 18 weeks (SD = 3 weeks) on average. Delivery information was collected for 180 women (no data for one maternal death and one woman with unknown delivery location). There were 167 term deliveries, 8 preterm deliveries, 3 preterm/stillbirth deliveries, and one preterm/early neonatal death. The results of follow-up and delivery can be seen in Figure 3.2

Measurements

Measurements taken at baseline, follow-up and delivery are summarized in Table 3.1. A description of ultrasound, biological and clinical measures relevant to the proposed analyses follows as previously described (Landis 2007).

Ultrasound measures

Ultrasound measurements were taken on the GE Logicbook Ultrasound System. Ultrasound images were stored on CD-ROM for blinded quality control at University of North Carolina-Chapel Hill. Victor Lokomba, an obstetrician-gynecologist from Clinique Universitarie in Kinshasa with extensive training in ultrasound technique, performed all ultrasound measures.

Fetal biometric measures

All fetal biometric measures were measured using standard techniques. Measurements of head circumference (HC) and biparietal diameter (BPD) were from an

image that displayed the fetal head in an axial plane that included the thalamus and cavum septum pellucidum. The BPD was measured by placing the calipers from leading edge to leading edge (outer to inner skull table) and the HC was measured using an ellipse trace of the outline of the fetal head. Abdominal circumference (AC) was measured from an image in which the junction of the umbilical vein and portal sinus were visible. The ellipse function was used to trace the extreme perimeter of the fetal abdomen. Femur length (FL) of the femoral diaphysis was also measured. A 10% sample of ultrasound images was assessed for quality by a maternal-fetal medicine physician at the University of North Carolina - Chapel Hill. Ninety-two percent of reviewed images were rated adequate for clinical assessment; 7% of questionable quality and 1% of poor quality (i.e. not all biometry landmarks clearly visible; shadowing in the image; or, poor tracing of the length of circumference). Intra-operator variability in measuring fetal biometry was assessed in ten patients. The correlation between two independent measurements on the same fetus was r=0.99 for each of BPD, HC, and FL, and r=0.98 for AC.

Uteroplacental blood flow

Color Doppler ultrasound used standard techniques to identify the left and right uterine arteries and umbilical artery. For each of the uterine arteries, color flow settings identified the external iliac artery and uterine artery located perpendicular and medial to it. Flow velocity waveforms were obtained from the uterine artery near the iliac vessel prior to division of the uterine artery into branches. For the umbilical artery, the flow velocity waveform was obtained from the free flowing portion of the umbilical cord approximately midway between placental and fetal insertion sites. Left/right uterine and umbilical artery measures included the systolic-diastolic (SD) ratio, resistance index (RI) and pulsatility index (PI). For the uterine arteries, there was a qualitative assessment of

the presence of early diastolic notching. For the umbilical artery, there was an assessment of end-diastolic flow, with absent or reversed end-diastolic (ARED) flow noted.

<u>Biological and clinical measures</u>

Malaria

At baseline, follow-up visits, interim visits and at delivery, blood was collected and prepared as a thick and thin smear, and applied to filter paper, using standard techniques. Thick and thin smears were taken from the site of a finger puncture, were dried and Giemsa-stained. Five dried blood spots were collected by lightly touching a filter paper to a large blood drop from the sight of finger puncture. Filter papers with dried blood spots were placed in individual plastic bags with desiccant; stored at -20° C; and, were transported to UNC. Malaria parasitemia was assessed in two ways: microscopy and real-time PCR.

Microcopy-Determination of Peripheral Malaria Parasitemia

Peripheral malaria parasitemia was assessed from Giemsa-stained thick smears on-site by a trained microscopist. Parasite sub-type was determined and density was quantified by counting the number of parasites against 200 white blood cells and converted to numbers of parasites per µl, assuming 6000 WBC per µl. All specimens were sent to the Ecole Sante Publique de Kinshasa for a second assessment of sub-type and density. If discrepancies occurred, a third reader assessed the slide. For quality assurance purposes, a random sample of 10% of all blood smears was blindly assessed by a second trained microscopist.

Real-time qualitative PCR-Determination of Peripheral Malaria Parasitemia

At University of North Carolina – Chapel Hill, three 0.6 cm diameter punches were punched from each dried blood spot filter paper card and were deposited into a single well of a 96-well deepwell plate. Genomic DNA was extracted from plates of punches using the QIAamp 96 DNA Blood Kit (Qiagen, Germantown, Maryland, USA) with a vacuum manifold according to the manufacturer's protocol. Genomic DNA was eluted into 150uL of eluate and stored at 4C. Primer and probe sequences to the block 9 region of the gene encoding the small subunit (18S) of plasmodia ribosomal RNA were modified from a published protocol which detected between 1 to 10 copies of the target DNA and was specific to plasmodia 18S DNA. qPCR was carried out in 25uL reactions consisting of 2uL of DNA, 12.5uL of 2x TaqMan Universal PCR MasterMix (Applied Biosystems, Foster City, California, USA), forward and reverse primers at 1000nM and VIC-labeled minor-groove binding probe at 200nM. Cycling conditions on an Applied Biosystems 7300 System were $50°C$ for 2 minutes, $95°C$ for 10 minutes and 40 cycles of $95°C$ for 15 seconds and $60°C$ for 1 minute. The ability of the assay to detect different malaria species and strains was evaluated by testing the assay with genomic DNA from Plasmodium falciparum strain 3d7 (MRA-102G, MR4, ATCC, Manassas, VA), genomic DNA from *P. vivax* strain Nicaragua (MRA-340G; MR4), and plasmids containing the 18S ribosomal DNA gene from *P. falciparum, P. vivax, P. ovale* and *P. malariae* (MRA-177, MRA-178, MRA-179, MRA-180, respectively; MR4). The sensitivity of the assay was determined by evaluating dilutions of *P. falciparum* genomic DNA (Taylor, Juliano et al. 2010).

Maternal anthropometrics

Standard techniques (NHANES 1998) were used to assess maternal weight, height and mid-upper arm circumference (MUAC). Maternal weight was measured on a UNICEF digital scale (SECA Model 890) to the nearest 0.1 kilogram. Height (without foot

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or head-wear) was measured to the nearest 0.1 cm. MUAC was measured with cloth tapes on the right arm at the midpoint between the acromial and olecranon processes of the scapula and ulna, respectively. The measurement was made to the nearest 0.1 cm with the arm hanging freely; the cloth tape snug to the skin; and, without compressing underlying tissue. There were technical problems with the digital scale halfway through the study period; as such, MUAC measurements were used for all proxy measures of maternal nutritional status in this dissertation.

Infant anthropometrics

Standard techniques (NHANES 1998) were used to assess infant anthropometrics within 24 hours of delivery. Birth crown-heel and crown rump length were measured using a pediatric length board; head and abdominal circumference were measured to the nearest 0.1 cm using a measuring tape; and, birth weight was recorded to the nearest gram on a LARIO Scale (Soc Curion & Co., Como, Italy).

Data Management and Analysis

Data entry was completed in the Democratic Republic of Congo using an EpiInfo database designed specifically for the Project ECHO study, with multiple quality control mechanisms, including 1) routine checking for complete data at the maternity prior to the end of each appointment; 2) pre-programmed ranges of plausible values for all 33 continuous numeric data fields (e.g., temperature, height, weight); 3) preprogrammed ranges of allowable values for all categorical responses; 4) preprogrammed skip patterns; 5) required data entry fields (such as ID numbers and dates) that must be entered to continue with entry; and vi) re-entry of a 10% sample of forms by an independent data entry clerk for comparison to the larger database using the EpiInfo Data Compare function.

The EpiInfo data base was transferred into SAS and SPSS datasets for data cleaning. Data cleaning steps included: 1) identifying data that is missing from required fields and attempting to locate that data if possible; 2) checking to ensure that skip patterns were properly followed; 3) descriptive statistics of all continuous variables to identify outlier values; 4) ensuring that dates match up on all forms for a given visit; 5) translating dates into American format (DD/MM/YYYY); and, 6) adding descriptive labels and user defined formats to each variable.

All information in the Project ECHO database is de-identified.

Specific Aim 1 Analyses

Using longitudinal Doppler ultrasound cohort data, Aim 1 examined the effect of malaria parasitemia on changes in uterine artery (UtA) and umbilical artery (UA) resistance indices (RI) over time.

Study population

Five participants were HIV-positive and were excluded from the analysis, leaving a final analytic sample of 177 women, with a total of 1120 antenatal visits. HIV-positive women were not different from the final analytic sample of women by model covariates. Participants had a median of 6 visits (interquartile range [IQR]: 5, 7) and 4 ultrasound scans (IQR: 4, 5).

Variables

Exposure:

Malaria: The primary malaria exposure variable was based on qPCR for *P. falciparum* malaria from peripheral dried blood samples. 'Malaria' was a binary, time-

dependent measure representing whether a woman was positive for *P. falciparum* malaria at the visit in which the ultrasound measure of uteroplacental blood flow were taken. Malaria was treated dichotomously: 0 (no malaria) and 1 (malaria). We removed suspected recrudescent malaria cases if parasitemia recurred within two weeks of treatment for a blood smear identified infection (6 episodes). Due to the substantial misclassification of microscopy-determined malaria parasitemia in the current dataset (Taylor, Juliano et al. 2010); the higher detection threshold of microscopy; and, the potential for poor microscopy sensitivity and specificity (Ohrt, Obare et al. 2007), for all proposed analyses the primary time-dependent malaria exposure was defined as qPCR-positive *Plasmodium falciparum* parasitemia from peripheral blood.

Outcome:

Outcome variables for placental blood flow included the continuous, time-dependent mean of the left and right uterine artery (UtA) and the umbilical artery (UA) resistance index (RI) after 20 weeks' gestation. The RI is defined as: (peak systolic velocity – end diastolic velocity) / peak systolic velocity. The RI is constrained between 0 and 1; demonstrates the least variance of the Doppler indices under identical hemodynamic conditions; is common in clinical settings; and, has a truncated normal distribution (Maulik 2005). Placental location influences the uterine artery waveform and there are differences in the Doppler indices of the placental and non-placental uterine arteries, generally with lower indices on the placental side (Kofinas, Penry et al. 1988). We utilized the mean of the two uterine artery resistance indices at each study visit to minimize the effects of placental location on the resistance index.

Time metric:

Gestational age was back-calculated from the first ultrasound using Hadlock's formula: Gestational age = 10.85 + 0.060*HC*FL + 0.6700*BPD + 0.1680*AC (Hadlock, Deter et al. 1984). As previously discussed, the derivation of gestational age from early ultrasound assumes no significant variability in growth at the pregnancy dating ultrasound and will bias estimates for the large fetus (upward) or small fetus (downward). However, ultrasound dating is still considered a reasonably accurate estimate of gestational age (Chervenak, Skupski et al. 1998). The 'gestational age' variable was centered in order to achieve a meaningful intercept that can be interpreted as the resistance index occurring at gestational week 28.

Hypothesized effect measure modifiers:

Gestational age: The interaction between the malaria exposure and gestational age were investigated to determine if the effects of malaria on UA RI and UtA RI vary over the course of pregnancy.

Primigravidity: As malaria is known to have a differential effect on birth outcomes in primigravidae (Desai, ter Kuile et al. 2007), gravidity was explored as a potential effect measure modifier. Primigravidity was coded dichotomously as primigravidae (1) or multigravidae (0).

Fetal sex: Fetal sex has never, to our knowledge, been investigated as a modifier of the relationship between malaria and pregnancy outcomes. However, recent work by Clifton et al. regarding the sex-specific adaptations of the placenta in asthma, pre-eclampsia, and preterm delivery (Clifton 2010) inspired our investigation of fetal sex a potential effect measure modifier. Fetal sex was coded dichotomously as female (1) or male (0).

Baseline maternal upper arm circumference: As maternal nutritional status has been shown to modify the effects of malaria on pregnancy outcomes (Landis, Lokomba

et al. 2009), we explored mid-upper arm circumference (MUAC) as a modifier of the relationship between malaria and pregnancy outcomes. MUAC is an indicator of maternal nutritional status that is relatively unaffected by gestational age and is a better proxy for pre-pregnancy weight than maternal BMI (Krasovec and Anderson 1991). Further, as there was a malfunction in the digital scale approximately halfway through the study period, we utilized MUAC for all proxy variables for maternal nutritional status in all analyses. Women with baseline MUAC in the lowest quartile (<24.5 cm) were classified as 'Low MUAC.'

Covariates:

Potential confounders were identified through a review of the literature and were analyzed via a directed acyclic graph (DAG) (Figure 3.3). Only relationships between the exposure, outcome and covariables are shown; relationships between covariates were considered, but are not depicted to simplify the graph. DAG confounders included maternal age, low education (<secondary vs. ≥secondary); low socioeconomic status (SES) (low vs. high). We did not consider antimalarials, ITN use, or number of ANC visits in the models as we did not find that they were associated with our outcomes in the literature.

Maternal age: Maternal age was measured continuously, but was categorized in the analysis (18-24, ≥30 years vs. 25-29 years) to better fit the well-described u-shaped relationship with adverse pregnancy outcomes.

Low education: 'Low educational status' was a binary variable defined as women who had less than a secondary education vs. women with a secondary education or higher.

Socio-economic status (SES): 'Low SES' was a binary composite variable, with women meeting all of the following criteria categorized as 'low SES': currently

unemployed; living in a home without toilet facilities; no access to a nearby water source; and, no electricity.

Data analyses

<u>Univariate and bivariate analyses</u>

We conducted exploratory analyses to examine cross-sectional and longitudinal relationships in the data. We plotted individual outcome measurements over time and fit ordinary least squares and nonparametric trajectories. We examined polynomial transformations of gestational age visually to determine which individual change trajectory best described the relationship between time and the outcome variables. To explore inter-individual differences in change, we graphed a random sample of 20 smooth nonparametric and OLS regression trajectories. We also smoothed average trend lines for covariate subgroups to investigate group differences in trajectories. Finally, we plotted individual profiles with the smoothed average trend line using a spline routine.

Standard descriptive statistics were examined for the entire dataset and aggregated by subject. For continuous measures we examined standard measures including means, medians, standard deviations, and quartiles. For categorical variables, we examined frequencies and percents at each level of the covariate, including missing values. We examined the proportion of missing values for all variables and outliers. We examined descriptive statistics for our continuous outcome variables, without consideration of repeated measures.

For bivariate analyses, we examined graphs of nonparametric growth trajectories separately for time-independent categorical covariates. We examined associations between outcomes, exposures, and covariates, using standard bivariate analyses. We

fitted linear mixed effect (LME) models to estimate simple mean differences and corresponding 95% confidence intervals (CIs) in UtA RI and UA RI by covariates, adjusting for gestational age (Laird and Ware 1982).

Linear mixed effect models:

As we utilized LMEs for our multivariable analyses, a brief overview of LME methods is included. LME models are appropriate for modeling the association among repeated measurements. LMEs can be thought of as a series of nested models, representing within-subject change and between-subject change. If Y_{ij} represents the continuous outcome for subject *i* at measurement occasion *j*; and, T_{ij} represents the time metric for subject *i* at measurement occasion *j*.

The within-subject change model (level 1) is:

$Y_{ij} = \beta_{0j} + \beta_{1j}T_{ij} + \varepsilon_{ij}$ where $\varepsilon_{ij} \sim N(0, \sigma^2_{\varepsilon_{ij}})$.

And, the between-subject difference in change model (level 2) is:

$\beta_{0j} = \gamma_{00} + \mu_{0j}$

$\beta_{1j} = \gamma_{10} + \mu_{1j}$

where $\begin{pmatrix} \mu_{0j} \\ \mu_{1j} \end{pmatrix} \sim MVN\left(\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \sigma^2_{\beta_{0j}} & \sigma_{\beta_{0j}, \beta_{1j}} \\ \sigma_{\beta_{0j}, \beta_{1j}} & \sigma^2_{\beta_{1j}} \end{pmatrix} \right)$

Then, the nested LME model with random intercepts and slopes is:

$Y_{ij} = (\gamma_{00} + \gamma_{10}T_{ij}) + (\mu_{0j} + \mu_{1j}T_{ij}) + \varepsilon_{ij}$

The average subject-specific intercept is represented by γ_{00}; μ_{0j} represents the subject-specific random effect (i.e. the random intercept) and is $\sim N(0, \sigma^2_{\beta_{0j}})$. The average subject-specific slope is represented by γ_{10}; μ_{1j} represents the between-

subject random effect (i.e. the random slope) and is ~ N(0, $\sigma^2_{\beta_{1j}}$). The random effects are assumed to be independent from ε_{ij}, but are not independent of one another, with covariance $\sigma_{\beta_{0j}, \beta_{1j}}$.

Multivariable analyses

We utilized LME models to estimate the unadjusted and adjusted mean differences in the mean uterine artery resistance index and in the umbilical artery resistance index with corresponding 95% confidence intervals (CI) (Laird and Ware 1982). LME models are flexible regression tools to represent change processes in longitudinal data and account for the correlation between repeated measures in an individual (Laird and Ware 1982). Strengths of LME models include handling data with subjects observed at different time points; use of all available data; providing valid standard errors and efficient statistical testing.

Multivariable model building occurred in several steps. We began with a fully adjusted LME model, including the dichotomized exposure (malaria), the time metric, the product interaction term between the exposure and the time metric, and hypothesized product interaction terms and covariates that were potential confounders of the association between malaria and the outcomes. We included a random intercept for each woman. First, we assessed linearity between gestational age and the outcome by examining polynomial transformations of gestational age graphically and using -2 log-likelihood (-2LL) tests for nested models using maximum likelihood [ML] estimation (a priori cutoff of $p < 0.10$).

Second, we examined the contribution of additional random effects to model fit using -2LL tests for nested models with ML estimation and a 50:50 mixed chi-square distribution (a priori cutoff of $p < 0.05$). Based on -2LL test results during model building,

random intercepts and slopes were included for each woman. Random intercepts allow each woman to differ in her level of the outcome; random slopes allow the outcome to change differently over gestation for each woman

Next, we used a backward elimination approach to examine effect measure modification (EMM) and confounding. We conducted -2LL tests to evaluate the contribution of product interaction terms for malaria and potential EMMs on mean differences in UtA RI and UA RI (ML estimation; a priori cutoff of $p < 0.10$). If a hypothesized modifier was not identified as an EMM, it was assessed as a potential confounder. Potential confounders were identified through a review of the literature and through analysis via a Directed Acyclic Graph (DAG) for the model (Greenland, Pearl et al. 1999; Rothman, Greenland et al. 2008). Covariates were considered confounders and retained in the model if the change in the DAG model mean estimate was greater than 10% within strata of EMMs (Rothman, Greenland et al. 2008).

Finally, we estimated model effects using restricted maximum likelihood (REML), as ML estimation can result in biased random effect estimates in small samples (Fitzmaurice 2009). We verified model assumptions by conducting a residual and iterative influence analyses. Model outputs include fixed effect parameters, which quantify the effects of predictors on the population average change trajectories, and random effect parameters, which assess the variability at the two levels across different subjects. The regression coefficient for malaria in the proposed linear mixed model can be interpreted as the difference in the outcome between women with and without malaria. Following the specification of the final model, we examined the unadjusted effect of malaria on the outcomes. We plotted population average growth curves to describe the unadjusted and adjusted effect of malaria on uteroplacental blood flow. All analyses were performed using SAS software (SAS, Cary, NC).

Sensitivity analyses

We performed simple sensitivity analyses to examine the robustness of model estimates to changes in the definition of our outcome and in our time metric, ultrasound estimated gestational age.

We examined the difference in weeks between last menstrual period and ultrasound estimates of gestational age (last menstrual period estimated gestational age – ultrasound estimated gestational age). We report the mean and standard deviation for the calculated difference in gestational age difference. As bias in ultrasound dating may be introduced if a fetus is larger or smaller for gestational age than expected, we examined the mean differences in gestational age dates across strata of maternal and fetal characteristics from our multivariable models known or theorized to be associated smaller fetuses (including malaria parasitemia at dating ultrasound; gravidity; fetal sex; and low maternal MUAC at baseline). Finally, we examined robustness of the UtA RI and UA RI final models to changes in the time-metric, ultrasound estimated gestational age in two sensitivity analyses in which we reran the final models using last menstrual period dating (S1); and, among the subset of women with 'certain' gestational age (i.e. LMP within ±14 days of the ultrasound derived date) (S2).

For the uterine artery resistance outcome, we conducted a third sensitivity analyses in which we examined the effect of using the resistance index value from the highest uterine artery rather than the mean value of the left and right uterine arteries (S3). Results for sensitivity analyses can be found in Appendix 1.

Specific Aim 2 Analyses

Using longitudinal Doppler ultrasound cohort data, Aim 2A estimated the effect of malaria in early pregnancy on subsequent uterine artery and umbilical artery resistance

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indices over time. Aim 2B estimated the effect of malaria in early pregnancy on the subsequent risk of intrauterine growth restriction. For Aim 2, we left-truncated person-time data at 21 weeks' gestation, resulting in 548 visits with Doppler ultrasound and fetal biometric measurements (Figure 3.4). We examined IUGR after 20 weeks' gestation for three main reasons: 1) we were interested in examining the "chronic" effects of early pregnancy malaria exposure for the duration of pregnancy; 2) we had very few Doppler ultrasound data points prior to 21 weeks' gestation as uterine artery and umbilical artery blood flow were not measured at the baseline study visit; and 3) there is little variation in fetal weight through the first trimester and IUGR is often not seen until the second trimester.

Study population

For this, we excluded 5 HIV-positive participants and 49 participants with no malaria exposure data ≤ 20 weeks' gestation, leaving an analytic sample of 128 women, with 548 antenatal visits after 20 weeks' gestation that included fetal biometric measures and interrogation of uterine and umbilical blood flow. Excluded participants did not significantly differ from those women enrolled later in pregnancy in age, gravidity, fetal sex, or socioeconomic status (SES), but were more likely to have a low level of education and to be undernourished (data not shown). Participants in the analytical sample were enrolled at a median of 18 weeks' gestation (interquartile range [IQR]: 16, 19) and were followed up for a median of 19 weeks (IQR: 17, 21). Study participants had a median of 7 follow-up visits (IQR: 6, 8), with 5 ultrasound scans (IQR: 4, 5) from enrollment to delivery.

Variables

Exposure:

Early pregnancy malaria: The primary malaria exposure variable was based on real-time quantitative (q)PCR for *P. falciparum* malaria from peripheral dried blood samples. The exposure variable, 'early pregnancy malaria,' was a binary, time-independent measure representing whether a woman was ever qPCR positive for peripheral *P. falciparum* malaria parasitemia at ≤20 weeks' gestation. Early pregnancy malaria was treated dichotomously: 0 (no early pregnancy malaria) and 1 (early pregnancy malaria).

Outcomes:

Uterine artery resistance index and umbilical artery resistance index were defined as described in Aim 1. In addition, we examined subsequent intrauterine growth restriction.

Intrauterine growth restriction: IUGR was defined as a time-varying binary outcome of <10th percentile of SEFW for attained gestational age using the longitudinally derived sex-specific Johnsen fetal standard curve (Johnsen, Rasmussen et al. 2006). IUGR was coded as yes (1) or no (0) at each ultrasound visit. SEFW in grams, was calculated according to the Hadlock algorithm: log(EFW)=1.3596 - 0.00386*AC*FL + 0.0064*HC + 0.00061*BPD*AC + 0.0424*AC + 0.174*FL (Hadlock, Harrist et al. 1985). Hadlock's formula is among those with the highest accuracy and precision in several recent reviews of the algorithms for estimated fetal weight (Kurmanavicius, Burkhardt et al. 2004; Dudley 2005; Anderson, Jolley et al. 2007). At each visit, women were classified as having an IUGR episode if, at that visit, their fetus was <10th percentile of SEFW for gestational age in completed weeks, using the longitudinal, sex-specific Johnsen SEFW nomogram (Johnsen, Rasmussen et al. 2006). We also examined repeated episodes of sonographically-identified IUGR. "Repeat IUGR" was a binary,

time-dependent outcome defined as IUGR (as previously defined), with ≥ 2 total IUGR episodes during pregnancy.

Time metric:

As in Aim 1, the continuous time metric, gestational age in weeks, was back-calculated from the first ultrasound using Hadlock's algorithm (Hadlock, Deter et al. 1984).

Effect measure modifiers:

Hypothesized effect measure modifiers were the same as those described in Aim 1.

Covariates:

Covariates assessed as potential confounders in the models were identical to Aim 1.

Data analysis

The data analysis plan for Specific Aim 2 was identical to the plan for Specific Aim 1 for continuous variables. As in Aim 1, we utilized linear mixed-effect models. Here, LME models were used to estimate the effect of early pregnancy malaria on UtA RI and UA RI. However, for Aim 2, we utilized a log-binomial generalized estimating equation (GEE) regression model (Liang and Zeger 1986) with an exchangeable correlation matrix for the binary outcome, intrauterine growth restriction. We estimated the unadjusted and adjusted risk ratios for IUGR with corresponding 95% confidence intervals. Like LME models, GEE models are appropriate for repeated measures

outcomes as they account for correlation between repeated measures (Liang and Zeger 1986).

For the log-binomial GEE model for IUGR, we began with crude models separately fitted for the early pregnancy malaria exposure variable and theorized effect measure modifiers. Next, we constructed simple log-binomial models with early pregnancy malaria, a theorized modifier, and their product interaction term. We examined risk ratios for the effect of early pregnancy malaria on IUGR within strata of the modifying variable. If the product interaction term was significant using the Wald test (a priori cut-off of 0.10), it was considered an effect measure modifier and was included in a multivariable model.

Multivariable model building occurred in several steps. We began with a fully adjusted log-binomial model, including the early pregnancy malaria exposure, the exposure modifier product interaction terms and covariates that were DAG confounders of the association between malaria and IUGR. Potential confounders were identified through a review of the literature and through analysis via a DAG for the model (Greenland, Pearl et al. 1999; Rothman, Greenland et al. 2008), and included maternal age, socioeconomic status, and education level (Figure 3.5). EMMs that were not found to modify the exposure outcome were evaluated as confounders. Covariates were considered confounders and not retained in the model if the change in the DAG model risk ratio was <10% within strata of EMMs (Rothman, Greenland et al. 2008).

Finally, we estimated the adjusted risk ratios. We reported the unadjusted and adjusted risk ratios for the effect of early pregnancy malaria on IUGR with corresponding 95% confidence intervals. The risk ratios for the final model can be interpreted as the risk of IUGR in women with early pregnancy malaria compared to women without early pregnancy malaria. All analyses were performed using SAS software (SAS, Cary, NC).

Sensitivity analyses

We performed sensitivity analyses to examine the robustness of model estimates to changes in model parameters. Results for all sensitivity analyses can be found in Appendix 2. In our first set of sensitivity analyses for LME models, we determined the robustness of model estimates to the definition of our time metric, ultrasound estimated gestational age. For all LME models, we examined the effect of using LMP estimated gestational age (S1) and we restricted the analysis to the subset of women with 'certain' gestational age (i.e. LMP within ±14 days of the ultrasound derived date) (n=145, with 608 study visits) (S2). For the uterine artery resistance LME model, we examined using the highest resistance index of the two uterine arteries as our outcome variable (S3). We also examined the use of a previously recommended cut-offs for maternal under nutrition (i.e. baseline MUAC <23 cm (S4)). Sensitivity analyses for umbilical artery resistance and uterine artery resistance can be found in Figures A2.1 and 2.2, respectively.

For the estimation of sonographically estimated fetal weight, we examined how robust our models were to changes to the algorithm for sonographically estimated fetal weight. We examined the association between early pregnancy malaria and SEFW estimated by algorithms developed by Combs (Combs, Jaekle et al. 1993) and Woo (Woo, Wan et al. 1985). Changes to the algorithms gave different estimates for SEFW, but did not have a noteworthy effect on LME model estimates for the effect of early pregnancy malaria on EFW (data not shown).

To determine how robust our IUGR findings were to our choice of fetal standard curve, we examined the use of two additional ultrasound-derived fetal standard curves in a sensitivity analyses. First, we considered Hadlock's fetal standard curve (Hadlock, Harrist et al. 1985). Hadlock's curve provides good fit to Project ECHO data at the 10th percentile (Landis 2007); is not sex-specific; and, was previously used in an analysis of

IUGR in Project ECHO data (Landis, Lokomba et al. 2009). We also considered

Gallivan's fetal standard curve. Gallivan's curve is prospective and is not sex-specific

(Gallivan, Robson et al. 1993). Results for sensitivity analyses for early pregnancy

malaria and IUGR can be seen in Appendix 2, Figure A2.3. Characteristics of the fetal

standard curves utilized can be seen in Appendix 2, Table A2.1.

Approach to Missing Data

Missing data and loss to follow-up were minimal in Project ECHO. Study

protocols were in place should a woman miss a scheduled appointment or deliver in a

different hospital. Two women were lost to follow-up during the study, including one

maternal death and one woman who moved out of the study area. Of 182 women,

delivery data are missing for one maternal death and for one woman who completed all

study visits, but couldn't be located for delivery (1% or 2/182). There are partial delivery

data for 10 women (6% or 10/180) who did not delivery at Binza Maternity Hospital, with

no infant size measurements. The proportion of missing data for predictors ranges from

0% for time-invariant measurements to <1% for fetal biometric measurements to

approximately 6% for uterine artery Doppler measures. The probability of missingness in

Project ECHO data is likely unrelated to unobserved concurrent outcomes (UtA RI, UA

RI, SEFW and IUGR), conditional on all observed outcomes. Thus, we expect Project

ECHO data to adhere to the "missing at random" (MAR) assumption for Aims 1 and 2,

providing unbiased results, and negligible losses in precision. In LME models (Aims 1 &

2), model estimates have been shown to be relatively robust to small deviations from the

assumption of MAR (Singer and Willett 2003). For the binary outcome variable IUGR

(Aim 2), the very low level of missing data (<1%) is unlikely to significantly bias study

results.

Strengths and Limitations

Project ECHO data uniquely provide the ability to examine several critical gaps in the field of malaria and pregnancy, namely, the relationship between the timing of malaria episodes and both placental circulations and *in utero* fetal size. However, there are several factors that limit the validity or generalizability of these data. These analyses focus on women in urban Kinshasa, receiving antenatal care, and enrolled in a prospective cohort. Enrolled women received two doses of IPT and were treated at monthly or interim visits for malaria. Thus, our findings may not be directly applicable to rural women receiving lower levels of antenatal care; who do not receive IPT; and, who are not actively diagnosed and treated for malaria infection. There were several unmeasured variables that may confound the relationship between malaria and study outcomes, including: time elapsed between pregnancy for multigravidae and possibly sickle-cell trait. Additionally, accurate measures of maternal weight were not available for all time points, so our ability to use maternal weight as a time-varying covariate was limited.

There may be important systematic and random error in estimates of gestational age and fetal weight. While bias and random error are inherent in regression equations, we have attempted to select commonly used formulae, with low levels of bias and high levels of precision. We utilized a longitudinally derived fetal growth curve from an industrialized country setting that distinguished between fetal genders for the identification of IUGR. The selection of an appropriate nomogram can affect the proportion of IUGR fetuses identified in a population (Appendix 2, Table 2.1). As such, we felt a sensitivity analysis of study findings using a different fetal growth curves would be useful.

We had limited power to detect differences. This study was initially designed as a pilot study to prepare laboratory, ultrasound and clinical operating procedures for a larger subsequent trial. Thus the sample size was selected for convenience, rather than to maximize power. Power may be particularly limited for the analyses of binary outcomes (Specific Aim 2). However, by coding outcome variables continuously for Aims 1 & 2, we attempted to increase power to detect reasonably small differences.

There are also important strengths of these data, including the prospective nature of the data, by which we have the ability to infer causality to our findings. We had reasonable reliable and precise estimates of gestational age, which have historically been a challenge in malaria endemic areas. Most critically to our analyses, we have prospective serial measures of Doppler fetal biometrics and EFW; blood flow velocity waveforms; and incident malaria parasitemia. While qPCR is considered a highly sensitive method to identify malaria, the test characteristics of qPCR have not been compared to the gold standard of placental histology. Positive qPCR malaria results that were discordant from microscopy were rerun; methods were used to prevent and detect contamination issues. Finally, we had very low levels of missing data.

Figure 3.1 Recruitment results for longitudinal Doppler ultrasound study. Kinshasa,

Democratic Republic of Congo, 2005-2006
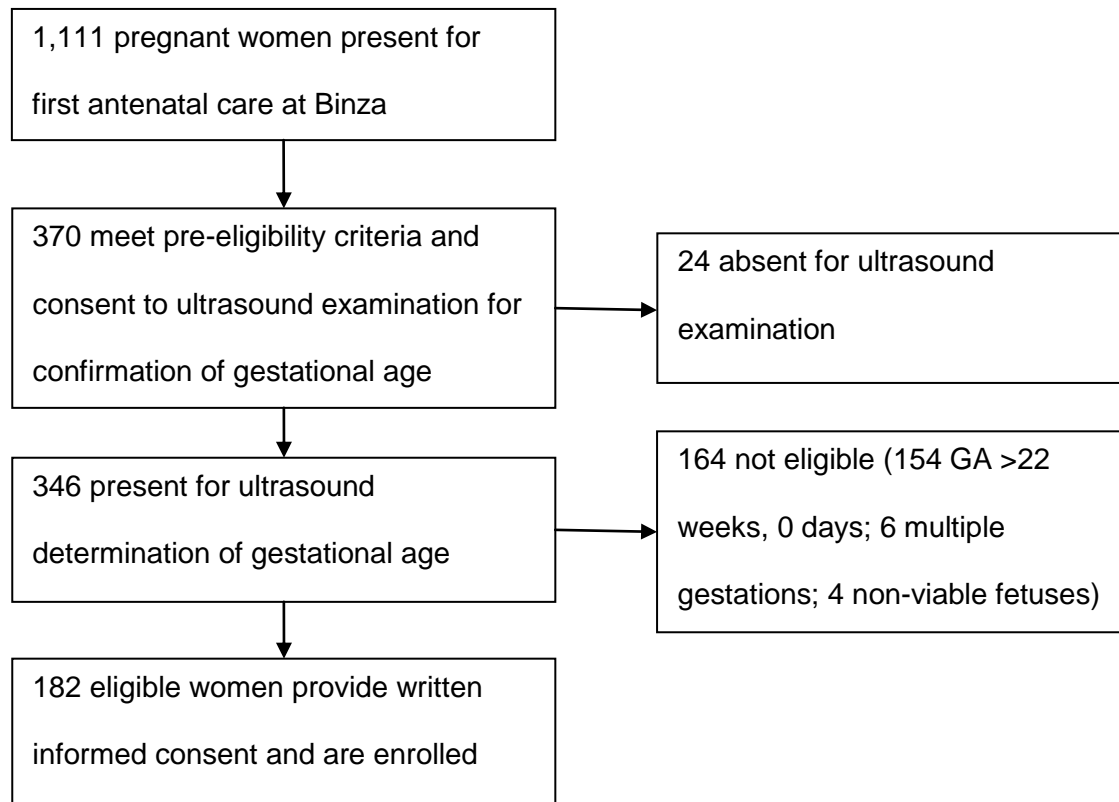
Figure 3.2 Results of follow-up and delivery for longitudinal Doppler ultrasound study.

Kinshasa, Democratic Republic of Congo, 2005-2006.

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┌─────────────────────────────┐
│ Baseline visit (N=182)      │
└─────────────────────────────┘
            │
            ▼
┌─────────────────────────────┐        ┌──────────────────────────────────────────┐
│ Follow-up visit 1 (N=182)   │───────▶│ 1 maternal death; 1 loss to follow-up      │
└─────────────────────────────┘        └──────────────────────────────────────────┘
            │
            ▼
┌─────────────────────────────┐        ┌──────────────────────────────────────────┐
│ Follow-up visit 2 (N=180)   │        │ 10 term births; 4 preterm deliveries; 3    │
└─────────────────────────────┘        │                                            │
            │                          │ preterm / stillbirth; 1 preterm / neonatal │
            ▼                          │                                            │
┌─────────────────────────────┐        │ death; 1 loss to follow-up                 │
│ Follow-up visit 3 (N=180)   │───────▶│                                            │
└─────────────────────────────┘        └──────────────────────────────────────────┘
            │
            ▼
┌─────────────────────────────┐        ┌──────────────────────────────────────────┐
│ Follow-up visit 4 (N=161)   │───────▶│ 85 term births; 2 preterm deliveries       │
└─────────────────────────────┘        └──────────────────────────────────────────┘
            │
            ▼
┌─────────────────────────────┐        ┌──────────────────────────────────────────┐
│ Follow-up visit 5 (N=74)    │───────▶│ 54 term births; 1 preterm delivery         │
└─────────────────────────────┘        └──────────────────────────────────────────┘
            │
            ▼
┌─────────────────────────────┐        ┌──────────────────────────────────────────┐
│ Follow-up visit 6 (N=19)    │───────▶│ 18 term births; 1 preterm delivery         │
└─────────────────────────────┘        └──────────────────────────────────────────┘
```
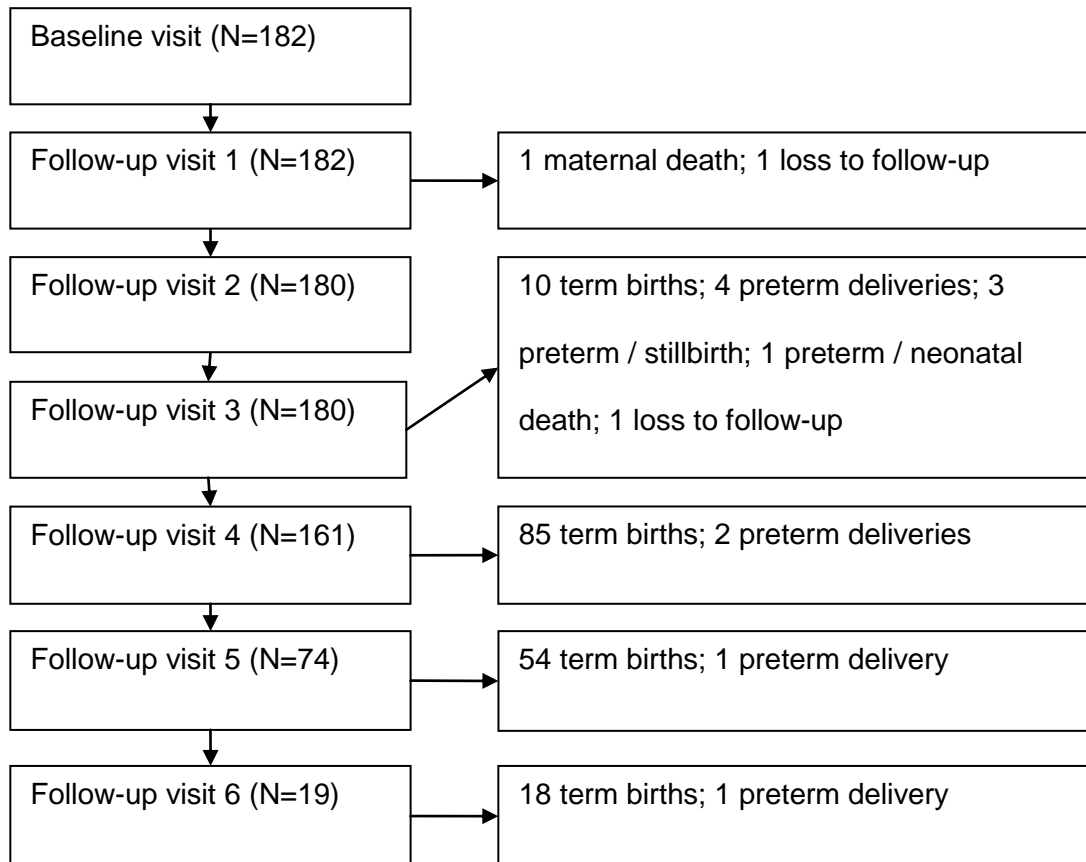
Table 3.1 Summary of ultrasound, clinical and laboratory measurements. Democratic

Republic of Congo, 2005-2006. From (Landis 2007)

Visit	Measurements	
Baseline	Clinical history	Filter paper sample
	Sociodemographics	1st dose of intermittent
	Last menstrual period	preventive treatment
	HIV testing and counseling	Height, weight, mid-upper arm
	Ultrasound examination	circumference
	Fundal height	BP, pulse
	Peripheral parasitemia and	Hematocrit
	assessment for fever	Urine test
		Assessment for edema
Follow-up	Ultrasound examination	Weight, mid-upper arm
(monthly)	Fundal height	circumference
	Peripheral parasitemia and	BP/pulse
	assessment for fever	Hematocrit[a]
	Filter paper sample	Urine test
	2nd dose of intermittent	Assessment for edema
	preventive treatment	
Delivery	Women:	Infants:
	Peripheral parasitemia	Birth weight
	Filter paper sample	Length (crown-heel, crown-
	Placental biopsy	rump)
	Hematocrit test	Head circumference
	Maternal mortality	Abdominal circumference
		Gestational age
		Infant mortality

a. Hematocrit tests were performed at every other follow-up visit

Figure 3.3 Simplified directed acyclic graph for malaria and uterine artery resistance

index (UtA RI) and umbilical artery resistance index (UA RI) (Aim 1).
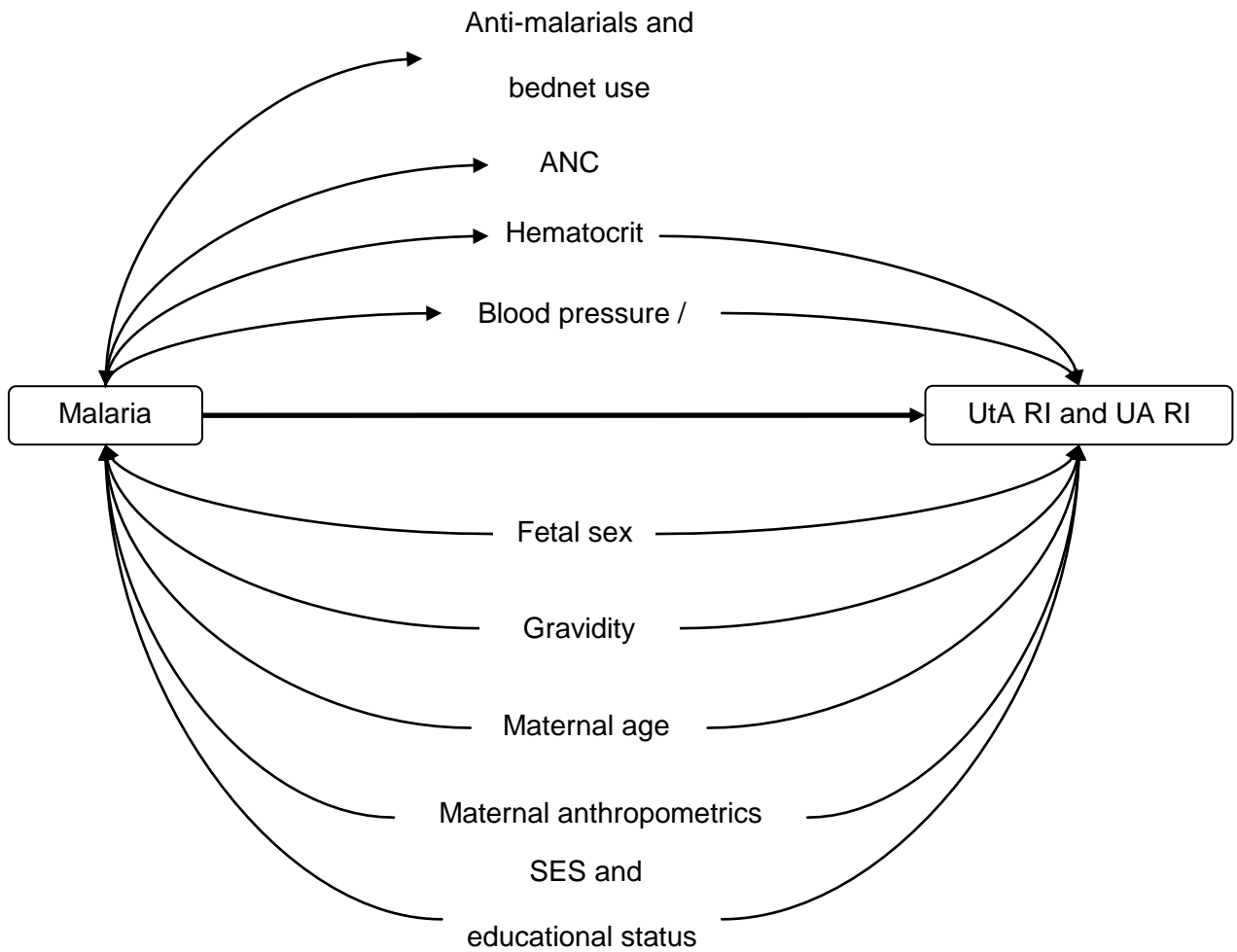
Figure 3.4 Timeline for key pregnancy events and study variables. Black arrow denotes gestational age in weeks. The temporal relationships between key events during pregnancy and peak malaria prevalence are shown (above black arrow). The temporal relationships between early pregnancy malaria exposure, subsequent placental blood flow and fetal growth outcomes, and typical IPTp dosing are shown (below black arrow).
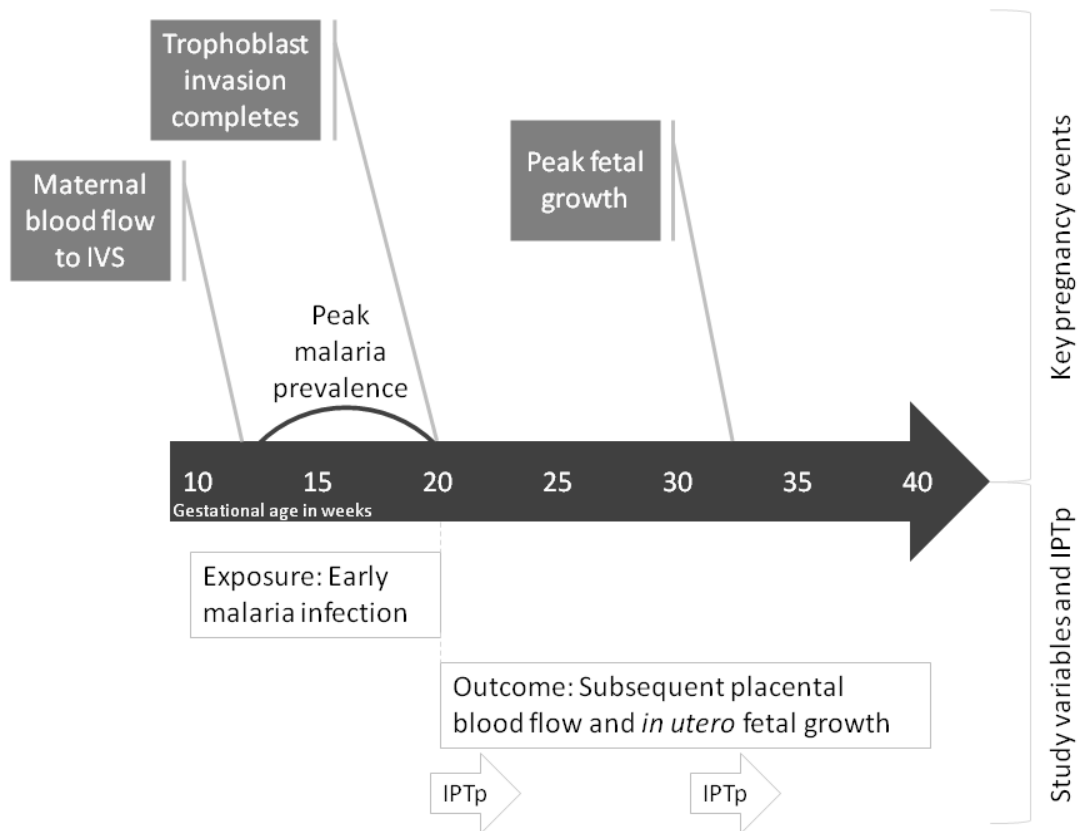
Figure 3.5 Simplified directed acyclic graph for malaria and fetal growth outcomes, estimated fetal weight and intrauterine growth restriction (Aim 2).
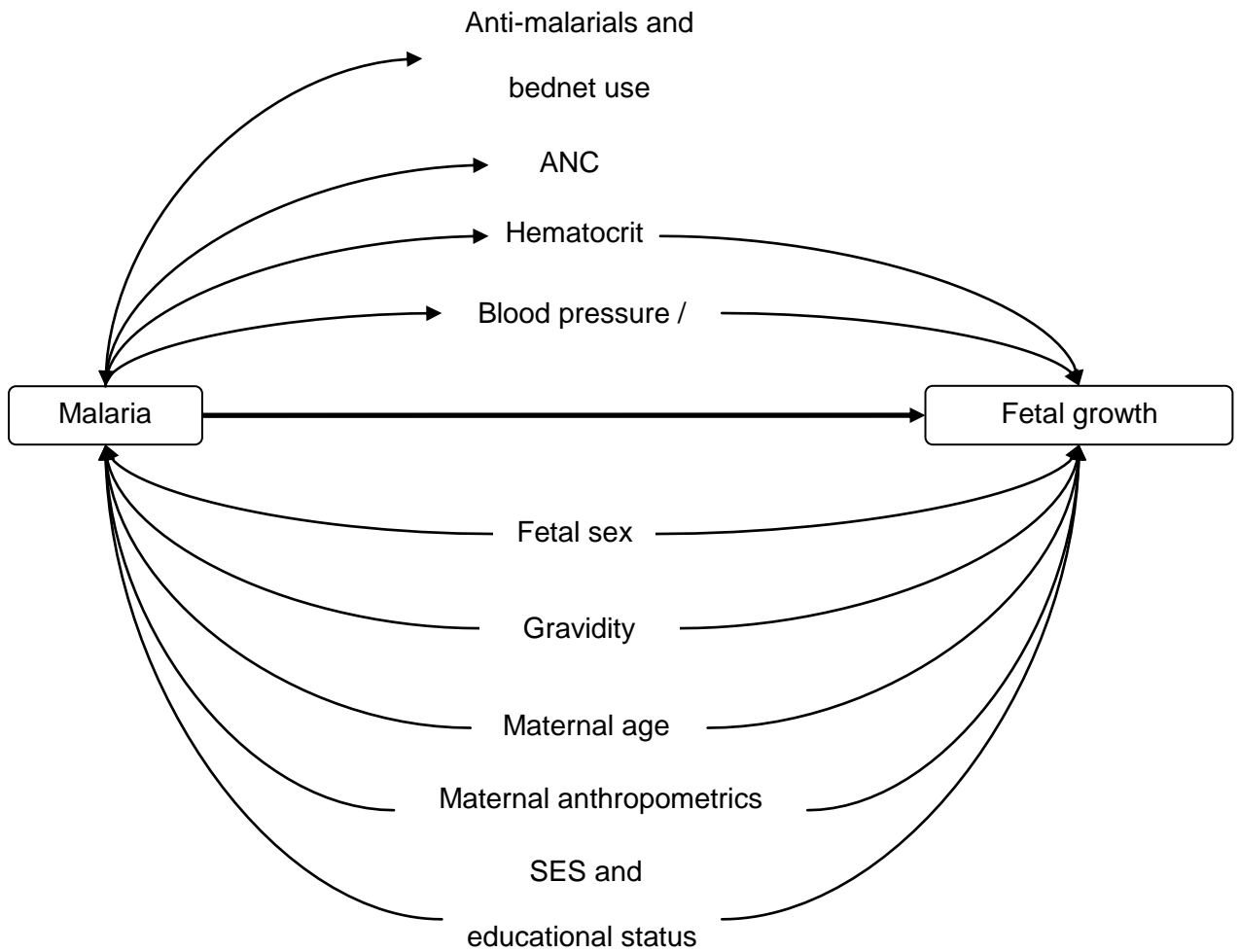
CHAPTER 4 - MALARIA IN PREGNANCY AND UTERO- AND FETOPLACENTAL BLOOD FLOW: GRAVIDITY AND FETAL SEX-SPECIFIC EFFECTS

Abstract

Malaria during pregnancy leads to low birth weight, possibly due to reduced uterine and umbilical artery blood flow. The relationship between malaria and uterine and umbilical artery blood flow has not been examined longitudinally. Malaria parasitemia was measured and Doppler ultrasound interrogated uterine and umbilical artery resistance at monthly antenatal care visits for 177 pregnant women from Democratic Republic of Congo. Women ever having high uterine artery or high umbilical artery resistance had smaller infants at delivery. Malaria reduced both uterine and umbilical artery blood flow. Compared to multigravidae with no malaria, primigravidae with malaria had an 11-13% increase in uterine artery resistance. The effect of malaria on fetoplacental blood flow was sex-specific. Compared to women with no malaria and a male fetus, women with malaria and a female fetus had a 10-14% increase in umbilical artery resistance, while women with malaria and a male fetus had a 2-3% increase. Reduced blood flow is a likely mechanism by which malaria leads to intrauterine growth restriction. The effects of malaria on fetoplacental blood flow are sex-specific, possibly via sex-specific placental cytokine responses to malaria parasitemia.

Introduction

From a public health perspective, *Plasmodium falciparum* malaria in pregnancy is among the most important preventable causes of low birth weight in sub-Saharan Africa (Kramer 1987; Steketee, Nahlen et al. 2001; Guyatt and Snow 2004). While there are multiple mechanisms by which malaria in pregnancy leads to low birth weight (Rogerson, Hviid et al. 2007), one is thought to be the reduction of utero- and fetoplacental blood flow (Arbeille, Carles et al. 1998; Dorman, Shulman et al. 2002).

Uterine artery blood flow (from the mother to the placenta) and umbilical artery blood flow (from the fetus to the placenta) increase over pregnancy to meet the metabolic demands of the growing fetus. Pathophysiological events in the placenta can decrease blood flow in the uterine or umbilical arteries, leading to a reduced capacity to transport oxygen and nutrients and, ultimately, to restricted fetal growth. Malaria in pregnancy has previously been found to be associated with abnormal utero- and fetoplacental circulation in the late second and early third trimesters (Arbeille, Carles et al. 1998; Dorman, Shulman et al. 2002).

Doppler assessment of the uterine and umbilical arteries allows for the non-invasive evaluation of blood flow and resistance in the maternal and fetal compartments of the placenta, respectively. Increased uterine artery resistance is associated with preeclampsia, restricted fetal growth and adverse pregnancy outcomes (Trudinger, Giles et al. 1985; Irion, Massé et al. 1998), while increased umbilical artery resistance is associated with fetal distress and restricted fetal growth (Giles, Trudinger et al. 1985; Trudinger, Giles et al. 1985).

To date, the effect of malaria on utero- and fetoplacental blood flow and resistance has not been examined longitudinally over pregnancy. The objective of this

longitudinal analysis was to examine the effect of malaria parasitemia on changes in the uterine artery (UtA) and umbilical artery (UA) resistance indices (RI) over time.

Methods

Study population

From May 2005 to May 2006, pregnant women seeking antenatal care at Binza Maternity Hospital were screened for pre-eligibility (n=1,111). One-third (n=370) met pre-eligibility requirements (≥ 18 years of age; not hypertensive; and <23 weeks' gestation by last menstrual period dating). Pre-eligible women had gestational age estimated via ultrasound (Hadlock, Deter et al. 1984) within 3-5 days of pre-eligibility screening to determine study eligibility. Of these, 182 women met screening criteria (healthy, singleton pregnancy ≤22 weeks' gestation by ultrasound dating); provided written informed consent; and were enrolled in the study. Enrolled women received intermittent preventive therapy with sulfadoxine-pyrimethamine twice (between 16-27 and 28-32 weeks) and an insecticide treated bed net. Five HIV-positive women were excluded from the analysis, leaving a final sample of 177 women, with a total of 1120 antenatal visits. Participants had a median of 6 visits (interquartile range: 5, 7) and 4 ultrasound scans (interquartile range: 4, 5).

This study was approved by the Institutional Review Boards of the University of North Carolina at Chapel Hill and the Kinshasa School of Public Health.

Clinical, Laboratory and Ultrasound Procedures

Data were collected at baseline in a socio-demographic interview; a medical examination (maternal anthropometrics, blood pressure, pulse, temperature); an ultrasound examination (estimation of gestational age and fetal weight); and laboratory

testing (urine protein, hematocrit, malaria thick and thin smears, and filter paper dried

blood spot samples) as previously described (Landis, Lokomba et al. 2009). At follow-up

visits, ultrasound examinations and medical and laboratory examinations (including

malaria thick and thin smears and filter paper dried blood spot samples) were repeated.

Gestational age and fetal biometrics were measured using standard procedures as

previously described (Landis, Lokomba et al. 2009). Quantitative real-time polymerase

chain reaction (qPCR) was conducted to detect all *Plasmodium* species from dried blood

spot samples and positive samples were speciated as previously described (Taylor,

Juliano et al. 2010).

Color pulsed-wave Doppler ultrasound was used to interrogate the flow velocity

waveforms in the left and right UtAs and the UA using standard techniques. The external

iliac artery and UtA located medial to it were identified using color flow settings. Flow

velocity waveforms were obtained from the UtA near the iliac vessel prior to division of

the UtA into branches. For left and right UtA analyses, Doppler indices were measured

from the Doppler signal, with qualitative assessment of early diastolic notching. For the

UA, the flow velocity waveform was obtained from the free flowing portion of the

umbilical cord and Doppler indices and presence of absent or reversed end-diastolic flow

were recorded. All ultrasound measurements were taken by a single, trained

obstetrician-gynecologist (V.L.) on the GE Logicbook Ultrasound System.

Variable definitions

The malaria exposure was a binary, time-dependent measure representing

whether a woman was qPCR positive for *Plasmodium falciparum* malaria at the visit in

which Doppler measures were taken. We utilized qPCR of peripheral dried blood spots

rather than blood microscopy due to the higher detection threshold of microscopy and

the potential for poor microscopy sensitivity and specificity (Ohrt, Obare et al. 2007). In a

secondary analysis, we examined a categorical, time-dependent malaria exposure comparing microscopic (microscopy positive, qPCR positive) and submicroscopic (microscopy negative, qPCR positive) malaria to women with no parasitemia at the visit in which Doppler measures were taken.

Outcome variables included the continuous, time-dependent mean of the left and right UtA RI and the UA RI. Commonly used Doppler indices include the resistance index, the pulsatility index and the S/D ratio. We selected the resistance index for our outcome for several reasons: RI values are constrained between 0 and 1; demonstrate the least variance of the Doppler indices under identical hemodynamic conditions; are frequently used in clinical settings; and have a truncated normal distribution, making the RI amenable to parametric statistical analyses (Maulik 2005). The RI is defined as: (peak systolic velocity – end diastolic velocity) / peak systolic velocity. We also examined the time-dependent, binary outcome unilateral UtA notching after 20 weeks' gestation. Early diastolic notching is normal in early pregnancy, but in later pregnancy reflects extreme resistance to uterine artery flow.

The time metric, gestational age in weeks, was back-calculated from the first ultrasound using Hadlock's formula (Hadlock, Deter et al. 1984).

Hypothesized effect measure modifiers were binary, time-independent variables and included: primigravidae (vs multigravidae); female fetal sex (vs male); and low baseline mid-upper arm circumference (MUAC) (vs 'normal' MUAC). MUAC is an indicator of maternal nutritional status that is relatively unaffected by gestational age and is a proxy for pre-pregnancy weight (Krasovec and Anderson 1991). Women with baseline MUAC in the lowest quartile (<24.5 cm) were classified as 'Low MUAC.' Potential confounders were identified in the literature and analyzed via a directed acyclic graph. Confounders included maternal age (18-24, ≥30 years vs. 25-29 years), education level (<secondary vs. ≥secondary); and socioeconomic status (SES) (low vs.

high). 'Low SES' was defined as a binary composite variable, with women meeting all of the following criteria categorized as 'low SES': currently unemployed, living in a home without toilet facilities, no access to a nearby water source, and no electricity.

We also examined UtA RI and UA RI as binary, time-independent predictors of perinatal outcomes, in which women who ever experienced UtA RI or UA RI >90th percentile for gestational age were defined as 'ever high' UtA RI or UA RI, respectively (Merz and Bahlmann 2005). We examined the time-independent binary outcomes LBW (<2500 grams), small for gestational age (<10th centile of Wilcox standard curve for attained gestational age) (Wilcox, Gardosi et al. 1993), and preterm (<37 weeks' gestation). We examined the continuous, time-independent outcomes: gestational age at delivery (days), birthweight (grams), head circumference (cm), abdominal circumference (cm), and crown-rump length (cm), which were measured using standard techniques, as previously described (Landis 2007).

Statistical analyses

We described the baseline characteristics of study population and fitted linear mixed effect (LME) models to estimate simple mean differences and corresponding 95% confidence intervals (CIs) in UtA RI and UA RI for covariates, adjusting for gestational age (Laird and Ware 1982). We explored the change of UtA RI and UA RI over time by plotting individual growth trajectories with population average smoothed trend line. We utilized LME models to estimate the unadjusted and adjusted mean differences in UtA RI and UA RI with corresponding 95% CIs. LME models are flexible regression tools that account for the correlation between repeated measures in an individual (Laird and Ware 1982). We plotted population average growth curves to describe the unadjusted and adjusted effect of malaria on utero- and fetoplacental blood flow. We estimated unadjusted risk ratios (RRs) and 95% CIs for binary perinatal outcomes using log-

binomial regression and estimated unadjusted mean differences and 95% CIs for continuous perinatal outcomes using linear regression.

For multivariable modeling of the effect of malaria on UtA RI and UA RI, we began with a fully adjusted LME model with a random intercept. Linearity between gestational age and the outcome variables was considering by using -2 log-likelihood (-2LL) tests for nested models using maximum likelihood estimation (a priori cutoff of $P <$ 0.10). We tested the addition of additional random effects using -2LL tests with a mixed chi-square distribution (a priori cutoff of $P < 0.05$). In the final model we included random intercepts and slopes for each woman. We used backward elimination to assess effect measure modification and confounding. We used -2LL tests to evaluate the contribution of product interaction terms for malaria and potential modifiers (gestational age, gravidity, fetal sex, and maternal MUAC) to the models (a priori cutoff of $P < 0.10$). Potential modifiers that did not modify the association between malaria and the outcome were assessed as potential confounders. Covariates were considered confounders if the change in the coefficient of the main exposure was greater than 10%, within strata of modifiers. Restricted maximum likelihood was used for final model estimates, as is common practice (Fitzmaurice 2009). Following the specification of the final model, we examined the unadjusted effect of malaria on the outcomes. All analyses were performed using SAS software (SAS, Cary, NC).

Results

Maternal characteristics and uteroplacental blood flow (Table 4.1)

The median age of the 177 pregnant women was 27 years (interquartile range: 23, 31) and approximately one-quarter of participants were primigravidae. Greater than half of pregnancies were with a female fetus. Most women had low socioeconomic

status (86%), and half had a low level of education. One-quarter of women met our definition as having a low MUAC. Uterine artery resistance was elevated in women 30 years of age and older and in women with a female fetus. Umbilical artery resistance was elevated in primigravidae, women with a female fetus, and women with a low level of education.

The 177 women were enrolled at a median gestational age of 19 weeks (interquartile range: 16, 21) and were followed for a median of 18 weeks' (interquartile range: 16, 20). During this time, thirty percent of women had malaria parasitemia, with the prevalence of parasitemia falling during pregnancy from 20% during the early second trimester to 8% during the late third trimester. Fewer than 10% (93/1105) of antenatal visits were positive for malaria parasitemia. Women with less than a secondary level of education had an increased risk of ever having malaria parasitemia during follow-up (risk ratio [RR]: 1.7; 95% CI: 1.0, 2.7) compared to women with a secondary level of education or higher. Maternal age, gravidity, fetal sex, low SES, and having low MUAC at baseline were not significantly associated with risk of ever having malaria parasitemia during follow-up.

Utero- and fetoplacental blood flow (Figure 4.1)

We plotted individual trajectories of the continuous outcomes, UtA RI and UA RI, in order to examine the intra- and inter-subject variability over gestational age (Figure 4.1). The steeper negative change in UtA RI until approximately 26 weeks' gestation (Panel A) reflects trophoblast invasion and remodeling of the spiral arterioles. After 20 weeks' gestation, there were 13 episodes of unilateral notching and 2 episodes of bilateral notching of the uterine arteries among 12 women. UA RI began higher and had a steeper downward slope over the course of pregnancy (Panel B), due to angiogenesis in the villous structure of the placenta. In the most extreme cases, absent or reversed

end-diastolic flow may be seen in the umbilical artery. This phenomenon was uncommon in this cohort, with 4 episodes occurring after 20 weeks' gestation.

High utero- and fetoplacental blood and perinatal outcomes (Table 4.2)

Forty-one percent of women ever had a high UtA RI and 41% ever had a high UA RI. UtA RI and UA RI were not strongly associated (Prevalence ratio: 1.2; 95% CI: 0.9, 1.8). Women with high UtA RI or UA RI had neonates with increased risk of small for gestational age, while neonates with high UA RI were also had increased risk of low birth weight, although this finding was not statistically significant. Compared to neonates with normal UtA RI, neonates with high UtA RI were smaller, with decreased birth weight, crown-rump length and abdominal circumference. There was also a trend toward decreased head circumference. Neonates with high UA RI had decreased birth weight, decreased abdominal circumference and crown-rump length. There was no association between high UA RI and head circumference. Neither gestational age at delivery nor preterm delivery was associated with high Doppler indices.

Malaria and uteroplacental blood flow (Figure 4.2)

There was no crude effect of malaria on uterine artery resistance. However, in the adjusted model, the effect of malaria on UtA RI varied by gravidity. Compared to the referent group of multigravidae with no malaria, the mean difference in UtA RI for malaria was -0.0073 (95% CI: -0.033, 0.018) and for primigravidae was -0.0034 (95% CI: -0.028, 0.021). The joint effect of malaria and primigravidae was 0.063 (95% CI: -0.0088, 0.13), or approximately 0.7 standard deviations. In other words, compared to the referent group, malaria increased UtA RI by 11-13% among primigravidae, but malaria did not increase UtA RI among multigravidae. We did not find a crude association between malaria and uterine artery notching after 20 weeks' gestation (p = 0.9), though this has

been previously reported in primigravidae (Dorman, Shulman et al. 2002). Fetal sex and maternal nutritional status did not modify the relationship between malaria and UtA RI.

Malaria and fetoplacental blood flow (Figure 4.3)

In the unadjusted model, malaria caused a 0.032 increase in UA RI (95% CI: 0.010, 0.055), compared to the referent of no malaria. In the adjusted model, the effect of malaria on UA RI was found to vary by fetal sex. Compared to the referent group of male fetuses with no malaria, the mean difference in UA RI for malaria was 0.016 (95% CI: -0.019, 0.050) and for female fetal sex was 0.021 (95% CI: 0.0065, 0.035). The joint mean difference in UA RI for women with current malaria and a female fetus was 0.076 (95% CI: 0.044, 0.11), or approximately four-fifths of a standard deviation. These findings signify a 10-14% increase in UA RI among women with malaria and a female fetus, and a 2-3% increase in UA RI among women with malaria and a male fetus, compared to the referent group of women with no malaria and a male fetus. We did not find that gravidity or maternal nutritional status modified the relationship between malaria and UA RI.

Discussion

In this longitudinal, pulsed-wave Doppler study, we report that malaria decreases utero- and fetoplacental blood flow. To our knowledge, our results are the first to identify that fetal sex modifies the pathological effects of malaria in pregnancy, with malaria significantly increasing umbilical artery resistance among female fetuses only. As expected (Giles, Trudinger et al. 1985; Trudinger, Giles et al. 1985; Irion, Massé et al. 1998), high UtA RI and UA RI were associated with decreases in fetal growth.

Malaria caused an acute increase in uterine artery resistance among primigravidae, as previously reported (Dorman, Shulman et al. 2002). We did not find increased uterine artery resistance among multigravidae. This is consistent with observations showing worse malaria-associated birth outcomes among primigravidae in areas of stable transmission (Desai, ter Kuile et al. 2007). Uterine artery blood flow to the placenta could be slowed by infected erythrocytes and inflammatory infiltrates in the intervillous spaces of the placenta, which can be massive, especially among primigravidae (Ordi, Ismail et al. 1998; Ordi, Menendez et al. 2001). In our cohort, microscopic, but not submicroscopic, parasitemia was associated with increased uterine artery resistance among primigravidae. Peripheral parasitemia detectable by microscopy has been found to be associated with higher densities of placental infection (Mockenhaupt, Ulmen et al. 2002). Together these findings suggest that mechanical obstruction of blood flow via the accumulation of infected erythrocytes and inflammatory infiltrates in the intervillous spaces of the placenta leads to increased uterine artery resistance.

We report that the pathological effects of malaria during pregnancy are modified by fetal sex, with malaria significantly increasing umbilical artery resistance among female fetuses only. Studies of malaria in pregnancy from the 1960s noted an increased prevalence of placental malaria in female fetuses (McLaren and Ward 1962; Jelliffe 1968), although this direction of research appears to have been abandoned. Today, there is growing evidence for sex-specific adaptive mechanisms in adverse placental environments, such as maternal asthma (Murphy, Gibson et al. 2003; Scott, Hodyl et al. 2009), pre-eclampsia (Stark, Dierkx et al. 2006; Stark, Clifton et al. 2009), and preterm delivery (Stark, Clifton et al. 2008).

Malaria during pregnancy may produce differential effects on fetoplacental blood flow via sex-specific immune responses, such as increased inflammatory responses

among female fetuses (Scott, Hodyl et al. 2009). During placental malaria, there is increased expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-8, which are also associated with low birth weight (Fried, Muga et al. 1998; Moormann, Sullivan et al. 1999; Abrams, Brown et al. 2003). IL-8 is associated with decreased umbilical artery blood flow (Trudinger, Wang et al. 2002; Wang, Athayde et al. 2003) and has been shown to be expressed in greater concentrations (in addition to other pro-inflammatory cytokines) in female placentas compared to male placentas with asthma (Scott, Hodyl et al. 2009). There may also be sex-specific responses along the hypothalamic-pituitary-adrenal axis and/or the insulin-like growth factor (IGF) axis, both previously shown to be associated with placental malaria (Umbers, Boeuf et al. ; Bouyou-Akotet, Kombila et al. 2004). Compared to male fetuses in pregnancies complicated by asthma, female fetuses have decreased growth, with increased cortisol levels and decreased cortisol metabolism (Stark, Wright et al. 2009). Female fetuses exposed to maternal asthma and cigarette use have decreased IGF-1, while males fetuses exposed to maternal asthma have increased IGF-1 (Clifton, Hodyl et al.). More research is needed to understand the modifying role of fetal sex in the pathology of placental malaria.

The antenatal diagnosis of placental malaria is problematic as the assessment of placental malaria is unavailable prior to delivery and placental infection can occur in the absence of peripheral infection (Rogerson, Chaluluka et al. 2000). At delivery, PCR has been found to be highly sensitive for placental malaria identified by blood smear microscopy (Malhotra, Dent et al. 2005; Mockenhaupt, Bedu-Addo et al. 2006); however, specificity may be lower (Mockenhaupt, Ulmen et al. 2002). To date, evidence regarding the clinical relevance of submicroscopic infections has been inconsistent, with submicroscopic infections predictive of poor birth outcomes in some studies (Malhotra,

Dent et al. 2005), but not others (Mankhambo, Kanjala et al. 2002; Mockenhaupt, Ulmen et al. 2002).

Malaria reduces blood flow from the mother to the placenta and from the fetus to the placenta from the early second trimester until delivery. Reduced utero- and fetoplacental blood flow is a likely mechanism by which malaria leads to intrauterine growth restriction. Adequate malaria prevention and control measures should be implemented to prevent the adverse effects of malaria during pregnancy. The effects of malaria on fetoplacental blood flow are sex-specific, possibly via sex-specific placental cytokine responses to malaria parasitemia. Research should attempt to clarify the sex-specific pathological effects of malaria in pregnancy.

Table 4.1 Characteristics of pregnant women, with estimated mean differences and 95% confidence intervals (CI) in uterine artery resistance index (UtA RI) and umbilical artery resistance index (UA RI). Democratic Republic of Congo, 2005-2006.

Characteristic		n (%)	UtA RI[a] Mean difference	(95% CI)	P	UA RI[b] Mean difference	(95% CI)	P
Maternal age	≥30	64 (36)	0.011	(-0.012, 0.034)	0.35	-0.0015	(-0.019, 0.016)	0.86
	25-29	57 (32)	Ref.	-		Ref.	-	
	<25	56 (32)	-0.018	(-0.042, 0.0057)	0.16	0.00050	(-0.018, 0.019)	0.96
Primigravidae	Yes	46 (26)	-0.0078	(-0.030, 0.014)	0.48	0.026	(0.010, 0.42)	0.002
	No	131 (74)	Ref.	-		Ref.	-	
Fetal sex	Female	81 (53)	0.024	(0.005, 0.43)	0.01	0.022	(0.008, 0.036)	0.003
	Male	93 (47)	Ref.	-		Ref.	-	
Low SES	Yes	153 (86)	-0.0087	(-0.036, 0.019)	0.53	-0.0011	(-0.022, 0.020)	0.92
	No	24 (14)	Ref.	-		Ref.	-	
Low education	Yes	88 (50)	-0.0086	(-0.028, 0.011)	0.37	-0.019	(-0.033, -0.0050	0.008
	No	89 (50)	Ref.	-		Ref.	-	
Low MUAC	Yes	45 (25)	0.00010	(-0.022, 0.022)	0.99	-0.0027	(-0.019, 0.014)	0.75
	No	132 (75)	Ref.	-		Ref.	-	

a. The standard deviation for UtA RI is 0.088, without considering repeated measures
b. The standard deviation for UA RI is 0.090, without considering repeated measures

Table 4.2 Perinatal outcomes by high uterine artery resistance index (UtA RI) and umbilical artery resistance index (UA RI)

(n=163).Democratic Republic of Congo, 2005-2006.

Perinatal outcomes	High UtA RI Ever n (%)	Never n (%)	RR (95% CI)	P	High UA RI Ever n (%)	Never n (%)	RR (95% CI)	P
Low birth weight	8/72 (11)	8/102 (8)	1.4 (0.6, 3.6)	0.46	10/72 (14)	6/102 (6)	2.4 (0.9, 6.2)	0.07
Small for gestational age	34/72 (47)	25/100 (25)	1.9 (1.2, 2.9)	0.002	34/72 (47)	25/100 (25)	1.9 (1.2, 2.9)	0.002
Preterm (<37 weeks)	5/72 (7)	6/102 (6)	1.2 (0.4, 3.8)	0.77	6/72 (8)	5/102 (5)	1.7 (0.5, 5.4)	0.36
	Mean	Mean	Difference (95% CI)	P	Mean	Mean	Difference (95% CI)	P
Gestational age at delivery (days)	274	275	-1 (-5, 2)	0.40	274	275	-1 (-3, 4)	0.80
Birth weight (grams)	2860	3069	-210 (-346, -74)	0.003	2874	3060	-185 (-322, -48)	0.009
Head circumference (cm)	33.7	34.3	-0.7 (-1.4, 0.02)	0.07	34.0	34.2	-0.2 (-0.9, 0.5)	0.54
Abdominal circumference (cm)	27.4	28.2	-0.8 (-1.5, -0.04)	0.04	27.5	28.1	-0.5 (-1.3, 0.2)	0.15
Crown-rump length (cm)	32.7	33.5	-0.8 (-1.3, -0.2)	0.009	32.9	33.4	-0.5 (-1.1, 0.04)	0.06

RR = risk ratio; CI: confidence interval

Figure 4.1 Time plots of mean uterine artery resistance index and umbilical artery resistance index against gestational age (in weeks), with population average smoothed trend line (in black). (n=177, with 765 visits) (Democratic Republic of Congo, 2005-2006. A. Uterine artery resistance index against gestational age. B. Umbilical artery resistance index against gestational age.
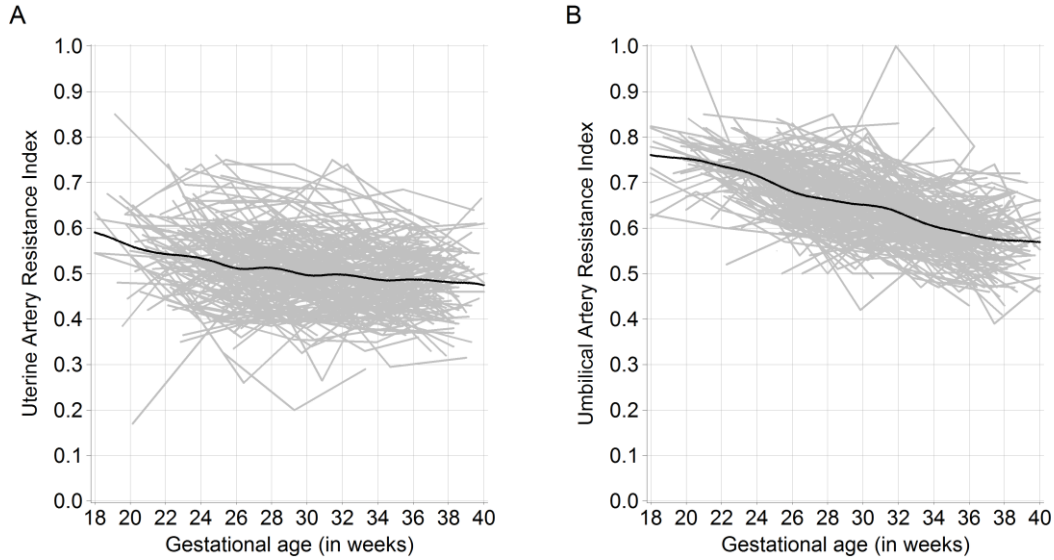
Figure 4.2 Linear mixed effect model estimated population average growth curves for the mean uterine artery resistance index by malaria against gestational age (in weeks). Democratic Republic of Congo, 2005-2006. A. The unadjusted effect of malaria on uterine artery resistance (n=759 visits). B. The adjusted effect of malaria on uterine artery resistance by gravidity. Model adjusted for maternal age and low education level; product interaction term (malaria * primigravidae) $P = 0.038$, with 1 df (n=759 visits). The standard deviation for UtA RI is 0.088, without considering repeated measures.
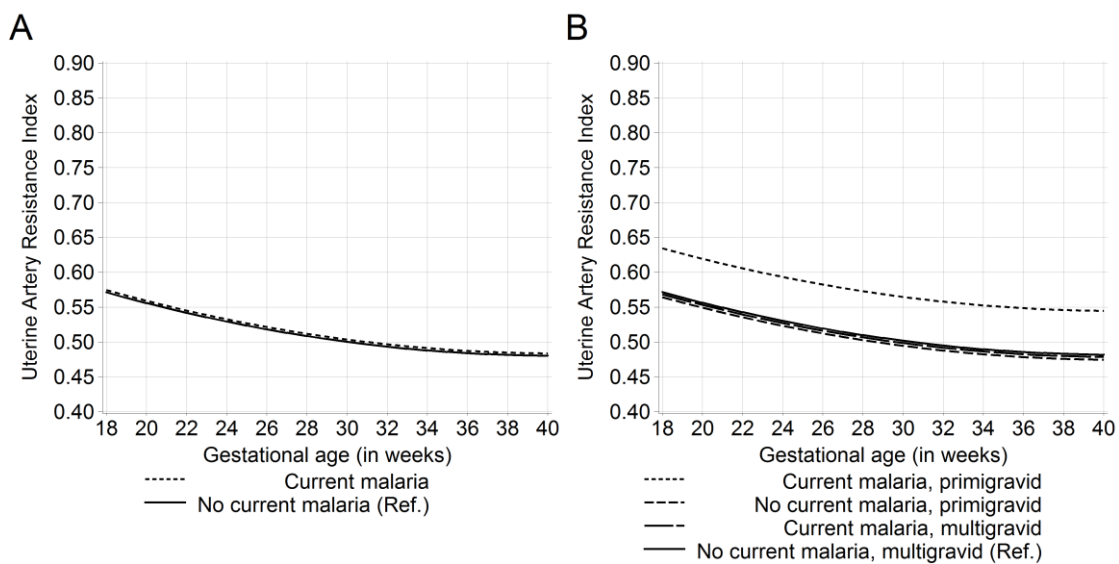
Figure 4.3 Linear mixed effect model estimated population average growth curves for the umbilical artery resistance index by malaria against gestational age (in weeks). Democratic Republic of Congo, 2005-2006. A. The unadjusted effect of malaria on umbilical artery resistance (n= 760 visits). B. The adjusted effect of malaria on umbilical artery resistance by fetal sex. Model adjusted for gravidity; product interaction term (Malaria * fetal sex) P = 0.065, with 1 df (n=755 visits). The standard deviation for UA RI is 0.090, without considering repeated measures.
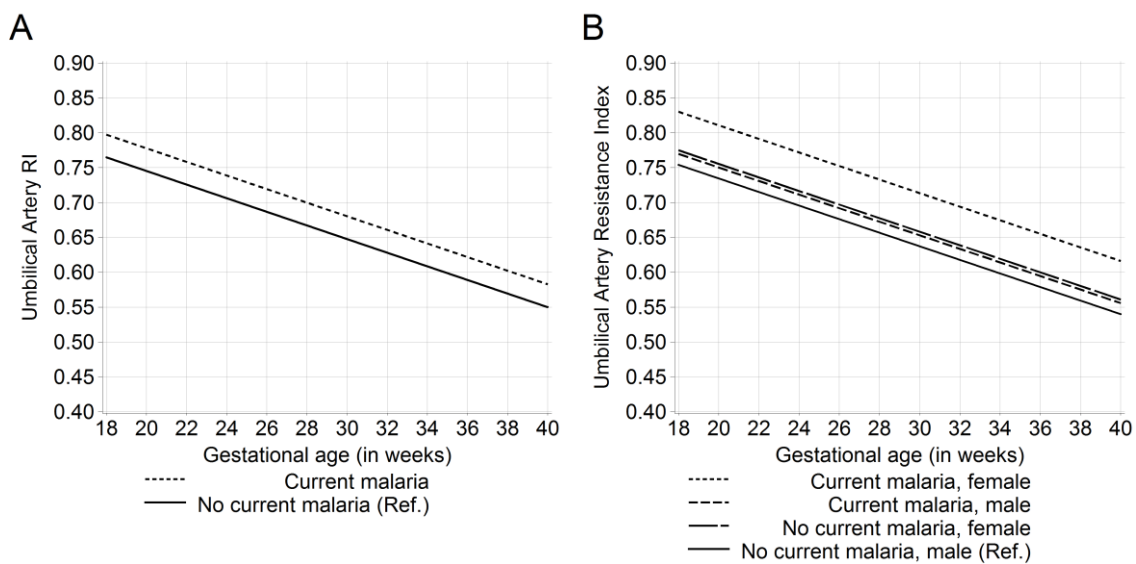
CHAPTER 5 - THE EFFECT OF MALARIA IN EARLY PREGNANCY ON
PLACENTAL BLOOD FLOW AND FETAL GROWTH: A LONGITUDINAL
ULTRASOUND STUDY FROM THE DEMOCRATIC REPUBLIC OF CONGO

Abstract

Objective: The objective of this analysis was to examine the hypothesis that early
pregnancy malaria parasitemia leads to subsequent changes in uteroplacental blood
flow and fetal growth.

Design: Data were analyzed from pregnant women from Democratic Republic of Congo
who participated in a longitudinal Doppler ultrasound study. 128 women had 548
antenatal visits after 20 weeks' gestation that included fetal biometric measures and
interrogation of uterine and umbilical blood flow.

Methods: Using linear mixed effects models, we examined the effect of early pregnancy
malaria (≤20 weeks' gestation) on subsequent changes (>20 weeks' gestation) in the
mean uterine and umbilical artery resistance indices and sonographically estimated fetal
weight. We used log-binomial models with generalized estimating equations to estimate
effect of early pregnancy malaria on the risk of subsequent IUGR.

Results: The effect of early pregnancy malaria on UtA RI varied by maternal nutritional
status Among nourished women, early pregnancy malaria decreased UtA RI (-0.036;
95% CI: -0.065, -0.0058), but among undernourished women, early pregnancy malaria
increased UtA RI (+0.022; 95% CI: -0.031, 0.074). The effect of early pregnancy malaria
on UA RI varied by gravidity. Among primigravidae, early pregnancy malaria decreased

UA RI. Primigravidae with early pregnancy malaria had 3.6 times the risk of subsequent IUGR (95% CI: 2.1, 6.2) compared to the referent group of multigravidae with no early pregnancy malaria. Early pregnancy malaria was associated with a small increased risk of IUGR among multigravidae that was not significant.

Conclusions: Early pregnancy malaria affects uterine and umbilical artery blood flow, indicating alterations in placentation and angiogenesis, respectively. Among primigravidae, early pregnancy malaria leads to decreased fetal growth and increased risk of IUGR. Our findings support the initiation of malaria prevention and control efforts earlier in pregnancy.

Introduction

In malaria endemic areas, the WHO recommends prevention and control strategies for malaria in pregnancy, including case management of malaria and anemia; insecticide-treated nets; and, at least two doses of intermittent preventive treatment (IPTp) in pregnancy with sulfadoxine-pyrimethamine after the awareness of fetal movement (WHO 2004) (from approximately 17-19 weeks' gestation (O'Dowd and O'Dowd 1985)).The peak prevalence of malaria in pregnancy occurs from 13 to 20 weeks' gestation (Brabin 1983), mostly prior to the first dose of IPTp (WHO 2004).

During this critical period of early pregnancy, the placenta develops to meet the growing metabolic demands of the fetus. Extravillous trophoblasts invade and remodel the uterine spiral arteries, increasing uterine artery blood flow to the maternal side of the placenta. Concurrently, villous angiogenesis leads to increased umbilical artery blood flow to the fetal side of the placenta.

Doppler ultrasound allows the non-invasive investigation of utero- and fetoplacental blood flow and resistance. The assessment of uterine artery blood flow reflects the extent of trophoblast invasion of the spiral arteries (Prefumo, Sebire et al. 2004; Sebire and Sepulveda 2008). Abnormal uterine artery resistance is associated with preeclampsia, IUGR and adverse pregnancy outcomes (Trudinger, Giles et al. 1985). Increased umbilical artery resistance is associated with fetal distress and IUGR (Giles, Trudinger et al. 1985; Trudinger, Giles et al. 1985). In addition, fetal biometry measurements can be used to estimate fetal weight and to identify IUGR fetuses by comparing estimated fetal weights to population growth charts.

It has been previously hypothesized that malaria in early pregnancy disrupts trophoblast invasion (Dorman, Shulman et al. 2002), leading to diminished uteroplacental blood flow and, ultimately, intrauterine growth restriction (IUGR). The

objective of this analysis was to examine the hypothesis that early pregnancy malaria leads to subsequent changes in uterine artery (UtA) and umbilical artery (UA) resistance indices (RI). We also examined the effect of early pregnancy malaria on the risk of subsequent IUGR.

Methods

Study population

The longitudinal cohort consisted of pregnant women presenting for first antenatal care (ANC) at Binza Maternity Hospital in Kinshasa, Democratic Republic of Congo (DRC) between May 2005 and May 2006, as previously described (Landis 2007; Landis, Lokomba et al. 2009). In brief, 182 pregnant women ≥ 18 years, with non-hypertensive, non-anomalous singleton pregnancies; and, ultrasound-derived gestational age of ≤22 weeks were enrolled and followed until delivery. Enrolled women were provided with ITNs and received IPTp with sulfadoxine-pyrimethamine twice during pregnancy (between 16-27 and 28-32 weeks' gestation) regardless of malaria status. All women with microscopy positive malaria were treated throughout follow-up.

Enrolled women provided written informed consent. This study was approved by the Institutional Review Boards of the University of North Carolina at Chapel Hill and the Kinshasa School of Public Health.

For the current analysis of early pregnancy malaria exposure, we excluded 5 HIV-positive participants and 49 participants with no malaria exposure data ≤ 20 weeks' gestation, leaving an analytic sample of 128 women, with 548 antenatal visits after 20 weeks' gestation that included fetal biometric measures and interrogation of uterine and umbilical blood flow. Excluded participants did not significantly differ from the analytic population in age, gravidity, fetal sex, or socioeconomic status (SES), but were more

likely to have a low level of education and to be undernourished (data not shown). Participants in the analytical sample were enrolled at a median of 18 weeks' gestation (interquartile range [IQR]: 16, 19) and were followed up for a median of 19 weeks (IQR: 17, 21). Study participants had a median of 7 follow-up visits (IQR: 6, 8), with 5 ultrasound scans (IQR: 4, 5) from enrollment to delivery.

Clinical, Laboratory, and Ultrasound Procedures

Baseline data were collected in an interview (socio-demographics, medical history); medical examination (maternal anthropometrics, blood pressure, pulse, temperature); ultrasound examination (estimation of gestational age and fetal weight); and laboratory testing (urine protein; hematocrit; malaria thick and thin smears; and, filter paper dried blood spot samples of peripheral blood) as previously described (Landis, Lokomba et al. 2009). At monthly antenatal care visits women repeated ultrasound, medical, and laboratory examinations (including malaria thick and thin smears and filter paper dried blood spot samples). Quantitative real-time polymerase chain reaction (qPCR) was conducted to detect all *Plasmodium* species from dried blood spot samples and *Plasmodium* positive samples were speciated as previously described (Taylor, Juliano et al. 2010).

Color pulsed-wave Doppler ultrasound was used to interrogate the flow velocity waveforms in the left and right UtAs and the UA using standard techniques. The external iliac artery and UtA located medial to it were identified using color flow settings. Flow velocity waveforms were obtained from the UtA near the iliac vessel prior to division of the UtA into branches. For left and right UtA analyses, Doppler indices were measured from the Doppler signal, with qualitative assessment of early diastolic notching. For the UA, the flow velocity waveform was obtained from the free flowing portion of the umbilical cord and Doppler indices and presence of absent or reversed end-diastolic flow

were recorded. All ultrasound measurements were taken by a single, trained obstetrician-gynecologist (V.L.) on the GE Logicbook Ultrasound System.

Variable definitions

The exposure variable, 'early pregnancy malaria,' was a binary, time-independent measure representing whether a woman was ever qPCR positive for peripheral *P. falciparum* malaria parasitemia ≤20 weeks' gestation. We utilized qPCR of peripheral dried blood spots rather than blood microscopy due to the higher detection threshold of microscopy and the potential for poor microscopy sensitivity and specificity (Ohrt, Obare et al. 2007).

Outcome variables for placental blood flow included the continuous, time-dependent mean of the left and right uterine artery (UtA) resistance index; and, the umbilical artery (UA) resistance index (RI) after 20 weeks' gestation. The RI is defined as: (peak systolic velocity – end diastolic velocity) / peak systolic velocity. We selected the resistance index for our outcome for several reasons: RI values are constrained between 0 and 1; demonstrate the least variance of the Doppler indices under identical hemodynamic conditions; are frequently used in clinical settings; and have a truncated normal distribution, making the RI amenable to parametric statistical analyses (Maulik 2005).

We also examined IUGR after 20 weeks' gestation. IUGR was a binary, time-dependent outcome. At each visit, women were classified as having an IUGR episode if, at that visit, their fetus was <10[th] percentile of estimated fetal weight for gestational age in completed weeks, using Hadlock's algorithm to estimate fetal weight (Hadlock, Harrist et al. 1991) and the ultrasound-derived, longitudinal, sex-specific Johnsen fetal growth standard (Johnsen, Rasmussen et al. 2006). We also examined repeated episodes of IUGR. "Repeat IUGR" was a binary, time-dependent outcome defined as IUGR (as

previously defined), with ≥ 2 total IUGR episodes during pregnancy. As this analysis was focused on IUGR, fetal weight was not considered.

The continuous time metric, gestational age in weeks, was back-calculated from the first ultrasound using Hadlock's algorithm (Hadlock, Deter et al. 1984). Ultrasound estimated gestational age has been shown to provide a better estimate of gestational age, even when last menstrual period dates are considered certain (Mongelli, Wilcox et al. 1996).

We hypothesized that the effects of early pregnancy malaria on uteroplacental blood flow and IUGR would be modified by primigravidity (vs. multigravidity), female fetal sex (vs. male), and baseline maternal mid-upper arm circumference (MUAC) ≤24.3 cm (i.e. the lowest quartile of the full study population)(vs. 'normal' MUAC). MUAC is a proxy for pre-pregnancy weight (Krasovec and Anderson 1991). Potential confounders included maternal age, education, fetal sex, and socioeconomic status (SES). 'Low SES' was defined as a binary composite variable, with unemployed women (or their partners) living in a home with few assets (no toilet, no water, no electricity) categorized as 'low SES.'

Statistical Analysis

For linear mixed effect (LME) models, we reported mean differences and plotted population average growth curves to describe the unadjusted and adjusted effect of early pregnancy malaria on UtA RI and UA RI. To describe the effect of early pregnancy malaria on IUGR, we estimated unadjusted and adjusted risk ratios (RRs) and 95% CIs using log-binomial regression generalized estimating equation (GEE) regression models with an exchangeable working correlation matrix. LME and GEE regression models are appropriate for longitudinal analyses as they account for the correlation between repeated measures in an individual (Laird and Ware 1982; Liang and Zeger 1986).

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For multivariable modeling of the effect of early pregnancy malaria on UtA RI and UA RI, we began with a fully adjusted LME model with a random intercept. Linearity between gestational age and the outcome variables was considering by examining polynomial transformations of gestational age using -2 log-likelihood (-2LL) tests for nested models using maximum likelihood [ML] estimation (a priori cutoff of $p < 0.10$). We tested the addition of additional random effects using -2LL tests with a mixed chi-square distribution (a priori cutoff of $p<0.05$). In the final UtA RI and UA RI models we included random intercepts and slopes for each woman. For log-binomial GEE models, we began with a crude model fitted for the early pregnancy malaria exposure variable. Next, we constructed simple log-binomial models with early pregnancy malaria, a theorized effect measure modifier, and their product interaction term. If the product interaction term was significant using the Wald test (a priori cut-off of 0.10), it was considered an effect measure modifier and was included in the multivariable model. For multivariable modeling, we began with a fully adjusted log-binomial model, including the early pregnancy malaria exposure, the significant product interaction terms and potential confounders of the association between malaria and IUGR. First, we utilized a backward elimination approach to assess effect measure modification. In LME models, -2LL tests were used to assess the contribution of the product interaction terms to the model using a maximum likelihood (ML) approach; in GEE models, Wald tests were used (*a priori* cutoffs of $p < 0.10$.) Potential modifiers that did not modify the association between malaria and the outcome were assessed as potential confounders. Covariates were considered confounders if the change in the coefficient of the main exposure was greater than 10%, within strata of modifiers. Restricted maximum likelihood (REML) was used for final LME model estimates, as is common practice (Fitzmaurice 2009). All analyses were performed using SAS software (SAS, Cary, NC).

Results

Baseline characteristics of the study population (Table 5.1)

Among the analytical sample of 128 pregnant women, 30% were ever qPCR positive for malaria and 21% had early pregnancy malaria. The mean age of the study population was 27.6 years (SD: 5.1; range 18-42). Approximately one-quarter of women were primigravidae and one in five women had a low baseline MUAC. More than half of women were pregnant with a female fetus. Women with and without early pregnancy malaria had approximately balanced gravidity, fetal sex, SES and low baseline MUAC. There was a non-significant trend toward greater risk of early pregnancy malaria among younger women and women with less education.

Uteroplacental blood flow and fetal growth (Figure 5.1)

We plotted individual trajectories of the continuous outcomes, UtA RI and UA RI, in order to examine intra- and inter-individual variability over gestational age in weeks. There is a slightly negative slope for UtA RI over gestational age (Panel A). UA RI was relatively higher in early pregnancy and decreased more rapidly over the course of pregnancy (Panel B), due to villous angiogenesis (Abramowicz and Sheiner 2008). Of the 44% (55/128) women ever having an IUGR episode after 20 weeks' gestation, 50% had one episode and one-third had two episodes, with 10 women having 3 or more episodes. Most IUGR episodes occurred in the late second and early third trimesters.

Early pregnancy malaria and uterine artery resistance (Figure 5.2)

There was no crude effect of early pregnancy malaria on UtA RI (mean difference: -0.017; 95% CI: -0.045, 0.011)(Panel A). In the adjusted model, the effect of early pregnancy malaria on UtA RI varied by maternal nutritional status (Panel B).

Compared to the referent group of women with no early pregnancy malaria with normal MUAC, the mean difference in UtA RI was -0.032 (95% CI: -0.062, -0.0006) for early pregnancy malaria and -0.011 (95% CI: -0.042, 0.019) for low MUAC. The joint mean difference in UtA RI for women with early pregnancy malaria and low MUAC was +0.022 (95% CI: -0.031, 0.076), or approximately one-fourth of a standard deviation (-2LL test for product interaction term $p = 0.026$). In other words, compared to the referent group, early pregnancy malaria caused a chronic increase in UtA RI of 4% among undernourished women, but caused a decrease in UtA RI of 6% among nourished women. Fetal sex and gravidity were not found to modify the association between early pregnancy malaria and UtA RI.

Early pregnancy malaria and umbilical artery resistance (Figure 5.3)

There was no crude effect of early pregnancy malaria on UA RI (Panel A). In the adjusted model, the effect of early pregnancy malaria on UA RI varied over time and by gravidity (Panel B). Compared to the referent group of multigravidae with no early pregnancy malaria, the mean difference in UA RI for early pregnancy malaria varied from 0.038 (95% CI: 0.0065, 0.069) at 21 weeks' gestation to -0.0087 (95% CI: -0.04, 0.03) at 39 weeks' gestation. The mean difference in UA RI was 0.032 (95% CI: 0.012, 0.052) for primigravidae. The mean difference in UA RI for the joint effect of early pregnancy malaria and primigravidity varied from 0.012 (95% CI: -0.030, 0.055) at 21 weeks' gestation to -0.032 (95% CI: -0.079, 0.011) at 39 weeks' gestation. Thus, multigravidae with early pregnancy malaria had elevated UA RI that decreased to levels similar to unexposed multigravidae during the third trimester. Among primigravidae, early pregnancy malaria led to decreased UA RI during the late third trimester, particularly compared to primigravidae with no early pregnancy malaria.

Early pregnancy malaria and intrauterine growth restriction (Table 5.2)

In the unadjusted model, early pregnancy malaria was associated with 1.8 times the risk of subsequent IUGR during pregnancy (95% CI: 1.1, 2.9). In the adjusted model, the effect of early pregnancy malaria was found to vary by gravidity (Wald test for product interaction term: p = 0.035). Primigravidae with early pregnancy malaria had 3.6 times the risk of subsequent IUGR compared to the referent group of multigravidae with no early pregnancy malaria (95% CI: 2.1, 6.2). Early pregnancy malaria was associated with a small increased risk of IUGR among multigravidae; this finding did not reach statistical significance. In the unadjusted model for repeat IUGR, early pregnancy malaria was associated with 2.2 times the risk of repeat IUGR episodes. In the adjusted model, the risk of repeat IUGR episodes among primigravidae with early pregnancy malaria was 5.6 (95% CI: 2.8, 11.3). The increased risk for repeat IUGR with early pregnancy malaria likely reflects the better ability of repeated episodes of <10th percentile SEFW to capture truly IUGR fetuses than a single episode.

Discussion

This is the first study to demonstrate that early pregnancy malaria leads to altered placentation and intrauterine growth restriction. These observations have important implications for the timing of malaria prevention and control efforts. The effects of early pregnancy malaria could be seen on both uterine and umbilical artery blood flow, indicating altered placentation and villous angiogenesis, respectively. Primigravidae with early pregnancy malaria had greater than three times the risk of subsequent IUGR and greater than five times the risk of repeat IUGR episodes compared to multigravidae with no early pregnancy malaria. The effects of early pregnancy malaria on fetal growth were less pronounced among multigravidae, suggesting that the effects of early pregnancy

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malaria are worse for primigravidae than multigravidae, as described at term (Desai, ter Kuile et al. 2007).

We found early pregnancy malaria increased UtA RI among women with low MUAC, but decreased UtA RI among women with normal MUAC. These differential effects of early pregnancy malaria on UtA RI between women with low and normal MUAC suggest important interactions between malaria and nutritional status on placental development (Prefumo, Sebire et al. 2004; Sebire and Sepulveda 2008). The effects of malaria on fetal growth have previously been shown to vary by nutritional status (Landis, Lokomba et al. 2009), but the joint effects of malaria and nutritional status during pregnancy on the fetus are not well understood.

Extravillous trophoblast invasion is highly regulated and is critical to establishing physiologic uteroplacental blood flow. Malaria in the placenta could dysregulate trophoblast invasion via relative placental hypoxia (Genbacev, Zhou et al. 1997; Arbeille, Carles et al. 1998); increases in inflammatory cells and mediators (such as TNFα) (Yui, Garcia-Lloret et al. 1994; Ordi, Menendez et al. 2001; Renaud, Postovit et al. 2005); functional folate deficiency (Williams, Bulmer et al. ; Brabin, Alexander Fletcher et al. 2003); and/or, increased complement activation (Conroy, McDonald et al. ; Girardi, Bulla et al. 2006).

We found increased uterine artery resistance with early pregnancy malaria among undernourished women. This finding indicates that placental malaria may be associated with diseases of trophoblast hypo-invasion, such as preeclampsia (Muehlenbachs, Mutabingwa et al. 2006). We also found evidence consistent with increased trophoblast invasion among nourished women with early pregnancy malaria. Placental bed biopsies are necessary to determine if the effects of early pregnancy malaria on trophoblast invasion are physiological or pathological, as in retained placenta or placenta accreta (Hung, Shau et al. 1999). Our findings demonstrate that malaria

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infection during early pregnancy affects placentation, likely via dysregulation of extravillous trophoblast invasion, and may contribute to diseases of both trophoblast hypo- and hyper-invasion.

Among primigravidae, early pregnancy malaria led to decreased umbilical artery resistance, indicating increased angiogenesis in the villous tree of the placenta (Abramowicz and Sheiner 2008). Adaptive villous branching and capillarization occur in hypoxic placental environments, including term preeclampsia (Kingdom and Kaufmann 1997), high altitude (Jackson, Mayhew et al.), smoking (Pfarrer, Macara et al. 1999), and anemia (Kadyrov and Kosanke 1998). Branching angiogenesis is associated with increased VEGF expression (Cao, Linden et al. 1996), previously shown to be associated with placental malaria and maternal hypertension (Muehlenbachs, Mutabingwa et al. 2006). Increased villous angiogenesis may explain the previously reported high placental to fetal weight ratio among primigravidae with placental malaria (Brabin, Romagosa et al. 2004), potentially linking malaria in early pregnancy to future cardiovascular disease (Barker, Bull et al. 1990).

Primigravidae with early pregnancy malaria had an increased risk of IUGR, compared to multigravidae with no early pregnancy malaria. Our findings support limited, but growing, evidence that malaria infection adversely affects birth weight (Kalilani, Mofolo et al. ; Taha, Gray et al. 1993; Cottrell, Mary et al. 2007; Huynh BT 2011). We found that the adverse effect of early pregnancy malaria on fetal growth in primigravidae occurred despite a blood flow profile consistent with normal placentation and increased angiogenesis. This suggests that the effects of early pregnancy malaria on fetal growth occur at the time of infection and are mediated via other mechanistic pathways, such as inflammatory cells and cytokines (Fried, Muga et al. 1998), anemia (Menendez, Ordi et al. 2000), or acute changes in blood flow during active parasitemia (Dorman, Shulman et al. 2002).

The prevalence of malaria parasitemia peaks in early pregnancy (Brabin 1983) and malaria prevention and control measures are infrequently initiated (WHO 2004) during this critical period in placental development. Our findings support the initiation of malaria prevention and control efforts earlier in pregnancy. The risks and benefits of anti-malaria drug treatment in early pregnancy must be carefully investigated.

Table 5.1 Characteristics of 128 mothers at baseline by early pregnancy malaria status.

Kinshasa, Democratic Republic of Congo, 2005-2006.

Characteristic	Total (n=128)	Early pregnancy malaria (n=27)	No early pregnancy malaria (n=101)	P^a
	N(%)	N(%)	N(%)	
Maternal age				
≥30	48 (38)	6 (22)	42 (42)	0.13
25-29	40 (31)	9 (33)	31 (31)	
<25	40 (31)	12 (44)	28 (28)	
Gravidity				
Primigravidae	34 (27)	6 (18)	28 (82)	0.56
Multigravidae	94 (73)	21 (22)	73 (78)	
Fetal sex				
Female	70 (55)	17 (24)	53 (76)	0.36
Male	57 (45)	10 (18)	47 (83)	
Low SES				
Yes	109 (85)	23 (21)	86 (79)	0.99
No	19 (15)	4 (21)	15 (79)	
Low education				
Yes	55 (43)	16 (29)	39 (71)	0.054
No	74 (57)	11 (15)	62 (85)	
Baseline MUAC <24.3				
Yes	28 (22)	6 (21)	22 (79)	0.96
No	100 (78)	21 (21)	79 (79)	

SES, socioeconomic status; MUAC, mid-upper arm circumference
a. p-values are for the comparison of women with early pregnancy malaria to women with no early pregnancy malaria.

Table 5.2 GEE log-binomial model estimated unadjusted and adjusted risk ratios (RR) for the effect of early pregnancy malaria on intrauterine growth restriction (IUGR) and repeat IUGR. Adjusted models stratified by gravidity. (n=544). Kinshasa, Democratic Republic of Congo, 2005-2006.

| | Early pregnancy malaria | | No early pregnancy malaria | |
	RR	(95% CI)	RR	(95% CI)
IUGR				
Unadjusted	1.8	(1.1, 2.9)	1.0	Ref.
Adjusted[ab]				
Primigravidae	3.6	(2.1, 6.2)	1.1	(0.6, 1.9)
Multigravidae	1.4	(0.8, 2.5)	1.0	Ref.
Repeat IUGR				
Unadjusted	2.2	(1.1, 4.2)	1.0	Ref.
Adjusted[cd]				
Primigravidae	5.6	(2.8, 11.3)	1.2	(0.5, 3.0)
Multigravidae	1.4	(0.6, 3.6)	1.0	Ref.

GEE = Generalized estimating equation
a. Adjusted for fetal sex and low baseline MUAC
b. Interaction term (early pregnancy malaria*primigravidae) p = 0.035
c. Adjusted for fetal sex and low baseline MUAC
d. Interaction term (early pregnancy malaria*primigravidae) p = 0.06

Figure 5.1 Time plot of mean uterine artery resistance index and umbilical artery resistance index against gestational age (in weeks), with population average smoothed trend line (in black). (n=128, with 544 visits) Kinshasa, Democratic Republic of Congo, 2005-2006. A. Uterine artery resistance index against gestational age. B. Umbilical artery resistance index against gestational age.

Figure 5.2 Estimated population average growth curves for the mean uterine artery resistance index (UtA RI) by early pregnancy malaria against gestational age (in weeks). Kinshasa, Democratic Republic of Congo, 2005-2006. A. The unadjusted effect of early pregnancy malaria on UtA RI (n=547 visits). B. The adjusted effect of early pregnancy malaria on UtA RI by maternal mid-upper arm circumference (MUAC). Model adjusted for gravidity and fetal sex; product interaction term (early pregnancy malaria * low MUAC) -2LL test p = 0.026, with 1 df (n=544 visits). The overall standard deviation for UtA RI was 0.089.
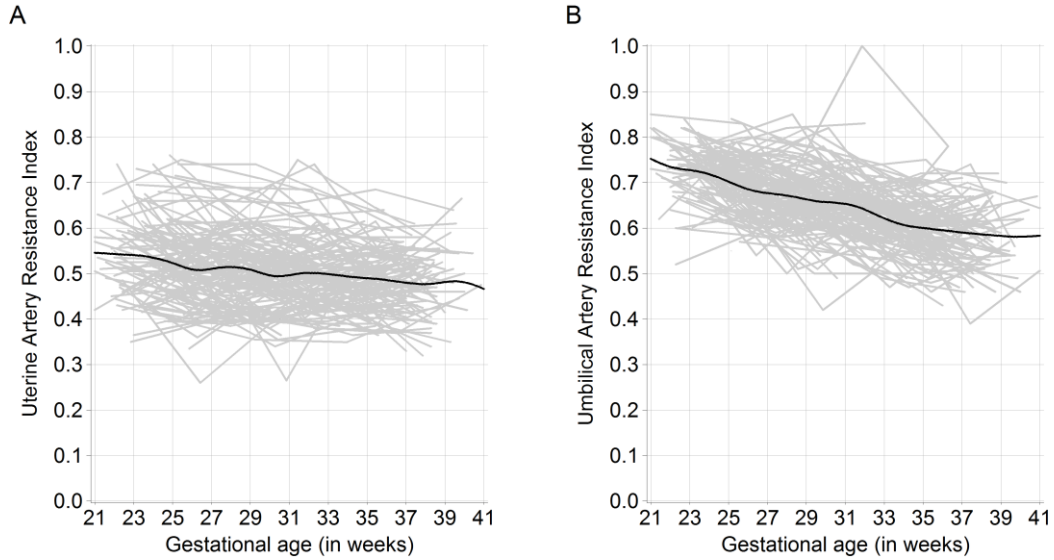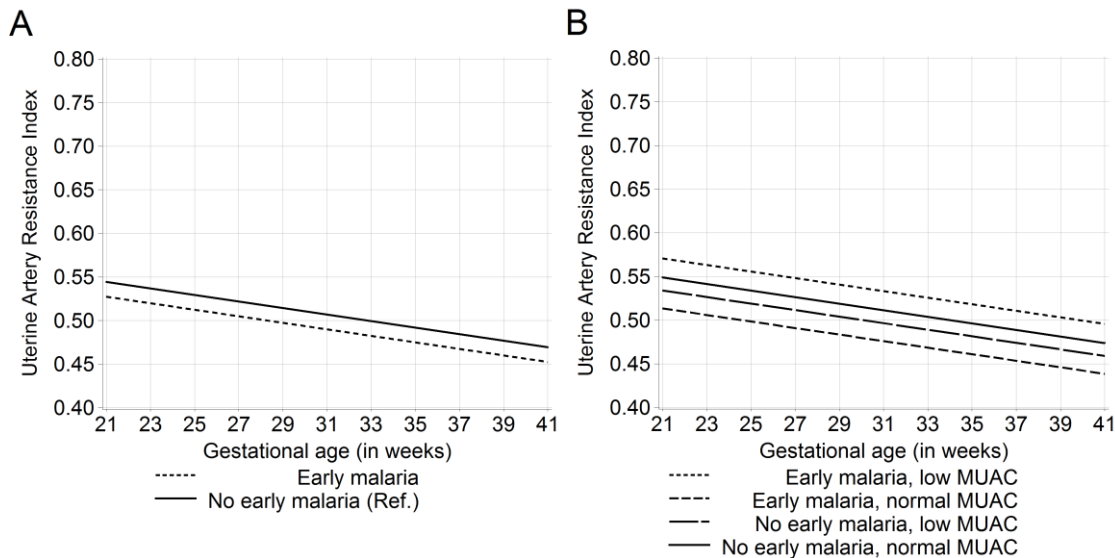
Figure 5.3 Estimated population average growth curves for the umbilical artery resistance index (UA RI) by early pregnancy malaria against gestational age (in weeks). Kinshasa, Democratic Republic of Congo, 2005-2006. A. The unadjusted effect of early pregnancy malaria on UA RI (n= 547 visits). B. The adjusted effect of early pregnancy malaria on UA RI by gravidity. UA RI model adjusted for fetal sex and low education; interaction terms: (early pregnancy malaria * gestational age in weeks) -2LL test p = 0.025; (early pregnancy malaria * primigravidae) -2LL test p = 0.025, with 1 df (n=540 visits). The overall standard deviation for UA RI was 0.089.
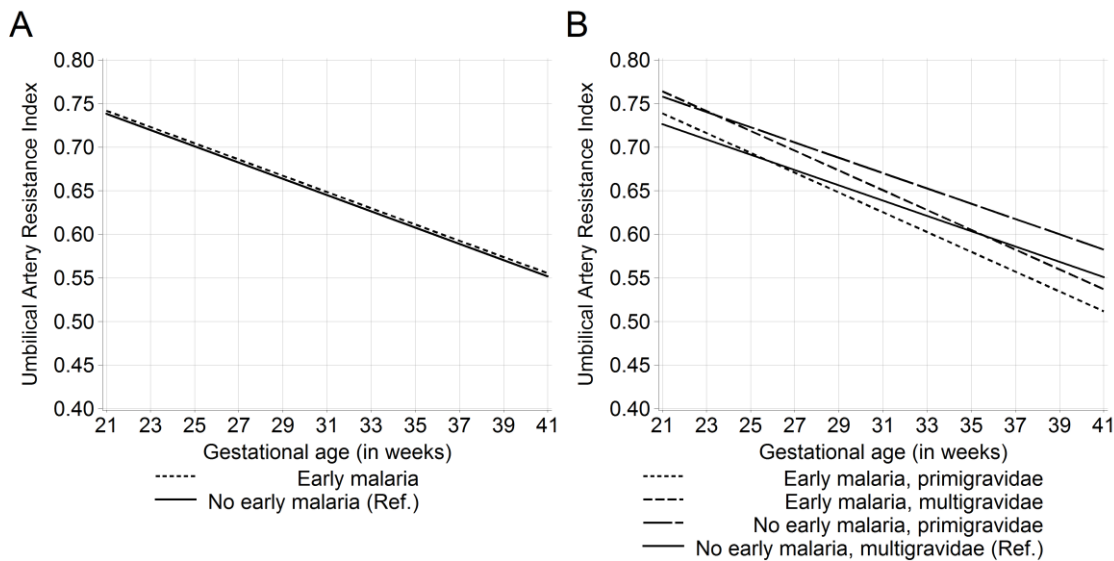
CHAPTER 6 - DISCUSSION

Overview

Nearly a century after the first reports linking malaria during pregnancy to adverse pregnancy outcomes (Clark 1915), malaria in pregnancy remains a significant public health problem in much of the world. Sub-Saharan Africa carries the bulk of the burden of malaria in pregnancy, with 30 million pregnancies and 21 million births occurring annually in areas with stable *Plasmodium falciparum* transmission (WHO 2003; Dellicour, Tatem et al. 2010).

In order to prevent the adverse outcomes of malaria during pregnancy in endemic settings, the World Health Organization recommends prevention and control strategies, including case management of malaria and anemia; ITNs; and, at least two doses of IPTp with sulfadoxine-pyrimethamine after the awareness of fetal movement (WHO 2004). These interventions have been shown to reduce the burden of disease and improve pregnancy outcomes in endemic areas (Gamble, Ekwaru et al. 2007; ter Kuile, van Eijk et al. 2007)

Unfortunately, despite high uptake of antenatal care in sub-Saharan Africa, IPTp and ITN coverage remain unacceptably low. A recent review found that while nearly all countries in sub-Saharan Africa had policies in place regarding ITNs and IPTp for pregnant women, coverage with ITNs is fewer than one in five pregnant women and coverage for a single dose of IPTp is one in four women (van Eijk, Hill et al.). Although progress has been made in a handful of countries, the Roll Back Malaria Partnership goals for 2010 of 100% coverage of IPTp and at least 80% coverage with ITNs in at-risk

areas were not met. To further complicate the issue of control and prevention of malaria in pregnancy, IPTp in its current manifestation is threatened by increasing resistance of *Plasmodium falciparum* malaria to SP (Harrington, Mutabingwa et al. ; ter Kuile, van Eijk et al. 2007; Mockenhaupt, Bedu-Addo et al. 2008).

Clearly, there is much work that needs to be done to reduce the burden of malaria in pregnancy. Studies of pathological processes during pregnancy resulting from malaria provide information that can be used to inform the type and timing of public health interventions. In this dissertation, we explored the effect of malaria on two *in utero* processes: utero- and fetoplacental blood flow and fetal growth. The goal of our work was to better understand the effect of malaria on pathological changes in pregnancy that lead to adverse pregnancy outcomes, in the hope of informing public health practice.

Summary of findings

For the first specific aim, our longitudinal analyses focused on the effect of concurrent malaria parasitemia on utero- and fetoplacental blood flow. The few previous studies investigating this association have indicated that concurrent malaria is associated with abnormal uterine artery (Arbeille, Carles et al. 1998; Dorman, Shulman et al. 2002) and umbilical artery blood flow (Arbeille, Carles et al. 1998). Our study confirmed previous findings that malaria parasitemia leads to increased uterine artery resistance among primigravidae (Dorman, Shulman et al. 2002); we did not find increased uterine artery resistance among multigravidae with malaria. The variation we observed in the effect of malaria on uterine artery blood flow by gravidity is consistent with data supporting worse pregnancy outcomes among primigravidae with malaria (Desai, ter Kuile et al. 2007). We also investigated the effect of concurrent malaria on umbilical artery blood flow. To our knowledge, our results are the first to identify that fetal sex modifies the pathological effects of malaria in pregnancy, as malaria significantly

increased umbilical artery resistance among women pregnant with female fetuses only. This finding points to a sex-specific response to malaria parasitemia in pregnancy, perhaps due to mechanisms such as sex-specific differences in placental cytokine expression, macrophage responses, growth factors, and/or responses to stress hormones such as cortisol, as found in asthma (Clifton 2010). Our research also confirmed the importance of utero- and fetoplacental blood flow to optimal fetal growth; as expected, high uterine and umbilical artery resistance were associated with decreases in fetal growth.

In our second specific aim, we focused on the effect of early pregnancy malaria (≤21 weeks' gestation) on changes in utero- and fetoplacental blood flow, as well as on fetal growth. The effect of early pregnancy malaria on uterine artery blood flow varied by nutritional status, with undernourished women having increased UtA RI with early pregnancy malaria and relatively nourished women having decreased UtA RI with early pregnancy malaria. As uterine artery resistance is reflective of the degree of extravillous trophoblast invasion of the spiral arteries, our results indicate that early pregnancy malaria affects placentation and that this relationship is modified by maternal nutritional status. We also examined the effect of early pregnancy malaria on umbilical artery resistance. We found that early pregnancy malaria led to decreased umbilical artery resistance among primigravidae, reflecting increased villous angiogenesis due to relative hypoxia in the placental environment with malaria infection. Finally, we found that early pregnancy malaria led to decreased fetal weight and increased risk of IUGR. Primigravidae with early pregnancy malaria had greater than three times the risk of IUGR compared to multigravidae with no early pregnancy malaria. There was a small, non-significant risk of IUGR among multigravidae with early pregnancy malaria. Our findings provide the first evidence that early pregnancy malaria affects placentation and fetal growth.

Together these analyses have contributed to the understanding of the pathological effects of malaria in pregnancy on utero- and fetoplacental blood flow and fetal growth. Our findings point to two distinct effects of malaria in pregnancy on utero- and fetoplacental blood flow: the acute effects of concurrent parasitemia and the effects of early pregnancy malaria on placental development. Our results indicate that the timing of malaria during pregnancy is important, with early pregnancy malaria infection leading to changes in placentation, villous angiogenesis, and fetal growth.

Public health significance

Our findings regarding the effects of malaria on utero- and fetoplacental blood flow occurred in the context of monthly antenatal care and extensive malaria prevention and control measures during pregnancy, as defined by the WHO, including high levels of ITN use, two doses of IPTp with SP, and case management of malaria infection and anemia. Yet, we found that both malaria parasitemia early in pregnancy and acute malaria parasitemia affected feto- and uteroplacental blood flow. Further, there was an increased risk of IUGR among primigravidae with early pregnancy malaria.

Changes in feto- and uteroplacental blood flow are thought to be a mechanism by which malaria during pregnancy causes fetal growth restriction. Our study provides the first longitudinal evidence that acute malaria parasitemia decreases uterine artery blood flow in primigravidae and umbilical artery blood flow among women with a female fetus. Our findings emphasize the public health importance of clinical management of malaria during pregnancy to prevent the acute effects of malaria on utero- and fetoplacental blood flow. Uterine artery blood flow provides oxygen and nutrients to the placenta in order to meet the metabolic demands of the fetus, while umbilical artery blood flow from the fetus to the placenta allows for the exchange of oxygen and nutrients to the fetus. Increased uterine artery and umbilical artery resistance has been shown to

lead to restricted fetal growth in animal models and in human studies, as well as in the current analysis.

Considering current recommendations by the WHO regarding the timing of IPTp during pregnancy (frequently after 20 weeks' gestation); existing knowledge of the peak prevalence of parasitemia (prior to 20 weeks' gestation); and timing of the process of placentation, we explored the effects of early pregnancy malaria infection on utero- and fetoplacental blood flow and fetal growth. A handful of previous studies have shown an adverse effect of early pregnancy malaria on birth weight (Kalilani, Mofolo et al. ; Taha, Gray et al. 1993; Cottrell, Mary et al. 2007; Huynh BT 2011). Given our findings that early pregnancy malaria alters placentation and leads to subsequent IUGR, this study provides strong evidence for the initiation of interventions for malaria during pregnancy prior to 20 weeks' gestation. Early pregnancy malaria infection could be found to have huge, unexpected public health implications should it be found to be involved in fetal programming and future metabolic and cardiovascular disease (Barker, Bull et al. 1990; Barker 2006).

The optimal type and timing of prevention and control efforts will need to be explored in intervention trials. The use of chemoprophylaxis for malaria infection in the first trimester is riddled with difficulties due to the potential teratogenic effects of anti-malarials. SP has a good safely profile after the first trimester. However, with increasing SP resistance and little data regarding other anti-malarials early in pregnancy, there is much to be done. It will be necessary to weigh the risks and benefits of early interventions to the pregnant woman and her fetus. As maternal blood begins to perfuse the intervillous spaces of the placenta at approximately 12 weeks' gestation, potentially allowing for parasite sequestration in the placenta, it is possible that interventions, particularly ITNs, for malaria in pregnancy would need to begin as early as 12 weeks'

gestation in order to prevent the effects of malaria in pregnancy on placentation and fetal growth.

Our findings also highlight the importance of the methods used to detect malaria during pregnancy to studies of pathogenesis and in the management of patients. As we previously reported, in the Project ECHO cohort, peripheral blood smear microscopy had 68% sensitivity and 91% specificity for the identification of peripheral malaria compared to qPCR (Taylor, Juliano et al. 2010). PCR of peripheral blood has been shown to have nearly 100% sensitivity with placental blood smear as the referent; however, specificity is lower (75%) (Mockenhaupt, Ulmen et al. 2002). The "false positives" found in the comparison of PCR of peripheral blood to placental blood smear microscopy may actually be due to: 1) low sensitivity of placental blood smear microscopy (estimated to be 76-78% when compared to the gold standard referent of placental histology) (Rogerson, Mkundika et al. 2003); peripheral parasitemia in the absence of placental infection; and/or remnant DNA in the peripheral blood from resolved placental infection. To date, PCR of peripheral blood at delivery and the gold standard of placental histology have not been compared. While qPCR is highly sensitive and specific, the clinical relevance of submicroscopic infections is under debate (Mankhambo, Kanjala et al. 2002; Malhotra, Dent et al. 2005; Mockenhaupt, Bedu-Addo et al. 2006).

Future research directions

The study of malaria in pregnancy combines challenges from infectious disease epidemiology and perinatal epidemiology. The identification of placental malaria in antenatal studies of malaria in pregnancy is problematic. While studies of malaria in pregnancy are most important in sub-Saharan Africa and Asia, most commonly used tools for perinatal epidemiology have been developed in the United States and Europe. These tools include algorithms for the estimation of gestational age, and nomograms for

the clinical classification of placental blood flow and fetal growth as normal or abnormal. In addition to several research directions that could improve the methods available for the study of malaria in pregnancy, several interesting study questions regarding potential pathological mechanisms emerged from our analyses of malaria in pregnancy on utero- and fetoplacental blood flow and fetal growth.

Improving exposure definitions for malaria during pregnancy:

A better understanding is needed of the diagnostic test characteristics used in the identification of placental malaria, including placental blood smear, rapid tests, immunohistochemistry, qPCR and nested-PCR, among others. Our understanding of the diagnostic test characteristics for placental malaria can be biased by the utilization of an imperfect gold standard. However, there are statistical tools, including latent class analysis (LCA) and Bayesian techniques, which can be used to compare the test characteristics in the absence of a gold standard test. Peripheral measures at term can be compared to placental measures to find improved antenatal measures of placental malaria. As diagnostic tools become more sensitive, their clinical relevance may decrease; thus, the predictive value of different diagnostic methods for relevant birth outcomes should also be explored. It is worth noting that higher levels of sensitivity may remain relevant for studies of pathogenesis. Improving our understanding of diagnostic methods for placental malaria infection and antenatal peripheral malaria, as well as their correlation would be an important contribution to the field of malaria in pregnancy.

Developing longitudinally-derived nomograms for utero- and fetoplacental blood flow

Similarly to the estimated fetal weight nomogram developed from Project ECHO data (Landis, Ananth et al. 2009), Doppler ultrasound data from Project ECHO can be used to develop longitudinally-derived nomograms for Doppler resistance indices. To our

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knowledge, these would be the first longitudinal nomograms for uterine and umbilical artery resistance in sub-Saharan Africa. Doppler ultrasound nomograms could be an important tool in antenatal studies of placental blood flow and fetal growth, as well as an important clinical tool in sub-Saharan Africa.

Exploring the modifying effects of fetal sex on the malaria and pregnancy outcome relationship

As fetal sex is commonly noted in studies, there is a great potential for secondary analyses on the modifying effects of fetal sex on the relationship between malaria, the placenta, the fetus, and pregnancy outcomes. There may be important sex-specific differences in placental cytokine expression, macrophage responses, growth factors, and/or responses to stress hormones. Should it be found to be an important modifier of the relationship between malaria and pregnancy outcomes, fetal sex will need to be considered in future research questions and study design.

Sex-specific responses to malaria can also be viewed through the lens of evolutionary biology. Sub-Saharan Africa has the highest male to female ratios in the world (Nigeria>1.050), as well as the lowest (<1.00 in DR Congo and Zambia). The sex ratio at birth is highest in younger pregnancies in sub-Saharan Africa, as well as among women with lower parity. Female fetuses have been found to have higher prevalence of placental infection at delivery than male fetuses. It would be interesting to investigate the hypothesis that malaria in pregnancy plays a role in sex-selection (likely via early spontaneous abortion) in sub-Saharan Africa.

Early pregnancy malaria and placentation

Due to our findings regarding the effects of early pregnancy malaria (<20 weeks' gestation) on placentation and angiogenesis, we hypothesized that early pregnancy

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malaria could have an effect on another aspect of placentation: placental location. Preliminary analyses suggest that malaria in early pregnancy is associated with an increased risk of low-lying placenta and decreased risk of a posterior site of placental attachment. Placental location is associated with adverse pregnancy outcomes, including preterm, IUGR, previa, and placenta accreta. It may be found that placental location explains some of the effects of early pregnancy malaria on changes in utero- and fetoplacental blood flow and pregnancy outcomes. Similarly, studies exploring the effects of early pregnancy malaria on the depth of trophoblast invasion would better elucidate whether early pregnancy malaria leads to alterations in placentation that are pathological or within physiologic norms.

Early pregnancy malaria and chronic disease

Sub-Saharan Africa is said to bear the double burden of infectious and chronic disease. In Aim 2, we found that early pregnancy malaria altered placentation and villous angiogenesis. Increased villous angiogenesis may explain the relatively high placental to fetal weight ratio among primigravidae with placental malaria. High placental to fetal weight ratios are predictive of future cardiovascular disease (Barker, Bull et al. 1990). Evidence increasingly supports a relationship between fetal "programming" in the placenta and health in later years. It would be interesting to explore the relationships between malaria exposure (and its timing) and patterns of non-communicable chronic disease.

Intervention for malaria early in pregnancy

Future studies should further explore the impact of prevention and control measures earlier in pregnancy. The utilization of ITNs and chemoprophylaxis for malaria in early pregnancy may be protective for pregnancy outcomes.

<u>Examining the effect of malaria during pregnancy on a more specific definition of IUGR:</u>

In utero small for gestational age (SGA) is the most commonly used proxy measure for IUGR; however, not all SGA fetuses are IUGR and not all IUGR fetuses are SGA. In addition to EFW, there are other fetal biometric manifestations of SGA useful for the identification of IUGR, such as abdominal circumference and the HC/AC ratio. Regardless of the measure used, estimates of fetal size alone have limited ability to distinguish the at-risk, pathologically growth restricted fetus from the healthy, but constitutionally small fetus. Currently, the combination of a small fetal abdominal circumference or EFW with increased umbilical artery resistance is considered most specific diagnosis of placenta-based IUGR, and best identifies small fetuses at risk of adverse outcomes (Baschat and Weiner 2000; Ott 2002). It would be useful to investigate the relationship between malaria and these, more specific, definitions of IUGR. Particularly, as a more specific definition of IUGR would be expected to better predict adverse birth outcomes.

Appendix 1: Sensitivity Analyses for Aim 1.

We conducted simple sensitivity analyses for the effect of concurrent malaria on UtA RI and UA RI by examining the effect of changing the choice of our time metric and our outcome definition on our final model estimates.

There are two primary methods to estimating *in utero* gestational age (GA): 1) the first day of the last menstrual period (LMP); and, 2) ultrasound dating via fetal biometry parameters. While straightforward and inexpensive, the validity of LMP is decreased by the uncertain recall of LMP dates; digit preference; and, biologic variation in the timing of ovulation, the timing of fertilization, and non-menstrual bleeding (Lynch and Zhang 2007). Ultrasound dating has been shown to provide a superior estimate of gestational age (Savitz and Terry 2002), even when LMP dates are considered certain (Mongelli, Wilcox et al. 1996). The main limitation of ultrasound estimation of gestational age is that estimates of gestational age will be biased if the fetus is either large (upward or older) or small (downward or younger).

We examined the difference between last menstrual period (LMP) estimated gestational age (GA) and ultrasound (US) estimated GA among the 150 women who reported LMP dates. The median LMP estimated GA at enrollment was 19.0 weeks, while the median US estimated GA was 19.7 weeks. Compared to LMP estimates, US GA was less variable (standard deviation of 2.8 vs 3.3 weeks' gestation). We examined the frequency distribution of the difference between LMP GA and US GA at enrollment (Figure A2.1). Positive values indicate that UA GA estimates are younger, while negative values indicate that UA GA estimates are older compared to ultrasound based estimates. The mean difference between LMP and US estimates of gestational age was -0.16 weeks (or -1.2 days), with a standard deviation of 2 weeks. The mean difference ranged from -5.6 weeks (-39 days) to 8.3 weeks (58 days). There was a trend toward

positive mean differences among all covariates that might be risk factors for a smaller

fetus at enrollment, although none of the mean differences were significant (Table A1.1).

This finding indicates that ultrasound estimated gestational age may have

underestimated the gestational age of older and younger women (vs 25 to 29 year olds);

women with a female fetus (vs male); and primigravidae (vs multigravidae).

Finally, we conducted a simple sensitivity analysis to determine how robust our

model estimates for the effect of malaria on UtA RI (Figure A1.2) and UA RI (Figure

A1.3) were to changes in our definition of gestational age. The estimated fixed effects

from the final models using ultrasound estimated gestational age are shown in both

tables (M). For both UtA RI and UA RI outcomes, final model estimates were robust to

use of LMP estimated gestational age (S1) and certain dates (S2). Our UtA RI model

was robust to the use of the highest value from the left and right uterine arteries, rather

than using the mean value (Figure A1.2; S3). Our models are robust to the choice of

time metric and outcome definitions. We conclude that while ultrasound estimated

gestational age may have introduced a small bias to our models, increased sample size

and precision makes use of ultrasound estimated gestational age a better choice for our

models. Further, mean UtA RI estimates were more precise than high UtA RI estimates.

The increased precision attained using the time metric and outcome definition from our

final selected models (M) makes our chosen definitions preferable to the alternates.

Figure A1.1 Frequency distribution of the difference between last menstrual period and ultrasound estimated gestational age at baseline in days. (n=150) Democratic Republic of Congo, 2005-2006.
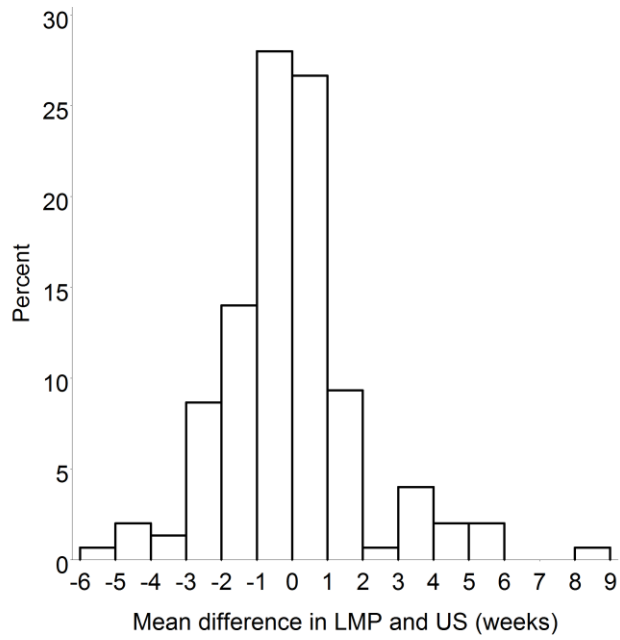
Table A1.1 Mean difference in LMP and ultrasound estimates of gestational age at enrollment by selected covariates at baseline.

(n=150) Democratic Republic of Congo, 2005-2006.

Characteristic		N (%)	LMP – ultrasound estimated gestational age in weeks		
			Mean difference	(95% CI)	*P*
Malaria at enrollment	Yes	26 (17.3)	0.36	(-0.48, 1.20)	0.40
	No	124 (82.7)	Ref.	-	
Maternal age	≥30	55 (36.7)	0.52	(-0.22, 1.26)	0.17
	25-29	54 (36.0)	Ref.	-	
	<25	54 (36.0)	0.50	(-0.30, 1.30)	0.22
Gravidity	Primigravidae	41 (27.3)	0.35	(-0.36, 1.10)	0.34
	Multigravidae	109 (72.7)	Ref.	-	
Fetal sex	Female	82 (55.0)	0.44	(-0.18, 1.07)	0.16
	Male	67 (45.0)	Ref.	-	
Low education	Yes	70 (46.7)	-0.26	(-0.90, 0.37)	0.42
	No	80 (53.3)	Ref.	-	
Low baseline MUAC	Yes	35 (23.3)	0.10	(-0.64, 0.85)	0.79
	No	115 (76.7)	Ref.	-	

Figure A1.2 Sensitivity analyses for effect of concurrent malaria on uterine artery resistance index (UtA RI). M: main analysis. S1: LMP estimated gestational age (n = 150, visits = 641). S2: restricted to women with certain gestational age only (n=115, visits = 489). S3: use of highest UtA RI value.
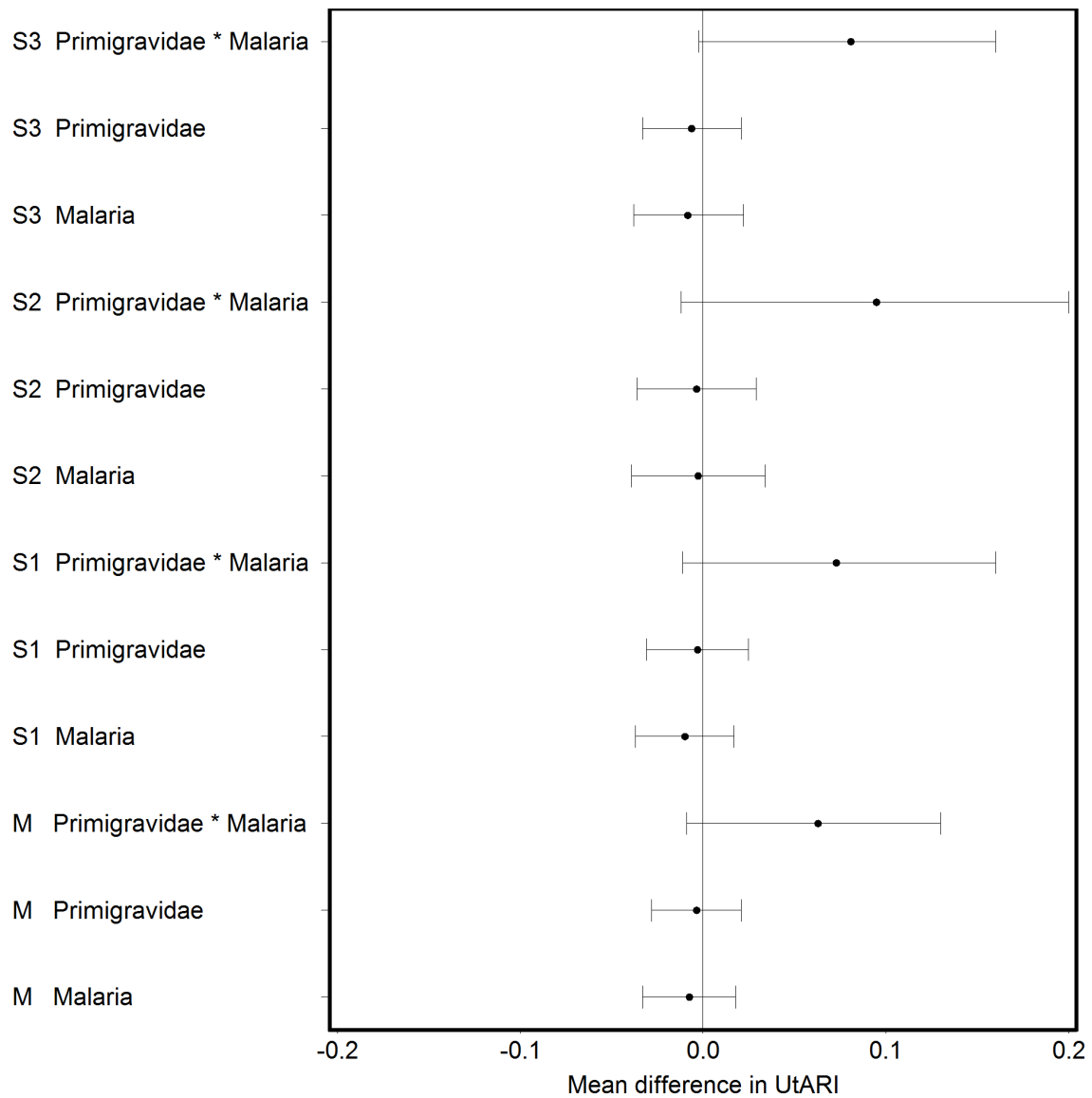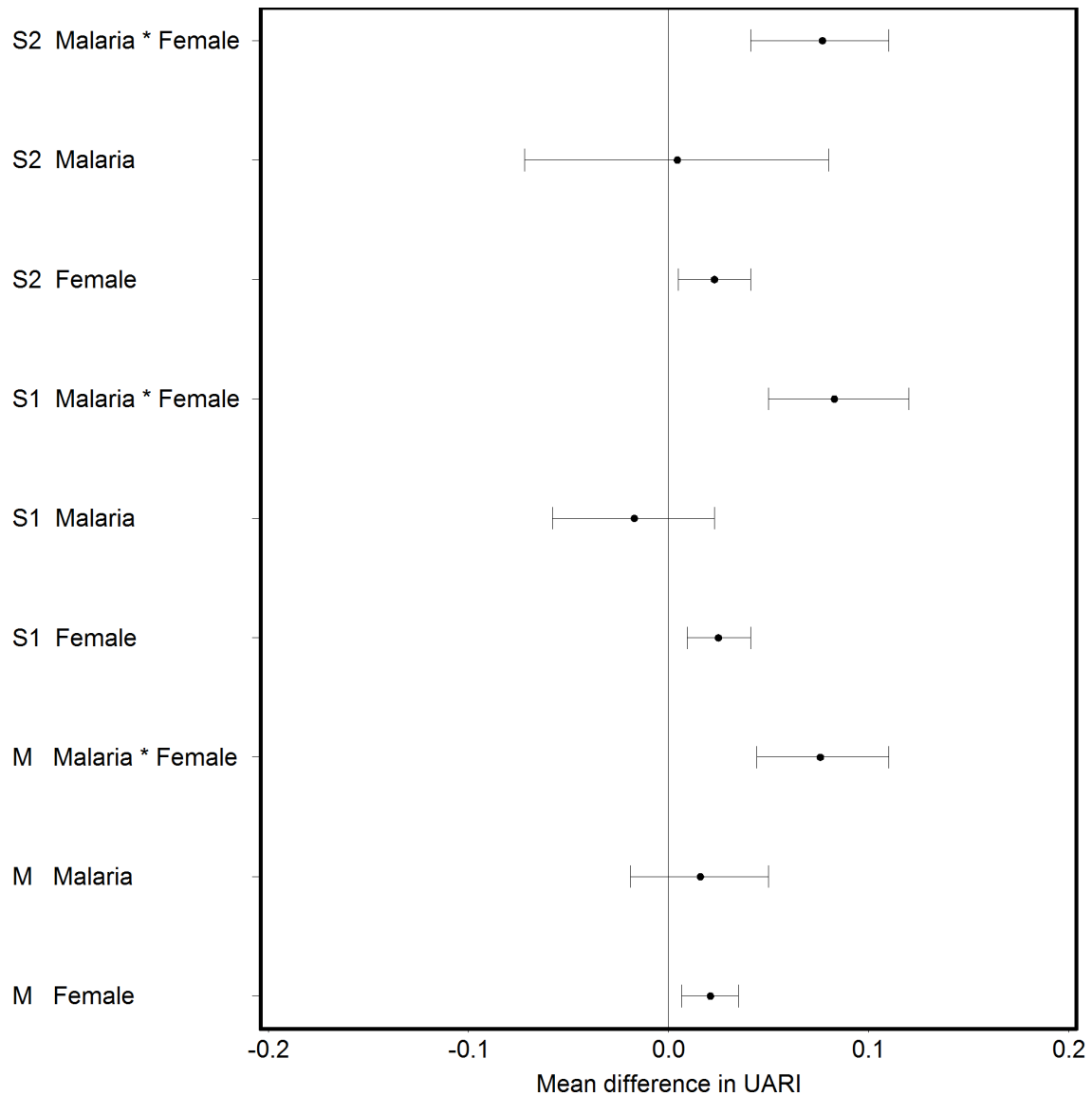
Figure A1.3 Sensitivity analyses for effect of concurrent malaria on umbilical artery resistance index (UA RI).M: main analysis. S1: LMP estimated gestational age (n=149, visits = 637). S2: restricted to women with certain gestational age only (n=115, visits = 489).

Appendix 2: Sensitivity Analyses for Aim 2.

We also conducted a simple sensitivity analysis to determine how robust our model estimates for the effect of early pregnancy malaria on UA RI (Figure A2.1) and UtA RI (Figure A2.2) were to changes in model definitions. The estimated fixed effects from the final models using ultrasound estimated gestational age are shown in both figures (M). For UA RI outcomes, final model estimates were robust to use of LMP estimated gestational age and certain dates (Figure A2.1, Panels B & C). Our UtA RI model was not robust to the use of the highest value from the left and right uterine arteries, rather than using the mean value (Figure A2.2; S3), but was robust to use of a more stringent definition of our modifying variable. The IUGR models were not robust to the use of LMP dates. We again conclude that while ultrasound estimated gestational age may have introduced a small bias to our models, increased sample size and precision makes use of ultrasound estimated gestational age a better choice for our models. The increased precision attained using the time metric from our final selected models (M) makes our chosen definitions preferable to the alternates.

Figure A2.1 Sensitivity analysis examining the effect of alterations in the definition of the time metric for umbilical artery resistance models. Linear mixed effect model estimated population average growth curves for the effect of early pregnancy malaria on umbilical artery resistance by gravidity against gestational age (in weeks). All models adjusted for fetal sex and low education. A. Using ultrasound estimated gestational age (M – final model). B. Last menstrual period estimated gestational age C. Certain gestational age (i.e. LMP within ±14 days of the ultrasound derived date).
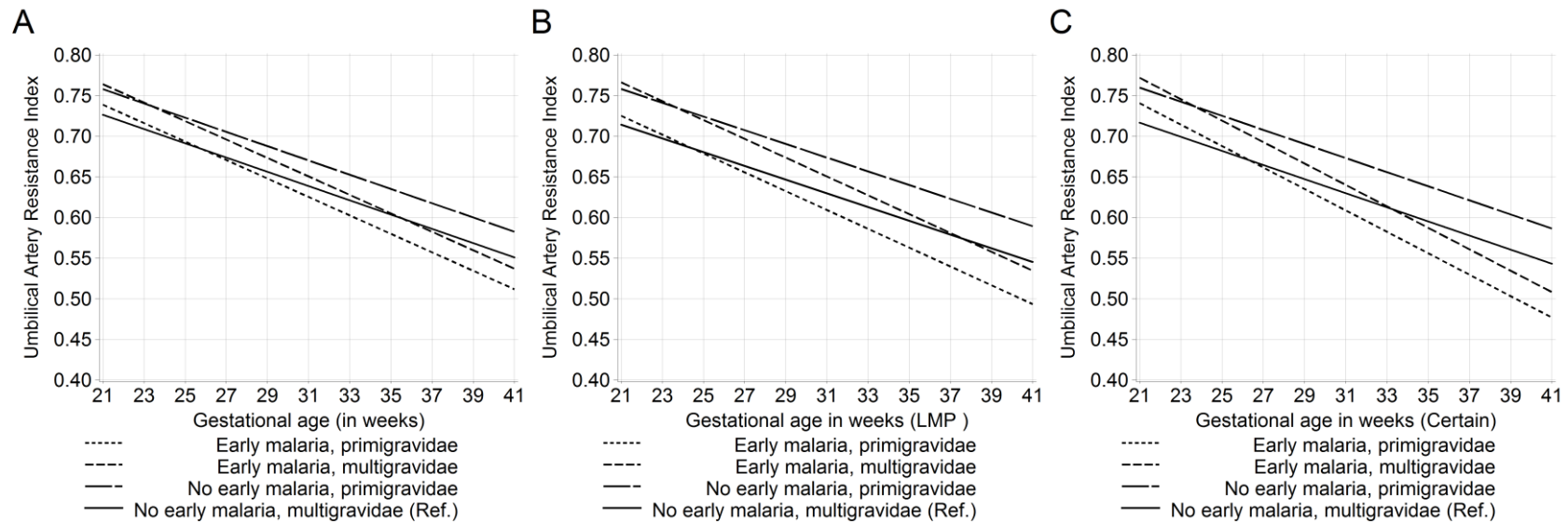
Figure A2.2 Sensitivity analyses for effect of early pregnancy malaria on uterine artery resistance index (UtA RI). M: main analysis. S1: LMP estimated gestational age (n = 109, visits = 463). S2: restricted to women with certain gestational age only (n=81, visits = 361). S3: use of highest UtA RI value. S4: Modifying variable defined as baseline MUAC <23 cm.

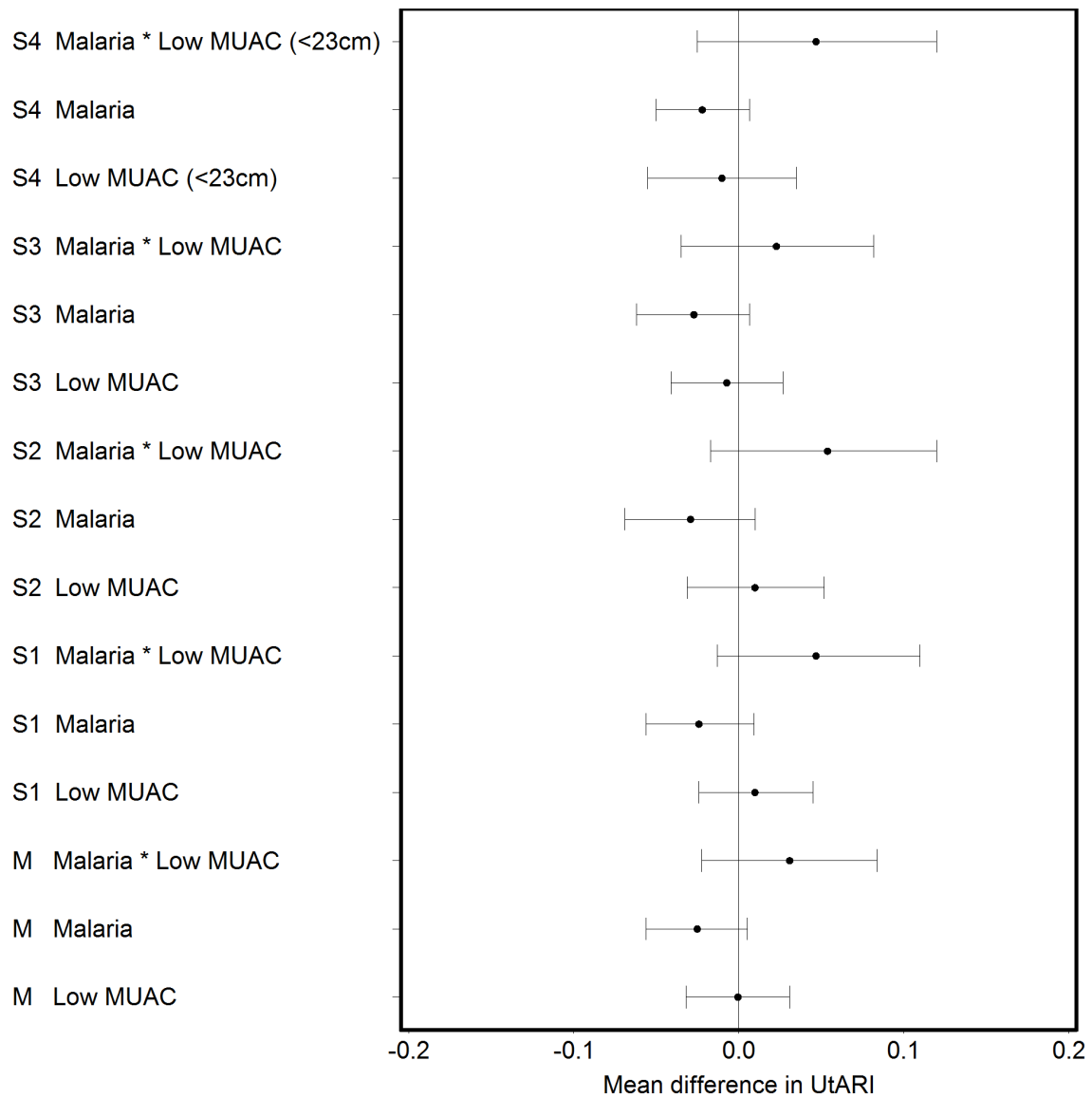Figure A2.3. Sensitivity analyses for effect of early pregnancy malaria on IUGR. M: main analysis. S1: LMP estimated gestational age used to derive EFW (n = 109, visits = 463). S2: Hadlock fetal weight standard. S3: Gallivan fetal weight standard.
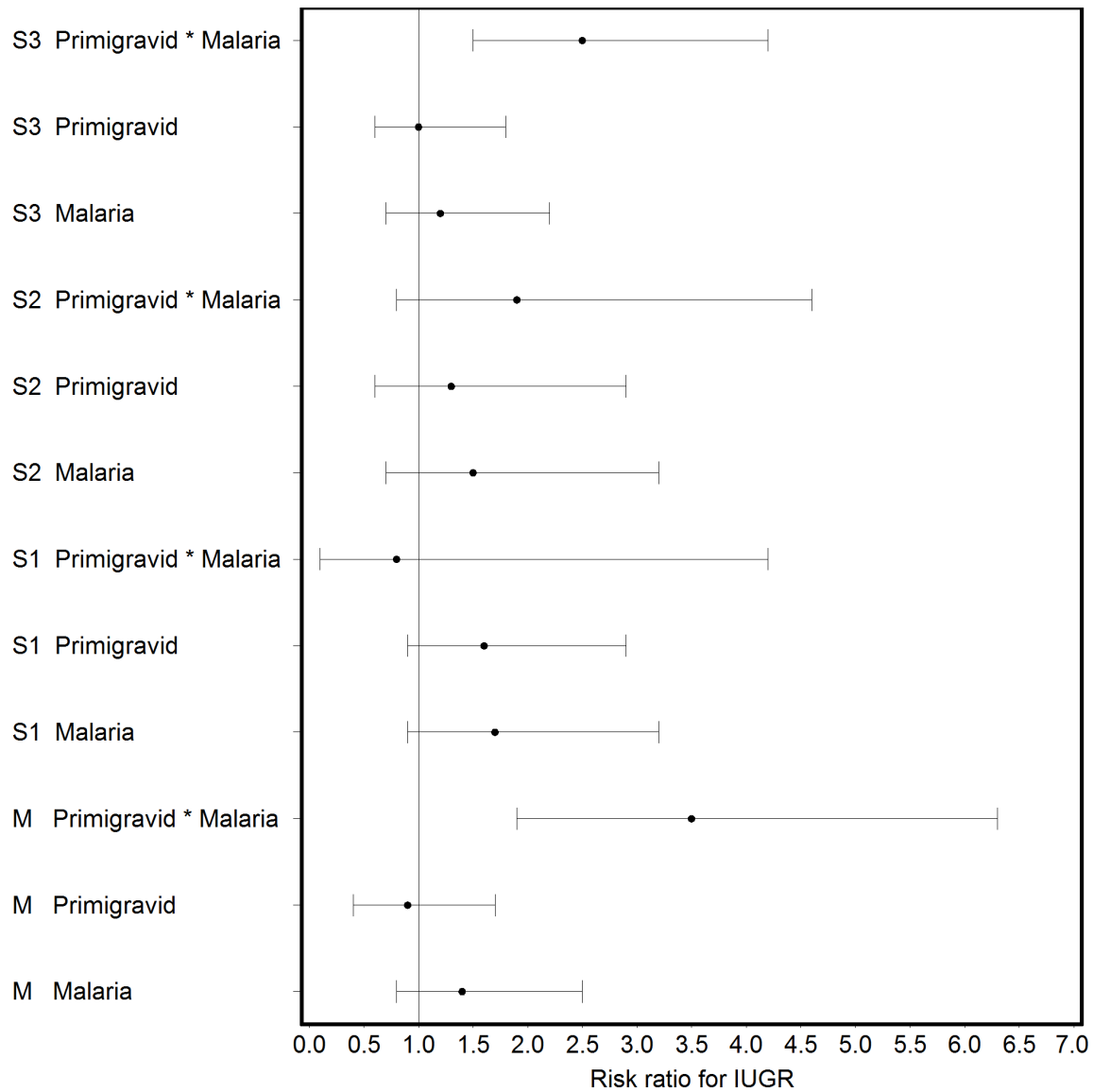
Table A2.1 Comparison of select fetal weight standards for the identification of IUGR. Democratic Republic of Congo, 2005-2006.

Nomogram	N	Country	Gestational age	EFW algorithm used	Longitudinal	Sex-specific	Number of measurements (%) identified as IUGR		
							Male	Female	Total
(Johnsen, Rasmussen et al. 2006)	643 women; 1799 examinations	Norway	Certain LMP (Ultrasound dating ±14 days)	(Combs, Jaekle et al. 1993)	Y	Y	50/265 (19%)	46/321 (14%)	96/586 (16%)
(Hadlock, Harrist et al. 1991)	361 women; 361 visits	United States	Certain LMP (not defined)	(Hadlock, Harrist et al. 1985)	N	N	23/293 (8%)	48/357 (13%)	71/650 (11%)
Gallivan (Gallivan, Robson et al. 1993)	67 women; 434 scans	England	LMP (BPD and FL <7 days discrepant)	(Hadlock, Harrist et al. 1985)	Y	N	46/264 (17%)	85/320 (26%)	131/584 (22%)

LMP = last menstrual period; Note: All populations were healthy women who had received ANC at a clinic or hospital.

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