IN UTERO B CELL RESPONSES TO PLASMODIUM FALCIPARUM AND RISK OF MALARIA DURING THE FIRST YEAR OF LIFE

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DEDICATION

This work is dedicated to my father,

Mr. SYLVESTER TASSI YUNGA

Words cannot express how much you have invested in my academic and professional journey.

You lovingly taught and encouraged me to pursue excellence in everything I do.

I could not have done it without you. Thank you papa!

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ABSTRACT

The fetal origin of disease hypothesis asserts that childhood predisposition to disease can be conditioned by intrauterine factors which, thus far, have not been well characterized. In malaria-endemic areas, fetuses of women infected with the malaria parasite *Plasmodium falciparum* (Pf) can be exposed to Pf antigens *in utero* during the second and third trimesters of gestation. The present dissertation comprises pioneer studies investigating the timing of acquisition antibody responses to Pf antigens *in utero* and the influence of the amount Pf antigen to which the fetus is exposed, on susceptibility to malaria during the first year of life. Our data show that the human fetus is not immunologically hyporesponsive as specific IgM to a wide breadth of Pf antigens were made by fetal B cells as early as 22 weeks of gestation and fetal B cells were capable of class-switching from Pf IgM to Pf IgG production towards term gestation. We also demonstrated that exposure to low and not high amount of Pf antigens *in utero* is associated with increased susceptibility to malaria since infants born to mothers with low placental parasitemia had a significantly higher number of infections during the first year of life and shorter time to first postnatal infection compared to infants of uninfected mothers or of mothers with high placental parasitemia. In addition, the infants of low placental parasitemia mothers did not make antibodies to Pf antigens during the first postnatal infection, while infants of high placental parasitemia mothers made a recall antibody response characterized by boosting of IgG and not IgM to the Pf MSP1 antigen. The data suggest that Pf-specific memory B cells can be generated *in utero* but a threshold amount of Pf antigen exposure may be required. Collectively, our data confirm that prentatal factors can modify risk of malaria after birth and demonstrate that low placental parasitemia at birth is as a major predictor of increased susceptibility of infants to malaria. Therefore, malaria control interventions during pregnancy should aim to eliminate and not reduce placental Pf parasitemia.

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LIST OF ABBREVIATIONS AND SYMBOLS

µL	microliter
Ab	antibody
Ag	antigen
AHR	adjusted hazard ratio
AMA	apical membrane antigen
APC	antigen-presenting cell
BSA	bovine serum albumin
CBMC	cord blood mononuclear cells
CD	cluster of differentiation
CDR	complementarity-determining region
CI	confidence interval
cm	centimeter
CSA	chondroitin sulfate A
CSP	circumsporozoite protein
DENV2-E	dengue virus envelop protein
DENV-NS1	dengue virus nonstructural protein 1
EBA	erythrocyte binding antigen
EIR	entomological inoculation rate
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FcRn or nFc-R	fc neonatal receptor
FcγRIIb	fc gamma receptor 2b

FIRS	fetal inflammatory response syndrome
FoxP3	forkhead box P3
GA	gestational age
GEE	generalized estimating equation
GPI	glycosylphosphatidylinositol
HRP2	histidine-rich protein 2
IC	immune complex
IE or iE	infected erythrocytes
IFN	interferon-gamma
Ig	immunoglobulin
IL	interleukin
IVS	intervillous space
kDa	kilodalton
LBW	low birth weight
LMP	last menstrual period
LSA	liver stage antigen
MFI	median fluorescence intensity
MSP	merozoite surface protein
MyD88	myeloid differentiation primary response gene 88
NFκb	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
OR	odds ratio
pABA	para-aminobenzoic acid
PAMP	pathogen-associated molecular patterns

PCR	polymerase chain reaction
PCV	packed cell volume
Pf	*Plasmodium falciparum*
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein-1
pg	picogram
PM	placental malaria
PM-	placental malaria negative
PM+Hi	placental malaria positive with high parasitemia
PM+Lo	placental malaria positive with low parasitemia
PRR	pattern recognition receptors
qPCR	quantitative polymerase chain reaction
RESA	ring-infected erythrocyte surface antigen
SD	standard deviation
SIRS	systemic inflammatory response syndrome
TLR	toll-like receptor
TNF	tumor necrosis factor
Tregs	T regulatory cells
VNTR	variable number of tandem repeats
wk	week

CHAPTER 1

INTRODUCTION

BURDEN OF MALARIA IN PREGNANCY AND INFANCY

Malaria is a mosquito-borne infectious disease caused by protozoan parasites of the genus Plasmodium, with *P. falciparum* (Pf) being the most lethal species. An estimated 3.2 billion people – about half of the world's population – residing in 91 countries and territories are at risk of malaria. A scale-up of malaria control interventions between the years 2010 and 2015, resulted in a 21% decrease in the number of new malaria cases and 29% decrease in malaria mortality worldwide. However, the burden of malaria remains high, as over 212 million malaria cases and 429,000 malaria-related deaths were reported for the year 2015 by the World Health Organization [1].

The burden of malaria is not evenly distributed across geographical regions and age groups. Children and pregnant women in sub-Saharan Africa are the most affected. For every 10 malaria cases or deaths in 2015, 9 occurred in sub-Saharan Africa where malaria is endemic. More than 70% of the reported deaths occurred in children before their fifth birthday. Natural partial immunity to malaria is acquired slowly after repeated Pf exposures [2], thus children are more susceptible to Pf infection and to severe malaria disease than adults.

Malaria during pregnancy is a major public health problem as it poses significant risks not just to the mother but also to her developing fetus. A combination of physiological and behavioral changes occurring during pregnancy place pregnant women at higher risk of Pf infections than their nonpregnant counterparts in the same region [3]. In addition, the pathogenesis of malaria in pregnancy is very unique. Unlike any other known pathogen, Pf has a

distinct ability to sequester in the intervillous spaces (IVS) of the placenta. This pathologic condition, known as placenta malaria (PM), occurs as a result of the adhesion of Pf-infected erythrocytes (IEs) to placental villi [4]. In pregnant women, Pf parasites express a specific variant of Pf-erythrocyte membrane protein 1 (PfEMP1) called VAR2CSA on the surface of IE which is capable of binding to chondroitin sulfate A on placental villi [5]. The sequestered IE stimulate resident macrophages to produce chemokines that recruit monocytes into the IVS leading to placental inflammation and adverse pregnancy outcomes [6–8]. PM is more common and more severe in primigravid women (first-time mothers) who have not yet acquired antibodies to VAR2CSA than in multigravid women who have acquired these antibodies as a result of multiple exposures to VAR2CSA during successive pregnancies [9].

CONSEQUENCES OF MALARIA IN PREGNANCY ON THE FETUS AND INFANT

Infection by Pf during pregnancy is associated with significant morbidity in the developing fetus, as well as in the neonate and infant.

Placental malaria and adverse pregnancy outcomes

Pregnancy outcomes associated with PM vary with the intensity of malaria transmission. In low transmission areas where pregnancy-related immunity to malaria is low in both primigravid and multigravid women, PM has been linked with spontaneous abortions and stillbirths [10,11]. In high transmission areas, adverse fetal comes include intrauterine growth retardation (small for gestational age), preterm delivery (PTD) (less than 37 complete weeks of gestation) and low birth weight (LBW) (less than 2,500g) [12]. In these high transmission areas, the poor fetal outcomes occur more frequently in primigravid women. For example, Tako and colleagues, showed that the adjusted odds of having a LBW baby in a high transmission setting of Cameroon was two times higher in primigravid compared to multigravid women [13].

Multiple mechanisms contribute to PM-associated pregnancy outcomes including maternal anemia and dysregulation of placental angiogenesis which decrease oxygen delivery to the fetus, and placental inflammation with trophoblastic dysfunction which reduces nutrient transport across the placenta [14].

Exposure of the fetus to *Plasmodium falciparum in utero*

When a pregnant woman is infected with Pf, her fetus can be exposed to intact IE or parasite products that cross the placenta. The term 'congenital malaria' specifically describes cases where live IEs are present in cord or peripheral blood of neonates. Congenital malaria is rare, occurring in only 0–5% of neonates in West and Central Africa [15,16]. The low prevalence of congenital malaria can be explained in part by high fetal hemoglobin content at birth which is not conducive for Pf growth [17,18]. The mechanism by which IEs cross the placental barrier is largely unknown. In contrast to other parasites that cause congenital infection by invading and multiplying in placental trophoblastic cells (e.g., *Toxoplasma gondii*) and chorionic cells (e.g., *Trypanosoma cruzi*) [19], Pf does not directly infect placental tissue. It is thought that some Pf-infected maternal red cells may gain access to the fetal circulation via microabrasions in the placenta during the process of labor. PM may also directly compromise the integrity of the placental barrier and provide a pathway for fetal infection. Crocker and collaborators, observed trophoblastic degradation and localized destruction of villi in placental tissue sections from women with placental Pf infection [20]. In a rural area of Malawi, the likelihood of congenital malaria was associated with high parasite density in the placenta [21].

Although congenital malaria is not very common, data suggest that many more babies in malaria-endemic regions are exposed to Pf *in utero*. Pf DNA and/or soluble parasite proteins have been detected in about 55% of newborns in sub-Saharan Africa [15,22]. Also, Metenou and

colleagues, demonstrated the release of Pf-specific IgG *in vitro* from peripheral mononuclear cells of 78% of Cameroonian neonates [23]. Because the mononuclear cells were isolated from cord blood at delivery, these neonates were primed with Pf antigens *in utero*. The discordance between the prevalence of neonates with congenital malaria and the prevalence of neonates with Pf-sensitized lymphocytes, suggests that other mechanisms are involved in prenatal Pf exposure besides intact IEs crossing through a breached placenta. Using human *ex vivo* placenta models, May and co-workers, demonstrated that Pf merozoite surface protein 1 (MSP1) crosses the placenta bound to MSP1-specific IgG as immune complexes [24]. Therefore, maternal IgG – which is physiologically transcytosed across the placenta with the help of Fc neonatal receptors [25–27] – provides a route for Pf antigens in the IVS to 'hitch a ride' into the fetal circulation.

Prenatal origin of infant susceptibility to malaria

Fetuses of PM-positive mothers appear to be programmed *in utero* for increased susceptibility to malaria and non-malarial infectious disease during childhood. In addition to its immediate adverse outcomes, PM is associated with increased number of Pf infections and shorter time-to-first infection in infants during the first year of life [28,29]. Heterogeneity of Pf transmission intensity can influence the risk of malaria in children but when adjustments were made for differences in climatic conditions and differences in bed net use, the association between PM and early infant malaria were still significant [30,31]. Therefore PM is an independent predictor of risk of early Pf infection after birth. Other authors have shown an association between PM and higher risk of severe malaria, anemia and non-malarial febrile episodes, as well as lower ability to cope with cognitive tasks during childhood [28,32–35].

Although these long-term health outcomes have been described, the underlying mechanisms linking PM with increased infant susceptibility to malaria have not been well

elucidated. Nevertheless, the findings support the fetal origin of disease concept which purports that childhood and adulthood predisposition to disease is conditioned, to some extent, by the intrauterine environment. The concept was first described in 1995 by the British epidemiologist David Barker after he noticed an association between mortality from coronary heart disease in adults and disproportionate fetal growth [36]. In his study of 16,000 adult men and women born between 1911 and 1930, Barker demonstrated that death rates from coronary artery disease were highest in adults who weighed less than 2,500g at birth, not as a result preterm delivery but because of intrauterine growth retardation [37,38].

DEVELOPMENT OF THE FETAL IMMUNE SYSTEM

The fetus is not immunologically dormant. Development of the immune system in humans begins during the embryonic period and continues throughout gestation. Beginning at 2 weeks of gestation, the yolk sac produces primitive hematopoietic precursors that migrate to the aorta gonad mesonephros region and to the liver. The fetal liver is the primary site of hematopoiesis from 6 weeks of gestation to about 10 weeks when the bone marrow takes over. Well-differentiated neutrophils, macrophages, NK cells, dendritic cells, T cells and B cells have been detected in fetal tissues by 10–12 weeks of gestation [39]. Many components of the adult innate and adaptive immune systems are present during early intrauterine life.

Ontogeny of the fetal innate immune system

The intra-uterine environment is relatively sterile as the fetus enjoys the privilege of being semi-walled off from the maternal genital tract by fetal membranes and from maternal circulation by the placenta. The placenta is fully formed with IVS by week 13 of gestation and continues to grow in size throughout pregnancy to attain a weight of about 1 pound at birth [14]. Other fetal organs such fetal skin, lung mucosa and gut mucosa serve as second-line physical and

chemical barriers against pathogens. These organs produce several anti-microbial peptides in amounts that increase with gestational age [40]. Thus, preterm babies are at higher risk of severe mucosal diseases such as necrotizing enterocolitis than full term babies and adults.

The number and functional capacity of fetal innate blood cells also vary with gestational age. In a rare study of differential cell counts in blood obtained by cordocentesis from 316 fetuses of gestational ages ranging from 18 to 40 weeks, Davies and colleagues [41], found that average total leukocyte counts increased from approximately 3,000/µL at 18 weeks to 5,000/µL at 30 weeks and 12,000/µL at 40 weeks. Average neutrophil counts remained under 1,000/µL until 32 weeks and increased exponentially to about 8,000/µL at 40 weeks. White blood cells in preterm fetuses also have a limited ability to extravasate into infected tissues because of decreased expression of integrins, such as leukocyte functional antigen (LFA-1), another reason why preterm newborns are more susceptible to bacterial infections than term newborns [42]. Monocytes, but not neutrophils, are important in the control of malaria parasitemia. Monocyte numbers and expression of pro-phagocytic markers like CD32, are higher in term compared to preterm newborns [41,42]. However, this may not affect Pf parasitemia of preterm and full-term newborns because prenatal and congenital malaria rarely occur in the first place.

Like in adults, the fetal innate immune system is equipped to recognize specific microbial molecular signatures called pathogen-associated molecular patterns (PAMPs). Expression of many known cell surface and cytosolic pattern recognition receptors (PRRs) has been confirmed in the fetus [43–45]. All known human Toll-like receptors (TLR) are expressed in the fetus including those that recognize the Pf PAMPs glycosylphosphatidylinositol (TLR 1, 2, 4 and 6) and Pf DNA/hemozoin pigments (TLR9) [46]. Downstream TLR signaling molecules such as the adaptor protein MyD88 and transcription factor NFκb have also been detected in fetal

membranes and other fetal tissues [45]. With regards to cytokine production, the fetal environment tends to be skewed towards an anti-inflammatory state. Upon TLR stimulation, cord blood monocytes and antigen-presenting cells (APC) express reduced levels of tumor necrosis factor-alpha (TNFα), interferon gamma (IFNγ), interleukin (IL) 12, and IL1β mRNA, but higher levels of IL10 mRNA compared to adult monocytes and APC [47]. IL10 production declines to adult levels at 1 to 2 years of post-natal life. The anti-inflammatory bias *in utero* may be important in preventing preterm labor and miscarriages.

Fetal inflammatory response syndrome

Even though the intrauterine environment is largely anti-inflammatory, the fetus is capable of mounting strong inflammatory responses when exposed to significant amounts of aggression. A classic example of prenatal inflammatory response is fetal inflammatory response syndrome (FIRS), described by Gomez and coauthors in 1998 [48]. The syndrome is characterized by a systemic activation of the fetal innate immune system and systemic release of pro-inflammatory cytokines, particularly IL6, but also, IL8, TNFα, and IL1β. The hematologic profile [49], leukocyte transcriptomic pattern [50], monocyte phenotypic changes and metabolic alterations [51] during FIRS are similar to those observed in systemic inflammatory response syndrome (SIRS) of adults. The most common etiologies of FIRS are microbial infections, such as group B *Streptococcus*, that invade the amniotic cavity from the maternal genital tract after preterm prelabor rupture of fetal membranes [52]. Increase in levels of pro-inflammatory cytokines in cord blood has also been observed in the absence of intra-amniotic infection, suggesting that hematogenous agents that invade the placenta can induce FIRS. Cytomegalovirus infection has been shown to elicit FIRS in animal models [53]. It is not yet clear if fetal exposure to Pf or Pf products elicits FIRS.

Ontogeny of the fetal adaptive immune system

The fetal thymus is formed at about 6 weeks of gestation and is seeded by T cells by 10 weeks [39]. Unlike mice in which transitional T cells are not released from the thymus until after birth, recent thymic immigrants are present in human fetal blood from 12 weeks of gestation. Peripheral T-cell subsets in the fetus are polarized towards T regulatory cells (Tregs). About 15–20% of CD4+ T cells of secondary lymphoid organs in the fetus are CD4+CD25+FoxP3+, compared to less than 5% in the newborn and adults [54,55]. Fetal Tregs play a role in exerting peripheral tolerance against maternal alloantigens that the fetus may be exposed to as result of maternofetal microchimerism [55,56]. The relatively high frequency of Tregs before birth and reversal thereafter also point to a layered immune system [57] in which sequential waves of peripheral immune cells with different polarization fates are produced at different times during development from distinct precursors. T cells produced during early development may therefore arise from precursors that are different from adult T-cell precursors, hence explaining the variation in the polarization of effector functions of fetal and adult T cells.

In addition to peripheral tolerance by Tregs, the developing fetus may become tolerant to alloantigens *in utero* through central mechanisms. Sir Peter Brian Medawar found that 8-week old mice did not reject grafts from genetically unrelated adult mouse donors if the graft recipients were inoculated with live cells from the same unrelated donor before birth [58,59]. These observations led to the idea that self and non-self recognition is defined *in utero* and that tolerance to foreign antigens can be actively acquired when the immune system is exposed to the antigens before birth. The tolerance observed by Medawar was later attributed to thymic deletion of T-lymphocyte clones that are reactive to donor antigens [60]. However, recent advances do not fully endorse these original conclusions. As explained later in this chapter, some newborns

possess T cells that proliferate and release cytokines in response to challenge by antigens derived from pathogens their mothers were infected with during pregnancy. Clearly these T cells had been sensitized to the antigens *in utero* and were not clonally deleted or suppressed by Tregs.

Like T lymphocytes, B lymphocytes develop early during fetal life. By 8 weeks of gestation, pre-B cells expressing cytoplasmic IgM can be detected in the fetal liver [39]. The pre-B cells become immature B cells expressing surface IgM by 10–12 weeks gestational age. At 12 weeks, B cells expressing IgM, IgD, CD19 and CD20 are present in peripheral circulation. Over 90% of circulating B cells in the human fetus express CD5 [61,62], the characteristic phenotypic marker of murine B-1 cells. Mouse B-1 cells spontaneously produce poly-reactive, predominantly IgM antibodies in a T-independent manner, in contrast to conventional B-2 cells that are T-dependent and antigen-specific. Based on the high prevalence of CD5+ B cells *in utero*, it was originally considered that human fetuses were incapable of mounting antigen-specific B-2 responses. However, recent advances refute this concept because human B cells that are functionally identical to mouse B-1 cells do not share the same phenotype as mouse B-1 cells. That is, human B-1 cells express the phenotype $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ and comprise less than 10% of total umbilical cord blood B cells [63,64]. Thus, conventional B-2 responses can be mounted by the human fetuses. The mouse-human discordance in fetal B-1/B-2 cell proportions and the absence of T cells in mouse peripheral lymphoid organs until after birth (whereas present in human fetal spleens and lymph nodes at 12 weeks of gestation) are two examples highlighting disparities between mouse and human fetal immunobiology. Moreover, the duration of gestation in humans (about 280 days) is approximately 14 times longer than in mice (about 20 days). Overall, the newborn mouse can be likened immunologically to a human fetus at very early stage of prenatal development.

Peripheral lymphoid organs in the fetus are populated with B cells by 16–21 weeks of gestation [65]. Initially, the B cells are diffusely spread within the lymphoid tissue, but become organized around resident dendritic cells to form primary follicles at approximately 17 weeks in lymph nodes and 24 weeks in the spleen. However, secondary follicles with germinal centers are thought to be absent in the fetus. Germinal centers are specialized immunologic microenvironments where immunoglobulin gene recombination leading to class switch from IgM to other antibody isotypes, and somatic hypermutations leading to affinity maturation and differentiation of high affinity B cells into long-lived plasma cells and memory B cells occur [66,67]. Al Barzanji and collaborators, studied spleen sections of 235 stillbirths and found no secondary follicles [68]. Splenic germinal centers were observed only in babies who died no less than 2 weeks after birth and the frequency of germinal centers increased with post-natal age [69].

The observed absence of germinal centers *in utero* may suggest that the fetal immune system is not mature enough to make the germinal centers or may simply reflect a relative lack of antigenic exposure *in utero*. Antigenic stimulation is important for germinal center formation and direct contact with a very diverse microbial environment after birth procures more antigenic exposure than a fetus would acquire *in utero* through the mother. In addition, maternal infections were unlikely etiologies of stillbirth in the study by Al Barzanji and colleagues [68] which was conducted in the United Kingdom where the burden of infections during pregnancy is low. Furthermore, some evidence that germinal centers may be produced *in utero* if the fetus had the right amount of antigenic exposure was provided by Wittenbrink and co-workers [70]. Peripheral blood samples of preterm babies born at 25–29 weeks were collected at different time points after birth. Premature contact with extrauterine environmental antigens was found to stimulate class-switch recombination and somatic hypermutations in immunoglobulin genes. However,

mutation frequencies were significantly low in newborns compared to adults [71] and preterm babies – despite exposure to environmental antigens – had low numbers of mutations until they reached a post-conception age corresponding to full term pregnancy [72]. Therefore, the generation of germinal centers *in utero* appears to be both antigen exposure-dependent and maturity-dependent.

Class-switch recombination occurs *in utero,* but the ability of the switched immunoglobulins to respond to a wide variety of antigens is shaped by the diversity of the immunoglobulin repertoire. Repertoire diversity is achieved through recombination in the VDJ segments of immunoglobulin variable region genes and N-nucleotide additions between segments. Rechavi and colleagues [71] reported an increase in the diversity of B cell repertoire with gestational age after conducting a cross-sectional study of four neonates born at 12, 13, 22 and 26 weeks. Other investigators have reported a higher occurrence of features of repertoire immaturity such as short complementarity determining regions (CDR) and over-representation of certain VDJ segments, as well as a slower rate of diversification of IgG repertoire after birth in premature than term babies [72]. The enzyme required for N-nucleotide addition is also expressed only late during fetal life, thereby further restricting the B cell repertoire during early gestation. Gestational age-related restrictions in T cell receptor repertoire have also been reported [71,73].

Even though there are limitations in the repertoire diversity and affinity maturation of antibodies produced *in utero*, the fetus benefits from maternal antibodies that are transferred across the placenta. Passive transfer of IgG from maternal to fetal circulation increases with gestational age as the placenta matures. The rate of increase in total IgG in fetal plasma is slow before 22 weeks, moderate between 22 and 26 weeks, and very rapid after 26 weeks [39].

Maternal IgG is transported across the placenta with the help of Fc-neonatal receptors [26,74,75]. IgG in the IVS of the placenta are trapped into pinocytotic vesicles at the maternal surface of syncytiotrophoblastic cells. While other contents of the pinocytotic vesicles may be digested, the IgG is not because it binds to FcRn. At low pH on the fetal side of the syncytiotrophoblast, the vesicles fuse with the cell membrane and IgG is released from the FcRn into the fetal circulation. The efficiency of IgG transplacental transport also depends on the subclass of IgG, with IgG1 being the most efficiently transported [76]. FcRn do not bind IgM, therefore maternal IgM is not transferred to the fetus. Hence, IgM detected in cord blood is made by the fetus. This fact has been explored in investigations of fetal antibody responses to prenatal antigenic exposure, including exposure to Pf.

FETAL IMMUNE RESPONSES TO *PLASMODIUM FALCIPARUM*: TOLERANCE VERSUS SENSITIZATION

As described above, the fetal immune system is not inert but there are differences in the quality, quantity and breadth of immune responses the fetus can make compared to adults and the quality of the response also changes with increasing gestational age. Yet, these differences are not necessarily, or solely, reflective of an immature fetal immune system; but also result from a relative lack of antigenic exposure *in utero* particularly during normal gestation. For example, the fetus is inclined towards maintaining an anti-inflammatory environment, but severe bacterial and viral infections can induce FIRS [48,52,53].

In addition, fetal T lymphocytes are polarized towards regulatory T cell responses, but several studies have demonstrated prenatal T-effector cell sensitization and non-regulatory T cell responses in some neonates exposed to pathogens *in utero*. Cord blood mononuclear cells (CBMC) from Brazilian newborns of mothers with Chagas disease show greater proliferative

response *to T. cruzi*-derived antigens compared to CBMC from newborns of healthy mothers [77]. In other studies, CD4 T cells of neonates exposed to hepatitis C virus *in utero* produced more IFNγ than CD4 T cells of control neonates [78] and higher frequencies of IFNγ-expressing and IL4-expressing CD4+ T cells were found in newborns of *Ascaris lumbricoides* infected mothers than in newborns of uninfected mothers [79]. In a region experiencing an influenza outbreak, T-cell proliferation in response to influenza hemagglutinin was observed in 19% of newborns [80]. Likewise, in a highly tuberculosis-endemic area of Kenya, purified protein derivative-induced cytokine release from cord blood CD4 T cells was detected in 30% of newborns [81]. Finally, filarial antigen-driven T effector responses were demonstrated in about 50% of newborns whose mothers were infected with *Wuchereria bancrofti* during pregnancy [82]. Thus, the fetus can develop antigen-specific T-cell responses prior to birth.

In many areas of sub-Saharan Africa where the burden of placenta-trophic infections such as Pf malaria is high, fetuses can be significantly exposed to Pf antigens *in utero*. The question then is whether Pf-exposed fetuses develop tolerance or sensitization to Pf antigens. There are data in support of tolerance on one hand and data depicting sensitization on the other hand. The data on tolerance demonstrate Pf-induced expansion of fetal Tregs and Treg-mediated suppression of sensitized T cells. For instance, the frequency of Tregs was found to be higher in newborns from areas of high malaria transmission compared to North American newborns [83] and in newborns of PM-positive mothers compared to newborns of PM-negative mothers [84]. Furthermore, *in vitro* culture of cord blood T cells with malarial antigens resulted in the expansion of both malaria-specific and polyclonal CD4+CD25hiFoxP3+ T cells [85]. Lastly, depletion of Tregs from CBMC of newborns resulted in a decrease in antigen-driven IL10 production, increase in antigen-driven IFNγ production, and increase in MHC class I and II

expression on monocytes [83,84]. Thus, a Treg-driven anti-inflammatory fetal response to malarial antigens has been reported.

On the other hand, strong Pf antigen-driven CD4 T-helper responses without signs of Treg suppression have been detected in a considerable proportion of newborns from some studies sites across Africa. Investigators have reported increased T cell proliferation and release of IFNγ, IL2, IL13, IL4 and/or IL5 in response to Pf antigen stimulation of CBMC from 25–60% of Kenyan and Cameroonian newborns. The T cell responses were not induced by nonspecific activation, as another study demonstrated responses that were finely specific to defined epitopes on alleles of the Pf $MSP1_{33}$ protein [86]. In addition, Pf-specific antibody responses have been detected in newborns indicating that fetal B cells were sensitized to Pf *in utero*. Total IgM in cord plasma is higher in newborns born to PM-positive compared to PM-negative mothers [87]. Several other studies have detected IgM that binds specific Pf antigens (or Pf whole parasite extract), in the cord plasma 2–25% of term African newborns [88–95]. IgM is not passively acquired from the mother implying that B cells of the newborn IgM responders encountered Pf antigens *in utero* and differentiated into IgM-secreting plasma cells.

Some studies have demonstrated *in utero* tolerance and sensitization in the same population of neonates exposed to Pf *in utero*. In a group of 366 newborns of PM-positive mothers from coastal Kenya, 67% had predominantly Pf-induced T cell responses characterized by Pf antigen-driven T cell proliferation and IFNγ, IL-2, IL-13, and/or IL-5 production while 23% had reduced T cell proliferation, reduced IFNγ, IL-2, IL-13, and/or IL-5 production, increased IL10 production and high expression of CD4+CD25+FoxP3 Treg phenotype [96]. Post-natal follow-up of the newborns revealed that risk of infant malaria is associated to whether the infant was sensitized or tolerized to Pf *in utero*. Compared to the sensitized group, the

tolerized group experienced delayed acquisition of functional anti-malarial antibodies and an almost two-fold increase in risk of malaria infection during the first three years life [96,97].

The reason why lymphocytes are tolerized to Pf in some exposed fetuses while in other exposed fetuses the lymphocytes are Pf-sensitized and appear to overcome Treg suppression, is not known. The direction the fetal adaptive immune response takes in response to Pf exposure could be related to several factors including: timing of Pf exposure during gestation (second trimester versus third trimester); the number of times exposed; the duration of each Pf exposure; the intensity of Pf exposure (amount of antigen to which the fetus is exposed at a given time); the type of exposure (antigen-only versus antigens and PAMPs); and the breadth of Pf antigens to which the fetus is exposed. Other non-malarial factors such as co-exposure to other pathogens and co-morbidities such as fetal undernutrition and intra-uterine growth retardation may also contribute in shaping prenatal responses to Pf antigens.

Direct assessment of fetal exposure to Pf is difficult because fetal samples are not accessible during the course of pregnancy. Nonetheless, evaluation of serially collected maternal samples over the course of pregnancy along with placental and cord blood samples collected at delivery can provide indirect estimates on the timing, frequency, chronicity and intensity of fetal exposure to Pf.

FIRST YEAR OF LIFE ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM*

For infectious agents that are not transplacentally transmitted such as enteric pathogens, infections during the first year of life constitute primary encounters of the infant with the corresponding antigens. The infant will therefore be expected to mount primary immune response to such antigens during the

first post-natal exposure. In the case of malarial antigens, primary exposure can occur *in utero* in endemic areas and post-natal infections may constitute secondary exposures. It is not known if prenatal exposure to Pf will induce recall antibody responses in the infant at first post-natal infection.

Circulating antibodies in infant plasma are comprised of maternal antibodies that were translocated across the placenta via FcRn and antibodies produced by the infant in response to environmental antigen exposure. The passively acquired maternal IgG decline over time in infant plasma and are replaced by actively acquired antibodies. Maternal IgG are believed to wane completely by 6 months of life but there is not much evidence supporting the assumption particularly in the case of Pf IgG. In addition, the relevance of maternal Pf IgG in protecting the infant from malaria is not well established. Some studies have reported protection from infection [89,98] while others have reported no association with protection [99–102] or an increased risk of Pf in infants who acquired high levels of maternal Pf IgG [100,103,104]. The question if maternal IgG are surrogates of protection from Pf during infancy or markers of infection still needs to be addressed in studies of well characterized Pf-resistant and Pf-susceptible infants.

CONCLUSION AND SCOPE OF DISSERTATION

In summary, infection with the malaria parasite during pregnancy can result in fetal exposure to Pf antigens. The fetal immune system is biased against inflammation and towards immune tolerance but Pf exposure *in utero* can sensitize fetal T and B lymphocytes.

Important knowledge gaps still exist and need to be systematically addressed. More studies on prenatal lymphocyte responses to Pf have focused only on T cells, even though immunity to malaria is primarily mediated through antibody responses. Also, the majority of

studies on T and B cells were done using umbilical cord blood samples only from full-term newborns. The characteristics of Pf-specific fetal adaptive responses at different times during gestation in relation to maternal Pf infection have not been investigated. In addition, it is not known if long-lived plasma cells or memory B cells are generated *in utero* in response to Pf sensitization and if these impact post-natal immune responses to Pf. Prenatal exposure to Pf may modify the risk of malaria during early childhood but specific exposure-related factors responsible for increased post-natal malaria risk are not well-known

Therefore, the main goals of the current study were to:

1. Investigate the timing of fetal antibody responses to Pf antigens to determine if gestational age influences the prevalence, isotype, amount, and breadth of fetal antibody responses to Pf antigens.
2. Investigate fetal origins of increased susceptibility to malaria by specifically determining if placental parasite density predicts the time to first Pf infection and total number of Pf infections experienced during the first year of life.
3. Determine the influence of prenatal exposure to malaria parasites on the antibody response to merozoite antigens during the first post-natal falciparum infection.

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CHAPTER 2

TIMING OF THE HUMAN PRENATAL ANTIBODY RESPONSE TO *PLASMODIUM FALCIPARUM* ANTIGENS

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ABSTRACT

Plasmodium falciparum (Pf)-specific T- and B-cell responses may be present at birth; however, when during fetal development they are produced is unknown. Accordingly, cord blood samples from 232 preterm (20-37 weeks of gestation) and 450 full-term (≥37 weeks) babies were screened for IgM to MSP1, MSP2, AMA1, EBA175 and RESA. Overall, 25% of the 682 newborns were positive for IgM to ≥1 Pf antigens with the earliest response occurring at 22 weeks. Interestingly, the odds of detecting Pf IgM in cord plasma decreased with gestational age (adjusted OR [95% CI] at 20–31 weeks = 2.55 [1.14–5.85] and 32–36 weeks = 1.97 [0.92–4.29]); however, preterm and term newborns had similar levels of Pf IgM and recognized a comparable breadth of antigens. Cord blood Pf IgM was associated with placental malaria (adjusted OR [95% CI] = 2.37 [1.25–5.54]). To determine if *in utero* exposure occurred via transplacental transfer of Pf-IgG immune complexes (IC), IC containing MSP1 and MSP2 were measured in cord blood. The proportion of cord samples with Pf IC increased with gestational age but was not associated with Pf IgM, suggesting that fetal B cells early in gestation had not been primed by IC. Finally, when cord mononuclear cells from 64 term newborns were cultured in vitro, only 11% (11/64) of supernatants had Pf IgM; whereas, 95% (61/64) contained secreted Pf IgG. These data suggest fetal B cells are capable of making *Pf*-specific IgM from early in the second trimester and undergo isotype switching to IgG towards term.

INTRODUCTION

In malaria-endemic regions, the fetus may be exposed to *Plasmodium falciparum* (Pf) antigens (Ags) that cross the placenta. Pf-infected erythrocytes (iE), DNA and/or soluble parasite proteins have been detected in umbilical cord blood samples of <1% to 55% of newborns in sub-Saharan Africa [1,2]. The risk of congenital parasitemia increases with high density of iE in the placental intervillous space [3] and placental pathology [4,5]. The fetus can also be exposed to Pf Ags in the form of immune complexes (IC) [6,7] that are transferred across the placenta via Fc-neonatal receptors (FcRn) [8]. Both IgG1 and IgG3, the main IgG subclasses produced against Pf Ags (9, 10), are efficiently transported by FcRn [11–13]. Since the IVS is formed by the end of the first trimester [14,15] and FcRn have been detected in human placentas as early as 8–12 wks of gestation [16], fetuses could be exposed to Pf Ags throughout the second and third trimesters.

At delivery, newborn lymphocytes exposed *in utero* to Pf can respond to Pf Ags. For example, cord blood mononuclear cells (CBMC) from 25-60% of Kenyan and Cameroonian newborns secreted IFNγ, IL2, IL13, IL4 and/or IL5 in response to stimulation with Pf Ags [17–22]. Not surprisingly, cytokine profiles were altered in cord plasma of Tanzanian neonates born to mothers with placental malaria (PM)-associated anemia [23]. In malaria-endemic areas, newborns have larger spleens compared to newborns in non-endemic areas [24], suggesting splenic lymphocyte proliferation takes place in response to Pf *in utero*. B cell responses also occur *in utero* as Pf-specific IgG and IgM have been detected in supernatants of *in vitro* CBMC cultures (16, 18) and in cord plasma, respectively. Since maternal IgM does not cross the placental barrier, Ag-specific IgM in cord plasma is a suitable biomarker for assessing development of the fetal B cell response. Higher total cord plasma IgM levels have been reported in babies born to placental malaria-positive (PM+) compared to PM-negative (PM-)

mothers [25], and other studies have detected IgM to an extract of iE and to individual Pf Ags in cord plasma of 2-25% of African newborns (16, 20, 24–29). Together, data from previous studies performed predominantly in term newborns, show that the fetus is not immunologically naïve as T and B cell responses can be developed prior to birth. It remains unclear, however, how the fetus responds to Pf Ags at different gestational ages (GA).

Development of the human immune system begins during the embryonic period and continues throughout gestation. Hematopoietic precursors are present in the yolk sac and diffuse B lymphocytes have been identified in fetal liver, peripheral blood and spleen by 8–12 wks, 12 wks, and 13-23 wks of gestation, respectively [32]. Primary follicles begin developing in lymph nodes and spleen from 17 and 23 wks, respectively [33], but organized germinal centers that are important in class-switching and affinity maturation, were not detected in spleens of European stillbirths [34,35]. Absence of germinal centers, however, could be due to low antigenic exposure *in utero* in developed countries and does not necessarily imply fetuses cannot make germinal centers. Total lymphocyte counts increase steadily from 20 wks through 40 wks [36] and the B cell receptor repertoire diversifies throughout gestation [37,38]. Thus, the GA at which the fetus is exposed to Ag may influence the resulting antibody (Ab) response.

In the present study, fetal IgM Abs to five Pf Ags were assessed in 232 preterm (GA range, 20–36 wks) and 450 term newborns (≥ 37 wks) in malaria-endemic areas of Cameroon. The influence of GA on the percentage of newborns with Pf-specific IgM, the amount of Pf IgM Ab present, and the number of Ags recognized (breadth) was investigated. Since maternal IC can cross the placenta and potentially stimulate a fetal Ab response, IC containing MSP1 and MSP2 Ags were also measured in cord blood and their association with Pf IgM was examined. Finally, Ab class-switching from Pf-specific IgM to IgG was evaluated using *in vitro* CBMC cultures.

MATERIALS AND METHODS

Study population and sample selection

The archival plasma samples and clinical data used were collected between January 1998 and August 2001 at the Central Hospital Maternity in Yaoundé, Cameroon. Yaoundé is a malaria-endemic area with perennial *P. falciparum* transmission. At the time of the study, individuals received an estimated 1 or 2 infective mosquito bites per month [39,40]. Since the samples were collected before the implementation of bed nets and intermittent preventive treatment for pregnant women in 2004 [41,42], women most likely were infected several times with Pf during pregnancy and their fetuses could have been exposed to Pf iE and/or Ags.

Preterm and term deliveries were defined as births before and after 37 completed weeks of gestation, respectively, calculated from the first day of the last menstrual period. All mother-newborn pairs with complete clinical data, and born ≥20 gestational weeks were used, providing a sample size of 682 newborns. Among the 682 babies, there were 40 sets of twins; however, cord blood from only one of each twin was used, so the number of samples (682) equals the number of deliveries. Cord plasma from 100 American newborns whose mothers had not traveled to a malaria-endemic region during pregnancy were obtained from the University of Hawaii Biospecimen Repository and used as negative controls.

For *in vitro* Ab studies, cord blood samples were also collected between July and November 2014 from 64 Cameroonian term newborns. The neaonates were born during peak malaria transmission periods in the Nkolbisson (July to October) and Maroua (October to November) health districts of Cameroon. Newborns of HIV-positive mothers were excluded. About 5 mL of blood was drawn from the umbilical vein after clamps had been placed at the

sectioned and placental insertion ends of the cord. Blood was collected into EDTA tubes and transported on ice to the laboratory for processing and analysis.

Ethical approval for samples collected between 1998 and 2001 was obtained from the Ethics Committee of the Ministry of Public Health, Cameroon, and the Institutional Review Board of Georgetown University, Washington, D.C. Human subject research exemption for use of archival Cameroonian and North American samples was obtained from the Human Studies Program of the University of Hawaii (CHS#21752). The 2014 study was approved by the University of Hawaii Institutional Review Board (CHS#21691) and the Cameroon National Ethics Committee for Human Health Research (No2014/02/416/L/CNERSH/SP).

Parasitological and hematological studies

Smears of maternal peripheral and placental IVS blood were prepared, stained with Diff-Quick (Baxter Scientific, Inc., Deerfield, IL), and examined for Pf iE by two microscopists. The mother was PM+ if iE were detected in either the peripheral or placental smear or both. Placental parasite density was calculated as the percentage of iE per 2,000 total erythrocytes in the IVS. Maternal anemia was defined as having a packed cell volume (PCV) of less than 30%.

Testing for admixture of maternal and fetal blood

Polymorphisms at three mini-satellite loci (D1S80, YNZ22 and ApoB) were examined using variable number of tandem repeat (VNTR) PCR assays. Paired maternal peripheral and cord blood samples were evaluated to verify that cord blood was not contaminated with maternal blood cells, i.e., to confirm that Pf IgM responses in cord plasma were made by fetal B cells and not by maternal B cells. Mother-stillborn pairs, in whom risk of admixture was presumed to be higher than in mother-livebirth pairs, were selected for the VNTR experiments. To increase

sensitivity of maternal DNA detection, DNA was extracted – using the NucleoSpin Blood QuickPure kit, (Macherey-Nagel, Germany) – from cord-blood buffy coat containing a much higher concentration of blood cells than whole blood. About 100 µL of buffy coat was pre-diluted in 100 µL of PBS.

For D1S80, 5′-GAAACTGGCCTCCAAACACTGCCCGCCG-3' forward primer and 5'GTCTTGTTGGAGATGCACGTGCCCCTTGC-3' reverse primer were used [43]. For YNZ22, 5'-AGGGAGAGAAAGGTCGAAGAGT-3' f-primer and 5'-GCCCCATGTATCTTGTGCAGTG-3'r-primer were used [44]. Finally, f-primer 5′-ATGGAAACGGAGAAATTATG-3' and r-primer 5'-CCTTCTCACTTGGCAAATAC-3' were used for ApoB [45]. Each 50 µL PCR reaction mix was comprised of 25 µL of 2X GoTaq Green Master Mix (Promega, Madison, WI), 2 µL each of f-primer (100ng/µL) and r-primer (100ng/µL), 4 µL DNA extract and 17 µL of nuclease-free water. The amplification conditions for D1S80 [20], were used for both D1S80 and YNZ22 PCR. ApoB PCR was performed as previously described [45], with the addition of an initial denaturation step at 94°C for 3 min. PCR products were electrophoresed on 2% agarose gel at 5V/cm. For each locus, a mother-newborn pair was informative if the mother possessed two alleles, one of which is not identical to the paternal allele in the newborn. For informative sample pairs, detection of both maternal alleles in the newborn indicated the presence of maternal DNA contamination in cord blood.

Malarial and control antigens

A panel of malarial Ags was used, including recombinant proteins of the FVO and 3D7 alleles of 42 kDa C-terminal merozoite surface protein-1 ($MSP1_{42}$) expressed in *Escherichia coli*; the FC27 and 3D7 alleles of MSP2 expressed in *E. coli*; the 3D7 allele of 83 kDa apical membrane antigen-1 (AMA1) expressed in yeast cells; the 60 kDa conserved region II of

erythrocyte binding antigen-175 (EBA175) expressed in yeast cells, and synthetic peptide of the 2.5 kDa ring-infected erythrocyte surface antigen (RESA) containing five EENV repeats. Three control Ags were used, including a recombinant dengue virus non-structural protein (DENV-NS1), dengue virus serotype 2 E protein (DENV2-E) and bovine serum albumin (BSA).

Coupling of antigens to microplex beads

Each Ag was covalently coupled to microplex polysterene beads (Bio-Rad, USA) with distinct spectral addresses as previously described [46]. To ensure bead surfaces were saturated with Ag, the following amounts of Ag were used per million beads: 1 µg (MSP1-FVO), 1 µg (MSP1-3D7), 0.2 µg (MSP2-FC27), 0.2 µg (MSP2-3D7), 1 µg (AMA1-3D7), 1 µg (EBA175), 10 µg (DENV-NS1), 10 µg (DENV2-E) and 1µg (BSA). Coupled beads were stored in the dark at $4^{o}C$ at a concentration of 25,000/µL of blocking-storage buffer (PBS pH 7.2, 1% BSA, 0.02% Tween 20, and 0.05% sodium azide).

Measuring Pf IgM in cord plasma

A bead-based multi-analyte platform assay was used to measure IgM to Pf and control Ags in cord plasma of Cameroonian and North American newborns. Plasma samples were diluted 1:100 in PBS-1% BSA and 50 µL was added to 50 µL of a mixture of equal numbers (3,000 each) of MSP1-FVO, MSP1-3D7, MSP2-FC27, MSP2-3D7, AMA1, EBA175, and RESA-coupled beads in filter plate wells (Millipore, Germany). After 1 h of incubation on a 500-rpm shaker at room temperature (RT) and in the dark, the plates were washed twice with PBS-0.05% Tween 20 and once with PBS-1% BSA. Next, 100 µL of 2 µg/mL phycoerythrin (PE)-conjugated donkey anti-human IgM Ab (Jackson ImmunoResearch, USA) was added to each well and incubated on the shaker for 1 h. After three washes, 100 µL of PBS-1% BSA was added to each well and beads were re-suspended on a shaker for 5 min before analysis in the LiquiChip

100 (Luminex Corp., Austin, TX). Amount of Ab was quantified as the median PE fluorescence intensity (MFI) of 100 beads per spectral address. Cameroonian cord plasma were considered positive for IgM to Pf Ags if the MFI was greater than the mean + 2 standard deviations (SD) of American cord plasma for the corresponding Ag.

To confirm the binding specificity of Pf IgM, 45 Cameroonian cord plasma samples that were positive for MSP1-FVO IgM were tested for IgM to non-malarial antigens that the newborns were unlikely to have been exposed to *in utero*, i.e., BSA, DENV-NS1 and DENV2-E.

Pf antigen-IgG immune complex assays

IC were measured in 484 maternal peripheral and cord plasma samples, i.e., 242 mother-newborn pairs, including 143 preterm pairs and 99 term pairs. Cord samples for IC assays were randomly selected from newborns whose mother's peripheral plasma were also available.

To capture IC containing malarial Ags, polyclonal anti-MSP1 and anti-MSP2 were used. Polyclonal MSP1 and MSP2 antisera were generated by immunizing rabbits with GST fusion protein containing full length recombinant Pf MSP1 or 2. Abs were further purified by protein G column chromatography and the purity was checked by SDS-PAGE. The Abs were coupled to beads at a concentration of 10 ng of anti-MSP1 and 10 ng of anti-MSP2 per million beads. Coupled beads were incubated for 1 h at RT with a 1:50 dilution of maternal and cord plasma samples. Beads were washed as described above, treated for 1 h with 100 µL of 2 µg/mL of PE-conjugated goat anti-human IgG (Fcγ fragment specific) (Jackson ImmunoResearch, USA) and washed again. The beads were then re-suspended in 100µL of PBS-1% BSA and analyzed in the Liquichip 100. Plasma from 20 American adults were also tested to establish MSP1 and MSP2 IC cut-offs (mean + 2SD).

CBMC cultures for in vitro IgM and IgG production

CBMC were isolated from freshly-collected cord blood of 64 term newborns by ficoll density gradient centrifugation. Mononuclear cells were harvested, washed three times with 10 mL of 2% Fetal Bovine Serum (FBS) and resuspended at 2 x 10^6 cells/mL of RPMI-1640 supplemented with 10% Serum Replacement-3 (Sigma-Aldrich), 0.2 M L-glutamine, 0.02 M HEPES, 100 IU/mL penicillin G, 100 µg/mL streptomycin sulfate and 0.1 M sodium pyruvate. Then, 200 µL of CBMC suspension (4 x 10^5 CBMC)/well was incubated in triplicate wells in 96-well U-bottom plates (Corning, USA) for 5 days at 37°C and 5% CO_2. Wells containing culture mediun only were also incubated for 5 days for comparison. All supernatants were harvested and stored at -80°C until analyzed.

Measurement of Pf IgM and IgG in culture supernatants

To determine whether Ab-secreting cells in CBMC had isotype-switched *in utero*, culture supernatants were tested for IgM and IgG Abs to Pf Ags. The multiplex assay used was similar to the plasma assay described above, except: 1) Ags were coupled on Magplex magnetic beads (Bio-Rad, USA), 2) culture supernatants were used undiluted, 3) PE-conjugated donkey anti-human IgG (Jackson ImmunoResearch, USA) was used to detect IgG bound to Pf Ags, and 4) the MagPix analyzer (Luminex Corp., Austin, TX) was used to quantify MFIs. Cut-off for *in vitro* IgM and IgG was the mean of the media-only + 3SD.

Statistics

Fisher's exact test was used to compare proportions and the Chi-square test for trend was used to identify significant trends in Pf IgM seropositivity rates across GA groups. Paired t test was used to compare levels of IgM to Pf Ags with IgM to control Ags measured in the same samples. Changes in levels of IgM or IC across GA groups were analyzed using one-way Analaysis of Variance (ANOVA) including post-test analyses for GA-related trends. Determinants of the presence Pf IgM in cord plasma, specifically IgM to MSP1 or MSP2, were assessed using logistic regression models. Exposure-related variables, i.e., PM and presence of Pf IC in cord plasma, as well as GA, were included a priori in the model. Maternal anemia was also included in the model because anemia was associated with the breadth of Pf IgM in cord plasma. Interaction between PM and GA was also examined.

RESULTS

Preterm newborns

A total of 232 out of 682 mothers (34%) had preterm deliveries. Having a preterm baby was associated with higher placental parasite density (median percent iE [25th–75th percentile] was 1.7% [0.6–6.1%] for preterm and 1.0% [0.3–3.4%] for term mothers, $p = 0.042$), suggesting that maternal malaria contributed to preterm births. Other maternal factors associated with preterm delivery were younger age (mean ± SD for preterm was 25.6±6.7 years, term = 27.4±6.1 years, $p = 0.001$); primigravidity (30% of preterm mothers, 22% of term, $p = 0.025$); and maternal anemia (38% of preterm mothers, 20% of term, $p<0.001$).

Based on the World Health Organization's classification of preterm births [47], 16% of the 232 preterm newborns were categorized as extremely preterm (<28 complete wks of gestation), 25% were very preterm (28–31 wks), and 59% were moderate to late preterm (32–36 wks). As expected, preterm singleton newborns had lower birthweights (2,089±710g) than term newborns (3,284±554g), $p<0.001$. Only 6% (43/682) were stillborn, but clinical information indicated they were not macerated and their placentas were macroscopically intact.

Confirmation that admixture of fetal and maternal blood had not occurred

Maternal IgM does not cross the placenta and previous studies have demonstrated that maternal blood does not significantly mix with fetal blood during normal vaginal deliveries at term [18,20]. However, 16% of premature newborns were stillbirths (38/232) compared to 1.5% (7/450) of term neonates ($p<0.001$). To evaluate if admixture had occurred, DNA was extracted from peripheral buffy coat of 37 stillbirths and their mothers and examined for polymorphisms at three mini-satellite loci. Of the 37 mother-stillborn pairs that were genotyped, maternal DNA

was not detected in 35 cord blood samples (95%) (S1 Fig. gel A and B) and the other two sample pairs were inconclusive as the mother and newborn were either homozygous or had the exact same heterozygous alleles (S1 Fig., gel C). Because maternal DNA was not detected in any of the stillborn infants, maternal:fetal blood admixture did not have a major influence on the study. Hence, detection of Pf IgM in cord blood indicated that the IgM was produced by fetal B cells.

Pf IgM in cord plasma

As expected, some Cameroonian newborns had IgM to Pf Ags (Fig. 1A), indicating that they had been exposed Pf Ags *in utero* and made IgM. Based on the cut-off of mean + 2 SD of 100 American newborns, 6.6% of Cameroonian newborns were IgM positive for MSP1-FVO, 7.5% for MSP1-3D7, 17.8% for MSP2-FC27, 17.4% for MSP2-3D7, 5.7% for AMA1, 7.2% for EBA175 and 8.5% for RESA. Overall, 25% (169/682) had IgM to one or more Pf Ags.

To confirm the IgM responses were Pf Ag-specific, cord plasma from 45 newborns who tested positive for IgM to MSP1-FVO were re-screened for IgM to an irrelevant Ag (BSA) and a viral pathogen (DENV) that is uncommon in Yaoundé, Cameroon [48] and does not frequently cross the placenta [49,50] (Fig. 1B). MFIs were significantly lower for BSA (mean±SD = 105±189) compared to malarial Ags MSP1 (991±1,262, p <0.0001), MSP2 (1,130±1,811 MFI, p = 0.0003) and AMA1 (1,018±1,763 MFI, p = 0.0009). IgM levels for DENV-NS1 (91±157 MFI, p = 0.013) and DENV2-E (81±103 MFI, p = 0.012) were also lower than MSP1 IgM. These data show the IgM response to malarial Ags was antigen-specific and not due to cross-reactivity.

Timing of Fetal Pf IgM response to Pf antigens

Stratification of the IgM response by GA showed a strong trend for a decrease in the proportion of IgM-positive newborns with increasing GA, i.e., the highest percentage of Pf IgM+

were in the extremely preterm, followed by very preterm, moderate to late preterm, and term newborns (Fig. 2A). This trend was particularly significant for IgM to MSP1 ($p = 0.008$), MSP2 ($p = 0.0006$) and RESA ($p = 0.002$). The proportion of newborns who were positive for IgM to ≥1 Pf Ags also decreased steadily from 37% at 20–27 wks, to 33% at 28–31 wks, and 31% at 32–36 wks, followed by a sharp decline to 21% at ≥37 wks. These results demonstrate that B-cell sensitization occurred early in gestation and was not impaired in premature fetuses.

The level of IgM to Pf Ags in Ab-positive newborns was comparable in preterm and term newborns (Fig. 2B). Average IgM levels did not differ significantly among the different GA groups (one-way ANOVA: MSP1-FVO ($p = 0.960$), MSP2-FC27 ($p = 0.887$), AMA1 ($p = 0.826$), EBA175 ($p = 0.566$) and RESA ($p = 0.145$). Therefore, sensitized B cells of preterm and term fetuses produce similar levels of Pf IgM.

Since the overall B cell repertoire increases during fetal development, the number of Pf Ags recognized with increasing GA was evaluated (Fig. 3). Among the 169 newborns who were IgM-positive for ≥1 Pf Ag, 15% of preterm (11/75) and 14% (3/94) of term newborns made IgM to all 5 Pf Ags, i.e., MSP1 (FVO or 3D7), MSP2 (FC27 or 3D7), AMA1, EBA175 and RESA. In addition, 21.4% (3/14) of extremely preterm newborns had IgM to all 5 Pf Ag compared to 14% of term newborns, but the difference was not significant ($p = 0.433$). These findings suggest that the repertoire of B cell clones to Pf Ags was not restricted when the fetuses were exposed to Pf. A significant association was found between maternal anemia and presence of cord IgM to 3–5 Ags (56% were anemic) versus 1–2 Ags (29%) ($p = 0.001$) (S1 Table).

In summary, these data demonstrate that preterm fetuses can produce similar amount of IgM to as many Pf Ags as term fetuses. Interestingly, the proportion of IgM responders for each and at least one Pf Ag significantly decreased with GA.

Timing of fetal exposure to Pf antigen-IgG immune complexes

Peripheral plasma samples from 242 mothers who had preterm (n = 143) and term (n = 99) deliveries were tested for IC containing MSP1 and MSP2. Overall, 77/242 mothers (32%) had IC containing MSP1or MSP2. Since these mothers had the potential of transferring IC to their fetuses, corresponding cord blood samples were tested for IC (Fig. 4). Results showed that IC were uncommon in cord plasma prior to 32 weeks of gestation, but rose quickly, such that 65% and 55% of term newborns had IC containing MSP1 and MSP2, respectively (Fig. 4A). Likewise, the amount of IC in cord plasma rapidly increased after 32 weeks of gestation (Fig. 4B). No significant differences in IC levels were found between PM+ and PM- mothers (data not shown). Altogether, the GA-related pattern observed for cord IC (Fig. 4) was essentially the reverse of that found for cord IgM Ab (Fig. 2).

Factors associated with presence of fetal Pf IgM

To determine which factors were associated with presence of IgM to Pf Ags in cord blood, a logistics model was employed including GA, PM status, presence of IC, and maternal anemia (which was associated with breadth of the IgM response) (Table 1). In the adjusted model, odds of being Pf IgM positive were significant in neonates born very prematurely (adjusted OR [95% CI]: 20-31 weeks; 2.55 [1.14-5.85]) and born to PM+ mothers (2.37 [1.25–4.54]). When an interaction between GA and PM status was evaluated, neonates born to PM+ mothers with GA of 20-31 weeks (very premature) had an adjusted risk of 7.87 [2.1-32.2] and those with GA of 32-26 weeks had an adjusted risk of 3.46 [1.02-12.5]. Neither IC nor maternal anemia were associated with Pf IgM.

CBMC of term newborns produce more IgG than IgM

When CBMC from 64 Cameroonian term newborns were cultured for 5 days and supernatants were tested for both Pf IgM and IgG, supernatants from only 11% contained IgM ≥ 1 Pf Ag; whereas 95% contained Pf IgG (Table 2). The *in vitro* IgM levels correlated with the plasma IgM levels (MSP1-FVO $r = 0.712$, $p<0.001$; MSP2-FC27 $r = 0.890$, $p<0.001$; AMA1 Pearson $r = 0.395$, $p = 0.002$; and EBA175 $r = 0.880$; $p<0.001$) supporting the conclusion that IgM in cord plasma was produced by B cells of fetal origin. No significant difference in the proportion of *in vitro* cultures containing Pf IgM or IgG was found between neonates of PM+ and PM- mothers. The finding of Pf IgG responses in newborns of PM-negative mothers confirms that prenatal Pf exposure does not occur exclusively close to term delivery but also earlier in gestation.

DISCUSSION

The human fetus develops in an intra-uterine environment with relatively little exposure to foreign Ags. Therefore, little is known about the timing and nature of acquired immune responses during fetal development. Based on mid-20th century observations that fetal mice inoculated with cells from unrelated mouse strains were less likely to reject grafts from the donor strains after birth [51,52], it was originally thought that fetuses recognize all foreign Ags as 'self' and became tolerant to them. However, it is clear today that the fetal immune system can recognize and respond to pathogens. Pattern recognition receptors for pathogen-associated molecular patterns have been identified on various fetal cells [53–55] and the fetal inflammatory response syndrome has been describe following microbial invasion of the amniotic cavity [56]. In addition, Ag-specific T and B cell responses have been detected in term newborns exposed *in utero* to Pf [17,19,20,22,26,29–31,57]; to other pathogens including rubella virus, hepatitis B virus, *Toxoplasma gondii*, and *Trypanosoma cruzi* [58–60]; and to inhalant allergens [61,62] . However, data on when the fetus can respond to Ags and produces an adaptive response during development has been difficult to determine.

Malaria during pregnancy presents a unique opportunity to study the timing of fetal immune responses , since: i) women in high transmission areas test positive for Pf in peripheral blood smears an average of 4–5 times during the second and third trimester [63], ii) Pf-infected erythrocytes sequester in the intervillous space leading to placental pathology and sometimes congenital infection [3,64]; iii) p sequestration of Pf in at the maternal:fetal border can occur from 8–12 wks to term [14,15]; iv) mathematical modeling studies calculate that primigravid women can harbor chronic placental infections throughout pregnancy [65]; and v) Pf Ags can be transported across the placenta as immune complexes [6]. Although not every infective mosquito

bite results in PM and transplacental Pf Ag transfer, it is highly probable that many fetuses in endemic regions are exposed to Pf Ags from the end of the first trimester through term. The present study is the first comprehensive investigation of fetal Ab responses to Pf Ags in a large sample size of neonates (n = 682) born with GAs ranging from 20–44 wks, 34% of whom were preterm (<37 wks, n = 232).

Having Pf IgM in cord plasma was strongly associated with PM (Table 1). Overall, 25% (169/682) of Cameroonian newborns, including a 22-wk preterm newborn, were positive for IgM to Pf Ags, but not to Ags such as BSA and DENV proteins that they were unlikely to be exposed to *in utero* (Fig. 1A, B, C). Since the Ab response was Ag-specific, the IgM Abs had to be produced by conventional B-2 cells and not by B-1 cells that produce 'innate' polyreactive IgM. Although ~90% of human cord blood B cells express the mouse B-1cell marker CD5 [66,67], Griffin et al., [68–70] showed that human B-1 cells are $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ and only comprise about 6% of cord blood B cells. Thus, the potential for B-2 cell responses to Pf develops early in humans.

The ability of the acquired immune system to specifically respond to Ags depends, in part, on the diversity of T and B cell receptors. Rechavi et al., [38] reported an increase in fetal BCR and TCR repertoire diversity with increasing GA. Thus, one might expect preterm newborns to make IgM to fewer Pf Ags than term newborns. Interestingly, the preterm newborns in this study made similar amounts of IgM and recognized as many, if not more, Pf Ags than term newborns (Fig. 2B and 3). The actual breadth of Pf IgM in premature and term fetuses may have been underestimated, given that only 5 out of >700 *P. falciparum* proteins made by blood-stage parasites [71]) were used to detect Pf IgM in cord blood. Xi et al. [29] reported that cord plasma with high levels of IgM to Pf-infected erythrocytes detected >30 Pf protein bands on

Western blots. Thus, it is likely preterm and term newborns made IgM to additional Pf Ags. GA-related restriction in BCR repertoire diversity does not appear to be the limiting factor in determining the breadth of the fetal Pf IgM response.

Maternal anemia was associated with increased breadth of Pf IgM independently of GA. Maternal anemia has previously been linked with higher placental weight to birth weight ratios [72,73], meaning that the placentas of anemic compared to non-anemic women, have a large surface area. An increase in surface area could translate into an increase in the number of FcRn for IC transport resulting in an increase in transcytosis of diverse IC. Therefore, B cells in fetuses of anemic women may be exposed to more Pf Ags.

Pf IgM was detected in cord plasma from 22 weeks of gestation through term, demonstrating that *in utero* exposure to Pf occurring from this early age can elicit a B-cell response. However, 22 wks may not be the earliest time point because only one newborn in this study was younger (20 wks) and babies born before 20 wks were excluded from the study. Because, Pf iE begin sequestrating in the intervillous space from ~12 wks and fetal spleens are seeded with B cells over the next 2 months, it is plausible that Pf IgM would be produced by some fetuses aged 14–20 wks.

The proportion of newborns with IgM to Pf Ags decreased with GA (Fig. 3A). After adjusting for PM and anemia, early gestation (specifically 20–31 wks) and maternal PM were associated with higher odds of having Pf IgM (Table 1). This implies that for a given equivalent exposure to Pf, fetal B cells at term are less likely to make Pf IgM than B cells preterm. *In vitro* experiments showed that B cells in term Cameroonian newborns had class-switched from Pf IgM to IgG (Table 2). This finding supports previous reports of class-switch and somatic

recombination occurring *in utero* [20,74] and suggests that GCs may be formed in fetuses exposed to Pf Ags. It remains to be elucidated if IgG-secreting cells in term newborns are short-lived or long-lived plasma cells and if Pf-memory B cells are generated *in utero*.

The presence of Pf IC in fetal circulation increased with GA (Fig. 4), with a rapid increase during the third trimester. This increase corresponds to the period of rapid placental growth and an increase in surface area for transport of nutrients, Ab and other growth factors. Also IC transfer to the fetus may be influenced by the size of maternal IC. Since preterm mothers had higher parasite density, their IC most likely form under conditions of relative Ag excess. Such ICs are small, do not fix complement, and are not easily deposited on trophoblasts for transplacental transfer to the fetus. Greater Pf IC deposition occurs in placentas with relatively low parasite densities [75]. In the literature, the relevance of ICs in eliciting immune responses in the human and animal hosts is contentiously debated, as ICs can enhance Ag processing and T-cell stimulation or suppress responses by masking Ags and blocking T-cell/B-cell interactions or exert no effect at all [76]. Since Pf IgM occurred early and transfer of IC later in gestation, Pf IC may play a small role in that initial *in utero* priming if naïve B cells.

In conclusion, we propose a model for the timing of fetal exposure and Ab responses to Pf (Fig. 5). The fetus may be exposed more than once to Pf Ags, from early in the second trimester through term. In response to Pf Ags, both preterm (including extremely preterm) and term fetuses are capable of producing Abs that specifically recognize Pf Ags; however, the Ab response during the second trimester are predominantly IgM while term fetuses predominantly make IgG to Pf Ags.

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FIGURES AND TABLES

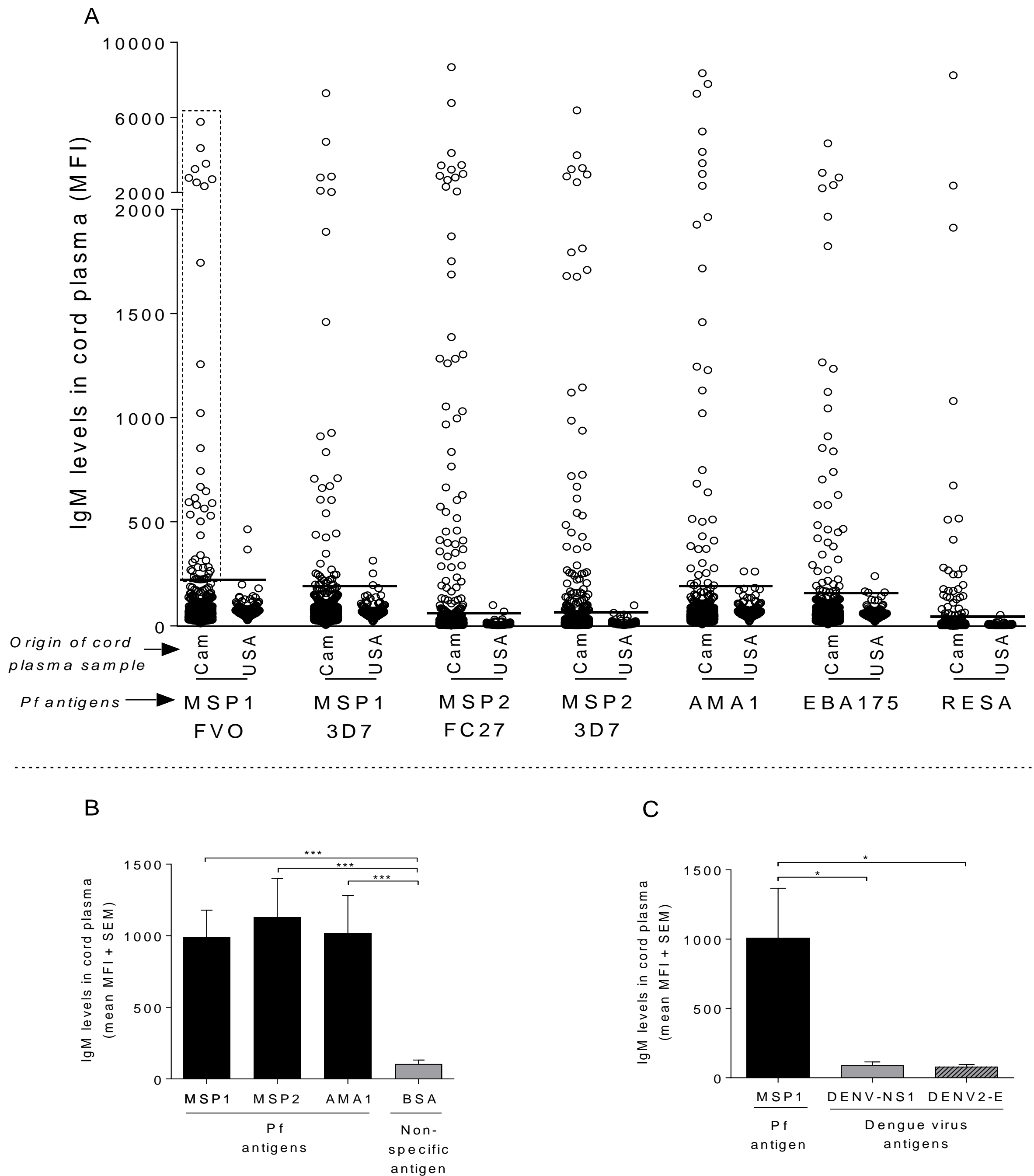


Figure 1. IgM to Pf antigens in Cameroonian and USA cord plasma samples. (A) The level of Pf IgM Abs in 682 Cameroonian (Cam) and 100 American cord plasma samples. The horizontal lines represent the cut-off for IgM positivity (mean + 2 standard deviations of the USA controls).

The vertical dotted box within Cam MSP1-FVO shows the 45 samples used in Fig. 1B and C. The binding specificity of 45 cord samples positive for IgM to MSP1 were tested on (B) BSA and (C) two Dengue virus proteins (DENV-NS1 and DENV-2 E). Mean MFI for a dengue IgM positive control was 4,003 MFI for DENV-NS1 and 847 MFI for DENV2-E (data not shown). Statistical significance was assessed using paired t test. The p values: *$p<0.01$ and ***$p<0.0001$. MFI = Median Fluorescence Intensity

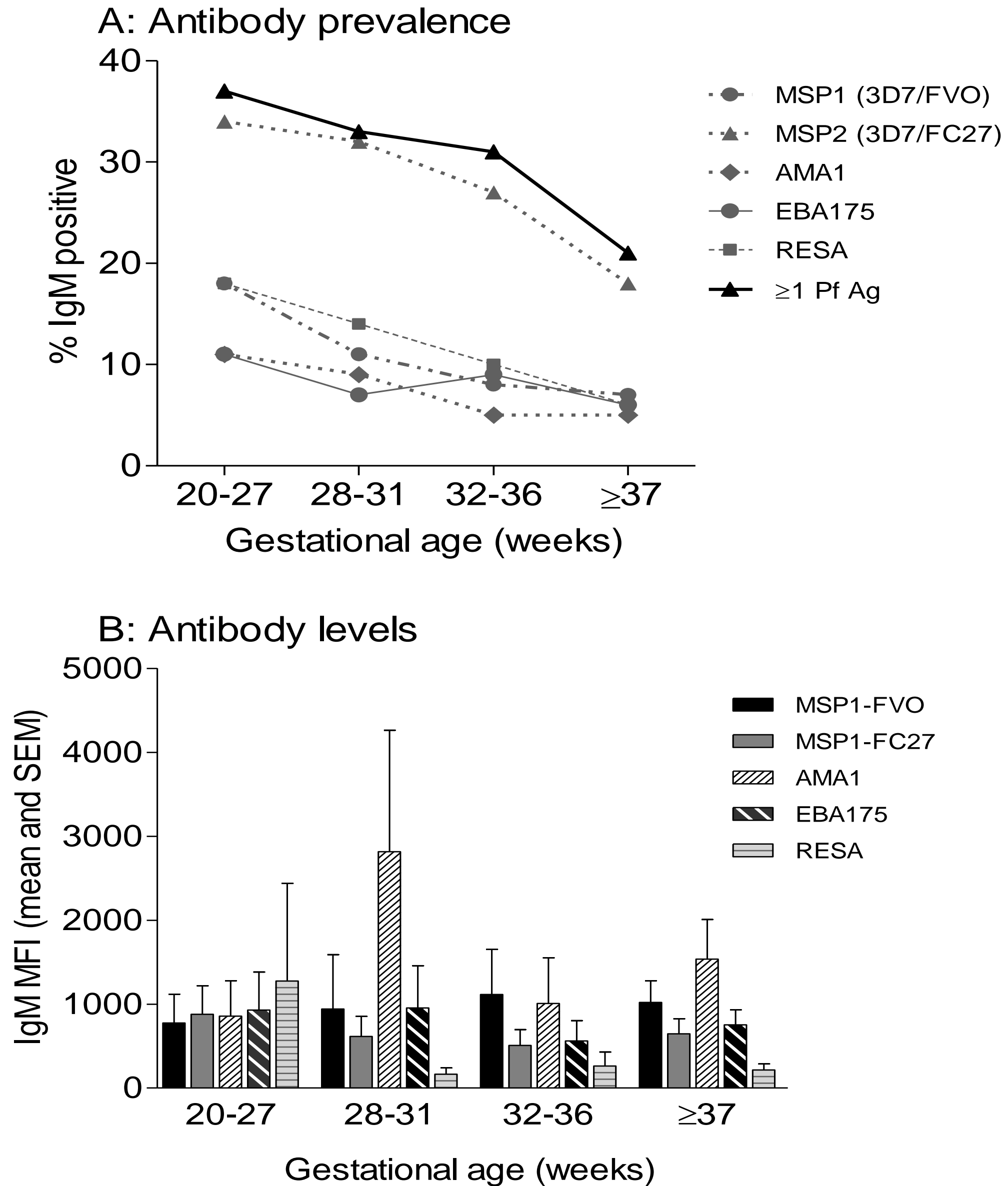


Figure 2. Timing of the prenatal Pf IgM response. (A) The percent of newborns (n = 652) positive for IgM to Pf Ags. The proportion of IgM-positive newborns decreased with gestational age, Chi-square test for trend: MSP1 (3D7 or FVO), p = 0.008; MSP2 (3D7 or FC27), p = 0.0006; AMA1, ns; EBA175, ns; RESA, p = 0.002. (B) Amount of IgM Ab present for newborns who were Ab positive for the specified antigen. Average IgM levels did not differ significantly with GA for any of the antigens (ANOVA).

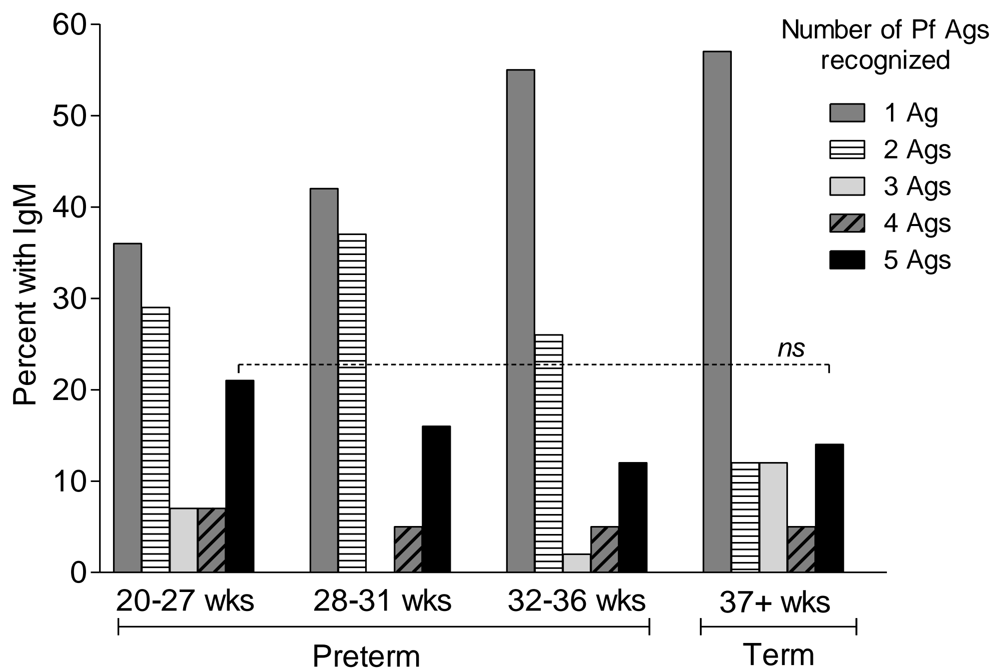


Figure 3. Breadth of Pf IgM response in different gestational age groups. The 169 Cameroonian newborns who were positive for cord IgM to ≥ 1 Pf Ag were included, i.e., n = 75 preterm [n = 14 (20-27 weeks), n = 19 (28-31 weeks), n = 42 (32-36 weeks)] and n = 95 term. For each GA group, the bars represent the proportion of positive newborns that produced IgM recognizing 1, 2, 3, 4 and 5 Pf blood-stage Ags. There were no significant (ns) differences between different GA groups.

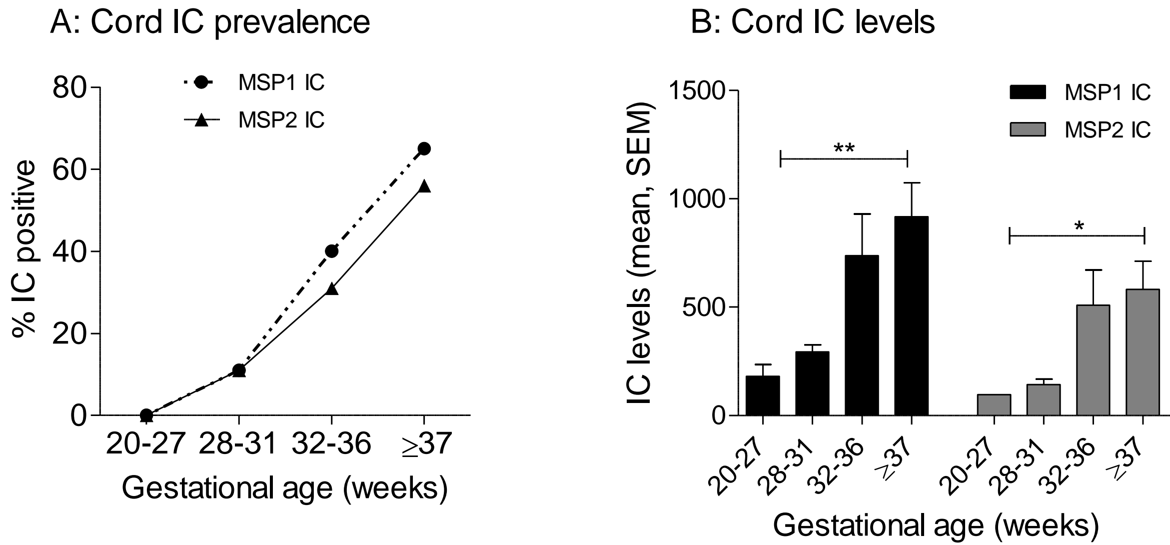


Figure 4. Timing of fetal exposure to maternal immune complexes (IC). Peripheral plasma samples from 242 mothers were tested for Pf-specific IC and 77/242 had MSP-1 C and 77/242 had MSP2 IC. Cord plasma from their 77 newborns were assessed for IC transfer. (A) The percentage of cord blood samples with IC increased with GA all neonates. B. Average levels of Pf-specific IC in cord plasma. P values were calculated using one-way ANOVA with post-test for linear trend: *p<0.01 and **p<0.001.

Table 1. Influence of gestational age and placental malaria on production of Pf IgM to MSP1 and MSP2 *in utero*

Criterion	Subgroup	% of n	Cord IgM (MSP 1 or 2)			
			Crude odds ratio		Adjusted odds ratio	
			OR	95% CI	OR	95% CI
GA, weeks [a]	20-31[b]	27%	1.76	[0.85-3.62]	**2.55**	[1.14-5.85]
	32-36	32%	1.55	[0.77-3.13]	1.97	[0.92-4.29]
	≥37	41%	Ref.		Ref.	
PM [a]	Yes	40%	**1.93**	[1.08-3.48]	**2.37**	[1.25-4.54]
	No	60%	Ref			
Cord IC (MSP1 or 2) [a]	Yes	16%	1.22	[0.55-2.58]	1.48	[0.63-3.35]
	No	84%	Ref		Ref.	
Maternal anemia [a]	Yes	33%	1.80	[0.99-3.26]	1.48	[0.79-2.75]
	No	67%	Ref		Ref.	
GA:PM interaction [c]	20-31: PM-yes	7%	**7.93**	[2.21-31.41]	**7.87**	[2.11-32.31]
	32-36: PM-yes	10%	**3.70**	[1.14-12.84]	**3.46**	[1.02-12.45]
	≥37: PM-yes	23%	2.06	[0.74- 6.31]	1.92	[0.69-5.94]
	20-31: PM-no	20%	1.79	[0.61-5.65]	1.85	[0.62-5.97]
	32-36: PM-no	22%	1.96	[0.70-6.05]	1.88	[0.66-5.92]
	≥37 : PM-no	18%	Ref		Ref.	

[a] Each variable was adjusted for all other variables (excluding the interaction variable) in the table. [b] Extremely preterm (20-27 wks) and very preterm (28-31wks) newborns were grouped together for this analysis. [c] Interactions between GA and PM were adjusted for maternal anemia and cord IC. Note: OR, odds ratio; CI, confidence interval; Ref., reference subgroup; GA, gestational age; PM, placental malaria; IC, immune complex; n = 242 newborns.

Table 2. Percent of Cameroonian neonates whose cord blood mononuclear cells produced Pf specific IgM and IgG *in vitro*

	Cord plasma IgM			**In vitro IgM**			**In vitro IgG**		
Antigen	PM neg. n=24	PM pos. n=35	All n=64	PM neg. n=24	PM pos. n=35	All n=64	PM neg. n=24	PM pos. n=35	All n=64
MSP1	0	6	3	0	3	2	67	80	77
MSP2	4	14	9	0	3	2	33	49	45
AMA1	4	17	11	4	3	3	83	80	81
EBA175	4	9	6	8	3	5	50	51	53
RESA	0	9	5	8	11	9	50	63	56
≥1 Pf Ag	8	20	14	12	11	11	92	97	95

Data are presented as percent of neonates. PM status was not available for 5 mothers. ≥1 Pf Ag = had Ab to one or more tested Ag in cord plasma or CBMC supernatant.

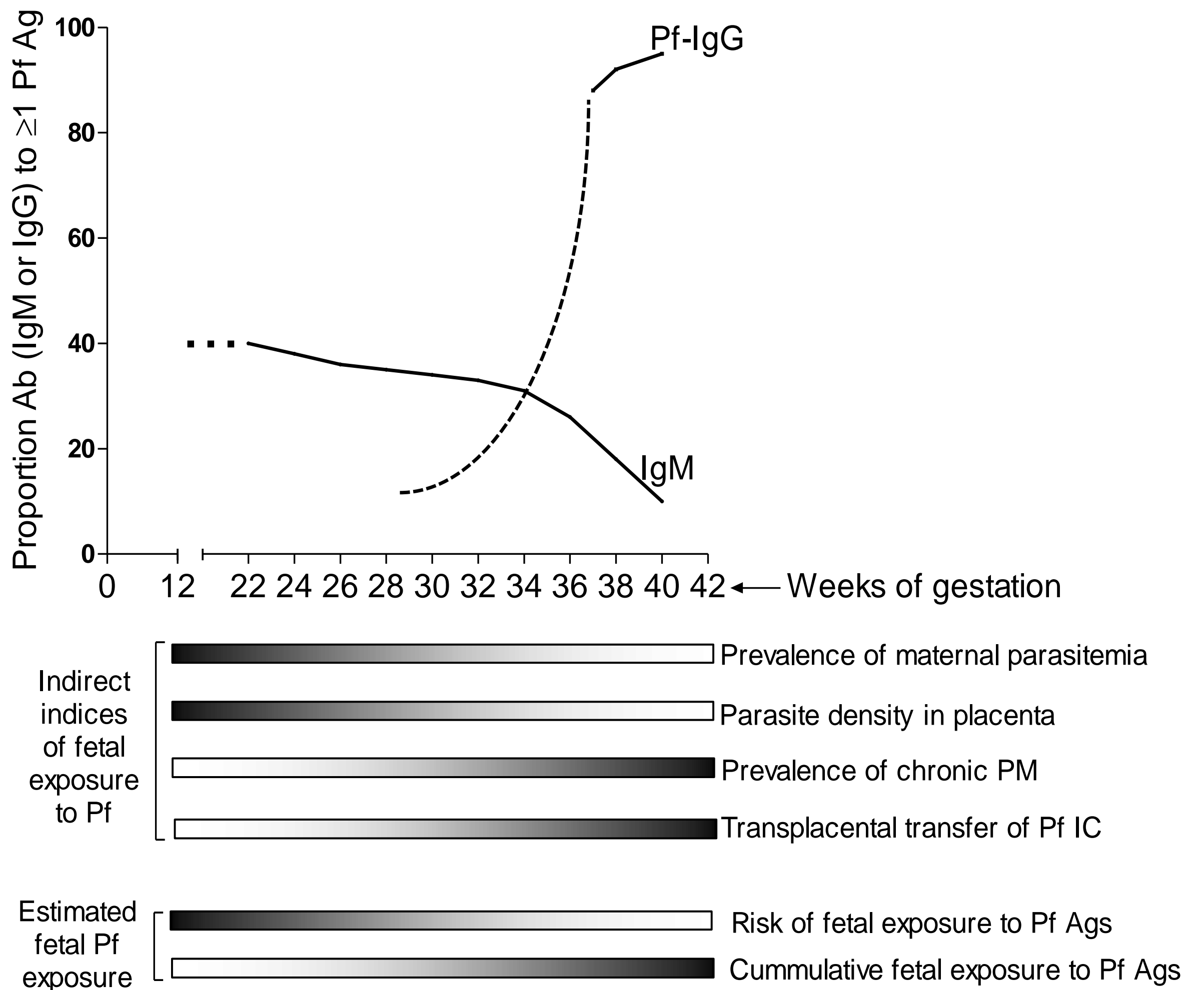


Figure 5. Model for the timing of fetal exposure and Ab responses to Pf Ags. The proportion of fetuses in this study making IgM or IgG to at least one Pf Ag is represented by solid and dotted lines. Solid portion of lines represent data from the present study. Dotted portion of lines are hypothetical projections. The timing of exposure to Pf Ags is shown in horizontal bars (Gray scale: Dark = High, Light = Low). Infected erythrocytes sequester in the IVS towards the end of the first trimester (~12 wks). The higher incidence of maternal malaria and higher placental parasitemia at 13-20 wks increase the risk of exposure and a significant proportion of fetuses respond by making Pf IgM during the second trimester. As gestation advances, fetal B cells

receive secondary Pf exposures and class-switching to IgG occurs. The proportion of fetuses with Pf IgM drops rapidly at term while the proportion of Pf IgG responders increases to almost 95% at 40 weeks.

Supplementary table 1. Factors associated with the breadth of Pf IgM in cord plasma

Factors	Number of Pf Ags recognized by Pf IgM responders		P Value	Adjusted P Value[a]
	1 or 2 Pf Ags	3–5 Pf Ags		
Number of deliveries	123	46		
Fetal factors				
Live births[b]	113 (92%)	42 (91%)	1.000	0.484
Twin delivery[b]	7 (6%)	0 (0%)	0.192	0.987
Birth weight in grams[c]	2675±936	2785±825	0.497	0.999
Placental weight in grams[c]	571 ±155	588 ±147	0.555	0.750
Preterm delivery[b]	58 (47%)	17 (37%)	0.297	0.277
Maternal factors				
Age in years[c]	26.9±6.7	26.2±6.7	0.516	0.500
Primigravid women[b]	29 (24%)	15 (33%)	0.242	0.228
Anemia[b]	34 (28%)	23 (50%)	**0.003**	**0.001**
Malaria positive[b]	35 (28%)	17 (37%)	0.351	0.552
Parasite density in %[d]	0.05 [0.01 – 0.73]	0.10 [0.01 – 0.43]	0.619	0.506

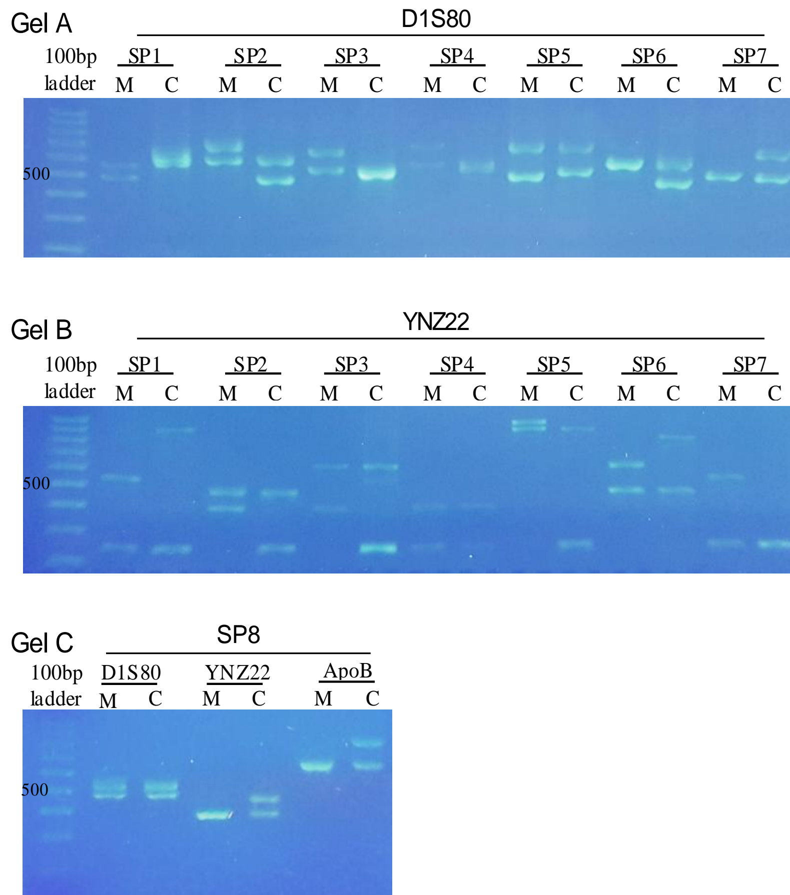
The analyses include 169 Cameroonian newborns who had IgM to at least one of 5 Pf Ags (MSP1-FVO or 3D7, MSP2-FC27 or 3D7, AMA1, EBA175, RESA).

[a] Multivariate analyses (logistic regression model including all variables in the table).

[b]Number percent (Fisher's exact test)

[c]Mean±standard deviation (Student t test)

[d]Median [25th – 75th percentile] (Man-Whitney U test)



Supplementary figure 1. Genotyping of VNTR loci for detection of maternal blood admixture with cord blood. Bands represent peripheral maternal blood (M) and cord blood (C) VNTR alleles for mother-stillborn sample pairs (SP). Eight SPs (out of 37 SPs analyzed) are represented. Gel A shows D1S80 alleles in SP1through SP7. Maternal DNA was not detected in cord blood compartments of SP1, 2, 3, 4 and 5. SP6 and SP7 were not informative at the D1S80 locus since the mothers were homozygous. Gel B shows YNZ22 alleles for the same SPs. SP1, 2, 3, 5, 6 and 7 were informative, showing no maternal DNA in cord blood. SP4 was not informative at the YNZ22 locus. Overall, analyses of both D1S80 and YNZ22 successfully ruled out maternal DNA in cord blood of 35 (out for 37) SPs. Gel C shows one (SP8) of the two SPs that were not informative after genotyping of all three VNTR loci (D1S80, YNZ22 and ApoB).

CHAPTER 3

INCREASED SUSCEPTIBILITY TO *PLASMODIUM FALCIPARUM* IN INFANTS IS ASSOCIATED WITH LOW, NOT HIGH, PLACENTAL MALARIA PARASITEMIA

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Key words: Malaria, parasite density, placenta, infant

FOOTNOTE

Potential conflicts of interest. All authors report no conflicts of interest.

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ABSTRACT

Background: Placental malaria (PM) may increase susceptibility to *Plasmodium falciparum* (Pf) in early childhood. The role of placental parasitemia at delivery as a predictor of susceptibility was evaluated.

Methods: Seventy-two newborns were classified as PM-negative (PM–), PM-positive with low parasitemia (PM+Lo) and PM-positive with high parasitemia (PM+Hi) based on placental parasitemia at delivery, and were monitored monthly through the first year of life for malaria.

Results: Time to first infection was significantly shorter in PM+Lo compared to PM– infants (p=0.001, by microscopy; p=0.002, by PCR), but not different between PM– and PM+Hi infants. The adjusted hazard ratios (95% confidence interval) for infections detected by microscopy/PCR were 2.8 (1.3–6.0)/3.9 (1.8–8.4) for PM+Lo and 1.5 (0.7-3.1)/1.5 (0.7-3.4) for PM+Hi, compared to PM– infants. Being PM+Lo at delivery was also associated with fewer maternal infections and lower peripheral parasitemia during pregnancy, and lower likelihood of Pf DNA in cord blood.

Conclusion: Low placental parasitemia is associated with reduced fetal exposure to Pf and increased risk of malaria during infancy. Therefore, malaria control regimens such as sulfadoxine-pyremethamine that reduce but do not eliminate placental Pf in areas of moderate drug resistance may increase the risk of malaria in infants.

INTRODUCTION

When pregnant women become infected with *Plasmodium falciparum* (Pf), parasitized erythrocytes adhere to placental villi and accumulate in the intervillous space (IVS), causing a condition referred to as placental malaria (PM) [1]. In addition to eliciting adverse pregnancy outcomes [2], PM has been identified as an independent risk factor of increased early childhood morbidity and mortality. In general, infants born to PM-positive (PM+) mothers have a shorter time to first infection [4,5] and first clinical episode of malaria [6,7], higher incidence of Pf infections early in life [3], lower hemoglobin levels [4], and higher risk of dying [5] compared to infants of PM-negative (PM–) mothers. However, risk of malaria is not homogeneous among infants of PM+ mothers, with some infants having similar risk outcomes as PM- infants [5–9]. There is therefore a need to identify specific prenatal factors associated with increased susceptibility to malaria in infants of PM+ mothers.

Factors thought to be important in modifying the susceptibility of infants include gravidity and the timing of infection during pregnancy. For example, infants of multigravid women in Tanzania had their first microscopic infection 12 weeks earlier than infants of primigravid women [6]. Likewise, the adjusted hazard ratio (AHR) for clinical episodes of malaria during the first 30 months of life was approximately two times higher in Gabonese infants born to multigravidae than primigravidae [10]. When maternal Pf infections occurred within 3 months prior to delivery, Ugandan infants were at higher risk of infection than when mothers were infected earlier in pregnancy [7]. Since multigravidae tend to have lower parasitemias than primigravidae and women infected only late in pregnancy would be infected for a short time, it is possible that exposure to low levels of malarial antigens, rather than higher

levels, influences early childhood susceptibility; however, no study has directly investigated the influence of placental parasite density on the risk of malaria after birth.

Therefore, the primary objective of the present study was to determine if susceptibility to Pf of Cameroonian infants was modified by maternal placental parasitemia at delivery. We hypothesized that infants born to mothers with low placental parasitemia (PM+Lo) would have shorter times to first Pf infection and experience more infections during the first year of life than infants whose mothers had higher parasitemias (PM+Hi) or mothers who were negative for Pf (PM–). The secondary objective was to determine if PM+Hi mothers had more infections and higher peripheral parasitemia during pregnancy that would, in turn, expose the fetus to higher levels of Pf. Fetal cord blood was also examined for evidence of Pf parasites and their products.

MATERIALS AND METHODS

Study Design

The study was conducted in the rural village of Ngali II, Cameroon, between January 2001 and May 2005. In this area, *P. falciparum* transmission is perennial with two rainy (March–May, September–November) and two dry (December–February, June–August) seasons and has an estimate entomological inoculation rate of 257 infective bites/person/year [11]. After obtaining written informed consent, 94 mother-newborn pairs were enrolled at delivery and infants were subsequently followed throughout the first year of life (Figure 1). Most women delivered at the village health center, at which time ~2 mL of peripheral and placental IVS blood, 5x5x5 cm placental tissue biopsy and ~5 mL cord blood were collected. Placental and cord samples were not collected for 14 home deliveries as the placentas were buried before the mother-newborn pair came to the health center.

Newborns were seen at 8 scheduled visits (Figure 1). In addition, they were monitored at home by four village health workers who arranged for sick children to be examined by the project physician. At all visits, the infant's axillary body temperature was measured and finger-prick blood collected (<0.5 mL <6 months old and 0.5–1 mL >6 months). Infants who were blood-smear positive were treated for malaria according to the government policy for treatment of childhood malaria.

The mothers of 26 infants had been followed monthly from ~14 weeks of pregnancy through delivery [11]. Peripheral blood samples were collected at each maternal visit.

Parasitological methods

During pregnancy, peripheral blood samples were stained with Diff-Quick (Baxter Scientific, IL) and examined for Pf-infected erythrocytes (iE) by microscopy. Pf parasitemia (iE/µL) was determined by counting the number of iE per 200 WBC and multiplying by the individual's WBC count.

At delivery, IVS and cord blood samples were examined for Pf by microscopy as described above. Placental tissue sections were also examined for Pf parasites and scored as presence/absence of Pf. PM+ was defined as the presence of Pf parasites in IVS blood and/or placental histosection. The amount of Pf DNA in cord blood was determined using nested quantitative PCR (qPCR), consisting of a conventional PCR step followed by qPCR. The assay was performed as previously reported [12], with minor modifications: DNA was extracted from 100 µL of packed red cells premixed with 100 µL of PBS; and GoTaq master mix (Promega, WI) and SsoAdvanced SYBR-Green Supermix (Bio-Rad, CA) were used for the PCR and qPCR steps, respectively. DNA from *in vitro* 3D7 parasites was quantified by Nanodrop and serially diluted (10 fold) to make standard series.

During the first year of life, Pf infections were detected by microscopy (as described above) and by nested PCR. DNA was extracted using the NucleoSpin kit (MN, Germany) from 200 µL of blood and the Pf 18S ribosomal RNA gene was amplified as described previously [13,14]. Amplicons were electrophoresed through 1.5% agarose gel (5V/cm) and SYBR-stained Pf bands were visualized. All Pf DNA-positive samples were evaluated for polymorphisms in allelic families of MSP1 block 2-4 (K1, MAD20, and R033) and MSP2 (FC27 and 3D7) [11,14]. An infant was considered to have a "new infection" if one or more Pf alleles were detected that

had not been present at the preceding visit. Finally, standard strip-based electrophoresis was used to assess hemoglobin genotypes HbAA and HbAS using infant blood samples

Immunological methods

To assess the degree of protection from placental malaria during pregnancy, the levels of IgG and proportion of high avidity IgG antibodies (Abs) to full-length VAR2CSA at mid-pregnancy (~5months) were measured as previously described [15].

To determine the level of passive immunity to Pf acquired by infants *in utero*, cord blood was tested for IgG to the merozoite antigens (Ags) MSP1 and EBA175 by Luminex assay. Briefly, 1 µg of each recombinant Ag [16] was coupled to 1 million beads with different spectral addresses and then 50 µL of beads (3000 of each Ag) was mixed with 50 µL of diluted plasma (1:100 in PBS-1%BSA) and incubated for 1 h on a shaker at room temperature. After two washes with PBS-0.05% Tween and one wash with PBS-1%BSA, 100 µL of 1:250 dilution of 2µg/ml. Phycoerythrin-conjugated goat anti-human IgG (Jackson IR, PA) was added to each well and incubated for 1 h. Beads were washed, resuspended in 100 µL of PBS-1%BSA and analyzed in the LiquiChip 200 (Luminex, TX). Results are reported as median fluorescence intensity (MFI).

Other laboratory methods

To further quantify Pf in cord blood, cord samples were tested for Pf-histidine rich protein-2 (HRP2) using CELISA kit (Cellabs, Australia). Whole blood was prepared by recombining equal volumes of cyropreserved plasma and packed RBC. The assay was conducted in duplicate using 100ul of blood per well following the manufacturer’s instructions. Serial

dilutions of the positive-control provided in the kit were used to generate standard curves and the cut-off for positivity were established as specified by the manufacturer.

Statistical methods

The susceptibility of infants to malaria was assessed using data from 72 infants who were followed for ≥4 months. The primary baseline predictor was placental parasitemia at delivery: PM– (no Pf detected, n = 36), PM+Lo (PM parasitemia <median of all PM+) and PM+Hi (PM parasitemia >median).

Study outcomes were time to first post-natal infection and number of times positive. Kaplan-Meier survival curves and log-rank tests were used to compare the median time to first infection. Right-censoring was done either at withdrawal from study, death, age 1 year, or end of study period, whichever occurred first. Cox regression was used to assess hazard ratios (HR) and 95% confidence interval (CI). HR was adjusted for covariates that could potentially influence susceptibility to infection, including gravidity [6,8]; residence and season at birth [11,17]; presence of fetal hemoglobin that influences parasite growth [18]; HbAA versus HbAS [19]; and Pf IgG in cord plasma [20,21]. Cord plasma Pf IgG levels were ranked as follows: rank 1 ($>67^{th}$ percentile), rank 2 (33rd to 66th percentile) and rank 3 ($<33^{rd}$ percentile). Ranking was performed because unit changes in IgG MFI (range 500 to 24,000 MFI) were not expected to have significant effect on infant outcome. Finally the average number of Pf infections was compared between groups using the Student t test.

For maternal data and parasite-related factors in cord blood, continuous variables were compared across study groups using student t tests and ANOVA post-test for linear trends. Categorical variables, such as proportion positive, were compared using Chi-square test.

RESULTS

Time from birth to first infection

Half of the mothers were PM+ (n=36) with placental parasitemia ranging from <14 to 10,521 Pf/μL of IVS blood, with a median parasitemia of 25 iE/μL. Among the PM+ mothers only 6% were multigravidae compared to 78% of PM– mothers, p = 0.03.

The mean duration of follow up for the 72 infants was 10.2 ± 2.9 months, with 60% followed until the last scheduled visit. One infant died at 10.3 months. At least one Pf infection was detected in 89% (64/72) of infants during the first year of life, with 45% (29/64) of first infections being submicroscopic. Time to first infection detected by microscopy was 5 months (95% CI = 4.1–6.2); whereas, first infections detected by PCR occurred significantly earlier at 3.3 months (2.9–4.1), p = 0.01.

The median time to first microscopic infection was significantly shorted in infants born to PM+ mothers (4.1 months) compared to PM– mothers (6.4 months), p = 0.009 (Figure 2A). Further stratification showed that infants in the PM+Lo group experienced a significantly shorter time to first microscopic infection compared to the PM– (3.4 versus 6.4 months, respectively, p = 0.001); however, there was no difference in time to first infection between the PM+Hi and PM– groups (5.1 versus 6.4 months, p = 0.2) (Figure 2B). A similar pattern was observed for time to first PCR-detected infection (Figure 2C and 2D), i.e., 2.8 months versus 4.0 months for PM+Lo and PM– (p=0.002), with no difference between PM+Hi (4.1 months) and PM– (4.0 months). By 4 months, all babies in the PM+Lo group had experienced ≥1 Pf infections, whereas only 50% of infants in the other two groups had become infected. Clinical episodes of malaria (fever plus microscopic parasitemia) were diagnosed in 17 infants (24%), but no differences in time to

malaria episodes were seen between the groups (data not shown). Since the mean duration of infant follow-up was similar across all groups, i.e., 10.2 ± 2.5 (PM–), 10.7 ± 3.1 (PM+Lo) and 9.6 ± 3.5 (PM+Hi) months (p = 0.494), variations in follow-up duration did not account for the PM+-associated differences in infection frequency. Thus, infants in the PM+Lo group had an increased susceptibility to malaria compared to infants in the other groups.

Multivariate assessment of the risk of infection

After adjusting for multiple covariates, the HR of infants experiencing a microscopic infection was 2.8 (1.3–6.0) for PM+Lo and 1.5 (0.7–3.1) for PM-Hi (Table 1). Adjusted HR of infections detected by PCR were 3.9 (1.8–8.4) and 1.5 (0.7–3.4) for PM-Lo and PM+Hi, respectively. Thus, infants born to PM+Lo mothers had a 2.8 times and 3.9 times higher risk of having microscopic and PCR infections, at different time points during the first year of life compared to infants in the PM– group.

Number of times infants were positive for Pf

The 72 infants had a total of 180 microscopic and 253 PCR infections, with 29% (73/253) being submicroscopic. The mean number of new microscopic infections was significantly higher in PM+Lo (3.4 ± 2.3) compared to PM– infants (2.1 ± 2.0), p = 0.04 (Figure 3A). The PM+Lo also experienced more PCR infections (4.7 ± 2.2) than the PM– (3.1 ± 2.1, p = 0.02) and PM+Hi (2.7 ± 2.1, p = 0.01) groups (Figure 3B). Together, time to first infection, number of infections and hazard ratios show that having low placental parasitemia at delivery is associated with increased susceptibility of infants to malaria.

Determining how maternal parasite burden during pregnancy relates to placental Pf parasitemia at delivery

Overall, PM+Hi mothers had more Pf infections (55% of longitudinal peripheral blood samples were slide Pf positive) and higher average parasitemia (9,193 Pf/µL of peripheral blood) during the second and third trimesters compared to the PM+Lo (38% and 3,161 Pf/µL) and PM– mothers (15% and 1,629 Pf/µL) (Figure 4A and 4B). In contrast, mid-pregnancy levels of IgG to VAR2CSA and proportion of high avidity anti-VAR2CSA Ab were lower in PM+Hi (MFI = 4,055 ± 2,989; percent high avidity Ab = 6.4 ± 2.4%) compared to PM+Lo (5,544 ± 3,323; 8.8 ± 6.3%) and PM– (9,279 ±6,060; 21.0 ±12.2%) women (Fig. 4C and 4D).

Furthermore, the proportion of newborns with Pf-DNA in cord blood increased with placental parasitemia (4.8% for PM+Lo versus 38.9% for PM+Hi, p = 0.0006) (Table 2) and a case of congenital infection (154 Pf/µL) was identified in the PM+Hi group. Cord HRP2 positive rates and amount/ml did not differ significantly between the PM+Lo and PM+Hi groups. Collectively, the chance of fetal exposure to Pf and/or malarial Ags was higher in PM+Hi than PM+Lo pregnancies.

DISCUSSION

The present study supports the concept that infant susceptibility to malaria can be conditioned by prenatal exposure to Pf. The current findings add to the growing body of evidence that PM modifies the risk of malaria during infancy and demonstrate for the first time that placental parasite density is a major determinant for susceptibility to Pf infections. The observation that survival of infants without infection is shorter in PM+Lo group than in all PM+ combined, shows that much of the PM-associated infant susceptibility to malaria is paradoxically accounted for by low placental parasitemia cases.

Samples of IVS blood cannot be obtained during pregnancy; however, based on longitudinal peripheral blood smear data, PM+Lo mothers seemed to have had a relatively low exposure to Pf during pregnancy. The PM+Lo were infected less frequently and experienced lower average parasitemia compared to PM+Hi women (Figure 4). Also, ~67% of the PM+Lo women were multigravidae compared to only 20% of the PM+Hi women. Mathematical modeling of gravidity-dependent immunity to PM suggests that placental iE are more rapidly cleared in multigravid than primigravid women [22], which further indicates that the PM+Lo women were likely to have had lower placental Pf densities through pregnancy.

Placental parasite density can affect fetal exposure to Pf. As shown here and previously in studies in Malawi [23], detection of iE in cord blood by microscopy is associated with high placental Pf parasitemia. Also, the PM+Hi newborns were more likely to have Pf-DNA presence in their cord blood. HRP2 rates were similar between the PM+Hi and PM+Lo groups, possibly because HRP2 can circulate weeks after parasites and Pf DNA have been cleared [24]. In general, newborns in the PM+Lo group were most likely exposed to lower amounts of Pf Ag and Pf pathogen associated molecular patterns (PAMP) compared to the PM+Hi newborns.

From an immunological standpoint, the question of how Pf exposure programs the fetus for increased or reduced susceptibility to malaria is pertinent. Peripheral tolerance to Pf Ags induced by T regulatory cells (Tregs) may be responsible for some of the increase in susceptibility. Developmental $CD4^+$ T cells are biased towards Tregs which constitute ~15-20% of $CD4^+$ T cells of fetal lymphoid organs compared to <5% in adults [25,26]. Pf Ag exposure can induce the expansion of fetal Tregs which, in turn, suppresses Ag-driven responses by T effector cells [27–29]. In contrast, other studies have shown strong Th1 and Th2 effector responses to Pf Ags in newborns of PM+ mothers, indicating that *in utero* Pf exposure can also result in T cell sensitization [16]. Interestingly, a study in Kenya demonstrated T cell sensitization to Pf in some newborns and T cell tolerance in other newborns of the same cohort [30]. The tolerant newborns had ~2-fold increase in risk of malaria during the first three years of life compared to the sensitized newborns. The specific factors responsible for the dichotomy in fetal immune response to Pf Ag have remained elusive. We propose that the outcome of sensitization or tolerance to Pf *in utero* is influenced by the amount of Pf to which fetuses of PM+ mothers are exposed i.e., low exposure inducing tolerance and increased risk of infant malaria and high exposure inducing sensitization and reduced risk of malaria.

Data from animal studies support our proposition. The Ag dose required to stimulate the suppressive functions of Tregs in mice is much lower than needed for T effector cells to proliferate [31], thus a relatively low Pf Ag concentration *in utero* would stimulate more Tregs than T effectors leading to overall suppression of response to Pf. In addition, mouse transgenic T cells with OVA TCR, proliferated very little in the presence of low OVA doses and Tregs, but overcame Treg suppression when the OVA dose was increased ~100 fold [32]. Therefore high amounts of Pf may also be required for T effectors to escape Treg suppression *in utero*.

In summary, results from the current and other studies provide evidence that placental parasitemia may be a good indicator of the amount of prenatal Pf exposure and can be used to predict susceptibility to malaria during infancy. In this study, infants were dichotomized into two groups, i.e., below and above the median parasitemia of 25/µL. Clearly, additional studies are needed to determine the best cut-off parasitemia if that threshold should be based on Pf densities in IVS blood, impression smears, or tissue sections. Nevertheless, infants of women who are Pf-negative by blood smear of IVS blood, but positive by histology are potentially at high risk of malaria during infancy.

Although the relatively small sample size of infants in the study was a limitation, the current study reproduced results from larger studies that reported increased risk of infant malaria in infants born to PM+ compared to PM– women. The study was unique in many ways. For example, Pf in infants was detected by PCR, in addition to microscopy, which increased sensitivity of parasite detection and provided better information on time to first infection. In addition, new infections were distinguished from persistent infections by parasite genotyping. Previous studies measuring age-specific rates of Pf may have overestimated the frequency of new infant infections by scoring all slide-positive infections as new [3,6].

In conclusion, the translational implications of the current findings are significant. If low placental parasitemia increases the risk of infant malaria, then malaria control interventions during pregnancy that do not clear Pf from the placenta, and only reduce the Pf density, could increase the risk of malaria in the offspring. This is particularly important because there is are reports from parts of Africa of decreasing efficacy of sulfadoxine-pyremethamine in eliminating parasites in pregnant women [33,34]. More effective interventions that clear Pf in pregnant women are needed.

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FIGURES AND TABLES

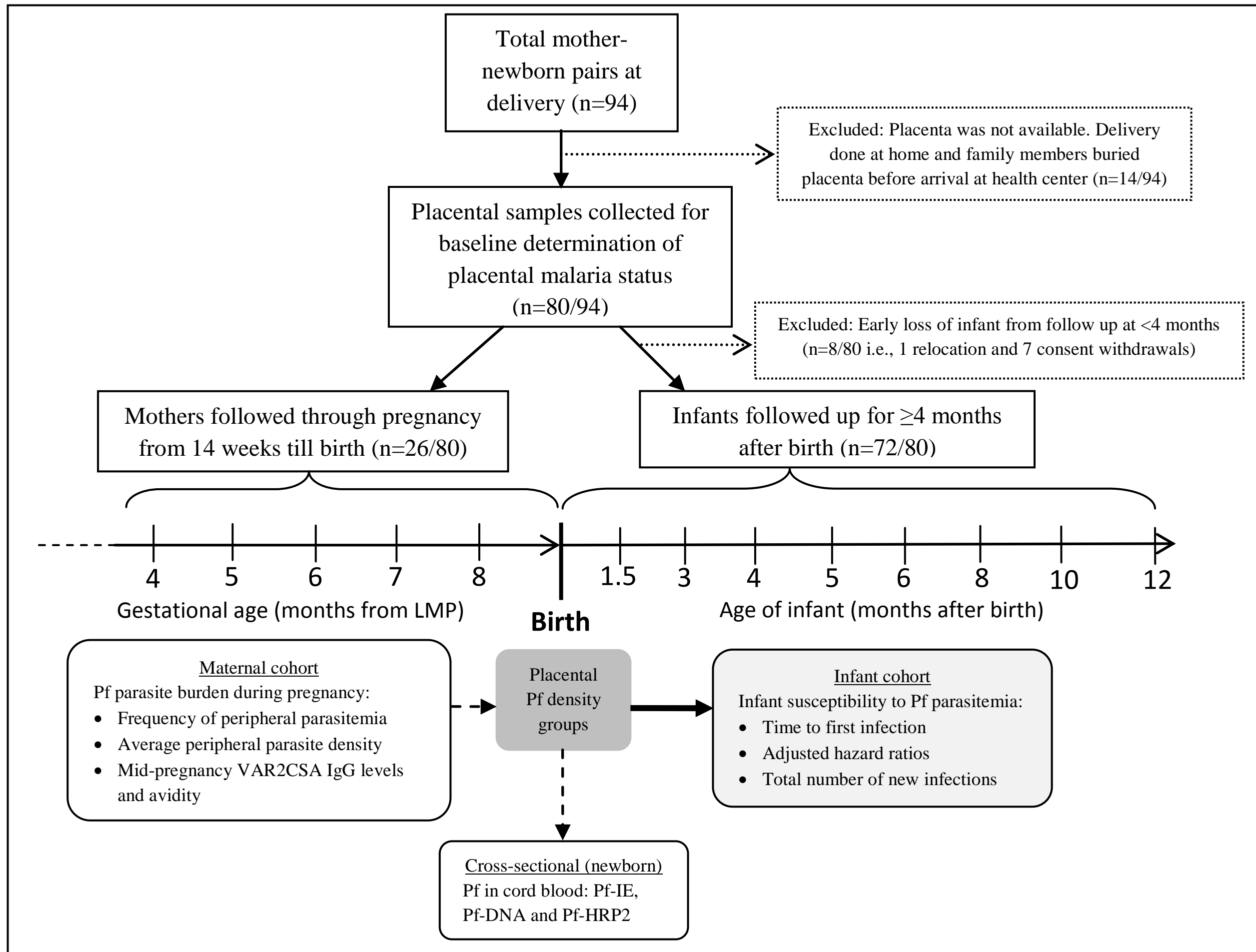

Figure 1. Study design. Placental Pf parasitemia was used to classify newborns into 3 groups: babies born to PM– (no placental Pf), PM+Lo (<median placental parasitemia), and PM+Hi (>median) mothers. Seventy-two infants were prospectively monitored from birth through the first year life to assess differences in susceptibility to Pf. Infant follow-up was conducted at scheduled time points and in-between if any illness was reported. To estimate the exposure of the mother and fetus to Pf during pregnancy, mothers of 26 infants had been followed through pregnancy and assessed for Pf infections. Also, cord blood samples were tested for Pf-IE, DNA and HRP2. LMP, last menstrual period; IE, infected erythrocytes.

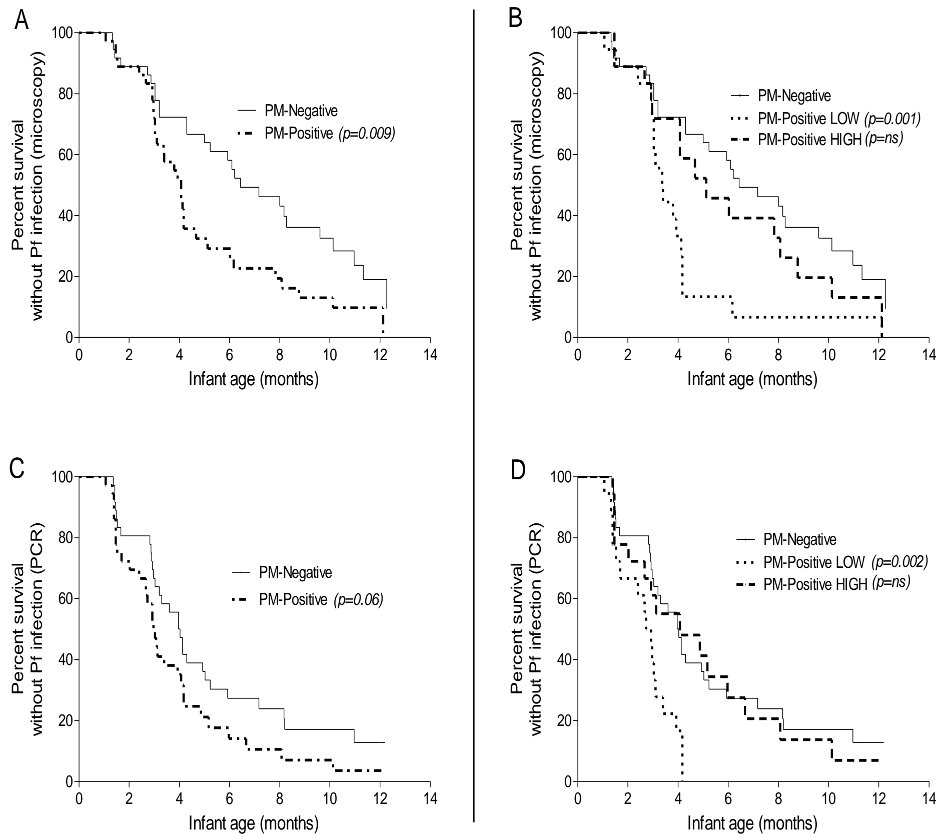

Figure 2: Kaplan-Meier curves showing time (in months) that infants survived without experiencing Pf infections that were diagnosed by microscopy (A and B) and by PCR (C and D). The left panels (A and C) show curves for infants born to PM– mothers (thin solid lines, n=36) and PM+ mothers (thick dash-dotted lines, n=36). In the right panels (B and D), infants born to PM+ mothers were further stratified into PM+Lo (thick dotted lines, n=18) and PM+Hi (dashed lines, n=18). P-values were obtained by log-rank test and represent significant differences in median time to first infection, between the PM+ groups and the PM– reference group. ns, not significant.

Table 1. Multivariate Cox regression analysis showing hazard ratios for Pf parasitemia in nfants

Variable	Subgroup	% of 72 infants	Microscopy		PCR	
			HR (95% CI)	Adjusted HR (95% CI)	HR (95% CI)	Adjusted HR (95% CI)
Placental Pf parasitemia at delivery	High (PM+Hi)	24	1.5 (0.8-2.8)	1.5 (0.7-3.1)	1.1 (0.6-2.1)	1.5 (0.7-3.4)
	Low (PM+Lo)	26	2.6 (1.3-4.8)	2.8 (1.3-6.0)	2.8 (1.5-5.4)	3.9 (1.8-8.4)
	No Pf (PM–)	50	Reference		Reference	
Residence[ϕ]	Zone B	56	0.7 (0.4-1.2)	0.6 (0.3-1.2)	1.1 (0.6-1.7)	1.0 (0.6-1.9)
	Zone A	44	Reference		Reference	
Gravidity	Primigravid	14	1.3 (0.6-3.0)	1.0 (0.4-2.4)	1.0 (0.5-2.3)	0.9 (0.4-2.4)
	Multigravid	69	Reference		Reference	
Season at birth	Heavily rainy	15	0.6 (0.3-1.5)	0.3 (0.1-0.9)	0.7 (0.3-1.6)	0.5 (0.2-1.3)
	Dry	20	0.9 (0.4-1.9)	0.6 (0.2-1.7)	0.8 (0.4-1.7)	1.0 (0.3-2.9)
	Rainy	36	0.7 (0.4-1.3)	0.4 (0.2-1.0)	0.5 (0.3-1.0)	0.6 (0.2-1.2)
	Very dry	29	Reference		Reference	
Hb genotype	HbAS	25	1.0 (0.6-1.9)	1.4 (0.7-2.8)	1.2 (0.7-2.1)	1.3 (0.6-2.5)
	HbAA	75	Reference		Reference	
Cord MSP1 IgG levels	Upper tertile	33	0.7 (0.4-1.5)	0.5 (0.2-1.3)	1.3 (0.7-2.4)	1.4 (0.6-3.3)
	Middle tertile	33	0.7 (0.3-1.4)	0.6 (0.3-1.2)	1.0 (0.5-1.9)	1.0 (0.5-2.1)
	Lower tertile	33	Reference		Reference	

[¥]Zone A, Central Ngali II village; Zone B, Satellite villages (Okoa, Abondo, Ntouessong)

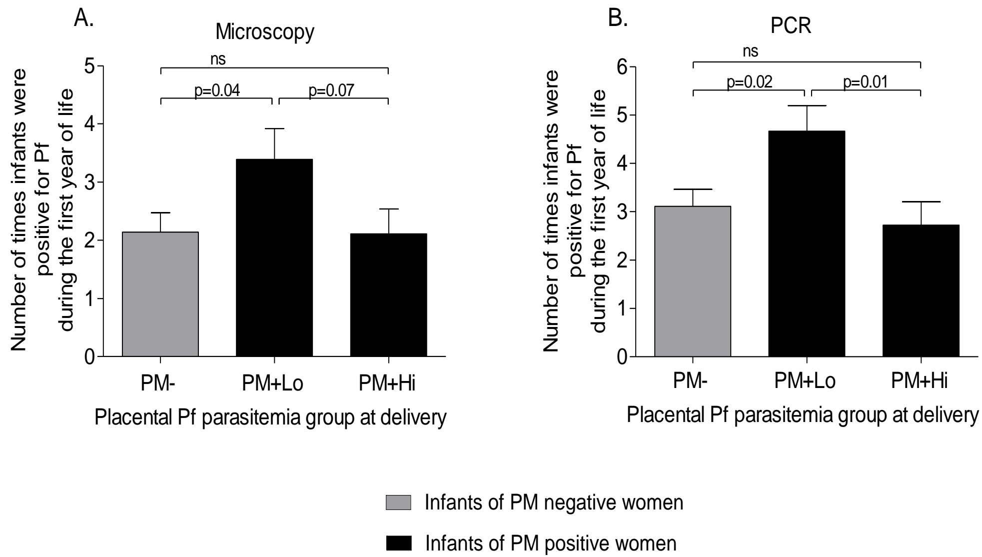

Figure 3. The number of Pf infections infants had during the first year of life. The presence of Pf in infant peripheral blood samples was diagnosed by microscopy (A) and by PCR (B). Data is represented as mean number of infections plus standard error for each group. Pf genotyping was used to distinguish new from persistent infections. Note that the mean duration of infant follow-up was similar for all groups: PM- (10.2 ± 2.5 months of follow up), PM+Lo (10.7 ± 3.1 months) and PM+Hi (9.6 ± 3.5 months), p = 0.494. Abbreviations: PCR, polymerase chain reaction; ns, not significant; Pf, *Plasmodium falciparum*; IVS, intervillous space; PM, placental malaria.

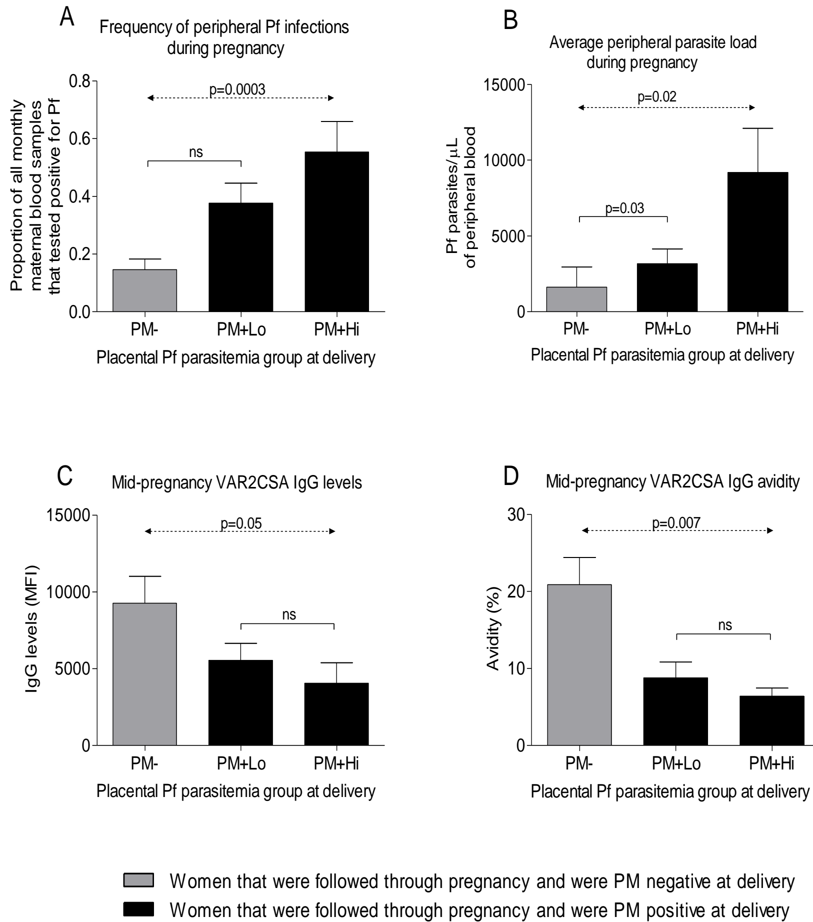

Figure 4. Relationship between factors associated with Pf burden during pregnancy and placental parasitemia at delivery. Twenty-six mothers (12 PM-, 9 PM+Lo and 5 PM+Hi were followed monthly during pregnancy. (A) Shows the percentage of visits they were peripheral blood smear-positive for Pf. (B) Average peripheral parasitemia (IE/µL) of all positive visits. (C) The levels IgG antibody to full-length VAR2CSA at 5 months of pregnancy. (D) The proportion of high avidity Ab to VAR2CSA. Data are shown as mean and standard error. P values represent significant difference between the PM+Lo and PM+Hi groups (continuous line) and the trend across all three groups (dashed line). ns, not significant.

Table 2. Relationship between placental Pf parasitemia and Pf products in cord blood

Analysis of cord blood	Placental Pf parasitemia at delivery			P value
	No Pf (PM–)	Low parasitemia PM+Lo)	High parasitemia (PM+Hi)	
Number of newborns tested	31	21	18	n/a
iE (microscopy)				
Percent slide-positive	0%	0%	5.9%	n/a
iE/µL	-	-	154	n/a
Pf DNA (qPCR)				
Percent positive	3.2%	4.8%	38.9%	0.0006
Concentration*, pg/mL	0.04	0.27	0.84 ± 1.23	n/a
HRP2 (ELISA)				
Percent positive	3.2%	19.0%	22.2%	ns
Concentration*, ng/mL	0.20	2.77 ± 2.85	2.85 ± 3.07	ns

*Concentration data represented as mean ± SD. No data range is shown where only one newborn was positive for the corresponding analyte. P values compare low versus high Pf density groups. iE, Pf-infected erythrocytes; n/a, not applicable; ns, not significant

CHAPTER 4

INFLUENCE OF PRENATAL EXPOSURE TO MALARIA ON THE ANTIBODY RESPONSE TO MEROZOITE ANTIGENS DURING THE FIRST POST-NATAL FALCIPARUM INFECTION

To be submitted to Infection and Immunity

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ABSTRACT

Although assumed to be protective, there is no solid evidence that maternal IgG to *Plasmodium falciparum* (Pf) antigens provide protection from malaria. It is also unknown if prenatal Pf exposure modifies an infant's initial antibody (Ab) response to malaria. In a Cameroonian birth cohort, infants of mothers with known placental parasitemia were classified into three groups based on their susceptibility to Pf infections and first year of life Ab profiles for 8 Pf antigens were evaluated. Maternal Pf IgG Abs were not associated with resistance to Pf infections as no significant differences in antibody levels at birth were found between susceptible and resistant infants. Using longitudinal regression models that incorporated time-varying covariates, infants were found to produce Ab to MSP1-42 (3D7 and FVO), MSP2 (FC27) and LSA1 following first infection, but not to AMA1 (FVO and 3D7), EBA175, MSP3, CSP or RESA. The interaction effect of placental parasitemia at birth and first post-natal Pf infection showed a boosting of MSP1 IgG, but not MSP1 IgM, in infants exposed to high amounts of Pf *in utero* and not in those exposed to low antigen amounts. The data show that secondary immune responses to MSP1 are generated during primary post-natal infection and suggest that high Pf antigen dose *in utero* sensitizes fetal B cells leading to Pf-memory B cell generation.

INTRODUCTION

Antibodies (Abs) are important mediators of host immunity to *Plasmodium falciparum* (Pf) as demonstrated by the effectiveness of passive administration of purified IgG, from malaria-experienced African adults, in treating severe malaria in Thai subjects (1). Ags (Ags) expressed by the merozoite stage of the parasite are considered to be key targets of the anti-malarial IgG (2, 3). In infants, circulating IgG to Pf merozoite Ags are either passively acquired maternal Abs or Abs produced by the infant in response to active infections. However, the kinetics of decline of maternal Pf Abs and the natural acquisition of Abs by the infant have not been well studied.

During pregnancy, Pf IgG are transported from maternal circulation to the fetal circulation via neonatal Fc-receptors (nFc-R) in placental trophoblasts (4). The nFc-R is an active transport system and explains why IgG levels in cord blood correlate with, and are sometimes higher than, IgG levels in paired maternal plasma (5, 6). Pf IgG has also been detected in breast milk (7, 8) but the IgG acquired through breast-feeding is largely degraded in the gastro-intestinal tract (9). Passive transfer of IgG into the fetal circulation is, therefore, not sustained after birth resulting in a decline of maternal Pf IgG levels in infant plasma over time. The decline is believed to be complete at age 6 months, but there is insufficient evidence in support of this assumption. A few studies have systematically examined the decline of maternal Pf IgG, but most studies were marred by limitations, such as long follow-up intervals and measurement of IgG to a few Pf Ags (10–13).

The role of maternal Abs in protection of infants from Pf infection is also controversial, as hitherto reviewed by Riley et al. (14) and by Dobbs et al.
15). During the first few months of life, infants are relatively protected from high parasite loads

and symptomatic malaria, which has been accredited to protective maternal Abs, but other factors could be involved (16–19). A study in the Southern region of Cameroon found an association between the presence of Pf IgG in cord plasma and absence of Pf infections during the first 6 months of life (20); however, several other studies either did not find any association or observed an increased risk of Pf infection in infants who had high levels of antimalarial IgG in cord plasma (13, 19, 21, 22). If Pf IgG levels during the first year of life reflect protection from Pf or are markers of infection warrant more investigation.

Furthermore, fetuses in malaria-endemic areas can be exposed Pf Ags *in utero* (23–25), meaning that the first post-natal infection in some infants actually constitutes a secondary exposure to parasite Ags. Yet it is unclear, if infants exposed to Pf before birth generate a recall antibody response during the first infection after birth. Pf-specific IgG Abs have been detected in some newborns (26, 27) suggesting prenatal priming to Pf occurs, but it is unknown if the prenatal Ab response is accompanied by the production of Pf-specific memory B cells. A careful assessment of Pf IgM and IgG response patterns during the first post-natal infection in infants of women with well characterized placental Pf infection status can contribute in determining if B cell memory to Pf is generated *in utero* and what prenatal exposure-related factors are involved.

The aims of the present study were to: 1) investigate the natural decline of maternal Abs to Pf Ags in a longitudinal cohort of Cameroonian infants, 2) determine if maternal Abs are associated with protection from malarial infections during the first year of life by examining Ab profiles of infants classified as resistant, intermediate and susceptible to malaria based on number of infections and time to first infection during the first year of life; and 3) explore if placental malaria (PM) and placental parasite density are associated with boosting of IgG and/or IgM levels in infants during their first Pf infection.

MATERIALS AND METHODS

Study design

The study was performed in the Ngali II locality situated about 40 km from the city of Yaoundé, Cameroon. The predominantly rural community has a total population of approximately 1,200 inhabitants and malaria transmission in the village was considered high as entomological inoculation rates (EIR) of up to 257 bites per person per year have been recorded (28). From May 2001 to November 2004, pregnant women were informed about the project and the newborns of women who signed informed consent forms were included as study participants. At enrollment, delivery information was recorded and placental samples were collected for diagnosis of PM. After birth, infants were monitored regularly during programmed visits at 7 days, 6 weeks, 3, 4, 5, 6 8, 10 and 12 months of life. At each follow-up visit, infant peripheral blood samples were collected by finger prick and used for parasitological and Ab studies. Samples collected from 70 infants who were not lost from follow up before the 8th month visit were included in analyses.

To study the decline of maternal Pf Ab in the absence of infection, plasma and clinical data were obtained from 35 infants born and raised in Yaoundé, Cameroon, a low transmission area. The infants were recruited and followed as described above. Longitudinally-collected blood samples showed the infants were PCR-negative for malaria at all time points. Thus, a total of 105 infants in high and low transmission areas of Cameroon were included in the overall study.

Parasitological studies

Thick smears of placental intervillous space (IVS) blood were stained with Diff-Quick and visualized under a microscope. Histo-sections from placental biopsies, stored in 10%

buffered formalin, were prepared and stained with Hematoxylin-Eosin before microscopic examination. PM was defined as the presence of Pf parasites in IVS blood smear and/or in histo-sections. Placental parasite density was considered low (PM-positive-low) if <median density of all PM-positive cases or high (PM-positive-high) if ≥ median.

Pf infections during the first year of life were detected in peripheral blood samples of infants by microscopy and PCR. PCR was performed by amplifying the 18S ribosomal subunit gene of Pf using primers and protocols that have been described previously (29, 30).

Antibody studies

Abs to 5 Pf merozoite-stage Ags (MSP1, MSP2, MSP3, AMA1, EBA175), one ring-stage erythrocyte surface Ag (RESA), and 2 exo-erythrocytic-stage Ags (CSP and LSA1) were measured in infant plasma samples using a multiplex Luminex assay. The description of each Ag including the amount of Ag used to coat seroMAP bead surfaces is provided in Supplemental Table 1. Beads with distinct spectral addresses were used for each Ag. A bead mix containing 100 of each Ag-coupled bead type per µL of PBS-1%BSA was made and 50 µL of the mix was added to 50 µL of diluted plasma

1/100 in PBS-1%BSA) in filter plate wells (Multiscreen BV, Millipore, Billerica, MA). After one hour of incubation on a 500-rpm shaker (Lab-line, Melrose Park, IL) in the dark at room temperature, the plate wells were washed 5 times with PBS-0.05% Tween 20 using a vacuum manifold (ZIP Plate Micro SPE plate, Millipore, Billerica, MA). Then, 0.1 mg (100 µL) of R-phycoerythrin conjugated to the F(ab') 2 fragment of goat anti-human IgG or to the F (ab') 2 fragment of donkey anti-human IgM (Jackson Immunoresearch, West Grove, PA) was added to each well. After 30 mins of incubation, the plates were washed again 5 times and the beads were resuspended in 100µl PBS-1%BSA. The plates were then read in the Liquichip M100 instrument

(Qiagen, Valencia, CA) which detected the spectral address of each bead type (hence corresponding Ag) and the median intensity fluorescence (MFI) of phycoerythrin (i.e., amount of Abs bound). Appropriate positive and negative controls were included on each plate

Statistics

Based on the time from birth to first infection and the total number of times Pf was detected by PCR during the first year of life, the 70 infants in the high transmission birth cohort were categorized as shown in Fig. 1: resistant to Pf infections (no Pf infection occurred during the first five months of life, n = 16); intermediately resistant to Pf included babies with infections during the first 5 months but their total number of infections during the entire follow-up period was <5, n = 31); and susceptible to Pf (total number times infected ≥5). Baseline descriptive data were compared between the resistant and susceptible groups using Fisher's exact test for categorical variables, such as percent PM-positive, and Student t test for continuous variables, such as birth weight (Table 1). Resistant and susceptible infants had similar baseline characteristics except that resistant infants were more likely to have been born to mothers with high placental Pf density than susceptible infants (p = 0.06). To determine if Ab levels in infants were markers of resistance or susceptibility to infection, average antibody levels at each timepoint were plotted for infants of each group and the profiles were compared with a focus on differences between the resistant and susceptible infants.

Mathematical modeling was used to determine if Pf IgG and/or IgM levels during infancy were significantly boosted at first Pf infection and if PM status influenced the response. A set of regression models capable of handling longitudinal outcomes and predictors were generated. The models were built using Ab levels tracked from day 7 of life to the time of first Pf infection or to the last follow-up visit if no infection had occurred. Antibody MFI was regarded as a count-type

repeated measure outcome variable in a predictive model that was based on generalized estimating equations (GEE), with first Pf infection as the predictor of primary interest. Adjustments were made for covariates, such as baseline Ab level, length of time from baseline to infection and for grouping variables such as PM status. Models were fitted for six different scenarios: no grouping variable, grouping by PM-positive/PM-negative, and by placental Pf density (PM-positive-low, PM-positive-high, PM-negative). The models containing grouping variables also included group interaction terms with first Pf infection. These models were fitted with log link function with AR-1 type correlation structure for specifying the correlation among repeatedly measured antibody levels. Note that the GEE approach estimates a marginal model that is robust against possible misspecification of correlation structure.

RESULTS

Infant susceptibility to Pf infections

Infants were divided into 3 groups based on the number of PCR-detected infections they had during the first year of life (Table 1). No significant differences were found between the groups in any of the parameters evaluated, including months of follow-up, except for placental Pf density at birth. Overall, 60% of the PM-positive mothers who gave birth to resistant infants had high placental parasitemia compared to only 8% of mothers whose infants were highly susceptible to malaria. Thus, having a mother with high placental parasitemia may be an advantage for infants.

Decline of maternal Pf IgG

The natural decline of maternal Ab in the absence of malaria infection during the first year of life was Ag-dependent. IgG waned, reaching its lowest levels at 6 months of age for MSP1 and at 8–12 months for EBA175 and AMA1 (Fig. 2, low transmission panels). Infants were born with minimal amounts of Ab to MSP2 that were gone by 3 months of age.

Acquisition of Pf IgG through the first year of life

Ab levels for infants of the susceptible and resistant high transmission groups were similar at birth (Fig. 2) and declined at similar rates among the infants. A comparison of results Pf prevalence (in Fig. 1) and Ab profiles (Fig. 2) showed that Ab to MSP1 IgG increased in parallel with infection. For example, MSP1 IgG levels begin rising at 6 months, 4 months, and 3 months for resistant, intermediate and susceptible infants, respectively, corresponding to timepoints where the prevalence of infections increased in the corresponding groups.

Collectively, there was no evidence that maternal MSP1 IgG or IgG to the other Ags was associated with protection from infection.

On the other hand, IgG to EBA175 and AMA1, declined steadily in resistant, intermediate and susceptible infants, even though the infants became infected with malaria multiple times. The rate of decline of EBA175 IgG was not related to amount present at birth, as infants in the all 3 groups lost their maternal IgG until 10 months of age at a rate like that of unexposed infants. MSP2 maternal IgG declined faster than Ab to EBA175 or AMA1 reaching lowest levels at about 4 months. Thereafter, MSP2 IgG remained low until about age 8 months, when the infant began producing its own MSP2 IgG. The MSP3 profiles were similar to those of MSP2, but the MFIs were lower (supplemental Fig. 1). Compared to all the other Ags, very little or no IgG to RESA, CSP and LSA were passively acquired by infants from their mothers.

Of great interest, Ab levels to Ags other than MSP1 did not increase in parallel with infection (compare Fig. 1 and Fig. 2). Thus, it appears that some of the babies did not produce significant amounts of Ab to most of the Ags upon repeated infection during the first year of life.

Antibody response at first post-natal infection

Using GEE longitudinal regression models, significant boosting of IgG to the two the MSP1 variants, MSP2-FC7 and to LSA1, but not the other Ags was observed at the first infection (Fig. 3A). No interaction effect of being born from a PM-positive mother and experiencing the first Pf infection during infancy was found (Fig 3B). However, when interaction effects with placental parasite density were examined, infants of PM-positive-high had a strong boost in their IgG levels to MSP1 3D7 and MSP1-FVO; whereas, this effect was absent in infants born to PM-positive-low mothers or PM-negative mothers (Fig. 3C and D).

Taken together, infants made IgG to MSP1 during their first infection, and the boosting effect was higher if they were born to mothers with high placental parasite density.

The longitudinal models were also applied for IgM to MSP1-3D7 and MSP1-FVO, and to EBA175. At the first occurrence of infection, IgM levels for the two MSP1 proteins were also boosted in addition to IgG (Fig. 4A). Interestingly, the infants made IgM, and not IgG, to EBA175 showing that the Ab response to EBA175 at first infection was a primary response. Again, general PM and low placental parasite density did not have significant effect on IgM or IgG levels for any of the tested Ags. However, PM-positive-high infants had a significant boost of IgG and not IgM to the MSP1 variants. Therefore, infants born to PM-positive-high mothers generate a secondary Ab response during the first Pf infection after birth.

DISCUSSION

The study extensively profiled Ab levels to broad set of Pf Ags during the first year of life and used robust modeling methods to evaluate Ab responses during the first post-natal infection with attention to prenatal exposure to Pf. Important findings include: 1) maternally acquired Pf IgG are not correlates of resistance to Pf infections during the first year of life; 2) the pattern of acquisition of Abs to Pf during the first year of life in general and specifically during the first post-natal infection is Ag-dependent; and 3) infants born to PM-positive mothers with high placental parasite density are more likely to mount a secondary recall Ab response to Pf than infants born to PM-negative or PM-positive mothers with low placental Pf density.

The hemochorial architecture of the human placenta and its Fc-neonatal receptors allow the efficient transfer of maternal IgG to the fetus, but the effective role of maternal IgG in protecting the infant from malarial infections is currently under debate. Results of the current study show that infants who are resistant and susceptible to Pf infections have similar baseline levels of maternal IgG to Pf Ags. Therefore, serologically measured levels of maternal Ab in the infant do not predict resistance or susceptibility to infections. The data corroborate with results from a Ghanaian cohort that showed no association between IgG to MSP-1, MSP-2, AMA-1, and RESA in newborn plasma and resistance to Pf infection during infancy (22).

Still, a protective role of maternal antibodies cannot be completely ruled out particularly during the first 3 months, since the prevalence of Pf infections before 3 months was significantly lower than age-related prevalence after 3 months, even in susceptible infants. Perhaps the levels of functional maternal Abd, and not total Abs are better correlates of resistance to Pf in the infants, as contrasting profiles of Abs measured by serology and functional assays have been reported in a Kenyan cohort (31). It is also possible that relative protection from malaria during

the first 3 months is accounted for by multiple factors mentioned in previous reviews (14, 15). These other factors include: fetal hemoglobin which constitutes the high percentage of infant hemoglobin during the first two months of life and can inhibit intra-erythrocytic growth of parasites (32) and breast-feeding which deprives the parasite of para-aminobenzoic acid (pABA). pABA is an essential nutrient for parasite growth but breast milk naturally contains little or no pABA. Reduced exposure to Pf during the first 3 months of life can also be due to the fact that younger babies were culturally better taken care of and were better covered with clothes than older babies in the Ngali II community, thus the older infants will be more susceptible to mosquito bites. Maternal Abs together with these factors may contribute to reduced prevalence of malaria infections during the first couple of months but there remains no solid evidence that maternal Abs are correlates of protection from malaria during the first year of life.

The overall pattern of acquisition of Pf Abs was unique for each Ag, but two patterns stood out. First, the Ab acquisition pattern of MSP1 antibodies very well mirrored the prevalence of Pf at each time-point. Infants tend to make IgG first to MSP1 before making IgG to other Ags. In contrast, the profiles for AMA1 and particularly EBA175 were different, characterized by a steady drop of maternal IgG up to age 8–12 months irrespective of number of infections the infant receives. MSP2 pattern was similar but a faster drop of maternal IgG was observed. The infants did not seem to actively produce anti-AMA1 and EBA175 IgG in response to many infections. A possible explanation for the differential dynamics of infant Ab profiles between MSP1 and EBA175 could be that the higher and longer lasting levels of maternal EBA175 IgG exert an negative feedback effect on the infant's EBA175-specific B cells via inhibitory Fc gamma receptor 2b (FcγRIIb) (33). Another reason could be related to *in utero* priming. Because MSP1 is the most abundant Ag in the merozoite surface, fetuses will hypothetically be more

exposed and primed to MSP1 than EBA175 or AMA1 which are proteins found in relatively small amounts in the micronemal regions of the merozoite. Therefore, infants would more likely to generate a recall response to MSP1 early during infancy than to EBA175 or AMA1. In addition, it was observed that infants made IgM and not IgG to EBA at their first infection supporting the idea that post-natal response to EBA175 is a primary immune response and not a secondary recall response. Thirdly, MSP1 could be more immunogenic than EBA175 or AMA in infants. However, Ab levels to AMA1 are usually higher than MSP1 levels in older children and adults (34), thus it remains puzzling why MSP1 will be more immunogenic infants. In all, the differential patterns of antibody acquisition should be taken into consideration in the design of anti-malarial vaccines that target infants.

A principal finding of the study is that the MSP1 IgG response at the first Pf infection after birth is modified by placental parasite density. The fact that a boosting of MSP1 IgG at first infection was seen only in infants born to PM-positive women with high placental parasite density and the finding that MSP1 IgG boosting in the PM-positive-high group was not accompanied by IgM production, strongly suggest that secondary Ab responses MSP1 are generated during primary post-natal infection if exposure to high, and not low, amounts of Ag occurred before birth. The findings provide indirect evidence that memory B cells can be generated *in utero* and persists through early to mid-infancy. As demonstrated in *in vitro* models with mouse transgenic T cells, high Ag dose is necessary for T helper cells to escape suppression by T regulatory cells (T-regs) (35). Given that fetal T cells are biased towards T-regs (36–38), it is plausible that the threshold antigen dose needed to activate fetal T effector cells will be high *in utero*. Since cognate interaction with activated T cells is required for B-cell activation and migration into the germinal centers for differentiation into memory B cells and long-lived plasma

cells (39), Ag amount *in utero* may also predict if antigen-specific memory will be generated. Although germinal centers are scarcely identified in lymphoid tissue of stillbirths (40), somatic hypermutations have been detected in preterm babies after exposure to environmental Ags (41), suggesting that with the right threshold of exposure, germinal centers and hence memory B cells could also be made *in utero*.

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FIGURES AND TABLES

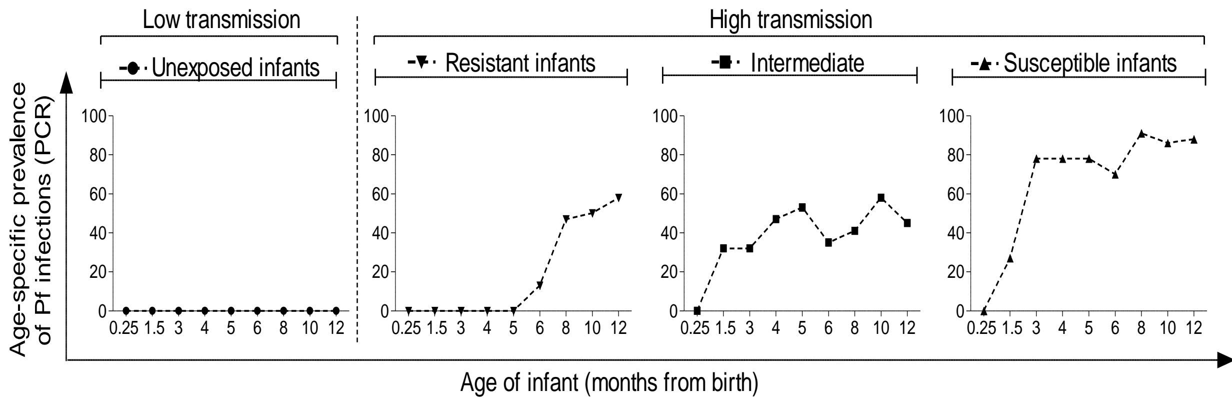


Figure 1. Classification of infants. Age-specific prevalence of Pf infections detected by PCR are shown for a total of 70 infants living in high Pf transmission village of Ngali II, Cameroon. The infants were further divided into 3 groups based on susceptibility to Pf infections during the first year of life. Resistant infants experienced no Pf infections until age 6 months (n=16), As from age 3 months, 70–90% of susceptible infants (n=23) and 30–60% of intermediate infants (n=31) were positive for Pf every month. The low transmission controls (n=35) from the city of Yaounde were not positive for Pf any time during the first year of life.

Table 1. Description of infants from the high transmission birth cohort of Ngali II

Description	Resistant infants	Intermediate infants	Susceptible infants	P Value*
Number of infants	16	31	23	n/a
Duration of follow-up (months from birth)	11.4±1.4	11.1±2.4	11.4±1.5	0.93
% residing at the outskirts of Ngali II village	56%	61%	52%	1.0
% born during the rainy season	63%	42%	48%	0.52
Gestational age at birth (weeks)	39.5±1.9	39.3±2.0	39.0±2.2	0.51
Birth weight (grams)	3292±456	3099±501	3221±479	0.68
Number with available placental samples	12	29	18	n/a
% Placental malaria (PM) positive	42%	41%	61%	0.46
% PM-positive with high placental Pf density	60%	33%	8%	0.06

*Resistant versus susceptible

n/a, not applicable

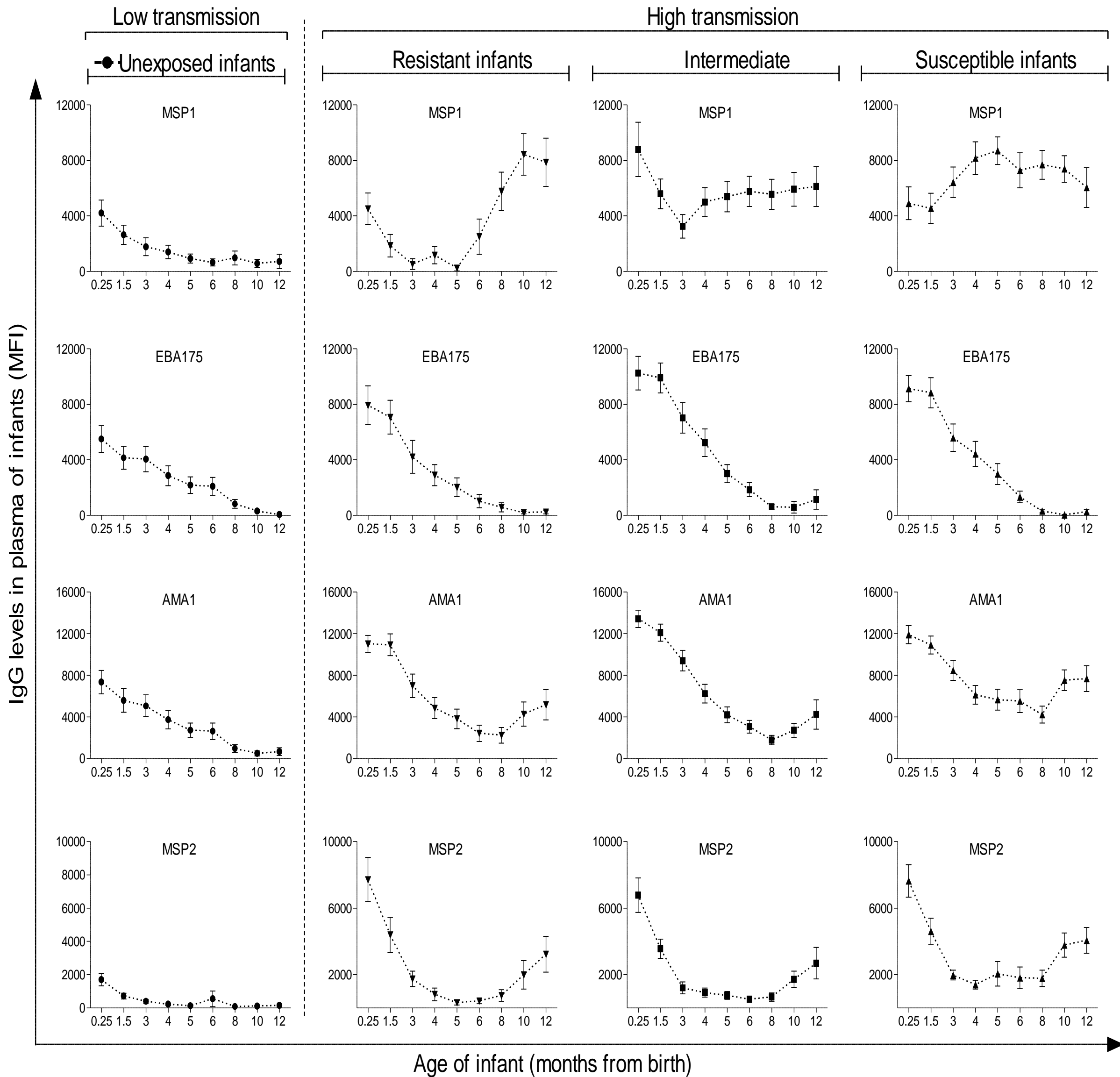


Figure 2. Kinetics of circulating IgG to Pf MSP1, EBA175, AMA1 and MSP2 during the first year of life. Antibody levels at each time point are represented as mean and SEM. Similar IgG patterns were observed for variants of the same antigen, so data are shown for only one variant of each antigen (MSP1-FVO, AMA1-FVO and MSP2-FC27).

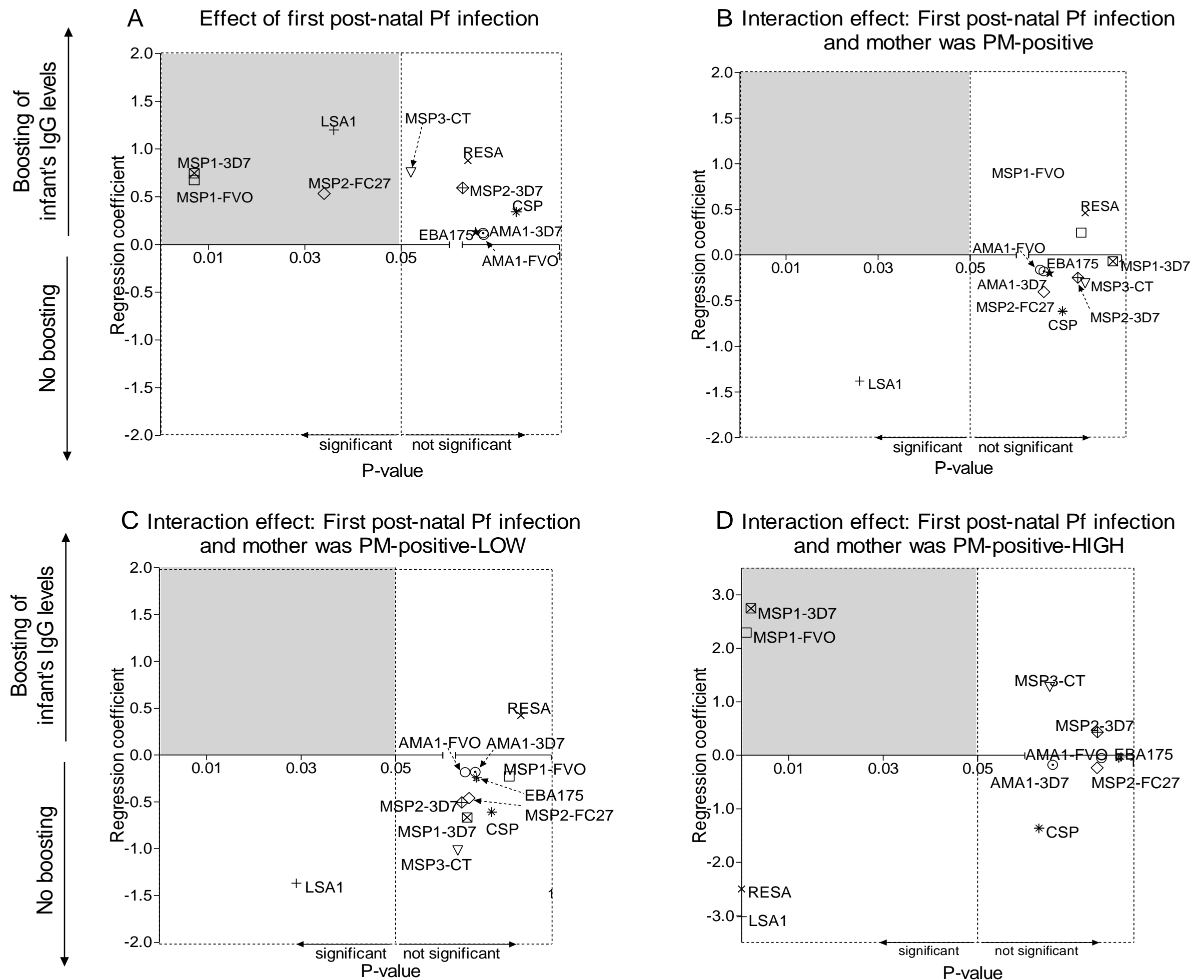


Figure 3. Assessment of Pf IgG boosting at first infection. GEE longitudinal regression models were used to determine if antibody levels at first infection were significantly higher than preceding visit level. Adjustments were made for covariates including baseline IgG at the start of infant follow up and time from birth. Each panel shows the effect of first Pf infection or the interaction between the occurrence of first infection and a PM subgroup (PM-positive, presence of Pf parasites in the placenta at birth; PM-positive-low, placental parasite density <median; PM-positive-high, placental parasite density>median). The shaded area shows area where the regression coefficients are positive and significant. The antibodies to antigens within the shaded area were significantly boosted

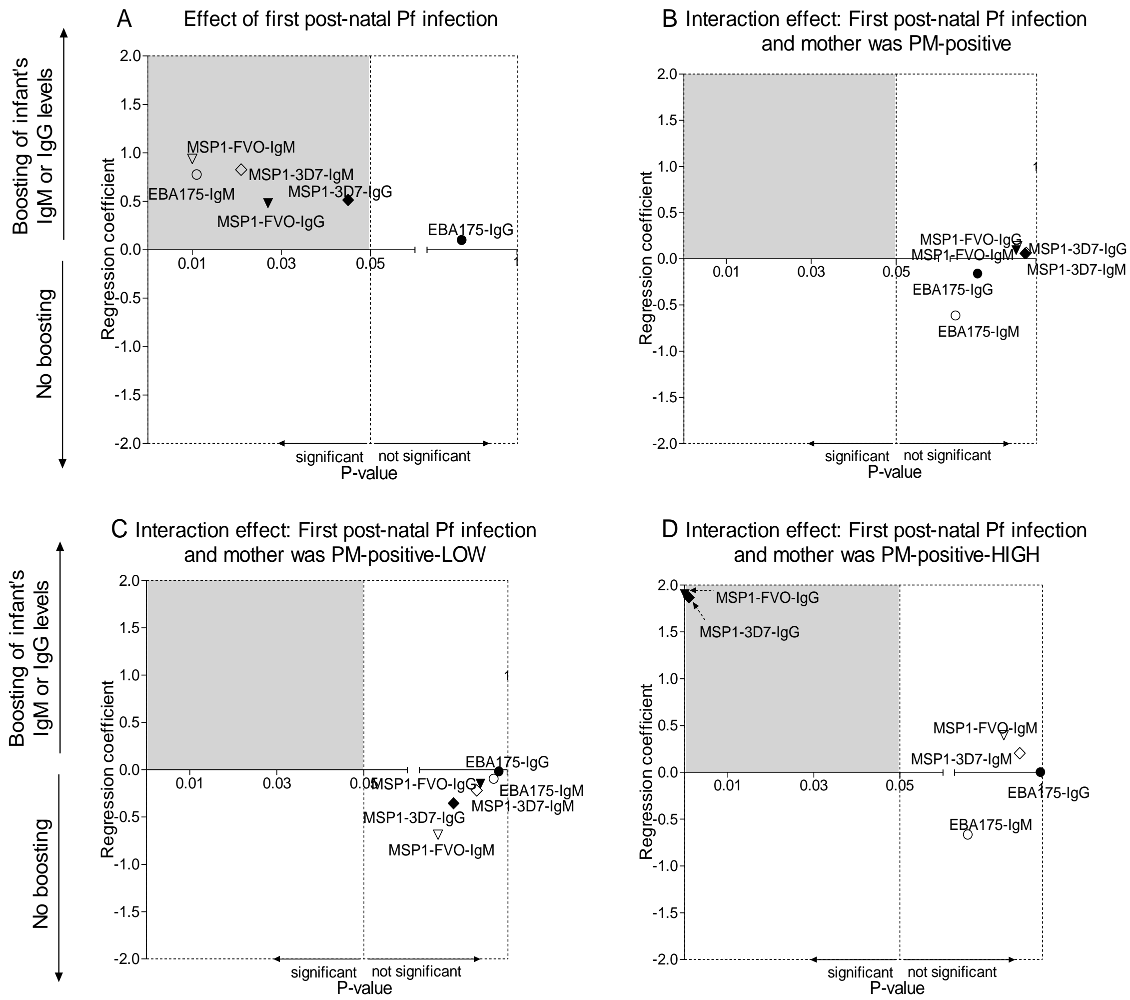
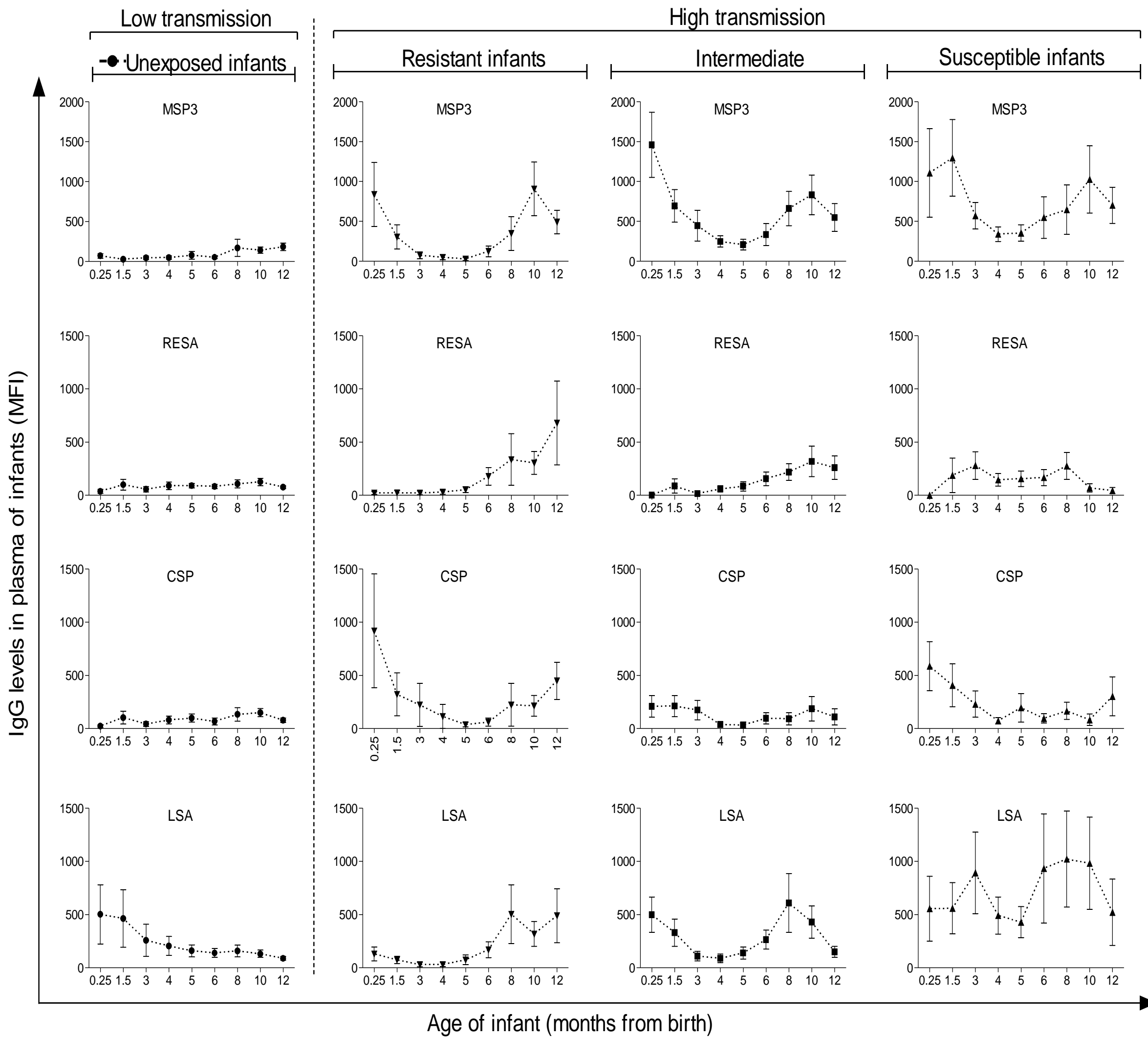


Figure 4. Assessment of Pf IgG and IgM boosting at first infection. In addition to IgG, IgM boosting was assessed for MSP1-3D7, MSP1-FVO and EBA175.

Supplementary table 1. Recombinant and synthetic antigens used in antibody assays

Antigen	Strain or sequence	Type	Expression system	Antigen amount used per million beads
$MSP1_{42}$	FVO	Recombinant	*Escherichia coli*	1 µg
$MSP1_{42}$	3D7	Recombinant	*Escherichia coli*	1 µg
MSP2	FC27	Recombinant	*Escherichia coli*	1 µg
MSP2	3D7	Recombinant	*Escherichia coli*	1 µg
MSP3	3D7, C-Terminal	Recombinant	*Escherichia coli*	0.2 µg
AMA1	FVO	Recombinant	Yeast cells	1 µg
AMA1	3D7	Recombinant	Yeast cells	1 µg
EBA175	Region II	Recombinant	Yeast cells	0.2 µg
RESA	[EENV]5-BSA	Synthetic	n/a	2.5 µg
CSP	[PNAN]5-BSA	Synthetic	n/a	2.5 µg
LSA1	-BSA	Synthetic	n/a	4 nmol

Source of antigens:
--MSP1, MSP2 and AMA1 provided by Carole A. Long, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases (Rockville, MD)
--EBA175 obtained from Science Applications International Corp. (Frederick, MD)
--RESA and CSP synthesized by AnaSpec, Inc. (San Jose, CA)
--LSA peptide synthetized by Genosys-Sigma-Aldrich (Saint Louis, MO)
n/a, not applicable



Supplemental figure 1. Kinetics of circulating IgG to Pf MSP3, RESA, CSP and LSA1 during the first year of life.

CHAPTER 5

DISCUSSION

The burden of malaria is disproportionately high in children and pregnant women. In 2015, 69% of 438,000 malaria-related deaths reported worldwide occurred in children less than 5 years old. Annually, about 30 million pregnant women are at risk of malaria, and infections during pregnancy can lead to severe complications for the mother and her baby. Besides the well documented malaria-associated pregnancy complications such as preterm delivery and low birth weights, in endemic areas, it is now being recognized that fetuses of *Plasmodium falciparum* (Pf)-infected women can be exposed to Pf *in utero*. The dynamics of prenatal Pf exposure and its repercussions on the health of infants after birth are not well understood.

Data from immunological studies provide some explanation for the overly high malaria burden in children and pregnant women. Immunity to malaria is never sterile, but significant protection from severe forms of malaria and high parasite loads can be attained by individuals in high transmission areas after years of repeated exposures to the parasite. Thus, acquired immunity to malaria is more robust in adults compared to children, resulting in the latter being more susceptible to Pf infections and severe disease. However, most immuno-epidemiological studies on malarial immunity in children, or lack of, have focused on children aged 2 to 10 years. Little is known about how infants acquire immunity to malaria during their first year of life.

Like many adults in malaria-endemic areas, women of child-bearing age have high levels of antibodies to Pf blood-stage antigens but these women face novel challenges in combating Pf parasites when they become pregnant for the first time. In pregnant women, Pf-infected erythrocytes express a distinct surface protein, VAR2CSA, which binds to CSA on placental villi

and allows infected erythrocytes to accumulate in perivillous spaces. This condition, known as placental malaria (PM), is the hallmark pathologic feature of malaria in pregnancy. Research on immunity to PM has advanced greatly over the past decade as anti-VARCSA antibodies, that inhibit placental sequestration of infected erythrocytes, have been identified and the natural acquisition of these antibodies during the course of pregnancy and over successive pregnancies has been extensively studied. PM also facilitates exposure of the fetus to Pf antigens, but relatively little is known about the development of antibody responses *in utero*.

Taken together, the threat of malaria to maternal and child health is undisputed and much research effort has been invested into understanding the determinants of susceptibility to infection and the process of acquiring immunity to Pf in pregnant women and children. But two developmental periods during pregnancy and childhood have received less research attention, i.e., the fetal period and the first year of life. The present dissertation uniquely and specifically focused on Pf exposure and induced antibody responses in fetuses and infants. If immune responses are mounted against Pf *in utero*, post-natal acquisition of immunity to Pf will potentially be influenced by the nature of the prenatal responses. A good understanding of the process of acquisition of immunity to Pf in infants is necessary in the design of anti-malarial vaccines that will be efficient when administered to this age group.

There are a few reasons why research progress on fetal and infant immune responses to Pf has been slow. First, fetal samples are difficult to obtain. Fetal tissue is not accessible for routine sampling during gestation. Percutaneous umbilical cord blood sampling, also referred to as cordocentesis, can be performed before birth, but this procedure is invasive and sometimes induces premature labor. Besides, many health care providers in countries where malaria is endemic do not have the skills to perform such specialized procedures. Therefore, much of what

is known about fetal responses to Pf emanate from studies done using umbilical cord blood collected at birth. These studies have demonstrated Pf antigen-driven cord blood T cell proliferation and cytokine production *in vitro,* Pf-specific IgM in cord plasma and IgG *in vitro*, all pointing to the possibility of T- and B-lymphocyte priming to Pf *in utero*. However, because these studies were conducted in full term newborns, it was not clear when during fetal development immune priming occurred and if the quantity or quality of the prenatal immune response to Pf was influenced by gestational age. Some of the research conducted as part of this dissertation constitutes pioneer work of fetal antibody responses to Pf at gestational ages ranging from 20 weeks to 40 weeks. The work was possible because of the availability of a unique and impressive archive of samples collected from Yaoundé, Cameroon at a period when local malaria transmission and the prevalence of preterm birth were a lot higher than are today. Between 1998 and 2001, 34% of deliveries in Yaoundé were preterm, i.e., birth before 37 complete weeks of gestation, but in the years 2014 and 2015 the prevalence dropped to about 12% (Tassi Yunga et al., unpublished).

Second, the advancement of scientific knowledge on immune responses to Pf during the fetal period and during infancy have been hindered by populist views and dogmas that are either not evidence-based or are based on archaic scientific findings. For example, it was widely believed that fetuses cannot mount any significant effector responses to foreign antigens because of a bias towards tolerance *in utero* and that infants do not need to mount responses to environmental antigens because they are protected by passive immunity acquired transplacentally or from breast milk. Our current studies question some of these stereotypes as we investigated, among other things, the components, timing and amount of exposure to Pf *in utero*; if preterm newborns, including some extremely premature (20–28 weeks) newborns

exposed to Pf *in utero* made antibodies to Pf; if the amount, breadth and isotype of these antibodies were different from that of exposed term newborns; and if maternal Pf-specific IgG in cord blood offer any protection from Pf infections during the first year of life. We also investigated associations between the amount of prenatal Pf exposure and post-natal outcomes, such as the time to first infection and antibody response profile at that first infection.

The current studies confirm that the developing fetus can be exposed to whole parasites and parasite products including DNA, secreted proteins and antigens bound to Pf IgG as immune complexes (IC). Intact parasites, free parasite DNA and proteins are thought to be transported across the placenta via microabrasions that may be induced by PM. Pf-DNA and Pf-glycosylphosphatidylinositol (GPI) – an endotoxin-like anchor for some Pf antigens – are pathogen-associated molecular patterns (PAMP). Therefore, both Pf antigens and PAMPs that respectively provide the first and second signals needed for efficient T helper cell activation are transferred across the placenta into the fetal circulation. Since antigen-presenting cells and lymphocytes have previously been identified in fetal lymphoid organs from 10–12 weeks of gestation, and pattern recognition receptors for Pf PAMPs (i.e., TLR9 for DNA and TLR1/2, TLR4, TLR2/6 for GPI) have been detected in prenatal tissue, proof that Pf antigens and PAMPs are present in cord blood suggests T- and B-cell sensitization can occur to Pf *in utero*.

With regards to which form of Pf exposure is predominant *in utero*, our data show that while intact parasites that contain multiple Pf antigens and PAMPs are rarely detected in cord blood (1 case out of 70 newborns) up to 25% of Cameroonian newborns (169 out of 682) had immunologic evidence (Pf IgM in cord blood) of exposure to one or more Pf antigens *in utero*. This suggests that exposure to individual antigens in the form of IC could be the primary mechanism by which fetuses encounter Pf *in utero;* however, there was absolutely no association

between being positive for Pf IgM and having Pf IC in cord blood, which brings into doubt the relevance IC as a mechanism of exposure to Pf. On the other hand, 27% (46 out of 169) of the cord IgM positive newborns recognized a wide breadth of Pf antigens indicating exposure to multiple antigens as are present in intact infected erythrocytes. Also, Pf was more commonly detected in cord blood by PCR than by microscopy, indicating that sub-microscopic amounts of Pf parasites were present in cord blood. Therefore, intact infected erythrocytes cannot be ruled out as a major source of Pf antigens for priming *in utero*. The fact that neonates born as early as 22 weeks were positive for Pf IgM to all tested antigens shows exposure to Pf parasites can occur any time from the second trimester through full term and induce a B cell responses.

Our data also demonstrate for the first time that placental parasite density is a proxy for the overall level of maternal and fetal exposure to Pf during pregnancy. It is impossible to directly quantify Pf exposure in the fetus throughout pregnancy. Thus, indirect measures of parasite load during pregnancy, such as number of times pregnant women were positive for Pf and average peripheral parasite density, were used. We demonstrated that frequent infections with high density parasitemia during the second and third trimesters are associated with higher parasite loads in the placenta at delivery, which in turn, is associated with a higher likelihood of cord blood parasitemia. This is a novel finding and it implies that quantification of parasite load in the placenta at birth provides insight on the level of exposure to Pf that newborns encounter during gestation.

Limitations in the number of circulating lymphocytes and in the diversity B cell receptor repertoire have been previously reported in preterm babies, but restricted diversity was not reflected in our Pf antibody data. Fetal antibody responses to Pf were not restricted in amount or in breadth for preterm newborns compared to full term newborns. Still, it is possible that

potential differences in breadth of IgM response were not detected in the present studies because of the small number of Pf antigens used to screen cord plasma for IgM. Nonetheless, we found that the ability of at least one fetal B cell clone to be primed for Pf antibody production *in utero* is not altered during early to mid pregnancy. Remarkably, the proportion of newborns that made IgM to Pf *in utero* decreased with increasing gestational age. The observed trend was most likely due to class-switch from Pf-IgM to Pf-IgG production since lymphocytes of term newborns overwhelmingly secreted Pf IgG *in vitro*.

The finding of antibody class-switching *in utero* suggests, but does not confirm, that Pf-specific memory B cells are generated in exposed fetuses. The Pf IgG may have been produced by short-lived plasma cells which have undergone extrafollicular class-switch recombination. Yet, it was fascinating to find a secondary antibody response to the MSP1 antigen at the first infection after birth in some infants, which is indirect evidence that MSP1-specific memory B cells were produced *in utero* and persisted into the post-natal period. Studies specifically aimed at identifying polyclonal and antigen-specific memory B cells in cord blood are needed to provide direct evidence of Pf-memory B cell generation *in utero*. We have received a pilot grant via University of Hawaii Centers of Biomedical Research Excellence (COBRE) and the New Mexico IDeA Networks of Biomedical Research Excellence (NM-INBRE) for a study of the B cell transcriptome of Cameroonian newborns exposed to Pf *in utero,* which may provide information on Pf-induced changes in fetal B cells. RNA obtained from sorted B cells of PM-positive and PM-negative newborns as well as North American control newborns will be deep sequenced for differential gene expression analyses.

With regards to our first year of life antibody studies, we have demonstrated that not all passively acquired maternal Pf IgG disappear by 6 months of life and that maternal IgG are not

correlates of protection from infection as previously assumed. The pattern of decline of Pf IgG during the first year of life was not identical for all Pf antigens. While levels of maternal IgG to many merozoite antigens completely waned before 6 months, maternal IgG to EBA175 and AMA1 remained in infant circulation until 8–12 months of life. On the other hand, infants typically produced IgG to MSP1 from their first postnatal infection, but little or no IgG to EBA175 and AMA1 were produced by infants in response to infections. We speculate that persistent maternal IgG to AMA1 and EBA175 may be inhibiting active acquisition of IgG to these antigens by the infant. The differential pattern of acquisition of antibodies to Pf antigens may have significant translational impact on vaccine designs. AMA1 and EBA175 are vaccine candidates, but based on the present data, administration of a vaccine based on these two antigens during the first year of life may not induce any active immune response because not much natural immunity to these antigens is acquired by infants in endemic areas

Finally, the current studies contribute significantly to the field of fetal origins of disease susceptibility. Prior to our work, many authors had repeatedly observed that offspring of PM-positive women had higher odds of becoming infected with Pf than offspring of women who did not have PM, but the underlying mechanisms were unclear. Our studies present maiden findings that the amount of exposure to Pf, reflected by placental parasite density modifies PM-related risk malaria in infants. The infants that were most at risk of experiencing a short time to first postnatal infection were those whose mothers had relatively low placental parasite densities. Infants of high PM parasitemia mothers had similar, and in some instances, better outcomes than infants of PM-negative mothers. Our explanation for this finding is that, in the intrauterine environment where T regulatory cell suppression of T effector functions is high, a threshold amount of Pf antigen (and PAMPs) is required for T helper cells to overcome T-reg suppression

and become sensitized to Pf. Our additional findings that PM-positive-high and not PM-positive-low infants had a recall antibody response to MSP1 during the first post-natal infection corroborate with an antigen threshold requirement for activation of fetal T and B lymphocytes.

Overall, the key take-home messages from these studies are that fetuses are not immunologically naïve to Pf but are capable of mounting Pf-specific responses *in utero* and the impact of prenatal exposure to Pf on post-natal malaria susceptibility depends on the amount of malarial antigen exposure *in utero* – low dose antigen exposure having a worse outcome than relatively high dose exposure. In translational terms, this means that malaria control measures that reduce placental parasite density, but do not prevent PM, will inadvertently increase the risk to Pf infections after birth. The currently used regimen for intermittent preventive treatment of malaria in pregnancy (sulfadoxine-pyremethamine) is reportedly ineffective in some parts of Africa in clearing placental parasites because of rising drug resistance. Based on our findings, health policy makers should consider replacing sulfadoxine-pyremethamine with more effective drugs in areas where resistance is being reported to prevent low antigenic exposure of fetuses.

The present dissertation does not exhaustively investigate early immune responses to Pf. Looking forward, there are interesting research questions that could be addressed. Because infants acquired antibodies to MSP1 before making antibodies to other Pf antigens, it will be important to further investigate the fine specificity of MSP1 IgG in infants. Not all IgG to MSP1 are functionally important in controlling Pf parasites – some of the MSP1 IgG inhibit erythrocyte invasion by merozoites, while others block this inhibitory process, and other MSP1 IgG are simply neutral. Also, in some of our side studies of antibody profiles in individuals aged 5–80 years old, we found an age-related pattern of acquisition of Pf antibodies which is opposite to the pattern during the first year of life. That is, in children 5–10 years old, high levels of IgG to

AMA1 and EBA175, comparable to adult levels, were detected, while MSP1 IgG were relatively low in this age group and continued to rise with age up to 15–20 years. Therefore, in the age gap of 1–5 years, it is unclear at what point EBA175 and AMA1 IgG production overtake MSP1 IgG production and its bearing to age-dependent changes in childhood susceptibility to malaria. Furthermore, children of the 2001–2005 birth cohort are currently in their teenage years. It will be important to investigate if the differences in immune response to Pf and susceptibility to malaria seen during the first year of life in relation to prenatal exposure persist through late childhood.