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Maternal-fetal conflict during placental malaria: hypertension, trophoblast sVEGFR1 expression and maternal inflammation

Atis Muehlenbachs

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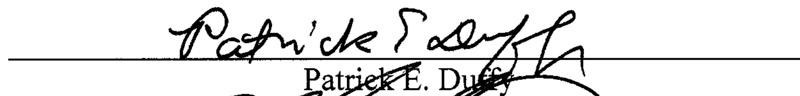

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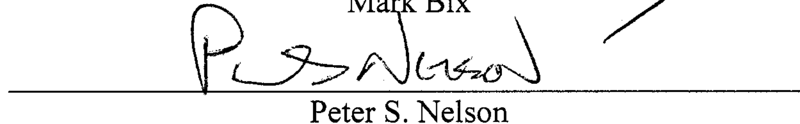
Mark Bix



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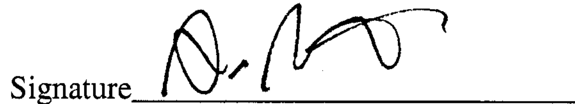
Signature 

Date 26 May 2006

University of Washington

Abstract

Maternal-fetal conflict during placental malaria: Hypertension, trophoblast sVEGFR1 expression and maternal inflammation.

Atis Muehlenbachs

Chair of the Supervisory Committee:
Affiliate Associate Professor Patrick E. Duffy
Molecular and Cellular Biology Program

The maternal-fetal relationship is hypothesized to be in genetic conflict over nutrient allocation. Fetal mechanisms that elevate maternal blood pressure increase blood flow to the placenta, nourishing the fetus. This relationship becomes pathological during hypertensive diseases in pregnancy, such as preeclampsia. During placental malaria, a third organism, *Plasmodium falciparum*, occupies the maternal side of the placenta, and maternal inflammation ensues which results in low birth weight. Both placental malaria and preeclampsia are more common during first pregnancies. This dissertation examines the maternal fetal relationship during placental malaria, specifically regarding maternal blood pressure. Chapter 1 is a review of the literature. Chapter 2 presents the epidemiology of placental malaria and hypertension based on data collected from the Mother Offspring Malaria Project in Muheza, Tanzania. Chapter 3 presents data that placental malaria involves vascular endothelial growth factor (VEGF) and its soluble inhibitor sVEGFR1, a preeclampsia biomarker. Chapter 4 characterizes the maternal inflammatory response in placental malaria, and finds correlation with sVEGFR1 expression.

In summation these chapters present data that hypertension occurs in first time mothers with chronic placental malaria, and that genetic conflict occurs between mother and fetus during placental malaria, whereby maternal macrophages in the placenta express the proinflammatory mediator VEGF, and the fetal trophoblast expresses its soluble inhibitor sVEGFR1. Because placental inflammation causes poor fetal outcomes, fetal mechanisms that promote sVEGFR1 expression may be under selective pressure in malaria endemic areas, particularly during first pregnancies, and may have influenced the evolution of preeclampsia.

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LIST OF ABBREVIATIONS

ANOVA- Analysis of variance

IFNG- Interferon-gamma

KRT7- Cytokeratin 7

MAP- Mean arterial pressure

PlGF- Placental growth factor

PM- Placental malaria

PlGF- Placental growth factor

RT-PCR- Reverse transcription polymerase chain reaction

VEGF- Vascular endothelial growth factor

sVEGFR1- Soluble vascular endothelial growth factor receptor 1

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DEDICATION

To my parents.

Chapter 1: Introduction

The placenta

The placenta was revered by the ancient Egyptians as being the "seat" of the soul, and the pharoah's placenta was regarded as a deity (Boyd et al. 1970). In a survey of pre-industrial societies worldwide, Jones noted that during delivery the placenta was consistently a "grave concern" because of the risk of placental retention and death to the mother (Jones et al. 1987). However there was a wide range of ritual regarding disposal of the placenta. In cultures with highly ritualized disposal, such as the Ganda of Africa, or the Pawnee of North America, the fate of the placenta was intimately connected to the future of the baby. In my dissertation these concepts remain fundamental: that the placenta has the ability to harm the mother, while an insult to the placenta during gestation harms the fetus.

Boyd and Hamilton compiled the history of knowledge concerning the placenta (Boyd et al. 1970). Aristotle identified the fetal membranes coining the term "chorion" and Hippocrates believed that blood transferred directly from the mother into the fetus, which was further promulgated by Galen. The placenta received little attention during the Middle Ages, until Leonardo da Vinci made his famous anatomical drawing (1510-1512) of a human fetus in utero, however erroneously with an ungulate's placenta. Realdus Columbus introduced the term "placenta," which is latin for cake, in his 1559 treatise "De Re Anatomica" (Colombo 1559). Aurantius 5 years later correctly but

controversially determined that the fetal and maternal vessels do not connect (Aranzi 1564), and William Harvey suggested that maternal blood flows through the placenta providing nourishment to the fetal circulation (Harvey 1651). William Hunter accurately and beautifully described placental anatomy (Hunter et al. 1774) and proved the separation of maternal and fetal blood by a wax injection experiment (Hunter 1786). William Turner in 1873 described the intervillous space, where maternal blood enters through the spiral arteries and exits the uterine veins, bathing the chorionic villi (Turner 1873). This structure was contested at the time because other mammals have dramatically different arrangements of placental tissue (Mossman 1937). After the advent of microscopy, Theodor Langhans in 1882 determined that the chorionic villi are covered by a fetally derived epithelial barrier without cell boundaries that separates the fetal blood vessels from the maternal blood (Langhans 1882). These cells were later termed "trophoblast" in a study of the hedgehog by Hubrecht, which he defined as cells from the blastocyst that provide nourishment to the embryo (Hubrecht 1889).

The placenta is a disc roughly 20 cm in diameter. There is a fetal surface, and a maternal surface that is attached to the uterus. Maternal blood enters the intervillous space through the spiral arteries. These are maternal blood vessels that have been invaded by invasive fetal trophoblast cells and transformed into low resistance vessels that the mother cannot limit blood flow through. Maternal blood exits the intervillous space through the uterine veins. Fetal blood enters the placenta through the umbilical artery, which branches and transverses the fetal surface of the placenta where further branches enter the chorionic villi that contain capillary trees, and the blood returns in a

venous path to the umbilical vein. If the chorionic villi are conceptualized as trees, the trophoblast would be the bark and the maternal blood would be air between the branches.

There are two major populations of trophoblast: first, the invasive trophoblast which invades the maternal lining of the uterus, the decidua, and also invades the spiral arteries, destroying smooth muscle and endothelium. Second, the villous trophoblast lines the chorionic villi, and consists of two layers. The outmost layer, termed the syncytiotrophoblast, is a true multinuclear syncytium. It is replenished by underlying cells with proliferative capacity, the cytotrophoblast. As a cytotrophoblast fuses with the syncytiotrophoblast, the cytotrophoblast nucleus undergoes apoptosis and clusters with other nuclei. These "syncytial knots" are pinched off into the maternal circulation. The entire process takes roughly two weeks. The syncytiotrophoblast is a remarkable cellular structure, roughly 10 m^2 in area contaning over 10 billion nuclei, through which all nutrient and gas exchange between mother and fetus occurs (Benirschke et al. 2000). In addition, the syncytiotrophoblast is the structure that *Plasmodium falciparum* infected erythrocytes adhere to during placental malaria.

The trophoblast and maternal adaptation to pregnancy

The trophoblast is critical for pregnancy maintenance. It has direct exposure to the maternal blood and secretes a wide range of steroid and peptide hormones. These hormones alter maternal physiology: they raise blood sugar, increase cardiac output

and modulate her immune system. This provides nourishment to the fetus and prevents allograft rejection.

The trophoblast interacts with diverse endocrine pathways in the mother. It participates in the synthesis of large amounts of estrogen and progesterone (Diczfalusy 1964), which maintain pregnancy (Albrecht et al. 2000) and may contribute to maternal hemodynamic changes (Pepe et al. 1995). The trophoblast secretes corticotrophin releasing hormone (Grino et al. 1987), which increases maternal cortisol synthesis which has pleiotropic effects during pregnancy. The trophoblast secretes human chorionic gonadotropin (hCG), the "pregnancy hormone" (Braunstein et al. 1973) which promotes progesterone synthesis (Braunstein 1996) and stimulates the mother's thyroid gland (Yoshimura et al. 1995). The trophoblast secretes the growth hormone analog, chorionic somatomammotropin, which causes insulin resistance, lipolysis, elevated blood sugar (Grumbach et al. 1973). In addition, the trophoblast secretes calcitriol (1,25-dihydroxyvitamin D) which increases calcium in the mother's blood (Seely et al. 1997).

Medawar described the fetus as an "allograft," with one half of its genetic material foreign to the mother, and proposed that the fetus induces tolerance in the mother to prevented graft rejection (Medawar 1953). Many pathways have been identified by which the placenta evades the mother's immune response. Maternal systemic cortisol and local progesterone synthesis act to inhibit the mother's inflammatory response against the conceptus (Siiteri et al. 1982). In addition, the placenta is a source of type 2 cytokines, including interleukin 4, which counteract the

effects of maternal tumor necrosis factor (TNF) and interferon (IFN)-gamma, which are harmful to pregnancy (Wegmann et al. 1993; Marzi et al. 1996). The trophoblast also suppresses maternal T-cell activity by catabolizing tryptophan (Munn et al. 1998). Further it displays non-classical MHC molecules (Kovats et al. 1990), and expresses the apoptosis inducing CD95L (Hunt et al. 1997).

During pregnancy, hemodynamic changes occur in the maternal cardiovascular system. These include an increase in plasma volume (Lindheimer et al. 1973), a decrease in total peripheral resistance(Spetz 1964), and an increase in cardiac output (Lees et al. 1967). Angiotensin II is a potent vasopressor, and normal pregnant women become resistant to its effects (Chesley et al. 1965). Maternal blood pressure falls during mid-pregnancy, but approaches normal levels near term (MacGillivray et al. 1969). Several mechanisms by which placenta effects maternal hemodynamic change have been suggested. The steroid hormones estrogen and progesterone are believed to increase the pliability of maternal blood vessels and increase blood flow to the uterus (Albrecht et al. 2000). hCG may act to increase plasma volume and decrease osmolarity (Davison et al. 1988). The placenta secretes a vasopressinase that clears maternal anti-diuretic hormone (Davison et al. 1993). In addition nitric oxide (Weiner et al. 1997) and prostacyclin (Knock et al. 1996) may cause vasodilation and decrease blood pressure. Also, serum from pregnant woman is anti-angiogenic, which may be related to the soluble vascular endothelial growth factor receptor (VEGFR1) that is secreted during normal pregnancy (Clark et al. 1998). These changes are important when considering preeclampsia which is marked by hypertension, pressor sensitivity

(Talledo 1966), increased cardiac output (Easterling et al. 1987; Easterling et al. 1990), and an increased anti-angiogenic state (Maynard et al. 2003).

Maternal fetal conflict

Trivers in 1974 wrote that parents and offspring are in genetic conflict- that what is best for the offspring is not necessarily best for the parent and the parent's future offspring (Trivers 1974). This theory predicts that offspring are selected to want more resources than the parents are selected to give. This conflict is genetic because offspring share only 50% of the mother's genetic material, and different genes would be associated with either giving or taking resources. The alleles of such genes could affect the fitness of the offspring or the fitness of the mother who may have multiple offspring. An illustrative example concerns weaning of suckling in baboons: an infant baboon, like a human, cries during weaning, however baboons are a species that are in general very quiet as to not to alert predators. Thus, the infant baboon puts its mother's life in danger in order to gain more milk (Trivers 1974).

In 1993 Haig applied these concepts to human gestation, stating that maternal-fetal relationship has been shaped by an evolutionary history of genetic conflict over nutrient allocation (Haig 1993). He stressed that neither mother nor fetus would benefit by a "*drastic change to the status quo*" but likened the situation to a

"*tug-of-war[, where] two teams attempt to shift a flag a small distance either way, yet there is high tension in the rope and the system would collapse if either side stopped pulling.*"

He suggested that these evolutionary dynamics may have resulted in diversification observed in placentas- the most morphologically diverse organ across mammalian species (Mossman 1937). Further, he suggested that the degree of cross-talk and redundancy in the endocrine interaction between mother and fetus was driven by genetic conflict.

Haig suggested that certain disorders of pregnancy are not the failure of both the mother and fetus to adapt to an environment, but are the result of maternal-fetal conflict: imbalances in the 'tug-of-war.' During gestational diabetes, excessive maternal insulin resistance leads to elevated blood sugar. During preeclampsia, the mother's blood pressure increases, increasing blood flow to the placenta. Conversely Haig suggested that conditions where the mother withholds resources could result in be spontaneous abortions and preterm deliveries. Pertinent to this dissertation, Haig and Karumanchi suggested that as a result of maternal-fetal conflict, the placenta secretes sVEGFR1 into the maternal circulation to increase placental blood flow (Yuan et al. 2005).

During placental malaria, a third organism, *Plasmodium falciparum*, occupies the maternal intervillous space of the placenta, and maternal inflammation ensues which results in low birth weight. What is the effect of this third organism on the "tug-of-war" between mother and fetus? Further, how could the evolution of this "tug-of-war" have been influenced by the burden of placental malaria during human history?

Hypertensive disorders of pregnancy

Hypertensive disorders of pregnancy affect about 10-15% of all pregnancies, and are estimated to cause 10-15% of all maternal deaths (Duley 1992). The most well described disorder is preeclampsia (Chesley 1978; Roberts et al. 2001; Redman et al. 2005). Preeclampsia is defined by hypertension and proteinuria during pregnancy, and affects 4-6% of all pregnancies. Eclampsia is characterized by convulsions or coma, and is especially deadly. Preeclampsia and eclampsia occur most frequently during first pregnancies, and are most common close to term. Other risk factors include advanced maternal age, obesity and diabetes. Both preeclampsia and eclampsia can cause death, which is rarely due to the clinical sign of hypertension, but it is often due to hepatic or renal failure or cerebral hemorrhage.

Leon Chesley chronicled the discovery of preeclampsia (Chesley 1978), and found no evidence that Hippocrates or the ancient Greeks were aware of eclampsia, and may have attributed it to epilepsy. Unawareness of eclampsia continued past the Middle Ages and through the Renaissance, largely because most deliveries were conducted by midwives who did not publish their observations. Yet even as late as the 18th century, major textbooks in obstetrics did not mention convulsions (Chesley 1978). Eclamptic convulsions were first described by Francois Mauriceau in 1694, who noted their preponderance in first pregnancy and their risk to the mother (Mauriceau et al. 1694). Importantly, he attributed their cause to the uterus. De Sauvages introduced the term "eclampsia" in 1739 (Boissier de la Croix de Sauvages et al. 1739). He attributed the term to Hippocrates, but the Greek root for "eclampsia"

was probably mistranslated at the time to mean "seizure," with the proper translation of "eclampsia" meaning "brightness" or "lightning" (Chesley 1978).

The clinical sign of edema was observed by Mauriceau in his first description of eclampsia (Mauriceau et al. 1694). 150 years later Lever identified proteinuria in eclampic patients, and proposed that eclampsia was due to compression of the renal veins by the pregnant uterus (Lever 1843). Screening of pregnant women for proteinuria by Sinclair and Johnson was successful in identifying women at risk for eclampsia at the Dublin Lying-in hospital during the 1850s (Sinclair et al. 1858). Tracking the pulse led Ballantyne in 1885 to believe blood pressure was elevated during eclampsia (Ballantyne 1885), which was formally demonstrated by Vaquez and Nobecourt 1897 (Vaquez et al. 1897). Cook and Briggs 1903 discovered that pregnant women with proteinuria often had hypertension and suggested that hypertension could be prognostic for eclampsia (Cook et al. 1903). These discoveries formed the basis of current practice: women at risk for preeclampsia or eclampsia are detected by screening for hypertension, proteinuria and edema.

At present there is debate over the exact definition of preeclampsia, which the International Society for the Study of Hypertension in Pregnancy defines as the onset of hypertension in the presence of proteinuria (Brown 2003). Many studies of preeclampsia have used different diagnostic criteria, such as the presence of either one of hypertension, proteinuria or edema. Further, the definition of hypertension or proteinuria often varies between study site, making comparisons or generalizations very difficult or near impossible (Davies 1971). In some regard, the differences in

diagnostic criteria reflect the difficulty in categorizing cases because measurements of blood pressure, urine protein and inflammatory markers fall into a spectrum that overlaps with normal pregnancy (Redman et al. 1999). A multitude of terms have been used in the literature including: "preeclampsia" (either mild or severe), "toxemia" (either preeclamptic or nephritic), "gestosis," "gestational hypertension," and "pregnancy induced hypertension". "Toxemia" once meant preeclampsia or eclampsia and sometimes included essential hypertension or edema. The term "preeclampsia" has even been contested because most patients, even if untreated, do not develop eclampsia (Chesley 1978), and the term is excluded in the Canadian classification system (Brown 2003).

Higher rates of preeclampsia have been observed in people of African ancestry. Studies have observed elevated risk in African Americans (Page et al. 1976; Eskenazi et al. 1991; Greenberg et al. 1993; Irwin et al. 1994; Coonrod et al. 1995; Mittendorf et al. 1996; Morris et al. 2001; Zhang et al. 2003; Shen et al. 2005), Africans in Europe (Knuist et al. 1998) and in South Africa (Naidoo et al. 1980). However Chesley (Chesley 1978) and Sibai (Sibai et al. 1995) found rates of preeclampsia in African Americans to be similar to Caucasian Americans. Yet in a larger trial Sibai found non-white race to be a risk factor (Sibai et al. 1997), but was corrected for by study center, body mass index and baseline blood pressure. The rates of chronic hypertension are higher in African Americans, which may contribute to the increased risk of preeclampsia (Samadi et al. 1996). People of South Asian ancestry have also been observed have increased rates of preeclampsia in Fiji (Bell et al. 1955), and Trinidad

(Magee 1961). Davies surveyed reports worldwide and found a high degree of variability. For example, in sub-Saharan Africa rates of preeclampsia ranged from 1 to 16 %. He attributed such variability to different diagnostic criteria and on the limitations of hospital based studies, concluding that no comparisons are informative for racial or geographic predisposition to preeclampsia (Davies 1971). However in a coordinated study, the World Health Organization found that geographic differences exist in the the incidence of preeclampsia between countries in Asia (WHO 1988), but no similar study has been performed to date involving sub-Saharan Africa.

The increased incidence of preeclampsia in people of African and South Asian ancestry would suggest that mechanisms that promote the development of preeclampsia may have been under selective pressure during recent human evolution. The data presented in this dissertation imply that malaria could have been such a selective factor.

Pathogenesis of preeclampsia

Zweifel in 1916 coined preeclampsia as the much quoted "disease of theories," ((Zweifel 1916) cited by (Chesley 1978)) of which there have been many: over-nutrition, under-nutrition, *Micrococcus eclampsiae*, helminthes, excess water, salt, and multiple metabolic products (Chesley 1978). Current understanding of the pathogenesis of preeclampsia involves two stages (Roberts et al. 2001; Redman et al. 2005). The first stage results from a defect in placental development, which may have a wide array of causes. This leads to a distressed or hypoxic placenta that secretes

substances into the maternal peripheral circulation. These substances cause the second stage of preeclampsia: the maternal syndrome, manifest by hypertension and proteinuria, which is marked by endothelial dysfunction (Roberts et al. 1989) and systemic inflammation (Redman et al. 1999).

Improper placentation, the first stage of preeclampsia was first suggested by Young who observed placental infarcts, suggestive of ischemia in eclampsia (Young 1914). In 1964, Brosens described the pathology of the uterine spiral arteries in preeclampsia, demonstrating shallow trophoblast invasion and a lesion he termed "acute atherosis" (Brosens 1964), which he suggested could be pathogenic in preeclampsia (Brosens et al. 1970). Poorly remodelled spiral arteries are also observed in intrauterine growth restriction (Brosens 1964). Page argued in 1972 the central role of hypoxia and placental insufficiency in the pathogenesis of preeclampsia (Page 1972). A genetic basis for improper placentation was recently proposed by Hiby to explain the association of preeclampsia with a strongly inhibitory combination of fetal HLA-C and maternal KIR alleles (Hiby et al. 2004). Spiral artery remodeling is complete by 20 weeks, suggesting that initial insult occurs before the onset of maternal symptoms.

Understanding of the maternal syndrome, the second stage of preeclampsia, began when examining the reaction of preeclamptic women to angiotensin II. Preeclamptic women are sensitive to angiotensin II (Talledo 1966), and angiotensin sensitivity occurs as early as 14 weeks gestation in women who develop preeclampsia, well before the onset of symptoms (Gant et al. 1973). This may be related to the

elevated cardiac output observed early in pregnancy in women who develop preeclampsia (Easterling et al. 1990). Pathologically, a kidney lesion unique to preeclampsia, glomerular endotheliosis, was identified by Spargo (Spargo et al. 1959). These discoveries, together with characterization of activation of the coagulation cascade, inspired the hypothesis that the maternal syndrome is secondary to generalized endothelial dysfunction (Roberts et al. 1989), explaining the clinical signs of hypertension, proteinuria and edema.

The maternal syndrome of preeclampsia is also characterized by systemic inflammation, which Redman proposes as causing a positive feedback loop that furthers placental damage (Redman et al. 1999). Trophoblast fragment deportation (Redman et al. 1999) and proinflammatory cytokines (Saito et al. 1999) have been proposed to contribute. Women with urinary tract infection (Schieve et al. 1994; Mittendorf et al. 1996), periodontal disease (Boggess et al. 2003) and anti-chlamydial antibodies (Heine et al. 2003) have been observed to be at greater risk for preeclampsia, suggesting a role for inflammation. Women with preexisting diabetes, obesity or hypertension are particularly at risk for preeclampsia. Because these conditions are associated with inflammation and endothelial pathology, these women may be particularly susceptible to endothelial dysfunction, therefore explaining their susceptibility to preeclampsia. By considering both fetal and maternal stages of disease, preeclampsia is perceived as a syndrome with many causes.

Characterization of the factors secreted by the preeclamptic placenta which cause the maternal syndrome has been pursued intensely. During the first half of the

20th century, various placental extracts and fractions were injected into animals to little avail (Chesley 1978). Trophoblast deportation, first observed in the maternal lungs of a woman who who died of eclampsia (Schmorl 1893), eventually lead to the hypothesis that trophoblast microparticles are toxic to maternal endothelial cells and induce inflammation (Redman et al. 1999). Other factors include TNF (Meekins et al. 1994), epinephrine (Manyonda et al. 1998), neurokinin B (Page et al. 2000), lipid peroxides (Zeeman et al. 1992), and agonistic anti-angiotensin II receptor antibodies (Dechend et al. 2005). Soluble vascular endothelial growth factor receptor 1 (sVEGFR1) is a soluble VEGF receptor (also termed fms-like tyrosine kinase 1 (sFlt1)). The evidence that sVEGFR1 contributes to the pathogenesis of preeclampsia is very compelling: it is elevated prior to the onset of disease (Levine et al. 2004), anti-VEGF antibodies result in hypertension and proteinuria in cancer patients (Kabbinavar et al. 2003; Yang et al. 2003). Further, sVEGFR1 administration to pregnant or non-pregnant rats resulted in hypertension, proteinuria and glomerular endotheliosis (Maynard et al. 2003). These findings make sVEGFR1 the only proposed factor to date that experimentally causes these three criteria for the maternal syndrome of preeclampsia. Further background to the VEGF and VEGFR1 system is provided in the introduction to **Chapter 3**, which concerns sVEGFR1 in placental malaria.

Two observations in preeclampsia are not sufficiently explained. First, why does preeclampsia occur primarily in first pregnancies? Second, why does preeclampsia occur primarily in humans? Pankow in 1906 observed differences in walls of uterine arteries between women who have never been pregnant to those that

have (Pankow 1906), suggesting that first pregnancies may encounter a different uterine environment from later pregnancies: that uterine arteries of first pregnancies would require a greater degree of remodelling. An additional theory suggests the risk in first pregnancies is actually a risk of first paternity (Robillard et al. 1993; Li et al. 2000), and that tolerance to paternal antigens, such as the length of premarital cohabitation (Robillard et al. 1994) or previous pregnancy, lowers the risk. Later studies found that the length of time between pregnancies corrected for the effect previously observed in changing partners- as advances in maternal age increase the risk of preeclampsia (Trogstad et al. 2001; Skjaerven et al. 2002).

Why does preeclampsia primarily occur in humans? Preeclampsia does not spontaneously occur in other mammals, with a few exceptions. Palmer described a preeclamptic state consisting of, edema, hypertension and proteinuria in 6% of patas monkeys which was more frequent in first pregnancies (Palmer et al. 1979). (Of note, no data is available for malaria parasites in patas monkeys, which live in the African savannah (Garnham 1966).) Benirschke has reported a case of placental infarcts, proteinuria and maternal death in an orangutan (Benirschke 2004) and convulsions and edema have been reported in three pregnant gorillas (Baird 1981; Thornton et al. 1992). There have been no reports on chimpanzees or other primates. Placental morphology has been attributed to explain why preeclampsia does not occur in rodents. Most rodents have epitheliochorial placentas, where the trophoblast is exposed to maternal uterine epithelium, not blood. However guinea pigs have a hemochorial placenta, like humans, but guinea pigs do not get preeclampsia. An argument has been

made that preeclampsia is an evolutionary consequence of the depth of invasion of the spiral arteries in humans, in order to support human brain size and length of gestation in comparison to other primates (Chaline 2003).

I propose an additional theory to the etiology of preeclampsia, first that placental malaria can lead to secretion of placental factors that lead to the maternal hypertension. I hypothesize that these factors protect the fetus from maternal inflammation. Applying the theory of maternal-fetal genetic conflict, human exposure to *falciparum* malaria may have influenced the evolution of preeclampsia, explaining its predilection for humans and first pregnancies.

Malaria

Malaria is a plague. Bruce-Chwatt chronicled the history of malaria (Bruce-Chwatt 1988): It was recorded in the Vedas and by the Ancient Egyptians as recurring fevers, splenomegaly and association with swamps. Hippocrates described intermittent fevers corresponding to the different malaria parasites. Malaria was scourge on the Romans, and was present around the Mediterranean until the 20th century: "Mal'aria" is Italian for "bad air". Malaria extended to Northern Europe such that Linnaeus and his wife suffered from it. It was until 1880 that Alphonse Laveran, a doctor stationed in Algeria performing autopsies on malaria victims, identified the malaria parasite in human blood (Laveran 1881). Marchiafava and Celli later determined that the parasite lives within erythrocytes (Marchiafava et al. 1884), where it completes its asexual life cycle (Garnham 1966).

Malaria is caused by the protozoa in the genus *plasmodium* phylum *Apicomplexa.* Four species of *Plasmodium* infect humans (Garnham 1966). *P. falciparum* is responsible for nearly all deaths, with *P. ovale, P. vivax* and *P. malariae* resulting in variable morbidity but minimal mortality. *P. falciparum* is estimated to be about 100,000 years old, with a recent population expansion 10,000 years ago, coincident with the advent of agriculture (Joy et al. 2003). Haldane postulated that malaria was a selective force on evolution of the human hemoglobinopathies (Haldane 1948). One appreciates the degree of selective force by the distribution of sickle trait (Allison 1954), where homozygosity is lethal. Based on these and other associations, Kwiatkowski described malaria to be the "strongest known force for evolutionary selection in the recent history of the human genome" (Kwiatkowski 2005). In the 20th century, malaria has been eradicated from the Mediterranean, parts of Asia and the Americas. However currently 1 billion people are at risk for malaria infection- which causes over 1 million deaths per year in sub Saharan Africa and much greater morbidity.

P. falciparum kills people by sequestering in the deep vascular beds of organs. Laveran observed his parasites in the vessels of the brain, however Marchiafava and Bignami in 1894 described selective sequestration based on the neuropathology of "ring hemorrhages," where infected erythrocytes remain sequestered in a cerebral capillary and uninfected erythrocytes bleed into the parenchyma (Marchiafava et al. 1894). Sequestration has been observed in heart muscle, gut, pancreas, periadrenal and abdominal fat, skin, bone marrow and placenta (Taliaferro et al. 1937; Boonpucknavig

et al. 1988). Intense inflammatory responses to the parasites occur in the vascular spaces of these organs, particularly the spleen, liver, bone marrow, and placenta, involving a striking accumulation of phagocytes engorged with malarial pigment (Taliaferro et al. 1937; Boonpucknavig et al. 1988).

Remarkably, *P. falciparum*'s closest relative, *P. reichenowi* which infects chimpanzees, does not sequester (Garnham 1966), whereas sequestration has been observed in the monkey parasite *P. knowlesi* and rodent parasite *P. berghei,* which are more closely related to the non-sequestering human malaria species (Garnham 1966). *P. knowlesi* experimentally sequestered in the placenta of an infected macaque (Das Gupta 1939), and *P. berghei* experimentally sequesters in the placenta of rats (Desowitz et al. 1989). Whether *P. berghei* infected erythrocytes adhere directly to endothelium has not been shown and may be mediated by immunological factors (Wright 1968). Other *Plasmodium* species are not known to sequester.

Data presented in dissertation suggests that malaria may be a selective force on the fetus, particularly during first pregnancies. *Falciparum* malaria may have influenced the evolution of preeclampsia- particularly its occurrence primarily in humans and first pregnancies.

Malaria during pregnancy

Duffy and Desowitz chronicled the history of pregnancy malaria and wrote "As long as woman walked the earth malaria may have stalked her."(Duffy et al. 2001). Hippocrates described intermittent fever during pregnancy, Mercado in 1614 further

described such fevers (Mercado 1614), for which Torti later recommended quinine (Torti 1814). Laveran, who discovered the parasite, described malaria causing anemia and cachexia during pregnancy (Laveran 1898). Parasites were first observed to be sequestered in the intervillous space by Bignami in 1898 (Bignami 1898)

Two patterns of pregnancy malaria occur depending on malaria endemicity of the area in which women live. Wickramasuriya, an obstetrician described epidemic malaria in a non-immune population during the 1934/1935 epidemic in Sri Lanka (Wickramasuriya 1936). Placental malaria resulted in miscarriages and neonatal deaths, which were as high as 80% in some areas, and maternal mortality of all cases was 13%. The effects on the mother were anemia, preeclampsia-eclampsia, cerebral malaria and cachexia. Wickramasuriya's series of case reports described women of all parities and he believed that the effects of malaria were worse in pregnant women than in non-pregnant women.

Pregnancy malaria is different in endemic areas. In endemic areas, people have a considerable amount of immunity against *P. falciparum* by adolescence and are able to limit both parasitemia and severe disease (Wilson et al. 1950). Pregnancy malaria is less severe for the mother, but Bruce-Chwatt observed it to cause low birth weight (Bruce-Chwatt 1952). Canon discovered that malaria occurs most in first pregnancies (Cannon 1958), and first pregnancies were previously known to have high rates of prematurity and low birth weight in endemic areas (Archibald 1956; Archibald 1958). Over subsequent pregnancies, women become resistant to malaria infection during pregnancy (McGregor 1984). At present, rates of placental infection have been

reported to be as high as 70% in first time mothers in certain regions of sub-Saharan Africa (Brabin 1983; Steketee et al. 1987; Jackson et al. 1991; Mvondo et al. 1992). Further, low birthweight due to malaria is estimated to cause 200 thousand infant deaths each year in sub-Saharan Africa.

In the late 20th century Fried and Duffy discovered why pregnant women are susceptible to malaria. Infected erythrocytes (IE) from infected pregnant women are phenotypically different than IE from non-pregnant hosts: IE isolated from placentas adhere to chondroitin sulfate A (CSA) which is present on the trophoblast surface, and not CD36 (a host receptor involved in IE vascular sequestration), the opposite of what is observed with IE from non-pregnant individuals (Fried et al. 1996). In addition, IE from pregnant women do not agglutinate in sera from immune multigravid women (Fried et al. 1998), nor do they rosette uninfected red cells (Maubert et al. 1998), like IE from non-pregnant individuals. These findings demonstrate that IE during placental malaria have a unique phenotype. Importantly antibodies from exposed multigravid women are able to block IE adherence to CSA (Fried et al. 1998), which explains why women in their first pregnancies are susceptible to infection and poor outcomes, whereas women in later pregnancies are not.

The relationship between mother, fetus and parasite is demonstrated in placental histology, which can reveal varying, and striking degrees of living parasites and maternal cellular infiltrates (Garnham 1938). Maternal inflammatory cells can accumulate to such an extent leading Garnham to comment that "In certain phases of malaria, the contents of the space are an almost solid mass of R.E. cells [macrophages]

and it is difficult to understand how the fetus is nourished." Which has later been termed "massive chronic intervillositis" (Ordi et al. 1998). Garnham, by analyzing analyzing 553 infected placentas (some inoculated), determined the time course of infection (Garnham 1938). Acute infection occurs within one week where IE are present without immune cells in the intervillous space or malaria pigment deposited in extracellular fibrinoid. In about one week, immune cells accumulate which begin to clear the IE. Relapses he characterized as having inflammatory cells present with abundant parasites. In past or resolved infections, malarial pigment remains in extravillous fibrinoid after parasites are cleared. Garnham observed that immune women (which were presumably multigravidae) did not get inflammation, but rapidly cleared parasites.

This maternal infiltrate is primarily composed of monocytes and macrophages, with a smaller proportion of lymphocytes (Ordi et al. 2001). Infected placentas from first-time mothers have a much greater degree of maternal immune infiltration than from later pregnancies (Meuris et al. 1993; Ordi et al. 1998). Epidemiologically, this maternal infiltrate is more closely associated with low birth weight than parasite density or pigment (Leopardi et al. 1996; Sullivan et al. 2000). The pro-inflammatory cytokines TNFa and IFNG are elicited in the placenta during malaria infection, and are associated with low birth weight(Fried et al. 1998). Infiltrating monocyte macrophages are the source of TNF, in addition to chemokines by histology (Moormann et al. 1999) and cell isolation (Suguitan et al. 2003). This suggests a model where the maternal inflammatory infiltrate, in response to infection, damages

the placenta which causes low birth weight. Hypoxia has also been postulated to occur during placental malaria and to lead to low birth weight (Watkinson et al. 1983). The specific roles of hypoxia, inflammation and their interaction to cause low birth weight due to malaria have not been elucidated.

Considering that fetus could survive maternal inflammation that occurs to the extent it astonished Garnham, and considering the 'tug-of-war' between mother and fetus as described by Haig, how does the fetus respond to this maternal assault?

The fetal response to placental malaria is incompletely characterized. Morphologically, there is cytotrophoblast proliferation, synctyial knotting, and syncytial necrosis has been observed (Walter et al. 1982). These are non-specific features thought to be related to hypoxia and are also observed in preeclampsia and intrauterine growth retardation. Explant studies suggest that the placenta may be a source of interferon gamma (IFNG) during malaria infection (Fievet et al. 2001; Suguitan et al. 2003), and other authors have observed upregulation of intercellular adhesion molecule 1 (ICAM1) on the trophoblast surface (Sartelet et al. 2000), and trophoblast expression of migration inhibitory factor (MIF) (Chaisavaneeyakorn et al. 2005). IFNG, ICAM1 and MIF are pro-inflammatory molecules, suggesting that the fetal compartment may promote maternal inflammation. Conceptually, if the fetus secretes these molecules in response to maternal inflammation, this would cause a positive feedback loop, and the ultimate result would be fetal demise. Based on the maternal-conflict theory of Haig (Haig 1993), the fetal component would be expected

to counter maternal inflammation. However molecules that limit inflammation have not been reported to be expressed by the trophoblast during placental malaria.

Current knowledge about the interaction between hypertension and placental malaria is scant and is detailed in the introduction of **Chapter 2**, where I show that hypertension occurs in young first-time mothers with chronic placental malaria. My dissertation provides evidence that during the 'tug-of-war' between mother, fetus and parasite, maternal inflammatory cells express the inflammatory mediator VEGF while trophoblast produces its soluble inhibitor sVEGFR1. This suggests fetal trophoblast tries to limit maternal inflammation, and in doing so causes maternal hypertension.

Chapter 2. The interaction between placental malaria and hypertension

Summary

Malaria and hypertension are major causes of maternal mortality in tropical countries, especially during first pregnancies, but evidence for a relationship between these syndromes is contradictory. We examined the epidemiologic relationship between PM and hypertension among Tanzanian women. We show that hypertension develops in first-time mothers with chronic placental malaria (PM). In contrast, PM during later pregnancies is associated with decreased blood pressure. Hypertension correlated to lower parasite densities and heavier pigment deposition in infected first-time mothers. Thus, chronic PM appears to induce hypertension among young first-time mothers.

Introduction: placental malaria and hypertension

Malaria decreases blood pressure in non-pregnant individuals, attributable to vasodilatation and hypovolemia (Warrell 2002). With severe malaria, people are at risk for hypotension and shock. The relationship between PM and hypertension is not clear (Brabin et al. 2005). During the 1935 malaria epidemic in Sri Lanka, the obstetrician Wickramasuriya wrote:

"Chronic malarial subjects show a marked predisposition to pre-eclampsia, eclampsia and 'nephritic' toxemia. This fact became particularly evident during the aftermath of the epidemic, when there was a greatly increased incidence of pre-eclampsia and eclampsia in hospital. Indeed, it may be said that there was an 'epidemic' of such toxaemic pregnancies following in the wake of the malaria epidemic." (Wickramasuriya 1936)

Hypertension was reported in 20%, Edema in 40% and albuminuria in 50% of 357 infected women. He made no comment on parity, although one half of 15 cases he described of infection with preeclampsia-eclampsia were in their first pregnancy. Prior to Wickramasuriya, Watson 1844 (Watson 1844) and Manson 1855 (Manson 1855) described convulsions, and Aubinais in 1850 described edema together with convulsions (Aubinais 1850) (Cited by (Duffy et al. 2001)). However these are not specific symptoms: cerebral malaria causes convulsions and *P. malaria* causes nephrotic syndrome with marked edema (Warrell 2002). Several case reports exist: a case of pregnancy malaria with preeclampsia was reported in Tanzania (Mrita 1965), and the Centers for Disease Control reported two cases of imported pregnancy malaria with preeclampsia, one of whom died of respiratory distress (Barat et al. 1997; Williams et al. 1999).

Curiously, an 'epidemic' of preeclampsia was reported in the Sakaeo refugee camp near the Cambodian border in Thailand, where preeclampsia rates are normally less than 10%. In November 1981 they peaked to about 25%, and decreased in the following months, and this did not occur in four other camps. Retrospectively, cases

had lower hematocrit than controls, but malaria was not suspected (Quillan et al. 1983).

In endemic areas the relationship between placental malaria and hypertension is less clear. In sub-Saharan Africa, the rates of preeclampsia and eclampsia increase during the rainy season, when malaria transmission is highest. The rainy season has been associated with increased rates of eclamspia in Mozambique (Bergstrom et al. 1992), Zimbabwe (Crowther 1985), and Nigeria (Agobe et al. 1981), Increased rates of preeclampsia in Senegal (Sartelet et al. 1996), and Zimbabwe (Wacker et al. 1998) and deaths due to eclampsia in The Gambia (Anya 2004) and Senegal (Etard et al. 2003). Neutra reviewed a large series of papers on the seasonality of eclampsia (Neutra 1974) and cited reports of increases during the cool damp months in India (pre-1940), Germany (pre-1950), and Italy (pre-1960). However studies in non-malarious areas have also observed preeclampsia to increase during the cooler months (Neutra 1974; Magnu et al. 2001). Evidence that placental malaria is related to preeclampsia is conflicting. In Senegal preeclamptic placentas were three times more likely to have malaria infection by histology (Sartelet et al. 1996), however in Kenya, no association between preeclampsia and peripheral parasitemia (Dorman et al. 2002) or placental malaria (Shulman et al. 2001) was observed. Other studies of placental malaria have not reported on hypertension (Bray et al. 1979; McGregor 1984), or have excluded hypertensive women (Crocker et al. 2004).

Understanding the epidemiological relationship between PM and preeclampsia is made difficult by the overlapping clinical presentation of both syndromes. Common features to PM and preeclampsia include headache, pulmonary edema, thrombocytopenia, seizures, and death. Wickramasuriya wrote:

"thus 18 out of 19 cases of cerebral malaria treated in the hospital during the epidemic had 'fits' sometime or other during their illness. Many had repeated fits and the clinical picture was often indistinguishable from that of true eclampsia" (Wickramasuriya 1936).

Such a challenge is illustrated in a recent study in Tanzania where 20 maternal deaths were attributed to cerebral malaria and only one out of 77 was attributed to eclampsia, otherwise a leading cause of maternal death (Olsen et al. 2002). Studies examining the relationship between PM and preeclampsia are further made difficult by the poor sensitivity of peripheral blood microscopy: it fails to diagnose about half of PM cases (Shulman et al. 2001; Mockenhaupt et al. 2002; Rogerson et al. 2003).

Brabin reviewed the relationship between preeclampsia and placental malaria (Brabin et al. 2005), and suggested that infected erythrocytes in the placenta might predispose to preeclampsia. He highlighted common mechanisms of pathogenesis which he suggested may act synergistically: in particular oxidative stress, placental ischemia and systemic inflammation in the mother.

During the epidemic, Wickramasuriya found that malaria was more severe in a pregnant woman than in her non pregnant counterpart (Wickramasuriya 1936). In addition, the risk of death or morbidity from malaria in the mother is greatest near term

(Edmonds 1899; Laffont 1911; Clark 1915; Wickramasuriya 1936; Menon 1972), cited by (Duffy et al. 2001). Most cases of preeclampsia also occur near term. Near term one can predict a high degree of maternal-fetal conflict because the fetus has a rapidly increased growth rate. These clinical observations suggest that placental factors elicited during PM, and not the infected erythrocytes *per se*, may be responsible for maternal morbidity in placental malaria.

In my dissertation I reach a similar conclusion to Wickramasuriya, that women with chronic malaria develop hypertension. Further I describe that parasite density has the effect to lower blood pressure, which may be why other studies have not found a relationship with infection and hypertension. In **chapter 3** I provide evidence that suggests trophoblast sVEGFR1 expression may mediate hypertension during placental malaria.

Methods

Human subjects. Placental samples and clinical information were provided by Tanzanian women age 18 to 45 years delivering at the Muheza Designated District Hospital, Muheza, Tanga region, in an area experiencing intense malaria transmission. Sample donors were among those recruited to participate in a birth cohort study known locally as the Mother-Offspring Malaria Study. Women provided signed informed consent before joining the study, and those with chronic debilitating disease and their children were excluded. Clinical information was collected by project nurses and assistant medical officers on standardized forms. Study procedures involving human

subjects were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the US National Institutes of Health, and ethical clearance was obtained from the Institutional Review Boards of Seattle Biomedical Research Institute and the National Institute for Medical Research in Tanzania.

Study Procedures. Hypertension was defined as systolic or diastolic blood pressure greater or equal to 140 or 90 mmHg, respectively. Blood pressure was abstracted from hospital antenatal cards as the maximum measurement recorded during the delivery hospitalization, Mean arterial pressure (MAP) was calculated as (systolic+2*diastolic)/3. If available, blood pressure measurements were also abstracted from prenatal clinic records. Mother's parity was calculated as number of previous term pregnancies subtracted by the number of miscarriages. Gestational age was calculated from the date of last menstrual period, and values less than 32 weeks or greater than 44 weeks were excluded for reliability. Mothers' heights and weights were recorded within 24 hours after delivery. Body mass index (BMI) was calculated as weight (kg)/(height (m) ^2). Urine protein assessment was not routinely performed in the hospital. Immediately prior to delivery, peripheral blood was collected in citrate phosphate dextrose, and serum was separated and frozen at -80 C. The placenta was collected at delivery, and a full thickness biopsy from the middle third of the placental disc was frozen in liquid nitrogen and stored at -80 C. Placental malaria was detected

by microscopy of Giemsa-stained thick and thin smears of placental blood extracted from placental tissue by mechanical grinding

Histopathologic analysis. Cryosections (5um) of placental tissue were Giemsa stained and assessed by examining greater than 90 60X fields per section. Hemozoin deposition in fibrinoid was quantified by determining the proportion of fields with hemozoin present. Inflammation was qualitatively scored by the presence of inflammatory cells in the intervillous space

Statistical analysis. Analyses were performed using Statview (SAS Institute). Student's t-test was used for the analysis of continuous variables. Peripheral plasma sVEGFR1 concentration and parasitemia (n+1) were log-transformed prior to analysis. Fisher's exact test was used to analyze differences in proportions of categorical variables. Odds ratios were calculated using logistic-regression analysis. Regression coefficients were calculated using multiple regression analysis. Interactions between variables were determined using factorial ANOVA. Spearman rank correlation was used to examine the correlation between sVEGFR1 quantitative PCR and ELISA measurements.

Results

We examined the relationship between placental malaria and blood pressure in women delivering at the Muheza Designated District Hospital, in north eastern Tanzania, an area experiencing intense malaria transmission. Analyses included 887 women with viable deliveries who joined the study between September 2002 and March 2005. Twins were excluded. Blood pressure data were available for 688 women. Women without blood pressure measurements did not differ in malaria status, age or parity, but had 70g smaller babies **(Table 1)**. This is a two percent difference in mass, and cannot be explained by the datar: all deliveries were viable and singleton, and the difference persists after excluding cesarean deliveries. However, the difference is corrected for after adjusting for either maternal age or parity by ANOVA.

Table 1. Clinical data for the women who did or did not have blood pressure recorded. PM rate is reported as percent, all other data as mean (SD). p-values were calculated using Chi-squared test for rate of infection, all others using unpaired t-tests. BP= blood pressure

		BP recorded	BP not recorded	p
PM rate	(%)	12.3	10.5	0.476
	(n)	85/688	21/199	
Age	(Years)	26.2 (6.3)	25.7 (6.2)	0.329
	(n)	687	197	
Parity	(#)	1.87 (1.85)	1.72 (1.84)	0.377
	(n)	685	197	
Infant weight	(kg)	3.20 (0.43)	3.13 (0.42)	0.032
	(n)	629	183	

As has been previously observed, placental malaria was associated with lower parity, younger age, lower birth weight and lower gestational age **(Table 2)**. In addition, women in their first pregnancies were more likely to be infected and had heaver placental parasite densities than women of later pregnancies **(Table 3)**.

Table 2. Maternal and fetal characteristics associated with placental malaria. MAP= mean arterial pressure. All data as Mean(SD) and p-values calculated using student's t-test and chi-square test for rate of hypertension.

		PM positive	PM negative	p
Maternal age	(Years)	23.3(5.1)	26.6(6.4)	<0.001
	(n)	85	602	
Parity	(#)	0.89(1.11)	2.00(1.89)	<0.001
	(n)	85	600	
MAP	(mmHg)	88.7(8.2)	89.0(8.4)	0.715
	(n)	85	603	
Hypertension	(%)	12.9	12.1	0.826
	(n)	11/85	73/603	
Body mass index	(kg/m^2)	23.2(3.0)	23.6(2.9)	0.376
	(n)	76	533	
Infant weight	(kg)	2.95(0.41)	3.24(0.42)	<0.001
	(n)	77	552	
Gestational age	(Weeks)	39.0(2.6)	40.0(2.4)	0.013
	(n)	48	346	

Table 3. Features of placental malaria in first versus later pregnancies. Data presented as median(IQR). P values calculated student's t-test and log (n+1) transformed parasite density.

		First Pregnancy	Later Pregnancy	p
Infection rate	(%)	18.8	9.7	0.001
	(n)	38/202	47/483	
Parasite density	(%)	3.1(1.0-6.0)	0.9(0.4-2.2)	0.004
	(n)	38	47	

Mean arterial blood pressure (MAP) and the rate of hypertension did not differ between PM-positive and PM-negative women **(Table 2)**. However, hypertension occurred in two different populations depending on whether women had PM. PM-positive women with hypertension were younger, of lower parity, and delivered smaller babies of lower gestational age, compared to PM-positive women without hypertension **(Table 4)**. By contrast, PM-negative women with hypertension were of greater parity, age and BMI and delivered heavier babies compared to PM-negative women without hypertension. The interaction effect between PM and hypertension (factorial ANOVA) was significant for maternal age, birth weight, gestational age and approached significance for parity.

Table 4. Clinical data for the women in the study, stratified by PM status and hypertension. Data presented as mean (SD). HT=hypertensive, NT= normotensive, MAP= mean arterial pressure. p-values calculated by unpaired t-test between groups, and by ANOVA for the interaction between PM and hypertension.

		PM (+)			PM (-)			Interaction: PM&HT
		HT	NT	p	HT	NT	P	P
Maternal age:	(Years)	19.9(2.1)	23.8(5.3)	0.017	27.8(6.5)	26.4(6.3)	0.079	0.014
	(n)	11	74		73	529		
Parity:	(#)	0.36(0.51)	0.97(1.15)	0.09	2.45(2.18)	1.94(1.84)	0.031	0.074
	(n)	11	74		73	527		
MAP:	(mmHg)	103.9(4.9)	86.4(5.8)	NA	104.6(5.4)	86.9(6.1)	NA	NA
	(n)	11	74		73	530		
Body mass index:	(kg/m^2)	23.0(2.5)	23.3(3.1)	0.798	24.4(3.6)	23.4(2.8	0.012	0.234
	(n)	11	65		65	468		
Infant weight:	(kg)	2.76 (0.40)	2.98(0.41)	0.104	3.30(0.43)	3.23(0.42)	0.182	0.049
	(n)	10	67		67	485		
Gestational age:	(weeks)	36.7(3.4)	39.3(2.4)	0.038	40.1(2.4)	39.9(2.4)	0.759	0.029
	(n)	5	43		35	311		

Blood pressure measurements of women attending antenatal clinic visits were available for a subset of women. One of four PM-positive women with hypertension at term was hypertensive during her first antenatal visit, and two out of 21 PM-negative women were hypertensive during their first antenatal visit. The sample sizes are insufficient for statistical analysis, however the numbers suggest that the majority of women are not hypertensive early in pregnancy and that the onset of hypertension occurs during pregnancy.

Because hypertension occurred in two different populations based on PM status, we tested whether PM was associated with hypertension after stratifying by parity and by median age of first-time mothers **(Table 5)**. PM significantly increased the odds of hypertension in first-time mothers aged 18 to 20 years (n=142), but not in those that were older (n=60), or in mothers of later pregnancy (n=322). Indeed, in PM-positive women with more than one prior pregnancy, none had hypertension. Because younger age contributed to the risk of PM-related hypertension in first-time mothers, adolescents may be particularly susceptible, and this hypothesis should be a priority for future research.

Table 5. Rates of hypertension in women with and without PM, stratified by parity and age. Odds ratios and p-values calculated by logistic regression and reflect odds of hypertension with PM. * p-values calculated by Fisher's exact test.

Pregnancy		Rate of hypertension (%)				Odds of hypertension if infected	
		PM positive		PM negative		OR [95%CI]	P
First	All ages	18.4	(7/38)	11	(18/164)	1.83 [0.71-4.76]	0.214
	Age 18-20	25	(7/28)	9.7	(11/114)	3.12 [1.08-8.99]	0.035
	Age 21+	0	(0/10)	14	(7/50)	NA	0.589*
Second	All ages	13.3	(4/30)	10.8	(14/130)	1.28 [0.39-4.19]	0.689
	Age 18-20	40	(2/5)	5	(1/20)	12.67 [0.86-187]	0.065
	Age 21+	8	(2/25)	11.8	(13/110)	0.65 [0.14-3.08]	0.586
Third or later	All ages	0	(0/17)	13.4	(41/306)	NA	0.143*
	Age 18-20	0	(0/1)	0	(0/2)	-	
	Age 21+	0	(0/16)	13.5	(41/303)	-	0.240*

Women in their first pregnancy, are more likely to suffer from chronic malaria. We examined placental pigment deposition, parasite density, and whether inflammatory cells were present in the intervillous space **(Table 6)**.

Table 6. Relationship between hypertension and placental parasite density and malarial pigment in PM-positive women. Placental malarial pigment as proportion of positive 60X fields (Mean (SD)) and parasite density presented as % IE (Median (IQR)). p-values calculated by Student's t-test. Parasite density was log (n+1) transformed prior to testing. Whether or not inflammation was present was tested using Fisher's exact test.

Pregnancy		Pigment deposition			Parasite density			Cellular infiltrate	
		Proportion of fields	(n)	P	%IE	(n)	P	Rate (%)	p
First	HT:	0.46 (0.15)	7	0.008	1.00 (0.83-1.45)	7	0.026	67 (4/6)	
	NT:	0.25 (0.18)	27		4.50 (1.65-9.25)	31		67 (18/27)	1.0
Later	HT:	0.38	1	NA	0.60 (0.30-1.05)	4	0.275	100 (1/1)	
	NT:	0.15 (0.13)	33		1.02 (0.43-2.88)	43		34 (11/32)	0.364

Malarial pigment is a heme-derived product that persists in acellular fibrinoid in the intervillous space. We quantified pigment deposition in PM-positive women **(Table 6)** as a measure of disease chronicity. Pigment deposition was greatest in first-time mothers, and was negatively correlated with birth weight (R=-0.525, n=32, p=0.002). PM-positive first-time mothers with hypertension had significantly increased amounts of pigment compared to PM-positive first-time mothers without hypertension.

Because placental malaria during first pregnancies is also characterized by maternal inflammation in the intervillous space, we examined the relationship between inflammation by histology and hypertension. Inflammation was best described categorically as there was a lot of heterogeneity in frozen tissue morphology. Women with PM were more likely to have intervillous inflammation if they were in their first

pregnancy (chi square p=0.014). However, women with inflammation were no more likely to be hypertensive **(Table 6).** Massive intervillositis was present in 6 first-time mothers, all of whom were normotensive (not significant).

We examined placental parasite density (percentage of IE) in relation to hypertension **(Table 6)**. Hypertension was associated with low placental parasite density, particularly in first-time mothers. These results were unexpected, but considering that malaria infection lowers blood pressure in non-pregnant individuals, I examined whether parasite density may have an effect to lower blood pressure. Placental parasite density was associated with mean arterial pressure (MAP) by ANOVA after adjusting for first pregnancy (p=0.041). Categorically, parasite density above 2.0% decreased MAP in all parities **(Figure 1)** and PM of any parasite density significantly decreased MAP among women of third or later pregnancy. Conversely, parasite density below 2.0% among first-time mothers, but not other women, was associated with significantly increased MAP. Since women with PM-related hypertension had low parasite density, these women are most likely to be misdiagnosed as uninfected by peripheral blood smear, which may explain why previous studies using peripheral blood microscopy failed to find a relationship between PM and hypertension.

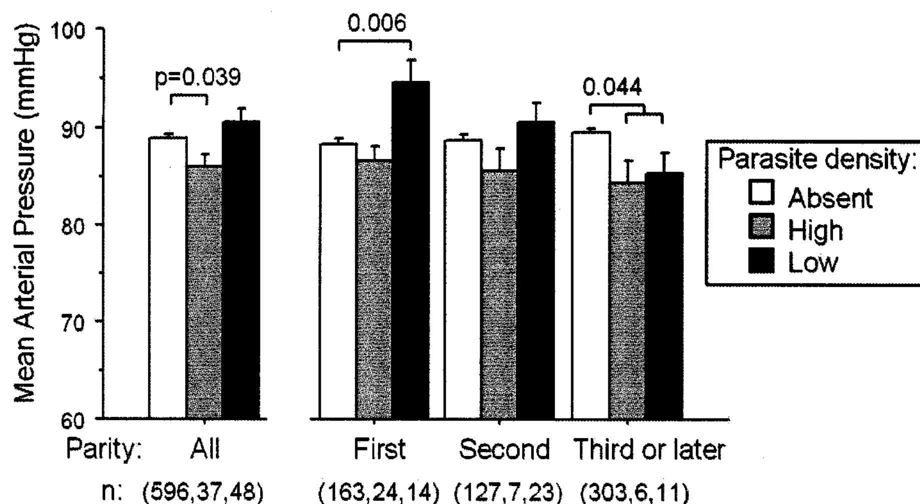

Figure 1. The relationship between MAP (Mean (SEM)), parity and PM. PM-positive women were stratified as having placental parasite density above (high density) or below (low density) 2.0% IE. P-values calculated using Student's t-test.

Because heavy pigment deposition was associated with hypertension during placental malaria, I wanted to determine if pigment, per se, is associated with hypertension. Women who have been exposed to malaria prior during pregnancy, but have resolved the infection, can have malarial pigment persist in intervillous fibrin: a "past" infection. The placentas of 96 first-time mothers were examined by histology, and based on pigment were categorized as having past infections (47%) **(Table 7).** Women with past infections were younger than women who had no pigment, but there was no difference in birth weight. Women with pigment deposition had lower rates of hypertension and significantly lower mean arterial pressures. However because susceptibility to hypertension increases with age, we corrected for maternal age by ANOVA, and found no association between pigment and MAP.

Table 7. Characteristics of PM-negative first time mothers, stratified by past infection. MAP=mean arterial pressure. P-values calculated by student's t-test and chi-square for catergorical variables. * = Adjusted for maternal age by factorial ANOVA.

		Pigment	No pigment	p-value
Age	(years)	19.6 (1.9)	21.5 (3.4)	0.002
	(n)	45	51	
Birth weight	(kg)	3.05 (0.51)	3.07 (0.47)	0.850
	(n)	44	50	
Hypertension	(%)	6.7	17.6	0.105
		3/45	9/51	
MAP	(mmHg)	86.5 (8.2)	90.0 (7.9)	0.037
	(n)	45	51	0.921*

Discussion

Malaria in our population is consistent with other reports from malaria-endemic areas. Almost 20% of first-time mothers have placental malaria, and rates decrease with increasing parity. The limitations to this study are that proteinuria measurements were not performed and we did not have access to baseline blood pressure measurements so preeclampsia could not be diagnosed. Further our data is cross-sectional at term, so we can only infer the burden of infection over time based on placental histology.

Hypertension occurs in two separate populations depending on PM-status. PM-related hypertension occurs in women who are younger and of lower parity- the same

population at risk for preeclampsia. These women also had smaller babies. Hypertension in the absence of PM occurred in women who were older and of greater parity. Although advances in maternal age increase the risk of preeclampsia, these women are also more likely to have chronic hypertension. Notably they also tended to have larger babies. Indeed, young first-time mothers were more likely to have hypertension if they were infected. This suggests that malaria may cause hypertension in this population.

I observe two opposing effects on blood pressure by placental malaria. The parasite density data indicate that blood pressure usually decreases in women with PM, but increases in first-time mothers who have features of chronic malaria such as low parasitemia and pigment deposits. We propose a model in which parasitemia decreases blood pressure by a similar mechanism as in non-pregnant individuals. However this hypotensive effect of parasitemia is overcome when chronic placental inflammation ensues in young first-time mothers and results in hypertension. In **Chapter 3**, I suggest that the placenta secretes factors in the maternal circulation during chronic placental malaria that lead to hypertension.

This effect of parasitemia may be mediated directly by the parasite, as observed with dog heartworm *Dirofilaria immitis* (Kaiser et al. 1990), or indirectly by host responses such as elevated nitric oxide levels. Because PM was associated with decreased blood pressure in multiparous women, who suffer minimal inflammation, it is more likely that a parasite factor directly decreases blood pressure, perhaps by vasodilatation. What would be the purpose of such a factor? Perhaps it induces

vasodilation that increases blood flow to capillary beds thereby facilitating parasite sequestration.

Chapter 3. Maternal VEGF and trophoblast sVEGFR1 in placental malaria

Summary

Malaria and hypertension are major causes of maternal mortality in tropical countries, especially during first pregnancies, but evidence for a relationship between these syndromes is contradictory. Here we show that chronic placental malaria in first-time mothers is related to elevated levels of soluble vascular endothelial growth factor receptor 1 (sVEGFR1), a preeclampsia biomarker. In contrast, PM during later pregnancies is associated with normal sVEGFR1 levels. In first-time mothers with PM, maternal macrophages in the placenta express the inflammatory mediator VEGF, while the fetal trophoblast expresses its soluble inhibitor sVEGFR1, suggesting maternal-fetal conflict. Because placental inflammation causes poor fetal outcomes, fetal mechanisms that promote sVEGFR1 expression may be under selective pressure in malaria endemic areas, particularly during first pregnancies, and may have influenced the evolution of preeclampsia.

Introduction: VEGF and VEGFR1

Vascular endothelial growth factor (VEGF) was independently discovered as vascular permeability factor (Senger et al. 1983; Keck et al. 1989) and vascular endothelial cell-specific growth factor (Ferrara et al. 1989; Leung et al. 1989). VEGF is a potent endothelial cell survival factor (Keck et al. 1989), it stimulates angiogenesis(Leung et al. 1989), and also regulates nitric oxide synthesis and vasodilatation (He et al. 1999).

Shibuya cloned fms-like tyrosine kinase 1 (FLT1) in 1990(Shibuya et al. 1990), which was later found to be a VEGF receptor (de Vries et al. 1992), VEGFR1. VEGFR1 is a strong affinity receptor with weak signaling capacity, and is thought to be a negative VEGF regulator in endothelial cells (Park et al. 1994). VEGFR2 on the other hand has weak affinity but strong signaling capacity (Terman et al. 1992). There are four VEGF isoforms, VEGF-121 which is soluble, and VEGF-165, -189 and -209 which are basic and bind extracellular matrix. There are also VEGF homologs: VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF). PlGF and VEGF-B are specific for VEGFR1, whereas the other VEGF homologs do not interact with VEGFR1 (reviewed in (Shibuya 2001)).

Kendall and Thomas identified a soluble isoform of VEGFR1 (Kendall et al. 1993), which was later found to be expressed at high levels by the placenta (Clark et al. 1998). Serum levels of placentally derived sVEGFR1 are elevated prior to (Levine et al. 2004) and during(Koga et al. 2003; Maynard et al. 2003; Tsatsaris et al. 2003; Levine et al. 2004) preeclampsia. sVEGFR1 may cause systemic endothelial

dysfunction by binding and sequestering free serum VEGF and placental growth factor (Maynard et al. 2003; Levine et al. 2004). In earlier studies, hypertension and proteinuria developed in rats administered sVEGFR1 (Maynard et al. 2003), and in cancer patients who received monoclonal anti-VEGF therapy (Kabbinavar et al. 2003; Yang et al. 2003). Conversely, hypotension was a limiting factor to VEGF administration in patients (Henry et al. 2003). sVEGFR1 levels were elevated in first compared to second pregnancies(Wolf et al. 2005). Of note, this difference in sVEGFR1 levels between first and second pregnancies was greatest in women of Hispanic ancestry compared to European. The role of sVEGFR1 in healthy pregnancy is not known, however VEGF administration caused pregnancy loss, decreases in birth weights and increased placental fibrin deposition in mice (He et al. 1999).

Limitations to the theory that sVEGFR1 causes the maternal syndrome of preeclampsia is that some women with preeclampsia have normal sVEGFR1 levels (Levine et al. 2004; Powers et al. 2005), and sVEGFR1 experimentally does not cause seizures. However reports worldwide have found an association between sVEGFR1 levels and preeclampsia. This includes reports from North America (Chaiworapongsa et al. 2005), Europe (Dimitrakova et al. 2004; McKeeman et al. 2004; Krysiak et al. 2005; Nadar et al. 2005; Staff et al. 2005; Wathen et al. 2006), Asia (Park et al. 2005), and Africa (Muy-Rivera et al. 2005). Further reduced urinary PlGF, a marker for elevated serum sVEGFR1 levels in preeclampsia, has been observed in United States (Levine et al. 2005) and in India (Aggarwal et al. 2006).

VEGF has been implicated in the pathogenesis inflammatory conditions, including atherosclerosis (Ferrara et al. 1991) and allograft rejection (Torry et al. 1995; Pilmore et al. 1999). VEGF administration to pregnant mice caused embryo resorption and decreases in birthweight (He et al. 1999). VEGF (Clauss et al. 1990; Clauss et al. 1996; Heil et al. 2000) and PlGF (Clauss et al. 1996) induce monocyte activation and chemotaxis, by signalling through VEGFR1 (Shen et al. 1993; Barleon et al. 1996; Clauss et al. 1996). sVEGFR1 has been used experimentally to limit monocyte activation and chemotaxis in reducing the severity of atherosclerosis (Zhao et al. 2002). Monocytes synthesize VEGF in response to a hypoxia and a range of inflammatory stimuli (McLaren et al. 1996; Hoper et al. 1997; Xiong et al. 1998; Perez-Ruiz et al. 1999; Melter et al. 2000; Inoue et al. 2001; Sakuta et al. 2001; Malaguarnera et al. 2004; Walczak et al. 2004).

VEGF and VEGFR1 have been examined in cerebral malaria (Deininger et al. 2003). VEGFR1 was localized to extracellular deposits in Durk granulomas, and VEGF was observed in astrocytes. However, neither VEGF nor VEGFR1 were observed in CD68-positive monocytes. VEGF and VEGFR1 have not been examined in any other severe malaria conditions. I provide evidence that during placental malaria, the maternal inflammatory cells express VEGF while the trophoblast expresses sVEGFR1.

Methods

Human Subjects and Study Procedures. These are outlined in **Chapter 2.**

Enzyme-linked immunosorbent assay. Soluble VEGFR1 levels in peripheral plasma were determined in duplicate by ELISA (R&D) (Maynard et al. 2003; Levine et al. 2004). Levels were corrected for dilution volume in anticoagulant, and over-diluted samples were detected by low potassium concentration as measured by Easylyte plus (Medica) and excluded. The cut-off was <2.6 mM, indicating a greater than 66% dilution based on a reference potassium of 4.0 mM. 9 out of 132 samples fit this criterion.

Quantitative RT-PCR. Total RNA was extracted from frozen cryosections using RNeasy minikits (Qiagen.) No women with cesarean sections were included. RNA quality was assessed by Agilent 2100 Bioanalyzer, resulting in 28/18s ratios of 1.1 to 1.5. cDNA was synthesized using Superscript III enzyme (Invitrogen) and anchored oligodT20 primers. Exon-spanning primers were designed for sVEGFR1, membrane VEGFR1, VEGF various isoforms, PlGF and KRT7 **(Table 8)**. Real-time PCR was performed in duplicate using SYBR green master mix and an ABI Prism 7000 (Applied Biosystems). with an annealing temperature of annealing at 60C. Threshold cycles (C_T) were normalized to C_T of KRT7, and t-tests performed on normalized C_T values. Data is presented as fold-difference from control gene, calculated by 2^(-normalized C_T).

Table 8. Primers used in **Chapter 3.**

		Fw	size (bp)
sVEGFR1	Fw:	GGGGAAGAAATCCTCCAGAA	63
	Rv:	AGCCTTTTTGTTGCAGTGCT	
VEGFR1	Fw:	AGGGGAAGAAATCCTCCAGA	73
	Rv:	GAGGTTTCGCAGGAGGTATG	
VEGF (all)	Fw:	CCCACTGAGGAGTCCAACAT	106
	Rv:	TGCATTCACATTTGTTGTGC	
VEGF-121	Fw:	AGGCCAGCACATAGGAGAGA	101
	Rv:	GCCTCGGCTTGTCACATT	
VEGF-165,-189,-205	Fw:	CAAGATCCGCAGACGTGTAA	162
	Rv:	GAGAGATCTGGTTCCCGAAA	
PlGF	Fw:	ACGTGGAGCTGACGTTCTCT	134
	Rv:	GTGGCAGTCTGTGGGTCTCT	
KRT7	Fw:	GGCTGAGATCGACAACATCA	103
	Rv:	CTTGGCACGAGCATCCTT	
RPS13	Fw:	CCTGGAGGAGAAGAGGAAAGAG	126
	Rv	TTGAGGACCTCTGTGTATTTGTCAA	

Immunofluorescence and immunohistochemistry. Cryosections (5 um) were fixed in paraformaldehyde, blocked in chicken and goat sera (Santa Cruz Biotechnology, Amersham) and probed with mouse anti-VEGFR1 extracellular domain (R&D) at 1:400, which detects both the membrane-bound and soluble isoforms of VEGFR1, mouse anti-VEGF clone VG1 (Chemicon) at 1:200 and rabbit anti-placental lactogen (Dako) at 1:1000 dilution. Secondary antibodies included Alexafluor 488 chicken anti-mouse antibody (Molecular Probes) and TRITC- goat anti rabbit antibody (Sigma). DAPI (Sigma) was used to define nuclear DNA. For immunohistochemistry, sections

were treated as above, except indirect DAB staining was used (Envision +, Dako) and counterstained with Giemsa.

Statistical analysis. Analyses were performed using Statview (SAS Institute). Student's t-test was used for the analysis of continuous variables. Peripheral plasma sVEGFR1 concentration was log-transformed prior to analysis. Regression coefficients were calculated using multiple regression analysis. Interactions between variables were determined using factorial ANOVA. Spearman rank correlation was used to examine the correlation between sVEGFR1 quantitative PCR and ELISA measurements.

Results

We measured sVEGFR1 levels in peripheral plasma by ELISA **(Figure 2A)**. sVEGFR1 levels in PM-negative normotensive first-time mothers were comparable to levels in healthy first-time mothers from non-endemic countries (Maynard et al. 2003; Levine et al. 2004). Compared to uninfected first-time mothers, sVEGFR1 levels were elevated in first-time mothers with PM, hypertension, or both. This pattern was absent during later pregnancies, where sVEGFR1 levels did not differ with PM. We determined sVEGFR1 transcript abundance in placental tissue of first-time mothers by quantitative RT-PCR. Placental transcript levels correlated with peripheral protein levels (Pearson Rho=0.611, $p<0.001$, n=43), and were significantly elevated in first-time mothers with PM, hypertension, or both **(Figure 2B)**. sVEGFR1 transcript levels

were specifically elevated in tissue that had maternal inflammatory cells in the intervillous space **(Figure 2C)**.

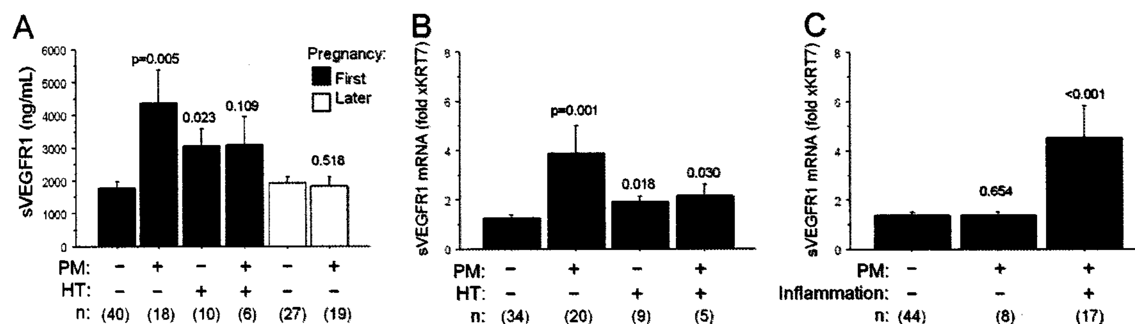

Figure 2. sVEGFR1 levels. **(A)** Peripheral plasma sVEGFR1 levels (Mean (SEM)). Levels are indicated for first-time mothers stratified by PM and hypertension (HT), and normotensive mothers of later pregnancies stratified by PM. **(B to D).** Placental sVEGFR1 mRNA abundance (Mean (SEM)) in first-time mothers. Levels indicate fold-increase over trophoblast-specific cytokeratin 7 (KRT7) mRNA. For sVEGFR1, **(B)** women were stratified by PM and hypertension or **(C)** by PM and intervillous inflammation. P-values were calculated (unpaired t-test) for comparisons with parity controls using log-transformed sVEGFR1 levels or corrected C_T values.

I also determined placental transcript levels of the membrane bound isoform of VEGFR1, which had a similar pattern to sVEGFR1, that levels were increased in first-time mothers with PM, hypertension or both **(Figure 3)**. This suggests that during PM, both isoforms are co-regulated.

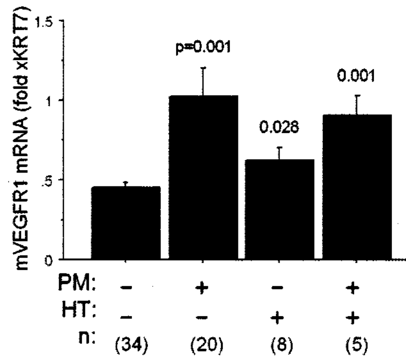

Figure 3 Placental VEGFR1 transmembrane isoform transcript levels. (Mean(SEM)) in first-time mothers. Levels indicate fold-increase over trophoblast-specific cytokeratin 7 (KRT7) mRNA. Women were stratified by PM and hypertension P-values were calculated (unpaired t-test) using corrected C_T values.

Because sVEGFR1 levels increase during gestation, at an estimated rate >100ng/week (Levine et al. 2004), gestational age is a confounder. In our study, PM was associated with decreased gestational age, indicating that if we were to correct for gestational age, the differences observed would be greater. However, gestational age data was not available for enough women for statistical analysis. Available gestational ages for corresponding samples are reported in the following **Table 9.**

Table 9. Gestational ages for samples used in sVEGFR1 measurements. Samples are stratified by parity, PM, hypertension status. Data presented as mean(SD). NT=normotensive, HT=hypertensive. p-values were calculated using Student's t test for comparisons with parity controls.

Pregnancy		Samples for ELISA			Samples for QPCR		
		Gestational age			Gestational age		
		weeks	(n)	p	weeks	(n)	P
First	PM(-) NT	40.0(2.0)	22		40.4(2.4)	20	
	PM(+) NT	38.6(2.3)	12	0.07	39.4(2.3)	12	0.236
	PM(-) HT	38.5(3.0)	5	0.196	40.4(2.2)	5	0.995
	PM(+) HT	34.6(4.7)	4	0.001	37.9(4.24)	2	0.181
Later	PM(-) NT	39.9(2.7)	19				
	PM(+) NT	38.3(2.4)	11	0.126			

sVEGFR1 mRNA levels were normalized to a trophoblast-specific control gene, KRT7. This was because PM-positive tissues had a wide range of immune cells present. GAPDH and RPS13 are "House keeping" genes that are commonly used in quantitative PCR experiments. These were significantly more abundant in RNA extracted from inflamed tissues. **(Figure 4)** Data for GAPDH is not shown. An explanation is that inflammatory cells may be more metabolically and transcriptionally active than placental cells. Further, the threshold cycle for KRT7 did not different between study groups, and hence was chosen to be the normalizing gene.

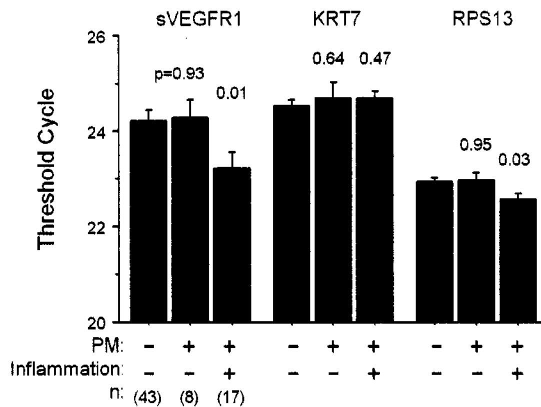

Figure 4. Non-normalized threshold cycles of placental mRNA determined by quantitative PCR (Mean (SEM)) in first-time mothers. Women stratified by PM and intervillous inflammation. P-values were calculated (unpaired t-test) using corrected C_T values.

We explored whether sVEGFR1 expression occurred in villous trophoblast cells, which are of fetal origin (**Figure 5**). In uninfected placentas, VEGFR1 immunoreactivity in villous trophoblast was not observed. In infected placentas from first-time mothers who were hypertensive, or had intense intervillous inflammation, VEGFR1 immunoreactivity co-localized with trophoblast, and not maternal inflammatory cells.

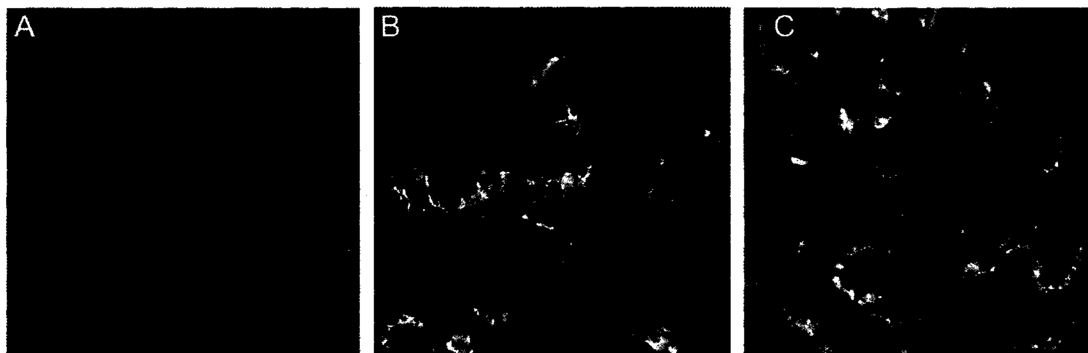

Figure 5. VEGFR1 localization. Immunofluorescence of placental cryosections from first-time mothers for VEGFR1, trophoblast (red), and nuclear DNA (blue). All fields are 200X magnification. Cryosections from **(A)** PM-negative normotensive pregnancy; **(B)** PM-positive normotensive pregnancy with intervillous inflammation; **(C)** PM-positive hypertensive pregnancy.

Placental transcription of VEGF was also elevated during PM in first-time mothers **(Figure 6A)**, suggesting a counter-regulatory response. Further, in first-time mothers with PM, maternal inflammatory cells were immunoreactive with VEGF **(Figure 6B)**. These cells had the morphology of malarial-pigment containing macrophages **(Figure 6C)**. These data indicate that during PM, VEGF is produced by the maternal cells while its soluble receptor is produced by the fetus.

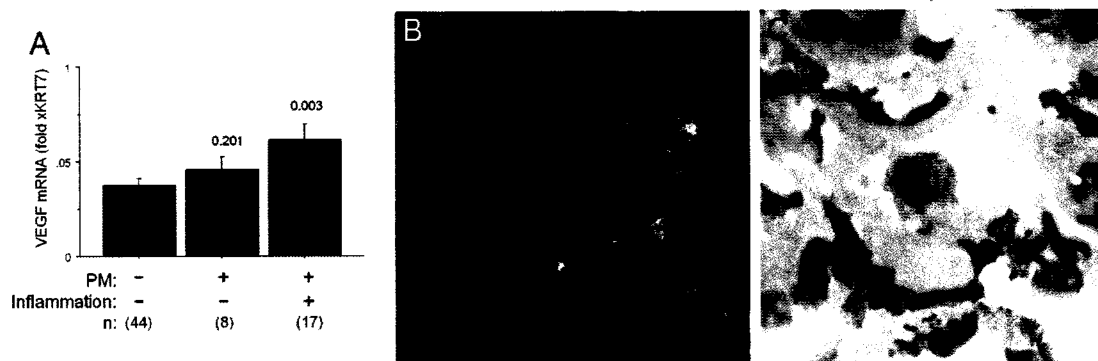

Figure 6. VEGF transcript levels and localization. **A)** Placental VEGF mRNA abundance (Mean (SEM)) in first-time mothers. Levels indicate fold-increase over trophoblast-specific cytokeratin 7 (KRT7) mRNA. women stratified by PM and intervillous inflammation. P-values were calculated (unpaired t-test) using corrected C_T values. **B)** Immunofluorescence of a PM-positive placental cryosection from a first-time mother for VEGF (green) and trophoblast (red), and nuclear DNA (blue). 200X magnification, digitally amplified 1.6X. and **C)** Immunohistochemistry for VEGF and counterstained with Giemsa. 400X magnification.

VEGF exists in several isoforms. I detected elevated levels of transcripts for VEGF-121 the soluble isoform, and VEGF-165,-189,-205, the basic isoforms in first time mothers with PM **(Figure 7)** but not elevated levels of PlGF.. Placental extravillous fibrinoid, in women with and without PM was immunoreactive for VEGF **(Figure 8)**. The basic isoforms of VEGF may be associated with placental fibrinoid, as they have strong affinity for hyaluronic acid.

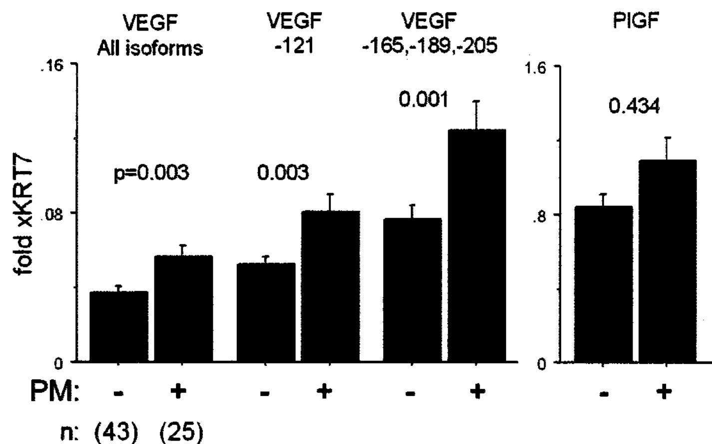

Figure 8. VEGF isoforms and PlGF. Placental mRNA abundance (Mean (SEM)) in first-time mothers of VEGF isoforms and PlGF. Levels indicate fold-increase over trophoblast-specific cytokeratin 7 (KRT7) mRNA. women stratified by PM. P-values were calculated (unpaired t-test) using corrected C_T values.

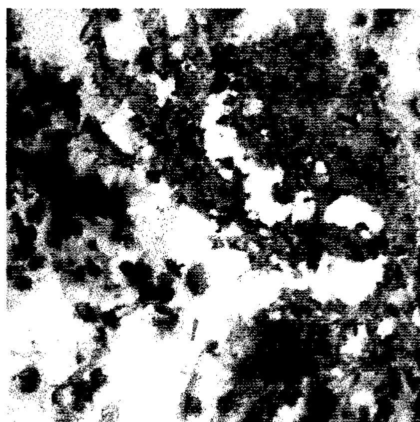

Figure 9. VEGF localization in extravillous fibrinoid. Immunohistochemistry for VEGF and counterstained with Giemsa of a PM-positive first time mother. Placental fibrinoid (upper right corner) contains malarial pigment and pigment-containing macrophages. The fibrinoid is immunoreactive for VEGF. 200X magnification.

Discussion

The specific effect of chronic PM to increase blood pressure in first-time mothers may be mediated by sVEGFR1. We speculate that the hypotensive effects of high parasite density early in PM may counter-balance the hypertensive effects of sVEGFR1, thus explaining why some PM-positive women with elevated sVEGFR1 do not manifest hypertension. Because placental parasite density diminishes over time, the effect of sVEGFR1 to induce hypertension would therefore be unmasked in first-time mothers with chronic PM.

The potential role of VEGF in placental malaria may recruit and activate monocytes. Various stimuli promote its expression including hypoxia (Xiong et al. 1998; Ramanathan et al. 2003) and toll-like receptor ligands (Perez-Ruiz et al. 1999). Extravillous fibrinoid stained strongly for VEGF. VEGF in this compartment may also lead to maternal monocyte accumulation. Further, because the maternal intervillous space is devoid of maternal endothelial cells, and some endothelial cells are derived from circulating hematopoietic precursors (Asahara et al. 1997), it is possible that inflammatory neovascularization may occur during chronic PM where the mother attempts to restablish circulation in the intervillous space.

Whether VEGF is involved in other malaria syndromes is not known, although astrocyte VEGF expression has been reported in cerebal malaria (Deininger et al. 2003). In these syndromes VEGF may function to recruit and activate monocytes. VEGF is also a survival factor for endothelial cells, so it may also preserve vessel

integrity during intravascular inflammation in the presence of a sequestered mass of parasitized erythrocytes.

Pro-inflammatory cytokines (Fried et al. 1998) and hypoxia (Watkinson et al. 1983) have been implicated in PM, and may contribute to trophoblast sFlt1 expression. Experimentally, hypoxia upregulated trophoblast sFlt1 (Nagamatsu et al. 2004), but the effect of pro-inflammatory cytokines has not been explored. In **Chapter 4** I identify several inflammatory mediators that are correlated with sFlt1 transcript levels.

Because sVEGFR1 during PM is expressed from fetal alleles, and VEGF is expressed from a maternal alleles, the system is in genetic conflict (Haig 1993). VEGF is a mediator of inflammation and sVEGFR1 is its soluble inhibitor. Poor fetal outcomes due to PM are more closely related to inflammation than to parasite density, suggesting that the fetus expresses sVEGFR1 in an effort to limit the maternal inflammatory response. If so, increased sVEGFR1 production may be under selective pressure in endemic areas. sVEGFR1 is implicated in the pathogenesis of preeclampsia and may cause maternal hypertension. Selection for fetal sVEGFR1 expression by malaria could have contributed to the increased prevalence of preeclampsia that has been observed among people of African or South Asian ancestry. Furthermore, sVEGFR1 levels are known to be elevated in healthy first versus second pregnancies (Wolf et al. 2005). Human exposure to *P. falciparum* explains the evolutionary selection of preeclampsia to occur during first pregnancy: the beneficial effect of sVEGFR1 to protect the fetus from PM outweighs its deleterious effect on the

mother during first pregnancy but not later pregnancies. *P. falciparum* would also explain why preeclampsia is an exclusively human condition.

Chapter 4. The maternal assault

Summary

I report the first genome wide analysis of malaria infected human tissue. Characterization of the inflammatory response in placental malaria suggests novel inflammatory pathways, and that inflammation is correlated to sFlt1 expression.

In first time mothers with chronic placental malaria, macrophages commonly accumulate in the intervillous space. Intervillositis consisting of macrophages has been reported in women in non-malarious areas and has been associated with autoimmunity. Malaria infection is associated with hypergammaglobulinemia, autoantibodies, circulating immune complexes, and a demographic association with Burkitt's lymphoma. By microarray and quantitative RT-PCR analysis of placental tissue of women with chronic PM we identified genes associated inflammation. Notably some of the most differentially expressed genes were associated with B-cells. We detected immunoglobulin light and heavy chain transcription, and by immunohistochemistry observed IgG3 and IgM deposition, plasma cells and proliferating B-cells in the intervillous space. The Fc binding molecules FcGRIa, FcGRIIIa and C1Q were upregulated. Macrophages expressed FcGRIa, FcGRIIIa and also expressed the B-cell chemoattractant CXCL13 which was over 1000-fold upregulated in inflamed tissue. In addition we identified various chemokines and cytokines that have been characterized in chronic inflammation and autoimmunity:

CCL18, CXCL9, CXCL16, BAFF, IL1B, IL18. Transcript levels of hepcidin, which mediates the anemia of inflammation, were upregulated. Transcript levels of immunoglobulin, CXCL13, and BAFF correlated inversely with birth weight. Transcript levels of CXCL16, hepcidin, IgG and IFNG correlated with sVEGFR1 in PM-positive women. We propose a pro-inflammatory feedback loop that contributes to chronic PM consistent with a type III hypersensitivity reaction. Whereas specific antibodies in women with previous pregnancies protect against PM, non-protective antibodies in first-time mothers may be pathogenic by activating and attracting phagocytes, which in turn recruit B-cells to further produce antibodies in the intervillous space.

Introduction

In first time mothers with chronic placental malaria, the accumulation of macrophages is a common feature. In non-malarious areas intervillositis consisting of primarily monocyte-macrophages is uncommon, but has been associated with autoantibodies (Salafia et al. 1997; Boyd et al. 2000), systemic lupus (Jacques et al. 1993), recurrent abortion (Doss et al. 1995) and preeclampsia (Jacques et al. 1993). If intervillositis in malarious and non-malarious has similar etiologies, it would suggest that the inflammation observed during chronic PM involves positive-feedback loops. Such positive-feedback loops could perhaps be triggered in the absence of malaria, perhaps by autoimmune mechanisms.

Immunoglobulin and parasite antigen deposition has been observed in the basement membrane of endothelial cells during cerebral malaria (Oo et al. 1987) and trophoblast during PM (Yamada et al. 1989; Maeno et al. 1993), This was postulated to activate complement and production of proinflammatory cytokines. Malaria infection is associated with B-cell abnormalities: primarily hypergammaglobulinemia (McGregor et al. 1956), autoantibodies (Greenwood et al. 1970), circulating immune complexes (Adam et al. 1981; Mibei et al. 2005), and a demographic overlap with Burkitt's lymphoma (Dalldorf et al. 1964).

Type III hypersensitivity reactions, as defined by Coombs and Gell (Gell et al. 1963), are mediated by immune complexes: they deposit in tissue, activate complement and phagocytes leading to tissue destruction. Immune complexes play a role in chronic inflammatory autoimmune diseases such as rheumatoid arthritis (Firestein 2003). A central role of phagocytic Fc receptors is suggested by the observation that FcGRIIIa (CD16) is absolutely required for a spontaneous mouse model of autoimmune arthritis (Ji et al. 2002). Aberrant B-cell regulation is involved in the etiology and persistence of autoimmune diseases. Proliferating B-cells in follicular structures, termed ectopic germinal centers, have been observed in tissues affected by inflammatory autoimmune disease, such as the synovium of rheumatoid arthritis patients (Weyand et al. 2003), salivary glands of Sjogren's patients (Salomonsson et al. 2002) and in the CSF during neuroborreliosis (Narayan et al. 2005). The chemokine CXCL13 is chemotactic for mature B-cells signaling through CXCR5, the Burkitt's lymphoma receptor (Gunn et al. 1998). CXCL13 is essential for

lymph node development in mice (Ansel et al. 2000), and is expressed in ectopic germinal centers (Shi et al. 2001; Salomonsson et al. 2002; Narayan et al. 2005).

Genome wide analysis of the host response during malaria infection has been examined in animal models of malaria infection (Sexton et al. 2004; Ylostalo et al. 2005) and in human peripheral blood (Griffiths et al. 2005). Garnham regarded changes observed in the peripheral blood to be "merely a mild reflection of the real mechanism occurring in the internal organs" (Garnham 1938). Here I report the first genome-wide analysis of malaria infected tissue in humans, and present data that suggests a localized type III hypersensitivity reaction and B-cells contribute to pathology, similar to observations of chronic autoimmune disease.

Methods

Human subjects and study procedures. These described in **Chapter 2.**

Sample processing. The placenta was collected at delivery, and a full thickness biopsy from the middle third of the placental disc was made. Tissue was frozen in liquid nitrogen and stored at -80 C, and additional tissue was collected in RNAlater(ambion) and stored at -80 C. Placental blood was extracted from placental tissue by mechanical grinding. Placental malaria was detected by microscopy of Giemsa-stained thick and thin smears of placental blood

Placental Histopathology. For analysis, 5 um cryosections of placental tissue were fixed in methanol and Giemsa stained. Sections were assessed by examining greater than 90 60X fields per section. Hemozoin deposition in fibrinoid was quantified by determining the proportion of fields with hemozoin present. Immune infiltrates within the intervillous spaces were qualitatively scored as (-) for none or very few inflammatory cells present, (+) for inflammatory cells present and (++) for having an extensive accumulation of inflammatory cells.

Microarray analysis. For microarray analyses, fine villi preparations were dissected at <0.5mm^3, excluding large vessels, stem villi, infarcts, fetal membranes or decidua from RNAlater preserved tissues. Total RNA was extracted using RNeasy minikits (Qiagen). RNA quality of representative samples was assessed by Agilent 2100 Bioanalyzer, resulting in 28/18s ratios of 1.1 to 1.5. Microarray assays were performed at the Center for Expression Arrays at the University of Washington. Biotinylated target cRNA was prepared and hybridized to Affymetrix Human Genome U133 plus 2.0 GeneChip with minor modifications from the Affymetrix recommended procedures. This procedure, in short, utilized 5ug total RNA to create first strand cDNA using a T7-linked oligo(dT) primer. Once second strand synthesis was complete, an in vitro transcription reaction was performed using biotinylated UTP and CTP in the Affymetrix IVT Kit or IVT Kit by Enzo Diagnostics. Labeled cRNA was then processed further as recommended by Affymetrix, where 15ug of cRNA was fragmented, a hybridization cocktail was assembled with the addition of spike-in

controls, and chips were hybridized for 16 hours. The chips were then washed and stained with streptavidin-phycoerythrin using the Affymetrix GeneChip System, and scanned using the GeneChip Scanner. Each GeneChip genome array hybridization performed underwent GCOS absolute expression analysis. Raw data from the scanned image (.DAT), files containing probe set information (.CEL), and files containing the absolute analysis, (.CHP) as well as quality control reports. Data was normalized using GC-RMA algorithm, and t-test and cluster analyses were performed using Acuity 4.0 (Axon) software.

Quantitative RT-PCR. For quantitative PCR, total RNA was extracted from frozen cryosections using RNeasy minikits (Qiagen.) RNA quality of representative samples was assessed by Agilent 2100 Bioanalyzer, resulting in 28/18s ratios of 1.1 to 1.5. cDNA was synthesized using Superscript III enzyme (Invitrogen) and anchored oligodT20 primers. Real-time PCR was performed in duplicate using SYBR green master mix and an ABI Prism 7000 or 7500 (Applied Biosystems). Initial denaturation was at 95C for 10 minutes, annealing at 60C for 30s, extension at 72C for 30s followed by denaturation at 95C for 15s. Exon spanning primers (except for CXCL9 which lies within an exon) were designed using Primer3 (MIT) (except primers for TNF were designed by Angela Collie (UW)). Primers are listed in **Table 10.** All primers yielded single products, and amplification was linear on serial dilutions of cDNA samples. Threshold cycles (C_T) were calculated and normalized to C_T of KRT7, and t-tests

performed on normalized C_T values. Data is presented as fold-difference from control gene, calculated by 2^(control C_T -gene C_T).

Table 10. Primers used in **Chapter 4.**

Target		Primers	Product size (bp)
IGGH (all)	Fw:	CAAGTGCAAGGTCTCCAACA	138
	Rv:	AGGCTGACCTGGTTCTTGGT	
IGMH	Fw:	ACCAGCACACTGACCATCAA	186
	Rv:	GGTGGACTTGGTGAGGAAGA	
IFNG	Fw:	TGACCAGAGCATCCAAAAGA	147
	Rv:	TGTATTGCTTTGCGTTGGAC	
IL1B	Fw:	CTGTCCTGCGTGTTGAAAGA	178
	Rv:	CTGCTTGAGAGCTGCTGATG	
IL18	Fw:	TGCATCAACTTTGTGGCAAT	220
	Rv:	ATATGGTCCGGGGTGCATTA	
TNF	Fw:	CACGCTCTTCTGCCTGCT	161
	Rv:	CAGCTTGAGGGTTTGCTACA	
TNFSF13B (BAFF)	Fw:	CGTTCAGGGTCCAGAAGAAA	115
	Rv:	AAAGCTGAGAAGCCATGGAA	
HEPC (Hepcidin)	Fw:	GACCAGTGGCTCTGTTTTCC	193
	Rv:	CTACGTCTTGCAGCACATCC	
CXCL10 (IP10)	Fw:	CCACGTGTTGAGATCATTGC	180
	Rv:	CCTCTGTGTGGTCCATCCTT	
CXCL9 (MIG)	Fw:	GAAGCAGCCAAGTCGGTTAG	75
	Rv:	TGGAAGGAGGTTTCCACATC	
CXCL13 (BLC)	Fw:	GGGAATGGTTGTCCAAGAAA	213
	Rv:	CAGAGCAGGGATAAGGGAAG	
CXCL16 (SR-PSOX)	Fw:	GCCCTTTCCTATGTGCTGTG	121
	Rv:	AGCTTCCATTCTTGGCTCAG	
CCL5 (RANTES)	Fw:	CGCTGTCATCCTCATTGCTA	196
	Rv:	ACACACTTGGCGGTTCTTTC	
CCL4 (MIP1b)	Fw:	CTTCCTCGCAACTTTGTGGT	88
	Rv:	GCTTGCTTCTTTTGGTTTGG	
CCL18 (PARC)	Fw:	CCTGGCAGATTCCACAAAAG	126
	Rv:	CCCACTTCTTATTGGGGTCA	
KRT7	Fw:	GGCTGAGATCGACAACATCA	103
	Rv:	CTTGGCACGAGCATCCTT	

Immunohistochemistry. For immunohistochemistry, 5um cryosections wer fixed for 10 minutes in 4% paraformaldehyde (for cxcl13, cxcl16 IgG3, IgM), or acetone (for CD16, CD64, CD138). Antibodies and dilutions are listed in **Table 11.** Staining was either indirect using Envision + anti-mouse kit (Dako) or PicTure anti-goat kit (Zymed) (for cxcl16) according to manufacturer's directions, or direct (for IgG3 and IgM), with sections blocked in serum con-specific to the antibody. Sections were mounted using Permount (Fisher). For immunofluorescence, tissue was fixed for 10 minutes in methanol-acetone. Secondary antibodies included Alexafluor 488 chicken anti-mouse antibody (Molecular Probes) and TRITC- goat anti rabbit antibody (Sigma) 1:500. The sections were stained with DAPI (Sigma) at 1ug/mL for 1 minute to define nuclei. Sections were mounted in 80% glycerol, and visualized using a fluorescent microscope.

Table 11. Antibodies used in **chapter 4.**:

Host	Antigen	Dilution	Manufacturer
Mouse	CD16	1:500	Chemicon
Mouse	CD64	1:500	Chemicon
Mouse	CD138	1:1000	Chemicon
Mouse	cxcl13	1:50	R&D Systems
Mouse	IgG3-HRP	1:500	Zymed
Goat	IgM-HRP	1:1000	Chemicon
Mouse	CD20	1:400	Dako
Rabbit	Ki67	1:200	Chemicon
Mouse	Isotype (IgG1)	1:50	eBiosciences
Goat	CXCL16	1:200	R&D Systems

Results

For global gene expression analysis, placental samples from 20 first-time mothes were selected based on PM status, placental histology, RNA quality: 10 were PM-positive, 10 were PM-negative. Of the PM-negative women, five had placental pigment deposition, indicating a past PM episode. Clinical characteristics of the women are described in **Table 12.**

Table12. Clinical characteristics of women who donated samples examined for global gene expression. Data is presented as mean(SD) except parasite density is median(range). P-values were calculated using students t-test for continuous variables, and chi-square analysis for proportions.

		PM negative	PM positive	P
Age	(years)	22.5(3.0)	19.6(1.8)	0.018
	(n)	10	10	
Parity	(#)	0	0	1.0
	(n)	10	10	
Infant weight	(kg)	3.22(0.31)	2.80(0.26)	0.006
	(n)	10	9	
Female infants	(n)	4/10	6/10	0.498
Placental parasite density	(% IE)	0	1.5(0.6-63)	Na
	(n)	10	10	
Pigment-positive	(n)	5/10	10/10	Na
Inflammation-positive	(n)	1/10	7/10	0.01

In the overall dataset, PM was not the greatest source of variance in gene expression (Data not shown). But by a hierarchical clustering algorithm we detected a group of 752 probes (correlation coefficient: 0.870) representing 528 co-expressed genes **(Figure 9)** that were related to PM status. These included genes previously identified to be involved in PM, such as IFNG, TNF, CCL3, CCL4 and CCL5. No differences were observed between PM-negative women who did or did not have pigment deposition.

In a dataset limited to the cluster 752 probes that were related to placental malaria, hierarchical clustering of placental samples separated women who had inflammation present from those who did not **(Figure 9)**. Samples from three PM-positive women with minimal inflammation were grouped with PM-negative samples. In addition, one sample from a PM-negative woman with pigment deposition and inflammatory cells grouped with the PM-positive inflamed samples. The placental smear from this subject was confirmed to be PM-negative. These data suggest that transcriptional changes during PM are closely related to placental inflammation.

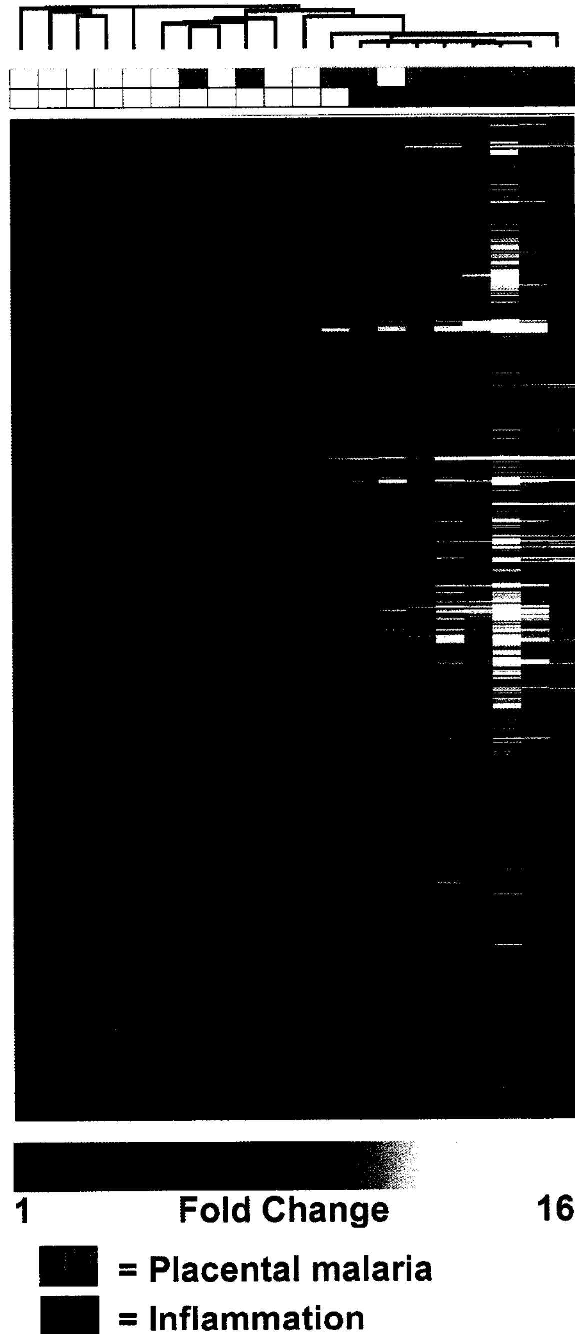

Figure 9. Hierarchical clustering of 728 probes associated with placental malaria and hierarchical clustering of samples. Data is presented as fold change over the mean of PM-negative samples.

Statistical testing for differences in gene expression between 9 PM-negative women without inflammation versus 7 PM-positive women with inflammation, revealed 234 genes with at least 2.5 fold elevation and p-values <0.01 **(Appendix 1)**. Selected genes are listed in **Table 13**, selection criteria including whether the genes have been characterized in other systems and are novel to malaria or encode soluble molecules. The most elevated gene was CXCL13 at 130-fold, followed by CCL18 at 47-fold, HLADQA1 at 37-fold, Ig light chain constant 2 at 33 fold, CXCL9 at 32-fold and Ig kappa constant at 25-fold.

Table 13. Expression data for selected genes upregulated during inflammation (at least 2.5 fold and p-value <0.01). P-values are calculated from student's t-test, not corrected for multiple comparisons.

Fold change	P-value	# of Probes	Accession	Gene Symbol	Gene Title
129.3	8.77E-08	1	NM_006419	CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
47.3	2.53E-10	2	Y13710	CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
33	5.51E-06	3	X57812	IGL@	immunoglobulin lambda locus
32	6.43E-09	1	NM_002416	CXCL9	chemokine (C-X-C motif) ligand 9
25	1.26E-05	3	AW575927	IGKC	immunoglobulin kappa constant
21.6	3.17E-10	1	AF301007	SIGLEC10	sialic acid binding Ig-like lectin 10
21.3	1.80E-05	1	M87790	IGLC2	Immunoglobulin lambda joining 3
13.7	6.03E-06	1	NM_002983	CCL3 /// L1	chemokine (C-C motif) ligand 3
13.5	1.27E-04	1	NM_002984	CCL4	chemokine (C-C motif) ligand 4
13.2	9.50E-08	1	NM_014479	ADAMDEC1	ADAM-like, decysin 1
12.8	6.15E-07	1	NM_001974	EMR1	egf-like module containing, mucin-like, hormone receptor-like 1
12.6	1.10E-07	2	AW872374	TLR8	toll-like receptor 8
12.2	5.24E-08	2	AW026543	FPRL2	formyl peptide receptor-like 2
11.8	1.54E-04	1	M87789	IGH@	immunoglobulin heavy locus
11.3	2.50E-08	2	NM_000295	SERPINA1	serpin peptidase inhibitor, clade A, member 1
10.5	4.40E-05	1	NM_004049	BCL2A1	BCL2-related protein A1
8.7	6.92E-09	2	AF134715	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b
8.1	4.18E-04	1	AV733266	IGJ	Immunoglobulin J polypeptide
8.1	1.56E-04	1	NM_001565	CXCL10	chemokine (C-X-C motif) ligand 10
7.9	3.95E-06	2	W46388	SOD2	superoxide dismutase 2, mitochondrial
7.4	2.35E-06	1	NM_013447	EMR2	egf-like module containing, mucin-like, hormone receptor-like 2
7.2	1.53E-06	2	NM_000022	ADA	adenosine deaminase
7.1	1.51E-04	1	NM_006144	GZMA	granzyme A
7.1	3.14E-04	2	U25677	LYZ	Lysozyme (renal amyloidosis)
6.2	2.46E-06	1	AA897516	PTGER4	prostaglandin E receptor 4 (subtype EP4)
6.2	5.52E-04	1	BG485135	IGKC	Immunoglobulin kappa variable 1-5
6.1	1.52E-03	1	AW404894	IGKV	Immunoglobulin kappa light chain variable
6.1	8.98E-06	1	NM_000570	FCGR3A /// 3B	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
5.8	5.61E-06	1	AL050262	TLR1	toll-like receptor 1
5.5	9.05E-04	1	U20350	CX3CR1	chemokine (C-X3-C motif) receptor 1
5.5	2.22E-05	1	NM_003151	STAT4	signal transducer and activator of transcription 4
5.4	1.58E-05	1	NM_003264	TLR2	toll-like receptor 2
5.3	5.44E-04	1	U65590	IL1RN	interleukin 1 receptor antagonist
5.3	5.35E-05	1	L25259	CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)
5.2	9.96E-08	2	BC002704	STAT1	signal transducer and activator of transcription

4.6	2.58E-05	1	NM_006682	FGL2	fibrinogen-like 2
4.4	2.02E-04	1	AI308863	CYBB	cytochrome b-245, beta polypeptide (chronic granulomatous disease)
4.3	1.05E-04	2	BC020763	FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide
4.3	2.25E-03	3	NM_002985	CCL5	chemokine (C-C motif) ligand 5
4.1	1.72E-04	1	NM_000579	CCR5	chemokine (C-C motif) receptor 5
4.1	3.81E-07	1	AF275260	CXCL16	chemokine (C-X-C motif) ligand 16
4	2.30E-05	1	NM_021175	HAMP	hepcidin antimicrobial peptide
3.9	3.79E-05	1	NM_001558	IL10RA	interleukin 10 receptor, alpha
3.6	2.49E-03	1	M15330	IL1B	interleukin 1, beta
3.6	3.82E-05	1	NM_000491	C1QB	complement component 1, q subcomponent, beta polypeptide
3.6	5.34E-03	1	J03189	GZMB	granzyme B
3.5	2.80E-03	1	BG536224	IGKV	Immunoglobulin kappa light chain VJ region
3.4	8.29E-05	1	NM_001562	IL18	interleukin 18
3.3	1.93E-03	1	NM_002964	S100A8	S100 calcium binding protein A8 (calgranulin A)
3.3	1.95E-03	1	NM_007115	TNFAIP6	tumor necrosis factor, alpha-induced protein 6
3.3	2.35E-05	1	J02959	LTA4H	leukotriene A4 hydrolase
3.3	5.46E-04	1	NM_002029	FPR1	formyl peptide receptor 1
3.2	6.51E-05	1	U62027	C3AR1	complement component 3a receptor 1
3.2	7.03E-06	1	AI073984	IRF8	Interferon regulatory factor 8
3.1	1.36E-03	1	J04162	FCGR3B	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)
3	1.93E-03	1	M80927	CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)
3	3.14E-04	1	NM_000064	C3	complement component 3
2.9	1.12E-03	2	NM_138557	TLR4	toll-like receptor 4
2.9	1.46E-03	1	X14355	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)
2.9	4.00E-03	1	NM_001736	C5R1	complement component 5 receptor 1 (C5a ligand)
2.9	7.91E-05	1	AF114013	TNFSF13	tumor necrosis factor (ligand) superfamily, member 13
2.8	2.05E-03	1	L03419	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)
2.8	3.48E-03	2	AI421071	CCR1	chemokine (C-C motif) receptor 1
2.8	2.66E-05	1	NM_000101	CYBA	cytochrome b-245, alpha polypeptide
2.8	4.06E-04	1	AI184968	C1QG	complement component 1, q subcomponent, gamma polypeptide
2.8	8.12E-07	1	NM_002032	FTH1	ferritin, heavy polypeptide 1
2.7	5.90E-04	1	NM_015991	C1QA	complement component 1, q subcomponent, alpha polypeptide
2.6	2.16E-04	1	NM_002133	HMOX1	heme oxygenase (decycling) 1
2.6	3.80E-03	1	U13700	CASP1	caspase 1 (interleukin 1, beta, convertase)
2.5	1.89E-04	1	NM_006417	IFI44	Interferon-induced protein 44

We validated the expression of a subset of genes by quantitative RT-PCR over a larger group of samples. Primers for IgG heavy chain were designed to amplify all classes (1 through 4). The level of gene expression correlated with the level of inflammation for all genes analyzed **(Table 14)**. Remarkably, CXCL13 was over 1000-fold upregulated in the placentas of women with massive intervillositis.

Table 14. Validation of array data by quantitative RT-PCR. Data is presented as fold-change calculated from mean differences in threshold cycle compared to KRT7 as control. Samples from all women who are PM-positive are compared to PM-negative women. P-values were calculated using student's t-test on corrected threshold cylces.

	PM-negative (n=24) compared to:				
	PM-positive (23)			PM-positive with (++) intervillositis (5)	
Gene	Fold change	P		Fold change	p
CXCL13	91.4	<0.0001		1242	<0.0001
CCL18	16.4	<0.0001		67	<0.0001
IGGH	5.4	0.0007		51	<0.0001
HEPC	8	<.0001		38	<0.0001
IFNG	6.6	<0.0001		37	<0.0001
IGMH	3.9	0.0013		34	<0.0001
TNF	6.7	<0.0001		19	<0.0001
CCL4	6	<0.0001		17	<0.0001
CXCL9	4	0.0004		14	0.0002
IL1B	5.5	<0.0001		13	<0.0001
CCL5	2.2	0.0054		9	<0.0001
IL18	2.8	<0.0001		8	<0.0001
CXCL10	2.5	0.0054		8	0.003
BAFF	2.3	0.0004		6	<0.0001
CXCL16	2.1	<.0001		3	<0.0001

Several genes, including IgG, IgM, BAFF, CXCL13, CCL18 and hepcidin were negatively correlated with birthweight in PM-positive women **(Figure 10)**. As a

measure of infection chronicity we quantified pigment deposition by microscopy in PM-positive women. Several genes, notably IgG, IgM, CXCL13 and BAFF were correlated with the degree of placental pigment deposition by histology **(Figure 11)**, indicating they may contribute to the chronicity of infection.

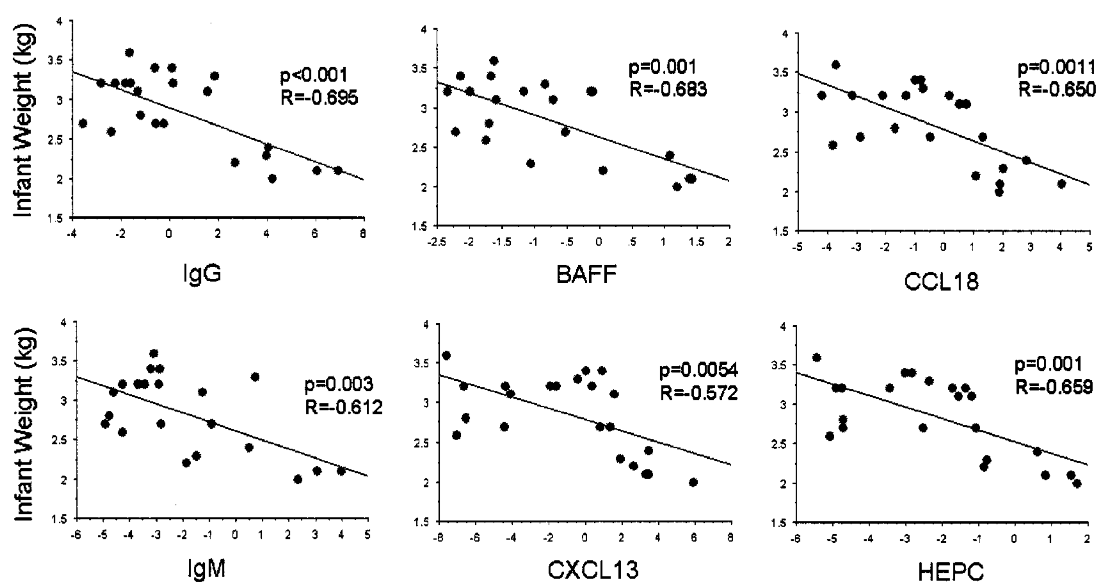

Figure 10. Correlation of gene expression by quantitative RT-PCR with birth weight for infected women. Gene expression is presented as 2^x fold expression over KRT7. Simple regression analysis was used to calculate R and p-values.

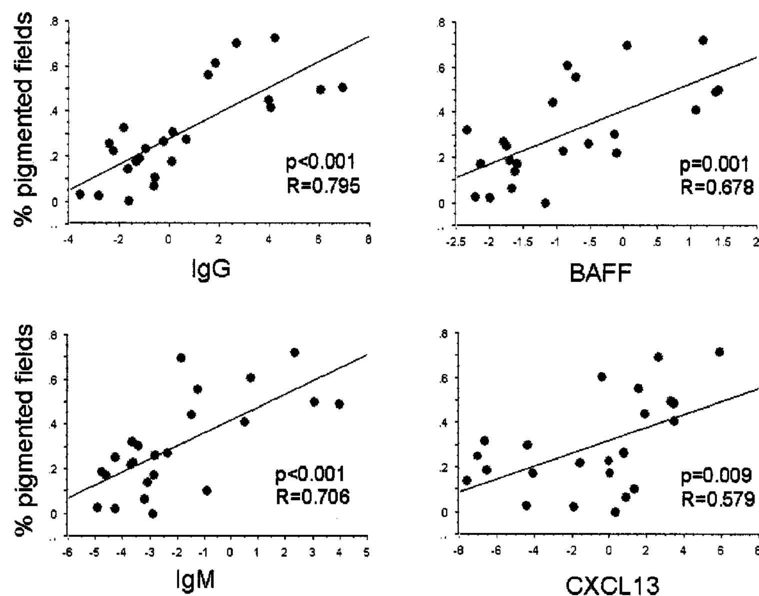

Figure 11. Correlation of gene expression by quantitative RT-PCR with placental malarial pigment deposition quantified by proportion positive fields by microscopy. Only infected women are analyzed. Gene expression is presented as 2^x fold expression over KRT7. Simple regression analysis was used to calculate R and p-values.

By immunohistochemistry, IgG3 and IgM stained strongly in the intervillous space and were associated with cellular infiltrate (**Figure 12**). IgG1 staining was not associated with the infiltrate (Data not shown). Infiltrating monocyte macrophages stained positively for the pro-inflammatory FcG receptors CD16 and CD64, in addition to the B-cell attracting chemokine CXCL13. Because of the high level of immunoglobulin transcription we tested for plasma cells, and identified CD138 positive cells present in the intervillous space during chronic placental malaria, but not in uninfected women. The villous trophoblast also stained positive for CD138, as the marker, syndecan-1, is also expressed by epithelial cells.

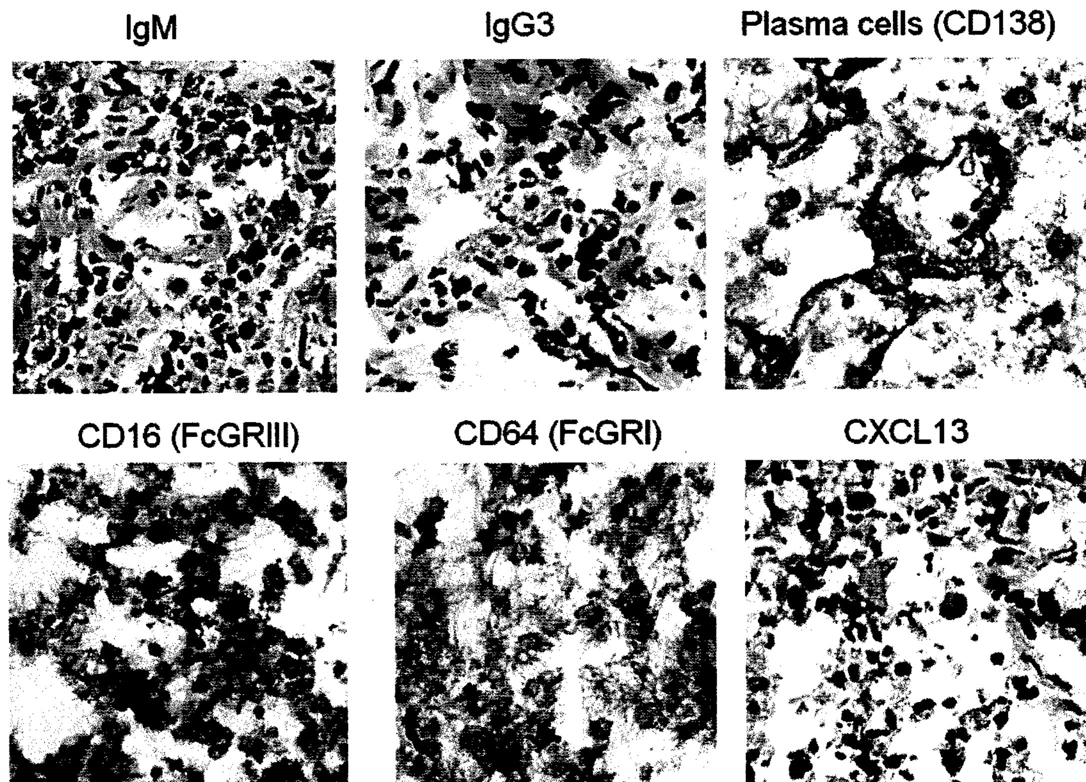

Figure 12. Immunohistochemistry of inflamed PM-positive tissues. All fields are 200X. IgM and IgG3 are in the intervillous space. CD138+ plasma cells are in the intervillous space, and the villous trophoblast is also CD138+. Maternal monocyte-macrophages, identifiable by ingested parasite pigment, are positive for CXCL13, BAFF CD16 and CD64.

Because B-cell attracting chemokines (CXCL13, CCL18) and B-cell activating factor (BAFF) have been associated with ectopic germinal center formation, we tested whether B cells were proliferating in the lesions of chronic placental malaria, indicating that they may be developing *in situ*. By double indirect immunofluorescence mitotic B-cells were identified in the intervillous space during chronic placental malaria, but not in uninfected women **(Figure 13)**.

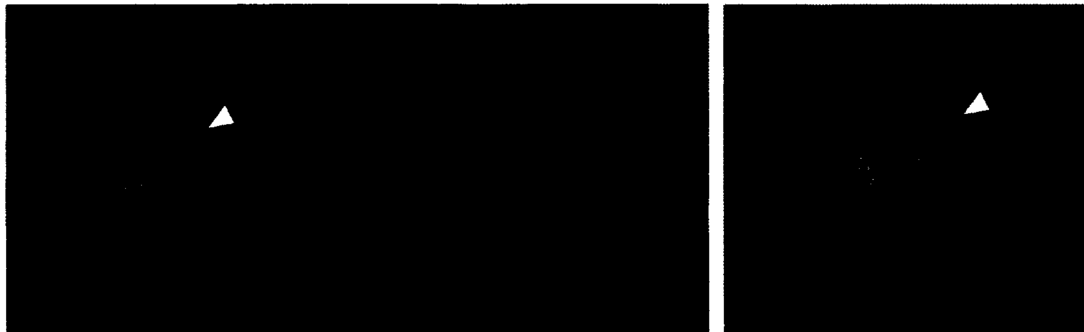

Figure 13. B-cell proliferation was detected by double indirect immunofluorescence using CD20 as a B-cell marker (green), Ki-67 (red) as a marker of mitosis, and DAPI to define nucear DNA (Blue). Arrowheads indicate proliferating B-cells. 400X magnification.

In **Chapter 3** I demonstrated that sVEGFR1 transcript levels are elevated in PM and were specifically elevated in women who had inflammation by histology. Microarray analysis did not identify VEGFR1 as being upregulated. Yet, transcript levels by quantitative PCR of several genes identified by array were positively correlated with sVEGFR1 (**Table 15**). CXCL16 showed the strongest correlation, followed by hepcidin, IgG heavy chain and IFNG. IL1B, IL18 and TNF transcript levels did not show correlation. Scatterplots are shown in **Figure 14**, where correlation occurs primarily in infected tissues. CXCL16 was immunolocalized to the placental trophoblast in infected placental tissue, but not uninfected tissue. **(Figure 15).**

Table 15. Correlation of sVEGFR1 transcript levels by quantitative RT-PCR with genes identified by array. P-values and rho calculated using Spearman rank correlation.

	Correlation with sVEGFR1 transcript levels (n=24)	
Gene	Rho	*P*
CXCL16	0.69	0.001
HEPC	0.515	0.014
IGGH	0.503	0.016
IFNG	0.458	0.028
CCL18	0.451	0.030
CCL5	0.411	0.035
IGMH	0.423	0.042
BAFF	0.41	0.051
CXCL13	0.365	0.080
CXCL10	0.356	0.088
CCL4	0.345	0.098
CXCL9	0.308	0.14
TNF	0.307	0.141
IL18	0.284	0.173
IL1 B	0.211	0.311

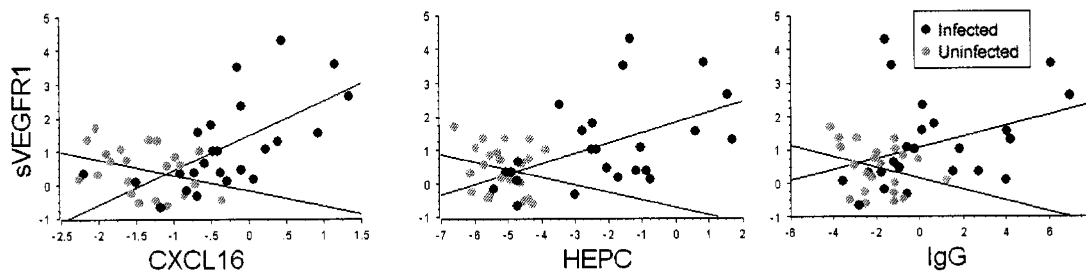

Figure 14. Correlation of sVEGFR1 with CXCL16, hepcidin and IgG transcript levels by quantitative RT-PCR with CXCL16, hepcidin and IgGH. Gene expression is presented as 2^x fold expression over KRT7. Regression line is plotted.

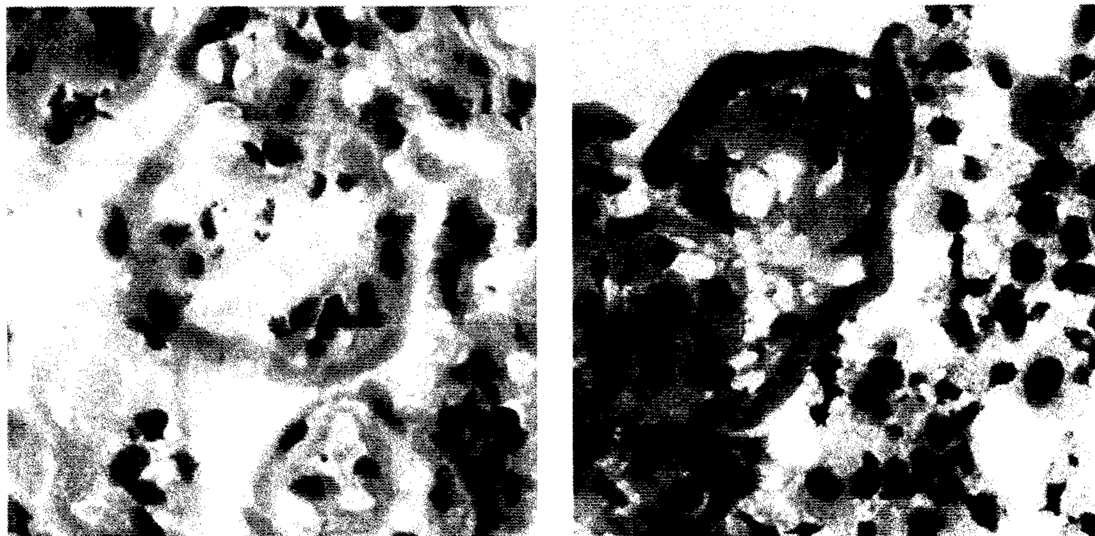

Figure 15. Immunohistochemistry for CXCL16. A. PM-negative placenta, B inflamed PM-positive placenta. Fields are 200X.

Discussion

This data suggests there is a bifunctional role of antibodies in placental malaria: while antibodies confer protection to multigravid women, antibodies contribute to pathology non-immune primigravid women. We propose a model where *P. falciparum* antigen-antibody complexes deposit in the intervillous space and activate monocyte-macrophages through Fc-receptor and complement activation. These monocyte-macrophages produce CXCL13, BAFF and CCL18, causing B-cell accumulation, proliferation and further antibody production. This mechanism may be a general phenomenon during malaria infection, and may contribute to the hypergammaglobulinemia, autoantibodies and Burkitt's lymphoma associated with malaria.

Cytokine and chemokine networks in chronic autoimmune inflammation have been partially characterized. IL1b and IL18 are primary drivers of inflammation in mice, elevated during chronic autoimmune diseases, and are therapeutic targets in rheumatoid arthritis (Dinarello 2004; McInnes et al. 2005). Elevated levels of the chemokines CCL18 (Schutyser et al. 2001), CXCL16 (van der Voort et al. 2005) and CXCL9 (Konig et al. 2000) are also observed, and are hypothesized to recruit lymphocytes to the site of inflammation. CCL18 is also expressed in germinal centers (Adema et al. 1997), and CXCL16 and CXCL9 are chemotactic plasma cells (Nakayama et al. 2003; Tsubaki et al. 2005). BAFF promotes B-cell survival and its overexpression in mice leads to hypergammaglobinemia and autoantibodies (Mackay et al. 2002). Elevated levels of BAFF are associated with autoantibodies in chronic autoimmune disease (Mackay et al. 2005).

Our data indicates that chronic PM involves similar mechanisms as chronic autoimmune disease, with the difference that in PM the immune response is directed against parasite antigens, not self antigens. Even though malaria is associated with autoantibody formation, inflammation does not persist after clearance of parasites. Examination of resolving infections may provide insight into the persistence of chronic autoimmune disease. It has been suggested that the incidence of autoimmune disease is lower in malaria endemic areas compared to non-endemic areas (Greenwood 1968), however longitudinal studies examining the interaction between autoimmune disease and malaria in endemic areas have not been performed. Whether malaria modulates the incidence or severity of autoimmunity should be examined in controlled trials.

We propose that eliciting a type III hypersensitivity reaction is a strategy of *P. falciparum* to prevent effective host development of specific antibodies. If this strategy is selected for, selection may contribute to the extensive polypyrimidine repeats and low complexity amino acid sequences present in *P. falciparum*. Similar repetitive sequences lead to polyclonal B cell activation and non-specific antibodies in experimental models. In addition malarial variant surface antigens genes have been proposed to act as polyclonal B-cell activators (Donati et al. 2004). Other parasite ligands may be important in influencing the host response: We detected upregulation of innate immune receptors: toll-like receptors -1,-2,-4,-8, and formyl peptide receptors -1,-L2 (**Appendix 1**) which may contribute macrophage and B-cell gene expression.

Transcript levels of CXCL16 correlated closely with sVEGFR1. CXCL16 is a pleiotropic molecule. It is a macrophage scavenger receptor (Shimaoka et al. 2000) that can mediate the phagocytosis of oxidized low density lipoprotein(Minami et al. 2001) and gram-positive or gram-negative bacteria (Shimaoka et al. 2003). It is induced by IFNG (Wuttge et al. 2004) and it signals via CXCR6 (Matloubian et al. 2000) which is present on mature lymphocytes, and recruits memory T cells (van der Voort et al. 2005) and plasma cells (Nakayama et al. 2003). CXCR6 and CXCL16 are also expressed by the trophoblast (Huang et al. 2006). CXCL16 is believed to be pathogenic in atherosclerosis (Minami et al. 2001; Wuttge et al. 2004) and rheumatoid arthritis (Nanki et al. 2005), but experimentally is required for allograft tolerance (Jiang et al. 2005). During PM, my data indicates CXCL16 synthesis occurs in trophoblast. CXCL16 may function to promote inflammation or it may function to

maintain fetal allograft tolerance. Based on a maternal-fetal conflict model, CXCL16 would be hypothesized to be protective for the fetus, and therefore may hypothesized to promote graft tolerance. Because it was correlated with sVEGFR1, CXCL16 may be functionally related to sVEGFR1 or transcript levels of the two genes may be regulated by similar mechanisms.

Anemia of inflammation occurs during chronic disease. Hepcidin is a cytokine-induced liver-expressed peptide (Nemeth et al. 2003) that is hypothesized to be a key mediator of the anemia of inflammation (Nemeth et al. 2004). Anemia is a major cause of maternal death during PM. We detected placental transcription of hepcidin during chronic malaria infection, suggesting that hepcidin may contribute to PM-related anemia. In a murine model, hepcidin was produced by macrophages in response to toll-like receptor stimulation (Peyssonnaux et al. 2006). Further studies should examine how placental hepcidin is related to maternal hemoglobin level, and examine whether hepcidin is produced locally at the site of inflammation in other chronic diseases.

This data suggests that placental inflammation and sVEGFR1 expression are related. Inflammation is a component of the maternal syndrome of preeclampsia (Redman et al. 1999). Women with urinary tract infection (Schieve et al. 1994; Mittendorf et al. 1996), periodontal disease (Boggess et al. 2003) and anti-chlamydial antibodies (Heine et al. 2003) are at greater risk for preeclampsia, and my data suggests that placental malaria can lead to hypertension. The expression data suggests that inflammation during placental malaria involves a type III hypersensitivity reaction

and antibody synthesis. Indeed immunoglobulin transcript levels were correlated with sVEGFR1. Women with autoimmune disease are at significantly greater risk for preeclampsia (Stamilio et al. 2000) as are women with anti-phospholipid antibodies (Pattison et al. 1993; Yasuda et al. 1995). CXCL16 expression was also correlated with sVEGFR1 expression, and CXCL16 was expressed by the fetal trophoblast. The correlation between sVEGFR1 (expressed by the fetus) and immunoglobulin (expressed by the mother) or CXCL16 (expressed by the fetus) highlights that the fetal response and the maternal response to chronic placental malaria are separate but closely related. These two responses are hypothesized to be in genetic conflict. Clinical and experimental studies examining the effects of placental malaria on sVEGFR1 regulation should provide insight into the pathogenesis of preeclampsia.

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Appendix: Table of genes associated with placental malaria

Expression data for genes identified by microarray to be upregulated during inflammation (at least 2.5 fold and p-value <0.01). P-values are calculated from student's t-test, not corrected for multiple comparisons.

Fold change	P-value	# of probes	Accession	Gene Symbol	Gene Title
129.3	8.77E-08	1	NM_006419	CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
47.3	2.53E-10	2	Y13710	CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
37.4	1.42E-07	3	BG397856	HLA-DQA1 /// A2	major histocompatibility complex, class II, DQ alpha 1
33.0	5.51E-06	3	X57812	IGL@	immunoglobulin lambda locus
32.0	6.43E-09	1	NM_002416	CXCL9	chemokine (C-X-C motif) ligand 9
25.0	1.26E-05	3	AW575927	IGKC /// IGKV1-5	immunoglobulin kappa constant
21.6	3.17E-10	1	AF301007	SIGLEC10	sialic acid binding Ig-like lectin 10
21.3	1.80E-05	1	M87790	IGLC2	Immunoglobulin lambda joining 3
19.9	2.32E-07	1	NM_006865	LILRA3	leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 3
18.3	1.43E-06	2	AF004231	LILRB2	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2
18.1	1.19E-07	3	AI681260	LILRB1	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3
16.8	9.24E-08	1	NM_017424	CECR1	cat eye syndrome chromosome region, candidate 1
14.0	1.45E-08	1	NM_002121	HLA-DPB1	major histocompatibility complex, class II, DP beta 1
13.7	6.03E-06	1	NM_002983	CCL3 /// CCL3L1	chemokine (C-C motif) ligand 3
13.5	1.27E-04	1	NM_002984	CCL4	chemokine (C-C motif) ligand 4
13.2	9.50E-08	1	NM_014479	ADAMDEC1	ADAM-like, decysin 1
12.8	6.15E-07	1	NM_001974	EMR1	egf-like module containing, mucin-like, hormone receptor-like 1
12.6	1.10E-07	2	AW872374	TLR8	toll-like receptor 8
12.3	3.62E-07	2	M27487	HLA-DPA1	major histocompatibility complex, class II, DP alpha 1
12.2	5.24E-08	2	AW026543	FPRL2	formyl peptide receptor-like 2
11.9	1.41E-06	2	S59049	RGS1	regulator of G-protein signalling 1
11.8	2.55E-07	1	X76775	HLA-DMA	major histocompatibility complex, class II, DM alpha
11.8	1.54E-04	1	M87789	IGH@	immunoglobulin heavy locus
11.4	3.70E-05	1	AL121985	SLAMF7	SLAM family member 7
11.3	2.50E-08	2	NM_000295	SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
10.9	1.08E-04	6	AI583173	HLA-DQB1	major histocompatibility complex, class II, DQ beta 1
10.5	4.43E-06	3	NM_002838	PTPRC	protein tyrosine phosphatase, receptor type, C
10.5	4.40E-05	1	NM_004049	BCL2A1	BCL2-related protein A1

10.2	2.65E-08	2	M60334	HLA-DRA	major histocompatibility complex, class II, DR alpha
10.2	1.81E-08	1	AW662189	C1orf162	chromosome 1 open reading frame 162
10.1	5.73E-08	4	NM_002125	HLA-DRB1	major histocompatibility complex, class II, DR beta 1
10.0	3.22E-04	1	AA601997	---	---
9.8	9.49E-07	2	AV734646	LOC441168	hypothetical protein LOC441168
9.3	1.18E-07	1	BC005926	EVI2B	ecotropic viral integration site 2B
8.7	6.92E-09	2	AF134715	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b
8.6	2.48E-06	1	M13975	PRKCB1	protein kinase C, beta 1
8.5	1.95E-07	1	NM_001778	CD48	CD48 antigen (B-cell membrane protein)
8.1	4.18E-04	1	AV733266	IGJ	Immunoglobulin J polypeptide
8.1	1.70E-06	1	BF439449	---	Transcribed locus
8.1	1.56E-04	1	NM_001565	CXCL10	chemokine (C-X-C motif) ligand 10
7.9	3.74E-06	3	NM_004079	CTSS	cathepsin S
7.9	3.95E-06	2	W46388	SOD2	superoxide dismutase 2, mitochondrial
7.4	2.13E-05	1	BG545653	GBP5	Guanylate binding protein 5
7.4	2.35E-06	1	NM_013447	EMR2	egf-like module containing, mucin-like, hormone receptor-like 2
7.3	2.92E-07	2	AW135013	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)
7.2	1.53E-06	2	NM_000022	ADA	adenosine deaminase
7.1	1.51E-04	1	NM_006144	GZMA	granzyme A
7.1	3.35E-05	1	NM_005601	NKG7	natural killer cell group 7 sequence
7.1	3.14E-04	2	U25677	LYZ	lysozyme (renal amyloidosis)
6.9	6.23E-05	2	NM_022136	SAMSN1	SAM domain, SH3 domain and nuclear localisation signals, 1
6.8	2.64E-04	2	X06557	TRA@ /// TRD@	T cell receptor alpha locus /// T cell receptor delta locus
6.8	9.84E-07	1	NM_003982	SLC7A7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7
6.6	3.66E-07	1	NM_021983	HLA-DRB5	Major histocompatibility complex, class II, DR beta 3
6.5	7.94E-06	1	BC004270	RASSF5	Ras association (RalGDS/AF-6) domain family 5
6.5	3.13E-06	1	AF020314	CD300A	CD300A antigen
6.2	2.46E-06	1	AA897516	PTGER4	prostaglandin E receptor 4 (subtype EP4)
6.2	1.91E-07	1	NM_003608	GPR65	G protein-coupled receptor 65
6.2	5.52E-04	1	BG485135	IGKC	Immunoglobulin kappa variable 1-5
6.1	1.52E-03	1	AW404894	IGKV	Immunoglobulin kappa light chain variable region (IGKV gene), clone 25
6.1	8.98E-06	1	NM_000570	FCGR3A /// B	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
6.0	1.31E-05	1	NM_002800	PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
6.0	1.27E-06	2	NM_000211	ITGB2	integrin, beta 2
6.0	1.05E-04	1	AF043179	TRBV	T cell receptor beta variable 21-1
5.9	5.32E-06	1	NM_005248	FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog

5.8	5.61E-06	1	AL050262	TLR1	toll-like receptor 1
5.8	1.09E-06	2	K01144	CD74	CD74 antigen
5.7	5.18E-06	2	NM_020125	SLAMF8	SLAM family member 8
5.7	1.38E-06	1	NM_001645	APOC1	apolipoprotein C-I
5.5	9.05E-04	1	U20350	CX3CR1	chemokine (C-X3-C motif) receptor 1
5.5	8.23E-06	2	U94592	UCP2	uncoupling protein 2 (mitochondrial, proton carrier)
5.5	2.22E-05	1	NM_003151	STAT4	signal transducer and activator of transcription 4
5.4	1.58E-05	1	NM_003264	TLR2	toll-like receptor 2
5.4	6.00E-07	1	L06633	PSCDBP	pleckstrin homology, Sec7 and coiled-coil domains, binding protein
5.3	5.44E-04	1	U65590	IL1RN	interleukin 1 receptor antagonist
5.3	5.35E-05	1	L25259	CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)
5.2	3.98E-06	6	AI735692	LST1	leukocyte specific transcript 1
5.2	9.96E-08	2	BC002704	STAT1	signal transducer and activator of transcription 1, 91kDa
5.2	1.21E-06	1	X77598	LPXN	leupaxin
5.1	1.41E-06	1	AI655467	---	CDNA FLJ41454 fis
5.1	6.07E-05	2	NM_002664	PLEK	pleckstrin
5.1	1.49E-03	1	M15564	TRBV19 /// TRBC1	T cell receptor beta variable 19 /// T cell receptor beta constant 1
5.0	2.46E-05	2	AB035482	C1orf38	chromosome 1 open reading frame 38
4.9	2.54E-06	1	NM_000161	GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)
4.7	1.06E-04	2	BE138888	RAC2	ras-related C3 botulinum toxin substrate 2
4.7	8.13E-06	1	D86964	DOCK2	dedicator of cytokinesis 2
4.6	2.58E-05	1	NM_006682	FGL2	fibrinogen-like 2
4.5	8.33E-04	2	AI669229	RARRES1	retinoic acid receptor responder (tazarotene induced) 1
4.5	5.28E-06	1	AI375915	---	---
4.5	1.05E-03	1	NM_015714	G0S2	G0/G1switch 2
4.5	9.21E-07	1	AW025572	HAVCR2	hepatitis A virus cellular receptor 2
4.5	1.23E-05	3	AB026043	MS4A7	membrane-spanning 4-domains, subfamily A, member 7
4.4	4.84E-04	1	AF283777	CD72	CD72 antigen
4.4	5.97E-06	1	BF740152	MYO1F	myosin IF
4.4	2.02E-04	1	AI308863	CYBB	cytochrome b-245, beta polypeptide (chronic granulomatous disease)
4.4	1.40E-03	2	NM_001995	ACSL1	acyl-CoA synthetase long-chain family member 1
4.3	1.38E-04	1	AF400600	CLEC7A	C-type lectin domain family 7, member A
4.3	1.05E-04	2	BC020763	FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide
4.3	2.25E-03	3	NM_002985	CCL5	chemokine (C-C motif) ligand 5 /// chemokine (C-C motif) ligand 5
4.3	4.68E-04	1	NM_020980	AQP9	aquaporin 9
4.2	2.87E-03	1	NM_001767	CD2	CD2 antigen (p50), sheep red blood cell receptor
4.2	3.31E-05	1	NM_021199	SQRDL	sulfide quinone reductase-like (yeast)

4.2	5.47E-05	1	NM_020179	FN5	FN5 protein
4.1	2.35E-05	1	U34690	CORO1A	coronin, actin binding protein, 1A
4.1	1.88E-05	1	NM_021822	APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
4.1	1.78E-04	1	BC008777	ITGAL	integrin, alpha L
4.1	6.72E-06	1	AI093231	APBB1IP	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein
4.1	1.73E-03	1	R34841	---	Transcribed locus
4.1	1.72E-04	1	NM_000579	CCR5	chemokine (C-C motif) receptor 5
4.1	3.81E-07	1	AF275260	CXCL16	chemokine (C-X-C motif) ligand 16
4.1	3.05E-03	2	M27331	TRGC2	T cell receptor gamma constant 2
4.1	9.89E-05	3	U19713	AIF1	allograft inflammatory factor 1
4.1	1.04E-03	1	AL022324	LOC91353	similar to omega protein
4.0	1.47E-04	1	N90866	CD52	CD52 antigen (CAMPATH-1 antigen)
4.0	7.74E-05	1	NM_173558	FGD2	FYVE, RhoGEF and PH domain containing 2
4.0	2.30E-05	1	NM_021175	HAMP	hepcidin antimicrobial peptide
3.9	1.29E-04	1	NM_006770	MARCO	macrophage receptor with collagenous structure
3.9	1.52E-04	1	U17496	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8
3.9	2.02E-06	1	NM_002115	HK3	hexokinase 3 (white cell)
3.9	1.48E-04	1	NM_001774	CD37	CD37 antigen
3.9	3.79E-05	1	NM_001558	IL10RA	interleukin 10 receptor, alpha
3.9	1.01E-06	1	AJ400843	PILRA	paired immunoglobin-like type 2 receptor alpha
3.9	2.57E-04	2	AI123251	LCP2	lymphocyte cytosolic protein 2
3.8	3.53E-04	2	Z22969	CD163	CD163 antigen
3.7	1.04E-03	1	NM_002110	HCK	hemopoietic cell kinase
3.7	3.49E-05	2	AI589086	LAPTM5	lysosomal associated multispanning membrane protein 5
3.7	1.34E-05	1	AV728268	C11orf32	chromosome 11 open reading frame 32
3.7	1.87E-03	2	NM_016816	OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa
3.7	1.93E-04	1	AW135176	---	MRNA; cDNA DKFZp686N07104
3.7	3.87E-05	1	NM_000690	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)
3.6	2.49E-03	1	M15330	IL1B	interleukin 1, beta
3.6	3.82E-05	1	NM_000491	C1QB	complement component 1, q subcomponent, beta polypeptide
3.6	1.59E-04	4	AF198052	FYB	FYN binding protein (FYB-120/130)
3.6	5.34E-03	1	J03189	GZMB	granzyme B
3.6	3.73E-04	2	NM_006748	SLA	Src-like-adaptor /// Src-like-adaptor
3.5	6.51E-04	1	NM_017718	DOCK10	dedicator of cytokinesis 10
3.5	7.11E-03	1	NM_014788	TRIM14	tripartite motif-containing 14
3.5	2.80E-03	1	BG536224	IGKV	Immunoglobulin kappa light chain VJ region
3.5	2.02E-06	1	D26054	FBP1	fructose-1,6-bisphosphatase 1
3.5	2.99E-07	1	AF005487	HLA-DRB6	major histocompatibility complex, class II, DR beta 6 (pseudogene)

3.5	3.55E-04	1	AI925518	ANKRD22	ankyrin repeat domain 22
3.5	4.44E-05	1	NM_004566	PFKFB3	6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3
3.5	2.73E-03	1	BE675995	CDC42SE2	CDC42 small effector 2
3.4	8.29E-05	1	NM_001562	IL18	interleukin 18 (interferon-gamma-inducing factor)
3.4	3.78E-04	1	NM_000041	APOE	apolipoprotein E
3.4	2.24E-06	1	NM_014210	EVI2A	ecotropic viral integration site 2A
3.4	2.98E-04	3	NM_017923	MARCH-I	membrane-associated ring finger (C3HC4) 1
3.4	1.19E-04	1	BE966604	SAMD9L	sterile alpha motif domain containing 9-like
3.4	4.41E-04	1	AF109683	LAIR1	leukocyte-associated Ig-like receptor 1
3.4	4.04E-03	1	AA045175	MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A
3.3	1.93E-03	1	NM_002964	S100A8	S100 calcium binding protein A8 (calgranulin A)
3.3	1.95E-03	1	NM_007115	TNFAIP6	tumor necrosis factor, alpha-induced protein 6
3.3	6.57E-06	1	NM_002118	HLA-DMB	major histocompatibility complex, class II, DM beta
3.3	5.84E-05	1	NM_012252	TFEC	transcription factor EC
3.3	2.35E-05	1	J02959	LTA4H	leukotriene A4 hydrolase
3.3	1.85E-05	1	NM_003332	TYROBP	TYRO protein tyrosine kinase binding protein
3.3	5.46E-04	1	NM_002029	FPR1	formyl peptide receptor 1 /// formyl peptide receptor 1
3.3	3.72E-04	1	N49935	RASSF4	Ras association (RalGDS/AF-6) domain family 4
3.2	3.34E-04	1	NM_001747	CAPG	capping protein (actin filament), gelsolin-like
3.2	1.43E-04	2	AK026747	LOC54103	hypothetical protein LOC54103
3.2	6.51E-05	1	U62027	C3AR1	complement component 3a receptor 1
3.2	9.61E-04	1	AF076642	RGS18	regulator of G-protein signalling 18
3.2	7.03E-06	1	AI073984	IRF8	interferon regulatory factor 8 /// interferon regulatory factor 8
3.2	1.41E-03	1	AI741188	ZNFN1A1	Zinc finger protein, subfamily 1A , 1 (Ikaros)
3.2	1.39E-05	1	AW575123	ARHGAP30	Rho GTPase activating protein 30
3.2	6.13E-04	1	BF591040	TAGAP	T-cell activation GTPase activating protein
3.2	3.84E-04	1	NM_002432	MNDA	myeloid cell nuclear differentiation antigen
3.1	4.08E-03	1	NM_001775	CD38	CD38 antigen (p45)
3.1	4.99E-03	2	T64884	MPEG1	macrophage expressed gene 1
3.1	1.67E-03	1	AV734646	---	---
3.1	2.41E-03	1	NM_002450	MT1X	metallothionein 1X
3.1	3.75E-03	1	NM_002003	FCN1	ficolin (collagen/fibrinogen domain containing) 1
3.1	3.11E-04	1	AI376997	FLJ13611	Hypothetical protein FLJ13611
3.1	9.26E-05	1	AI081246	SLIC1	Selectin ligand interactor cytoplasmic-1
3.1	7.42E-05	1	NM_001611	ACP5	acid phosphatase 5, tartrate resistant
3.1	1.36E-03	1	J04162	FCGR3B	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)

3.0	1.14E-04	1	BC001604	NCKAP1L	NCK-associated protein 1-like
3.0	1.38E-03	1	NM_021136	RTN1	reticulon 1
3.0	1.93E-03	1	M80927	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
3.0	1.01E-03	1	AI130690	---	Transcribed locus, weakly similar to NP_055301.1
3.0	2.95E-04	1	AV715309	C20orf118	Chromosome 20 open reading frame 118
3.0	1.16E-04	1	NM_004390	CTSH	cathepsin H
3.0	3.14E-04	1	NM_000064	C3	complement component 3
2.9	1.12E-03	2	NM_138557	TLR4	toll-like receptor 4
2.9	7.28E-04	1	NM_000560	CD53	CD53 antigen
2.9	9.13E-03	1	AI936034	---	Transcribed locus
2.9	5.99E-03	1	AF270513	EMILIN2	elastin microfibril interfacer 2
2.9	4.46E-05	2	AF498927	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta
2.9	1.46E-03	1	X14355	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)
2.9	4.00E-03	1	NM_001736	C5R1	complement component 5 receptor 1 (C5a ligand)
2.9	7.91E-05	1	AF114013	TNFSF13	tumor necrosis factor (ligand) superfamily, member 13
2.9	1.19E-03	1	NM_002971	SATB1	special AT-rich sequence binding protein 1
2.8	2.53E-05	1	NM_015474	SAMHD1	SAM domain and HD domain 1
2.8	2.05E-03	1	L03419	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)
2.8	3.48E-03	2	AI421071	CCR1	chemokine (C-C motif) receptor 1
2.8	2.66E-05	1	NM_000101	CYBA	cytochrome b-245, alpha polypeptide
2.8	4.06E-04	1	AI184968	C1QG	complement component 1, q subcomponent, gamma polypeptide
2.8	4.71E-04	1	U82278	LILRA2	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2
2.8	1.54E-03	1	U86453	PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide
2.8	1.74E-03	2	NM_002053	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa
2.8	4.94E-04	1	AW070431	MBP	myelin basic protein
2.8	2.22E-04	1	AI692703	KCNE3	potassium voltage-gated channel, Isk-related family, member 3
2.8	8.12E-07	1	NM_002032	FTH1	ferritin, heavy polypeptide 1
2.8	1.03E-03	1	NM_139018	CD300LF	CD300 antigen like family member F
2.8	5.53E-04	1	BF575213	MGC5618	hypothetical protein MGC5618
2.7	1.07E-03	1	M55905	ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial
2.7	4.54E-03	1	U11058	KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1
2.7	5.54E-03	1	AI659418	RCSD1	RCSD domain containing 1
2.7	1.66E-03	1	NM_018460	ARHGAP15	Rho GTPase activating protein 15
2.7	4.60E-04	1	AI431931	GIMAP2	GTPase, IMAP family member 2
2.7	5.90E-04	1	NM_015991	C1QA	complement component 1, q subcomponent, alpha
2.6	1.19E-04	1	AL023653	CXorf9	chromosome X open reading frame 9

2.6	3.41E-04	1	NM_003874	CD84	CD84 antigen (leukocyte antigen)
2.6	1.58E-04	1	NM_000061	BTK	Bruton agammaglobulinemia tyrosine kinase
2.6	4.88E-05	1	NM_001175	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta
2.6	2.16E-04	1	NM_002133	HMOX1	heme oxygenase (decycling) 1
2.6	4.58E-04	1	AF237772	PARVG	parvin, gamma
2.6	4.45E-04	1	AC003999	SCAP2	src family associated phosphoprotein 2
2.6	1.33E-03	1	AL515318	SH3BGRL	SH3 domain binding glutamic acid-rich protein like
2.6	4.71E-03	1	NM_004833	AIM2	absent in melanoma 2
2.6	6.18E-06	2	NM_003730	RNASET2	ribonuclease T2
2.6	3.80E-03	1	U13700	CASP1	caspase 1
2.6	9.43E-04	1	BG107203	RABGAP1L	RAB GTPase activating protein 1-like
2.6	3.18E-04	1	NM_004313	ARRB2	arrestin, beta 2
2.6	1.57E-04	1	BM976092	LOC400368	hypothetical gene supported by BC031266
2.6	1.41E-03	1	BF246115	MT1F	metallothionein 1F (functional)
2.5	1.16E-03	1	AF151074	MARCH-II	membrane-associated ring finger (C3HC4) 2
2.5	4.12E-04	1	AI912571	---	Full length insert cDNA clone YT94E02
2.5	9.16E-04	1	AW043782	LDLRAD3	low density lipoprotein receptor class A domain containing 3
2.5	1.28E-03	1	AW504569	---	Transcribed locus, moderately similar to XP_522527.1
2.5	1.89E-04	1	NM_006417	IFI44	interferon-induced protein 44
2.5	1.25E-05	1	AA477260	---	Transcribed locus, weakly similar to XP_510104.1
2.5	4.36E-04	1	NM_021626	SCPEP1	serine carboxypeptidase 1
2.5	2.19E-03	1	NM_015364	LY96	lymphocyte antigen 96
2.5	9.06E-06	1	AW130600	---	MRNA; cDNA DKFZp564O0862

VITA

Personal Information
Name: Atis Muehlenbachs
Date of Birth: November 22 1977

Educational Background

1995 to 1999	Biochemistry and Cell Biology, University of California at San Diego, Summa Cum Laude, BSc
1999 to present	Medical Scientist Training Program. University of Washington, School of Medicine. PhD pending 2005, MD pending 2007
2002 to present	Molecular and Cellular Biology Program, University of Washington. Graduate advisor: Patrick Duffy, MD

Training and Professional Experience

04/96 - 04/97 & 09/98 - 06/99	Assistant, Department of Pathology, neuropathology, University of California, at San Diego.
01-06/98.	Research Assistant, Scripps Institute of Oceanography, La Jolla CA.
06-09/96, 06-09/97 & 06-09/98	Research Assistant, Earth and Atmospheric Sciences, University of Alberta, Canada
07-08/01.	Internship at Instituto Nacional de Biodiversidad, Costa Rica.
08/01 to 06/02	Graduate Student Research Assistant, Department of Pathology, University of Washington. Advisor: Ray Yeung, MD
06/02 - present	Graduate Student Research Assistant, Molecular and Cellular Biology Program, University of Washington. (PhD Candidacy 04/04) Advisor: Patrick Duffy

Publications

1. **Muehlenbachs A**, Mutabingwa TK, Edmonds S, Fried M, Duffy, PE. "Hypertension and maternal-fetal conflict during placental malaria." *Submitted*

2. **Muehlenbachs A**, Mutabingwa TK, Fried M, Duffy, PE. "Cryptic infection with *Plasmodium falciparum* during pregnancy" *Submitted*

3. **Muehlenbachs A**, Mutabingwa TK, Fried M, Duffy, PE. "Genome wide expression analysis of placental malaria suggests B-cells and type III hypersensitivity contribute to inflammation." *In preparation.*

4. Yamamoto Y, Jones KA, Mak BC, **Muehlenbachs A**, Yeung RS. "Multi-compartmental distribution of the tuberous sclerosis gene products, hamartin and tuberin." *Arch Biochem Biophys*. 15;404(2):210-7, Aug 2002.

5. Powell HC, Garrett RS, **Muehlenbachs A**, Brett FM, Campbell IL. "Crystalloid inclusions in brain macrophages and hemopoietic tissue in GFAP-IL3 mice resemble inclusions identified in multiple sclerosis" *Ultrastrucural Pathology,* 23:285-297, Sep-Oct 1999. (with cover illustration)

6. Rowe D, **Muehlenbachs A.** "Low temperature thermal generation of hydrocarbon gases in shallow shales" *Nature* 398:61-63, Mar 1999.

Seminars and Presentations

Seattle Parasitology Conference, Seattle, WA, "Exploring the relationship between placental malaria and maternal blood pressure" May 2005

Washington State Obstetrical Association Annual Meeting, Seattle WA, "Exploring relationships between cytokine gene expression and pregnancy outcomes" Dec 2004

International Society for the Study of Hypertension in Pregnancy, Vienna, Austria, "Gene Expression Arrays to examine the pathogenesis of preeclampsia and placental malaria", November 2004.

North American Society for the Study of Hypertension in Pregnancy, Vancouver, Canada, "Malaria and pregnancy: The trophoblast response and hypertension?" July 2004.

International Congress American Chemical Society, Honolulu, Hawaii, "Electron microprobe analysis of mineral deposition in the brain" Muehlenbachs A, Campbell IL, Muehlenbachs K, Powell HC, Vinters HV. December 2000

(Featured in: Zubritzky E. "Finding fossils in the brain" *Analytical Chemistry* 74(5) pp. 129A-130A, March 2001)

Western Student Medical Research Forum, Carmel, CA. "Retinal hamartoma in the Eker rat model of tuberous sclerosis" Muehlenbachs A, Reh TA, Yeung RS, February 2001.

Western Student Medical Research Forum, Carmel, CA. "Tuberin and hamartin interaction: distribution between monomers and heteromers of two proteins involved in tuberous sclerosis" Muehlenbachs A, Aicher LD, Yeung RS, February 2000.

Scholarships, Fellowships, Honors, and Awards

8/05	Young investigator award, North American Society for the study of hypertension in pregnancy.
06/03	ARCS Foundation Fellowship
06/03	TA nomination, by Tribeta Biological Honors Society
06/99	Beta Kappa, University of California, at San Diego.
06/99	Provost's Scholar, University of California, at San Diego, Eleanor Roosevelt College.

Grants

12/05	American Heart Association Northwest Affiliate Predoctoral Fellowship
07/04.	Travel Grant to attend the 14th World Congress of the International Society for the Study of the Hypertension in Pregnancy, Vienna, Austria, November 2004.
02/04.	AMA Foundation Seed Grant ($2450) "The Role of Malaria in Triggering Gestational Hypertension."
11/03.	Marc Lindenberg Center International Mobility Grant ($1850) "Malaria in Pregnancy."
11/03.	Trainee, University of Washington Cardiovascular Pathology Training Grant.
12/02.	Washington State Obstetrical Association Project Grant ($5,000) "Exploring Relationships Between Cytokine Gene Expression in the Placenta and Pregnancy Outcomes."

Human Subjects Approval

Principal Investigator, "Placental Gene expression" UW IRB 02-4897-D01, Jan 31 2003.

Teaching Experience

01-03/03	Teaching assistant, University of Washington. Biology 200 "Introductory Biology," Three-hour laboratory sessions twice per week, 50 students.
03-06/03	Teaching assistant, University of Washington, Biology 401 "Cell Biology," seven-hours per week, project based learning in selected topics involving writing and presentations, 30 students.

06/04	Teaching assistant, East African Workshop, “The Pregnant Host,” two-week training workshop for East African students and scientists on basic and applied research on protozoan pathogens
07/05	Teaching assistant, East African Workshop, "Drug Trials in Africa"