Editorial Manager(tm) for The Journal of Infectious Diseases
Manuscript Draft

Manuscript Number:

Title: Placental malaria-associated inflammation disturbs the IGF axis of fetal growth regulation

Short Title: Placental malaria and the IGF axis

Article Type: Major Article

Section/Category: Parasites

Keywords: Placental malaria; Low birth weight; Fetal growth restriction; Insulin-like-growth factors.

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Manuscript Region of Origin: AUSTRALIA

Abstract: Background. The pathogenetic mechanisms of fetal growth restriction associated with placental malaria are largely unknown. We sought to determine whether placental malaria and related inflammation were associated with disturbances in the insulin-like-growth-factor (IGF) axis, a major regulator of fetal growth.

Methods. We measured IGF-I and IGF-II concentrations in plasma from 88 mother-neonate pairs at delivery, and IGFBP-1 and IGFBP-3 in cord plasma from a cohort of Papua New Guinean women with and without placental malaria. IGF-I, IGF-II and the IGF receptors (IGF1R, IGF2R) mRNA levels were measured in matched placental biopsies.

Results. Compared to uninfected pregnancies, IGF-I levels were reduced by 28% in plasma from women with placental P. falciparum and associated inflammation (P = .007) and by 25% in their neonates (P = .002). Levels of fetal IGFBP-1 were elevated in placental malaria with and without inflammation (P = .08 and P = .006 respectively) compared to uninfected controls. IGF-II and IGFBP-3 plasma concentrations and placental IGF ligand and receptor mRNA transcript levels were similar across groups.

Conclusion. Placental malaria-associated inflammation disturbs maternal and fetal levels of IGFs which regulate fetal growth. This may be one mechanism by which placental malaria leads to fetal growth restriction.

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Dr. Han's laboratory is focused on studying the molecular and cellular mechanisms of normal fetal and placental growth with the aim of understanding the pathobiological mechanisms of fetal growth restriction, and is an expert in role of the insulin-like-growth factor hormones in fetal growth

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Dear Dr. Hirsch

Re: Submission of a manuscript for evaluation

Please find the enclosed manuscript "Placental malaria-associated inflammation disturbs the IGF axis of fetal growth regulation" for publication evaluation as a major article in the Journal of Infectious Diseases.

Placental malaria, especially when placental inflammation is present, is strongly associated with low infant birth weight, but the physiological mechanisms underlying this association remain largely unknown. In this manuscript, we investigated whether placental malaria and related inflammation might affect levels of insulin-like growth factor 1 (the principle fetal growth regulating hormone) in maternal and fetal blood plasma from a cohort of pregnant women from Papua New Guinea.

In pregnancies affected by placental malaria with monocyte infiltrate we found significantly attenuated levels of IGF-I in both the maternal and the fetal circulation. In mothers, the extent to which IGF-I was reduced was related to the degree of placental monocyte infiltrate, implicating inflammation as an important determinant of reduced IGF concentrations. In the offspring, IGF-I levels were positively correlated with birth weight, and we observed the lowest IGF-I levels in low birth weight infants born from placentas with malarial inflammation. By contrast, levels of IGF-II (which principally controls fetal growth in early pregnancy) were not altered by malaria. Our study suggests that reduction in IGF-I may be an important mechanism underlying development of low birth weight in malaria, and it is a significant advance in our understanding of the pathogenesis of low birth weight in malaria during pregnancy.

This manuscript has not been submitted to any other journal. The authors fulfill the criteria outlined in the JID Authorship paragraph. All authors have contributed to, reviewed and approved the final submitted version of the manuscript. The authors have had no writing assistance in the compilation of this manuscript. The authors prefer to have the manuscript published in black and white only. The word counts for the abstract and manuscript are 197 and 3491 respectively.

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Kind regards,

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Footnote[1]

[1] No authors have any conflict of interest to declare. This project was supported by the NHMRC (Project Grant 509185 to SJR and JG and Project Grant 575534 to JB and SR). JB was supported by an Australian Research Council Future Fellowship. Corresponding author contact Alexandra Umbers a.umbers2@pgrad.unimelb.edu.a

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Running head: Placental malaria and the IGF axis

Title: Placental malaria-associated inflammation disturbs the IGF axis of fetal growth regulation

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Word count (abstract 197, body of text 3491)

Abstract

Background. The pathogenetic mechanisms of fetal growth restriction associated with placental malaria are largely unknown. We sought to determine whether placental malaria and related inflammation were associated with disturbances in the insulin-like-growth-factor (IGF) axis, a major regulator of fetal growth.

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Results. Compared to uninfected pregnancies, IGF-I levels were reduced by 28% in plasma from women with placental *P. falciparum* and associated inflammation (P = .007) and by 25% in their neonates (P = .002). Levels of fetal IGFBP-1 were elevated in placental malaria with and without inflammation (P = .08 and P = .006 respectively) compared to uninfected controls. IGF-II and IGFBP-3 plasma concentrations and placental IGF ligand and receptor mRNA transcript levels were similar across groups.

Conclusion. Placental malaria-associated inflammation disturbs maternal and fetal levels of IGFs which regulate fetal growth. This may be one mechanism by which placental malaria leads to fetal growth restriction.

Key words: placental malaria, low birth weight, fetal growth restriction, insulin-like-growth factors

Introduction

Placental malaria is a leading cause of low birth weight (LBW) in Africa, resulting in 75,000 - 200,000 infant deaths each year. In these settings, LBW is more commonly due to fetal growth restriction (FGR) rather than preterm delivery [1, 2]. One key feature of malaria in pregnancy is the sequestration of *Plasmodium falciparum* infected erythrocytes (IE) in the maternal intervillous spaces of the placenta, termed placental malaria. Placental malaria can trigger the recruitment of inflammatory cells and production of cytokines, which are strongly associated with LBW [3-5]. The biological mechanisms leading to LBW are not known, but placental insufficiency and endocrine disturbances may underlie the pathogenesis.

The placenta functions as an endocrine organ and transmits hormonal signals between the mother and the developing fetus to ensure adequate support for sustained fetal growth. Few studies have examined maternal endocrine profiles in the context of malaria in pregnancy [6-8], and no study has yet investigated the potential role of growth regulating hormones in the pathogenesis of low birth weight in placental malaria.

The insulin-like-growth-factor (IGF) system is the most influential growth promoting factor in fetal life [9], with roles in regulating placental development and function, transplacental exchange of nutrients, and fetal growth. In mice, genetic ablation of *Igf1* or *Igf2* decreases birth weight by 40% [10], and double knock out of *Igf1* and *Igf2*, or of the IGF receptor (*Igf1r),* further restricts fetal growth [10]. The liver is the main source of circulating IGF-I in postnatal life [11], but during pregnancy the placenta and the fetus secrete IGFs and regulatory proteins. Fetal IGF–I and -II promote fetal growth but have differential actions, which have been attributed to distinct interactions with receptors.

The IGF receptors (IGF1R and IGF2R) mediate IGF activity and are abundant in all placental cell types [12] and in the microvillous plasma membrane of the syncytiotrophoblast [13]. Activation of IGF1R stimulates cell signaling cascades [14] that lead to proliferation, survival and fetal growth promotion [9], whereas IGF2R lacks the cell signaling domain [11], acting as a sink to sequester free IGF-II, and is considered anti-mitogenic.

Bioactivity of the IGFs is modulated by the IGF binding proteins (IGFBPs), which transport circulating IGFs. IGFBPs have greater ligand affinity than IGFRs [15] and thus sequester circulating IGFs to restrict their interactions with the receptors. IGFBP-1 and -3 are expressed in abundance at the maternal-fetal interface and are regulated in a developmental and tissue specific way [15]. IGFBP-1 is an important negative short-term regulator of IGF bioactivity and levels fluctuate in response to maternal stress and nutritional intake [16-18].

Alterations in the absolute level and bio-availability of the IGFs in maternal, fetal, and placental compartments are implicated in other causes of FGR [9, 11, 19, 20]. However, the relationship between placental malaria, the IGF axis and birth weight has not been explored.

We investigated whether placental malaria and the associated inflammation impacted on the IGF system in the maternal-placental-fetal axis at delivery, and whether potential changes in the IGF axis were consistent with the IGF profile observed in FGR of other etiologies.

Methods

Study design. Pregnant women attending antenatal care at Alexishafen Health Centre, Madang, Papua New Guinea (PNG) between September 2005 and October 2007 were recruited to a longitudinal study of malaria in pregnancy, following written informed consent. At delivery, babies were weighed, Ballard scores were used to estimate gestational age, and maternal hemoglobin levels were measured. Maternal and cord blood was collected in K_2EDTA vials (BD), and separated plasma was frozen at -80°C. A random sampling of placental biopsies were either collected into 10% neutral buffered formalin for histological examination for placental malaria infection or frozen at -80°C in RNAlater (Ambion) for RNA analysis. The Medical Research Advisory Council of PNG and the Human Research Ethics Committee, Melbourne Health, Australia approved the study. Samples used in the present study are from a subset of participants recruited.

Placental histology and inclusion criteria

Placental histology was examined as described by Rogerson et al [3]. Full thickness Giemsa-stained biopsies were examined, and IE, monocytes containing malaria pigment and malaria pigment in fibrin were noted. Where IE were detected, 500 intervillous cells were counted, and placentas were classified either as placental malaria without inflammation (≥1% IE detected, <1% pigmented monocytes), denoted as placental malaria (PM), or placental malaria with monocyte infiltrate (PMM) (≥1% IE and ≥1% pigmented monocytes). Plasma samples from a subset of approximately 30 women from each group with live singleton deliveries were randomly selected for IGF assays.

Placental RNA extraction and real time qPCR.

RNA extraction from placental tissue biopsies stored in RNALater was performed using Trizol reagent (Invitrogen), according to the supplier's instructions. Removal of DNA contamination of RNA samples was achieved by DNAse-treatment (Ambion) and confirmed by a lack of amplification signal following real time qPCR. Two micrograms of RNA were reverse transcribed according to supplier's recommendations (Superscript III, Invitrogen) and cDNA was diluted two-fold in DNAse-free water and stored at -20°C until use. Real time qPCR was performed in duplicate using a SYBR Green master mix (Applied Biosystems) dispensed by liquid handling robot (Eppendorf), on an ABI 7900HT real-time PCR machine (Applied Biosystems). Primer sequences were derived from previous literature, or designed using Primer Express (supplementary Table 1A). Transcript levels of target genes (IGF-I, IGF-II, IGF1R and IGF2R) were quantified in 88 placental biopsies by real time qPCR using a ratio of transcription (R), as described in Table 3. R corrects for potential differences in PCR efficiency (E) between primer pairs, where (E) = 10^(-1/slope) and slope is calculated from the fluorescence generated by a seven point standard curve of pooled cDNA for each primer pair. The relative amplification efficiency of each target gene was > 93% of the housekeeping gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ).

Enzyme linked immunosorbent assay (ELISA)

Total IGF-I and IGF-II concentrations in cord and maternal plasma were determined by ELISA in 88 mother-infant pairs. Four additional maternal samples that met selection criteria but lacked the corresponding cord sample were included. Cord IGFBP-1 and IGFBP-3 were measured by ELISA in 87 (due to volume limitation) and 88 samples respectively, IGFBP-1 concentrations in cord plasma were log transformed to normalize the data before analysis. For all analytes, both intra- and inter-assay variability did not exceed 6%, except for IGF-II and IGFBP-3 ELISAs, which varied between assays by 8.5% and 6.5% respectively. All ELISA kits were purchased from Diagnostic Systems Laboratory Inc. (Webster, TX).

Statistical analysis

Univariate analysis was performed using GraphPad Prism Software version 4 (Graphware Software Inc, San Diego, CA, USA). Parametric data, represented as mean and standard deviation (SD), were compared using Student's t test, or Analysis of Variance (ANOVA) for three or more groups. Non-parametric data are shown as median and interquartile range (IQR), and were compared using the Kruskall-Wallis test (KW). Correlation analyses used Pearson's correlation r for parametric analysis or Spearman's rho (ρ) for non-parametric correlation. Linear regression was used to determine the slope of the correlation, and is expressed as the correlation co-efficient (ß) ± standard error. Multivariate analysis and odds ratio (OR) calculations were performed using Stata v.10 Software. In all cases $p \leq 0.05$ denoted statistical significance.

Results

Participant characteristics at delivery. Table 1 summarizes the clinical characteristics of the 92 participating women. In total, 58 women had placental malaria, of which 27 were positive for both placental malaria and monocyte infiltrate (PMM), and 31 had placenta malaria infection only (PM). Thirty four cases had neither malaria infection nor monocytes (uninfected). Maternal age at enrolment (P = .03), gravidity (P = .02), and gestational age at delivery (P = .05) of the women with PMM were significantly lower than those of the uninfected controls, while hemoglobin levels were unchanged between groups.

Placental malaria with inflammation is associated with decreased birth weight

Mean birth weight of infants from pregnancies with PMM was 370g less than for infants delivered in the absence of infection (P = .01; Table 2). Infants delivered from pregnancies with PM were on average 100g lighter than infants born from uninfected women. Women with placental malaria and monocyte infiltrate had three times the risk of delivering a LBW infant (<2500g) (OR 3.0, P = .08, 95%CI 0.9-10.4) compared to uninfected women, while there was no increased risk in women with placental malaria without monocyte infiltrate (OR 0.8, P =0.8 95% CI 0.2-3.4). Because PMM and uninfected women had significantly different maternal age, gravidity, and gestational age at delivery that can all have independent effects on birth weight, multivariate analysis was performed to determine the relative impact of placental malaria ± monocyte infiltrate on birth weight (Table 2). Adjusting for gestational age at delivery, PMM reduced mean birth weight by 200g compared to babies of uninfected women (P = .04). Furthermore, when we stratified women by

gravidity, and controlled for the influence of gestational age at delivery and maternal age, the reduction in birth weight with PMM was limited to primigravidae, who delivered infants nearly 300g lighter than those of matched uninfected women (P = .02; Table 2).

Placental malaria and inflammation reduce maternal IGF-I concentrations

We measured levels of IGF-I and IGF-II protein by ELISA in maternal plasma collected at delivery. The mean total IGF-I concentration in women with PMM was 28% lower than in uninfected women (P = .007) (Figure 1B), while IGF-II levels were similar across all cohorts (Figure 1A). In the whole cohort, maternal IGF-I concentration was not correlated with birth weight (P = .4), gestational age (P = .4), or gravidity (P =.2), but among women with PMM there was a significant negative correlation between the extent of monocyte infiltrate and maternal IGF-I levels women (XY=27, r=-.4, P =.05) (Figure 1C).

Placental malaria does not alter placental IGF transcript levels

We measured expression of mRNA transcripts for IGF-I, IGF-II, IGF1R and IGF2R in 88 placental biopsies by real time qPCR (Table 3). Mean Ct values for YWHAZ were not different between groups (KW test P = .7), validating the use of the housekeeping gene. Transcript levels of all four genes did not differ significantly between placentas with and without malaria infection and/or monocyte infiltrate.

Total levels and bioavailability of fetal IGF-I are reduced in placental malaria and monocyte infiltrates.

To evaluate the effect of placental malaria and the associated inflammation on the fetal compartment of the IGF axis, we measured concentrations of total IGF-I and IGF-II and important regulatory binding proteins IGFBP-1 and IGFBP-3 in cord plasma samples.

IGF-I was reduced by 25% in the PMM group compared to infants from uninfected placentas (P = .002; Figure 2B), whereas IGF-II concentrations in cord plasma were similar across the three groups (ANOVA 0.9, Figure 2A). Infants delivered from pregnancies with PM alone had intermediate IGF-I concentrations; these were not statistically different from either uninfected or PMM groups. Cord IGF-I concentration did not vary with gravidity (n=88), nor with placental parasitemia in babies of infected women (n=57) or with monocyte infiltrate in babies with PMM (n=26). Cord IGF-I concentration was weakly correlated with birth weight (XY 83, r= .2, P = .06; Figure 3A) and to a lesser extent gestational age (XY=, r= .2, P = .07). After adjusting for gestational age, the relationship between cord IGF-I concentrations and birth weight was substantially strengthened (R^2 =0.5, P = 0.001;).

Compared to infants born from uninfected pregnancies, median IGFBP-1 concentration was 80% higher in infants born in the presence of PM (P =.006), and 40% higher in infants delivered to PMM group (P = .08; Figure 2C). No significant differences were observed in cord plasma IGFBP-3 concentration between uninfected and PM categories (ANOVA F[2,81]=0.2, P = .9; Figure 2D). However, because of the biological relationship between ligand and binding protein, the ratio of log IGF-I/log IGFBP-3 (as proxy for free IGF-I [21]) was examined in relation to placental infection (Figure 2E). Compared to uninfected pregnancies, the mean estimated free

fetal IGF-I was reduced by 5% in PM (P =.02) and 10% in the presence PMM (P <.0001).

To further examine the possibility that diminished fetal IGF-I is a key pathogenic mechanism leading to LBW in placental malaria with inflammation, cord IGF-I levels were compared between normal birth weight (NBW, ≥2.5kg) and LBW (<2.5kg) infants delivered from uninfected pregnancies and those with PM and PMM (Figure 3B). Both NBW and LBW infants from the PMM group had lower IGF-I concentrations (P = .02 and P = .04 respectively) than the respective birth weight group from uninfected pregnancies (Figure 3B), and LBW infants from the PMM group displayed the most severe reduction in IGF-I concentration.

Discussion

Although the causal relationship between placental malaria and LBW is well recognized, little is known about the pathogenetic mechanisms leading to FGR in malaria during pregnancy. We investigated the role of IGFs as key endocrine regulators of fetal growth to determine if placental malaria and the associated inflammation were associated with disturbances in the IGF axis in the mother, placenta or fetus. In agreement with previous studies in malaria-endemic areas [3, 4], the greatest impact of placental malaria on birth weight was observed in infants born with placental malaria and monocyte infiltrate, which was most common in primigravidae.

We have identified changes in the IGF axis in mothers and infants with placental malaria and inflammation consistent with the endocrine profile of growth restricted infants [19, 22]. The most severe attenuation of growth-promoting IGF-I occurred in LBW infants delivered from placentas with malaria and inflammation, suggesting a pathogenetic link between inflammation and decreased IGF-I leading to LBW. We observed a strong negative association between maternal IGF-I and the extent of placental monocyte infiltrates. Although we did not find evidence of a statistical association between maternal IGF-I levels and birth weight, very few studies have demonstrated a direct relationship between circulating maternal IGF-I and birth weight [22, 23].

Although not transferred across the placenta, maternal IGFs promote fetal growth indirectly through modulation of placental nutrient partitioning. Nutrient transporters

are important down-stream targets of the IGF system, and physiological levels of IGF-1 stimulate *in vitro* glucose and amino acid transporter expression and activity in trophoblast cell cultures [24, 25]. The malaria-associated inflammatory events reducing maternal IGF-I may lead to impaired nutrient delivery to the fetus.

Several physiological explanations could account for the diminished maternal IGF-I concentrations we have observed in placental malaria with inflammation. Previous studies have shown the IGF-axis is influenced by hypoxia, nutrition, cortisol [26], and the inflammatory cytokines IFNγ , TNFα, IL-1β, and IL-6 [27-29]. Although there is no evidence that placental malaria causes hypoxia, [30], elevated levels of pro-inflammatory cytokines [5, 31-33] and glucocorticoids [6, 8] observed during malaria in pregnancy have the potential to influence the IGF axis. Whether these processes are responsible for the decreased IGF-1 levels (and therefore fetal growth) we have observed in placental malaria with inflammation deserves further investigation. Whatever the intermediate signal, the lack of a pronounced effect of parasite sequestration alone on circulating IGF-I, coupled with the strong negative association between inflammatory load and maternal IGF-I levels, suggests that inflammatory processes are the driving force reducing IGF-I in the maternal compartment at delivery.

Throughout pregnancy, the fetal liver synthesizes IGFs [15], which act in part to regulate materno-fetal glucose transfer according to fetal demand. IGFs also have a direct trophic effect in fetal target tissues, via activation of the IGF1R expressed in fetal organs. Decreased levels of fetal IGF-I, such as we have described in placental

malaria and inflammation, are likely to negatively affect fetal growth through decreased cellular proliferation, and indirectly, by limiting trans-placental passage of nutrients. Indeed evidence from our study supports a pathogenetic link between attenuated IGF-I and birth weight, as cord IGF-I was positively associated with birth weight, and LBW infants from PMM displayed the most severe reduction in IGF-I levels. These observations are consistent with other studies, in which cord blood IGF-I concentration at term is positively correlated with fetal growth [34-36], and is diminished in human FGR [9, 11, 20, 37]. The mechanisms by which fetal IGF-I is decreased in human studies remain unclear (although it may be inversely related to cortisol levels [37]), and in animal models restriction of maternal nutrition [38], placental blood flow [39] and oxygen [40] all decrease fetal IGF-I concentrations.

IGF-II is more abundant in fetal circulation than IGF-I [34], but the relationship between cord IGF-II levels and birth weight is inconsistent [20, 34-36, 41, 42]; cord IGF-II may be correlated with ponderal index and placental weight [36], but neither measure was available for the present cohorts. Our data are consistent with published studies in associating placental disease with decreased IGF-I, rather than IGF-II [20, 35, 36].

Elevated levels of cord IGFBP-1 that we observed in placental malaria may act to diminish the bioavailability of IGF-I. Similar IGFBP-1 profiles have been observed in cord blood of fetuses experiencing maternal stress [43]. We found no evidence of a relationship between IGFBP-1 concentration and birth weight, and it may exert its influence instead through its effect on IGF-I availability in babies whose IGF-I levels

are already compromised by placental malaria. The mechanisms causing elevated concentrations of IGFBP-1 in growth restricted human infants are not currently known.

Placental malaria did not affect total IGFBP-3 concentrations; indeed IGFBP-1 concentrations are more sensitive to hypoxia [40] and endocrine [43] regulation than IGFBP-3. The decreased ratio of IGF-I:IGFBP-3 we report in placental malaria is a surrogate measure of free IGF-I [21] and supports the notion that free, as well as total, IGF-I at delivery is reduced in infants born to mothers with placental malaria, and to a greater extent, in placental malaria with monocyte infiltrate.

By mRNA analysis of placental tissue, we did not find any significant changes in IGF-related gene transcription. In contrast, significantly increased placental IGF-I and/or IGF-II mRNA levels have been reported in several but not all human studies of FGR [44-48]. Some of these studies have examined placental mRNA transcript levels in smaller sized studies than ours [45, 47], suggesting that our study size had adequate power to detect any changes present. The observation that placental transcripts for components of the local IGF system remained unchanged with malaria infection and associated inflammation implies that autocrine effects of IGFs are perhaps not the locus for modulation of fetal growth in malaria affected pregnancies. Alternatively, closer examination of IGF levels in the syncytiotrophoblast layer by laser capture micro-dissection might reveal differences that are not apparent in biopsied placental tissue [30].

A potential limitation to this study is that the detection method used for IGF-I and IGF-II measured total (bound and unbound fractions) rather than bioactive (free) ligand, which constitutes approximately 1% of total IGF [20]. However, concentrations of total IGF correlate with free IGF [20, 34] and frequently associate with clinical indices of fetal growth [20, 35, 36, 45, 49]. As a proxy measure of free IGF-I we determined the ratio of IGF-I to IGFBP-3 in cord samples [21]. A further consideration is that phosphorylation state of IGFBP-1 during gestation affects the ligand binding activity [11] however, measurement of phospho-variants is both time and cost intensive and would have limited the study size. Instead, we quantified total IGFBP-1 by ELISA, which does not discriminate between specific phospho-isoforms but has been validated in other studies [20, 36, 49]. To minimize possible proteolytic degradation of IGFBP-3, which may over estimate IGFBP-3 levels (DSL product insert), we restricted samples to only one cycle of freeze/thawing, Future studies that employ more sophisticated IGFBP-1 phospho-isoforms detection, and that take into account free and total fractions of IGF-I and IGF-II, may reveal additional functional subtleties.

Current theories on the pathogenesis of LBW associated with placental malaria-induced inflammation centre on the conflicting immune environment between resolution of infection and the requirement for continued fetal growth. The release of pro-inflammatory cytokines [5, 31, 32] and monocyte infiltrates in the placental intervillous spaces [4] are associated with LBW, but the physiological impact of the inflammatory environment on placental function and fetal growth has not been studied extensively. To date, disturbances in the IGF system relating to fetal growth have been described in preeclampsia, asthma, nutritional deprivation, maternal stress,

and in diabetic pregnancies [44, 49]. Our study adds malaria to this list, and indicates that changes in this axis in a resource-poor setting, where mothers may suffer from acute or chronic undernutrition, are consistent with those described in resource-rich settings. Interventions that specifically target growth restriction through IGF therapy have been shown to prevent FGR in sheep [50], but have yet to be applied to human disease. Whether perturbations of the IGF system in humans are an underlying cause or response to growth restriction remains to be fully elucidated.

This study provides strong evidence of concordant disturbances in the maternal and fetal compartments of the IGF axis with placental malaria and associated inflammation, which have the potential to compromise nutrient partitioning to the fetus, and may play a central role in the development of LBW due to fetal growth restriction in malaria infected pregnancies.

Acknowledgements

The authors wish to thank the mothers and their families for participation in this study and Sr Valsi and the staff of Alexishafen Health Centre for their enthusiastic cooperation.

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Tables

Table 1. Univariate analysis of maternal characteristics according to placental malaria histopathology.

Malaria histopathology	Uninfected (n=34)	PM (n=31)	PMM (n=27)	ANOVA (P value)
Enrolment				
Maternal age (yrs)	24.7±5.2	24.7±4.2	21.9±4.0*	.03
Maternal weight (kg)	55.0±5.2	55.0±7.1	55.6±5.7	.9
Gravidity	2.3±1.4	2.4±1.3	1.6±1.0**	.02
Delivery				
Gestational age (wks)	38.4±2.3	37.6±2.5	36.8±2.6*	.05
Maternal Hb (g/dL)	9.2±2.3	9.5±1.7	9.1±1.8	.6

Data are mean ± SD. Women with placental malaria infection (PM, n=31) and monocyte infiltrate (PMM, n=27) were on average younger, primigravidae and delivered younger neonates than uninfected women (n=34). (*) and (**) indicate $P \leq .05$ and $P \leq .01$ respectively for PMM vs. uninfected controls (t-test).

Table 2. Multivariate analysis of the impact of placental malaria with and without monocyte infiltrate on birth weight.

Birth weight	PM ±M (n)	Average difference in birth weight compared to uninfected β (g)	95% CI	P value
Unadjusted	- (31)	-100	-285 to 156	NS
	+ (27)	-370**	-565 to -99	0.01
Adjusted for gestation	- (31)	5	-170 to 179	NS
	+ (27)	-200*	-394 to -6	0.04
Primigravidae adjusted for gestation, maternal age	- (9)	-13	-275 to 249	NS
	+ (19)	-291*	-532 to -51	0.02
Multigravidae adjusted for gestation, maternal age	- (22)	1	-240 to 242	NS
	+ (8)	45	-301 to 392	NS

Change in mean birth weight **(**β) in neonates delivered to women with placental malaria (PM) without monocytes (-) or with monocyte infiltrate (+) (PMM) compared to uninfected controls. On average both PM, and to a greater extent PMM, decreased birth weight compared to women without infection. Adjusting for the effect of gestational age on birth weight, this decrease remained significant in PMM group only. Stratifying for gravidity, and adjusting for maternal and gestational age, a significant reduction in birth weight was observed in primigravid women only. (*) $P \leq .05$, (**) $P \leq .01$ PMM vs. uninfected controls (t-test). NS non-significant, $P > .05$.

Table 3. IGF ligand and receptor mRNA levels in placental biopsies.

	Uninfected	PM	PMM	Kruskall Wallis (KW) P value
IGF-I	0.43 (0.32, 0.65)	0.42 (0.26, 0.63)	0.36 (0.29, 0.50)	KW =1.5 P = .5
IGF-II	0.85 (0.41, 1.87)	1.23 (0.45, 2.14)	1.40 (0.53, 3.0)	KW =1.6 P = .4
IGF1R	1.30 (1.01, 1.71)	1.44 (1.12, 2.00)	1.32 (1.17, 2.05)	KW= 0.7 P = .7
IGF2R	0.61 (0.47, 0.80)	0.57 (0.42, 0.80)	0.70 (0.58, 1.00)	KW=3.6, P= .2

Data are expressed as median and (IQR) transcript levels in uninfected (n=31), placental malaria (PM, n=31) and placental malaria with monocytes (PMM, n=26). In each placental sample, transcript levels for target and housekeeper genes were normalized to a reference sample of pooled cDNA (ΔCt=control Ct-sample Ct), where Ct is the cycle threshold, defined as the number of cycles required for a fluorescent signal from the PCR product to reach detection above background levels. The ratio (R) of target gene mRNA levels between samples were determined by the equation R = [E(target)^ΔCT]/[E(house keeper)^ΔCT], correcting for any potential differences in PCR efficiency (E) between primer pairs.

Supplementary information

Table 1A. Primer sequences used in real time qPCR

Gene symbol	Gene name	Accession number	Forward (F) and Reverse (R) sequence	Cross intron-exon boundary	Amplification efficiency	Reference origin
IGF-I	Insulin-like-growth-factor-1	NM_000618	(F) TGCCCAGCGCCACAC (R) TCCTACATCCTGTAGTTCTTGTTTCCT	Yes	1.96	V. Murphy Thesis
IGF-II	Insulin-like-growth-factor-2	NM_000612	(F) CCCCTCCGACCGTGC (R) TCATATTGGAAGAACTTGCCCA	Yes	1.96	Primer Express
IGF1R	Insulin-like-growth-factor-receptor-1	NM_000875	(F) TCGTGGGAGGGTTGGTGAT (R) CCAGCCTGCTGTTATTTCTCTTTC	Yes	2.10	Hu et al 2008
IGF2R	Insulin-like-growth-factor-receptor-2	NM_000876	(F) ATCCAACTTTCCTCCATCACAAG (R) TGCTGATCGTTGGGCTTCA	No	2.10	Hu et al 2008
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_003406	(F) ACTTTTGGTACATTGTGGCTTCAA (R) CCGCCAGGACAAACCAGTAT	No	1.96	Vandesompele, Genome Biology 2002

Primer sequences are in the (5'-3') direction. Amplification efficiency (E) was calculated using 7 point serial dilution of pooled cDNA where (E) = 10^(-1/slope of standard curve).

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Figure
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Figures

FIGURE 1

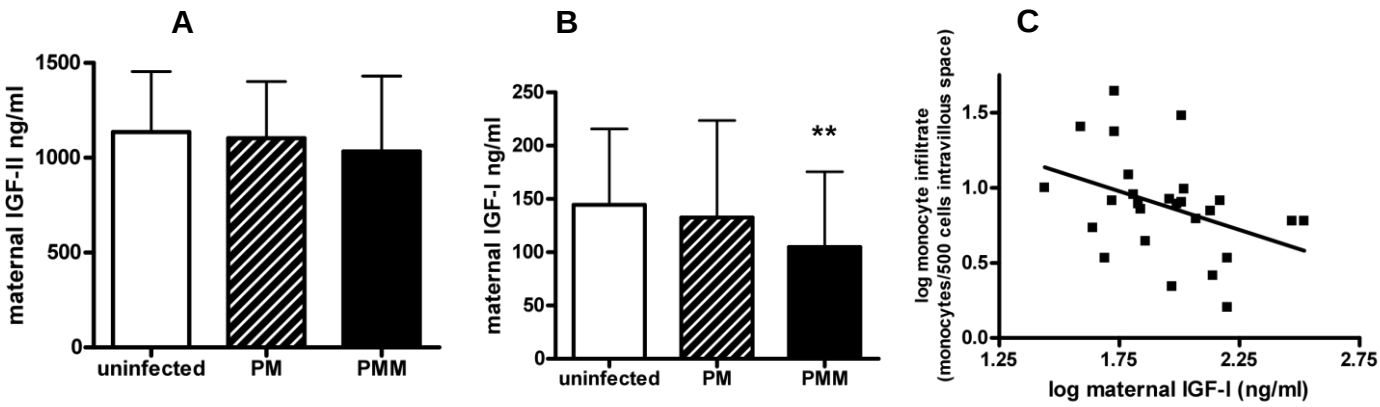

Figure 1. Maternal IGF concentrations in women with and without placental malaria. Maternal IGF-I and IGF-II (mean + SD) were measured in plasma of uninfected women (n=34), and those with placental malaria (PM; n=31) and placental malaria with monocyte infiltrate (PMM; n=27) (***A***) Maternal IGF-II concentration did not differ between groups (ANOVA P = .5). (***B***) Maternal IGF-I concentration differed significantly between groups (ANOVA P = .03), with levels in PMM significantly less than uninfected control (** P = .007 vs. uninfected, t-test). (***C***) Maternal IGF-I concentration from mothers with PMM (n=27) demonstrate a negative correlation with the degree of monocyte infiltration measured by placental histology (r =-0.4, P = .05).

FIGURE 2

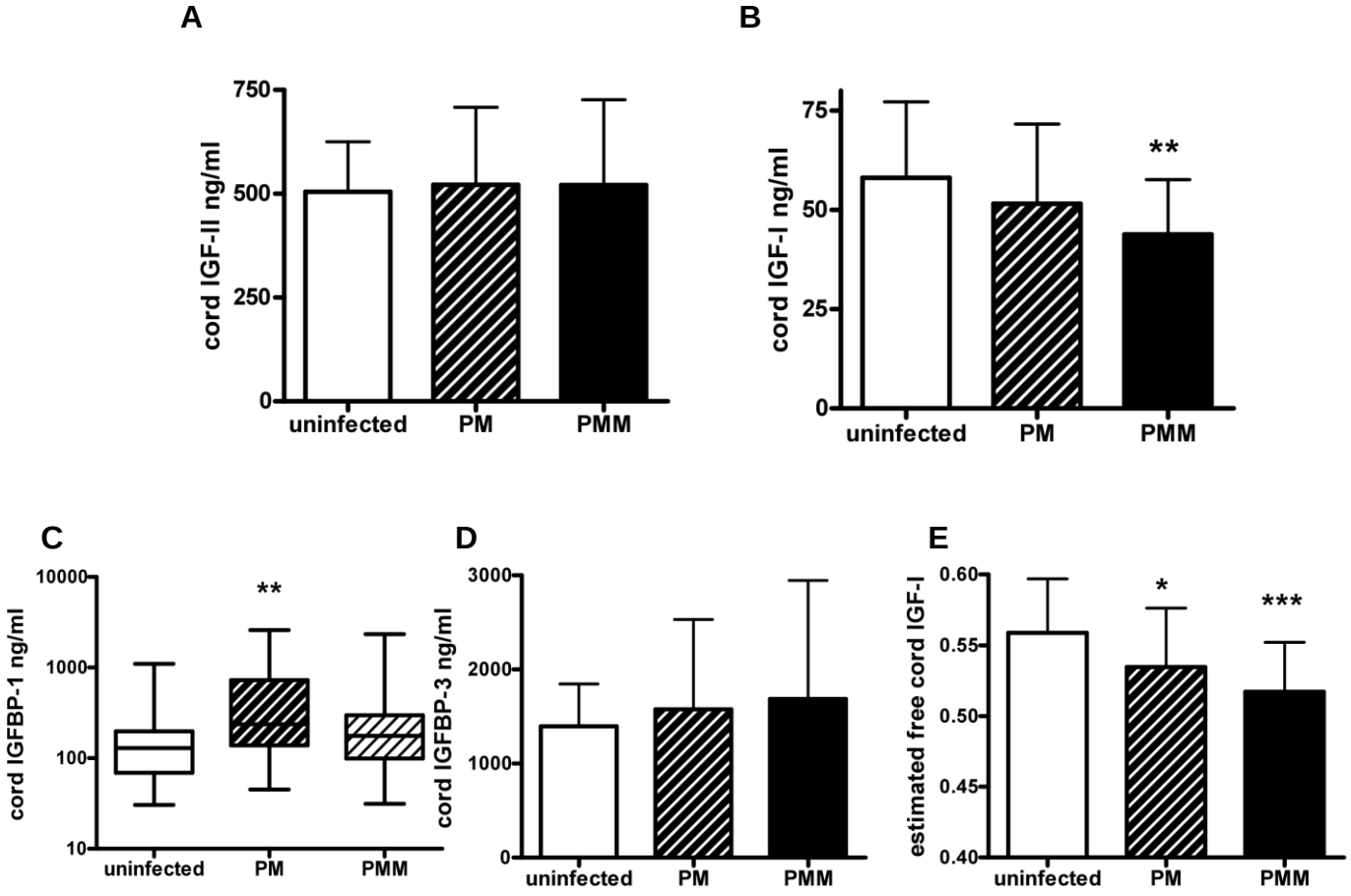

Figure 2. Fetal IGF and binding protein levels. Concentrations of IGF-I, IGF-II, IGFBP-1 and IGFBP-3 were measured in cord plasma in uninfected (n=31) placental malaria (PM, n=30 for IGFBP-1, and n=31 for other analytes) and placental malaria with monocyte infiltrate (PMM, n=26) *(A)* IGF-II (mean+ SD) did not differ significantly (ANOVA P = .9) whereas (*B*) cord IGF-I concentrations were significantly different between groups (ANOVA P = .007) ** P = .002 PMM vs. uninfected control (t-test) *(C)* IGFBP-1 median + IQR concentrations. IGFBP-1 concentrations were normalized by log transformation. Log IGFBP-1 values differed significantly between groups (ANOVA P = .005), **P = 0.006 PM vs. uninfected (t-test on log values), and PMM vs. uninfected P = .08 (t-test on log values) *(D)* IGFBP-3 (mean +SD) was not statistically different between groups (ANOVA P = .9) *(E)*

Estimated free IGF-I in cord blood (log ratio of IGF-I to IGFBP-3) differed significantly between groups (ANOVA P = .004) * P ≤ .02 PM and *** P < .0001 PMM vs. uninfected control respectively (t-test).

FIGURE 3

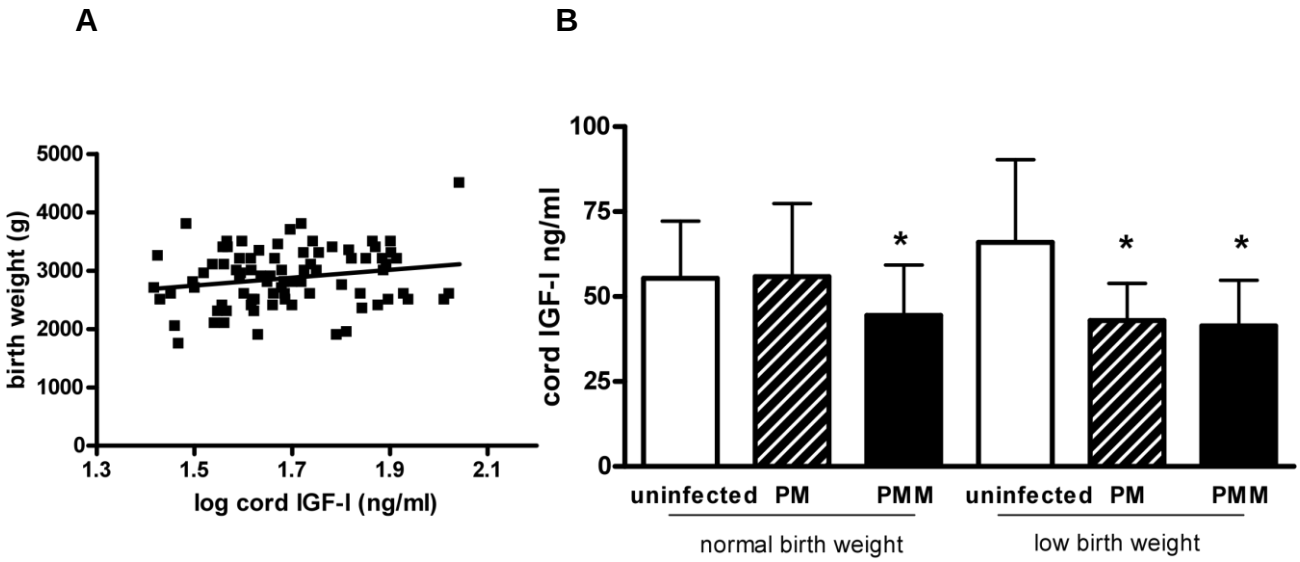

Figure 3. The relationship between fetal IGF-I and birth weight. ***(A)*** Among all deliveries in the groups (n=88), cord IGF-I and birth weight were positively correlated (r = .2, P = 0.06), ***(B)*** IGF-I concentration (mean+SD) differed significantly (ANOVA F(5, 77)=2.9, P = .02) in cord blood from uninfected, placental malaria (PM) and placental malaria with monocyte infiltrate (PMM) pregnancies according to birth weight category (normal birth weight NBW ≥ 2.5kg, and low birth weight LBW <2.5kg). In NBW: *P = .02 PMM vs. uninfected (t-test). In LBW: *P = .02 PM and *P = .04 PMM vs. uninfected control respectively (t-test).

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Umbers, AJ; Boeuf, P; Clapham, C; Stanisic, DI; Baiwog, F; Mueller, I; Siba, P; King, CL; Beeson, JG; Glazier, J; Rogerson, SJ

Title:
Placental Malaria-Associated Inflammation Disturbs the Insulin-like Growth Factor Axis of Fetal Growth Regulation

Date:
2011-02-15

Citation:
Umbers, A. J., Boeuf, P., Clapham, C., Stanisic, D. I., Baiwog, F., Mueller, I., Siba, P., King, C. L., Beeson, J. G., Glazier, J. & Rogerson, S. J. (2011). Placental Malaria-Associated Inflammation Disturbs the Insulin-like Growth Factor Axis of Fetal Growth Regulation. JOURNAL OF INFECTIOUS DISEASES, 203 (4), pp.561-569. https://doi.org/10.1093/infdis/jiq080.

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