International Journal for Parasitology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

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High numbers of circulating pigmented polymorphonuclear neutrophils as a prognostic marker for decreased birth weight during malaria in pregnancy

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ARTICLE INFO

Article history:
Received 3 September 2014
Received in revised form 1 December 2014
Accepted 2 December 2014
Available online xxxx

Keywords:
Malaria
Pregnancy
Haemozoin
Pigmented leukocytes
Neutrophil
Birth weight

ABSTRACT

During gestational malaria, *Plasmodium falciparum*-infected erythrocytes can sequester within the placenta, contributing to poor pregnancy outcomes, especially low birth weight. In children and non-pregnant adults, pigmented leukocytes may serve as markers of sequestered parasite burden and predict clinical outcomes. Here, we investigated circulating pigmented leukocyte numbers as predictors of clinical outcomes in pregnant women presenting with malaria at enrolment. The number of circulating pigmented neutrophils at enrolment negatively correlated with birth weight (Rho = −25, $P = .04$), suggesting these cells may have a pathogenic role in, and could serve as prognostic markers for, malaria-associated low birth weight.

During its erythrocytic life stages, *Plasmodium falciparum* degrades haemoglobin and converts the resulting free haeme into haemozoin, an insoluble crystal known as malaria pigment. Host phagocytes such as neutrophils and monocytes can acquire these pigments via phagocytosis of free haemozoin or of erythrocytes infected with late stage parasites. The ingested pigments are visible under the light microscope inside phagocytes, termed "pigmented leukocytes" (Supplementary Fig. S1).

Plasmodium falciparum-infected erythrocytes can adhere to host cells and sequester in deep cerebral vascular beds or in placental intervillous spaces, a process implicated in organ-specific manifestations of disease such as cerebral malaria and placental malaria, respectively. In these cases, the peripheral parasitaemia may not mirror total parasite biomass, which may complicate both the diagnosis and prognosis of malaria infection. Pigmented leukocytes circulate in the peripheral blood and may reflect sequestered parasite biomass (Nguyen et al., 1995). A previous study has detected several cases of pigmented monocytes in aparasitaemic pregnant women, suggesting that it could be used as an adjunct tool for malaria diagnosis (Hänscheid et al., 2009).In addition, haemozoin has been shown to activate pro-inflammatory signalling pathways in phagocytes (Dostert et al., 2009), thus increased levels of these pigmented cells may be a marker of inflammation in malaria, which in turn can be associated with the development of severe malaria (Schofield, 2007).

The number of pigmented leukocytes has been used to predict disease progression and clinical outcomes in malaria-infected patients. High numbers of pigmented leukocytes were associated with severe malaria in children, including cases of cerebral malaria and severe anaemia (Amodu et al., 1998; Lyke et al., 2003). In addition, among children with severe malaria, those who eventually succumbed to the disease were shown to have higher numbers of pigmented neutrophils compared with survivors (Lyke et al., 2003). Similarly, adults who later died of severe malaria had higher numbers of pigmented leukocytes at the time of admission

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http://dx.doi.org/10.1016/j.ijpara.2014.12.002
0020-7519/

Please cite this article in press as: Chua, C.L.L., et al. High numbers of circulating pigmented polymorphonuclear neutrophils as a prognostic marker for decreased birth weight during malaria in pregnancy. Int. J. Parasitol. (2015), http://dx.doi.org/10.1016/j.ijpara.2014.12.002

(Nguyen et al., 1995). This may be due to their inability to clear malaria infection, as an inverse relationship between the numbers of pigmented neutrophils and IL-12, a cytokine important in mediating protection against malaria, has been reported (Luty et al., 2000). In addition, the number of peripheral pigmented neutrophils was a better predictor of fatality compared with peripheral parasitaemia (Lyke et al., 2003). These studies suggest that the number of circulating pigmented leukocytes may serve as a simple, rapid and informative tool for better management of malaria infection.

The sequestration of *P. falciparum*-infected erythrocytes in maternal placental blood can trigger intervillositis, a local innate inflammatory response. When complicated by intervillositis, placental malaria is associated with an increased risk of maternal anaemia and intrauterine growth restriction, contributing to low birth weight (Rogerson et al., 2003b). Infants born with low birth weight suffer from an increased risk of mortality and morbidity. Thus, early identification of women who are at risk of poor clinical outcomes would allow them to be monitored and treated accordingly to reduce the prevalence of low birth weight. Because intrauterine growth restriction is most common and severe in the presence of intervillositis, circulating markers of intervillositis could serve as prognostic markers for poor birth outcomes.

Based on the positive association between high pigmented leukocyte numbers and malaria severity in non-pregnant individuals, we hypothesised that increased numbers of circulating pigmented leukocytes detected during gestation are associated with, and can predict, poor pregnancy outcomes, especially decreased infant birth weight.

The study was approved by the Papua New Guinea (PNG) Medical Research Advisory Council. Following written informed consent, pregnant women in their second or third trimester of pregnancy were enrolled from the Alexishafen Health Centre in Madang, PNG, between 2005 and 2007 and followed through to delivery (Supplementary Table S1). Maternal haemoglobin levels were recorded at enrolment and at delivery. Using peripheral blood smears collected at enrolment from 64 participants with peripheral blood parasitaemia, the numbers of pigmented neutrophils and pigmented monocytes were assessed with the aid of polarised light, as previously described (Lyke et al., 2003; Lell et al., 2005). Briefly, the numbers of pigmented neutrophils per 100 neutrophils in thin blood smears were counted and normalised to the percentage of neutrophils obtained from a differential leukocyte count. This was multiplied by an estimated leukocyte number of 8,000 cells/μL and expressed as the number of pigmented neutrophils/μL. To determine the number of pigmented monocytes, 200 mononuclear cells were counted in thick blood films. This was converted to the number of monocytes counted based on the proportions of monocytes and lymphocytes in differential leukocyte counts. The number of pigmented monocytes/μL was then calculated in a similar way to that for pigmented neutrophils.

The correlation between the numbers of pigmented neutrophils or monocytes and clinical parameters was determined using Spearman's rank correlation test. Comparisons between two groups were made using Mann–Whitney's test and between three groups using Kruskal–Wallis' test. All statistical analyses were performed using Graph Pad Prism (Version 5). $P < 0.05$ was considered statistically significant.

We first assessed whether the numbers of pigmented neutrophils and pigmented monocytes in maternal peripheral blood were useful markers of parasite density, birth weight and of maternal anaemia (Table 1). The numbers of pigmented neutrophils and monocytes were not associated with parasitaemia and maternal haemoglobin levels at enrolment ($P \geqslant 0.18$).Pregnant women were further grouped into those who were non-anaemic (>11 g/dL), mildly anaemic (9–11 g/dL) or moderately/severely anaemic (<9 g/dL). Similar numbers of pigmented leukocytes were found across women with different severities of anaemia at enrolment ($P = 0.9$ for both pigmented neutrophils and monocytes; Fig. 1A, B).

Next, we examined whether the numbers of pigmented neutrophils and pigmented monocytes in maternal peripheral blood at enrolment predicted maternal haemoglobin at delivery and infant birth weight. The numbers of pigmented neutrophils and monocytes at enrolment were independent of maternal haemoglobin levels at delivery ($P \geqslant 0.6$) and women classified into groups with different severities of anaemia at delivery had similar numbers of pigmented leukocytes measured at enrolment ($P \geqslant 0.1$; Fig. 1C, D).

The numbers of pigmented monocytes at enrolment did not correlate with infant birth weight ($P = 0.58$). We further categorised women into two groups: those with pigmented monocyte numbers higher or lower than the median number of pigmented monocytes in the cohort. Birth weights were similar between women with high or low numbers of pigmented monocytes ($P = 0.5$). In contrast, the numbers of pigmented neutrophils at enrolment were negatively correlated with birth weight (Rho = −0.25, $P = 0.04$; Fig. 2A). We further categorised women into two groups: those with pigmented neutrophil numbers higher or lower than the median number of pigmented neutrophils in the cohort. We found that women with higher numbers of pigmented neutrophils gave birth to babies with lower birth weights (median birth weight difference = 440 g; $P = 0.004$; Fig. 2B). The prevalence of low birthweight amongst women with high numbers of pigmented neutrophils (28.1%) was also higher ($P = 0.1$) than that in women with low numbers of pigmented neutrophils (12.5%).When women were grouped into primigravidae, secundigravidae and multigravidae (three or more pregnancies), we found no significant difference in birth weight or in pigmented neutrophil numbers across groups (Kruskal–Wallis test, $P \geqslant 0.1$).

In a previous study, the numbers of pigmented neutrophils and parasitaemias were both inversely correlated with levels of IL-12 (Luty et al., 2000),which can be secreted by neutrophils, suggesting that haemozoin phagocytosis might impair effective parasite clear-

Table 1
Correlations between the numbers of pigmented leukocytes at enrolment and clinical parameters at enrolment or at delivery in malaria-infected pregnant women.

	n	Spearman's rank correlation coefficient	95% confidence interval	*P* value
Pigmented neutrophils				
Parasitaemia at enrolment	55	−0.07	−0.33 to 0.2	0.59
Maternal haemoglobin level at enrolment	64	−0.06	−0.31 to 0.19	0.62
Maternal haemoglobin level at delivery	60	0.03	−0.22 to 0.28	0.80
Infant birth weight	64	−0.25	−0.47 to −0.004	0.04
Pigmented monocytes				
Parasitaemia at enrolment	55	0.18	−0.09 to 0.43	0.18
Maternal haemoglobin level at enrolment	64	−0.02	−0.27 to 0.23	0.85
Maternal haemoglobin level at delivery	60	0.06	−0.18 to 0.31	0.60
Infant birth weight	64	−0.06	−0.31 to 0.18	0.58

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