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PERIPHERAL PARASITAEMIA AND ITS ASSOCIATION WITH PLASMA CYTOKINES LEVELS IN MALARIA-INFECTED PREGNANT WOMEN IN ABA, ABIA STATE, NIGERIA

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Abstract

Background: Cytokines in pregnant female may not be a normal phenomenon as malarial infection is often associated with strong CD4+ cell activation and up-regulation of pro-inflammatory cytokines. We investigated the relationship between peripheral parasitaemia and plasma levels of cytokines among malaria infected pregnant women in Aba, Abia State, Nigeria.
Materials and Methods: A total of 206 non-HIV positive asymptomatic malaria parasitaemic (n=144) and non-parasitaemic (n=62) pregnant women were recruited for this study alongside 80 non-pregnant women who served as positive (n=40) and negative (n=40) controls. Blood samples were aseptically collected from each subject and tested for HIV and malaria parasites using standard methods. Also, plasma levels of cytokines were measured using Th1/Th2 human cytokine ELISA kits (Abcam, UK). Analysis of Variance and Student's t-test were used for Comparison of groups while Pearson's Correlation Coefficient was used for tests of association.
Results: The results revealed a mean parasite density of 685.56±484.55 parasites/µl of blood. Malaria infected pregnant subjects showed significantly higher levels of IFN-γ, TNF-α, IL-4, IL-6 and IL-10 when compared with their non-infected counterparts ($P< 0.05$). The cytokines evaluated were higher in moderate parasitaemia than mild parasitaemia. Positive correlation existed between peripheral parasite density (PPD) and IL-4 ($r= 0.24$, $P=0.004$), PPD and IL-6 ($r = 0.35$, $P = 0.001$) as well as PPD and IL-10 ($r = 0.29$, $P = 0.001$).
Conclusion: This study showed that increase in peripheral parasitaemia increased levels of some plasma cytokines (IL-4, IL-6 and IL-10) but not IFN-γ and TNF-α in the malaria infected pregnant women studied.

Keywords: Malaria, pregnancy, cytokines, woman, association

Introduction

Malaria, a mosquito-borne parasitic infection caused by species of *Plasmodium*, is the leading cause of pregnancy-associated death in sub-Saharan Africa (Sanyaolu *et al.*, 2013; Sonny-Johnbull *et al.*, 2014; Gething *et al.*, 2016; Maitland, 2016; Sued *et al.*, 2016). In sub-Saharan Africa, 25 million pregnant women are currently at risk for malaria, accounting for 10,000 maternal and 200,000 neonatal deaths per year (Matangila *et al.*, 2014; Mbah *et al.*, 2015). In such regions of high transmission and endemicity, sub-clinical malarial infection is common (Takem and D'Alessandro, 2013) and individuals living in such regions usually develop protective immunity to malaria by adolescence; however, this protection is partially abrogated in women during pregnancy, resulting in pregnancy-associated malaria (PAM) (Jaworowski *et al*., 2009; Takem and D'Alessandro, 2013). Pregnancy- associated malaria results in tremendous obstetrical and paediatric morbidity, including maternal anaemia, intra-uterine growth retardation, low birth weight, prematurity, miscarriage, and stillbirth (Tonga *et al.*, 2013; Mbah *et al.*, 2015; White, 2015; Chedraui, Daily and Wylie, 2016; Prahl *et al.*, 2016).

In maternal blood, malaria parasites induce immune response, which results in the secretion of cytokines (Prema *et al.*, 1982). Similarly, semi-allogenic foetal tissue is directly exposed to the maternal blood which incidentally invades the maternal decidua and results in the activation and alteration of immune system of the mother with the secretion of cytokines (Lashley *et al.*, 2011). Cytokines induce various transcription factors which in turn determine the fate of cells either for proliferation, differentiation and maturation or death (Quesenberry, 1995).

Under normal conditions, change in the cytokine profile occurs in the pregnant women and in the transformation that takes place at the materno-foetal interface to ensure successful delivery of healthy infants (Sacks *et al.*, 1998). As pregnancy progresses, there is the systematic transition from type1 to type 2 cytokine dominance because over-expression of type-1 may compromise the viability of the foetus (Wegmann *et al.*, 1993). In response to invading pathogens, the cytokine profile is truncated and reversed to Th1 cytokine bias. One of such condition that induces and reverses the profile to Th1 dominance is maternal malaria (Clark *et al.*, 2006). The concentration of cytokines in some pathological conditions may directly or indirectly correlate with the severity of diseases and could serve as prognostic marker (Santos-Rosa and Mota-Pinto, 2006). This study evaluated the level of parasitaemia and its association with cytokines in malaria infected pregnant women.

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Materials and Methods

This cross-sectional study was carried out among pregnant women in Aba, a cosmopolitan town in Abia State and second largest commercial city of South Eastern Nigeria. Aba town has dirty environs characterized by heaps of refuse dumps and stagnant water all over the city. This condition is ideal for the breeding of mosquitoes and largely account for high incidence of malaria cases in the area. Prior to the study, ethical approval for the use of human subjects was sought and received from the Research and Ethics Committee of the Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria. Also, informed consent was sought from each participant and those whose consents were received and who met the inclusion criteria were recruited into the study. A total of 286 individuals shared into four groups containing non HIV-positive asymptomatic malaria parasitaemic (n=144), non-parasitaemic (n=62) pregnant women, 40 non-pregnant malaria-positive women and 40 non-pregnant malaria-negative women. Pre-tested structured questionnaires were used to obtain basic socio-demographic information from each study participant. Eight millilitres (8 ml) of venous blood samples were aseptically collected from each participant, properly labelled in coded numbers and transported to the laboratory for immediate analysis.

Determine® HIV-1/2 (Inverness Medical Japan) and Unigold® HIV-1/2 (Trinity biotech PLC, Ireland) were used to determine the HIV status according to the manufacturers' specifications.

Thick blood smear from each of the study participants was made on a clean grease-free glass slide and stained with 10% Giemsa to determine by microscopy, the species of malaria parasites and parasite density according to earlier published protocol (Cheesbrough, 2006). All stained slides were examined by microscopy using 100 power fields under oil immersion. Also, Rapid Diagnostic Tests were performed on positive slides using Insta Test ™ Malaria Pf/Pv kits according to the manufacturers' instructions. Peripheral parasite density was calculated by using an assumed WBCs count of 8000/µL of blood (WHO, 2010). In the sub-sample of both pregnant women, commercially available Human Th1/Th2 Cytokine ELISA Kits (Abcam, UK) were used to measure plasma levels of IL-4, IL-6, IL-10, tissue necrosis factor- α (TNF-α) and interferon- gamma (IFN- γ) according to the manufacturer's instructions.

All statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 21. The results were expressed as mean and standard deviation. Analysis of Variance (ANOVA) and student's t-test were used for comparison of differences in various groups. Level of significance was set at $p<0.05$. The tests of association were performed using Pearson's correlation coefficient.

Results

The results showed that both pro-inflammatory and anti-inflammatory cytokines (IFNγ, TNFα, IL-4, IL-6 and IL-10) varied among the different groups studied with respect to malaria infection and pregnancy. Among the malaria-infected pregnant women, the mean levels of IFN-γ was significantly higher ($p<0.05$) than observed among the uninfected pregnant and uninfected non-pregnant groups. However, the malaria-infected non-pregnant group recorded significantly ($P<0.05$) higher mean levels of IFN-γ than the infected and uninfected pregnant groups. There was no significant difference ($P>0.05$) in the mean level of IFNγ between uninfected pregnant women and uninfected non-pregnant women, while IFNγ levels in the infected non-pregnant subjects was significantly higher ($P<0.05$) than observed in the uninfected non-pregnant subjects (Table 1).

Also, the malaria-infected pregnant subjects had significantly higher ($P<0.05$) levels of TNF-α when compared with the uninfected pregnant women as well as infected and uninfected non-pregnant groups. The mean level of TNFα in uninfected pregnant group was significantly lower ($P<0.05$) than the infected non-pregnant counterpart, but significantly higher ($p<0.05$) than observed in the uninfected non-pregnant group. Malaria infected pregnant women had significantly higher ($P<0.05$) levels of IL-4 than the uninfected pregnant women as well as the infected and uninfected non-pregnant women respectively. The mean level of IL-4 in uninfected pregnant women was significantly high ($P<0.05$) when compared with the infected and uninfected non-pregnant women. Infected non-pregnant women had significantly higher ($P<0.05$) mean level of IL-4 compared to their uninfected non-pregnant counterpart (Table 1).

With respect to the level of IL-6, the infected pregnant women had higher and more significant ($P<0.05$) levels compared with 8.68±8.41, 23.42±0.45 pg/ml and 2.33±0.58 pg/ml observed in the uninfected pregnant women, infected and uninfected non-pregnant subjects respectively. The mean level (35.19±28.82 pg/ml) of IL-10 in the infected pregnant women was significantly higher ($P<0.05$) compared with 14.76±6.17 and 6.45±4.15 pg/ml observed in the uninfected pregnant and uninfected non pregnant groups, but significantly lower in infected non-pregnant group. The mean level (14.76±6.17 pg/ml) of IL-10 in uninfected pregnant group was lower than 43.42±0.45 in the infected non-pregnant women but higher ($P<0.05$) when compared with 6.45±4.15 in the uninfected non-pregnant group (Table 1)

The results on parasitaemia, classified as mild (<1000 parasite/µl) and moderate (1000 - 10,000 parasite/µl) parasitaemia revealed that the mean level of IFNγ was significantly ($P<0.05$) lower (21.31±11.74 pg/ml) in mild parasitaemia than in moderate parasitaemia (29.14±14.48 pg/ml), while level of TNFα, IL-4 and IL-10 showed no significant ($p>0.05$) difference between mild and moderate parasitaemia. However, levels of IL-6 in mild parasitaemia was significantly ($P<0.05$) lower than observed in moderate parasitaemia (Table 2). A very strong positive correlation was observed between IL – 4 and parasite density ($r = 0.24$, $p = 0.004$), between IL-6 and malaria parasite density ($r = 0.06$, $p = 0.001$) and between IL – 10 and malaria parasite density ($r = 0.71$, $p = 0.001$) (Figures 1-3).

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Table 1: Mean Cytokines levels with regards to malaria infection and pregnancy among women in Aba, Abia State, Nigeria.

GROUP	Cytokines (pg/ml)				
	IFN-γ	**TNF-α**	**IL-4**	**IL-6**	**IL-10**
G1 (n = 144)	22.94 ± 12.71	21.12 ± 12.57	9.66 ± 7.05	32.11 ± 27.92	35.19 ± 28.82
G2 (n = 62)	5.98 ± 3.11	10.03 ± 3.04	7.17 ± 3.91	8.68 ± 8.41	14.76 ± 6.17
G3 (n = 20)	30.07 ± 0.39	13.17 ± 0.33	3.60 ± 0.29	23.42 ± 0.45	43.42 ± 0.45
G4 (n = 20)	4.69 ± 2.64	4.66 ± 0.78	2.13 ± 0.36	2.33 ± 0.58	6.45 ± 4.15
F(p) Value	64.00 (0.00)	30.26 (0.00)	15.17 (0.00)	23.12 (0.00)	21.47 (0.00)
G1 vs G2	0.001*	0.001*	0.008*	0.001*	0.001*
G1 vs G3	0.001*	0.001*	0.001*	0.002*	0.004*
G1 vs G4	0.001*	0.001*	0.001*	0.001*	0.001*
G2 vs G3	0.001*	0.001*	0.001*	0.001*	0.001*
G2 vs G4	0.277	0.001*	0.001*	0.001*	0.001*
G3 vs G4	0.001*	0.001*	0.001*	0.001*	0.001*

Key
α-level set at 0.05, *(P< 0.05) = Significant, P> 0.05= Not Significant, G1 = Malaria Infected Pregnant Subjects, G2 = Malaria Uninfected Pregnant Subjects, G3 = Malaria Infected Non-Pregnant Subjects, G4 = Malaria Uninfected Non-Pregnant Subjects

Table 2: Relationship between Mean cytokine levels and parasitaemia among malaria-infected pregnant women in Aba, Abia State, Nigeria (using student's t-test)

PARAMETERS	MILD PARASITAEMIA (<1000parasite/ul) n = 114	MODERATE PARASITAEMIA (1000- 10000 parasite/ul) n = 30	P-Values
IFNγ (pg/ml)	21.31 ± 11.74[a]	29.14 ± 14.48[b]	0.044
TNFα (pg/ml)	20.20 ± 12.29[a]	24.62 ± 13.19[a]	0.359
IL-4 (pg/ml)	9.42 ± 7.08[a]	10.58 ± 6.99[a]	0.851
IL-6 (pg/ml)	28.00 ± 24.77[a]	47.72 ± 33.71[b]	0.024
IL-10 (pg/ml)	31.95 ± 26.27[a]	47.49 ± 34.77[a]	0.120

Key:
α-level set at 0.05
Values not sharing the same superscript means there is a significant difference
Values sharing the same superscript means there is no significant difference

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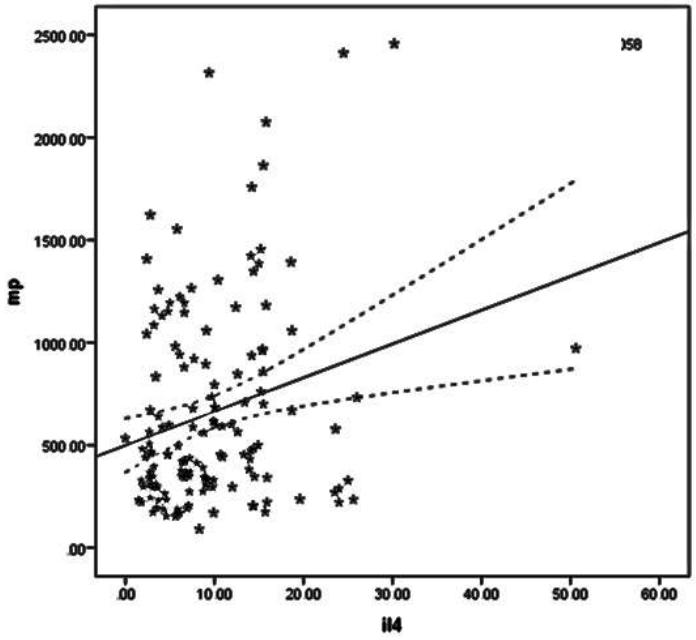

Figure 1: Positive Correlation between Parasite density and IL-4

(r= 0.24, p = 0.004)

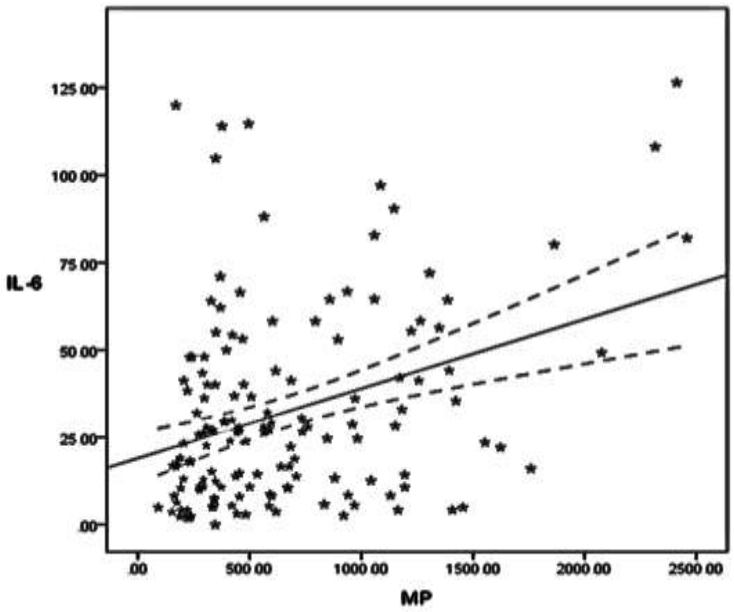

Figure 2: Positive correlation between parasite density and IL-6
(r = 0.35, p = 0.001)

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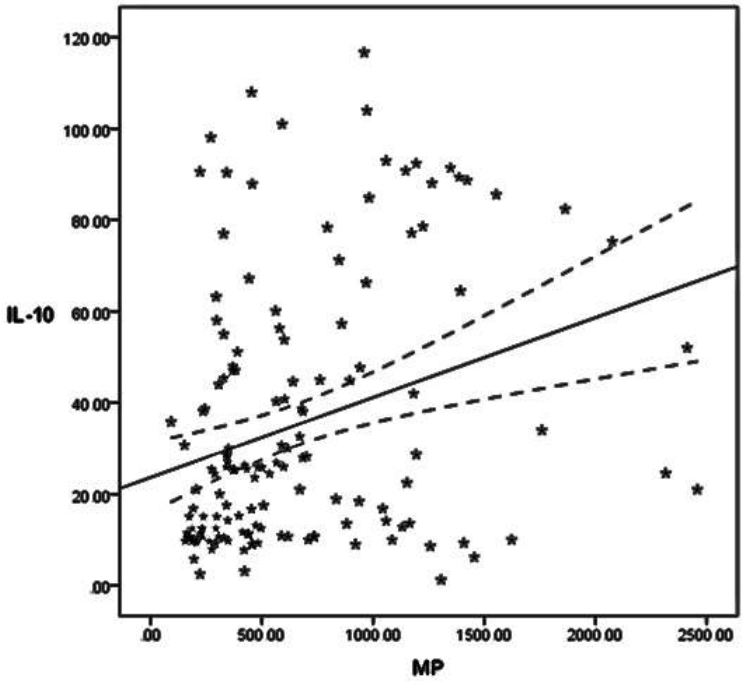

Figure 3: Positive correlation between parasite density and IL-10 (r = 0.29, p = 0.001)

Discussion

Malaria is an intractable disease and its clinical presentation and overall immune response may largely depend on the parasite density as well as the pregnancy status in women. The value of malaria parasite density in the peripheral blood recorded in this study was at variance with most values obtained in other studies: specifically, it was lower than what was obtained by Douamba *et al*. (2012) but was higher than that obtained by Onyenekwe *et al.* (2004), Achidi *et al.* (2007) and Akinboro *et al.* (2010) respectively. The differences in values recorded by different studies may suggest various levels of transmission in different geographical areas and the different methods of evaluation. Additional factor that may generally vary reports of parasite density is sequestration and adherence of the organism in the internal organs (Newbold *et al.*, 1999). Granted that this factor is species specific and most common with *P. falciparum* infection, it is well known that sequestration and adherence of malaria parasite to endothelial cells in tissues such as spleen, brain, kidney and placenta may reduce the parasite load in the peripheral circulation (Ockenhause *et al.*, 1992; Newbold *et al.*, 1999; Beeson *et al.*, 2000).

The finding presented herein agrees with earlier studies that malaria induces immune stimulation (Inigo and Manuel, 2002; Sacks *et al.*, 2003), resulting in increased secretion of cytokines. Phagocytosis of the parasite or the haemozoin, glycosilphosphatidylinositol (GPI) and the parasite toxin also causes immune stimulation evinced by up-regulation of cytokines (Venugopal, 2007; D'Ombrain *et al.*, 2008). Moreover, reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl (OH^-) and superoxide (O_2^-) generated during oxidative stress activates leucocytes with the release of more cytokines (Kumar *et al.*, 2010). Another factor that contributes in the secretion of cytokines in malaria could be the initiation of coagulation cascade. Coagulation factors are initiated in response to endothelial wall damage (Kawthalkar, 2008). The endothelial walls could be injured or damaged in the cause of sequestration and adherence of the parasite. It is pertinent to note that with damage of the endothelial walls, haemostatic activities are initiated for the repair of the injured vessels and preventing blood loss (Kawthalkar, 2008). Most plasma proteins especially the coagulation factors that are activated in the process also are inflammatory mediators and the release of an inflammatory mediator could lead to the activation, secretion and release of another (Kumar *et al.*, 2010). Such phenomenon may have resulted in the secretion of more cytokines recorded in the malaria-infected pregnant women.

The results observed in this study also agrees with the fact that pregnancy is considered to be a state of controlled maternal mild inflammation where levels of pro-inflammatory and regulatory cytokines are raised compared to non-pregnant states as suggested by (Szoba *et al.*, 2003). Data obtained in this study is similar to that obtained by Sacks *et al.* (2003), where the cytokine levels were higher in the pregnant compared to the non-pregnant subjects. The cytokines in the plasma or serum initially are predominantly type-1 because circulating monocytes are primed to produce Th1 cytokines crucial in immune-surveillance against pathogens (Szoba *et al.* 2003). However, as the pregnancy progresses, there is the systemic transition to type-2 cytokine because over-expression of type-1 could be harmful and may compromise foetal viability (Wegmann *et al.*, 1993).

Findings in this study of which IFN-γ, TNF-α, IL-6, IL-4 and IL-10 were significantly elevated in malaria infected pregnant than their uninfected pregnant counterparts is consistent with the findings of Szoba *et al.* (2003) and Nmorsi *et al.* (2010) respectively who reported that IFNγ and IL-6 were significantly elevated in malaria infected pregnant than the uninfected non-

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pregnant women. Also, our finding corroborates that of Torre *et al.* (2002) who also reported significantly elevated levels of IFNγ, TNFα and IL-10 in malaria infected pregnant women than their control counterparts. The same is true in comparison with Bayoumi *et al.* (2009) and Boston *et al.* (2012) who reported significantly increased level of IL-10 in malaria infected pregnant women than the uninfected pregnant control subjects. On the other hand, the finding did not agree with the finding of Bayoumi *et al.* (2008) who reported that IFNγ, IL-4 and IL-10 were elevated in the uninfected than the malaria infected peripheral blood. The discordance in the results could be the gap in sample size coupled with the fact that the later worked in an area of unstable malaria transmission.

As regards the degree of parasitaemia, this study revealed that pro-inflammatory and anti-inflammatory cytokines evaluated were more expressed in moderate parasitaemia than mild parasitaemia. This finding did not agree completely with that of Szoba *et al.* (2003), who reported that IL-4 and IL-10 were significantly elevated in mild infection than moderate infection. The difference could account to the fact that increased level of the anti-inflammatory cytokines in mild infection is possible as it reflects early and effective immune response but as infection progresses, more cytokines of interest are liberated.

On determining the relationship between parasite density and cytokines in malaria infected pregnant women, this study shows positive relationship between parasite density and IL-4, between parasite density and IL-6 and between the parasite density and IL-10. This finding agrees with the findings of other investigators Zeyrek *et al.* (2006), Adeoti *et al.* (2012) and Rodrigo *et al.* (2014), who observed positive association between the parasite density and IL-6 and, between parasite density and IL-10; although, Rodrigo *et al.* (2014) also observed an association between parasite density and TNF-α. The positive association recorded in this study suggests that the level of parasitaemia may induce a corresponding secretion of IL-4, IL-6 and IL-10. On the contrary, this study did not observe any significant association between parasite density and INF-γ as well as between parasite density and TNF-α. The reason for this presentation is not clear; however, it may suggest that increase in the parasite density may not always be a sole factor that determines the secretion of INF-γ and TNF-α. It is possible that other factors like genetic and host immunity may contribute to determine the amount of the respective cytokines secretion.

Conclusion

The observation made in this study has shown that pregnancy induces the secretion of cytokines. It also reinforced the aforementioned reports that malaria parasite induces increased secretion of cytokines. In addition, the parasite density was elevated with of IL-4, IL-6 and IL-10. Therefore, evaluation of these cytokines could serve as diagnostic markers in asymptomatic malaria infected pregnant women. The study has further highlighted the intriguing relationship between malaria infection and innate immune response in asymptomatic pregnant women.

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