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Association between parasite density and cytokines in malaria infected human placentas

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ABSTRACT

Background: Placental malaria is a major cause of infection induced adverse conditions in pregnancy and is attributed to the sequestration of malaria parasite in the intervillous space. We investigated if any relationship exists between the parasite density and cytokines in malaria parasite infected human placentas.
Methods: Sixty (60) malaria parasite infected placentas from apparently healthy immediate post-partum women and 40 malaria parasite uninfected placentas which served as control were studied. Blood from the human placenta was aseptically collected and tested for HIV and malaria parasite using standard methods. Interferon-Gamma (IFNγ), Tumor Necrosis Factor alpha (TNFα), Interleukin-4 (IL-4), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) were measured by Enzyme-Linked Immunosorbent Assay (ELISA) technique. Data were analysed using appropriate statistical tools.
Results: The result revealed *P. falciparum* with a mean parasite density of 762.47±459.62 parasite/µl of blood. The mean±SD (11.71±6.55pg/ml) and 55.57±43.13 pg/ml for IFNγ and IL-10 respectively for infected placenta was statistically higher on comparison with 5.58±2.86 pg/ml and 16.60±4.88 pg/ml for IFNγ and IL10 respectively for uninfected human placenta ($P<0.05$). Positive correlation existed between parasite density and IL-6 ($r = 0.59$, $p = 0.001$) and between parasite density and IL-10 ($r = 0.41$, $p = 0.024$).
Conclusion: The study showed upregulated levels of IL-6 and IL-10 which indicates disruption of normal immune balance in the parasite infected placenta and the amount of IL-6 and IL-10 secreted could reflect the level of parasitaemia and could serve for diagnostic assessment of placental malaria.
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Keywords: malaria, cytokines, parasite density, human placenta, post-partum women

INTRODUCTION

Malaria parasite adheres and sequesters in the endothelium of most organs particularly in the trophoblastic villus epithelium of the placenta (Ayres Pereira *et al.*, 2016). The sequestration of the parasite, mostly *P. falciparum* in the placenta is facilitated by adhesion molecules collectively known as Variant Surface Antigens (VSA) (Chan *et al.*, 2014; Ayres Pereira *et al.*, 2016). The parasite expresses the VSA on the surface of infected red cells which enables the infected and uninfected red cells to anchor to the vascular lining. The principal and most characterised group of VSA is *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP-1).

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P. falciparum erythrocyte membrane protein 1 is encoded by *var*-genes and is reported to be dominantly expressed during the asexual stage of the parasite life cycle (Agudelo *et al.*, 2014; Chan *et al.*, 2014) and also associated with pathogenicity of the parasite (Dörpinghaus *et al.*, 2020).

As for *P. falciparum,* the infected erythrocytes accumulate in the intervillous space and binds to Chondroitin Sulphate-A (CSA) in the syncytiotrophoblast (Rieger *et al.*, 2015; Ayres Pereira *et al.*, 2016; Pehrson *et al.*, 2016). The CSA is a glycosaminogen identified as host placental endothelial molecule associated with binding to VSA1, and this phenomenon favours the development of the parasite. Again, binding of the parasite, to the placental endothelium most times is accompanied by infiltration of mononuclear phagocytes in the intervillous space (Seitz *et al.*, 2019) of which most of the leucocytes contain hemozoin; the malaria parasite pigments (Sharma and Shukla, 2017). Hemozoin is composed of indigestible haem polymer.

The placenta is the primary link between the mother and the foetus. The organ nourishes the foetus, eliminates foetal wastes and produces pregnancy hormones (Oratz, 2014; Bronson and Bale, 2016; Sharma and Shukla, 2017). The placenta is prone to invasion by malaria parasite and high parasite infection of the placenta activates the immune cells resulting in some changes in the placenta. The changes include the consumption and reduction in the amount of oxygen, glucose and other nutrients required for foetal development, the deposition of fibrinoid materials which culminates in thickening and lesion of the cytotrophoblast membrane and disruption of normal immune balance due to increased secretion of cytokines (Sharma and Shukla, 2017). It is posited that the increased secretion of cytokines also results in drastic reduction and transport of nutrient to the foetus (Robertson *et al.*, 2015) and the pathological lesions also compromises the placental circulation. Overall, the increased synthesis of cytokines such as TNFα, IL-2, and IFNγ have been posited to adversely affect pregnancy (Maestre and Carmona-Fonseca, 2014; Sharma and Shukla, 2017).

The placental malaria induced adverse conditions include low birth weight, intra-uterine growth retardations, still birth, abortion, placental abruptio and other pregnancy-related abnormalities and sepsis (Omer *et al.*, 2017). In other words, to prevent these adverse conditions, the intermittent preventive treatment for malaria is usually administered during the second and third trimesters of pregnancy (Anto *et al.*, 2019). However, the efficacy of the intervention often may not be assured (Anchang-Kimbi *et al.*, 2020).

Similarly, Pregnancy is also associated with immune cells activation. The condition resembles an immunologic tolerance; the maternal subject accepts implantation of foetal allograft in her uterus (Robertson *et al.*, 2015). The foetal tissue is allogenic and invades the maternal decidua and stimulates immune cells subsequent to secretion of cytokines. Under normal conditions, two distinctive cytokine profiles; Type-1 and Type-2 cytokine profiles are secreted in the placenta. Type-1 cytokines are important in immune-surveillance against pathogens such as malaria infection whereas Type-2 is essential to regulate pro-inflammatory response and prevent damage of the placental barrier (Yockey and Iwasaki, 2018).

After conception, the profile in the placenta favours increased secretion of Type-1 cytokines such as IFNγ, TNFα and IL-6. But as pregnancy progresses, a transformation occurs in favour of Type-2 dominance with increased secretion of IL-4, Transforming Growth Factor beta (TGFβ) and IL-10. The change in the cytokine profiles at the maternofoetal interface is necessary in determining the fate of pregnancy as over-expression of Type-1 may compromise the viability of the foetus (Morelli *et al.*, 2015). However, in response to invading pathogens such as malaria infection, the Type-2 dominance is reversed to Type-1 and this development may result in adverse pregnancy conditions (Lufele *et al.*, 2017).

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This study was designed to assess the effect of malaria parasite infection of the placenta as well as if any relationship exists between parasite density and cytokine in malaria parasite infected human placentas. It is with the view to predict the basis for most placental malaria associated adverse conditions. Placental malaria induced adverse conditions appears to be prevalent in Aba. Findings are expected to proffer solution to ensure successful delivery of healthy infants.

MATERIALS AND METHODS

Study site

This study was carried out in Aba, Abia State, Nigeria. Aba is a cosmopolitan town and the second largest commercial city of South Eastern Nigeria. It is located on latitude 05^0 10^0 N and longitude 07^0 19^0 E. It is about 205meters (633ft) above sea level. Aba is 67km from Umuahia, the State capital (Ezeigbo *et al.*, 2014).

Study design

The study was conducted between 2015 and 2016. It was a cross-sectional research work involving the immediate post-partum women. The subjects were recruited by simple random sampling technique and placental blood collected shortly after delivery. The deliveries were without complications and via vaginal route. Sixty *P. falciparum*-infected and 40 malaria parasites uninfected placental blood which served as control were investigated. The detection of malaria parasite in the placental blood was the foremost criteria used in the study.

Ethical consideration

Prior to the study, ethical approval was obtained from the Research and Ethics Committee of Abia State University Teaching Hospital and Living Word Mission Hospital, Aba. The hospitals are of the tertiary status manned by qualified personnel and frequently used by pregnant women. Informed consent of the pregnant women from whom the blood was collected from their placenta after de-livery was obtained.

Data collection

The age of the post-partum women was between ages 17- 44 years. Before delivery, the pregnant women had their peripheral blood tested for HIV and all tested negative. Similarly, the blood from the placenta after delivery also tested negative to HIV. In addition, the subjects had no history of *T. gondii* infection or other infections. The tests for the infections were conducted to ensure that other sources of immune stimulation aside malaria were eliminated.

The placental blood was obtained by biopsy pool method: A block of tissue (5cm x 5cm x 5cm) was excised from the clean maternal side (surface) of the placenta, resulting in a large pool of intravenous blood at the incision site. About 8 ml of blood was quickly aspirated with a graduated sterile pipette. This was carried out by Doctors and Matrons. Out of the amount of blood collected, 2ml was dispensed into blood specimen container with 2 drops of 10% Ethylene Diamine Tetra Acetic acid (EDTA) and mixed for the examination of malaria parasite and estimation of the parasite density. The remaining 6mls were allowed to clot in a pyrogen free container and centrifuged at 3000rpm for 10mins. The serum was used for serological testing for Human Immuno-deficiency Virus (HIV) and cytokines: IFNγ, TNFα, IL-4, IL-6 and IL-10.

The parasite test was determined by Rapid Diagnostic Tests (RDT), Thick and thin Film Methods. The thin as well as thick films were used for the speciation of malaria parasite. The malaria parasite density was evaluated using the quantitative parasite count (Thick film). The HIV testing was done using the DetermineTM HIV 1/2, (Alere, Japan) and Unigold HIV 1&2 Test kits (Trinity Biotech PLC, Ireland). The cytokine kits were sourced from (Abcam Company, UK) and estimations were by ELISA technique. Procedure for each test was followed according to the manufacturer's instruction.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 21. The results were expressed as mean and standard deviation. Student's t-test was used for comparison

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between groups and Pearson's correlation coefficient used for Tests of Association. Level of significance was set at $p<0.05$.

RESULTS

Table 1 shows comparison of mean of cytokine levels between *P. falciparum*-infected and uninfected placenta. It was set to ascertain the response of placental immune cell to the parasite activation. Data showed varied levels of cytokines.

For IFNγ, the mean values for the parasite infected placenta were higher and statistically significant on comparison with that of uninfected placenta ($P<0.05$).

With regards to TNFα, the mean±SD (19.35±10.94pg/ml) for the malaria parasite infected placenta was higher but did not show any statistical significance when compared with 12.36±6.81pg/ml for uninfected placenta ($P>0.05$).

For IL-4, the mean values for the malaria parasite infected placenta showed no statistical difference on comparison with the value obtained for the uninfected placenta ($P>0.05$).

For IL-6, 34.27±13.78pg/ml obtained for the malaria parasite infected placenta was higher but showed no statistical difference when compared with 26.99±12.65pg/ml for malaria uninfected placenta ($P>0.05$).

With IL-10, the values obtained for the parasite infected placenta was higher and statistically significant when compared with that of uninfected placenta ($P<0.05$).

Correlations were between the parasite density and cytokines. It was set to showcase if any relationship exists between the parasite density and cytokine. Data showed no correlation between the parasite density and INFγ ($r= 0.22$, $P= 0.250$), between parasite density and TNFα ($r= -0.07$, $P= 0.729$) and between parasite density and IL-4 ($r= 0.34$, $P= 0.067$). On the other hand, significant positive correlation existed between parasite density and IL-6 ($r= 0.59$, $P= 0.001$) and between parasite density and IL-10 ($r= 0.41$, $P= 0.024$).

Scatter plots were used to represent statistically significant correlations and were between the parasite density and IL-6 and between parasite density and IL-10 (figures 1and 2 respectively). The scatterplots that didn't show any correlation were not represented.

DISCUSSION

Placental malaria is one of the causes of maternal and foetal morbidity and mortality. One of the findings of this study revealed that *P. falciparum* infects the placenta and this apparently supports earlier studies that the parasite infects and sequesters in the placenta sometimes in high density. The reason for this occurrence is that the placenta is rich in nutrients and provides an environment conducive for growth and multiplication of the parasite. Again, the parasite develops adhesion ligand that favours the sequestration of infected erythrocytes on host endothelial cells.

Sequestration is a phenomenon whereby malaria infected erythrocytes accumulate and adheres to the microvasculature of various organs (Adams *et al.*, 2014). In placental *P. falciparum* infection, there is selective adherence of parasites and infected erythrocytes to the syncytiotrophoblast by PfEMP-1. *P. falciparum* erythrocyte membrane protein 1 preferentially mediates binding to the placental CSA (Fried and Duffy, 2015; Ayres Pereira *et al.*, 2016), and this development causes the parasite to avoid the splenic clearance mechanism.

It is posited that Chondroitin Sulphate A is not accessible for binding to malaria parasites in other tissue beds elsewhere in the body except in the placenta (Pehrson *et al.*, 2016) and even if it has access and binds to CSA in other organs, such binding may not commonly affect the host. This is postulated to be the reason why non-pregnant women and adults do not succumb easily to malaria than the pregnant women.

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Table 1: Comparison of cytokine levels of *P. falciparum* infected placentas

Parameters	Malaria Infected Placenta n = 60	Placenta not infected with Malaria n = 40	p-value
IFNγ (pg/ml)	11.71 ± 6.55[a]	5.58 ± 2.86[b]	0.001
TNFα (pg/ml)	19.35 ± 10.94[a]	12.36 ± 6.81[a]	0.140
IL-4 (pg/ml)	14.86 ± 6.37[a]	12.03 ± 5.01[a]	0.100
IL-6 (pg/ml)	34.27 ± 13.75[a]	26.99 ± 12.66[a]	0.065
IL-10 (pg/ml)	55.57 ± 43.13[a]	16.60 ± 4.88[b]	0.001

a-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

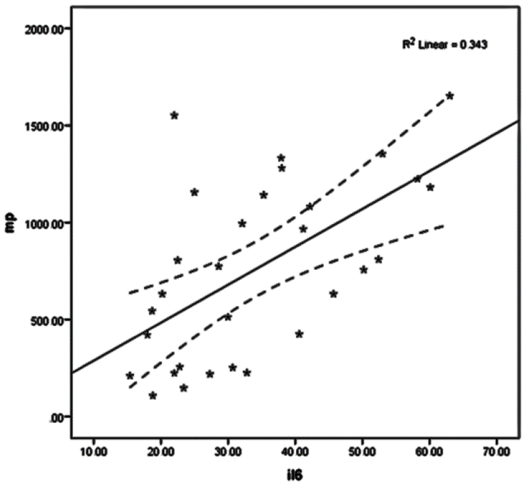

Figure 1: Correlation between parasite density and IL-6 (r = 0.59, p = 0.001)

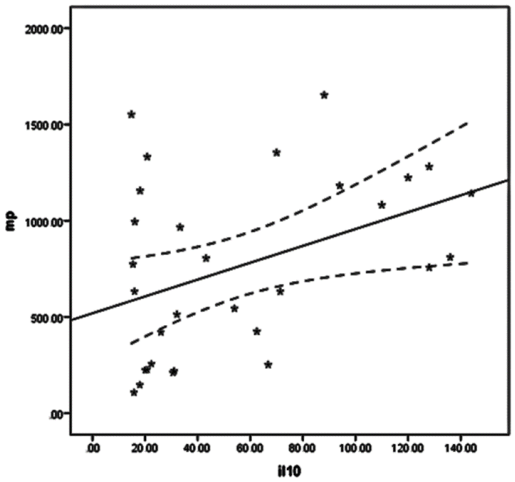

Figure 2: Correlation between parasite density and IL-10 (r = 0.41, p = 0.024)

Moreover, the impact of placental malaria could be reduced due to protection conferred by antibodies against placental malaria (Ndam *et al.*, 2015; Lufele *et al.*, 2017; Patel *et al.*, 2017). The antibodies prevent binding of the parasite to the host endothelial molecules thereby protecting the placenta from malaria. However, the protection varies with gravidity or parity as it is posited that the placental CSA antibodies are not developed in the primigravidae but where it does, are not acquired early enough; about 20 weeks of gestation, and therefore could not protect the placenta from the infection (Ndam *et al.*, 2015; Sharma and Shukla, 2017).

On the contrast, in the multigravida, antibodies are conferred early; at about 12 weeks of gestation and are high in successive pregnancies (Lufele *et al.*, 2017). Therefore, early onset and high antibody production in multigravida but absent or delayed in primigravidae appears to be the more apparent explanation as to why multigravida are less susceptible to placental malaria than the primigravidae (Lufele *et al.*, 2017; Sharma and Shukla, 2017). Overall, we speculate that value of the parasite density obtained in this study may be reduced as a result of the administration of Intermittent Preventive Treatment (IPTp) during pregnancy.

However, the study did not take into cognizance the type of IPTp administered considering that the study was not necessarily to access the efficacy of such interventions or to correlate the dose effect of the intervention with parasite density and cytokine secretion.

Another factor that causes placental malaria

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associated adverse conditions is cytokine dysregulation. Findings of this study showed that IFNγ, TNFα, IL-4, IL-6 and IL-10 were elevated in infected placentas as opposed to that of uninfected placentas. This finding is in consonance with studies by (Sharma and Shukla, 2017; Okamgba *et al.*, 2018; Lima *et al.*, 2019) who observed that IFNγ and TNFα were increased in the parasite infected than the uninfected placenta. On the contrary, it did not agree with the finding of Bayoumi *et al.* (2009) who observed that IFNγ, IL-4 and IL-10 were elevated in uninfected than infected placentas and Yasnot *et al.* (2013) who observed that IL-6 and IL-10 were decreased in the infected than the uninfected placenta. The disagreement with the findings of Bayoumi *et al.* (2009) could be that this study was conducted in an area of stable and endemic malaria parasite transmission in contrast with Bayoumi *et al.* who worked in an area of unstable malaria parasite transmission.

The area covered in this study experiences malaria transmission throughout the year. The stagnant water in the drainages provide adequate ecological habitat for the breeding of mosquitoes. The mosquitoes are less susceptible to insecticides and the majority of the study population sleep outside mosquito nets (Kalu *et al.*, 2012; Ezeigbo *et al.*, 2014). As for the discordance with that of Yasnot *et al.* (2013) the reason could be due to the different assay methods. Whereas this study evaluated cytokines by ELISA technique, Yasnot *et al.* (2013) measured the cytokines using Real Time Polymerase Chain Reaction (PCR).

The secretion of type-1 cytokine in placental malaria is increased and is important for clearance of intracellular pathogens (Djontu *et al.*, 2016). For instance, one of the major type-1 cytokines; IFNγ is elevated in the infected placenta. IFNγ is a potent activator of T-cells and macrophages. It contributes to eliminate the parasite and confers protective immunity against malaria (Sylvester *et al.*, 2018). Similarly, TNFα, another molecule of type-1 cytokine also confers immunity to malaria by the elimination of malaria parasite. On the other hand, the secretion of type-2 molecules in the infected placenta is increased and plays essential role in dampening the expression of pro-inflammatory cytokines and prevents damage on the maternofoetal placental barrier (Agudelo *et al.*, 2014). For example, IL-10; one of the major type-2 cytokines, is significantly expressed in malaria infected placenta than uninfected placenta and the finding is in conformity with (Agudelo *et al.*, 2014; Lima *et al.*, 2019).

IL-10 characterizes normal human pregnancy (Morelli *et al.*, 2015) and in spite of the shift towards type-1 cytokine profile in placental malaria, IL-10 is significantly elevated (Agudelo *et al.*, 2014) in order to suppress the effects of the increased levels of type-1 cytokines. As for IL-4, it is assumed that the molecule performs similar function as IL-10 by regulating pro-inflammatory responses. As for IL-6, the cytokine is secreted in immune responses and has pro-inflammatory as well as regulatory effects (Choy and Rose-John, 2017). It is posited that IL-6 inhibits Transforming Growth Factor beta (TGFβ) and IL-10 producing regulatory T. cell (Treg) differentiation (Choy and Rose-John, 2017) but together with TGFβ, it preferentially promotes the differentiation of IL-17 producing T-helper cells (Th 17) (Monin and Gaffen, 2018). IL-17 enhances the function of Th-17 helper T cells that promote further recruitment of neutrophils, eosinophils, monocytes and other immune cells.

Again, in placental infection, the number of mononuclear cells infiltrating the intervillous space is increased. The infiltration is common in malaria infection but rare in the absence of malaria (Sharma and Shukla, 2017; Seitz *et al.*, 2019). The mononuclear infiltrates on activation secretes cytokines which also contributes to up-regulate the cytokine levels in the placenta. This study essentially on determining relationship between the parasite density and cytokines in *P. falciparum* infected human placentas reveals statistically significant association between the parasite density and IL-6 and between parasite density and IL-10. But no association existed between parasite density and IFNγ, between parasite density and TNFα and between parasite density and IL-4.

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The finding suggests that the parasite density influences the secretion of IL-6 and IL-10. The parasite density increases alongside IL-6 and IL-10 and indicates the tendency for cytokine dysregulation, disruption of normal immune balance, placental defects and placenta associated adverse pregnancy condition.

The lack of association between parasite density and IFNγ was not expected given that the cytokine was significantly expressed in malaria infected than the uninfected, however, suggests that parasite density may not be the only factor that influences the level of IFNγ; probably, the dose and efficacy of the Intermittent Preventive Treatment and genetic composition and endocrine molecules may have influenced the secretion of the cytokine. The lack of association between parasite density and TNFα suggests that TNFα may be regulated by some exogenous factors as Intermittent Preventive Treatment and soluble factors such as progesterone concentration. Similarly, no association between parasite density and IL-4 observed in this study could depict that the expression of any cytokines in the placenta may depend on the cytokine of interest.

CONCLUSION

This study showed that IL-6 and IL-10 are immunologic markers for placental malaria and could serve as diagnostic indices in the assessment of *P. falciparum* placental infection, the administration of Intermittent Preventive Treatment notwithstanding. Again, the amount of IL-6 and IL-10 could reflect the parasite density in most parasite induced adverse pregnancy conditions.

COMPETING INTEREST

Authors declare that they have no competing interests.

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