Anti-inflammatory cytokines (TNF-α and IL-10) in malaria-infected “AA” and “AS” subjects in Enugu metropolis, South-East Nigeria

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ABSTRACT: Malaria has remained a public health issue in Nigeria and Africa, and increasing attention is being directed towards disease prevention. Cytokines have been reported to determine the outcome of malaria and Hb polymorphism has also been implicated in protection in endemic areas. This study investigated the relationship between some anti-inflammatory cytokines (TNF-α and IL-10) in Plasmodium falciparum malaria patients of AA and AS Hb genotypes in Enugu metropolis, South-East Nigeria. A total of 207 subjects aged 20-60 years were recruited for the study and divided into 4 groups. Determination of Hb genotype was done electrophoretically, while quantitation of parasites and estimation of parasite density were done microscopically. Estimation of TNF-α and IL-10 in serum was carried out using ELISA kit (Enzo® Life Sciences, U.S.A). All data were analyzed using Graph Pad Prism version 5 and SPSS version 20 computer software at 95% confidence level and results are expressed as mean ± SEM. A comparison of the test groups showed that AA test group had significantly higher (P<0.0001) MP density (3,906 ± 436.5/µl) than AS test group (1,293 ± 179/µl). The AA test group also had significantly higher TNF-α (36.31 ± 2.34 pg/ml), compared to AS test group (22.05 ± 1.22 pg/ml). IL-10 also showed significant increase (<0.05) in AA test subjects (21.78 ± 1.09 pg/ml) compared to the AS test subjects (17.21 ± 1.53). The cytokines were generally significantly higher (P<0.05) in the test subjects compared to the control. MP density however showed a significant positive correlation (P=0.0215) with IL-10 in AA test subjects. There was significant positive correlation (r=0.439, P=0.003) between IL-10 and TNF-α in AA test subjects. The high IL-10 and TNF-α in AA test subjects is suspected to have led to progression of the disease, as evidenced by the increased parasitemia in these subjects. There was neither any correlation in AA test subjects between gender and either IL-10 (r= 0.024, P= 0.873) or TNF-α (r=0.045, P=0.771) nor in AS subjects between gender and IL-10 (r= 0.116, P= 0.463) or TNF-α (r=0.176, P=0.271). The present study shows that cytokines actually contribute to P. falciparum malaria outcome in AA and AS subjects in Enugu metropolis. The predisposition of AA subjects to malaria may be linked to increased TNF-α and IL-10.

KEYWORDS: Anti-inflammatory cytokines, AA, AS, malaria, P. falciparum, genotype, Enugu.
INTRODUCTION

Malaria is presently endemic in a broad-band around the equator, in areas of the Americas, many parts of Asia, and much of Africa; in Sub-Saharan Africa, 85-90% of malaria fatalities occur (Ross and Smith, 2010). Infection with *Plasmodium falciparum* remains one of the most common infectious diseases worldwide and is still a major cause of morbidity and mortality in tropical regions (Bostrom et al. 2012). The expression of cytokines in general as well as the balance of pro-and anti-inflammatory response are supposed to be involved in malaria pathogenesis, but their relationship with the pattern and extent of vital organ dysfunction in malaria infection has not been well defined yet (Niikura et al. 2010). The presence of severe malaria has been strongly attributed to an exaggerated immune response of the host towards parasite antigens and several authors reported on the positive correlation between high TNF-α production and cerebral malaria in humans as well as in animal models (Achidi et al. 2013).

Early and effective inflammatory response, mediated by gamma interferon (IFN-γ) in the interleukin 12 (IL-12) and 18 (IL-18) dependent manner, seems to be crucial for the control of parasitaemia and resolution of malaria infection through the mechanisms of the tumor necrosis factor a (TNF-α) induction and enhanced release of the antiparasitic reactive nitrogen and oxygen radicals (McCall and Sauerwein, 2010). IFN-γ, a defining cytokine of Th1 cells expressing the transcription factor T-bet, has proven to be important for controlling the acute erythrocytic stage of *Plasmodium* infection in rodent models (Su et al. 2000) and IFN-γ from CD4 C T-cells has been shown to be important in maintaining strain-transcending blood-stage immunity (da Silva et al. 2013). Studies carried out in Africa associated defective IL-12 production with severe malarial anaemia, which is a common feature of *Plasmodium falciparum* infection in children in the continent (Achidi et al. 2013).

Sickle cell disease (SCD) is one of the most prevalent erythrocyte alterations, mainly in malaria-endemic regions. Previous study by Lopez et al., (2010) suggested that this is a protection mechanism against *P. falciparum* malaria and malarial mortality, possibly due to a selective advantage conferred by HbAS. Lopez et al (2010) have shown that HbS changes its nature in deoxygenating conditions and parasites become gravely affected, therefore showing that only erythrocyte mechanisms are sufficient for providing resistance “in vivo”. Also 90% of young parasites have been shown to be eliminated in HbAS cell populations, of which only < 60% are sickled or distorted and that the parasite contributes to conditions inducing sickling in its host cell (Lopez et al. 2010).

Williams et al. (2005) suggested that protection conferred by HbAS is remarkably specific to malaria, whereas other studies have shown that heterozygous people carrying the sickle-cell trait (HbAS) are protected against severe malaria (up to 90% in some populations) (Cabrera et al. 2005; Duffy and Fried, 2006). A recent study in Cameroon suggested that the protective effect of the sickle cell trait may be linked to the raised level of Transforming Growth Factor beta (TGF)-β, suggesting that some rare polymorphisms in candidate genes may have important implications for the susceptibility of Cameroonian to severe malaria (Apinjoh et al. 2014).

The present study assessed the relationship between some anti-inflammatory cytokines and malaria parasitemia in Nigerian subjects of AA and AS genotypes. The study intends to reveal if anti-inflammatory cytokines (TNF-α or IL-10) contribute to the protection or susceptibility and progression of malaria infection and pathology in Nigerian subjects of HbAA and HbAS genotype.

MATERIALS AND METHODS

Subjects:

The subjects comprised initially of a total of six hundred and twenty (620) subjects. Out of this population 413 were disqualified from the study for inability to meet the criteria for inclusion in the study. A total of two hundred and seven (207) individuals were finally included in the study. The test subjects for the study comprised of a total of one hundred and seven (107) patients (79 males and 28 females), (AA (n=53) and AS (n=54)) with symptomatic malaria infection. The patients were adults, belonging to the age range of 20-60 years. Each test group was further subdivided according to the degree of parasitemia (++, ++++) and malaria patients). All patients were reviewed to confirm that malaria was the sole or principal cause of their condition.

The degree of clinical impairment was assessed and clinical evaluation was done by experienced Medical Officers. Patients were classified as having malaria according to the WHO criteria (WHO, 2010). Concomitant infection with other agents was considerably excluded by clinical evaluation. The study test subjects were recruited from the out-patient departments of Enugu State University Teaching Hospital (ESUTH) G.R.A Enugu and University of Nigeria Teaching Hospital, (UNTH) Ituku-Ozalla, Enugu.
The control subjects were one hundred (100) apparently-healthy (70 males and 30 females), age-matched subjects (AA, n=50) and (AS, n=50), residing in Enugu metropolis.

### Inclusion criteria

Test subjects were included in the study if they were resident in Enugu, showed presence of symptomatic malaria and absence of typhoid, hepatitis B, hepatitis C, syphilis, pneumonia and clinical viral symptoms at the point of sampling.

### Exclusion criteria

Exclusion criteria include; documented or strong clinical signs suspecting of viral hepatitis (HBV, HCV), typhoid fever, chronic alcoholism, yellow fever, common cold, dengue, leptospirosis, tuberculosis, Hansen’s disease and visceral leishmaniasis. Others include documented or referred cancer and/or other chronic degenerative disease, and the use of hepatotoxic and immunosuppressive drugs. Widal tests were also carried out to exclude typhoid infection whereas rapid diagnostic tests (RDT) strips were used to rule-out HBV, HCV and Syphilis infections.

### Sample Collection and processing:

Blood samples were collected during the patients visit to the hospital. Peripheral venous blood samples (4mls) were collected from the ante cubital vein, by clean venepuncture with minimal stasis, while the subjects were in the sitting position. Exactly 1ml of the sample was transferred into ethylene diamino tetra acetic acid (EDTA) bottle for malaria parasite count and Hb genotype assay, whereas the remaining 3 mls were transferred into chemically clean plain tubes and allowed to clot. The clotted samples were centrifuged at 3000 rpm for 5 minutes and the serum samples were stored frozen for cytokine concentration measurement.

### Analytical Methods

The Hb genotypes of all the subjects included in the study were determined using electrophoresis on alkaline cellulose acetate paper (pH 8.6) (Cheesbrough, 2005), whereas malaria parasites were detected and quantitated by thick and thin peripheral blood smears using the method of Wroczynska et al. (2005). Parasite density was estimated using the method of WHO, (2010), whereas the parasite

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**Table 1: Mean ± SEM levels of cytokines in the different malaria groups in AA and AS genotypes.**

<table>
<thead>
<tr>
<th>MALARIA TEST GROUPS</th>
<th>N=107</th>
<th>TNF-α (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (+)</td>
<td>12</td>
<td>16.87 ± 1.97</td>
<td>14.37 ± 1.21</td>
</tr>
<tr>
<td>AA (++)</td>
<td>38</td>
<td>38.17 ± 2.05</td>
<td>22.94 ± 1.09</td>
</tr>
<tr>
<td>AS (+)</td>
<td>3</td>
<td>67.0 ± 1.15</td>
<td>28.33 ± 7.68</td>
</tr>
<tr>
<td>AS (++)</td>
<td>26</td>
<td>25.62 ± 2.00</td>
<td>18.47 ± 2.32</td>
</tr>
</tbody>
</table>

**Table 2: Correlation between Gender and the Assayed Cytokines in AA Test Subjects**

<table>
<thead>
<tr>
<th>AA Test (N=53)</th>
<th>Sex</th>
<th>TNF-α</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson Correlation</td>
<td>-0.045</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>P-Value (2-tailed)</td>
<td>0.771</td>
<td>0.873</td>
</tr>
</tbody>
</table>

**Table 3: Correlation between Gender and the Assayed Cytokines in AS Test Subjects**

<table>
<thead>
<tr>
<th>AS Test (N=54)</th>
<th>Sex</th>
<th>TNF-α</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson Correlation</td>
<td>0.174</td>
<td>0.116</td>
</tr>
<tr>
<td></td>
<td>P-Value (2-tailed)</td>
<td>0.271</td>
<td>0.463</td>
</tr>
</tbody>
</table>

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density were converted to absolute values thus: (+) = 1-10 parasites per 100 high power fields; (++) = 11-100 parasites per 100 high power fields; and +++= 1-10 parasites in every high power field (Cheesbrough, 1998). Serum concentrations of TNF-α was determined using ELISA method (Tijssen, 1985) whereas IL-10 was determine using the ELISA method of (Chard, 1990). Kit reagents were produced by Enzo® Life Sciences Inc, Farmingdale, NY, USA.

Analysis of Data

Data from the study was analyzed using Graph Pad Prism and SPSS version 20.0 computer software. Results were presented as mean ± standard error of mean (± SEM) with p<0.05 considered as significant. Comparison of means between and within different groups was done using one-way ANOVA and post-test Bonferroni’s multiple comparison Test. Correlation analysis between mp density and cytokines was done using Pearson’s correlation coefficient analysis whereas comparison between genotype groups (for MP density) was done using the Mann Whitney test.

RESULTS

The malaria parasite densities (mean ± SEM) of the AA and AS test groups were 3906 ± 436.5 and 1293 ± 179 respectively. The 25th percentile of the AA and AS test groups were 1200 and 280 respectively whereas their 75th percentile were 6000 and 2200, respectively. The comparison between the malaria parasite density of the two test groups showed statistically significant difference (P<0.0001). The MP density in AA test group was significantly higher than that of the AS test group. This implies that parasite multiplication was more in AA test subjects.

Table 1 shows the cytokine levels in different malaria parasite densities in the AA and AS test subjects. The table show that the highest concentration of TNF-α and IL-10 was recorded in AA (+++) group while the least concentration of the two cytokines were recorded in the AS (+) and AA (+) groups respectively. This suggests that these cytokines were increased with increased level of parasitaemia.

Tables 2 and 3 show the correlation analysis between the cytokines and gender in the AA and AS test subjects respectively. There was no correlation between gender and the cytokines in the two groups. However, there was positive correlation (r= 0.439, P=0.003) between IL-10 and TNF-α in AA test subjects. (Table 2).

The results of the TNF-α (pg/ml) is shown in Fig. 1. The concentrations of TNF-α in the AA test group, AA control group, AS test group and AS control groups were 36.31 ± 2.34, 11.22 ± 0.45, 22.05 ± 1.22 and 12.12 ± 0.58 respectively. Result of 1-way ANOVA showed that TNF-α was significantly different (F=61.64; P<0.0001) across the four groups analyzed.

Also individual comparisons of TNF-α concentration between AA test and control groups, AA and AS test groups and AS test and control groups (t=11.84; P<0.05), (t=6.960; P<0.05) and (t=4.540; P<0.05) respectively (Fig. 1).
The result of IL-10 estimation is shown in figure 2. The results showed that concentrations of IL-10 of the AA test group, AA control group, AS test group and AS control groups were $21.78 \pm 1.09$, $10.73 \pm 1.07$, $17.21 \pm 1.53$ and $11.18 \pm 0.62$ respectively. 1 way ANOVA showed statistically significant difference ($F=20.71; P<0.0001$) in IL-10 concentration across the groups. Individual comparisons between AA test and control groups, AA and AS test groups and AS test and control groups ($t=6.742; P<0.05$), ($t=2.880; P<0.05$) and ($t=3.570; P<0.05$) respectively. (Fig. 2.)

Figure 3 shows the correlation analysis between MP density and TNF-α in the AA test groups. The figure showed significant positive correlation ($r=0.8875; P=0.0453$) between MP density and TNF-α concentration (pg/ml) in the AA test groups. This implies that TNF-α increases with increase in MP density. The AS test subjects also showed significant positive correlation ($r=0.4166; P=0.0060$) between MP density and TNF-α. This also implies a highly significant increase in TNF-α with increase in MP density (Fig. 4).

![Figure 3: Correlation between MP Density and TNF-α in AA test subjects. ($r=0.8875; P=0.0453$)](image)

![Figure 4: Correlation between MP Density and TNF-α in AS test subjects. ($r=0.4166; P=0.0060$)](image)

![Figure 5: Correlation between MP Density and IL-10 in AA test subjects. ($r=0.3418; P=0.0215$)](image)

![Figure 6: Correlation between MP Density and IL-10 in AS test subjects. ($r=0.0.0969; P=0.5414$)](image)
The correlation analysis between MP density and IL-10 showed significant positive correlation (r=0.3418; P=0.0215) in AA test subjects. This shows that there was significant increase in IL-10 concentration (pg/ml) with increase in mp density in this group of subjects (Fig. 5).

Correlation analysis in the AS test subjects, however, showed no correlation (r=0.0969; P= 0.5414) between IL-10 concentration (pg/ml) and mp density in these subjects (Fig. 6.)

DISCUSSION

The present study evaluated the level of anti-inflammatory cytokines (TNF-α and IL-10) in *P. falciparum* malaria infection in subjects of AA and AS Hb genotypes in Enugu Metropolis, South-East Nigeria. The malaria parasite (MP) density in AA test subjects was significantly increased (P<0.0001) compared with the AS test group, suggesting that the AA test subjects were more predisposed to malaria infection.

IL-10 (pg/ml) in the present study was significantly increased in AA test subjects compared with AS subjects (P<0.05). Niikura et al. (2010) reported that production of IL-12 is suppressed by anti-inflammatory cytokine such as IL-10. Thus, enhancement of IL-10 production contributes to suppression of parasite killing, by suppressing IL-12 production. Furthermore, IL-10 inhibits the antigen presentation of monocytes/macrophages (Specht et al. 2010). Actually, IL-10 inhibits both the proliferation and the cytokine synthesis of CD4+ T cells (Groux et al. 1996). IL-10 is hence considered to play a detrimental role during *P. falciparum* infection (Specht et al. 2010).

TNF-α (pg/ml) was significantly increased in AA test subjects in the present study compared to AS test subjects (P<0.05). TNF-α stimulates neutrophils in order to increase parasite destruction, whereas overproduction of TNF-α predisposes to severe pathology (Laishram et al. 2012). Increased concentration of TNF-α has been implicated in increased parasitaemia, malaria anaemia and increased malaria pathology (Burl et al. 2011). The enhancement of TNF-α production might be associated with the aggravation of disease severity, such as severe anemia, by which phagocytosis of uninfected RBC (Lutz et al. 1996), or dyserythropoiesis (Clark and Chaudhri, 1988) occurs.

The values of the assayed cytokines in the present study was similar to those in a previous study by Bostrom et al. (2012) who recorded TNF-α of 23.02 pg/ml in *Plasmodium falciparum*-infected children of Dogon tribe in Mali. The concentration of IL-10 in the present study was however decreased compared to the study by Wroczynska et al. (2005) involving Polish adults, who recorded mean IL-10 concentrations of (45.19pg/ml) in severe malaria. The increase in TNF-α, resulting from increased parasitaemia in the AA test subjects may have contributed to increased malaria pathology in these subjects, since the concentration was decreased in AS subjects with decreased parasitaemia.

Positive correlation was also observed between TNF-α and IL-10 in AA test subjects whereas there was no correlation in AS test subjects. The correlation analysis between the different cytokines and gender showed no correlation (P>0.05) between gender and all the assayed cytokines in both AA and AS subjects, suggesting that there was no relationship between cytokines and gender in both the test and control subjects.

Conclusion

This study has shown that cytokines actually contribute to *P. falciparum* malaria outcome in AA and AS subjects in Enugu metropolis. The predisposition of AA subjects to malaria may actually be linked to increased level of TNF-α and IL-10. Decreased TNF-α and IL-10 may also be linked to the protective effect of sickle cell trait in AS subjects.

However, further extensive studies involving more cytokines, both severe and uncomplicated malaria patients and more subjects, including Hb SS genotypes on a larger scale is recommended to improve on the information from the present study. Such studies will give a clearer picture if screening for HIV infection and co-infection with helminthes can be excluded. Also, single nucleotide polymorphisms (SNPs) of the various cytokines can also be genetically evaluated to know the cytokines with the most amplified genes in malaria infection in different genotypes.

The present study had confounding factors such as lack of screening for co-infection with helminths and HIV screening (since the subjects would not voluntarily offer themselves for HIV screening, due to fear of the possible outcome). Also, undetected/ subclinical viral infections could also not be excluded. These confounders could have affected the study outcome.

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