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Effect Of Pro-inflammatory Cytokines (IFN-γ AND IL-12) In Malaria Infection In "AA" AND "AS" Subjects In Enugu Metropolis, South-east Nigeria. Does Gender Play Any Role?

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ABSTRACT

The present study was aimed at investigating the relationship between some *pro-inflammatory* cytokines (IFN-γ and IL-12) and *Plasmodium falciparum* malaria infection in subjects of AA and AS Hb genotypes in Enugu metropolis, South-East Nigeria. A total of 207 subjects aged 20-70 years were recruited for the study and divided into 4 groups. Blood samples were collected from each patient from the ante-cubital vein without venous stasis. Determination of Hb genotype was done electrophoretically, while quantitation of parasites and estimation of parasite density were done microscopically. Estimation of IFN-γ and IL-12 in serum was carried out using ELISA kit (Enzo® Life Sciences, U.S.A). 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 MP density (3,906 ± 436.5 parasites/µl of blood) than AS test group (1,293 ± 179 parasites/µl of blood) ($P<0.0001$). The AA test group also had significantly higher IFN-γ (pg/ml) (14.36 ± 0.56 pg/ml) compared to AS test group (10.69 ± 0.40 pg/ml). IL-12 however was significantly decreased ($P<0.05$) in AA test subjects (13.93 ± 0.62 pg/ml) compared to the AS test subjects (23.55 ± 1.20 pg/ml). All the assayed cytokines were significantly higher ($P<0.05$) in the test subjects compared to the control. MP density however showed significant negative correlation ($r=-0.3198$; $P=0.0389$) with IL-12 in AS test subjects. There was no correlation ($P>0.05$) between IFN-γ and IL-12 in both AA and AS test subjects. There was also no correlation between gender and the cytokines in both AA test subjects IFN-γ ($r=0.085$, $P=0.578$) IL-12 ($r= -0.091$, $P=0.552$) and AS test subjects IFN-γ ($r=0.000$, $P=0.998$) IL-12 ($r=0.019$, $P=0.904$). The low IL-12 in AA test subjects must have led to progression of the disease, whereas the high IL-12 in AS test subjects must have led to increased resolution of the disease. The present study shows that pro-inflammatory cytokines actually contribute to P. falciparum malaria outcome in AA and resolution AS subjects in Enugu metropolis. The protective effect of sickle cell trait may be linked to the levels of IL-12. However, gender played no role.

Key Words: Proinflammatory cytokines, malaria, Enugu, AA, AS, *P. falciparum* , genotype.

INTRODUCTION

Malaria parasites are micro-organisms that belong to the genus *Plasmodium.* More than 200 species of the genus Plasmodium have been identified that are parasitic to reptiles, birds, and mammals (WHO, 2010a). 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). For many years, the importance of effective acquired immune response to protect against severe *P. falciparum* infection has been known. In this sense, both innate and adaptative immune responses constitute a key component in subsequent *Plasmodium* challenges by reducing parasitaemia during the acute phase of the disease (Agudelo et al. 2012).

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). In agreement with this, 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). In addition to antibodies, cytokines such as interleukin (IL)-12, interferon (IFN)-γ and tumor necrosis factor (TNFα) have been shown to be involved in the control of *P. falciparum* infection (Rovira-Vallbona et al. 2012). However, the excessive production of

pro-inflammatory cytokines, such as TNFα and IL-6, may damage host tissues, increase the expression of adhesion molecules on endothelial cells and enhance parasite cytoadhesion (Burl et al. 2011). Either a direct role of IL-12 defective production in erythropoiesis or its influence on protective immune responses in general, were suggested to be involved in the development of severe anaemia during malaria infection (Achidi et al. 2013).

Sickle cell disease (SCD) is one of the most prevalent erythrocyte alterations, mainly in malaria-endemic regions. Several studies have 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). Different mechanisms proposed to explain how HbAS confers protection against severe malaria include, accelerated sickling of parasite-infected HbAS erythrocytes, low growth rates and parasite invasion in HbAS erythrocytes (in low oxygen conditions) and increased phagocytosis of infected HbAS erythrocytes (Lopez et al. 2010).

However, more recent experimental data mainly support two of them. Firstly, it has been shown that intra-erythrocytic parasite growth is greatly inhibited by HbS polymerization when oxygen levels drop below 5%. Secondly, higher parasite-infected sickle erythrocyte phagocytosis by host immune cells has been observed when compared to infected normal erythrocytes (Lopez et al. 2010).

The present study is aimed at assessing the effect of some pro-inflammatory cytokines in malaria parasite infection in Nigerian subjects of AA and AS genotypes. The study intends to reveal if the protection and increased resolution of malaria infection and pathology or susceptibility and progression of malaria infection in Nigerian subjects of HbAA and HbAS genotype is partly contributed by pro-inflammatory cytokines IFN-γ or IL-12.

MATERIAL 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) malaria patients, (AA (n=53) and AS (n=54)) with malaria infection, and showing visible signs and symptoms of malaria disease. The patients were adults, belonging to the age range of 20-70 years. 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, 2010b). 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.

Control Subjects:

The control subjects were one hundred (100) apparently-healthy, age-matched subjects (AA, n=50) and (AS, n=50), residing in Enugu. Controls were recruited after exclusion of acute disease or infection by physical clinical examination and completion of a questionnaire, which was also used to out rule previous history of malaria. The study was approved by the Ethical Committee of ESUT Teaching Hospital (ESUTH), Enugu, and informed consent was obtained from all the subjects before being included in the study.

Inclusion and Exclusion criteria

Subjects who were resident in Enugu and showed presence of symptomatic malaria with absence of typhoid, hepatitis B, hepatitis C, syphilis, pneumonia and clinical viral symptoms at the point of sampling were included in the study. Subjects who with 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, visceral leishmaniasis,

documented or referred cancer and/or other chronic degenerative disease, and the use of hepatotoxic and immunesupressive drugs were excluded from the study. 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.

Study Design:

The study comprised of a total of 207 subjects (107 test subjects and 100 control subjects) of AA and AS Hb genotypes. The test subjects were grouped into 2 main groups (AA (n=53) and AS (n=54) malaria patients). Each test group was further subdivided according to the degree of parasitemia ((+), (++), (+++) malaria patients). The control subjects were also grouped into 2 main groups (AA (n=50) and AS (n=50) subjects).

Sample Collection and processing:

Peripheral venous blood samples (4mls) were collected from the ante cubital vein, by clean vene-puncture with minimal stasis, while the subjects are 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 3mls were transferred into chemically clean plain tubes and allowed to clot. The clotted samples were immediately centrifuged and the serum samples were stored frozen for measurement of cytokine concentration.

Analytical Methods:

The Hb genotypes of all the subjects were determined using electrophoresis on alkaline cellulose acetate paper (pH 8.4-8.6) (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, (2010b), whereas serum concentrations of cytokines (IFN-γ and IL-12) were determined using ELISA method (Tijssen, 1985; Chard, 1990; Hayes et al. 1995). Kit reagents were produced by Enzo® Life Sciences Inc, Farmingdale, NY, USA.

Statistical Analysis.

Data from the study was analyzed with 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 with Mann Whitney test.

RESULTS AND DISCUSSION

Tables 1 and 2, show the demographic profiles of the test and control subjects respectively. The tables show that the test subjects comprised of (79 males and 28 females) whereas the controls were made up of (70 males and 30 females), with their different age ranges.

TABLE 1: Demographic Profile of the Test Subjects.

	N= 107	(%) 100
Ethnicity		
Igbo	107	100
Gender		
Males	79	74
Females	28	26
Marital Status		
Single	68	64
Married	39	36
Age Range		
20 – 30 yrs	61	57
31 - 40 yrs	39	36
41 – 50 yrs	06	06
51 – 60 yrs	01	01
61 – 70 yrs	00	00
Religion		
Christian	**107**	**100**

TABLE 2: Demographic Profile of the Control Subjects.

	N=100	(%) 100
Ethnicity		
Igbo	100	100
Gender		
Males	70	70
Females	30	30
Marital Status		
Single	46	46
Married	54	54
Age Range		
20 - 30 yrs	46	46
31- 40 yrs	34	34
41 - 50 yrs	18	18
51 - 60 yrs	02	02
61 - 70 yrs	00	00
Religion		
Christian	100	100

Table 3 shows the cytokine levels in AA and AS test subjects with different malaria parasite densities. The table show that the highest concentration of serum IFN-γ was recorded in AA (+++) group while the least concentration of the cytokine was recorded in the AS (+) and AA (+) groups respectively. This suggests that IFN-γ was increased with increased level of parasitaemia. However, IL-12 (pg/ml) was recorded with the highest concentration in AS (+) and the least concentration in AA (++) (Table 3). Tables 4 and 5 show the correlation between the cytokines and Gender in AA and AS subjects respectively. There was no correlation between gender and the cytokines in the 2 groups of test subjects.

TABLE 3: MEAN ± SEM Levels of Cytokines in the Different Malaria Groups in AA and AS Genotypes.

MALARIA TEST GROUPS	N= 107	IFN-g (pg/ml)	IL-12 (pg/ml)
AA (+)	12	13.88 ±1.25	18.75 ±0.99
AA (++)	38	14.41 ± 0.64	12.65 ± 0.61
AA (+++)	3	15.0 ± 3.21	15.67 ± 2.84
AS (+)	28	10.19 ± 0.37	27.38 ± 1.76
AS (++)	26	11.19 ± 0.70	19.71 ± 1.17

TABLE 4: Correlation Analysis between Gender and the Assayed Cytokines in AA Test Subjects

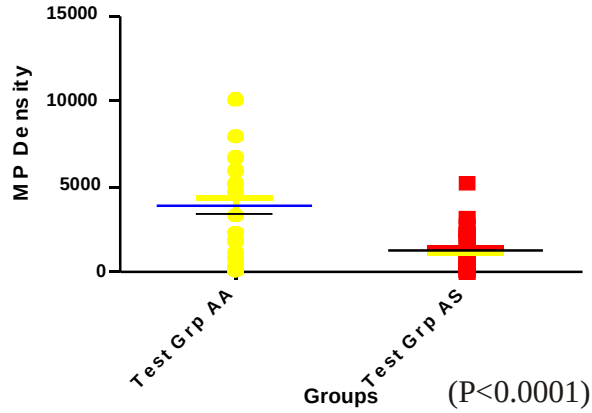
AA Test (N=53)	Sex	INF-γ	IL-12
Sex			
Pearson Correlation	1	0.085	0.578
P-Value (2-tailed)		-0.091	0.552
INF-γ			
Pearson Correlation	0.085	1	-0.067
P-Value (2-tailed)	0.578		0.663
IL-12			
Pearson Correlation	-0.091	-0.067	1
P-Value (2-tailed)	0.0552	0.663	

TABLE 5: Correlation Analysis between Gender and the Assayed Cytokines in AS Test Subjects

AS Test (N=54)	Sex	INF-γ	IL-12
Sex			
Pearson Correlation	1	0.000	0.019
P-Value (2-tailed)		0.998	0.904
INF-γ			
Pearson Correlation	0.000	1	-0.096
P-Value (2-tailed)	0.998		0.545
IL-12			
Pearson Correlation	0.019	-0.096	1
P-Value (2-tailed)	0.904	0.545	

Figure 1 shows the malaria parasite density in the AA and AS test groups. The malaria parasite density (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 that the MP density in AA test group was significantly higher than that of the AS test group ($P<0.0001$). The (mean ± SEM) of serum IFN-γ (pg/ml) in the AA test group, AA control group, AS test group and AS control groups were (14.36 ± 0.56), (8.14 ± 0.43), (10.69 ± 0.40) and (8.03 ± 0.42) respectively (Fig. 2). The 25th percentile of IFN-γ concentration in

the four groups were (11.5, 6.5, 9 and 6) respectively whereas their 75th percentile of the groups were (16.6, 10, 12 and 9) respectively. Result of 1-way ANOVA showed that the concentration of IFN-γ was statistically significant across groups (F= 41.78; P<0.0001). Individual comparisons between AA test and control groups, AA and AS test groups and AS test and control groups (t= 9.499; P<0.05), (t= 5.792; P<0.05) and (t= 3.943; P<0.05) respectively (Fig. 2). The highest concentration was recorded in AA test group, followed by AS test group. The least concentration however, was in AS control group.



(P<0.0001)

FIG. 1: Malaria Parasite Density across the Two Test Groups

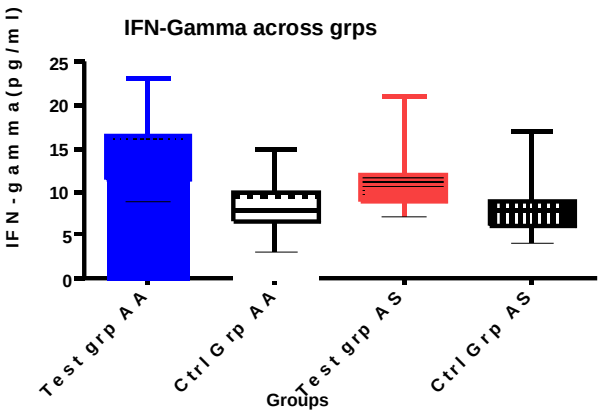


FIG. 2: IFN-γ Across the Test and Control Groups

The (mean ± SEM) of IL-12 (pg/ml) in the AA test group, AA control group, AS test group and AS control group were (13.93 ± 0.62), (5.41 ± 0.47), (23.55 ± 1.20) and (5.61 ± 0.51) respectively (Fig. 3). The result of 1-way ANOVA showed statistically significant difference (F=116.7; P<0.0001) in IL-12 concentration across the groups. Individual group comparisons showed significant differences between AA test and control groups, AA and AS test groups and AS test and control groups (t=7.689; P<0.05), (t=8.975; P<0.05) and (t=15.69; P<0.05) respectively (Fig. 3). The figure shows that the highest level of IL-12 was recorded in AS test group, followed by the AA test group. The least concentration was observed in AA control subjects.

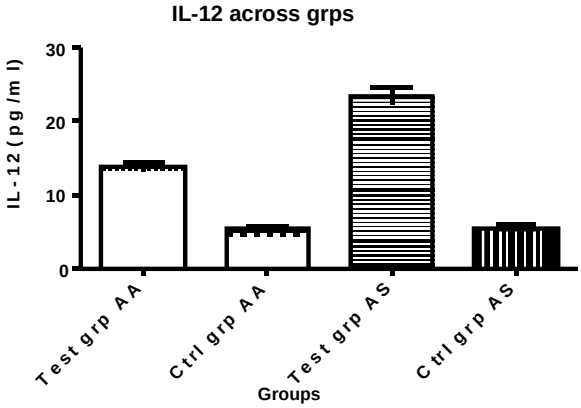
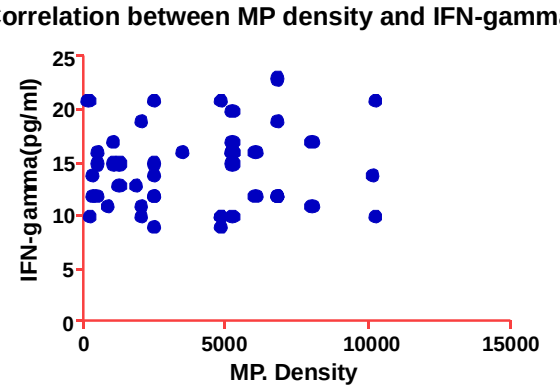


FIG. 3: IL-12 Across the Test and Control Groups

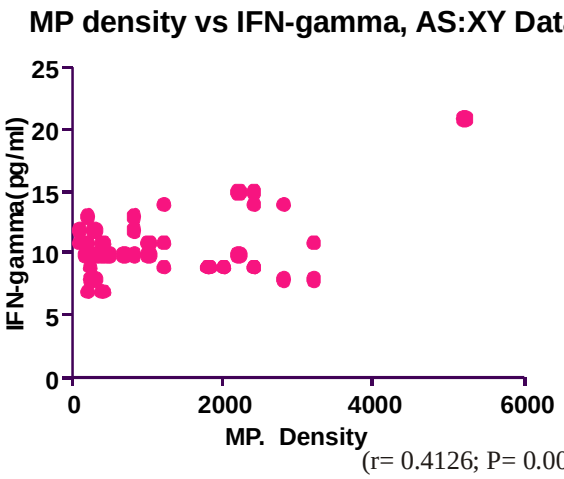


(r= 0.0957; P= 0.5316)

FIG. 4: Correlation between Mp Density and IFN-γ in AA Test Subjects

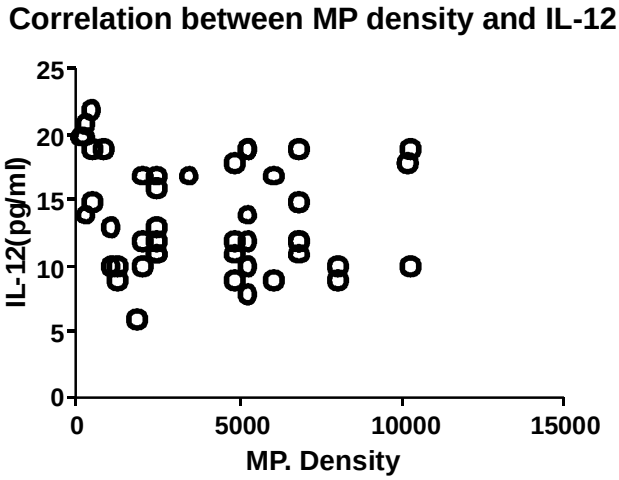
Fig. 4 shows the correlation analysis between the MP density and IFN-γ in AA test group. The result showed no correlation (r= 0.0957; P= 0.5316) between mp density and IFN-γ in the AA test subjects. There was however, significant positive correlation (r= 0.4126; P= 0.0066) between MP density and IFN-γ concentration (pg/ml) in the AS test subjects. This implies that there was a significant increase

in IFN-γ with increase in MP density in AS test subjects (Fig. 5).



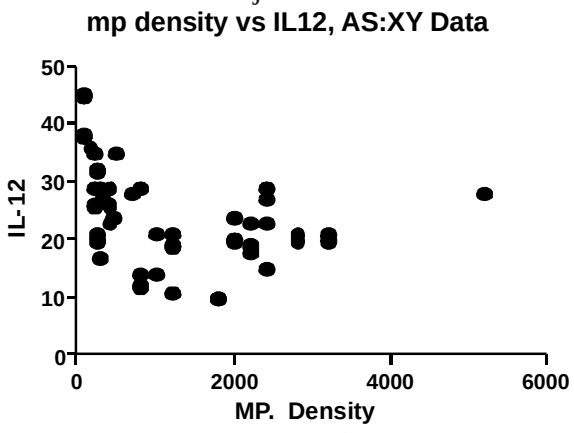
(r= 0.4126; P= 0.0066)

FIG. 5: Correlation between Mp Density and IFN-γ in AS Test Subjects



(r = -0.1673; P= 0.2718)

FIG. 6: Correlation between Mp Density and IL-12 in AA Test Subjects.



(r= -0.3198; P= 0.0389)

FIG. 7: Correlation between Mp Density and IL-12 in AS Test Subjects.

The result of correlation analysis between MP density and IL-12 concentration in AA test subjects is shown in Fig. 6. The result showed a non-significant negative (inverse) correlation (r= -0.1673; P= 0.2718) between MP density and IL-12 in AA test subjects, showing a non-significant decrease in IL-12 with increase in MP density (Fig. 6).

AS test subjects, however showed significant negative (inverse) correlation (r= -0.3198; P= 0.0389) between MP density and IL-12 concentration (pg/ml) (Fig. 7). This shows that IL-12 decreased significantly with increase in MP density.

In this study we evaluated the level of pro-inflammatory cytokines (IFN-γ and IL-12.) in *P. falciparum* malaria infection in subjects of AA and AS Hb genotypes in Enugu Metropolis, South-East Nigeria. The result shows that AA test subjects had significantly increased (P<0.0001) parasite (MP) density compared with the AS test group (Figure 1). This suggests that the AA test subjects were more predisposed to malaria infection.

The present study also showed decreased concentration of IL-12 (pg/ml) in the AA (13.93 ± 0.62) compared to the AS test subjects (23.55 ± 1.20) (P<0.05) (Table 3). This decrease may partly contribute to the increased degree of parasitaemia in AA test subjects compared to AS test subjects, since IL-12 has been reported to play a crucial role in the clearance of intracellular pathogens (Niikura et al. 2010). Inflammatory responses in the interleukin 12 (IL-12) - dependent manner, seems to be crucial for the control of parasitaemia through enhanced release of anti-parasitic reactive nitrogen and oxygen radicals (Niikura et al. 2010; Burl et al. 2011). IL-12 is a necessary factor for clearance of non-lethal *Plasmodium* parasites, suggesting that IL-12 plays an important role in protective immunity via IFN-γ production in murine malaria (Niikura et al. 2011). Also, low levels of IL-12 production have been observed in young African children with severe anaemia during infection with *P. falciparum* (Niikura et al. 2011).

IFN-γ (pg/ml) was significantly increased in AA test subjects in the present study (14.36 ± 0.56) compared to AS test subjects (10.69 ± 0.40) respectively, (P<0.05). Increased INF-γ in

AA test subjects may have contributed to the increased severity of the disease. INF-γ stimulates neutrophils in order to increase parasite destruction (Laishram et al. 2012). However, overproduction of IFN- γ predisposes to severe pathology (Laishram et al. 2012).

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 IFN-γ of 35.12 pg/ml in *Plasmodium falciparum*-infected children of Dogon tribe in Mali. The concentration of IFN-γ in the present study was however decreased compared to the study by Wroczynska et al. (2005) involving Polish adults, who recorded mean IFN-γ concentration of (23.71pg/ml) in severe malaria. The same study recorded very high concentration of IL-12 (102.49pg/ml), in severe malaria subjects (Wroczynska *et al*., 2005). This was in contrast with the IL-12 values of (13.93 pg/ml) and (23.55 pg/ml) in AA and AS malaria patients respectively in the present study.

Previous study by Neboh et al (2017) reported significantly increased level of IL-10 (pg/ml) in AA test subjects compared with AS subjects ($P<0.05$). TNF-α (pg/ml) was significantly increased in the same study, in AA test subjects in the present study compared to AS test subjects ($P<0.05$) (Neboh et al. 2017). Niikura et al. (2010) reported that production of IL-12 is suppressed by anti-inflammatory cytokine such as IL-10. Enhancement of IL-10 production contributes to suppression of parasite killing, by suppressing IL-12 production. IL-10 is hence considered to play a detrimental role during *P. falciparum* infection (Specht et al. 2010; Neboh et al. 2017). 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 (Neboh et al. 2017).

Neboh et al (2017) also observed positive correlation was also observed between TNF-α and IL-10 in AA test subjects whereas there was no correlation in AS test subjects. However the present study showed significant negative (inverse) correlation ($r= -0.3198$; $P= 0.0389$) between MP density and IL-12 concentration (pg/ml) in AS subjects (Fig. 7). This shows that IL-12 decreased significantly with increase in MP density.

Our result also showed no relationship between the different assayed cytokines ($P>0.05$) and gender showed no correlation ($P>0.05$) between gender and all the assayed cytokines in both AA and AS subjects. This suggests that there was no relationship between cytokines and gender in both the test and control subjects.

CONCLUSION

Our study has shown that the protective effect of sickle cell trait may be linked to the increased levels of IL-12 in AS subjects with *P. falciparum* infection. The decreased level of IL-12 may have predisposed the AA subjects to malaria pathology. However, gender appears to play no role in the relationship between cytokines and malaria infection. 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. Pharmaceutical industries may consider manufacture of cytokines such as IL-12 for administration to malaria-prone individuals to help in resolution of malaria pathology via the same mechanism as cytokines in-vivo.

LIMITATIONS

Lack of screening for co-infection with helminths and HIV screening, were not done (since subject 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. 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. All these are confounding factors that could have affected the outcome of the study.

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