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Pro-inflammatory Cytokines Establish a Balance in Symptomatic and Asymptomatic Malaria in *Plasmodium falciparum* Endemic Regions

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Authors' contributions

This work was carried out in collaboration among all authors. Author OCU designed the study and drafted the manuscript. Author CEO performed part of the experimental work. Authors INI and HOO contributed to the manuscript writing. Authors JA and GOU contributed to the manuscript writing and provided critical comments. All authors read and approved the final manuscript.

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ABSTRACT

Background and Objectives: Pro-inflammatory cytokines are key in the control of malaria. This study profiled the pro-inflammatory cytokines: tumor necrosis factor-α (TNF-α), Interleukine–2 (IL-2), Interleukine–6 (IL-6) and Interleukine–12 (IL-12) in symptomatic and asymptomatic *P. falciparum* malaria in Abuja with the aim of elucidating their role in symptomatic and asymptomatic malaria.

Methodology: In the study, 246 plasma samples each from symptomatic and asymptomatic malaria volunteers were assayed for TNF-α, IL-2, IL-6 and IL-12 using Enzyme-Linked Immunosorbent Assay (ELISA) technique and values expressed in pictogram (pg).

Results: The study population comprised 48% male within which 50.4% were symptomatic and 49.6%) asymptomatic, 51.2% was female comprising 49.6% symptomatic and 50.4% asymptomatic. The median plasma concentration of TNF-α in symptomatic and asymptomatic groups of the population were 2.75pg and 2.04pg respectively and differed significantly (P<0.001), likewise IL-6: 4.83pg and 3.01pg (P = 0.014). IL-2 and IL-12 differed significantly in symptomatic and asymptomatic groups but the asymptomatic group showed higher levels of the cytokines: 3.20pg and 3.07pg (P<0.001) for IL-2, and 6.17pg and 3.40pg respectively (P<0.001) for IL-12. When segregated by study areas and age groups, TNF-α, IL-6 and IL-12 showed significant difference between the symptomatic and asymptomatic cases in age group 10-20years; but IL-2 showed no significant difference. TNF-α and IL-6 levels were higher in symptomatic than asymptomatic cases (2.80pg/2.05pg, P<0.001, and 5.2pg/2.42pg, P = 0.023 respectively), IL-12 was higher in asymptomatic case than symptomatic case (3.44pg/5.65pg, P<0.001). In age group 21-39years, TNF-α was significantly higher in symptomatic group than asymptomatic group (2.49pg/2.03pg, P< 0.001) however, IL-2 and IL-12 are significantly higher in asymptomatic than symptomatic cases (3.03pg/3.17pg, P<0.001 and 3.40pg/6.34pg, P < 0.001 respectively). In age group 40 years and above, only IL-2 and IL-12 showed significant difference with asymptomatic cases showing higher plasma levels of the cytokines (3.03pg/3.17pg, P<0.001, and 3.22pg/5.52pg, P<0.001) respectively.

Conclusion: The different patterns exhibited by the pro-inflammatory cytokines (TNF-α and IL-6 that were elevated in symptomatic cases, and IL-2 and IL-12 that were elevated in asymptomatic cases) show interplay among pro-inflammatory cytokines to regulate malaria manifestation in *P. falciparum* endemic place.

Keywords: Cytokines; symptomatic malaria; asymptomatic malaria; immunity; malaria.

1. INTRODUCTION

Plasmodium falciparum, like other species that cause malaria in humans produces malaria as a result of immunologic “war fare” between the host immune system and the parasite upon the expression of the plasmodial antigens and toxins in the host system [1]. These immune responses involve interplay between innate (cellular) and acquired (humoral) arms of immune response. The cellular activities aid in limiting the parasite proliferation, and stimulating humoral responses [2], which is marked by the expression of pro-inflammatory cytokines and complement factors to eradicate the parasites [1]. These responses are aimed at controlling the invading parasites, however, excess production of pro-inflammatory cytokines leads to malaria symptoms and are influenced by some factors such as host genetic factors, parasite endemicity/transmission among others [3]. People living in *Plasmodium falciparum* endemic places are exposed to high transmission rates, as a result are believed to be immuned against *P. falciparum*, and generate high level of pro-inflammatory cytokines upon the parasite infestation due to secondary immune responses.

Surprisingly, people in *P. falciparum* endemic places respond differently to the parasite infestation, where some come down with clinical symptoms (symptomatic malaria) and some present no clinical symptoms (asymptomatic malaria) at similar parasite densities. These different presentations in *P. falciparum* malaria in endemic places are of both medical and epidemiological importance. Asymptomatic status could lead to complications or fatal consequence as the parasite could proliferate and cause severe damages without being noticed. In addition, it constitutes silent carriers that aid transmission from person to person. On

the other hand, symptomatic cases could imply poor resistance to the disease or poorly developed immunity to the disease in an endemic place. This work therefore, profiled some important pro-inflammatory cytokines in symptomatic and asymptomatic *P. falciparum* cases in Abuja to aid in understanding the role of pro-inflammatory cytokines in symptomatic and asymptomatic malaria.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Federal Capital Territory (FCT), Abuja, Nigeria considered a malaria hyperendemic place with perennial transmission status [4]. The predominant *Plasmodium* specie in this region is *Plasmodium falciparum* whose transmission is known to peak at the beginning and ending of rainy seasons [5]. The region is divided into six area councils namely: Abaji, Abuja Municipal Area Council (AMAC), Bwari, Gwagwalada, Kuje and Kwali. The AMAC has the highest population among the area councils, followed by Bwari area council with populations of 776298 (55.2%) and 229,274 (16.3%) respectively [6]. AMAC and Bwari area councils of Abuja give good representation of Abuja as they have good population, are strategically located and host different institutions that attract people from all nook and cranny of Abuja.

2.2 Study Population

The study population comprises persons who have lived in Abuja for 5 years or more. Two hundred and forty-six (246) symptomatic and asymptomatic volunteers were selected for the study. The symptomatic volunteers were recruited from people seeking medical care in Defence Head Quarters Medical Centre (DHQMC), Asokoro, in AMAC; and Kubwa General Hospital (KGH) in Bwari Area Council. The DHQMC is a tertiary health facility used by military personnel and civilians from various parts of Abuja, and also handles referral cases while KGH is also a tertiary health facility accessed by people from all nook and cranny of Abuja and receives referred medical cases. The asymptomatic participants were recruited in both AMAC and Bwari area councils through advocacies in market places and churches for free malaria tests, and use of banners in open places imploring people to check their malaria status.

2.3 Inclusion and Exclusion Criteria

Participants included in this study were those who are 6 years old and above, and have lived in Abuja for 5 years or more. Symptomatic group are those who tested positive to *P. falciparum* upon microscopic examination of their thick blood film at the time of sample collection and showed one or more of these signs: headache, malaise, lassitude, fatigue, abdominal discomfort, muscle and joint pains, fever (axillary temperature ≥ 37.5 °C), chills, perspiration, anorexia or vomiting [7]. Asymptomatic group are those that tested positive to *P. falciparum* via microscopy but did not present signs associated with symptomatic malaria at the time of blood collection. Those that had the following conditions: pregnancy, inflammatory disease, immunodeficiency/ immunosuppressive disease(s), on immune suppressive drugs, have not lived in Abuja for 5 years or were less than 6 years of age were excluded in the study. The participants were selected after consenting and administration of questionnaire by screening volunteers for *P. falciparum* parasitaemia via microscopic examination of their thick blood film made from finger prick and stained with 10% Giemsa stain (Rapid staining) [8].

2.4 Sample Processing

Blood samples for *P. falciparum* detection were collected via finger prick and used for thick film preparation and staining using 10% Giemsa stain (Rapid staining) and microscopic examination following WHO standard [8]. 2ml of venous blood were aseptically collected from the volunteers who met the acceptance criteria into EDTA bottle and spun at 5000 rpm for 5 minutes to express plasma. The plasma samples were separated, screened for *Salmonella typhi* and *Salmonella paratyphi* A, B and C antibodies using TYDAL® Widal Antigen Set / Antigens for Slide and Tube Tests (Tulip Diagnostics (P) Ltd); to eliminate possible confounding factor due to salmonellosis. Samples that tested negative to Salmonella antibodies were selected and stored in cryovials at -80°C till used.

2.5 Cytokine Assay

The cytokine assays were carried out by ELISA technique using Human TNF-α ELISA kit, catalogue number: CT209A; Human IL-2 ELISA kit, catalogue number: CT202A; Human IL-6 ELISA kit, catalogue number: CT205A and Human IL-12p70, catalogue number: CT210A all

from *U-Cytech Biosciences* - Netherlands (www.ucytech.com) to assay for TNF-α, IL-2, IL-6 and IL-12 respectively following the manufacturer's instructions after bringing the plasma samples to room temperature. The absorbance of each of the cytokines in the samples were read and their values converted to the cytokine concentration in picogram (pg) using *Zigmaplot* software, version 8.

2.6 Data Analysis

The cytokine concentrations were tabulated and compared according to different disease status (symptomatic and asymptomatic), catchment areas of the study (AMAC and Bwari Area Councils) and age groups. Statistical analysis was performed using IBM SPSS and R software. Descriptive statistics was done to determine the normality and the different assumptions, as the data sets were not normally distributed, non-parametric test was used to determine the relationship, Mann-Whtiney U test was used to compare the median plasma cytokine concentrations between symptomatic and asymptomatic malaria groups, while Kruskai-wallis test was used to compare the median cytokine across the age groups in the different catchment areas. In all cases, $P < 0.05$ was statistically significant at 95% CI while $P > 0.05$ was statistically insignificant at 95% CI.

2.7 Study Design

Participants for the study were selected from AMAC and Bwari Area Councils of Abuja to give good representation of Abuja population. A total of 265 plasma samples each from symptomatic and asymptomatic malaria subjects who met the inclusion criteria were prepared from whole blood collected from the subjects. The samples were screened for *Salmonella* antibodies to rule out confounding factor due to Salmonellosis and the plasma pro-inflammatory cytokine concentrations measured in the symptomatic and asymptomatic malaria groups.

3. RESULTS AND DISCUSSION

The study population revealed 48% male (n = 238) within which 50.4% (n = 120) were symptomatic and 49.6% (n = 118) asymptomatic. On the other hand, 51.2% (n = 254) of the population is female in which 49.6% (n = 126) was symptomatic and 50.4% (n = 128) asymptomatic. The age range of the population is 10 – 55 years. The median pro-inflammatory cytokines (TNF-α, IL-2, IL-6 and IL-12) were grouped according to malaria cases (symptomatic and asymptomatic) Table 1 and stratified according to age (10-20 years, 21-39 years and 40years and above), segregated by catchment areas of the study (AMAC and Bwari) Table 2.

The TNF-α and IL-6 displayed similar pattern in the symptomatic and asymptomatic groups, both showing higher levels in the symptomatic group than asymptomatic group. The plasma concentration of TNF-α showed a statistically significant difference ($P < 0.001$) at median plasma levels of 2.75pg and 2.04pg in the symptomatic and asymptomatic groups respectively while IL-6 at plasma levels of 4.83pg and 3.01pg in symptomatic and asymptomatic groups respectively was also significantly difference ($P = 0.014$), Fig. 1. In a similar way, IL-2 and IL-12 showed a significant difference in symptomatic and asymptomatic groups but in a reverse pattern. The asymptomatic group in this case showed higher plasma levels of the cytokines. The asymptomatic group showed IL-2 median plasma level of 3.20pg and the symptomatic group 3.07pg ($P < 0.001$), while the IL-12 median plasma level in the asymptomatic and symptomatic group are 6.17pg and 3.40pg respectively ($P < 0.001$) Fig. 1.

With the cytokines levels segregated by age (10-20years, 21-39years and 40years+), and malaria cases in their catchment areas (AMAC and Bwari) Fig. 2, TNF-α, IL-6 and IL-12 showed statistically significant difference in symptomatic and asymptomatic cases in age group 10-20years, though IL-2 showed no significant difference. While TNF-α and IL-6 plasma levels were higher in symptomatic than asymptomatic cases (2.80pg/2.05pg, $P < 0.001$, and 5.2pg/2.42pg, $P = 0.023$ respectively), IL-12 was higher in asymptomatic case than symptomatic case (3.44pg/5.65pg, $P < 0.001$). In age group 21-39years, TNF-α was significantly higher in symptomatic group than asymptomatic group (2.49pg/2.03pg, $P < 0.001$), however IL-2 and IL-12 are significantly higher in asymptomatic than symptomatic cases (3.03pg/3.17pg, P 0.001 and 3.40pg/6.34pg, $P < 0.001$ respectively). In age group 40 years and above, only IL-2 and IL-12 showed statistically significant difference with asymptomatic cases showing higher plasma levels of the cytokines (3.03pg/3.17pg, $P < 0.001$, and 3.22pg/5.52pg, $P < 0.001$) respectively.

In the catchment areas, the cytokines did not differ significantly in AMAC except IL-12 that maintained statistically significant difference among all the age groups with the asymptomatic cases having higher plasma levels of the cytokine, Fig. 2. In addition, TNF-α also differed significantly between the two symptomatic and asymptomatic cases in age groups 10-20years and 21-39years with the symptomatic cases having higher levels of TNF-α (2.88pg/2.07pg, $P = 0.017$ and 2.54/2.08, $P < 0.001$) respectively.

In general, the pro-inflammatory cytokines (TNF-α, IL-2, IL-6 and IL-12) profiled in this work differed significantly in the symptomatic and asymptomatic cases. However, while TNF-α and IL-6 showed higher median values in the symptomatic group than the asymptomatic group, IL-2 and IL-12 were higher in asymptomatic group than the symptomatic group. The increased level of TNF-α is in agreement with the work in [9] which showed that symptomatic *P. falciparum* cases are associated with increased plasma level of TNF-α. In a like manner, TNF-α and IL-6 were elevated in symptomatic and acute malaria [10], indicating that theses pro-inflammatory cytokines are key in malaria symptoms. According to [11], circulating IL-6 increases when *P. falciparum* symptoms manifests (acute malaria) and conversely decrease with anti-malarial treatment and is associated with decreasing pyrexia. The work by [12] also supports this finding, showing that IL-6 level in the plasma is associated with symptomatic parasitaemia. TNF-α has been documented to be a marker of fever in inflammation and in the case of *P. falciparum*, the plasma level peaks at the rupture of schizonts [10]. However, in another publication, an increased TNF-α in asymptomatic *P. falciparum* has been demonstrated, though not in comparison with symptomatic cases [13]. Earlier findings show that TNF-α can induce the production of IL-6 and IL-6 in turn regulates TNF production possibly via a feedback mechanism [14], thus as TNF-α increases, IL-6 will increase to a point of balance. These two cytokines being pro-inflammatory, will induce symptoms. In addition, the low plasma level of TNF-α and IL-6 in the asymptomatic cases in endemic situation as in this work may be explained by the possible mop up of the cytokines. According to [15], soluble TNF and IL-6 receptors are elevated in asymptomatic cases, thus suggests that part of the tolerance strategies in endemic places may be mopping up of the pro-inflammatory cytokines by their corresponding receptors to limit disease manifestation.

The IL-2 and IL-12 like TNF-α and IL-6 differed significantly between symptomatic and asymptomatic cases, but unlike the former showed higher median values in asymptomatic groups than the symptomatic groups. This therefore is suggestive of the potential role of IL-2 and IL-12 in malaria manifestation. This result is in agreement with the work by [16], which demonstrated that IL-2 level is high in mild malaria and higher when symptoms disappear, but decreased in severe and cerebral malaria. According to [17], clinical immunity to *P. falciparum* is associated with persistent low parasite density associated with T-regulatory cells which are in turn under the influence of IL-2. This therefore suggests that the high level of IL-2 in the asymptomatic cases of this work was crucial in the maintenance of the asymptomatic status. In a like manner, increased IL-12 level in *P. falciparum* asymptomatic condition is in agreement with the work by [18] which revealed that IL-12 increased in asymptomatic *P. falciparum* malaria and negatively correlates with parasitaemia, while TNF and IL-10 increase in acute and severe malaria and positively correlates with parasitaemia. The IL-2 and IL-12 secretion by the mediator cells (dendritic cells, macrophages, monocytes and neutrophils) is seen to be supressed by haemozions, (the parasite product from haemoglobin digestion) in acute and severe malaria as the pigmented mediator cells are seen to increase in symptomatic *P. falciparum* malaria with decreased IL-2 and IL-12 level, but decrease with increased IL-2 and IL-12 levels in asymptomatic cases [19]. This implies that the expression of haemozions suppresses IL-2 and IL-12 production, but allows the production TNFα which in turn induces the secretion of IL-6 whose increased concentration supresses TNF-α. This therefore explains the result obtained here and shows that stimulus that led to increased production of TNF-α and IL-6 leads to decrease in IL-2 and IL-12 production. In line with this, the removal of the haemozion stimulus, possibly due to parasite clearance by TNF-α allows for increased IL-2 and IL-12 production observed in asymptomatic status.

When result of this work was segregated by age (10-20years, 21-39years and 40years+) and catchment areas (AMAC and Bwari) TNFα and IL-12 showed a similar pattern in all the age groups and the catchment areas.

Table 1. Pro-Inflammatory cytotines in symptomatic and asymptomatic malaria in Abuja

Parameter	Symptomatic	Asymptomatic	*P*-Value
TNF (pg/mL)	2.75	2.04	<0.001
IL-2 (pg/mL)	3.07	3.20	<0.001
IL-6 (pg/mL)	4.83	3.01	0.014
IL-12 (pg/mL)	3.40	6.17	<0.001

Table 2. Pro-inflammatory cytotines in symptomatic and asymptomatic malaria in abuja segregated by age, malaria case and catchment area

	AMAC				BWARI			
	TNF (pg/mL) Sym/Asy	**IL-2 (pg/mL) Sym/Asy**	**IL-6 (pg/mL) Sym/Asy**	**IL-12 (pg/mL) Sym/Asy**	**TNF (pg/mL) Sym/Asy**	**IL-2 (pg/mL) Sym/Asy**	**IL-6 (pg/mL) Sym/Asy**	**IL-12 (pg/mL) Sym/Asy**
10-20yrs	2.80/2.05 *P* < 0.001	3.13/3.33 *P* = 0.21	5.20/2.42 *P* = 0.023	3.44/5.65 *P* < 0.001	2.88/2.07 *P* = 0.017	3.20/3.26 *P* = 0.52	6.24/1.89 *P* = 0.27	3.68/6.9 *P*= 0.0036
21-39yrs	2.49/2.03 *P* < 0.001	3.03/3.17 *P* < 0.001	4.78/3.68 *P* = 0.56	3.40/6.34 *P* < 0.001	2.54/2.08 *P* < 0.001	3.09/3.17 *P* = 0.29	3.54/2.90 *P* = 0.79	3.29/6.01 *P* < 0.001
40yrs+	2.89/2.03 *P* = 0.11	3.03/3.17 *P* < 0.001	9.36/2.42 *P* = 0.71	3.22/5.52 *P* < 0.001	2.81/2.10 *P* = 0.078	3.71/3.07 *P* = 0.14	7.56/6.35 *P* = 0.5	3.44/6.92 *P* < 0.001

Sym: Symtomatic, Asy: Asymtomatic

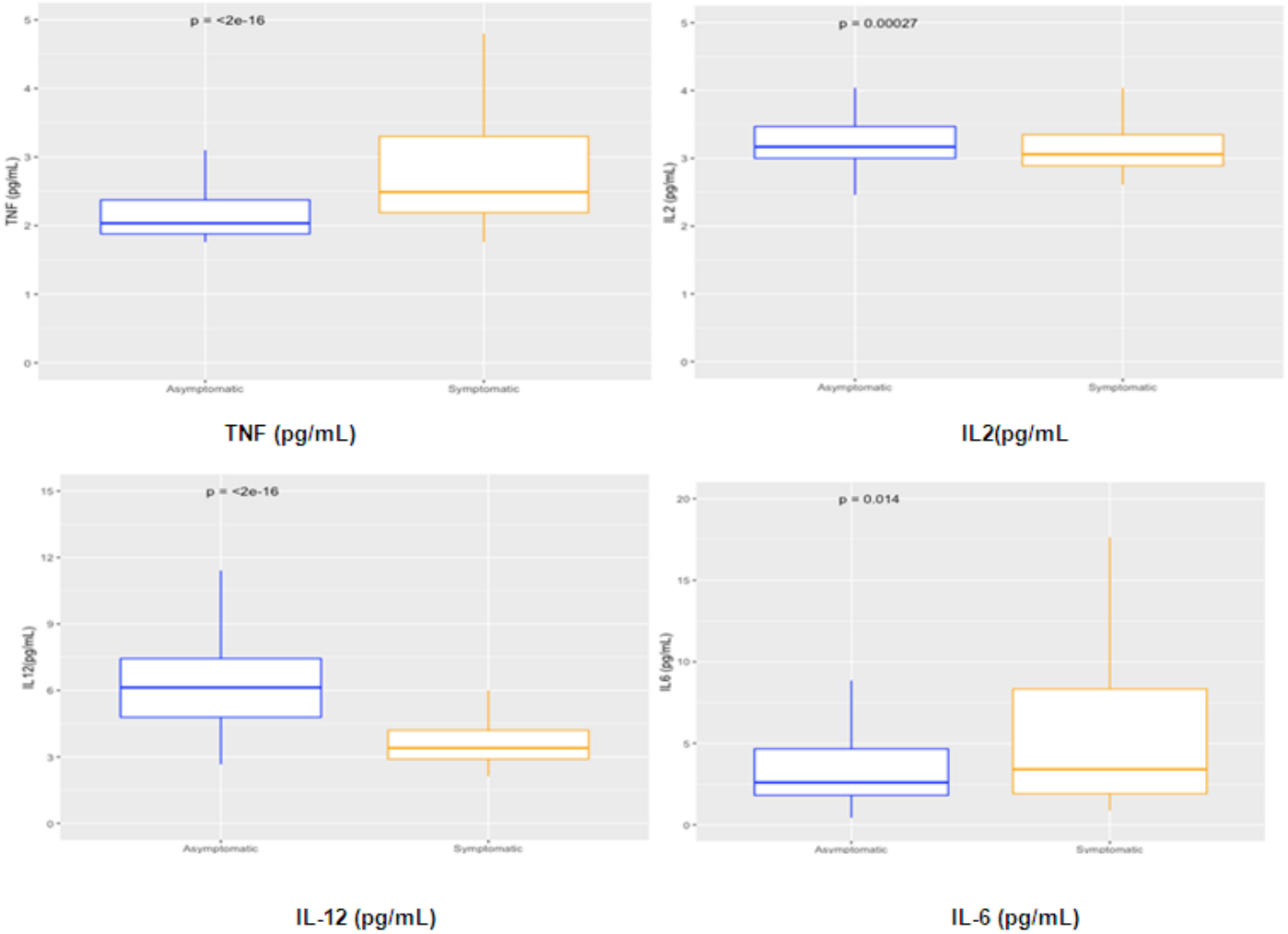

Fig. 1. Pro-Inflammatory cytokines in symptomatic and asymptomatic malaria

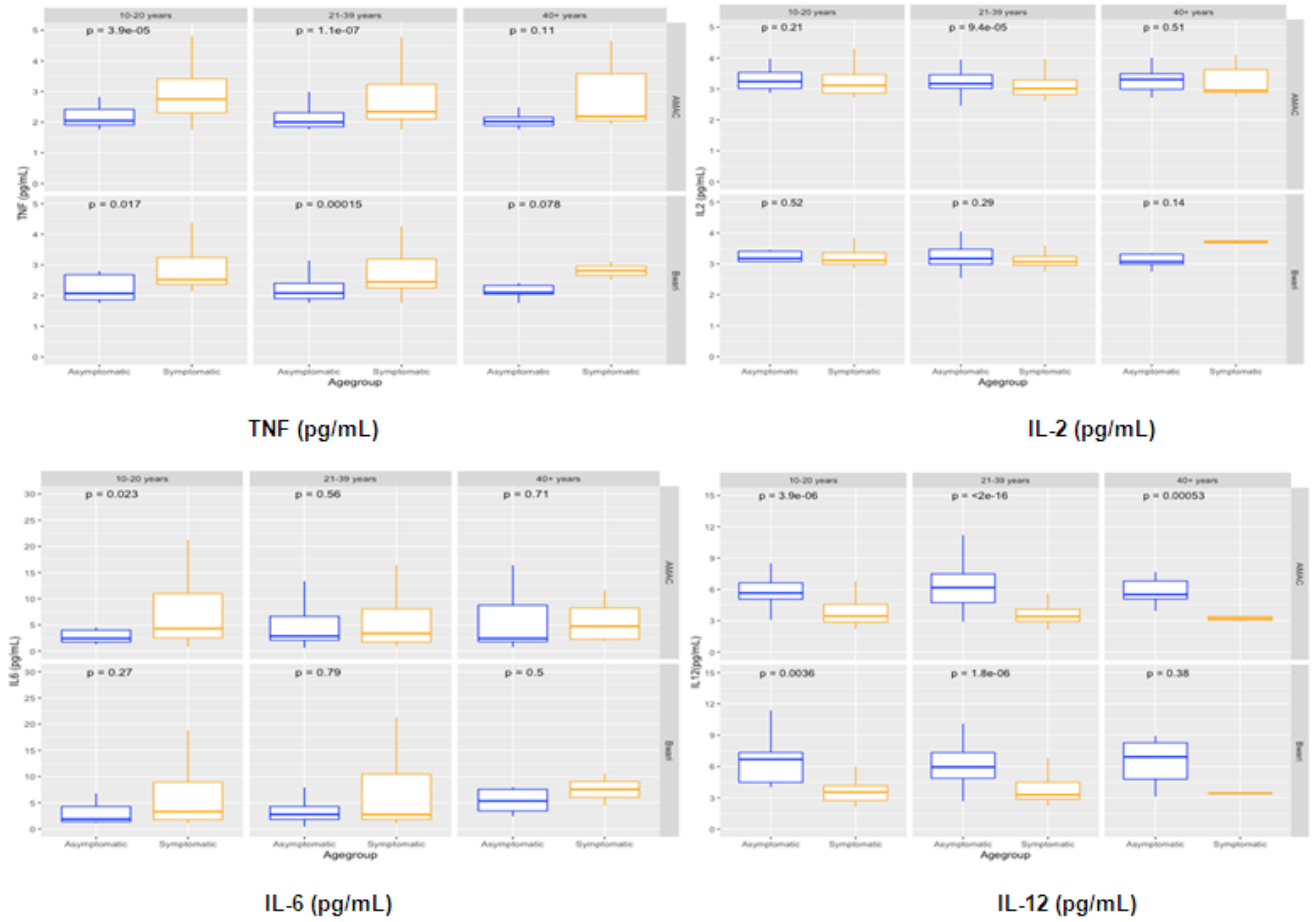

Fig. 2. Pro-Inflammatory Cytokines in Symptomatic and Asymptomatic Malaria Groups Segregated by Age Groups and Catchment Areas (AMAC and Bwari Area Councils)

Plasma TNF-α and IL-12 levels in age groups 10-20years and 21-39years were higher in symptomatic than asymptomatic groups with a statistically significant difference ($P < 0.05$), however, no significant difference was observed in TNF-α levels in age group 40years and above in both catchment areas. This pattern was simila to the observation without segregation. The TNF-α and IL-12 taking similar pattern could also be as a result of the influence of TNF on IL-12. According to [20], TNF primes neutrophils and regulates IL-12 production by macrophages and serves as a co-factor for IL-12-induced IFN-γ production. This indicates that there are other factors that influence symptoms manifestation in *P. falciparum* malaria.

While IL-2 showed a statistically significant difference in age groups 21-39years and 40years+, but no difference in age group 10-20years in AMAC catchment area between symptomatic and asymptomatic groups, it did not show any statistically significant difference in all the age groups in Bwari Catchment area. This however, may suggest a difference in the demographic, immunologic or exposure pattern in the two catchment areas. Although, as other parameters are not in line with this pattern, it may not be of immunological importance. However, the age-associated difference may be of importance as some publications support this result. As published in [21], *P. falciparum*-induced IL-2 differed significantly in school children and older people from infants, suggesting that the production of IL-2 improves with age.

Similarly, IL-6 displays some resemblance to IL-2 in Bwari catchment area, showing no significant difference between symptomatic and asymptomatic cases across all the age groups.

Bwari area council consists of the satellite towns/suburbs of Abuja while AMAC comprises mainly the major city areas of Abuja, although different attraction centres in AMAC pull people from all parts of Abuja to AMAC, thus people from other areas could have contributed to the AMAC study population. This thus suggests a stable *P. falciparum* immunity in AMAC area in the older people (21-39years and 40years+) as high plasma IL-2 level is associated with asymptomatic malaria. This is also in line with the significantly higher IL-6 observed in the symptomatic age group 10-12years in the AMAC population but not Bwari. IL-6 increases with TNF till a level where it supresses TNF production and its level also drops with resultant asymptomatic status. Life styles, different malaria intervention programmes and attitude to malaria case management may vary in these populations and may account for the slight difference in cytokine levels observed in the two study areas. According to [22], various malaria control measures such as insecticide-treated bed nets control, infected human's treatment control, sterile mosquitoes technique control and use of control on pregnant women and newborn births are control strategies which decrease malaria incidence, thus exposure. This thus implies that in case of non-uniform control measures in different parts of Abuja, some level of variations in immune responses are likely to occur.

Limitations of this study include: Other cytokines that may have influence on malaria status were not measured.

4. CONCLUSION

The interplay among different pro-inflammatory cytokines are key in the manifestation of *P. falciparum* malaria. Though pro-inflammatory cytokines are known to support inflammatory diseases, there interaction at different plasma concentrations support different malaria status, symptomatic and asymptomatic in *P. falciparum* endemic place. While TNF-α and IL-6 are high in symptomatic status, IL-2 and IL-12 are high in asymptomatic status. Since these cytokines are all produced in the same *P. falciparum* infestation, this produces a fragile balance between symptomatic and asymptomatic status that can easily lead to status change from symptomatic to asymptomatic and vice versa. Age appear to have influenced the pro-inflammatory cytokine responses to *P. falciparum* status in the studied areas though there was no specific trend in the cytokine pattern across different age groups. The two catchment areas in this study also reveals relatively similar cytokine responses in symptomatic and asymptomatic *P. falciparum* malaria. People in satellite towns and city centres in Abuja demonstrate similar immune responses to *P. falciparum*, thus endemicity and transmission rate may be uniform in both city centres and the satellite towns.

CONSENT

Informed consent of the clients were obtained in writing (signed/thumb printed) from the volunteers or parents/guardian in cases of dependents.

ETHICAL APPROVAL

Ethical approval for this research was obtained from the Federal Capital Territory Health Research Ethical Committee (FCT HREC), with the number: FHREC/2018/01/15/12-02-18.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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