

# Association Between IL-10 Levels With Plasmodium Falciparum Related to Age Groups and Density of Infection Among Sudanese Patients- Khartoum State- Sudan

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**Abstract:** A complex parasite such as human Plasmodium is likely to generate a variety of substances that injure the hosts directly or cause immunopathology. In malaria, a blood concentration of anti-inflammatory cytokines, such as interleukin (IL-10) is increased. The present study was performed to analyze IL-10 levels in patients with malaria falciparum and healthy controls individuals and correlate with malaria density infection as well as age groups. It is a cross sectional study was carried out in Khartoum state /Sudan, a total of 70 febrile patients and 20 healthy volunteers as controls were included in this study. Blood samples were collected from both patients and control candidates in (EDTA) containers for parasitological and immunological tests. Immunochromatography test and microscopic examination for both thick and thin blood films were used to detect the positive samples for Plasmodium falciparum. Enzyme linked Immunosorbant Assay (ELISA), was used for the determination of IL-10 levels. The result showed that of IL-10 levels in malaria patients were 1598.50 and SD 4771.64 while the levels in healthy participants the level was 10.39 and SD was 124.33 the difference was found to be statistically significant ( $P=0.007$ ). The levels of cytokines mentioned above were observed to be raised in malaria individual compared to healthy control candidates.

**Keywords**—IL-10; P.falciparum; ELISA; Parasite Density; Khartoum State

## 1. INTRODUCTION

Malaria remains a major global health threat in the 21<sup>st</sup> century. The number of human infections continues to increase in countries where the disease is endemic as well as in regions where the disease is not endemic [1]. The distribution and severity of malaria depends on the interaction of a number of factors. These include the size of the infective dose of sporozoites, nutritional status of the host, level of acquired immunity, host genetic factors, parasite growth rate, drug resistance status, socioeconomic conditions, standard of health care and education [2]. In areas with lower transmission, infections are less frequent and a larger proportion of the older children and adults have no protective immunity. In such areas, malaria can be found in all age groups. However, in areas with higher transmission, newborns will be protected during the first few months of life by maternal antibodies transferred through the placenta. As these antibodies decrease with time, young children (usually < 5 years of age become vulnerable to disease and consequently death by malaria [3]. Cytokines released during malaria may play both protective and

pathological roles. Cytokine production is triggered via schizont rupture leading to release of substances/molecules such as parasite antigens, merozoites, pigment, glycosylphosphatidylinositol anchor (GPI), and other soluble antigens or toxins that may induce cytokine production. Various cytokines have been shown to take part in malaria protection and/or pathogenesis. These include both the pro-inflammatory cytokines; IFN- $\gamma$ , IL-1, IL-6, TNF- $\alpha$  or anti-inflammatory cytokines, including IL-4, IL-10 and IL-5. The balance between pro- and anti-inflammatory cytokines may determine disease severity. IL-10 overproduction in response to TNF- $\alpha$  may play an important role in severe malarial anemia, but not apparently through down-regulation of TNF- $\alpha$  production [4,5]. Overall, an appropriate production of IL-10 is required to protect against the harmful effects of TNF- $\alpha$ , thereby reducing the possibility of severe anemia and mortality induced by the malaria. Potential protective effects of IL-10 were observed in adult Kenyan volunteers whose peripheral blood mononuclear cells, in response to the liver stage antigen 1 (LSA-1), produced increased levels of IL-10, which

correlated with subsequent resistance to re-infection with *P. falciparum* [6].

## 2. Materials and methods

### 2.1 Study design:

This is a cross sectional study.

### 2.2 Study area and study period:

The study was conducted in Khartoum state-Sudan during period from July 2011 to July 2014.

### 2.3 Study population:

A total of 70 febrile patients (cases) and 20 ages, sex matched healthy controls were included in this study.

### 2.4 Inclusion criteria:

Patients with history of fever proceeding in the past 24 hours with confirmed malaria parasite positive slides and ICT were included study participant's cases and healthy volunteers were included as the study controls. Patients without recent (at least 2 weeks prior to examination).

### 2.5 Exclusion criteria:

All patients who were negatively diagnosed for malaria parasites. Patients with any known concurrent chronic illness such as malnutrition, concurrent infection, tuberculosis or pneumonia. Patients who were on antimalarial drug treatment during the last two weeks.

### 2.6 Sample size:

The classical statistical method for determining sample size based on an unknown proportion of markers was used at 95 percent confidence level and 10 percent precision. Since there is no known figure for the prevalence of malaria, a prevalence rate of 50 percent was used to calculate the sample size using the formula below as described by Mendenhall *et al.* (1981) [7].

$$n = \frac{Z^2 P Q}{d^2} \quad \text{Or} \quad N = Z^2 P (100 - P) / d^2$$

Where n = sample size

P = prevalence rate

Z = 1.96 at  $\alpha = 0.05$  ( $\alpha$  = desired confidence level)

d = desired width of confidence (precision)

Q = 100-P

There for the sample size (n) was determined as:

$$n = \frac{1.96^2 \times 50 \times 50}{10^2}$$
$$n = 96.04 \approx 96$$

## 3. Methods

### 3.1 Sample collection and processing:

Capillary blood for thick and thin film and intravenous blood for serological tests was collected from each candidate.

### 3.2 Collection of samples:

Blood was collected aseptically after the thumb was cleaned with 70% alcohol before pricking. For thick and thin film, blood was collected from the tip of the finger by

pricking with a needle. After that blood was allowed to ooze out one drop of blood was collected on a clean, dust and grease free glass slide. For serological tests, 2.5ml venous blood was collected into the collection tube containing anticoagulant (EDTA) by vein puncture and then processed [8,9].

### 3.3 Preparation of thick film:

After collection of blood on a clean and grease free glass slide, thick film was made by spreading one drop of blood with a spreader evenly on an area about 15×15 mm in diameter. Care was taken to avoid rouleaux formation. Then, the slide was labeled properly and allowed to air-dry by keeping the slide on horizontal position. Precaution was taken during spreading and drying [8].

### 3.4 Preparation of thin film:

After collection of one drop of blood on a clean grease free slide, thin film was made by spreading the blood using a smooth edged slide or spreader at an angle of 45° from the horizontal plane. A well-prepared thin blood film was judged by having a smooth tail end and free of vertical lines and holes. The slide was then labeled properly and allowed to air-dry [8]. Absolute methanol or ethanol was used to fix the thin film. Following steps were taken for fixing the thin film as described by Cheeshbrough (1999) [8]:

- The slide was placed horizontally on a staining rack.

- A small drop of absolute methanol or ethanol was applied to the thin film.

- Then the slide was allowed to fix for 1-2 minutes.

Staining of the films:

The slide was first placed on a staining rack. Then 10% Giemsa stain having a pH of 7.2 was poured gently on the fixed thin film or de-hemoglobinized thick film until the slide was totally covered. Then the slide was allowed to stain for 30-45 minutes out of the sunlight. Then the stain was washed with clean water. Back of the slide was wiped and placed in a draining rack. The slide was then allowed for air-dry [8].

### 3.5 Microscopic examination of the stained film

#### Thick film:

The thick film was examined first by using the 40x objective, a well-stained part of the thick film was selected which was well populated with WBC. Then the selected portion of the film was examined with 100x oil immersion objective by moving along the width of the slide. At least 100 fields were examined before a slide was considered as negative for malaria parasite [8].

#### Thin film:

The thin film was examined by the 40x objective first followed by 100 x oil immersion objectives. After applying immersion oil the film was examined by moving along the edge of the film. Then moving the slide inward by one field, returning in a lateral movement and so on. At least 100 fields were examined before a slide was considered as negative for malaria parasite [8].

### 3.6 Determination of malaria parasitaemia:

**Parasites density determination:**

The method used is based on the number of parasites per  $\mu\text{l}$  of blood in a thick film which are counted in relation to a predetermined number of leucocytes. An average of 8000 leucocytes per  $\mu\text{l}$  is normally taken as the standard. Two tally counters were used to count parasites and leucocytes separately. In each case, the parasites counts in relation to leucocyte count were expressed as parasites per  $\mu\text{l}$  by the following formula as described by WHO (2010) [10].

$$\text{No. of Parasites per } \mu\text{l} = \frac{\text{No. of Parasites} \times 8000}{\text{No. of Leukocytes}}$$

**3.7 Detection of Antibody:**

Antibody was detected by immunochromatographic (ICT) method with Malaria *P. falciparum* *P.vivax* *P.ovale* and *P.malariae* Antibody onsite Rapid Screening Kit. The kit was manufactured by Standard Diagnostics, Inc. Korea. Lot no: 044013. Test procedure performed according to manufactories instructions.

**Principle of the test:**

The Malaria onsite Rapid test is a one step chromatographic immunoassay which specifically detects the antibodies to *P. falciparum*, *P.vivax*, *P.ovale* and *P. malariae* in human serum or plasma. The test utilizes *P. falciparum* specific antigen HRP-II and *P.vivax* antigen aldolase to ensure test specificity and sensitivity.

**Sample collection for serology:**

Two and half milliliters (2.5 mls) of venous blood were collected in EDTA containers at the time of recruitment of participants for determination of cytokines. The blood was then centrifuged immediately and plasma was collected and stored at  $-20^{\circ}\text{C}$  until analysis.

**3.8 Determination of IL-10:**

IL-10 levels expression were determined using specific monoclonal antibodies to IL-10 using sandwich ELISA at 450 nm wavelengths, according to the manufacturer’s instructions (RF).

**3.9 Ethical consideration and permission:**

Ethical approval was sought from the University of Alzaiem Alazhari and individual patients and the purpose and benefit of the study were explained.

**3.10 Data analysis:**

Data was first entered in Microsoft excel, then converted and analyzed using SPSS 15.0.

**4. Results**

The results showed that IL-10 levels in malaria patients was 1598.5 and SD was 4771, while the levels in healthy participants was 10.39 and SD was 124.33. The difference was found to be statistically significant ( $P= 0.007$ ) (Table 1). The results showed that there was positive relation between the parasite density and IL-10 as the correlation coefficient (rho) was found to be +0.072 and the result was found to be statistically insignificant at  $P=0.552$  (Table 2) implied that

when the parasite density was high the IL-10 was high (Figure 1).

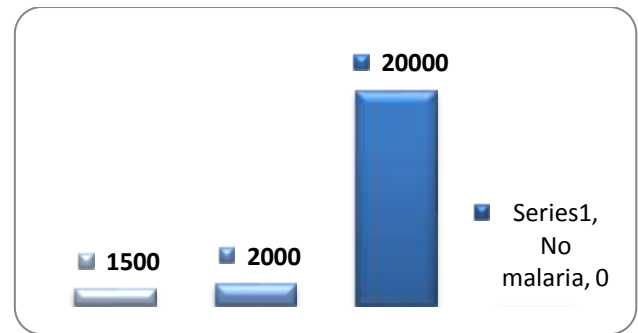
The results showed that there was positive relation between IL-10 and age groups as the correlation coefficient (rho) was found to be + 0.144 and the result was found to be statistically insignificant at  $P=0.235$  (Table 3) showing that the levels of IL-10 was increasing by age increased (Figure 2).

**Table 1:** Mean and SD levels of IL- 10 in patients and controls individuals

Group	Sample	IL-10 (pg/ml)	P.value
Patients	Mean	1598.50	0.007
	SD	4771.64	
Control	Mean	10.39	
	SD	4771.64	

**Table 2:** Correlation between parasite density and IL-10

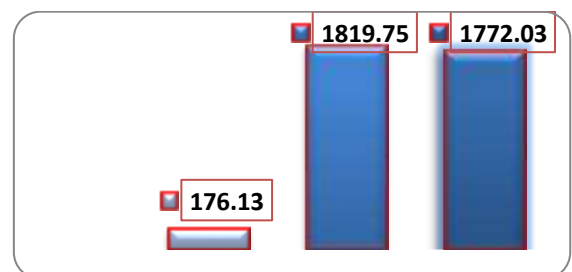
	Parasite Density (parasites/ $\mu\text{l}$ )	P.value
IL-10	+ 0.072 (rho)	0.552



**Figure 1:** Correlation between parasite density and IL-10

**Table 3:** Correlation between IL-10 and age groups

	Age groups	P.value
L-10	+0.144 (rho)	0.356



**Figure 2:** Correlation between IL-10 and age groups

## 5. Discussion

The study aimed to determine the existence of a correlation between IL-10 cytokine and malaria infection. The role of cytokines in the pathogenesis of malaria has been clearly confirmed, but not completely understood. It is thought that many factors impact on their level, which in the course of malaria depends on the circadian rhythm, and in accordance with this, there are large variations in the cytokine levels.

The anti-inflammatory cytokines, IL-10 levels were elevated in patients with malaria compared to healthy controls; older patients had much greater elevated levels of IL-10 compared to the other age groups.

The higher levels of IL-10 in older patients could be due to their ability to mount strong T-helper type 2 responses quite early in infection.

Experimental malaria studies performed exclusively in adult hosts indicated that both CD4+ and CD8+ T cells play an important role in the defense against malaria. To address the role of these cells in an age-dependent model, where young susceptible rats can be protected by the transfer of whole spleen cells from adult protected rats, TCR cells were transferred to young infected rats. These experiments indicated that 58% of young rats recovered from infection after T cell transfer [11].

Furthermore, IL-10 has an important role in immunoregulation down regulation of cytokines production (predominantly TNF- $\alpha$ , IL-6 and IL-12), which have Th1 function and as a result, Th1 functions are inhibited and natural killer cell activity is promoted [12].

This finding is consistent with our suggestion that the balance between the Th1 cytokine TNF- $\alpha$  and the Th2 cytokine IL-10 is critical in the pathogenesis of severe disease in *P. falciparum*-infected persons who live in areas in which malaria is endemic.

Consistent with the present results, study carried by Linke *et al.* (1996) [13] showed that IL-10 gene knockout mice with an intrinsic deficiency for IL-10 production when infected with *Plasmodium chabaudi chabaudi* succumb to severe disease and higher mortality than their heterozygote counterparts or normal mice. An enhanced Th1 response persisted throughout the course of infection in the IL-10-deficient mice, while in control mice a Th2 response was predominant. In another study carried by Kossodo *et al.* (1997) [14], the exogenous administration of IL-10 to susceptible CBA mice protected them from *Plasmodium berghei*-induced cerebral malaria while an in vivo neutralization of IL-10 in resistant BALB/c mice induced a neurologic syndrome. Collectively, these findings support the hypothesis that IL-10 is a critical factor in down-regulating the pathogenesis of severe malaria.

The younger children appear not to maintain IL-10 levels in response to inflammatory process, hence, probably the observed relative lower levels of cytokine compared to their older counterparts. This in turn may explain the younger children's relative higher susceptibility to anemia as reported

by Nussenblatt *et al.* (2001) [15] as they do not mount enough Th2 response to counteract the suppressive erythropoiesis effects of TNF- $\alpha$ .

## 6. Conclusion:

This study concluded that there was positive relation between IL-10 level and malaria infection among Sudanese patients related to age groups, showing that the levels of IL-10 was increasing by age increased.

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