

ELEVATED PLASMA LEVELS OF TNF-ALPHA, INF-GAMMA, IL-10 AND TGF-BETA IN MALARIA PATIENTS FROM TWO MALARIA NON-ENDEMIC REGIONS IN KARNATAKA, INDIA

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Abstract

Purpose: In India, *Plasmodium vivax* malaria is endemic and accounts for 50-55% of the total malaria burden in the country. There has been limited sero-epidemiological data available from malaria non-endemic regions in Karnataka state. In this study, we aimed to evaluate the plasma levels of Tumor necrosis factor- α (TNF- α), Interferon- γ (IFN- γ), Interleukin-10 (IL-10), and Transforming growth factor- β (TGF- β) and correlate with malaria parasitaemia and infection type in vivax and falciparum malaria cases reported from two study centres.

Methods: This hospital-based cross sectional observational study was conducted at BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapur, Karnataka and BGS Global Institute of Medical Sciences, Bengaluru, Karnataka during 2016 to 2019. A total number of 45 microscopy positive and molecularly confirmed malaria cases were included in the study. Plasma samples were analyzed for the concentrations of four cytokines by Enzyme-linked immunosorbent assay (ELISA). 20 uninfected healthy volunteers were used as controls. Correlation of cytokines and parasitemia was done using Pearson correlation analysis.

Results: The results show an overall significant elevation of plasma TNF- α ($p < 0.05$), IFN- γ ($p < 0.005$), IL-10 ($p < 0.001$), and TGF- β ($p < 0.001$) in malaria patients compared to healthy controls. Except TNF- α ($p < 0.001$), there was no significant difference in infection type specific immune responses. No significant correlation was seen among all the four cytokines with parasite load. A Receiver operating curve (ROC) was generated and showed that TNF- α , IL-10, and IFN- γ were the best individual predictors of malaria.

Conclusions: We conclude that significantly elevated plasma concentrations of TNF- α , IL-10, IFN- γ and TGF- β in both *P. vivax* and *P. falciparum* cases suggest their active involvement in mounting defensive immune response against malaria infection.

Keywords: Malaria, *Plasmodium vivax* malaria, TNF- α , INF- γ , IL-10, TGF- β , Karnataka

1. Introduction

Infection with malaria remains a major cause of significant morbidity, mortality and imposes significant economic loss across the globe making it a major global health care threat. It is endemic particularly in most of the tropical and subtropical countries where nearly half of the world's population is resided and are ever at the

risk of malaria infections^[1]. This devastating disease is caused by a hemoprotozoan, single-celled protozoan parasites of the genus called *Plasmodium* that spreads by the vector-female *Anopheles* mosquitoes. Among the 5 species of *Plasmodium* parasites, *P. falciparum* and *P. vivax* are the two major culprits of causing malaria. There were estimated incidences of more than 219 million clinical cases in the year 2017-18 and 435,000 deaths

were reported from 87 countries worldwide ^[2]. India is considered to be a major contributor to the worldwide *P. vivax* malaria, accounting for 50-55% of the total malaria burden in the country, and remainder cases being caused by *P. falciparum* and a few cases by *P. malariae* and of *P. ovale*. India accounts for approximately two-thirds of the confirmed malaria cases (~1.09 million clinical cases) and 331 deaths were reported in the subcontinent region ^[2]. National Vector Borne Disease Control Programme (NVBDCP) estimated a total of 0.84 million confirmed malaria cases and 194 related deaths in 2017 ^[3]. *Plasmodium vivax* malaria was once thought to be benign and also was long considered to cause low mortality, but recent studies reported from some geographical areas suggest it as a more virulent form and more common than previously thought leading to severe malaria and life threatening complications ^[4, 5].

Clinical manifestations of malaria infection differ and appear to be regulated by several factors such as age and the acquisition of immunity, host and parasite genetic polymorphisms, and regional variation ^[6-8]. Malaria is an inflammatory response-driven disease and immune responses against circulating parasite play key roles both in host protection and pathogenesis. Initial pro-inflammatory responses such as inflammatory cytokines are essential for clearing malaria parasites and a finely tuned balance is required between inflammatory and regulatory cytokine responses for controlling disease progression and parasite clearance ^[9]. Early production of proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-6, and other inflammatory cytokines allow faster inhibition and clearance of parasite and stimulate monocyte phagocytosis ^[10, 11]. As the infection progresses, pro-inflammatory responses are gradually downregulated with a parallel increase in anti-inflammatory responses such as IL-10 and transforming growth factor (TGF)- β resulting in balanced pro-/anti-inflammatory responses that regulate pathogenesis to protect against severe complications ^[12]. Findings from two studies suggested that *P. vivax* elicits greater host inflammation than *P. falciparum* ^[13, 14]. Contrary to these two reports, a study from Brazil reported similar levels of regulatory cytokines per parasitized red blood cell in both vivax and falciparum malaria ^[15]. A study from the central zone of India reported preliminary data on pro- and anti-inflammatory cytokine profiles and their association with clinical signs of mild anemia in *P. vivax* malaria patients ^[16]. Parasite specific factors like adhesion, sequestration, release of bioactive molecules and host inflammatory responses like cytokines, chemokines production and cellular infiltration are responsible for the pathogenesis of severe malaria ^[17, 18]. Thus, the analysis of clinical,

biochemical profile and a thorough understanding of the immunological responses in the serum/plasma of the patient are necessary to know the degree of morbidity and pathophysiological changes associated with malaria infection.

Most of the available studies on malaria outbreak in Karnataka state are from Mangaluru, a malaria endemic south-western city in India. However, there is a paucity of data on malaria outbreak and disease transmission in non-malaria endemic regions in Karnataka state. A detailed hospital-based cross-sectional observational investigation is needed to study malaria cases reported to tertiary care hospitals in such areas. The objective of the present study was to describe the sero-epidemiological features of the laboratory-diagnosed malaria cases and to analyse immunological responses shown by affected population in the non-malaria endemic regions, Vijayapur and Bangalore from Karnataka state. We analyzed plasma samples from malaria subjects together with samples from healthy individuals to diagnose malaria with species identification and to determine the concentrations of different cytokines.

2. Material and Methods

2.1 Study Area, Subjects, and Ethical approval

This hospital-based cross sectional observational study was conducted at two study centres, BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapur, Karnataka and BGS Global Institute of Medical Sciences Bengaluru, Karnataka. Geographically separated by around 530 km of distance, both the study centres are tertiary referral centres in Karnataka state where the patients reach not only from Bangalore and Vijayapur but also from the surrounding districts and bordering states. The study was conducted during 2016-2019 for a period of 3 years. All patients from 15 to 70 years age of both the genders that were presenting with symptoms of malaria were screened and cases that were smear positive or antigen positive for plasmodium parasite were included in the study. 600 blood samples from malaria suspected patients were screened and among them 45 malaria positive cases were further analyzed for infected parasite species, clinical conditions, and four key cytokines that are produced in response to infection. Samples from 40 uninfected healthy individuals were also included in the study as controls. The study protocol was approved by the institutional review committees of both the study institutions BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapur & BGS Global Institute of Medical Sciences Bengaluru. All the participants were orally explained about the study

and were recruited. Pregnant women, immunocompromised individuals, patients on anticancer drugs, chronic alcoholics, patients partially treated with antimalarial drugs in recent past, individuals with any underlying diseases like diabetes, rheumatoid arthritis were excluded.

2.2 Blood Collection, Plasma separation and Storage

Before giving any antimalarial medications, about 5 to 10 ml of venous blood was drawn aseptically and collected into sterile tubes containing EDTA anti-coagulant. Two aliquots were made for each collected blood sample. One aliquot was centrifuged to separate plasma and used for rapid malaria test and rest of the plasma was stored at -80°C till cytokine analysis by ELISA. The second aliquot of blood was labelled and stored at -80°C for further molecular studies.

2.3 Microscopy, Malaria diagnosis and Mean Parasite density

The malarial infections were confirmed by careful microscopic examination of Giemsa stained peripheral blood smears. Thick and thin blood smears were made, stained by Giemsa stain (4%) and examined for the parasitological evaluations of malaria parasites under 100X magnification using an oil immersion objective. The number of parasites was counted against 200 white blood cells (WBC). The parasite density per microliter of blood was determined by multiplying the number of parasites counted by number of WBC divided by 200 [19]. In addition to microscopy, Immunochromatographic principle based malaria rapid diagnostic tests (RDTs) using SD BIOLINE Malaria P.f / P.v test kit was done to detect the evidence of malaria specific antigen in the plasma of study subjects. In addition to the microscopy, PCR analysis was also carried out for malarial diagnosis of *Plasmodium vivax* infected cases.

2.4 Determination of Plasma Cytokine levels using ELISA

Plasma concentrations of Inflammatory cytokines TNF- α , IFN- γ , IL-10 and TGF- β were quantified using solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) kits (Dialclone, France) in strict adherence to the kit manufacturer's instructions. Recombinant lyophilized native human cytokines supplied in the kit were used to obtain standard curves ranging from 12.5 to 2000 pg/ml. All samples were tested in duplicates. Plasma samples added on to the wells in the ELISA plate coated with specific antibody against test cytokine, a biotin-conjugated primary antibody was added and incubated. After a wash step Streptavidin-HRP that binds to the biotin-conjugated primary antibody was added. Following the incubation and subsequent wash,

substrate solution reactive with HRP was added to the wells. Coloured products were formed and absorbance was measured at 450 nm.

2.5 Statistical analysis, Statistical methods

Data were analyzed using statistical software GraphPad Prism 8 for Windows (SPSS Inc., Chicago, IL, USA). All characteristics were summarized descriptively. Quantitative variables are shown as N, the mean \pm standard deviation (SD). For categorical data, the number and percentage were used in the data summaries. Pearson Correlation test was employed for correlation analysis. ROC analysis for was done to check sensitivity and specificity. P-value < 0.05 was considered to be statistically significant.

3. Results

3.1 Demographics of Study subject characteristics and Malaria diagnosis

After screening of a total of 600 blood samples from malaria symptomatic cases, a total of 45 patients were found to be positive for malaria by three diagnostic methods; microscopic observation, antigen detection by RDTs and molecular confirmation by PCR (PCR was done only for *P. vivax* cases). Out of these 45 malaria study participants, 33 (73%) were caused by *P. vivax*, 10 (22.2%) were *P. falciparum* cases and 2 (4.4%) were of mixed infection (*P. vivax* + *P. falciparum*) cases. The diagnostic results are summarized in the below Table-1. Nine cases that were negative by microscopy but positive by RDT were not included into the study. The study comprised 35 (77.8%) cases of males and 10 (22.2%) cases females and all these recruited subjects were adults with mean age of $29.58 \pm \text{SD } 9.40$ years. The mean parasitic density in terms of percentage was found to be $1.48 \pm \text{SD } 0.88$.

Table 1: Summary of malaria diagnosis results

Sample	Microscopy		RDT		PCR (Only <i>P. vivax</i> Cases)	
	Positive	Negative	Positive	Negative	Positive	Negative
Number of malaria confirmed cases (n=45)	45	6	51	0	33	0
Healthy Controls (n=20)	0	20	0	20	0	20

3.2 Inflammatory Cytokines Profile of the Study Participants

Both the selected pro-inflammatory (TNF- α and IFN- γ) and anti-inflammatory (IL-10 and TGF- β) markers in the present study were found to be significantly elevated in the plasma of malaria cases compared to healthy controls (Table-2, Figure-1). Mean values of IL-10, TGF- β , and IFN- γ was found not significantly different among

the type of malaria infection except TNF- α , which was significantly elevated in subjects with *P. vivax* infection compared to *P. falciparum* affected cases (Table-3, Figure-2). There was no significant difference in the levels of all the four inflammatory markers and mean parasite density between both the genders (Data not shown). Hence, gender was not a significant factor that affects the cytokine levels in malaria infected patients.

Table 2: Inflammatory cytokine levels between malaria cases and healthy controls

Parameter	Group	Mean \pm SD	p-value
TNF- α (pg/ml)	Healthy Control	6.19 \pm 4.34	p<0.05*
	Cases (Pv + Pf)	100.56 \pm 178.03	
IFN- γ (pg/ml)	Healthy Control	1.33 \pm 1.51	p<0.005*
	Cases (Pv + Pf)	76.48 \pm 83.21	
IL-10 (pg/ml)	Healthy Control	2.41 \pm 1.59	p<0.001*
	Cases (Pv + Pf)	224.07 \pm 240.74	
TGF- β (pg/ml)	Healthy Control	68.02 \pm 7.01	p<0.001*
	Cases (Pv + Pf)	79.71 \pm 15.88	

Note: * p-value < 0.05 was considered to be statistically significant.

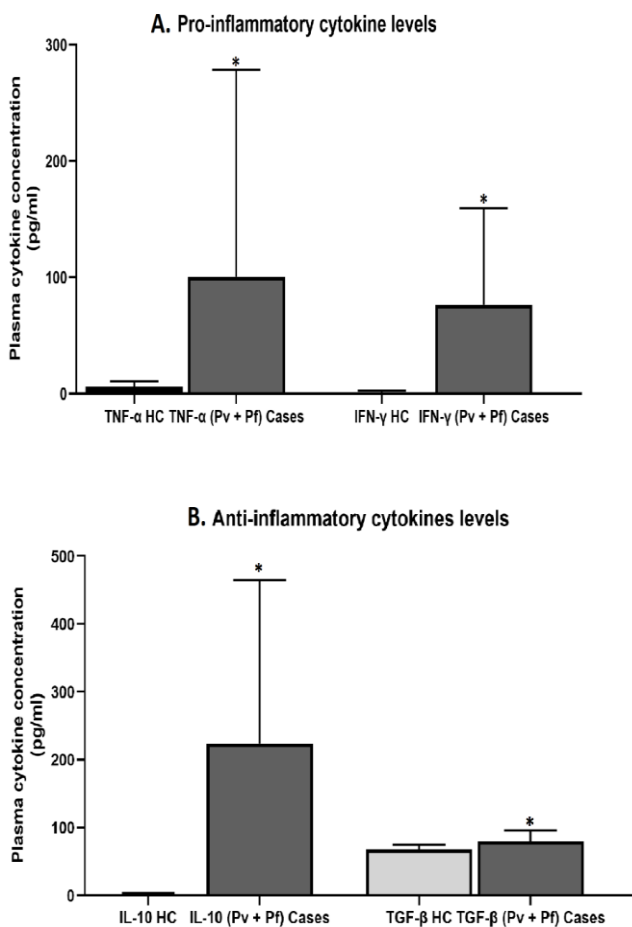


Figure 1: A. Pro- B. Anti-inflammatory cytokine levels between malaria cases and healthy controls

Table 3: Comparison of cytokine levels among the type of malaria infection

Parameter	Group	Mean \pm SD	p-value
TNF- α (pg/ml)	Pv malaria Cases	47.57 \pm 70.52	p< 0.001*
	Pf malaria Cases	266.19 \pm 306.65	
IFN- γ (pg/ml)	Pv malaria Cases	63.90 \pm 80.31	p= 0.053
	Pf malaria Cases	122.78 \pm 88.22	
IL-10 (pg/ml)	Pv malaria Cases	233.10 \pm 240.03	p= 0.524
	Pf malaria Cases	177.50 \pm 239.54	
TGF- β (pg/ml)	Pv malaria Cases	78.58 \pm 16.30	p= 0.342
	Pf malaria Cases	83.97 \pm 12.57	

Note: * p-value < 0.05 was considered to be statistically significant.

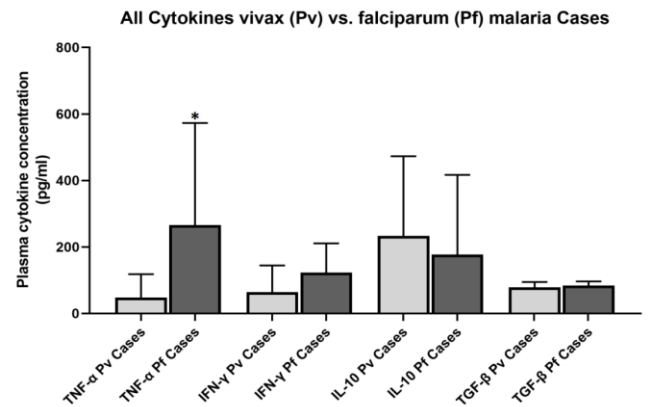


Figure 2: Comparison of cytokine levels among the type of malaria infection

3.3 Correlation among Parasite density and Cytokines
Correlation results have been tabulated below (Table-5). No significant correlation was seen among all the four cytokines with parasite load ($r = 0.253$ & $p = 0.094$, $r = -0.002$ & $p = 0.987$, $r = 0.087$ & $p = 0.569$ and $r = -0.050$ & $p = 0.743$ respectively).

Table 4: Analysis of Correlation among Parasite density and Cytokines

Pearson Correlation r with p-value	Parasite Density (%)	TNF- α All Cases	IFN- γ All Cases	IL-10 All Cases	TGF- β All Cases
Parasite Density (%)	$r = 1.000$	$r = 0.253$ $p = 0.094$	$r = -0.002$ $p = 0.987$	$r = 0.087$ $p = 0.569$	$r = 0.050$ $p = 0.743$
TNF- α All Cases	$r = 0.253$ $p = 0.094$	$r = 1.000$	$r = 0.202$ $p = 0.183$	$r = -0.122$ $p = 0.425$	$r = 0.210$ $p = 0.165$
IFN- γ All Cases	$r = -0.002$ $p = 0.987$	$r = 0.202$ $p = 0.183$	$r = 1.000$	$r = 0.230$ $p = 0.128$	$r = -0.030$ $p = 0.844$
IL-10 All Cases	$r = 0.087$ $p = 0.569$	$r = -0.122$ $p = 0.425$	$r = 0.230$ $p = 0.128$	$r = 1.000$	$r = -0.702$
TGF- β All Cases	$r = 0.050$ $p = 0.743$	$r = 0.210$ $p = 0.165$	$r = -0.030$ $p = 0.844$	$r = -0.702$	$r = 1.000$

Note: p-value < 0.05 was considered to be statistically significant.

3.4 ROC Analysis for studying association of parameters

A Receiver operating curves (ROC) were generated (Figure-4) and results (Table-6) showed IL-10 and IFN- γ

were found to be better and significant predictors of malaria than TNF- α and TGF- β .

Table 5: ROC Results

Test Variables	Result	Area under the curve	Std. Error	Sensitivity	Specificity	p-value
TNF- α		0.7956	0.056	82.22%	20%	<0.0001*
IFN- γ		0.8667	0.046	86.67%	55%	<0.0001*
IL-10		0.9561	0.026	97.78%	25%	<0.0001*
TGF- β		0.6922	0.063	NA	NA	<0.0139*

Note: * p-value < 0.05 was considered to be statistically significant.

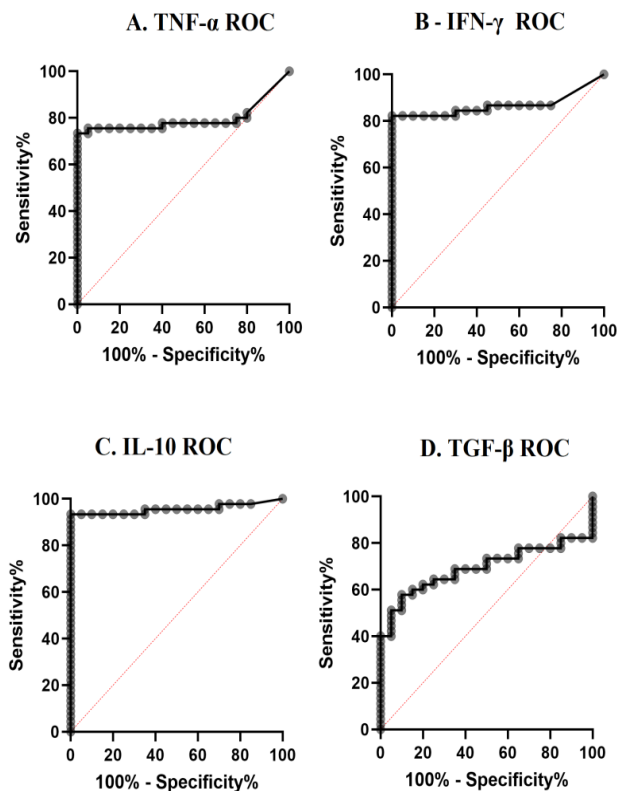


Figure 3: ROC Results A. TNF- α , B. IFN- γ , C. IL-10, and D. TGF- β

Discussion

Among the malaria infections caused by two predominant parasites *P. vivax* and *P. falciparum* in India, vivax malaria accounts for more than 53% of the estimated cases and continued as a substantial health and economic burden in the country^[20, 21]. The present study shows the higher incidences of *P. vivax* malaria (73%) over *P. falciparum* cases (22.2%) in the study regions. State National Vector Borne Disease Control Programme had earlier reported the very low slide positivity reflecting the very low transmission rates in 2008-2014 in Karnataka state^[22]. In our study, the mean parasitic density in terms of percentage was found to be

1.48 \pm SD 0.88. However, we could not see any differential changes in the parasite density among infection types and between genders.

The balance in the inflammatory and immunological responses especially, between the pro- and anti-inflammatory cytokines in the host is needed for malaria protection and parasite clearance. In the present study, we found the significantly elevated levels of both pro- and anti-inflammatory cytokines indicating the active host immune responses towards parasite growth. A study on murine and human models has shown the inverse correlation between TGF- β , an anti-inflammatory cytokine and malaria severity^[23]. Plasma levels of TGF- β in the subjects of present study could possibly explain its contribution for the balance between inflammatory marker levels. A study from Brazil reported the higher levels of interleukin IL-10 and an elevated IL-10/TNF- α ratio in the plasma of symptomatic vivax malaria patients compared to falciparum or mixed-species malaria patient groups^[15]. In contrast, though we observed an increase in the levels of IFN- γ , TNF- α , IL-10 and TGF- β in both types of malaria infections but, except TNF- α , we could not see any significant differences in the levels of other three cytokines between *P. vivax* and *P. falciparum* malaria patients. However, in our study, sample size of falciparum cases (n=10) are not matching with that of vivax malaria cases (n=33), hence, provided the matching sample size in both the groups cytokine profiling outcome would reveal different pattern of results.

The major limitation in the present study was the partial collection of clinical data owing to the lack of sufficient number of health personnel, difficulty to manage and properly execute the study at remotely located two different study centres. Severity of disease was not determined for the cases and biochemical and haematological investigation reports such as platelet abnormalities, thrombocytopenia, leukopenia and anemia were not collected. This study had additional limitations, including the categorization of samples into symptomatic or asymptomatic and lack of recording of the exact timing of infection during sample collection.

To conclude, our study shows that both the patient groups infected with *P. vivax* and *P. falciparum* had significantly elevated plasma concentrations of IL-10, TNF- α , IFN- γ and TGF- β compared to uninfected healthy controls. This suggests involvement of these inflammatory cytokines in the mounting of active immune response towards infection. However, except TNF- α , there was no significant difference in the inflammatory markers between infection types. Malaria cases in the present study were from non-endemic regions in Karnataka state and further studies are

needed to understand the role of parasite and host genetic diversities, their association with malaria disease severity and other clinical complications during the pathogenesis of the disease.

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