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Serum TNF alpha levels: a prognostic marker for assessment of severity of malaria

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Abstract. Complicated *Plasmodium falciparum* infection is associated with a 6.4% mortality rate in India, yet its prognostication is incompletely understood. The conventional prognostic markers of falciparum malaria include clinical, haematological and biochemical parameters. However these factors are non-specific. Hence there is a need of an accurate inexpensive objective marker for prognosticating falciparum malaria infection outcomes. Angiopoietins, angiogenic factors, eotaxins, adhesion molecules and inflammatory cytokines have been studied for prognostication of this common disease. Determination of the first four is technically difficult and requires a high level of expertise and equipment. Intermediary cytokines have the most promising role. This study was conducted with the aim to evaluate the serum level of TNF-α in patients with *P. falciparum* malaria and carry out statistical analysis of levels of serum TNF-α with parasite index, age, severity of anaemia, hypoglycaemia, hepatic and renal dysfunction. In our study the average TNF alpha level in 91healthy controls was 46.42 pg/ml whereas that in mild falciparum malaria was 100.45 pg/ml, in severe malaria – 278.63 pg/ml and in cerebral malaria it was 532.6 pg/ml. The mean TNF alpha level was significantly different in severe malaria and cerebral malaria compared to that in healthy controls ($p < 0.02$). The difference in levels of TNF alpha was significantly higher in falciparum malaria patients with anaemia, altered liver functions, hyperparasitemia, leucocytosis, hepatosplenomegaly and hypoglycaemia. The TNF levels did not correlate well with haemolysis markers and patients with altered renal function. Hence a raised TNF alpha can predict the likelihood of oncoming anaemia, hypoglycaemia, altered hepatic function and leucocytosis but not the grades of malaria. The duration of stay in hospital and change in parasite index between the 5th day and the 1st day of admission was used a clinical outcome marker in this study. The analysis showed that serum TNF alpha was raised significantly ($p= 0.001$) in patients with longer duration stay in hospital. The cytokine was significantly raised in patients having disorientation /cognitive disorder /coma and ARDS ($p= 0.001, 0.0023$ respectively). The study concluded that serum TNF alpha if done at time of admission and on day 3 can indicate the severity of disease and its complications.

INTRODUCTION

Malaria is one of the most important global health problems, potentially affecting more than one third of the world's population. Falciparum malaria infection is associated with a 7-32% mortality rate depending on organ/systems involved (Krishnan *et al.*, 2003). Falciparum malaria, a clinically complex syndrome, is associated with increased levels of pro-inflammatory cytokines like tumour necrosis factor (TNF)-α, interferon (IFN)-γ and lymphotoxin (Hunt *et al.*, 2003). Recent studies (Wassmer *et al.*, 2004) have shown that mechanical blockage due to sequestration of parasitized red blood cells (pRBCs), leukocytes and platelets (Deininger *et al.*, 2003), secretion of cytokines and chemokines, angiogenic failure, immune status/genetic background of the host and parasite factors (Hunt *et al.*, 2003) are involved in the pathogenesis of falciparum malaria. Severe malaria has been associated with high TNF-α plasma levels,

increased production of other cytokines (IFN-γ and IL-1β) (lyke *et al*., 2004) and decreased production of anti-inflammatory cytokines, notably IL-10 and TGF-β (Esamai *et al*., 2003). Pro-inflammatory Th1-type cytokines (e.g. TNF-α, IFN-γ, interleukin IL-1β, and IL-6) are thought critical for the control of exoerythrocytic and erythrocytic *Plasmodium falciparum* infection, but their increased production may also contribute to organ damage, particularly in the brain. The clinical studies (Beutler *et al*., 1985) demonstrated TNF to cause toxic side effects such as headache, nausea, vomiting, fever, chills and myalgia.

Moncunill *et al.* (2013) in a recent study showed that children infected with falciparum malaria had higher levels of TNF and IL-1β as compared to adults thereby suggesting a more effective ability of the young to regulate pro-inflammatory and anti-inflammatory responses. The authors have explained this due to constant exposure to parasite, a state of tolerance develops in adults. Zhou *et al.* (2012) concluded that secretion of TNF and IL-1β in a falciparum malaria patient is dependent on Fcγ receptor-mediated phagocytosis; for IL-1β, this occurs by activation of the inflammasome. The study (Zhou *et al*., 2012) showed that TNF alpha is potentially beneficial both in reducing parasitaemia via Fcγ receptor-dependent macrophage phagocytosis and in generating an effective pro-inflammatory response.

We investigated the serum TNF alpha levels in active falciparum malaria cases, and correlated these levels with differing clinical symptoms, signs and other biochemical and haematological parameters. The serum TNF alpha levels in the study group were compared to conventional prognostic markers and clinical outcomes including complications.

MATERIAL AND METHODS

This was a multicentric study carried out in three hospitals simultaneously. All primary cases of falciparum malaria diagnosed using light microscopy and immuno-chromatography were included in the study. Careful history taking inclusive of number of days of fever, associated complaints of chills/ rigors and intake of chemoprophylaxis was done. The general examination documented was: presence of icterus, pallor, CNS/ respiratory involvement, hepatomegaly and splenomegaly. The patients were analyzed for the following parameters on the day of admission, day-3 and day-5 of the hospital stay: haemoglobin, blood sugar, serum urea, creatinine, albumin, bilirubin, alanine aminotransferase (ALT), aspartate amino-transferase (AST), lactate dehydrogenase (LDH) parasite index and serum TNF-alpha levels. The immunochromatography for *P. falciparum* was done using First Sign Para view-2 Cards. TNF alpha in serum was estimated using the sandwich ELISA technique using kits of Immunotech (Beckman Coulter). 91 healthy controls with no history of malaria, fever or any source of infections were included as negative controls.

All cases of falciparum malaria were further classified based on WHO (2000) criteria into following: Mild Malaria: Febrile illness in a subject with asexual *P. falciparum* parasites in blood film, without any other satisfactory explanation for the fever, and without cerebral or severe malaria. Severe Malaria: Subjects with parasite index of more than 1% and one of the following - hypoglycaemia (blood sugar < 60 mg/dl), or severe anaemia (Hb< 7 gm/dl), serum creatinine > 2 mg/dl and serum transaminase > 80IU/L. Cerebral Malaria: Patients were considered to have cerebral malaria if they were comatose and had detectable *P. falciparum* asexual forms in peripheral blood and if there was no other recognized cause of alteration of consciousness.

Demographic variables were compared across the 3 study groups using analysis of variance (for continuous variables). Non-parametric test (Mann-Whitney rank sum test) was used for TNF alpha analysis. Normally distributed data were analysed using Student's t-test (all p values are two tailed). Pearson's correlation coefficient was used to evaluate the relationship within normally distributed variables. P values <0.05 were considered significant.

RESULTS

A total number of 154 cases of falciparum malaria were diagnosed during the 2 years. The average age of patients in this study was 32.51 years (range 22-67 years). There were 144 males and 10 females in the study. Based on defining criteria discussed in methodology the cases were divided clinically into 3 categories. The majority (138) had mild malaria; 14 cases had severe and 2 had cerebral malaria. The mean serum TNF alpha levels in these three groups were 100.45 pg/ml, 278.63 pg/ml and 532.6 pg/ml respectively.

The average duration of fever before the hospital admission was 2.88 days range (1-15 days). The average duration of hospital stay was 5.73 days range (2-15 days). Two cases in this study died during the course of treatment. The mean (range) of TNF-alpha in 91 healthy individuals was 46.42 (1.562-164.49)pg/ml. The levels of TNF were significantly different in severe malaria and cerebral malaria compared to that of healthy controls. However no statistical difference was found between the mild malaria serum TNF alpha levels and that of healthy controls (Table 2). This signifies that the raise in serum TNF alpha clearly depicts the severity of the disease.

DISCUSSION

Although more than 500 million *P. falciparum* malaria infections are estimated to occur each year, only few of the patients progress to severe and potentially fatal complications such as cerebral malaria (CM) (WHO, 2000). However, the mechanisms underlying CM are poorly understood, and limited prognostic tools are available to medical fraternity to determine which infected individuals will progress to cerebral complications (Dzeing *et al.*, 2005).

Table 1. Clinical findings in different groups of malaria patients

	Palpable spleen	Palpable Liver	Icterus	Pallor	Cognitive disorder/ Disorientation/ Coma	Respiratory Insufficiency
	No. of cases (%)					
Mild Malaria (n=138)	59 (42.75%)	47 (34.05%)	36 (26.08%)	14 (10.1%)	1 (0.72%)	0
Severe Malaria (n=14)	10 (71.4%)	10 (71.4%)	12 (85.7%)	4 (28.57%)	5 (35.7%)	0
Cerebral Malaria (n=2)	2 (100%)	2 (100%)	2 (100%)	0	2 (100%)	2 (100%)

Table 2. Statistical analysis (Mann-Whitney rank sum test) to test significance between the serum TNF alpha levels of healthy controls against various groups of malaria patients

Correlations of level of serum TNF alpha between 2 groups	P value
Serum TNF of 154 malaria patients Vs. 92 Healthy controls	4.211
Serum TNF of 2 cerebral malaria patients Vs. 92 Healthy controls	<0.0004479
Serum TNF of 14 severe malaria patients Vs. 92 Healthy controls	< 0.02781
Serum TNF of 138 mild malaria patients Vs. 92 Healthy controls	1.31

Endothelial cell activation and dysfunction have been implicated in the pathogenesis of CM (Turner *et al*., 1994), in which the endothelium responds to parasite-induced inflammation and mediates the parasitized erythrocyte sequestration, especially in vital organs such as the brain and kidney. Angiopoietin-1 (ANG-1) is constitutively expressed and acts to maintain vascular function. The ANG-1 stabilizing effect is antagonized by angiopoietin-2 (ANG-2), which primes the endothelial activation response and promotes vascular permeability (Fiedler *et al*., 2006; Parikh *et al*., 2006).

Elevated levels of TNF have also been associated with severe malaria (Akanmori *et al*., 2000) and were identified as a predictor of mortality in CM (Grau *et al*., 1993). However, other studies have challenged these findings and reported that TNF levels do not correlate with disease severity (Gimenez *et al*., 2000). Our study was carried out to test the hypothesis that TNF alpha is raised with higher grade of malaria and signifies worse outcome. In our study the mean age of patients was 32.51 years (range 22-67 years).

TNF alpha is produced and released by host cells following exposure to various malarial antigens. The increase of TNF alpha

Table 3.The changes in average values of various haematological and biochemical parameters of 154 malaria patients on Day 0, Day 3 and Day 5 of admission

(n=154)	Day 0: average (range)	Day 3: average (range)	Day 5: average (range)
Haemoglobin (gm/dl)	12.9 (6.8-15.8)	12.8 (7-15.6)	12.8 (6.7-15.5)
TLC (/cmm)	7698 (2600-14800)	7592 (2900-13700)	7693 (3200-13200)
ESR (mm fall 1st hr)	17.3 (6-58)	16.2 (6-42)	14.3 (4-40)
FDP (Pos/Neg)	4 cases positive	4 cases positive	2 cases positive
Serum Urea (mg/dl)	32 (17-85)	34.3 (18-133)	31.1 (18- 192)
Sr Creatinine (mg/dl)	0.98 (0.5-2.7)	0.96 (1-3)	0.9 (0.6- 6.4)
Sr Bilirubin(mg/dl)	0.9 (0.7-14)	1.6 (0.6-22)	1.53 (0.5- 22)
Sr Albumin (mg/dl)	3.9 (2.6-4.8)	3.9 (2.8-4.5)	3.8 (2.8- 4.5)
AST (IU/L)	55.4 (23-310)	46.9 (21-260)	42.1 (20- 182)
ALT (IU/L)	55.1 (22-370)	46.6 (20-270)	40.3 (20- 144)
LDH (IU/L)	375.1 (63-876)	339.8 (64-787)	275.7 (65- 656)
Blood Sugar (mg/dl)	92 (53-143)	90 (56-142)	89 (78- 156)

Table 4. The distribution of average haematological and biochemical parameters in different grades of malaria

Average	MM	SM	CM
Hb (gm/dl)	13.04	10.89	9.6
Blood Sugar (mg/dl)	90.6	84.85	70.5
Blood Urea (mg/dl)	31.2	31.88	36
Serum Creatinine (mg/dl)	0.86	1.26	4.1
Serum Bilirubin (mg/dl)	1.16	2.47	18.83
Serum Albumin (gm/dl)	3.9	3.7	3.93
AST(IU/L)	41.9	112.3	194
ALT(IU/L)	42.1	100.9	166.6
LDH (IU/L)	81.67	402.11	397.1
Parasite Index (infected RBC/1000 RBC)	0.14	0.764	3.83
TNF alpha (pgm/ml)	100.45	278.63	532.6

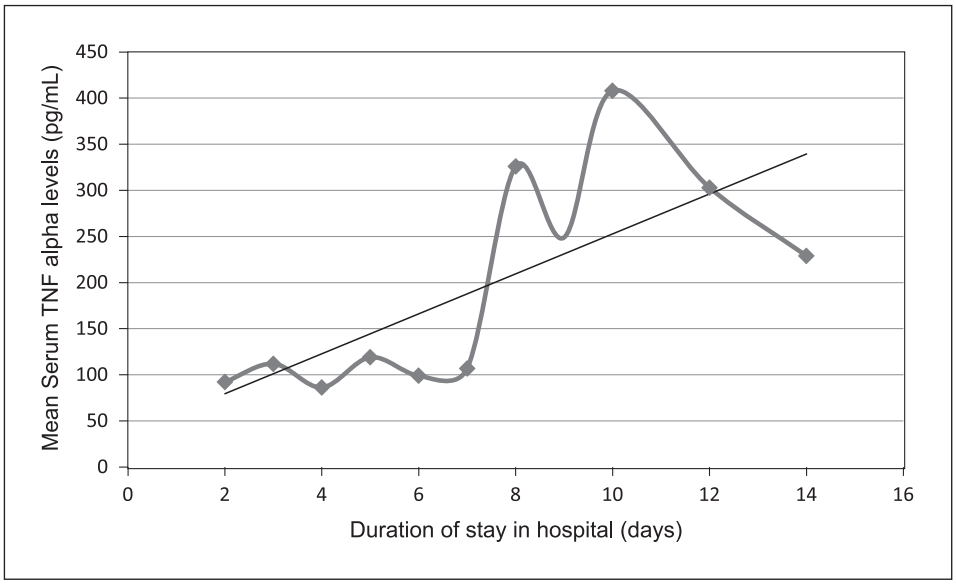

Figure 1. Mean serum TNF alpha levels of in-patients against duration of stay in hospital

release is responsible for the over-expression of adhesion molecules. Kwiatkowski *et al.* (1990) found plasma levels of tumour necrosis factor (TNF) were significantly higher in 178 Gambian children with uncomplicated malaria due to *P. falciparum* than in 178 children with other illnesses. 110 children with cerebral malaria were studied shortly after admission to hospital; 28 subsequently died. Compared with the children with uncomplicated malaria, mean plasma TNF levels were twice as high in cerebral malaria survivors and ten times as high in the fatal cases. Although high TNF levels were associated with high parasitaemia and with hypoglycaemia, they predicted fatal result in cerebral malaria independently of parasitaemia and glucose concentrations. Raised levels of interleukin-1 alpha, but not interferon gamma, also correlated to the severity of malaria.

In our present study, it was found that the there was a significant difference in TNF levels of severe malaria and cerebral malaria compared to the average values in healthy controls ($p < 0.02$). However no statistical difference was found between the mild malaria serum TNF alpha levels and serum TNF alpha levels of 91 healthy controls ($p=1.31$). This signifies that the raise in serum TNF alpha clearly depicts the severity of the disease.

Anaemia in malaria is due to various causes (i) destruction of parasitized erythrocytes, the shortened survival of unparasitized erythrocytes (ii) bone marrow dyserythropoiesis (White and Ho, 1992). Univariate regression analysis in 273 children at Uganda revealed that age, log (10) erythropoietin levels, IL-10/TNF-alpha ratio was each significantly associated with haemoglobin levels at baseline. Haemoglobin concentrations were inversely correlated with the erythropoietin level (Nussenblatt *et al* 2001). Sartorello *et al.* (2010) found that that, in addition to haemozoin formation, the falciparum parasite has evolved two new mechanisms for dealing with haem toxicity, namely, the uptake of haem into a cellular compartment where haemozoin is formed and haem-oxygenase activity. In our study, both mild malaria and severe malaria anemics had significantly higher TNF alpha compared to non anemics in the group. No statistical analysis was carried out for cerebral malaria as both the cases were anaemic. On applying unpaired student's 't' test it was realized that anemics had significantly higher TNF alpha compared to non-anemics ($p=0.0122$).

The incidence of jaundice in falciparum malaria varies between 10 to 45% in different reports, and is seen more in adults than in children (Trang *et al*., 1992), Presence of jaundice in falciparum malaria indicates a more severe illness with higher incidence of complications. Tender hepatomegaly and splenomegaly are common findings in all human malaria, and most commonly in young children (Tran *et al*., 1992). Jaundice in severe *P. falciparum* malaria is multi-factorial (i) intravascular haemolysis of parasitized RBCs, (ii) possibly micro-angiopathic haemolysis associated with DIC, (iii) associated haemoglobinopathies, (iv) drug-induced haemolysis and (v) G6PD deficiency. In the present study, serum transaminitis and hyperbilirubinemia was found in 85.7% of severe malaria, compared to 36.9% in mild malaria ($p< 0.05$). The evidence of haemolysis was based on detection of unconjugated hyper-bilirubinemia and raised LDH at least two times of normal= > 600 IU/L. Malaria patients with altered liver function had significantly higher TNF alpha compared to patients with normal LFT ($p=0.001$). However such statistical significance was not noted between the patients with and without features of haemolysis ($p=0.583$).

Malarial acute renal failure (ARF) is commonly found in patients with falciparum malaria. Since the precise mechanism of malarial ARF is not known, several hypotheses include (i) mechanical obstruction by infected erythrocytes, (ii) immune mediated glomerular and tubular injury, (iii) fluid loss and (iv) change in the renal microcirculation have been proposed. Increased fluid administration, oxygen toxicity, and yet unidentified factors may contribute to pulmonary oedema, acute respiratory distress syndrome (ARDS), multi-organ failure and death (Das *et al*., 2008). Wenisch *et al.* (1996) showed that falciparum malaria patients with renal failure (n= 16) had higher levels of TNF than patients without renal failure (n = 29) (8116+1440 µg/l versus 9453+1017 µg/l; $P<0.05$). In our study, TNF alpha levels were significantly raised in cerebral malaria and severe malaria with altered renal functions compared to mild malaria. Patients with altered renal function had no significantly different TNF alpha levels when compared to patients with normal renal function ($p=0.09$). ARDS was seen in two patients with cerebral malaria. The TNF alpha levels were significantly raised in this group of ARDS patients ($p=0.002$).

Hypoglycemia in malaria occurs more frequently in children (up to 25%) than in adults having falciparum malaria (8%). There are several generally accepted risk factors, such as prolonged fasting, severity of the infection, young age, and malnutrition (Zijlmans *et al*., 2008). In a study by Manish *et al*. (2003), the mean glucose level of falciparum malaria patients at admission was 102.29 mg/dl (SD ¼ ±18.37 mg/dl). The lowest glucose value was 60.87 mg/dl. One of the factors implicated in the pathogenesis of hypoglycaemia is TNF-alpha. In our study, there was no statistical difference in levels of TNF alpha in cases of hypoglycaemia in the three groups. However as a whole, the levels of TNF alpha were higher in the combined cases of hypoglycemics compared to normo-glycemics ($p=0.0035$). Hence a raised tendency of TNF alpha can predict the likelihood of hypoglycaemia, though it will not aid in discerning the grades of malaria.

In our study an average stay of all the cases was 5.73 days. There is a significant increase in TNF alpha levels in malaria patients with average hospital stay of more than 5 days compared to patients with stay of less than 5 days depicting it to be a good marker for prognosticating the clinical outcome (0.025).The levels of TNF alpha on day 1, 3 and day 5 showed statistical significant correlation between the duration of stay and the TNF alpha levels ($p=0.00001$).

Hepatosplenomegaly caused by chronic exposure to malaria was clearly associated with increased circulating levels of pro-inflammatory mediators, with higher levels of regulatory modulators, and with tissue repair cytokines, perhaps being required to control the inflammatory response. The 60 cases in our study had either or both organomegaly and TNF levels were higher in cases with organomegaly as a whole group ($p=0.001$).

The difference between the serum TNF alpha levels between the parasite index < 1% and > 1% were significantly different. The parasite index on day 5 did not correspond well with serum TNF alpha levels. This can be explained by reduction in parasite load but corresponding fall of TNF does not take place which explains complications inspite of reduced parasite index. This further signifies TNF as a superior prognostic marker compared to parasite index. There was very good correlation between the parasite index and TNF levels on day 1, 3 but not on day 5 post-admission. Keeping parasite index as a marker of outcome of disease; the index change statistically correlated well with parameters like SGOT, bilirubin, blood urea and serum creatinine levels.

Looraseewan *et al*. (1999) in his study gave single doses (250, 500, 1,000, or 2,000 units/kg) of polyclonal-specific Fab fragment directed against TNF-alpha to 17 adult patients with severe falciparum malaria immediately before treatment with artesunate in a pilot study to assess safety and optimal dosage with a view to future studies. In the groups given Fab, there was a tendency for a faster resolution of clinical manifestations and reduction of fever but also a tendency towards longer parasite clearance times.

A number of prognostic markers for predicting falciparum malaria are known, including clinical and laboratory markers-(haematological/ biochemical), which have already been studied extensively for their application in the management of falciparum malaria cases. However this lack the advantage of objectivity and multiple factors can alter these variables, hence there is a need to explore the possibility of using some objective laboratory marker to predict the outcome of the falciparum malaria which might turn fatal if not attended to carefully. We used tumour necrosis factor alpha for this purpose as it has a vital role in immunological intermediate cascade of falciparum malaria. Since TNF alpha levels showed significant rise in falciparum malaria cases with hypoglycaemia, anaemia, hepatosplenomegaly and raised bilirubin/transaminases, this cytokine can prognosticate the complicated malaria cases with very high accuracy. Our study had a major limitation that all other possible reasons for raised TNF alpha were ruled out in healthy controls, severe malaria/ cerebral malaria but not in mild malaria cases. Future studies should address the potential prognostic value of the serum TNF alpha test using an objective scoring system and possible use of anti TNF- alpha antibody in severe malaria to counter the increased cytokine levels causing the damage. Our study concluded that the measurements of serum TNF alpha should be performed in the patients at time of their admission at the hospital and on day 3; this can indicate severity of disease and its complications. However, it is not possible to correlate this time of infection among all of them since the patients were not admitted at the hospital at the same time of their initial infectivity.

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