UNDER PEER REVIEW

RELATIONSHIP BETWEEN SERUM C-REACTIVE PROTEIN LEVELS AND SEVERITY OF *PLASMODIUM FALCIPARUM* MALARIA IN CHILDREN SEEN IN SOUTH-SOUTH NIGERIA.

ABSTRACT

Aim: to determine the relationship between serum C-reactive protein levels and severity of malaria infection in children aged six months to five years in a malaria endemic setting.

Study design: A prospective cross-sectional study.

Place and duration of study: This study was carried out in the Children Out-patient (CHOP) Clinic, Children Emergency Unit (CHEU), Child Welfare/Growth Monitoring Clinic, Immunization Centre and main Paediatric Ward of the University of Uyo Teaching Hospital (UUTH), Uyo in Akwa-Ibom State. It was conducted over a six month period, from November 2010, to April 2011.

Methodology: We studied three hundred and sixty (360) children with microscopically confirmed *Plasmodium falciparum* malaria and three hundred and sixty (360) healthy controls matched for age and gender. Their serum C-reactive protein levels were measured using the highly sensitive Enzyme Immunosorbent assay (ELISA). Data analysis was done using the Statistical Package for Social Sciences (SPSS) version 17.0.

Results: The serum C-reactive protein levels showed a positive correlation with the malaria parasite count ($r = 0.55$; $p < 0.001$). Children presenting with severe manifestations of malaria had significantly higher mean serum C-reactive protein levels (33.3 ± 16.88mg/l) than the 8.12 ± 9.09mg/l obtained in those with uncomplicated malaria ($t = -15.57$; $p = 0.001$.

UNDER PEER REVIEW

Conclusion: This study shows that C-reactive protein levels are increased in children with malaria, especially in severe malaria. Its estimation therefore holds potential usefulness as an adjunct for assessment of malarial severity.

Keywords: Malaria, severity, C-reactive protein, children, Nigerian.

INTRODUCTION

Malaria remains a major cause of morbidity and mortality worldwide and more than 90% of these deaths, occur in sub-Saharan Africa.[1,2] All cases of falciparum malaria are potentially severe and life-threatening, especially when treated inappropriately. A major reason for progression from mild to complicated or severe disease is missed or delayed diagnosis especially when initial test is negative.[3,4] Measurements of parasitaemia have drawbacks as markers of severity as the intensity of parasitaemia does not always correlate with disease severity.[1,5,6] Hyper-parasitaemia in itself does not necessarily have major prognostic significance in semi-immune individuals (individuals living in an endemic area and exposed to malaria several times), as they often tolerate high parasite counts without signs of disease or severe effects.[7]

The burden of malaria disease and its complications during childhood still constitutes a major public health problem that warrants more integrated and collaborative intervention towards its control.[5,7,8] Though microscopy is helpful, it is laborious, requires experienced technicians and is an imperfect 'gold standard'.[7,8] Prompt and accurate diagnosis is essential for proper case management. Efforts are being made to develop new procedures for malaria diagnosis, treatment and control.[2,5,6] The severity of an episode can be assessed by evaluating and combining a panel of criteria including fever, parasite load with *in vivo*

UNDER PEER REVIEW

measures of host factors which have rarely been assessed before as markers of malarial severity.[9-11] Kremsner et al[12] reported high plasma levels of CRP in patients with severe malaria than uncomplicated cases. Some researchers believe that CRP or its toxic effects may contribute to the pathology of severe forms of malaria, and that many of the malarial symptoms may be due to excessive CRP induction.[6,13,14] This was suggested because the control of *P. falciparum* parasitaemia is dependent on a pro-inflammatory response, and an uncontrolled inflammation is thought to cause severe symptoms. It is postulated that higher levels of circulating CRP could affect the delicate balance between pro- and anti-inflammatory responses, leading to a reduced control of parasitaemia.[14,15]

Host immune markers of inflammation, like C-reactive protein (CRP) have been found to play an important role in the immune responses to malaria. Its measurement has physiological relevance as an indirect measure of inflammatory events. In hyperendemic or holoendemic malarial areas like the sub-Saharan Africa, including Nigeria, these immunological markers may serve as useful indicators for active malarial episodes.[6,13,16] A prospective characterization of its level during malaria infection could provide additional insight to its response in the disease. This study sought to determine the importance of measuring C-reactive protein (CRP) levels in *falciparum* malaria in an area where it is endemic, and if this would prove valuable in the assessment of malarial severity.

UNDER PEER REVIEW

MATERIAL AND METHODS

This study was carried out in the Children Out-patient (CHOP) Clinic, Children Emergency Unit (CHEU), Child Welfare/Growth Monitoring Clinic, Immunization Centre and main Paediatric Ward of the University of Uyo Teaching Hospital (UUTH), Uyo in Akwa-Ibom State. It was conducted over a six month period, from November 2010, to April 2011. Uyo, the capital city of Akwa-Ibom State, is located in the south-south region of Nigeria.

The Teaching Hospital is the only tertiary healthcare facility and is located on the outskirts of Uyo six kilometres from the centre of the city. The hospital is a three hundred and fifty-five bed health facility. The Department of Paediatrics provides in-patient and out-patient services for all children. The Children Outpatient clinic provides services for an average of three hundred and fifty children per week, for various illnesses. The Children Emergency Unit has a twenty-two bed capacity with an average admission rate of forty children per month. The children's ward has a twenty-seven bed capacity with an average in-patient admission rate of eighty children per month. All children with features of severe malaria were admitted into the children emergency unit for treatment, and were thereafter transferred to the Paediatric ward when their clinical condition was stable.

UNDER PEER REVIEW

The study population were children aged six months to five years, and were made up of two groups, the patients and the controls. Children with fever or a history of fever in the previous 48 hours prior to presentation in the hospital with a parasitological proof of *P. falciparum* malaria, and those with one or more features of severe malaria such as prostration, repeated convulsions, hyperpyrexia, impaired consciousness, severe anaemia, respiratory distress, jaundice, haemoglobinuria were included.[2,17] Children with history of anti-malarial use in the preceding three days of presentation and those with any obvious foci of infection and/or a positive blood culture examination were excluded. Also, excluded were children with evidence of chronic illnesses, chronic liver disease, inflammatory diseases, viral exanthema and those receiving anti-inflammatory drugs.

Every consecutive patient, aged six to sixty months, who presented with fever, (axillary temperature $\geq 37.5^0$C) or a history of fever had a detailed history and physical examination, including examination of the ears and throat. Those with a positive smear for *Plasmodium falciparum* who met other inclusion criteria were ultimately studied.

The controls were afebrile, apparently healthy children aged six to sixty months, matched for age and gender with no clinically identifiable foci of infection after a detailed clinical examination. They were selected from children attending child welfare clinic for growth monitoring and those presenting for immunization.

The blood samples were analysed in the Microbiology/Parasitology and Chemical Pathology sections of the laboratory. The Laboratory equipment is standardized and all laboratory results were confirmed by qualified and skilled laboratory personnel. Serum C-reactive protein assay was done by the quantitative method using the highly sensitive

UNDER PEER REVIEW

Enzyme Linked Immunosorbent Assay (ELISA) method.[18] Two millilitres each of the patient's serum and control serum were diluted 100-fold prior to use and 10ul of CRP standards, diluted specimens and diluted controls were dispensed into the appropriate wells. The absorbance value (OD450) for each set of reference standards, controls and samples were obtained using the micro-plate reader. The obtained values of the patient samples and control sera were multiplied by the dilution factor of 100 to obtain CRP results in mg/l. Other ancillary investigations done in every child presenting with severe malaria included random blood glucose, full blood count and electrolytes/urea and creatinine.

Data analysis was done using the Statistical Package for Social Sciences (SPSS) version 17.0. Univariate and multivariate models were built. These helped to adjust for possible associations between malarial infection and the C-reactive protein response. P-value less than 0.05 ($p < 0.05$) was considered statistically significant.

Ethical approval was obtained from the University of Uyo hospital's ethics committee while a verbal informed consent was obtained from parents/guardian of children to be included in the study after initial assessment.

UNDER PEER REVIEW

RESULTS

Three hundred and sixty (360) patients, and three hundred and sixty (360) controls were studied. Of these, 200 (55.6%) each were males and 160 (44.4%) each were females with a male to female ratio of 1.25 : 1. The mean age of the male and female patients and that of the control group were similar (range 6 to 60 months). The mean weight of the male and female patients and the controls were also similar (range 6.5 to 18kg). The duration of fever in the patients was one to four days (mean 1.9 ± 0.8) and mean temperature 37.83 ± 0.89^0Celsius (range 36.5 – 40.1^0Celsius). No child in the control group had fever (Table I).

The clinical characteristics of all the patients at presentation showed fever as the most common clinical feature in 217 (60.28%) of patients. Others in order of frequency were poor appetite, in 118 (32.8%) of patients, hyperpyrexia 54 (15.0%), splenomegaly 52 (14.4%), hepatomegaly 16 (4.4%), severe pallor 15 (4.2%), convulsions 10 (2.8%), altered sensorium in seven (1.9%) of the patients. Only three (0.8%) of the patients had jaundice.

Five of the fifteen patients who presented with severe pallor, also had signs of congestive cardiac failure – tachycardia (with a gallop rhythm), dyspnoea and tachypnoea (*respiratory distress) and tender hepatomegaly. Of the seven patients who presented with altered sensorium, three were deeply comatose (Blantyre's score of 0 - 2), with a diagnosis of

UNDER PEER REVIEW

cerebral malaria, while four had light coma (Blantyre's score of 3 - 4) from hypoglycemia. The mean packed cell volume (%) in patients with uncomplicated malaria was 30.7 ± 2.40 greater than the 25 ± 9.03 obtained in those with severe malaria (t = 8.84; p = 0.001). The mean random blood sugar (mmol/l) in the patients with severe malaria was 3.09 ± 0.75 (range 1.60 to 4.80).-Table II. The relative frequency of the characteristics in patients presenting with severe malaria showed prostration as the most common sign in 31 (53.4%), and jaundice the least, in three (5.5%) of the patients (Table III).

The mean malaria parasite count in patients was 15,602.22 ± 61,641.86 with a range of 500 to 695,500 parasites/µl. *Plasmodium falciparum* was the only specie found in all patients. The mean malaria parasite count was significantly higher in children with severe malaria than in those with the uncomplicated type (t = -7.24; p<0.001). The mean serum level of C-reactive protein (mg/l) was 33.3 ± 16.88 significantly higher in children with severe than the 8.12 ± 9.09 obtained in the patients with uncomplicated malaria (t = -15.57; p<0.001) - Table IV.

The distribution of the malaria parasite count and serum C-reactive protein levels in the patients is shown in Table V. The patients with hyperparasitaemia (>250,000 parasites/µl)[5,6] had the highest mean serum C-reactive protein level. Using the one way analysis of variance (ANOVA), the differences in the mean serum C-reactive protein levels among the various parasitaemia groups was statistically significant, (F = 87.13; p = 0.001). Also, the mean parasite count (parasites/µl) of patients with hyperpyrexia was 67841 ± 141572.28 significantly greater than the 8774.69 ± 24789.74 in febrile patients, and the 3409.09 ± 5068.07 in the afebrile patients (F = 26.76; p = 0.001).

UNDER PEER REVIEW

There was a 55% relationship between the natural log of malaria parasite count and natural log of C-reactive protein and this was statistically significant. This was a positive correlation ($r = 0.55$; $p<0.001$). Thus, as the malaria parasite count increased, there was a concomitant increase in serum C-reactive protein. This is shown in Figure 1.

DISCUSSION

The findings from this study showed greater mean serum CRP concentrations in patients with manifestations of severe malaria, than in the uncomplicated type. There has been similar reports from other authors in African children.[19-21] The various authors documented an association of severe malaria with high circulating levels of inflammatory cytokines such as tumour necrosis factor-alpha, interleukin-1 and interleukin-6, as measured by immunoassay. These cytokines responsible for acute malarial illness, is also responsible for the stimulation, production and sustained release of C-reactive protein.

The significantly higher serum CRP level in the febrile patients than the afebrile ones in the present study, was also documented in other African children by Hurt et al[21] and Imrie et al.[22] In Tanzania, Hurt et al[21] found this trend of significantly increasing serum CRP levels in children with temperatures greater than 37.4^0C. The children who presented with hyperpyrexia in present study had relatively higher parasite densities. This higher density of parasitaemia, schizont rupture and merozoite release into the bloodstream, may have resulted in febrile symptoms at presentation. It is known that fever accompanies cytokine release, which results from macrophage response to invading parasitaemia. Fever is triggered by endogenous pyrogens like interleukins 1 and 6, and tumour necrosis factor which are also responsible for the stimulation of CRP in malaria.[23,24]

UNDER PEER REVIEW

The higher levels of mean serum CRP concentration between the patients and controls in the various age groups in present study reinforces the effect of malaria antigen in the induction of CRP production.[20,25,26] Mononuclear cells which are activated by the *plasmodium* during malaria, produce inflammatory cytokines and stimulate the hepatic synthesis of acute inflammatory proteins including CRP. This cytokine response is likely responsible for this observation.

The positive correlation of serum C-reactive protein levels in patients with higher levels of parasitaemia in present study is similar to trends in children of The Gambia,[19,20] Tanzania[21] and Zambia[25] which are also endemic for malaria. This finding may be related to the higher parasite burden in these children which triggers the inflammatory response. However, contrasting reports, from Papua New Guinea[22] did not document a consistent increase in the serum CRP level with increasing parasitaemia. This may be explained by the wider age group of the Papuan study population, which included adolescent children up to seventeen years of age permanently resident in the malaria hyperendemic region. Most of these adolescent children, had already acquired malarial immunity. They therefore elicited lowered immune responsiveness to malarial antigens, and a corresponding reduction in pro-inflammatory mechanisms.

Children in this study were less than five years of age, and have been recognized to elicit more marked immune responses to invading malaria parasites. This increased inflammatory response, increases their CRP levels. This documented trend continues till malarial immunity has been acquired, and then it later plays an important protective role in the modulation of the immune responses to invading parasites, including severity of malarial episodes.

UNDER PEER REVIEW

Limitations to this study include confounding factors for increased C-reactive protein which are viral fevers that can co-exist in children with malaria. Additional investigations such as viral screen studies were not available in our facility. This would have been employed to confirm any co-existing viral infection in the children with malaria.

UNDER PEER REVIEW

CONCLUSION

C-reactive protein levels are increased in children with *P. falciparum* malaria infection, greater in those with the severe manifestations. This study suggests the usefulness of C-reactive protein estimation as an adjunct for assessment of malarial severity. It holds potential benefits for future use in children with *falciparum* malaria as it will help in the identification and prompt treatment of such children.

COMPETING INTEREST

The authors declare that they have no competing interests.

REFERENCES

1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI: **The global distribution of clinical episodes of *Plasmodium falciparum* malaria.** *Nature* 2005, **434**:214-217.
2. Pasvol G: **The treatment of complicated and severe malaria**. *Brit Med Bull* 2006, **75**:29-47.
3. Philip JA, Hassan IST: **How easily malaria can be missed.** *J R Soc Med* 2006, **99**:201-202.
4. Gwer S, Newton CRJC, Berkley JA: **Overdiagnosis and co-morbidity of severe malaria in African children. A guide for clinicians.** *Am J Trop Med Hyg* 2007, **77**:6-13.

UNDER PEER REVIEW

297 5. Bassett MT, Taylor P, Buirakare J, Chiteka F, Govere E: **Clinical diagnosis of**
298 **malaria: can we improve?** *J Trop Med Hyg* 1991, **94**:65-69.
299 6. Gillespie SH, Dow C, Raynes JG, Behrens RH, Chiodini PL, McAdam KPWJ:
300 **Measurement of acute phase proteins for assessing severity of *Plasmodium***
301 ***falciparum* malaria.** *J Clin Pathol* 1991, **44**:228-231.
302 7. Collier JA, Longmore JM: **The reliability of the microscopic diagnosis of malaria**
303 **in the field and in the laboratory.** *Am Trop Med Parasitol* 1983, **77**:113-117.
304 8. Payne D: **Use and limitations of light microscopy for diagnosing malaria at the**
305 **primary health care level.** *Bull World Health Org* 1988, **66**:621-626.
306 9. Ochola LB, Marsh K, Lowe B, Gal S, Pluschke G, Smith T: **Estimation of the**
307 **sequestered parasite load in severe malaria patients using both host and**
308 **parasite markers.** *Parasitol* 2005, **131**:449-458.
309 10. Trape JF, Peelman P, Morault-Peelman B: **Criteria for diagnosing clinical**
310 **malaria among a semi-immune population exposed to intense and perennial**
311 **transmission.** *Trans R Soc Trop Med Hyg* 1985, **79**:435-442.
312 11. Graninger W, Thalltamer F, Holleinstein U, Zotter GM, Kremsner PG: **Serum**
313 **protein concentration in *Plasmodium falciparum* malaria.** *Acta Trop* 1992,
314 **52**:121-128.
315 12. Kremsner PG, Winkler S, Widling E, Prada J, Bienzie U, Graninger W et al: **High**
316 **plasma levels of nitrogen oxide are associated with severe disease and**
317 **correlates with rapid parasitological and clinical cure in *plasmodium***
318 ***falciparum.*** *Trans R Soc Med Hyg* 1996, **90**:44-47.
319 13. Hurt N, Smith T, Tanner M, Mwanskuye S, Bordmann G, Weiss NA et al:
320 **Evaluation of C-reactive protein and haptoglobin as malaria episode markers**

UNDER PEER REVIEW

in an area of high transmission in Africa. *Trans R Soc Trop Med Hyg* 1994, **88**:182-186.

14. Clark IA, Budd AC, Alleva LM, Cowden WB: **Human malarial disease: a consequence of inflammatory cytokine release.** *Malar J* 2006, **85**:1-32.

15. Artanavis-Tsakonas K, Tongren JE, Riley EM: **The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology.** *Clin Exp Immunol* 2003, **133**:145-152.

16. Hurt NM, Thein T, Bordmann H, Gallati N, Drees M, Weiss N: **Immunological markers of childhood fevers in an area of intense and perennial malaria transmission.** *Clin Exp Immunol* 1995, **100**:59-66.

17. World Health Organization: **Guidelines for treatment of malaria.** Geneva; 2006.

18. **High Sensitivity C-reactive protein Enzyme Immunoassay test kit.** Catalog number BC-1119, U.S.A.

19. Jakobsen PH, McCay V, N'Jie R, Olaleye BO, D'Alessandro U, Zhang GH, et al: **Decreased antitoxic activities among children with clinical episodes of malaria.** *Infect Immun* 1998, **66**:1654-1659.

20. Ree GH: **C-reactive protein in Gambian Africans with special reference to *P. falciparum* malaria.** *Trans R Soc Trop Med Hyg* 1971, **65**:574-580.

21. Hurt N, Smith T, Teuscher T, Tanner M: **Do high levels of C-reactive protein in Tanzanian children indicate malarial morbidity?** *Clin Diagn Lab Immunol* 1994, **4**:437-444.

22. Imrie H, Fowkes FJ, Michon P, Tavul L, Reeder JC, Day KP: **Low prevalence of an acute phase reactant in asymptomatic children from a malaria-endemic area of Papua New Guinea.** *Am J Trop Med Hyg* 2007, **76**:280-284.

UNDER PEER REVIEW

23. Gabay C, Kushner I: **Acute phase proteins and other systemic response to inflammation.** *N Engl J Med*. 1999, **340**:448-454.

24. Harpaz R, Edelman R, Wassermann SS, Levine MM, Davis JR, Sztein MB: **Serum cytokine profiles in experimental human malaria: Relationship to protection and disease course after challenge.** *J Clin Invest* 1992, **90**: 515-523.

25. Naik P, Voller A: **Serum C-reactive protein levels and *falciparum* malaria.** *Trans R Soc Trop Med Hyg* 1984, **78**:812-813.

26. Haghighi L: **C-reactive protein in malaria.** *J Clin Pathol* 1969, **22**:430-432.

Table I: General characteristics of the study population

	Patients			Controls		
Characteristics	**Male**	**Female**	**T (p-value)**	**Male**	**Female**	**T (p-value)**
Age (months)	24.24 ± 16.77	25.48 ± 17.97	-0.67 (0.50)	24.92 ± 17.24	24.71 ± 17.20	0.12 (0.91)
Weight (kg)	11.50 ± 3.01	11.58 ± 3.04	-0.25 (0.80)	11.59 ± 3.07	11.40 ± 2.97	0.59 (0.52)

UNDER PEER REVIEW

Height (cm)	82.2 ± 13.91	83.44 ± 14.49	-0.83 (0.40)	82.48 ± 14.09	83.14 ± 14.23	-0.44 (0.65)
*MUAC (cm)	13.42 ± 0.47	13.40 ± 0.45	0.31 (0.76)	13.43 ± 0.46	13.43 ± 0.43	0.09 (0.92)
#OFC (cm)	50.02 ± 3.09	49.87 ± 3.25	0.45 (0.66)	50.12 ± 2.97	49.82 ± 3.28	0.92 (0.36)
Temperature (^{0}C)	37.84 ± 0.94	37.79 ± 0.85	0.53 (0.59)	36.96 ± 0.22	36.94 ± 0.91	0.92 (0.36)

*MUAC - Mid Upper Arm Circumference
#OFC - Occipito-frontal circumference

Table IIa: Clinical symptoms of the patients at presentation.

SYMPTOMS#	N	(%)
Fever	217	60.28
Poor appetite	118	32.8
Vomiting	94	26.1
Convulsions	10	2.8

#Most children had more than one clinical symptom at presentation.

Table IIb: Clinical signs of the patients at presentation.

UNDER PEER REVIEW

SIGNS#	N	(%)
Hyperpyrexia	54	15
Splenomegaly	52	14.4
Prostration	31	8.6
Hepatomegaly	16	4.4
Severe Pallor	15	4.2
Altered sensorium	7	1.9
Rapid breathing*	5	1.4
Jaundice	3	0.8

#Most children had more than one clinical sign at presentation.

*Respiratory rate ≥ 50 cycles/minute in infants 2 to less than12 months and ≥ 40 cycles/minute in children 1 to 5 years.[123]

Table III: Relative frequency of characteristics of the 55 patients with severe malaria.

Variable*	Yes (%)	No (%)
Hyperpyrexia	54 (98.2)	1 (1.8)
Prostration	31 (53.4)	24 (46.6)
Severe anaemia	15 (27.3)	40 (72.7)
Multiple convulsions	10 (18.2)	45 (81.8)
Altered sensorium	7 (12.7)	48 (87.3)

UNDER PEER REVIEW

Hyperparasitaemia	7 (12.7)	48 (87.3)
Respiratory distress**	5 (9.1)	50 (90.9)
Cerebral malaria	3 (5.5)	52 (94.5)
Jaundice	3 (5.5)	52 (94.5)

*Most patients presented with more than one manifestation of severe malaria.

**Respiratory rate ≥ 50 cycles/minute in infants 2 to less than12 months and ≥ 40 cycles/minute in children 1 to 5 years.[123]

Table IV: Malaria parasite count and mean serum C-reactive protein levels in patients.

Clinical malaria	Malaria Parasite Count (parasites/µl) Mean ± S.D	C-reactive protein level (mg/l) Mean ± S.D
Uncomplicated malaria	5234.43 ± 50404.98	8.12 ± 9.09

UNDER PEER REVIEW

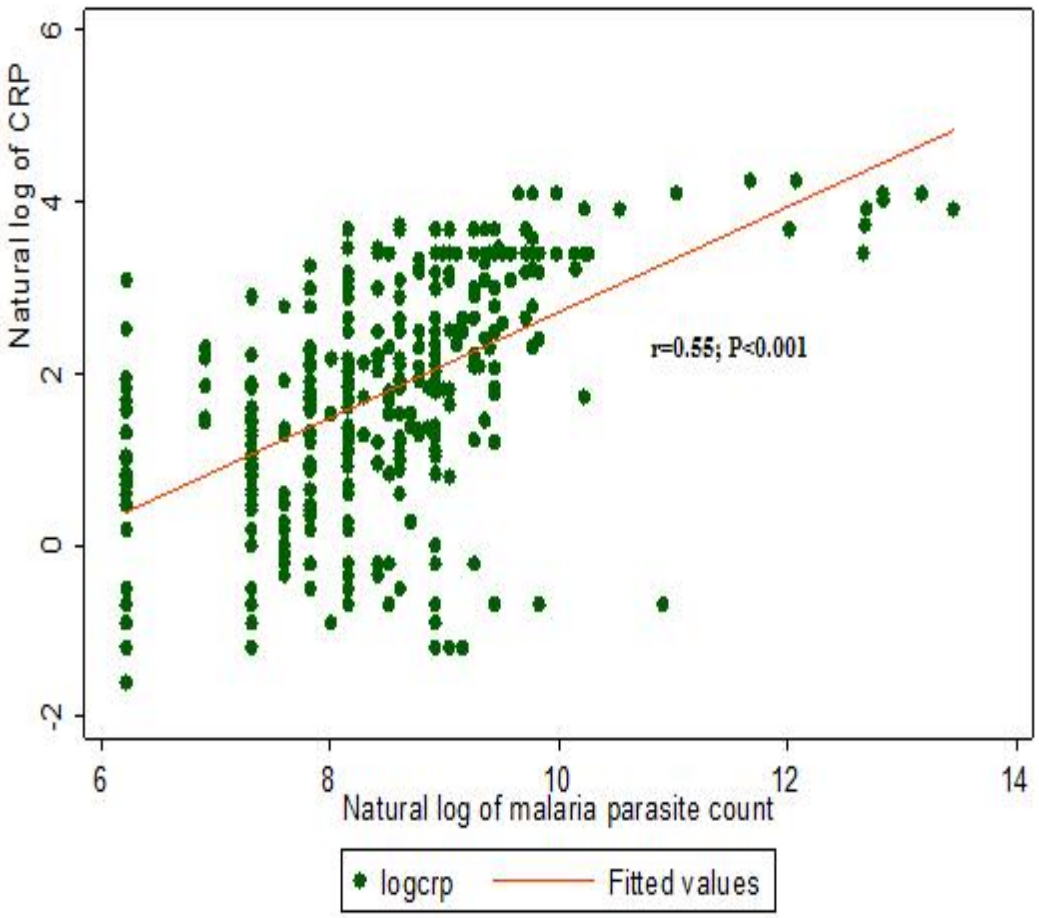
Severe malaria	73096.36 ± 145336.6	33.30 ± 16.88
T test (p-value)	-7.24 (0.001)*	-15.57 (0.001)*

*Statistically significant

Table V: Distribution of the malaria parasite count and serum CRP levels in the patients.

Malaria parasite count/µl (range)	**N**	**C-reactive protein (mg/l) Mean ± S.D**	**F (p value)**
500 – 5000	195	5.96 ± 7.01	87.13 (0.001)
>5000 – 100,000	86	11.43 ± 10.97	
>100,000 – 250,000	72	25.20 ± 16.73	
> 250,000	7	49.71 ± 10.79	
Total	360	11.97 ± 13.96	

ANOVA F = 87.13; p = 0.00

Figure 1: Relationship between the malaria parasite count and serum C-reactive protein levels in patients

UNDER PEER REVIEW