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Journal Name:	International Journal of TROPICAL DISEASE & Health
Manuscript Number:	2014-IJTDH_10912
Title of the Manuscript:	RELATIONSHIP BETWEEN SERUM C-REACTIVE PROTEIN LEVELS AND SEVERITY OF <i>PLASMODIUM FALCIPARUM</i> MALARIA IN CHILDREN SEEN IN SOUTH-SOUTH NIGERIA.
Type of the Article	Original article

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PART 1: Review Comments

	Reviewer's comment	Author's comment (if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)
Compulsory REVISION comments	Lines 87 to 94 are completely irrelevant. Line 181: Graphic results should be shown in Results section to make for greater clarity, especially regarding parasitemias and CRP values.	Lines 87 to 94 have been expunged. The table V is self- explanatory. It should not necessarily be represented graphically because it does not represent fractions. If we use the N (number of children with different levels of parasitaemia), we will lose information about the other aspects of the table, i.e the actual parasite counts and the p-value.
Minor REVISION comments	Line 32: <i>falciparum</i> should be in italic. Line 50: CRP shows up in the full text as an abbreviation without being written in full first. Line 59: no more need to define CRP after correction at line 50. Line 85: what does "south-south" mean? Line 122: ELISA test is not always highly sensitive. If you mean it is sensitive for CRP, then please provide reference. Line 127: was compensation for the naive serum OD done, or the results are just presented "as is"? Line 210: abbreviations should be kept consistent through the text. Use CRP.	Line 32 – <i>falciparum</i> has been put in italics Line 50 – CRP has been written in full (C-reactive protein) Line 59 – CRP written, as in line 50 Line 85 – "south-south" is one of the six presently recognized geo-political zones of the country, Nigeria. This is where the study site, Akwa Ibom State is located and the study conducted. Line 122 - The highly sensitive ELISA test kit (hsELISA) was used for this study. Please refer



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		"Reference 18". Line 127 - Using the absorbance value for each sample, the corresponding CRP concentration was determined from the standard curve. Line 210 – CRP used and kept consistent through text.
<u>Optional/General</u> comments	Line 57: it might be relevant to the reader and help increase text clarity if you further explain the relationship between CRP and inflammatory responses.	A brief paragraph, explaining the relationship between CRP and inflammatory response has been included in text. It follows line 51



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1 RELATIONSHIP BETWEEN SERUM 1 C-REACTIVE PROTEIN LEVELS AND
2 SEVERITY OF *PLASMODIUM FALCIPARUM* MALARIA IN CHILDREN SEEN
3 IN SOUTH-SOUTH NIGERIA.

4

5 **ABSTRACT**

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

8 **Study design:** A prospective cross-sectional study.

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

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

19 **Results:** The serum C-reactive protein levels showed a positive correlation with the malaria
20 parasite count ($r = 0.55$; $p < 0.001$). Children presenting with severe manifestations of
21 malaria had significantly higher mean serum C-reactive protein levels ($33.3 \pm 16.88\text{mg/l}$)
22 than the $8.12 \pm 9.09\text{mg/l}$ obtained in those with uncomplicated malaria ($t = -15.57$; $p =$
23 0.001).

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Conclusion: This study shows that C-reactive protein levels are increased 24 in children with
25 malaria, especially in severe malaria. Its estimation therefore holds potential usefulness as
26 an adjunct for assessment of malarial severity.

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

29

30 **INTRODUCTION**

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

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

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measures of host factors which have rarely been assessed before as 49 markers of malarial
50 severity.⁹⁻¹¹ Kremsner et al¹² reported high plasma levels of CRP in patients with severe
51 malaria than uncomplicated cases. Some researchers believe that CRP or its toxic effects
52 may contribute to the pathology of severe forms of malaria, and that many of the malarial
53 symptoms may be due to excessive CRP induction.^{6,13,14} This was suggested because the
54 control of *P. falciparum* parasitaemia is dependent on a pro-inflammatory response, and an
55 uncontrolled inflammation is thought to cause severe symptoms. It is postulated that higher



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56 levels of circulating CRP could affect the delicate balance between pro- and anti57
inflammatory responses, leading to a reduced control of parasitaemia.^{14,15}

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

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80 **MATERIAL AND METHODS**

81 This study was carried out in the Children Out-patient (CHOP) Clinic, Children Emergency
82 Unit (CHEU), Child Welfare/Growth Monitoring Clinic, Immunization Centre and main
83 Paediatric Ward of the University of Uyo Teaching Hospital (UUTH), Uyo in Akwa-Ibom



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84 State. It was conducted over a six month period, from November 2010, to April 2011. Uyo,
85 the capital city of Akwa-Ibom State, is located in the south-south region of Nigeria.

86

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

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The study population were children aged six months to five 98 years, and were made up of two
99 groups, the patients and the controls. Children with fever or a history of fever in the
100 previous 48 hours prior to presentation in the hospital with a parasitological proof of *P.*
101 *falciparum* malaria, and those with one or more features of severe malaria such as
102 prostration, repeated convulsions, hyperpyrexia, impaired consciousness, severe anaemia,
103 respiratory distress, jaundice, haemoglobinuria were included.^{2,17} Children with history of
104 anti-malarial use in the preceding three days of presentation and those with any obvious
105 foci of infection and/or a positive blood culture examination were excluded. Also, excluded
106 were children with evidence of chronic illnesses, chronic liver disease, inflammatory
107 diseases, viral exanthema and those receiving anti-inflammatory drugs.

108

109 Every consecutive patient, aged six to sixty months, who presented with fever, (axillary
110 temperature $\geq 37.5^{\circ}\text{C}$) or a history of fever had a detailed history and physical examination,
111 including examination of the ears and throat. Those with a positive smear for *Plasmodium*



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112 *falciparum* who met other inclusion criteria were ultimately studied.

113

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

118

119 The blood samples were analysed in the Microbiology/Parasitology and Chemical
120 Pathology sections of the laboratory. The Laboratory equipment is standardized and all
121 laboratory results were confirmed by qualified and skilled laboratory personnel. Serum C122
reactive protein assay was done by the quantitative method using the highly sensitive

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Enzyme Linked Immunosorbent Assay (ELISA) method.¹⁸ Two millilitres 123 each of the
124 patient's serum and control serum were diluted 100-fold prior to use and 10ul of CRP
125 standards, diluted specimens and diluted controls were dispensed into the appropriate wells.
126 The absorbance value (OD450) for each set of reference standards, controls and samples
127 were obtained using the micro-plate reader. The obtained values of the patient samples and
128 control sera were multiplied by the dilution factor of 100 to obtain CRP results in mg/l.
129 Other ancillary investigations done in every child presenting with severe malaria included
130 random blood glucose, full blood count and electrolytes/urea and creatinine.

131

132 Data analysis was done using the Statistical Package for Social Sciences (SPSS) version
133 17.0. Univariate and multivariate models were built. These helped to adjust for possible
134 associations between malarial infection and the C-reactive protein response. P-value less
135 than 0.05 ($p < 0.05$) was considered statistically significant.

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



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154 RESULTS

155 Three hundred and sixty (360) patients, and three hundred and sixty (360) controls were
156 studied. Of these, 200 (55.6%) each were males and 160 (44.4%) each were females with a
157 male to female ratio of 1.25 : 1. The mean age of the male and female patients and that of
158 the control group were similar (range 6 to 60 months). The mean weight of the male and
159 female patients and the controls were also similar (range 6.5 to 18kg). The duration of fever
160 in the patients was one to four days (mean 1.9 ± 0.8) and mean temperature $37.83 \pm$
161 0.89°C (range $36.5 - 40.1^{\circ}\text{C}$). No child in the control group had fever (Table I).

162

163 The clinical characteristics of all the patients at presentation showed fever as the most
164 common clinical feature in 217 (60.28%) of patients. Others in order of frequency were
165 poor appetite, in 118 (32.8%) of patients, hyperpyrexia 54 (15.0%), splenomegaly 52
166 (14.4%), hepatomegaly 16 (4.4%), severe pallor 15 (4.2%), convulsions 10 (2.8%), altered
167 sensorium in seven (1.9%) of the patients. Only three (0.8%) of the patients had jaundice.



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

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cerebral malaria, while four had light coma (Blantyre's score of 3 173 - 4) from hypoglycemia.

174 The mean packed cell volume (%) in patients with uncomplicated malaria was 30.7 ± 2.40

175 greater than the 25 ± 9.03 obtained in those with severe malaria ($t = 8.84$; $p = 0.001$). The

176 mean random blood sugar (mmol/l) in the patients with severe malaria was 3.09 ± 0.75

177 (range 1.60 to 4.80).-Table II. The relative frequency of the characteristics in patients

178 presenting with severe malaria showed prostration as the most common sign in 31 (53.4%),

179 and jaundice the least, in three (5.5%) of the patients (Table III).

180

181 The mean malaria parasite count in patients was $15,602.22 \pm 61,641.86$ with a range of 500

182 to 695,500 parasites/ μ l. *Plasmodium falciparum* was the only specie found in all patients.

183 The mean malaria parasite count was significantly higher in children with severe malaria

184 than in those with the uncomplicated type ($t = -7.24$; $p < 0.001$). The mean serum level of C185

reactive protein (mg/l) was 33.3 ± 16.88 significantly higher in children with severe than

186 the 8.12 ± 9.09 obtained in the patients with uncomplicated malaria ($t = -15.57$; $p < 0.001$) -

187 Table IV.

188

189 The distribution of the malaria parasite count and serum C-reactive protein levels in the

190 patients is shown in Table V. The patients with hyperparasitaemia ($>250,000$

191 parasites/ μ l)^{5,6} had the highest mean serum C-reactive protein level. Using the one way

192 analysis of variance (ANOVA), the differences in the mean serum C-reactive protein levels

193 among the various parasitaemia groups was statistically significant, ($F = 87.13$; $p = 0.001$).

194 Also, the mean parasite count (parasites/ μ l) of patients with hyperpyrexia was $67841 \pm$

195 141572.28 significantly greater than the 8774.69 ± 24789.74 in febrile patients, and the



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196 3409.09 $\pm$ 5068.07 in the afebrile patients ($F = 26.76$; $p = 0.001$).
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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.

202

203 **DISCUSSION**

204 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.

211

212 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 al²¹ and Imrie et al.²² In Tanzania, Hurt et al²¹ found this trend of significantly increasing serum CRP levels in children with temperatures greater than 37.4°C. 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}

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The higher levels of mean serum CRP concentration between the 223 patients and controls in 224 the various age groups in present study reinforces the effect of malaria antigen in the 225 induction of CRP production.^{20,25,26} Mononuclear cells which are activated by the 226 *plasmodium* during malaria, produce inflammatory cytokines and stimulate the hepatic 227 synthesis of acute inflammatory proteins including CRP. This cytokine response is likely 228 responsible for this observation.

229

230 The positive correlation of serum C-reactive protein levels in patients with higher levels of 231 parasitaemia in present study is similar to trends in children of The Gambia,^{19,20} Tanzania²¹ 232 and Zambia²⁵ which are also endemic for malaria. This finding may be related to the higher 233 parasite burden in these children which triggers the inflammatory response. However, 234 contrasting reports, from Papua New Guinea²² did not document a consistent increase in the 235 serum CRP level with increasing parasitaemia. This may be explained by the wider age 236 group of the Papuan study population, which included adolescent children up to seventeen 237 years of age permanently resident in the malaria hyperendemic region. Most of these 238 adolescent children, had already acquired malarial immunity. They therefore elicited 239 lowered immune responsiveness to malarial antigens, and a corresponding reduction in pro240 inflammatory mechanisms.

241

242 Children in this study were less than five years of age, and have been recognized to elicit 243 more marked immune responses to invading malaria parasites. This increased inflammatory 244 response, increases their CRP levels. This documented trend continues till malarial 245 immunity has been acquired, and then it later plays an important protective role in the 246 modulation of the immune responses to invading parasites, including severity of malarial 247 episodes.

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Limitations to this study include confounding factors for increased C 249 -reactive protein which
250 are viral fevers that can co-exist in children with malaria. Additional investigations such as
251 viral screen studies were not available in our facility. This would have been employed to
252 confirm any co-existing viral infection in the children with malaria.

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276 **CONCLUSION**

277 C-reactive protein levels are increased in children with *P. falciparum* malaria infection,
278 greater in those with the severe manifestations. This study suggests the usefulness of C279
reactive protein estimation as an adjunct for assessment of malarial severity. It holds
280 potential benefits for future use in children with *falciparum* malaria as it will help in the
281 identification and prompt treatment of such children.

282

283 **COMPETING INTEREST**

284 The authors declare that they have no competing interests.

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365 **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)

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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 (°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 366 Upper Arm Circumference

367 #OFC - Occipito-frontal circumference

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381 **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

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

383

384 **Table IIb: Clinical signs of the patients at presentation.**

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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 385 sign at presentation.

386 *Respiratory rate ≥ 50 cycles/minute in infants 2 to less than 12 months and ≥ 40

387 cycles/minute in children 1 to 5 years.¹²³

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

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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)

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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 402 of severe malaria.

403 **Respiratory rate ≥ 50 cycles/minute in infants 2 to less than 12 months and ≥ 40

404 cycles/minute in children 1 to 5 years.¹²³

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

Clinical malaria Malaria Parasite Count
(parasites/ μ l)

C-reactive protein level
(mg/l)

Mean $\pm$ S.D Mean $\pm$ S.D

Uncomplicated malaria 5234.43 $\pm$ 50404.98 8.12 $\pm$ 9.09

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19

Severe malaria 73096.36 $\pm$ 145336.6 33.30 $\pm$ 16.88

T test (p-value) -7.24 (0.001)* -15.57 (0.001)*

*Statistically 424 significant

425

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

Malaria parasite count/ μ l
(range)

C-reactive protein



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(mg/l)

N Mean $\pm$ S.D F (p value)

500 – 5000 195 5.96 $\pm$ 7.01 87.13 (0.001)

>5000 – 100,000 86 11.43 $\pm$ 10.97

>100,000 – 250,000 72 25.20 $\pm$ 16.73

> 250,000 7 49.71 $\pm$ 10.79

Total 360 11.97 $\pm$ 13.96

428 ANOVA F = 87.13; p = 0.00

429

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

UNDER PEER REVIEW

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432

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UNDER PEER REVIEW

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



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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.



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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 ($33.3 \pm 16.88\text{mg/l}$) than the $8.12 \pm 9.09\text{mg/l}$ obtained in those with uncomplicated malaria ($t = -15.57$; $p = 0.001$).

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

Comment [J1]: falciparum in italic



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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* measures of host factors which have rarely been assessed before as markers of malarial severity.⁹⁻¹¹ C-reactive protein is synthesized in the liver and is secreted in increased amounts within six hours of an acute inflammatory stimulus. Interleukin-6 (IL-6) plays a critical role in CRP induction. After effective treatment or removal of the inflammatory stimulus, levels can also fall. The only condition that interferes with the normal CRP response is severe hepato-cellular impairment.

Comment [J2]: Paragraph added to explain relationship between CRP and inflammatory response.



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Kremsner et al¹² reported high plasma levels of C –reactive protein (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}

Comment [J3]: CRP has been written in full

Host immune markers of inflammation, like CRP has 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.

Comment [J4]: CRP used as abbreviation

MATERIAL AND METHODS



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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.

Comment [J5]: South-south has been explained as in authors' comments

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.

Comment [J6]: Lines 87 to 94 expunged.

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.*



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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 $\geq 37.5^{\circ}\text{C}$) 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.



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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 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.

Comment [J7]: This is explained in authors' comments

Data analysis was done using the Statistical Package for Social Sciences (SPSS) version 17.0. Data was summarized into Tables and Graphs as appropriate. Discrete variables were expressed as numbers and percentages, while continuous variables expressed as mean and standard deviation. Normally distributed continuous variables were compared using the student's t-test. The Wilcoxon rank sum test was used for non-normally distributed continuous variables. Proportions were compared using the Chi-square (χ^2) test. 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.

Comment [J8]: Computations and tests done are as stated



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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.



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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 $37.83 \pm 0.89^{\circ}\text{Celsius}$ (range $36.5 - 40.1^{\circ}\text{Celsius}$). 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.



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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 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 $15,602.22 \pm 61,641.86$ with a range of 500 to 695,500 parasites/ μ l. *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.



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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 ($F = 26.76$; $p = 0.001$).

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-



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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 al²¹ and Imrie et al.²² In Tanzania, Hurt et al²¹ found this trend of significantly increasing serum CRP levels in children with temperatures greater than 37.4⁰C. 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}

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.



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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} Tanzania²¹ and Zambia²⁵ 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 Guinea²² 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.



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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.

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



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The authors declare that they have no competing interests.

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Table I: General characteristics of the study population

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Characteristics	Patients			Controls		
	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)
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 (°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



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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.

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 than 12 months and ≥ 40 cycles/minute in children 1 to 5 years.



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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)
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 than 12 months and ≥ 40 cycles/minute in children 1 to 5 years.



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

Clinical malaria	Malaria Parasite Count (parasites/ μ l) Mean $\pm$ S.D	C-reactive protein level (mg/l) Mean $\pm$ S.D
Uncomplicated malaria	5234.43 $\pm$ 50404.98	8.12 $\pm$ 9.09
Severe malaria	73096.36 $\pm$ 145336.6	33.30 $\pm$ 16.88
T test (p-value)	-7.24 (0.001)*	-15.57 (0.001)*

*Statistically significant



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

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



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ANOVA $F = 87.13; p = 0.00$

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



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