<u>Original Research Article</u>

C-Reactive Protein as an inflammatory Biomarker for the assessment of malaria parasitemia in a tertiary health care facility in Rivers State, Nigeria.

ABSTRACT

Aim: To use C-Reactive Protein (CRP) in assessing` malaria and malaria parasitemia among out-patients in the University of Port Harcourt Teaching Hospital (UPTH).
Study design: Cross-sectional descriptive study.
Place and Duration of Study: This study was carried out at the Out Patients Department (OPD) of the University of Port Harcourt Teaching Hospital, Port Harcourt, Rivers State, Nigeria. It was conducted between November 2017 and April 2018.
Methodology: This study was done on 400 subjects between the ages of 11 and 60years which consisted of 254 falciparum malaria infected patients and 164 non-infected patients. Quantitative analysis of Serum CRP was done using the High Sensitivity C-Reactive Protein (HsCRP) enzyme immunoassay test kit (Biocheck©) while malaria diagnosis was done using microscopy Giemsa thick and thin film prepared on separate slides, slides were observed under the ×10 and ×100 objectives of the light microscope. Slides viewed under a high power field with parasite density less than three (<3), between 3 and 10 (3-10), between eleven to nineteen (11-19) and greater or equal to 20 (≥20) were classified as scanty, one plus (+), two pluses (++) and three pluses (+++) respectively. Data analysis was done using statistical packages for social science (SPSS) version 21 and minitab version 15.
Results: The results showed a significant (p<0.001) overall mean high serum CRP concentration of 25.63±14.40mg/l in the malaria infected patients compared to 3.74±1.02mg/l in the non-infected group, with a direct increment of the CRP level from the scanty to the three pluses with 9.50±0.37mg/l, 13.51±1.6mg/l, 44.19±2.62mg/l and 53.84±1.75mg/l recorded for scanty, one plus, two pluses and three pluses respectively.
Conclusion: The abnormally elevated level of CRP in the infected patients showed that CRP is a positive biomarker for *Plasmodium falciparum* malaria and can be used as an indicator of the disease coupled with other febrile symptoms.

Keywords: Biomarker; Immunoassay; C-Reactive Protein; Malaria; Plasmodium falciparum.

1. INTRODUCTION

Biomarker, a portmanteau of 'biological marker' refers to a sub-category of medical signs that are objective indication of medical state observed from the outside of the patient which can be accurately measured [1]. They are characteristics that can objectively be quantified, analyzed and evaluated as indicators of normal physiological, biological, pathogenic as well as pharmacological responses to therapeutic interventions [2]. The use of biomarker in basic and clinical researches and practices had become so common that their primary end point in clinical trials is now acknowledged almost without any question or query [1]. Biomarker plays

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important role and function in drug improvement and developmental processes as well as in the biomedical research world, as the understanding of the relationship between these quantifiable biological processes and clinical outcomes had been documented to be vital to the expansion of the knowledge of treatments of all diseases as well as the broadening of the understanding of healthy and normal physiology [3].

CRP is a calcium-dependent ligand binding acute phase protein (APP) whose plasma levels rises or reduces in reaction to inflammation and had been documented to evidently show the occurrence or non-occurrence of infection, trauma and infection as a response by the innate immune system [4,5]. It activates the classical pathway of the complement system through direct binding to the C1q of the classical cascade. It also interact with the complement system and Fc receptors in order to activate immune response through its binding to the lysophosphatidylcholine molecule found on the surface of antigens, dead and dying cells leading to membrane attack complex (MAC) of the dead cells [6]. This innate response occurs through alterations in a group of heterogeneous proteins in response to infection, trauma, myocardial infarction, collagen tissue disorders which results in the production and liberation of Interleukin -6 (IL-6), Interleukin-1 (IL-1) and Tumor necrosis factor-alpha (TNF-α) [7].
The human CRP is the most studied acute phase protein, although produced in the liver, extra-hepatic synthesis had also been documented in macrophages, smooth muscle like cells and atherosclerotic plagues although these extra-hepatic productions had been documented not to quantitatively influence the plasma concentration of CRP [8] (Yasojima, *et al., 2001).* It has a plasma half-life of 18hours and provides the first line of defense against microbes, parasites and pathogen [5].

With 212 million morbidities and 429,000 mortalities in the year 2015, malaria still remains an important public health menace, contributing significantly to poverty in Africa with about $12billion spent annually on illness, treatment and management [9]. *P. falciparum* is the deadliest of the plasmodia species and it accounts for 99% of malaria cases in the year 2016 making it the most prevalent malaria parasite in the sub-Saharan nations [10]. Several researches had gone into its prevention, treatment, elimination and diagnosis but just a few researches had been done on diagnostic biomarker. Etuk, et al. [11] evaluated the relationship between serum CRP and *P. falciparum* severity among children in Uyo, South-South, Nigeria while Ridker, et al. [12] established its usage as a strong marker of cardiovascular diseases. Despite these researches, there is still paucity of information, especially its usage as malaria biomarker in Port Harcourt, a major oil producing city in the South-South region of Nigeria.

2. MATERIAL AND METHODS

2.1 Study Population and site

The study was carried out among 400 consenting patients who fitted in into the inclusion criteria in the Out-Patient Department (OPD) of the University of Port Harcourt Teaching Hospital (UPTH), Port Harcourt, Rivers State, Nigeria. It was conducted over 6-months duration (November 2017 to April 2018). Port Harcourt is a metropolitan city and the capital of Rivers State of Nigeria. With an estimated population of about 7million people (2006 census), Port Harcourt is one of the most populated city in Nigeria and a major oil producing state in the country [13], contributing greatly to the economy of the country. Geographically, it is located on latitude 4.75^0N and latitude 7.00^0E [14] and it is bounded by the Atlantic Ocean, Anambra state, Imo and Abia states, Akwa Ibom state, Bayelsa and Delta states to the south, North, East and West respectively. The exclusion list in this study includes obese patients (Using the Body Mass Index), patients with recent record of surgery, those with

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cardiovascular and renal diseases, smokers, patients with recent history of malaria treatment, Pregnant and lactating mothers as well as those on birth contraceptives which are criteria that could abnormally elevate the serum CRP hence leading to false positive results.

2.2 Data Collection

Using a 5ml syringe, 2ml of blood was collected into both the Ethylene Di-amine Tetra-acetic acid (EDTA) and plain bottles, following standard procedures and practice. The EDTA samples were used for malaria parasite test while the plain bottle samples were used for quantitative analysis of CRP. Information on socio-economic, demographic (excluding names) and risk factors were collected from every participant in the study using the standardized questionnaires given to adult and to the guardians of the subject below 18years.

2.3 Microscopic Examination and quantification of malaria parasite

Preparation of blood samples for malaria parasites examination was done using the thin and thick blood films prepared on separate slides, which were stained with 10% Giemsa stain as described by Cheesbrough [15]. The slides were examined under the ×10 and ×100 objectives of the light microscope. Stained slides with parasite density less than three (<3) in a high power field was recorded scanty; between 3 to 10 (3-10) as (+); ten to nineteen (10-19) as (++), 20 and above (≥20) as (+++) as described by Wogu et al. [14].

2.4 Quantitative Analysis of Serum CRP

Quantitative analysis of serum CRP assay was done using the high sensitivity Enzyme Linked Immunosorbent Assay (HsCRP ELISA) technique [16] described thus;

The 2ml of blood collected in the plain bottles were centrifuged at 3500r/min for 15minutes. The sera were collected and diluted in 100 fold by mixing 5µl of sera with 495µl of the sample diluent. The antibody coated wells were placed in the holders, and 10µl of the CRP standards, diluted specimens and the controls were dispensed into the wells. 100µl of the CRP enzyme conjugate were dispensed into each wells respectively. The wells were then mixed for 30seconds and later incubated for 45minutes at 22^0C. The incubated mixtures were removed by flicking the plate contents into the waste container. The microtiter wells were them rinsed and flicked 5 times with distilled water. The wells were sharply stricken on paper towels to remove all residual water droplets and then 100µl of Tetra-methybenzidine (TMB) was dispensed into the wells and gently mixed for 5seconds. The wells were subsequently incubated for another 20 minutes. The reactions were stopped by adding 100µl of Stop Solutions. The wells were then gently mixed for 30 seconds, making sure all the blue colour changes to yellow completely. The absorbance were read at 450nm with a fully automated Chemical Analyzer (Map Lab Plus Reader Inc ©) which is a microtiter well reader.

2.5 Statistical Analysis

Analysis was done using descriptive statistics such as frequency, mean, standard deviation, percentages and charts. Data were summarized into tables and graphs as appropriate.

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Continuous variables like CRP was expressed as mean ± S.D while discreet variables were expressed in numbers and percentages. A non-parametric statistical test, Chi-square test was used to test association between variables or the effect or impact or influence of the variables. Consequently, a parametric One-way and two-way analysis of variance (ANOVA) test was used to examine the significant difference between two or more group means at 0.05 α significant levels. These analysis was done with the aid of SPSS version 21 and Minitab version 15.

3. RESULTS

Malaria infection was recorded in 254(63.5%) patients compared to 146(36.5) patients who were reported negative (P<0.05) as *Plasmodium falciparum* was the only plasmodium species recorded in this study. Scanty, +, ++ and +++ parasitemia levels accounts for 28(11.02%), 134(52.76%), 54(21.26%) and 38(14.96%) of the 245 malaria infected patients respectively. Sex analysis showed that, 174(43.5%) females and 226(56.5%) males were examined, which denoted a female to male ratio of 1:1.3. Of the 174(100%) females recruited for this study, 114(65.52%) were positive for malaria compared to 140(61.95%) recorded in their male counterpart. There was no significant difference between the two sexes (*P*>0.05). Of the 114 infected females, scanty, +, ++ and +++ accounts for 8(7.02%), 52(45.6%), 30(26.3%) and 24(21.05%) respectively while in the male gender, scanty, +, ++ and +++ accounts for 20(14.28%), 82(58.57%), 24(17.14%) and 14(10.00%) of the 140 infected males as shown in Table 1. Chi-square analysis of sex showed a p-value of 0.06, which is greater 0.05 significant level, which implies that sex is not associated with the severity of malaria infection. Malaria was more prevalent in those in the 21-30 years age group with 32.28%, followed by 18.90% in the 41-50 years age group, 18.11% in the 11-20 years age group, 16.53% in 31-40 years age group and the least recorded in the 51-60 years group with 14.17%. A *p* value of 0.001 across the age groups showed that age is significantly associated with malaria infection as shown in Table1.

A mean CRP of 3.74 ±1.02mg/l was recorded in the non-infected patients compared to a higher mean value of 25.63 ±17.40mg/l reported in the infected subjects. A mean CRP range of 18.67±13.00mg/l to 27.61±18.37mg/l was recorded in the infected age groups compared to a range of 3.23±1.0mg/l to 4.28 ±1.02mg/l in the non-infected age groups (Table 2). The result of the analysis also showed a direct proportion from the non-infected group across the four parasitemia levels as 3.74±1.02mg/l, 9.50 ±0.37mg/l, 13.51 ±1.60, 44.19mg/l±2.62mg/l and 53.84±1.75mg/l were recorded as shown in figure 1.

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Criteria	NE (%)	NNI (%)	NI (%)	Scanty (%)	+ (%)	++ (%)	+++ (%)	X^2	*P*-value	Decision
Female	174 (100)	60(34.48)	114(65.52)	8(7.02)	52(45.61)	30(26.32)	24(21.05)	12.628	0.06	Not-Significant
Male	226 (100)	86(38.05)	140(61.95)	20(14.28)	82(58.57)	24(17.14)	14(10.00)			
Total	400 (100)	146(36.50)	254(63.50)	28(11.02)	134(52.76)	54(21.26)	38(14.96)			
11-20years	70(17.50)	24(16.44)	46 (18.11)	6(13.04)	22(47.83)	18(39.13)	0(0)	33.71	0.001	Significant
21-30years	120(30.00)	38(26.03)	82(32.28)	8(9.76)	40(48.78)	16(19.51)	18(21.95)			
31-40years	88(22.00)	46(31.51)	42(16.53)	6(14.29)	18(42.85)	10(23.81)	8(19.05)			
41-50years	70(17.50)	22(15.07)	48(18.90)	6(12.50)	26(54.17)	6(12.50)	10(20.83)			
51-60years	52(13.00)	16(10.96)	36(14.17)	2(5.56)	28(77.78)	4(11.11)	2(5.56)			

Table 1. Demography and malaria parasitemia of the study population
NE= Number examined; NNI= Number not infected; NI=Number infected
Scanty (<3 parasites per high power field), + (3-10 parasites per high power field), ++ (11-19 parasites per high power field) +++ (20 and above parasites in high power field.

Age group (Years)	NE	NNI	NI	Mean CRP± S.D (mg/l)		Mean CRP ± S.D (mg/l)			
				NNI	NI	Scanty	+	++	+++
11-20	70	24	46	3.23 ±0.75	25.74±16.45	9.80±0.05	13.58 ±1.93	45.75 ±2.11	-
21-30	120	38	82	3.69 ±0.75	27.61±18.37	9.31±0.47	13.33 ±1.57	44.25 ±2.37	53.04±1.79

Table 2: Mean CRP for the Non-infected and the parasitemia levels.

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31-40	88	46	42	3.81 ±0.82	26.52±17.99	9.61 ±0.03	13.67 ±1.30	41.91 ±1.14	54.91±1.01
41-50	70	22	48	3.80 ±1.19	26.57±18.29	9.58 ±0.28	13.76 ±1.75	43.16 ±3.44	54.37±1.86
51-60	52	16	36	4.28 ±1.78	18.67±13.00	8.89 ±0.00	13.38 ±1.50	42.64 ±1.55	54.15±0.73
Total	**400**	**146**	**254**	**3.74 ±1.02**^{a}	**25.63±17.40**^{b}	**9.50 ±0.37**^{c}	**13.51 ±1.60**^{d}	**44.19 ±2.62**^{e}	**53.84±1.75**^{f}

NE= Number examined; NNI= Number not infected; NI=Number infected

Rows with ± S.D with different alphabet superscript is significant.

Scanty (<3 parasites per high power field), + (3-10 parasites per high power field), ++ (11-19 parasites per high power field) +++ (20 and above parasites in high power-field)

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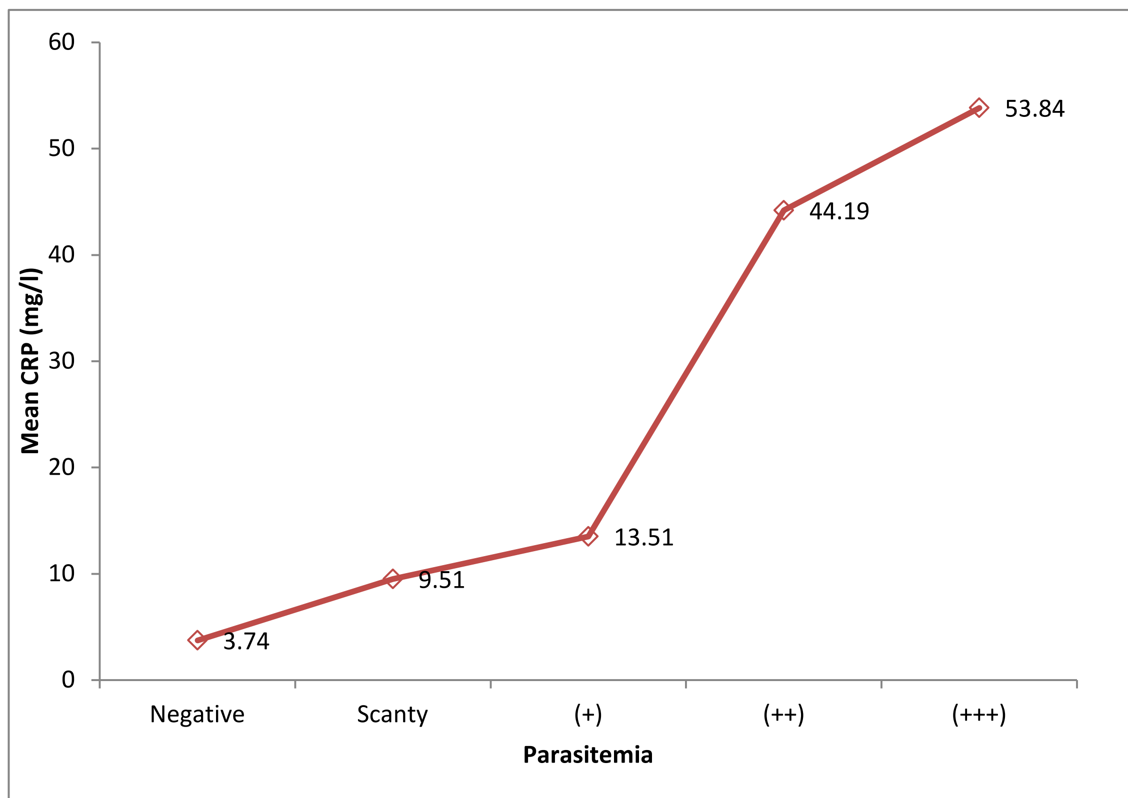

Figure-1:Line graph showing direct proportion between malaria Parasitemia and CRP

4. DISCUSSION

Malaria is a major infectious disease affecting nearly half of the world's population, with most morbidity and mortality coming from Saharan Africa, South East Asia, Latin America and the Middle East [17]. CRP is a non-specific biomarker of inflammation which plays a significant role in complement-mediated haemolysing of infected Red Blood Cells; it is involved in defense against pre-erythrocytic stage of malaria [18].
The findings from this study show greater mean serum CRP in malaria infected study subjects compared to the non-malaria subjects group. This is similar to the significantly increased CRP reported by Utuk et al. [11] in a research work done on children in Uyo, a city about 106km from Port Harcourt. These findings is also similar to researches by Hurt et al. [19] in a Tanzania study group, Imrie, et al. [20] in Papua New Guinea, Lubell, et al. [21] in South Eastern Asia and Dongho et al. [22] in Cameroon who all documented increased CRP in malaria infected subject compared to the non-infected control and hence concluded that CRP is an effective biomarkers for malaria infection. The higher levels of mean CRP in the malaria group compared to the non-malaria group buttress the roles played by *P. falciparum* antigen, Histidine Rich Protein 2 (HRP-2) in the production of CRP as highlighted by previous researches [11,23,24,25] as *P. falciparum* activates mononuclear cells which produce cytokines that stimulates the hepatic production of several inflammatory markers including CRP.
CRP increased with increase parasitemia as recorded by other studies [22, 26], who all documented proportional increase in serum CRP with increase in malaria parasitemia, but contradicted the findings of Imrie et al., [20] who reported an inconsistent increase in CRP with the severity parasitemia levels.

Limitations to this study includes underlying disease factors like undiagnosed renal and cardiac infections as well as other infections which could falsely elevate the level of CRP in the study group.

5. CONCLUSION

The results of this study not only showed that C-reactive protein levels were significantly elevated in malaria subjects compared to non-malaria subjects, but also showed that they are elevated with increase in *P. falciparum* parasitemia. Conclusively, C-reactive protein can serve as effective biomarker for assessing severity as well as parasite clearance in *P. falciparum* malaria hence it can serve both diagnostic and prognostic purpose in falciparum malaria management.

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CONSENT

Written informed consent form was obtained from patients above 18years and from the parents or guardians of those below 18years for the study.

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

Ethical clearance to undertake this research was gotten from the University of Port Harcourt Teaching Hospital Research Ethics Committee.
All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki

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