C-REACTIVE PROTEIN AS A LIKELY BIOMARKER FOR UNCOMPLICATED MALARIA.

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ABSTRACT

Introduction

Malaria is a public health disease that presents with symptoms that are similar to bacterial and viral infections that stimulates inflammation. C-reactive protein (CRP) is one of the markers of inflammation, which is identified to increase with high fever. The connection between malaria and C-reactive protein is still emerging. Therefore understanding the relationship between CRP and malaria parasitaemia is likely to guide its development as a possible marker for malaria. The objective of the study was to assess CRP and malaria parasitaemia in patients presenting with uncomplicated *Plasmodium falciparum* malaria in Lagos, Nigeria

Methodology: This was a cross-sectional study conducted in health facilities in Lagos State, Nigeria. The subsets of 100 patients enrolled in the study were randomly selected from participants who presented with fever or with history of fever in the last 48 hours. Patients presenting symptoms were captured in a designed case report form (CRF), blood smears done for malaria microscopy and plasma obtained from the 4mls of venous blood that was collected from each patient in Ethylenediaminetetraacetic acid (EDTA) bottles. Thick and thin blood smears were prepared for malaria microscopy on the same slide. Plasma separated from the blood in EDTA was used to run the CRP latex agglutination qualitative and semi quantification assay.

Result: This exploratory study recorded a positive correlation between CRP and parasitaemia which was statistically significant (Spearman's rho correlation coefficient= 0.845, $P<0.001$ and Pearson Correlation= 0.795, $P<0.001$), though with overall weak correlation ($R^2=0.4492$). Also, the highest level of CRP range (49->96mg/l) was seen in individuals with higher parasitaemia, which ranges from 244.4-21615.5mg/l.

Conclusion: High parasitaemia correlated positively with high CRP level though the overall correlation of all parasitaemia was weak. This is suggestive that CRP could be assessed for its potential as a biomarker for malaria using a larger sample

Key Words: C-Reactive Protein, Biomarker, Malaria

INTRODUCTION

Inflammation is a part of a complex biological response of the immune system to disease causing organisms, damaged cells or irritants which is grouped by five signs namely redness (rubor), swelling (tumor), heat (calor), pain (dolor) and loss of function (functiolaesa)[1]. Inflammation is a mechanism that helps the body get rid of foreign matter also known as non-self and dispose cells that are not useful, which could result to healing.

The immune system is a system that helps to protect the body from possible infectious agents as well as causing occasional harmful effects[2]. The immune system is composed of the innate or non-specific immune system and the adaptive or specific immune system[3,4.] During malaria infection, the immune system triggers an inflammatory response. As a result of these activities, certain proteins are formed to help fight off the infection, one of such protein is the C-reactive protein (CRP)[5-7].

CRP has been identified as a biomarker of inflammation[8], and a prognostic marker in malaria[7] that is known to induce adhesion molecule expression in human endothelial cells[9], ligand binding, activation of complement[6,8,10], opsonization and antigen presenting[11-12], protection against pre-erythrocytic stages of malaria[13], and could increase tolerance to malaria[14]. It has also been identified that CRP and the classical component acts together to promote non-inflammatory clearance of apoptotic cells[15]. These functionalities help in the prompt identification and clearance of the parasite by the immune system.

C reactive protein (CRP) level has been said to increase with the severity of malaria[16-19] and could be used as a diagnostic and management tool in malaria holoendermic areas like Nigeria to reduce disease burden[20-21]. CRP has also been identified to have adverse pathological effects like the clearance of RBC which results in severe anaemia[22-23], increasing susceptibility to *Plasmodium falciparum* malaria among Sudanese donors[24] and, playing a part in the expression of experimental cerebral malaria[25]. Malaria is a parasitic disease of importance in Nigeria and the better understanding of the "malaria-human immune" relationship will help the better management of the disease. In this study, we assessed the relationship between CRP and malaria parasitaemia in patients that presented with uncomplicated malaria with the aim of underscoring the utility of CRP as a potential biomarker for malaria diagnosis.

MATERIALS AND METHODS

Study Area and Participant Recruitment:

This study was carried out in Lagos state situated in the south-west zone of Nigeria where perennial malaria transmission occurs. Patients suspected to have uncomplicated malaria, including those with fever (>37.5^{0}C) or those with history of fever (<37.5^{0}C) in the last 48 hours were enrolled from four health facilities, namely: Regina Mundi Catholic Hospital, Mushin; Randle General Hospital, Surulere; Igando General Hospital, Igando; and Badagry General Hospital, Badagry, Lagos, Nigeria.

The symptoms of patients that consented to participate in the study were recorded in a case record form (CRF) after which venous blood was collected. Patients with signs of severe malaria and other severe diseases were excluded from the study.

Sample Collection:

Four millilitres of blood (4mls) was collected from each patient in EDTA containers. A total of 1,867 patients were enrolled from the four health facilities. The samples collected were transported in a cool sample carrier bag to the ANDI Centre of Excellence for Malaria Diagnosis, College of Medicine University of Lagos, Idiaraba, Lagos where they were processed for malaria microscopy and assay for CRP determination.

Malaria Microscopy

Thick and thin malaria blood films (MBFs) were made from newly-collected blood in EDTA bottle of each patient on the same slide and stained following standard procedures. The patient's absolute white blood cell count was used to determine the patients' parasite density per micro-litre of blood (parasite/µL of blood). Essentially, two independent certified Malaria Microscopist read each slide with a third certified Microscopist

that served as an arbiter where there was discrepancy in detection, stage and species of the parasite, and parasite enumerated.

Separation of Plasma and Serological Assay for C-reactive protein

The blood samples collected in the EDTA bottles were spurned at 4000 revolutions per minute (RPM) for ten minutes to separate the plasma. Separated plasma was stored in cryovials at -20°C from which aliquots were taken for the CRP serological assay. A sub-set of 100 malaria parasite positive and negative samples from the suspected malaria patients from the four health facilities was randomly selected for the assay.

The Biotec Cambridge CRP latex test kit for 100 tests was used to determine the presence of CRP in the separated sera following the manufacturer's instructions. A serial dilution of the plasma samples were done and calculated for. Using normal saline,100µL of saline was placed in about 5 tubes each and another 100µLof sample was placed in the first tube, which is mixed properly. Then, 100µL of mixed sample with saline is taken using a new pipette tip and mixed in the next tube containing normal saline; thus a serial dilution was done on each positive sample (Table 1). A drop (40µL) of reagent is placed in the circle of the slide and the 50µL of sample prepared via serial dilution is added, the reagent and the serum is spread round the circle and tilted backwards and forward approximately once every 2 seconds for 2 minutes. This procedure was to determine the highest dilution that will show reaction. Once this is determined, the estimated level of CRP in the sample is calculated and recorded (Table 1).

Table 1: Serial dilution procedure and calculation

Dilutions	*½*	*¼*	*1/8*	*1/16*	*1/32*	*1/64*	*1/128*
Sample Serum	100µL	-	-	-	-	-	-
Saline	100µL	100µL	100µL	100µL	100µL	100µL	100µL
Volume of Sample used on test slide	50µL	50µL	50µL	50µL	50µL	50µL	50µL
6 x Titer	6 x 2	6 x 4	6 x 8	6 x 16	6 x 32	6 x 64	6x128
Mg/ml	12	24	48	96	192	384	768

Ethical Considerations

All patients enrolled in this study gave written informed consent after the purpose of the study was explained. Patients who refused to participate also received standard care in the presenting facilities. The study protocol was reviewed and approved by the Research Ethics Committee of the College of Medicine, University of Lagos, Lagos.

Data Analyses

The data obtained were entered in a computer and validated to ensure quality. The Spearman's rho and Person's correlation was used in the determination of association as appropriate. P-values was set at <0.001 for significance.

RESULTS

The subset of 100 participants made up of 61 females and 39 males was assayed from the 1,867 patients that were enrolled for significant level of C-reactive protein (CRP >6). Of the 100 participants, 75 (75%) had documented fever (temperature >37.5°C) while 25 (25%) had normal temperature but with history of fever. A total of 13 participants (13%) were microscopy positive from both groups. The 13 microscopy positive samples that was tested for CRP, 8 (61.5%) had CRP level of 0-95mg/L while 5 (38.5%) had CRP level of 96-384mg/L (Table 2).

Table 2. Baseline characteristics of the sub-set of the study population assayed for CRP.

Variables	CRP (0-95 mg/L) (n=73)	CRP (96-384 mg/L) (n=27)	P value
Age (Years)			
Mean ± SD	31.01 ± 16.01	22.30 ± 13.4	0.013
Sex			
Male	24 (32.9%)	15 (55.6%)	0.039
Female	49 (67.1)	12 (44.4%)	
Malaria			
Negative	65 (89%)	22 (81.5%)	0.318
Positive	8 (11%)	5 (18.5%)	
Febrile (Fever)			
No	22 (30.6)	3 (11.1)	0.047
Yes	50 (69.4)	24 (88.9)	
CRP			
Geometric mean	0	137.52	<0.001
Median (Range)	0 (0-48)	96 (96-384)	<0.001

Note: N = number of samples. SD = standard deviation. CRP = C-reactive protein (mg/L). P values were based on Pearson Chi-Squared test or Exact Chi-Square for categorical variables and ANOVA for the comparison of the mean of the continuous variables.

CRP was associated with an increase in temperature in individuals with fever (88.9%) when compared to those without fever (11.1%). The relationship between CRP level and parasite density using the Spearman's rho correlation (coefficient t= 0.845, P<0.001) and Pearson Correlation (0.795, P<0.001) was significant (Figure 1). Higher level of CRP (96-384mg/l) was seen in individuals with high parasitaemia, which ranged from 244.4 to 21615.5 P/μL of blood, while those with low parasite density had lower concentration of CRP (Table 3).

Table 3. CRP concentration compared with malaria parasitaemia

CRP Concentration (g/dl)	Malaria Parasitaemia (P/ml)
6-12	28
13-24	33
25-48	Nil
49-96	244.4-3017.2
>96	1560-21615.5

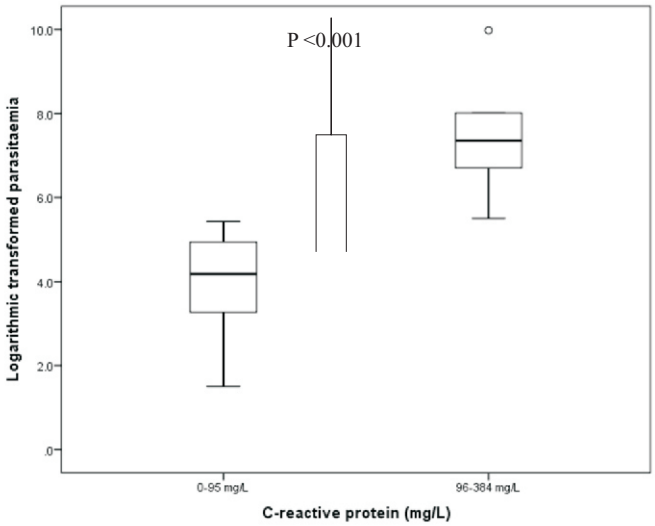

Figure 1. Logarithmic transformed parasitaemia level with CRP (mg/L). The boxes illustrate the total observations equivalent to the first quartile and the third quartile. The median is represented by the horizontal line. The outlier is shown as a circle point.

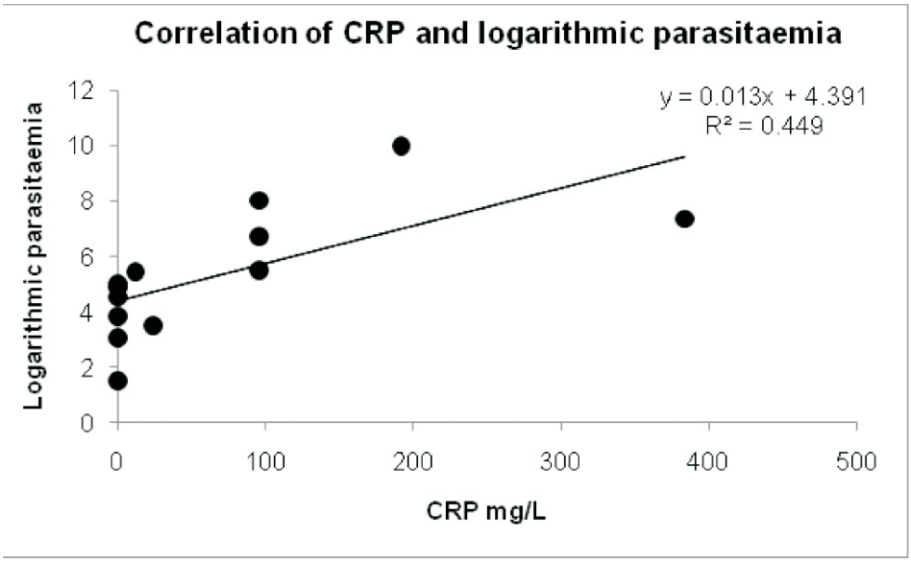

Figure 2: Overall correlation of CRP (mg/L) and Logarithmic parasitaemia

DISCUSSIONS AND CONCLUSIONS

CRP can be used as a prognostic marker since its concentration in human sera reduces with reduction in parasitaemia and increases with increased parasitaemia; this is supported by other studies[5,7] which affirmed that CRP can be used as an effective biomarker in assessing malaria severity and could also be used as a follow-up test for malaria patients to track recovery progress. Our study showed that CRP was associated with malaria since there was a significant increase in the level of CRP in participants with malaria as identified in other studies[5,14,23,26-27.]

This study further highlighted that CRP was associated with increased temperature as seen in the number of individuals without fever (<37.5^{0}C) at the point of sample collection that had high level of CRP when compared to those who presented with fever (>37.5^{0}C) at the point of sample collection (88.9%), thus identifying CRP as a marker of inflammation associated with fever since fever is a sign of systemic inflammation[8].

We noted that the highest level of CRP was seen in individuals with higher parasitaemia while those with low parasite density had lower concentration of CRP. This is consistent with some studies that reported increased C-reactive protein concentration with severity of malaria infection[16-19]. Consequently increased level of CRP and increased parasitaemia, which could be used for

diagnostic basis to differentiate malaria fever from non-malaria fever like Dengue fever in areas where other tests are not available[27].

CRP may also have an important role to play in the immune response to malaria since there is a possibility the marker is involved in the clearance of the parasite or the pathogenesis of the disease due to its high concentration during high parasitaemia and lower concentration in cases of low parasitaemia. CRP may play an important role in immune response to malaria through the process of inhibiting dendritic cells, neutrophils or complement regulatory proteins resulting in the clearance of red blood cells which results in severe anaemia[6,22,23]. Also C-reactive protein could bring about systemic autoimmunity through binding to apoptotic cells and protecting the cells from terminal complement Components, thereby sustaining an anti-inflammatory innate immune response[15].

Some other studies recorded a strong association between increase malaria susceptibility and presence of CRP-286 A-allele[24] and, the expression of experimental cerebral malaria (a sign of severe malaria) as being promoted by CRP[25]. Another study in Ghana suggested that CRP levels are positively related to immune responsiveness and malaria parasitaemia[28]. Furthermore, CRP can be used to track the acquisition of tolerance to malaria that is suggestive of a protective function in malaria patients[14]. In addition, CRP could be useful for malaria immunoepidemiology; however, it was not clear if CRP is beneficial or detrimental[18].

In this assessment study, we showed that the concentration of C-reactive protein in sera increased with increase in malaria parasite density in patients presenting with uncomplicated *Plasmodium falciparum* malaria in Lagos, Nigeria. This therefore, could indicate that CRP is associated with the functionality of parasite clearance in malaria patients since the protein level is seen to increase with an increase in parasite density or could also be involved in the disease pathogenesis of which further studies on the immunological role of CRP will help to clarify its precise immunological function. These observations suggest that CRP could be explored for its potential as a biomarker for malaria using a larger sample.

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