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Phospholipases Activities in Malaria Patients: Possible Effects of Drug Administration

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Abstract: Malaria is one of the most deadly diseases confronting sub-Saharan Africa especially Nigeria and results in the alteration of some biochemical parameters including enzymes as one of the biochemical consequences. The possible effects of drug administration on phospholipases activities in the plasma and the red blood cell membrane of malaria positive individuals were evaluated in this study. A total of 100 individuals (consisting of 87 malaria positive patients and 13 malaria negative individuals used as control) were recruited for this study. The medical history of the patients such as the duration of illness, age, gender and drug administered and social status were obtained. The degree of parasitaemia in the blood of malaria positive individuals was also determined. The result of this study showed that there was significant ($p<0.05$) increase in plasma phospholipase A_2 activities in malaria patients (1.15±0.04U/mg) when compared to the control (0.52±0.05U/mg) and this increase depends on the degree of parasitaemia. However, there was no significant ($p>0.05$) change in plasma phospholipase C activities in malaria patients (4.51±0.16U/mg) compared to the control (4.60±0.69U/mg). Also, there were significant ($p<0.05$) increase in RBC membrane phospholipase A_2 and phospholipase C activities in malaria patients compared to the control. The increase in phospholipase A_2 and phospholipase C activities were found to depend on the degree of parasitaemia and duration of illness of the malaria patients. The result of this study also showed no significant ($p>0.05$) change in plasma phospholipase C activity in the control individuals and the test patients with regards to drug administered, but there were significant ($p<0.05$) increases in the plasma phospholipase A_2 in the test patients on drugs when compared to the control individuals with malaria patients on chloroquine and fansidar having highest phospholipase A_2 activity. The RBC membrane phospholipase A_2 activity in the control individuals was significantly ($p<0.05$) lower when compared to the malaria patients administered drugs. Also, the phospholipase C in RBC membrane of control individuals showed significant decreased activities compared to the malaria patients on drugs with patients on fansidar having highest phospholipase C activity. It may be concluded from the study that phospholipases activities may likely increase in malaria patients, this increase may likely depend on the severity of parasitemia, duration of illness and type of drug administered.

Key words: Malaria · Antimalaria drugs · Phospholipase activities

INTRODUCTION

Malaria is the world's most common parasitic infectious disease which poses serious health challenges [1, 2]. A wide range of drug has been developed in combating malaria, including one of the first anti-malaria compounds quinine, chloroqiune, combination of sulphadoxine and pyrimethamine (fansidar), artesunate and recently the combination of artemisinine and lumefantherine (ACT). Nevertheless, there is continuous spread of drug-resistant parasite strain, which is partly responsible for the spread of malaria and require the development of new anti-malarial drugs. Unfortunately, the search for such anti-malaria compounds is hindered

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because the mechanism of action of most anti-malaria compounds is not fully known or poorly understood. It has been demonstrated that some anti-malaria drugs may act by inhibiting some plasmodial cellular enzymes that plays a role in the pathogenesis and infectivity of the parasite [3].

Phospholipases are group of enzymes that catalyse the cleavage of phospholipids, converting the phospholipids into fatty acids and other lipophilic substances. Phospholipases A_2 (E.C. number 3.1.1.4) catalyse the hydrolysis of the fatty ester bond of phospholipids at the sn-2 position to release unsaturated fatty acids and lysophospholipid. Phospholipase C (E.C. 3.1.1.11) catalyse the cleavage of glycerophosphate bond of phospholipid. Phospholipase A_2 and phospholipase C are involved in diverse cellular processes such as membrane homeostasis, nutrient acquisition, generation of bioactive molecules that mediates processes such as fever and high temperature, intricate modulation of host immune response and tissue invasion [4]. The activities of these enzymes increases during parasitic infection. Phospholipase A_2 has been reported in some intracellular parasites infection such as; *Trichomonas vaginalis, Toxoplasma gondii* where it is required for tissue invasion [5, 6]. The activity of the enzyme has been demonstrated to increase during malaria parasite infection [7], high levels of endogenous circulating phospholipase A_2 may contribute to hemolysis of plasmodial parasitized erythrocytes in patients with malaria with release of immature parasite or the release of mature infective parasite. This may be significant from the standpoint of host defence in that phospholipase A_2 may augment the action of TNF-α to enhance killing of parasite or to promote the infection of another erythrocyte by liberating mature plasmodium. The plasmodial phospholipase C might degrade host-derived lysophosphatidylcholine to supply the parasites with phosphocholine and/or monoacylglycerol for their efficient intra-erythrocytic growth since lysophosphatidylcholine is abundant in the human plasma, especially in forms associated with lipoproteins [8]. Lipid metabolism has been demonstrated to increase during malaria parasite infection [9 and 10]. Increased destruction of erythrocyte leads to anemia which is one of the commonest complication of plasmodial infections [11]. Thus, these enzymes may be exploited as a target for chemotherapeutic agent against malaria parasite infection and pathogenesis, thereby reducing the spread of the parasite. Therefore, in this study, we report the activities of phospholipase A_2 and phospholipase C in malaria patients and the possible effect of some antimalarial drugs on the activities of these enzymes.

MATERIALS AND METHODS

Subjects: Patients admitted to Central Hospital, Benin City and a private clinic (Time hospital) all in Benin City, Nigeria, with diagnosis of malaria were recruited for this study. The study was conducted in compliance with the Declaration on the Right of the Patient (WMA, 2000) after approval by the Ethical Committee of the Central Hospital, Benin City, Edo State, Nigeria. Before enrolment for the study, informed consent was obtained from the patients or their relatives. The study included a total of 100 patients, 87 were tested and were malaria parasite positive (test patients), while 13 were malaria parasite negative (control patients). Of the 87 malaria patient 42 (48%) were males, while 45 (52%) were females, the age of the patients range from 10 months to 65 years and mean age of 30 years. Maximum number (50%) of cases occur in 18-30 years age group. On admission, the medical history including; age, sex, social status, duration of illness and drug history of the patients were obtained and recorded.

Collection of Blood Sample: Blood sample was collected from patients by veinous puncture into 5ml lithium heparin and 5ml ethylenediamine tetraacetic acid (EDTA) anti-coagulant bottles from the patients and delivered to the laboratory within 3hrs after collection. The samples were taken for enzymatic assay and in the diagnosis and determination of degree of parasitaemia respectively.

Malaria Diagnosis: Malaria was diagnosed with blood smear staining method of Warhurst and Williams (1996) [12]. Malaria parasite detection was done by microscopic examination of thin blood films stained with 3% Giemsa stain. The parasitaemia was graded as: += mild (1-999μL^{-1}), ++= moderate (1, 000-9, 999μL^{-1}) and +++ = severe (>10, 000 μL^{-1}).

Enzymatic Assay

Enzymatic Assay of Phospholipase A_2: Phospholipase A_2 was estimated according to the procedure described in Sigma enzymatic assay manual, a modification of the phospholipase A_2 enzymatic assay as described by Henry *et al*., (1972). 0.35ml of Tris-HCl buffer and calcium chloride were pipette into test tubes labelled test and blanks, 0.35ml of lecithin and deoxycholate were added to both test-tubes and equilibrated at 37°C, 0.025ml of the sample solution was added only to the test only and 0.025ml of calcium chloride was added to blank tubes. The mixture was immediately mixed by inversion and incubated at 37°C for 5minutes. 0.1ml aliquot was removed from the mixture. To the 0.1ml aliquot, 0.75ml of ether was

added to both test tubes followed by the addition of 0.10ml of hydroxylamine (reagent G) and sodium hydroxide. The solutions were incubated at 25°C for 20 minutes. 0.15ml of hydrochloric acid (reagent I) and ferric chloride (reagent J) were added. The mixture was mixed by swirling and transferred to suitable cuvette and absorbance read at 570nm in spectrophotometer.

Enzymatic Assay of Phospholipase C: A modification of the method of MacFarlane and Knight (1981) was used. After hydrolysis of lecithin by the enzyme, the released inorganic phosphorylcholine is digested and measured as inorganic phosphorus. 2.0ml of 0.1M Tris-malate buffer pH 7.3 was pipette into two test tubes labelled blank and control, 0.5ml, 1.5ml and 1.0 ml calcium, lecithin and 1% albumin respectively were added to the test tubes. 0.1ml of sample solution was then added to the test solution. The test tubes were incubated for 30 minutes at 37°C and then 5.0ml 5% tri-chloroacetic acid was added to the test tubes. After centrifugation, 1.0ml portion of the supernatant was evaporated and 1.0ml of 5N sulphuric acid, 1.0ml of 2N nitric acid were added, the mixture was heated until fuming. It was cooled and 1.0ml water was added and then boiled in a water bath for 5minutes, allowed to cool and 1ml 2.5% ammonium molybdate and 6.5ml water added. At timed intervals, 0.4ml reducing agent was added, the absorbance was read after 10minutes in a suitable spectrophotometer set at 660nm. A standard curve was prepared using increasing concentration of potassium hydrogen tetra-oxophosphate (v).

Extraction and Purification of Lecithin from Egg Yolk: Phosphatidylcholine was isolated using the method described by Sreedevi *et al.* [13]. Firstly, egg yolk was separated from albumin manually. Chloroform: Methanol (2:1v/v) solution was added to the egg yolk and mixed well. The mixture was stirred well using magnetic stirrer for 2 hours, followed by transferring into a separating funnel. The clear solution which settle below the mixture contain the phospholipids and this solution was separated and concentrated, followed by the addition of ice cold acetone. The mixture was kept at very low temperature (-20ºC) for phosphatidylcholine to get precipitated out of the phospholipid solution. The precipitated mass was separated using vacuum filtration. The obtained mass was stored in an amber coloured bottle in the refrigerator until use.

Statistical Analysis: The statistical analysis of data was performed using the one way ANOVA and student's t-test with SPSS version 16. Significant level was set at $p<0.05$. The result values were expressed as mean ± standard error of mean. Also, correlation analysis and linear regression were plotted for the phospholipase A_2 and phospholipase C against the degree of parasitemia, duration of illness and age range of malaria patients.

RESULTS

The results obtained showed that there was statistical significant ($p<0.05$) increase in the mean phospholipase A_2 and phospholipase C activities of malaria patients compared to the control individuals with the exception of plasma phospholipase C in which there was no statistical significant ($p>0.05$) difference between that of malaria patients and control individuals at 4.51±0.16 and 4.60±0.69U/mg respectively (Table 1).

There was also significant increase in plasma phospholipase A_2 activity of the malaria patients with respect to their degree of parasitaemia when compared to the control individuals, with severe malaria patients having the highest phospholipase A_2 activity. However, the mean plasma phospholipase C activity in control individuals, mild and moderate malaria patients were not significantly different but were significantly lower when compared to that of severe malaria patients (4.60±0.54, 4.29±0.17, 5.20±0.37 and 6.63±1.10U/mg respectively). The RBC membrane phospholipase A_2 activity in the control and mild patients were not significantly ($p>0.05$) different but were significantly lower compared to moderate and severe malaria patients. The result for RBC membrane phospholipase C activity in the control, mild, moderate and severe patients were significantly ($p<0.05$) different, with both moderate and severe patients having highest activities (but not significantly different) compared to the control individuals (Table 2).

The result in Table 3 showed that the mean plasma and RBC membrane phospholipase A_2 activities in the malaria patients were significantly ($p<0.05$) different in relation to duration of illness with malaria patients of 13 days and above having highest value of enzyme activities. Also RBC membrane phospholipase C activity follow similar pattern, however, there was no significant difference between 1-4 days and 5-8 days groups at 11.56±0.82, 11.8±0.58U/mg, respectively. However, the plasma phospholipase C activity in the control individuals were not significantly different from that of other groups of patients except for 13 days and above.

Table 1: Phospholipase A_2 and phospholipase C activities in malaria patients

	Plasma		RBC Membrane	
Analytes/ Patients	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)
Control	0.52±0.05[a]	4.60±0.69[a]	0.55±0.0.04[a]	5.42±0.32[a]
Malaria Patients	1.15±0.04[b]	4.51±0.16[a]	1.22±0.05[b]	10.95±0.55[b]

Values in the same column with different superscripts differ significantly ($p<0.05$)

Table 2: Effect of degree of parasitaemia on phospholipase A_2 (PLA_2) and phospholipase C (PLC) activities in plasma and RBC membrane

	Plasma		RBC Membrane	
Degree of Parasitemia	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)
Control	0.52±0.05[a]	4.60±0.54[a]	0.54±0.04[a]	5.43±0.32[a]
Mild (+)	0.95±0.03[b]	4.29±0.17[a]	0.98±0.03[a]	9.98±0.30[b]
Moderate (++)	1.63±0.06[c]	5.20±0.37[a]	2.37±0.93[b]	15.09±0.94[c]
Severe (+++)	1.98±0.30[d]	6.63±1.10[b]	2.14±0.65[b]	16.38±4.25[c]

Values in the same column with different superscripts differ significantly ($p<0.05$)

Table 3: Effect of duration of illness on phospholipase A_2 (PLA_2) and phospholipase C (PLC) activities in plasma and RBC membrane

	Plasma		RBC Membrane	
Duration of Illness	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)
Control	0.52±0.05[a]	4.60±0.54[a]	0.54±0.04[a]	5.43±0.32[a]
1-4 days	1.16±0.08[b, c]	4.32±0.19[a]	1.16±0.10[b]	11.56±0.82[b]
5-8 days	1.07±0.05[b]	4.42±0.23[a]	1.14±0.06[b]	11.84±0.58[b]
9-12 days	1.25±0.14[b, c]	5.07±1.01[a]	1.36±0.25[b,c]	14.23±1.60[c]
13 days and above	1.33±0.46[c]	5.52±0.67[b]	1.55±1.17[c]	15.36±1.74[d]

Values in the same column with different superscripts differ significantly ($p<0.05$)

Table 4: Gender consideration of phospholipase A_2 (PLA_2) and phospholipase C (PLC) activities in plasma and RBC membrane

	Plasma		RBC Membrane	
SEX	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)
Control- Male	0.54±0.06[a]	4.80±0.23[a]	0.55±0.07[a]	5.60±0.35[a]
Test-Male	1.18±0.07[b]	4.24±0.28[a]	1.25±0.07[b]	12.60±0.73[b]
Control-Female	0.45±0.08[a]	4.32±0.19[a]	0.53±0.02[a]	5.70±0.87[a]
Test-Female	1.14±0.05[b]	4.65±0.20[a]	1.42±0.17[b]	11.90±0.61[b]

Values in the same column with different superscripts differ significantly ($p<0.05$)

Table 5: Effect of age on phospholipase A_2 (PLA_2) and phospholipase C (PLC) activities in plasma and RBC membrane

	Plasma		RBC Membrane	
Age	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)
Control	0.52±0.05[a]	4.60±0.54[a]	0.54±0.04[a]	5.43±0.32[a]
1-10 years	0.95±0.13[b]	4.64±0.69[a]	1.08±0.13[b]	11.12±1.67[b]
11-17 years	1.30±0.181[b]	3.99±0.28[a]	1.15±0.29[b]	10.19±0.40[b]
18-30 years	1.18±0.06[b]	4.81±0.26[a]	1.22±0.07[b]	12.27±0.59[b]
31 years and above	1.16±0.07[b]	4.11±0.13[a]	1.30±0.11[b]	12.81±0.94[bc]

Values in the same column with different superscripts differ significantly ($p<0.05$)

Table 6: Effect of drug on phospholipase A_2 (PLA_2) and phospholipase C (PLC) activities in plasma and RBC membrane.

	Plasma		RBC Membrane	
Drugs	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)	PLA_2 ($\times10^{-2}$ U/mg)	PLC ($\times10^{-2}$ U/mg)
Control	0.52±0.05[a]	4.60±0.54[a]	0.54±0.04[a]	5.42±0.32[a]
ACT	1.10±0.07[b]	4.11±0.16[a]	1.18±0.11[b]	11.91±0.98[b]
CQ	1.23±0.17[b, c]	5.35±0.60[a]	1.24±0.16[b]	14.55±1.29[c]
Fansidar	1.59±0.28[c]	4.21±0.04[a]	1.72±0.26[b]	17.36±2.32[d]
No drugs	1.16±0.06[b]	4.45±0.20[a]	1.28±0.08[b]	12.01±0.67[b]
Others	1.07±0.06[b]	5.18±0.76[a]	1.08±0.12[b]	10.59±0.98[b]

Values in the same column with different superscripts differ significantly ($p<0.05$)

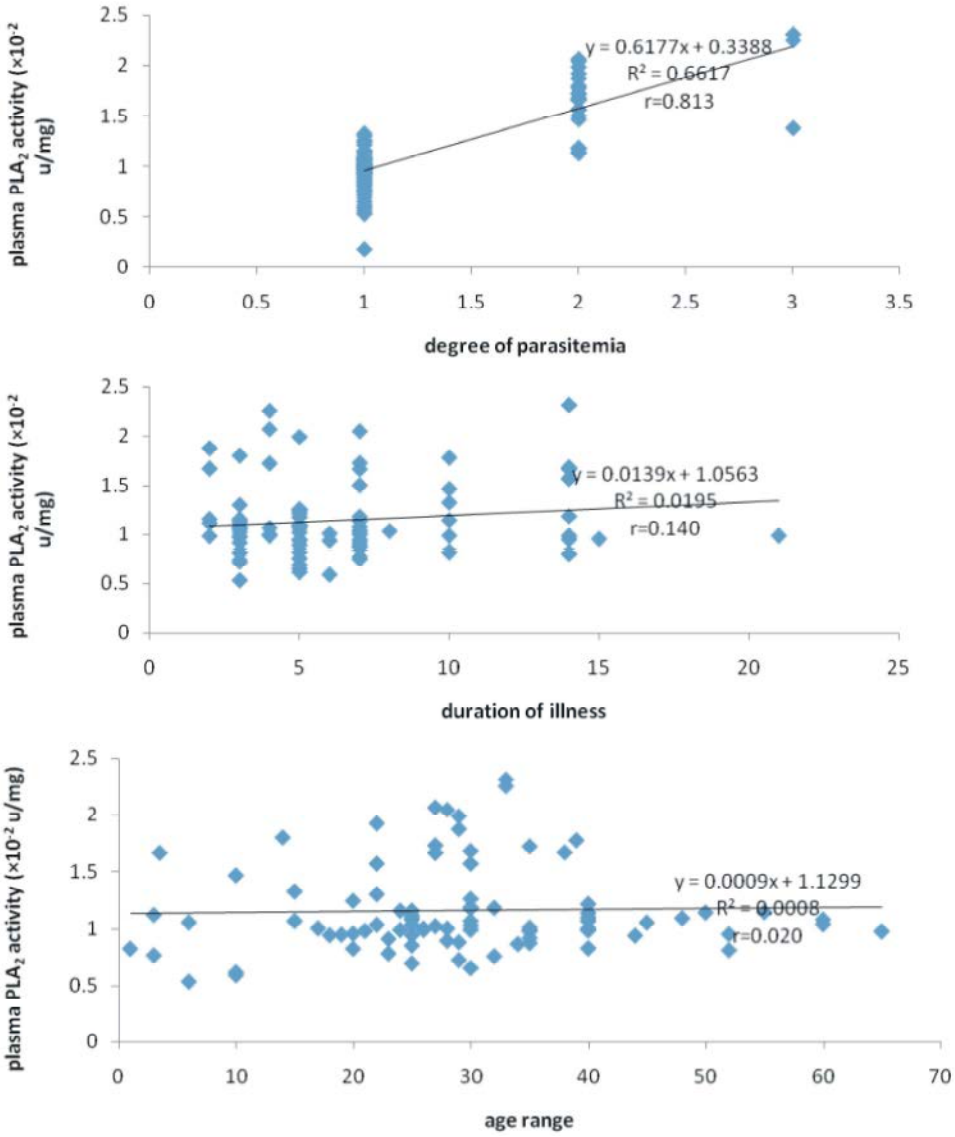

Fig. 1: Plot showing significant positive correlation (r=0.813, r=0.140) for plasma phospholipase A_2 activity against degree of parasitemia, duration of illness respectively and non-significant correlation (r=0.020) for plasma phospholipase A_2 activity against age range

In terms of gender consideration of the patients, the mean plasma phospholipase A_2 activity, RBC membrane phospholipase A_2 and phospholipase C activities were significantly elevated when ($p<0.05$) compared to the control individuals. However, the mean plasma phospholipase C activities value were observed not to be significantly different in the control individuals and the malaria patients according to their sex (Table 4).

Considering the age of the malaria patients, the result obtained showed that the mean plasma phospholipase A_2 activity, RBC membrane phospholipase A_2 and phospholipase C activities in the control group were significantly ($p<0.05$) lower compared to other age groups of the malaria patients. However, there were no statistical significant ($p>0.05$) difference among the age groups in their phospholipases activities. The plasma phospholipase C activities in the control group and the age groups of the malaria patients were not significantly different, although the plasma phospholipase C in age group 11-17 years was observed to be lowered than other age groups and the control individuals but was not statistically significant (Table 5) ($p>0.05$).

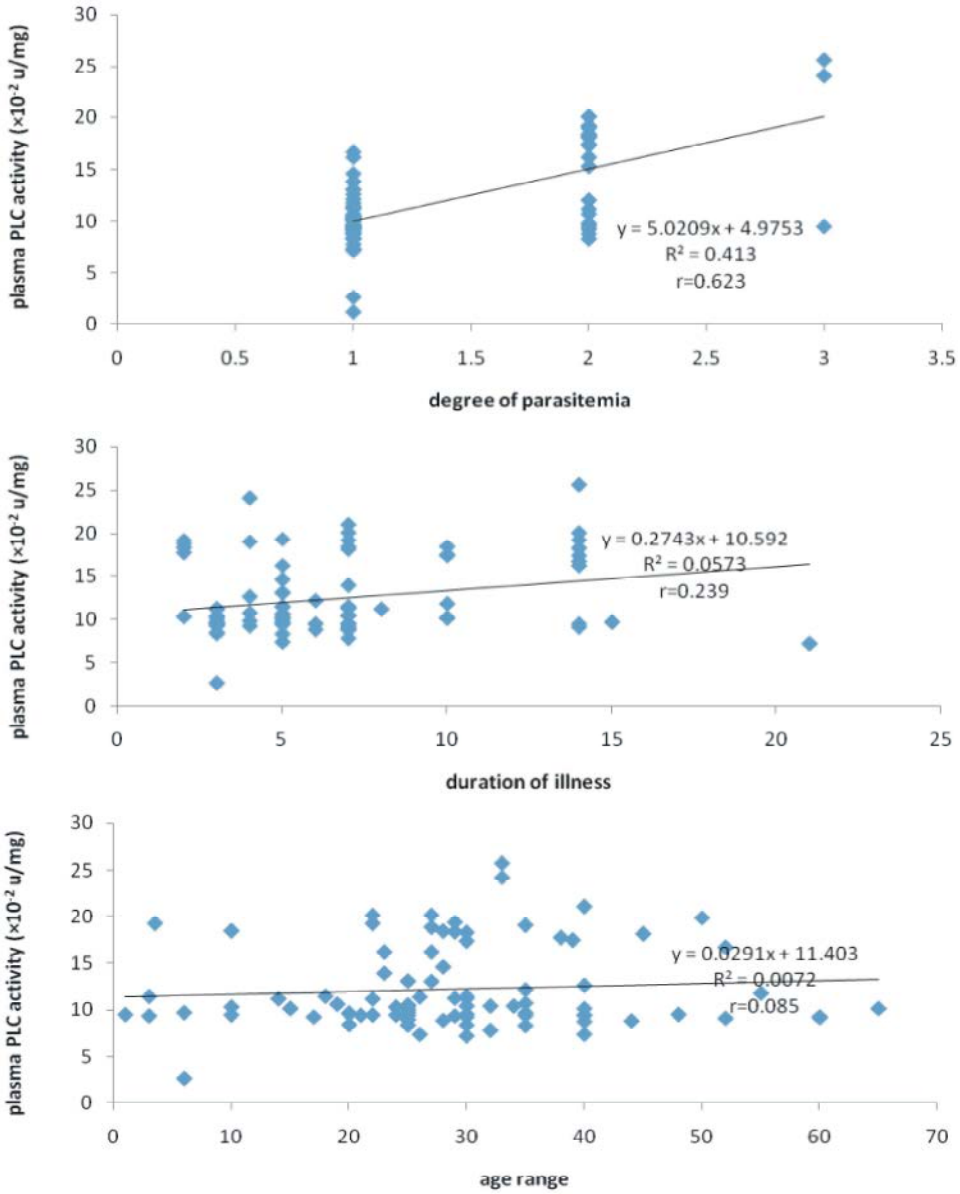

Fig. 2: Plot showing significant positive correlation (r=0.623, r=0.239) for plasma phospholipase C activity against degree of parasitemia, duration of illness and non-significant correlation (r=0.085) for plasma phospholipase C against age range

The results recorded after drug administration from this studies showed that the plasma phospholipase A_2 activities in malaria patients on chloroquine and fansidar were significantly ($p<0.05$) higher than those on other anti-malaria drugs such as ACT and other type of anti-malarials (whose enzyme activities show no statistical significant ($p>0.05$) difference) and the control individuals (which has lowest enzyme activity). However, the plasma phospholipase C activities showed no significant increase in all the groups when compared to control individuals. The result obtained also showed that the RBC membrane phospholipase A_2 in control individuals was significantly lower when compared to malaria patients on drug but shows no significant ($p>0.05$) difference with respect to the particular drugs administered to the malaria patients. The RBC membrane phospholipase C had the highest activities in malaria patients on fansidar. Also, patients on chloroqiune show significant increase in enzyme activities when compared to the control individuals and other malaria patients on other anti-malaria drugs (Table 6).

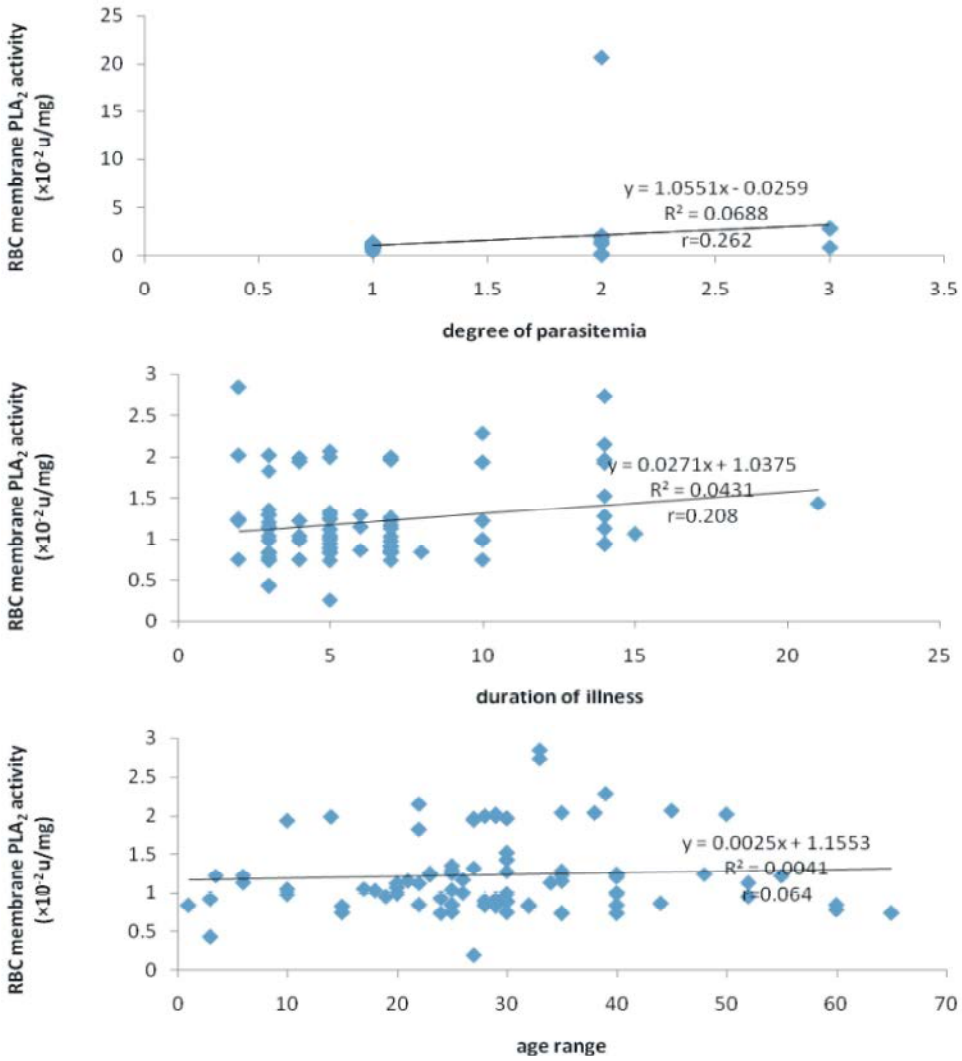

Fig. 3: Plot showing significant positive correlation (r=0.262, r=0.208) for RBC membrane phospholipase A_2 activity against degree of parasitemia, duration of illness and non-significant correlation (r=0.064) for RBC membrane phospholipase A_2 activity against age range.

The phospholipases activities correlate positively with degree of parasitaemia, duration of illness and age ranges. The highest significant positive correlation was observed in plasma phospholipase A_2, plasma phospholipase C, RBC membrane phospholipase C activities and degree of parasitaemia. However, there were no significant correlation for plasma phospholipase A_2 activity with age range and also, for plasma phospholipase C against age range.

DISCUSSION

The role of phospholipases most especially phospholipase A_2 and phospholipase C in the pathogenesis and growth respectively of some intracellular parasites including *plasmodium spp.* have been fully studied [14]. So far, among the genus *plasmodium*, it is only *P. falciparum* reported to produce phospholipase A_2 whose inhibition may be related to the therapeutic action of some anti-malarial drugs [15]. This study reports the possible effects of ant-imalarial drugs on phospholipase A_2 and phospholipase C activities in malaria patients.

In the present study, there were significant ($p<0.05$) increase in both plasma and RBC membrane-bound phospholipase A_2 activity in malaria patients as compared to the control individuals. These increase in enzyme activities were observed to increase with the degree of

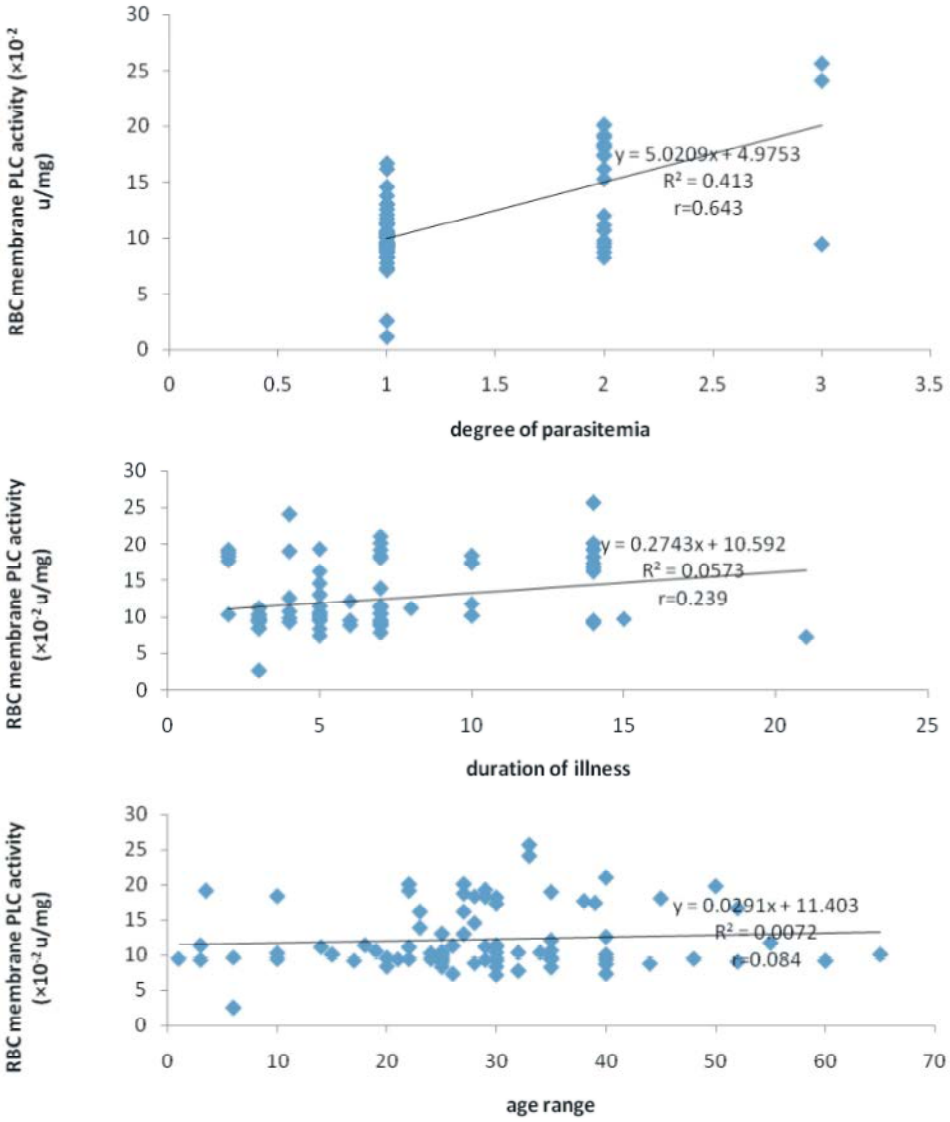

Fig. 4: Plot showing significant positive correlation (r=0.643, r=0.239) for RBC membrane phospholipase C activity against degree of parasitemia, duration of illness and non-significant correlation (r=0.084) for RBC membrane phospholipase C activity against age range.

parasitaemia and duration of illness of the malaria patients. This is in support of the report of the study by Vadas *et al.* [16] which observed abnormally elevated levels of circulating phospholipase A_2 activity in malaria infected humans. The mechanism of malaria induced increase in phospholipase A_2 activity have been proposed to be due to increased secretion of tumor necrosis factor α (TNF-α) by immune cells induced by malaria parasite endotoxin. Elevated levels of TNF-α is found in serum of severe malaria patients [16]. It has been demonstrated that part of the pathophysiological effects of TNF-α were TNF-α-induced synthesis and secretion of plasmodial phospholipase A_2 [17]. The increase in phospholipase A_2 activity may play a crucial role in the developments of symptoms observed in malaria patients. This is because phospholipase A_2 hydrolyse polyunsaturated fatty acids such as arachidonic acid of phospholipids, the released polyunsaturated fatty acid serve as precursor for eicosanoids through the cyclooxygenase pathway. The eicosanoids, majorly prostaglandin, mediate many physiological processes such as fever, high body temperature, etc. seen in malaria patients. Although, it has been suggested that endogenous circulating phospholipase A_2 may contribute to hemolysis of

parasitized erythrocytes in patients with malaria with release of immature parasites [18]. This may be significant from the stand point of host defence in that phospholipase A_2 may augment the action of TNF-α to enhance the killing of malaria parasites or reduce their infectivity to the mosquito vector. However, it may also play a role in the release of matured infective parasite from infected erythrocyte. The significant decrease in phospholipase A_2 in the control individual compared to the enzyme activities in malaria patients is in support of the report of Dise *et al.* [19], who reported no measurable amount of enzyme activities in normal erythrocytes. The result of this study showed that increase in duration of illness increase phospholipase A_2 activity and this suggests that the longer the duration of illness the more the enzyme released which may contribute to greater symptoms observed.

In this study phospholipase A_2 activities in malaria patients does not decrease significantly in response to drug administered including patients administered chloroquine. This is in contrast to the report by Zidovetzki *et al.* [15] which reported that anti-malarial drugs especially of the chloroquine-type inhibit phospholipase A_2 activity. The reason for this increase in the enzyme activity even in the presence of anti-malarial drug may likely be due to resistance by the plasmodium parasite to the anti-malarial drugs. This might likely be true considering that the enzyme activities were lowered in malaria patients administered ACT (arthemisinine combination therapy) to which there is a less resistance to by the plasmodium parasite, although the mechanism by which malaria parasite produce resistance to anti-malarial parasite leading to increase in phospholipase A_2 activities is not known.

The result of this study show that there is significant ($p<0.05$) increase in RBC membrane-bound phospholipase C activity in malaria patients compared to its activity in the control individuals. This increment in the phospholipase C activity was found to increase with the severity of parasitemia, duration of illness, social status of the patients as well as the drug administered. Although, the mechanism by which malaria parasite infection increase phospholipase C activity have not been accounted for, nevertheless, several studies have reported that phospholipase C may be involved in the lysing of erythrocytes during parasite invasion [20]. The present study also reveal that anti-malarial drugs do not appear to lower the activity of phospholipase C in malaria patients. This may likely be that some of these anti-malarial drugs do not exert their effect on phospholipase C in their mechanism of action.

The result of this study also showed that there is no significant increase in plasma phospholipase C activity in malaria patients compared to control individuals. This may be due to the fact that most of the characterised phospholipase C in human are intracellular and membrane bound and are not secreted directly into the blood as circulatory phospholipase C during malaria infection.

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