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Research Article

Responses of Selected Inflammatory, Kidney and Liver Function Markers in Serum of Nigerian Children with Severe Falciparum Malaria to Treatment with Artesunate/Artemether-Lumefantrine Combination Therapy

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ABSTRACT

Malaria tolerance is a defence strategy that limits the damage caused by Plasmodium species irrespective of pathogen burden. The mechanisms responsible for this, responses of these mechanisms and their impact on organs to treatment have not been extensively studied. Thus, in this study, serum levels of selected pro- and anti-inflammatory markers, liver and kidney function indices with leucocytes indices in 100 children (1-10 years) with severe falciparum malaria were determined before treatment, at 48 hours during treatment and 48 hours after treatment with WHO recommended dosage of artesunate/artemether-lumefantrine combination therapy using standard methods. Data were analysed using SPSS, differences were considered significant at $p<0.05$. The results revealed that the serum levels of interleukin-12 (IL-12), interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), C-reactive protein (CRP), nitric oxide (NO), creatinine, albumin, total protein and conjugated bilirubin were not significantly changed at higher parasite densities before treatment. Only serum IL-4, CRP, total bilirubin, urea and creatinine levels and alanine aminotransferase activity were significantly reduced below the ranges of those with severe malaria. The results suggest a self-protective feed-back control, indicating tolerance, which reduced the adverse effects of the disease on kidney and liver functions at higher parasite densities. The results also suggest serum IL-12, IL-4, TNF-α, IFN-γ, CRP and NO levels as immune-protective markers for tolerance and serum IL-4 level as an effective marker for disease severity and recovery from the disease in children with severe malaria

Keywords: Immunity, falciparum malaria, inflammatory markers, children

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INTRODUCTION

Children are easily exposed to malaria parasite at early stage of life in malaria endemic regions like Nigeria (Okoli and Solomon, 2014). Survival from exposure to malaria leads to development of immunity against the pathogen (Boutlis *et al*., 2005). However, the strength of the developed immunity depends on the number of exposure to the parasite which is also dependent on age (Boutlis *et al*., 2005). Young children especially less than 5 years living in malaria endemic areas have fewer exposures to the parasite in comparison to older children or adults (Boutlis *et al*., 2005; Medzhitov *et al*., 2012). Consequently, they have incomplete immunity against the parasite and can easily come down with the infection, especially the severe form of malaria (Penman and Gupta, 2008). However, some children develop tolerance to the pathogen and are able to house the parasite burden without concomitant signs or symptoms such as fever; this is termed "anti-fever" tolerance (Boutlis *et al*., 2005; Medzhitov *et al*., 2012). It is also termed, "anti-toxic" tolerance on the discovery that the immunity is against the putative glycosylphosphatidylinositol (GPI) toxin of *P. falciparum* (Perry *et al*., 2005). "Anti-fever" tolerance is more common in young children (Boutlis *et al*., 2005) as against the older children or adults that express "anti-parasite" immunity or resistance (Boutlis *et al*., 2006; Medzhitov *et al*., 2012). Resistance is defined as the ability to limit pathogen burden (Boutlis *et al*., 2006; Medzhitov *et al*., 2012). Increased serum

NO level has been reported as the mediator for "anti-fever" tolerance in children with asymptomatic malaria living in malaria endemic regions (Anstey *et al*., 1996; Jeney *et al*., 2014). Tolerance has also been described in children with mild uncomplicated or acute symptomatic malaria in the presence of increasing parasite burden (Boutlis *et al*., 2006, Medzhitov *et al*., 2012). This is termed "anti-severe" malaria tolerance (Boutlis *et al*., 2005). Pro- and anti-inflammatory cytokines are involved as mediators of "anti-severe" malaria tolerance (Amante and Good, 1997; *Fernandes et al., 2008; Couper et al., 2008; Langhorne et al., 2008).*

However, not all young children are able to express "anti-fever" or "anti-severe" malaria tolerance because about 1-2% of mild uncomplicated malaria cases progress to severe malaria (Penman and Gupta, 2008; Tiago *et al*., 2011). Moreover, it has been documented that clinical immunity against malaria is short-lived and requires repetitive exposure to the parasite to maintain it (Boutlis *et al*., 2005), otherwise, asymptomatic malaria could progress to mild symptomatic uncomplicated malaria and then to severe malaria if not promptly diagnosed and treated (WHO, 2010). Severe malaria is more prevalent and lethal in children who are less than 5 years of age and causes tissue pathology and even death because their acquired partial immunity which is due to their few exposures to the parasite causes the immune system to extremely respond to the pathogen it recognises without effectively controlling the inflammation (Penman and Gupta, 2008).

Nevertheless, there are individual differences in inflammatory and immune responses to severe *P. falciparum* and this affects the disease outcome because either survival or mortality has been recorded in some children irrespective of the level of hyperparasitaemia (Aye Aye *et al*., 2016). Survival from the disease suggests host expression of tolerance to the pathogen irrespective of the hyperparasitaemic level (Ayres and Schneider, 2008; Medzhitov *et al*., 2012), which may limit the adverse effects of the increased inflammatory and immune responses on host tissues or organs (Ayres and Schneider, 2008; Medzhitov *et al*., 2012) before effective treatment is administered. Thus, studying the responses of inflammatory markers in severe malaria, their effects on selected organs and their responses to treatment may give a better understanding of the underlying mechanisms responsible for either positive or negative outcomes observed after treatment of severe malaria and may also help in identifying new markers for disease tolerance, disease severity and recovery from the disease. Thus, this study was carried out to evaluate the responses of selected inflammatory, kidney and liver function markers in serum of Nigerian children with severe *falciparum* malaria to treatment with artesunate/artemether-lumefantrine combination therapy.

MATERIALS AND METHODS

Rapid diagnostic test (RDT) kits for hepatitis B and HIV screening were obtained from Standard Diagnostics, Korea. Unless otherwise stated, all the reagents used for this study were of analytical grade and were prepared in all glass-distilled water.

Study population: One hundred children with severe malaria aged 1 to 10 years, treated at the Paediatrics wards of Jos University Teaching Hospital (JUTH), a reference tertiary hospital in Jos, North-Central Nigeria, were recruited for this study from 28th April 2014 to 15th February, 2016.

Children who met the study's inclusion criteria were recruited consecutively for the study. The inclusion criteria were: (i) assent of the child and parent/caregiver's consent; (ii) children aged 1 to 10 years clinically presenting with severe malaria without any other ailment as diagnosed by the paediatrician (severe malaria was diagnosed as the presence of one or more symptoms of malaria complications e.g anaemia, respiratory distress, jaundice etc and detection of malaria parasite (hyperparasitaemia)/product in the patient's blood); (iii) children with confirmed hyperparasitaemia using microscopy; (iv) children confirmed by laboratory tests as presenting with only severe malaria after excluding other disease conditions such as septicaemia, helminthiasis, typhoid, shigellosis, glucose-6-phosphate dehydrogenase deficiency (G6PDD), sickle cell disease, human immune-deficiency virus (HIV) and hepatitis B; (v) children on admission in the hospital using mosquito bed-net; (vi) children on artesunate/artemether-lumefantrine combination therapy; and (vii) children that recovered and were discharged on the 7th day of admission.

Ethical Approval: This study was carried out in line with the ethics guiding research undertakings on human subjects as approved by the ethical committees of University of Ilorin (reference No. UERC/ASN/2014/013) and Jos University Teaching Hospital (reference No. JUTH/DCS/ADM/127/XIX/5933). In addition, informed consents of the children's parents or caregivers were obtained before enrolment after due explanation of the aims and procedures of the project.

Study Design: This was a prospective longitudinal hospital-based study.

Administration of drugs: The children were given 2.4 mg/kg body weight of artesunate intravenously at 0 hour and then 1.2 mg/kg body weight at 12, 24 and 48 hours (if the patient was able to swallow, the daily dose was given orally) by the paediatricians and nurses. This was followed by oral administration of artemether-lumefantrine combination therapy at doses of 5 to 24 mg/kg body weight of artemether and 29 to 144 mg/kg body weight of lumefantrine for 3 days as fixed doses (WHO, 2015).

Sample collection and laboratory analysis: After clinical assessment, 5 ml of blood was aseptically collected from the ante-cubital vein of the children using sterile needle and syringe. This was done before initiation of treatment on the day of admission (day 0), then 48 hour after initiation of treatment (day 2) (Watt *et al*., 1992). Another sample was collected 48 hours after the last dose of the combination therapy i.e 7th day of initiation of treatment (CATMAT, 2000). Three millilitres (3 ml) of the blood was dispensed into EDTA tube for screening tests for exclusion of other abnormalities, malaria parasite detection and haematological tests. Screening tests for exclusion of other abnormalities carried out include:

haemoglobin genotype for exclusion of sickle cell disease using electrophoresis as described by Roberts and Williams (2003), glucose-6 phosphate dehydrogenase (G6PD) test for exclusion of G6PD deficiency using the meth-haemoglobin qualitative method described by Brewer *et al*. (1962), hepatitis B surface antigen test for exclusion of viral hepatitis using lateral flow technique (rapid diagnostic test (RDT) and blood culture for exclusion of septicaemia by direct aseptic injection of the blood into brain-heart infusion broth and thioglycollate broth (at 1:20 dilution) as described by Cheesbrough (2005).
The remaining 2 ml of the blood was dispensed into screw-caped plain sample tube for serum preparation. It was allowed to clot and retract at room temperature (22-27 ^{o}C) for about 20 minutes. The serum was separated after centrifuging at 3000 revolutions per minute (rpm) for 5 minutes in a clinical bench top centrifuge (MSE minor, England) using pasteur pipette into a pre-cleaned, dried, metal and steroid free cryo-vials and was used for HIV screening using the immunochromatographic technique (RDT) and biochemical analysis. Tests were carried out in duplicate tubes.
Stool sample was collected once into a transparent stool container from the children for exclusion of helminthiasis and pathological enteric bacterial infection by stool microscopy and culture tests using normal saline method for microscopy and Selenite-F and dextrose-citrate agar for culture as described by Cheesbrough (2005).

Malaria diagnosis: Malaria parasite detection was carried out by microscopy using Giemsa stained duplicate thick and thin slides (Cheesbrough, 2010). The duplicate slides were blindly read by an expert microscopist who is involved in the study; this served as the internal quality control. The slides were also read by another expert microscopist who was not involved in the study (independent reader); this served as the external quality control. The degree of variation in the results was determined and statistical analyzed. Malaria diagnosis was based on identification of asexual stages of *Plasmodium* species in the thick blood smears. Film was reported as 'malaria parasite not seen' i.e. negative after examining about 100 fields. Thin films were used to identify species and stages of the parasite.

Malaria parasite density was determined as the number of parasites/µl of blood (thick film method) relative to respective patient's total white blood cell count (WHO, 2007). Hyperparasitaemia in children was defined as parasite count of >200 x 10^3 parasites/µl (Maina *et al*., 2010).

Children with severe malaria were grouped based on age. Parasite densities before treatment (day 0) were grouped into mild hyperparasitaemia (201 to 300 x 10^3 parasites/µl), moderate hyperparasitaemia (301 to 500 x10^3 parasites/µl) and marked hyperparasitaemia (501 to 800 x10^3 parasites/µl). Parasite densities during treatment (day 2) were grouped into malaria parasite not seen (treated malaria negative, 0.0 x10^3 parasites/µl), low parasitaemia (1 to 100 x10^3 parasites/µl) and moderate parasitaemia (101 to 200 x10^3 parasites/µl) while parasite densities after treatment (day 7) were grouped into malaria parasite not seen (treated malaria negative, 0.0 x10^3 parasites/µl) and low parasitaemia (1 to 100 x10^3 parasites/µl).

Haematological analysis: Total and differential leucocytes counts were determined manually (WHO, 1991).

Biochemical assays: Serum levels of tumour necrosis factor-α (TNF-α), interferon- γ (IFN-γ), interleukin-12 (IL-12), IL-4 and IL-10 were determined using standalone enzyme linked immunosorbent assay (ELISA) as described by Natsuko *et al*. (2011). C-reactive protein (CRP) and nitric oxide (NO) concentrations were determined using the methods described by Black *et al*. (2004) and Griess (1978) respectively. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities and the concetrations of total protein and albumin in the serum were determined using the methods described by Friedman *et al*. (2003). Total and conjugated bilirubin concentrations in the serum were determined using the method of Jendrassik and Grof (1938). Serum urea and creatinine concentrations were determined using the methods described by Amin *et al*. (2014).

Statistical Analysis: Results are expressed as means ± standard error of the mean (S.E.M.). Data were analysed using one-way ANOVA, followed by Duncan's multiple range test and the level of their relationships in children with severe malaria before treatment (day 0), 48 hours after initiation of treatment (day 2) and 48 hours after treatment (day 7) were determined using Pearson correlation. Differences were considered significant at $P<0.05$ when compared among the different parasite density ranges

RESULTS

Parasite density by number and age of children with severe malaria before, during and after treatment. The parasitaemia in children with severe malaria before treatment (day 0), 48 hours after initiation of treatment (day 2) and 48 hours after treatment (day 7) are shown in Table 1. The results revealed that artesunate/artemether-lumefantrine combination therapy caused significant reduction ($p<0.05$) in parasite density on days 2 and 7 compared to those of day 0 (Table 1).

Responses of selected white blood cell indices to change in parasite density in children with severe malaria before, during and after treatment : Responses of total white blood cell, absolute neutrophil, absolute lymphocyte, absolute monocyte and absolute eosinophil counts to change in parasite density in children with severe malaria before treatment (day 0), 48 hours of treatment (day 2) and 48 hours after treatment (day 7) are shown in Figure 1. There was relatively no significant change ($p>0.05$) in the white blood cell indices with increase in parasite density from 201 to 800 x10^3 parasites/µl before treatment; indicating that they are non-markers of disease severity (Figs. 1 A-E). Blood lymphocyte counts showed a strong significant correlation ($r = .012$) with parasite density. Total white blood cell, neutrophil, monocyte and eosinophil counts showed weak significant positive correlation ($r = .343$, $r = .378$, $r = .306$, $r = .189$ respectively) with parasitaemia (Figs. 1A-E).

Responses of selected inflammatory markers in serum to change in parasite density in children with severe malaria before, during and after treatment

There was significant increase ($p<0.05$) in serum levels of TNF-α, IFN-γ, IL-12, IL-4 and CRP with significant rise ($p<0.05$) in parasite density from 201 to 400 x 10^3 /µl (mild to moderate hyperparasitaemia) with no significant change ($p>0.05$) in their levels with further significant increase ($p<0.05$) in parasite density from 401 to 800 x 10^3 /µl (501 to 800 x10^3/µl for IL-4, CRP) (marked hyperparasitaemia) before treatment (Figs. 2A-F). The levels decreased significantly ($p<0.05$) during and after treatment (Figs. 2A-F). Serum TNF-α, IFN-γ, IL-12, IL-4 and CRP concentrations showed a significant positive correlation ($r=.593$; $r=.441$; $r=.574$; $r=.844$; $r=.803$ respectively; $p<0.05$) with parasitaemia, but IL-10 had a weak non-significant negative correlation ($r=-.079$; $p>0.05$) (Fig. 2A-F).

Response of serum nitric oxide to change in parasite density in children with severe malaria before, during and after treatment

The results revealed that there was a steady decrease in serum NO level with increase in parasite density from 201 to 500 x10^3 parasites/µl (mild to moderate hyperparasitaemia) which was significant ($p<0.05$) at parasite density of 501- 600 x 10^3 parasites/µl on day 0 compared to those of days 2 and 7 (Fig. 3). Following a further increase in parasite density to 501 to 600 x10^3 parasites/µl, there was significant increase ($p<0.05$) in serum NO concentration to the range of those of days 2 and 7. With further significant increase ($p<0.05$) in parasite density from 601 to 800 x10^3 parasites/µl (marked hyperparasitaemia), there was no significant change ($p>0.05$) in serum NO concentration compared to those of day 2 (Fig. 3). The level increased significantly ($p<0.05$) during and after treatment and correlated negatively ($r=-.510$; $p<0.05$) with parasitaemia and disease severity (Fig. 3).

Table 1:
Parasite densities by number and age of children with severe malaria before, during and after treatment with artesunate/artemether-lumefantrine combination therapy

Range of parasite density	Parasite density Mean ± SD	Number of Children	Age (years) Mean ± SD	Age range (years)
Day 0 (before treatment)				
Mild hyperparasitaemia				
201-300 (x10^3 parasites/µl)	237.64 ± 27.74	33 (33%)	4.63 ± 2.44	1.60 – 10.00
Moderate hyperparasitaemia				
301-400 (x10^3 parasites/µl)	361.69 ± 34.42	16 (16%)	2.53 ± 1.68	1.00 – 6.00
401-500 (x10^3 parasites/µl)	462.35 ± 22.03	17 (17%)	4.74 ± 2.11	1.60 – 10.00
Marked hyperparasitaemia				
501-600 (x10^3 parasites/µl)	532.0 ± 21.99	14 (14%)	3.89 ± 3.36	1.00 – 10.00
601-700 (x10^3 parasites/µl)	640.0 ± 14.14	2 (2%)	2.70 ± 0.00	2.70 – 2.700
701-800 (x10^3 parasites/µl)	793.89 ± 7.96	18 (18%)	4.21 ± 2.63	1.00 – 10.00
Day 2 (48 hours of treatment)				
MP not seen (treated MP- negative)				
0.0 (x10^3 parasites/µl))	0.00	7 (7%)	4.00 ± 2.97	2.00 – 10.00
Low parasitaemia				
01-100 (x10^3 parasites/µl)	83.33 ± 12.81	18 (18%)	5.38 ± 2.51	2.80 – 10.00
Moderate parasitaemia				
101-200 (x10^3 parasites/µl)	167.61 ± 20.41	75 (75%)	3.80 ± 2.31	1.00 – 10.00
Day 7 (48 hours after treatment)				
MP not seen (treated MP- negative)				
0.0 (x10^3 parasites/µl)	0.00	93 (93%)	3.93 ± 2.45	1.00 – 10.00
Low parasitaemia				
01-100 (x10^3 parasites/µl)	85.0 ± 14.43	7 (7%)	6.29 ± 2.69	1.00 – 10.00

MP = malaria parasite

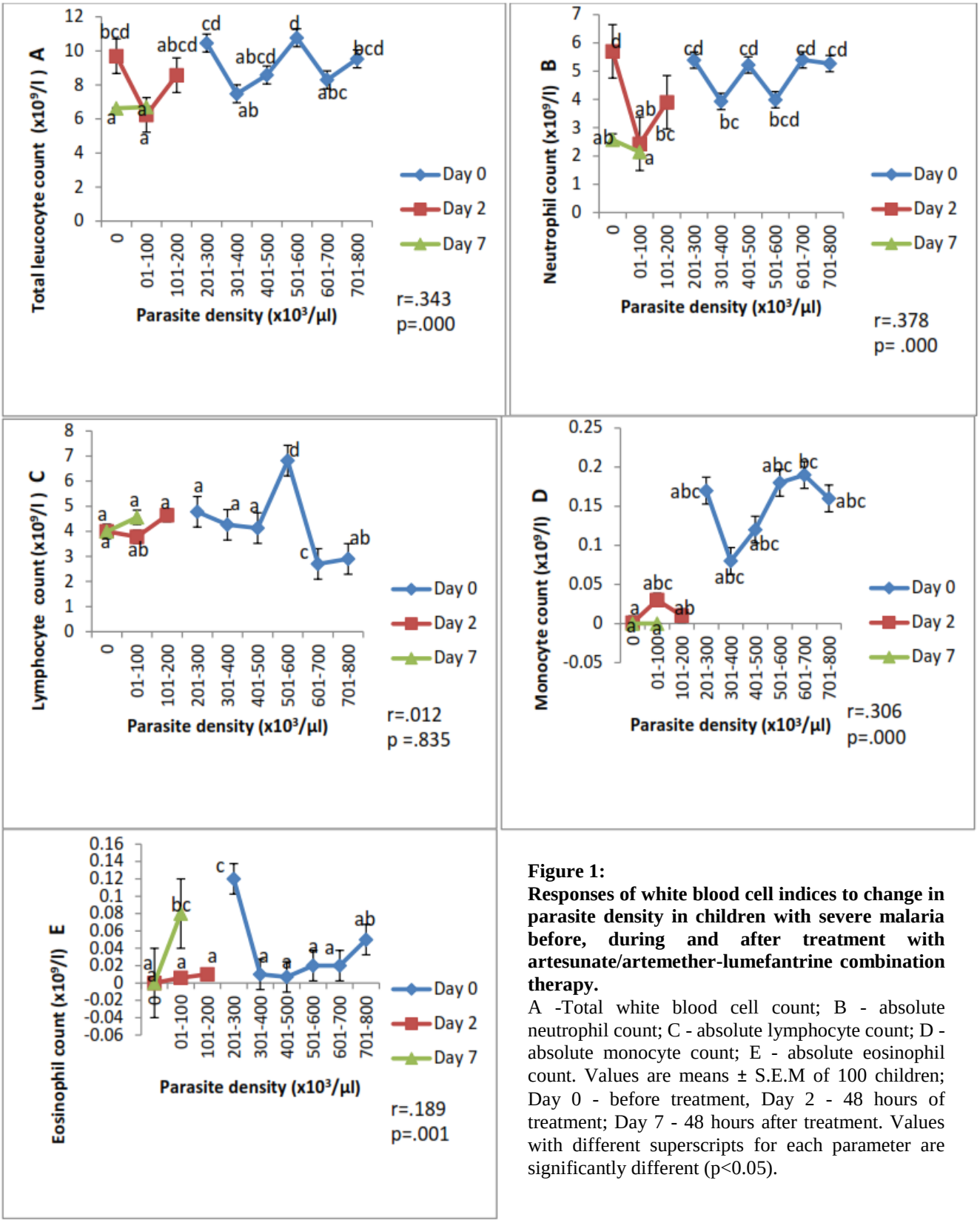

Figure 1:
Responses of white blood cell indices to change in parasite density in children with severe malaria before, during and after treatment with artesunate/artemether-lumefantrine combination therapy.
A -Total white blood cell count; B - absolute neutrophil count; C - absolute lymphocyte count; D - absolute monocyte count; E - absolute eosinophil count. Values are means ± S.E.M of 100 children; Day 0 - before treatment, Day 2 - 48 hours of treatment; Day 7 - 48 hours after treatment. Values with different superscripts for each parameter are significantly different ($p<0.05$).

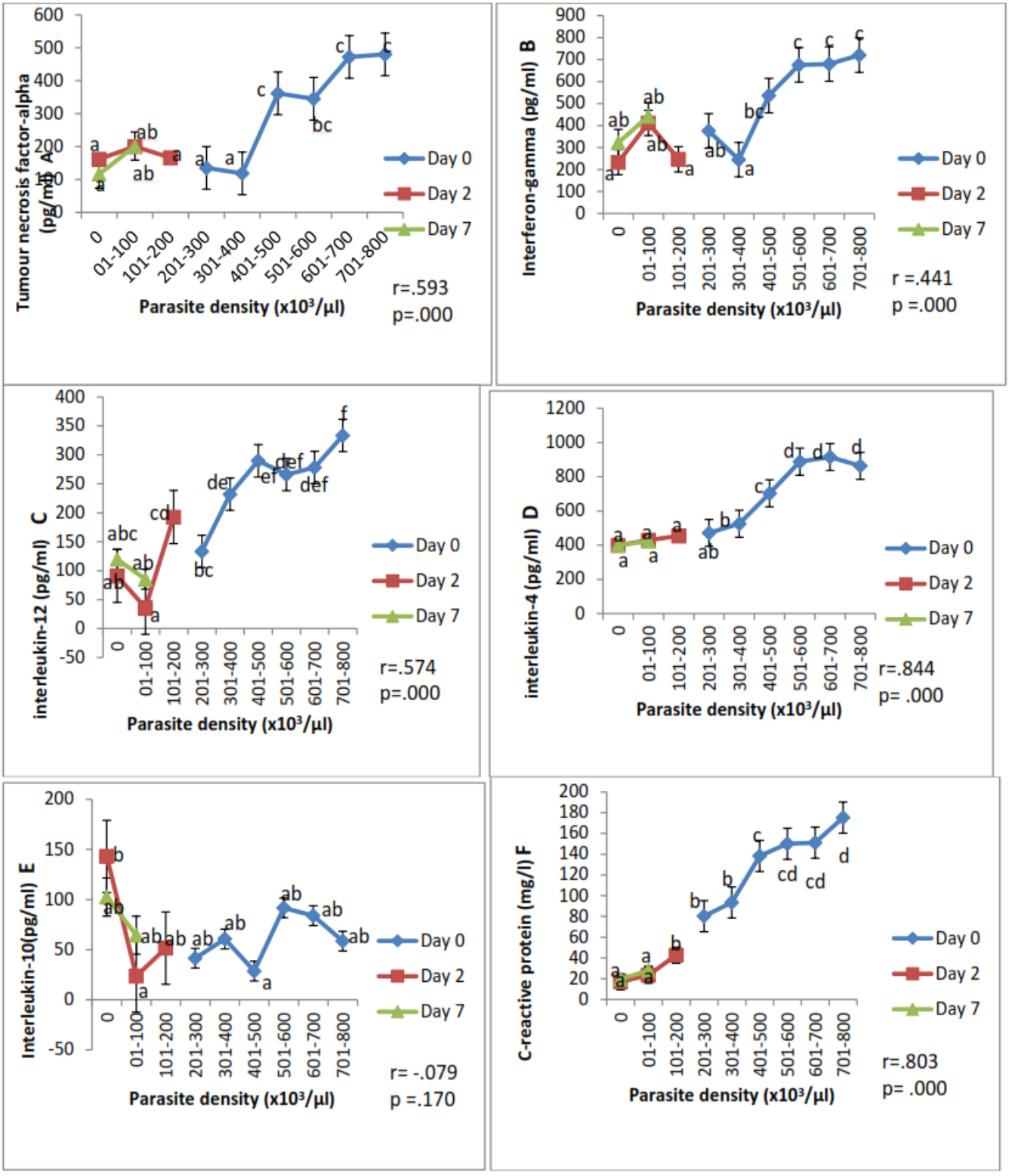

Figure 2:
Responses of selected inflammatory markers to change in parasite density in children with severe malaria before, during and after treatment with artesunate/artemether-lumefantrine combination therapy.
A –Tumor necrosis factor; B – interferon gamma; C – interleukin-12; D – interleukin-4; E – interleukin 10; F – C-reactive protein. Values are means ± S.E.M of 100 children; Day 0 - before treatment, Day 2 - 48 hours of treatment; Day 7 - 48 hours after treatment. Values with different superscripts for each parameter are significantly different ($p<0.05$).

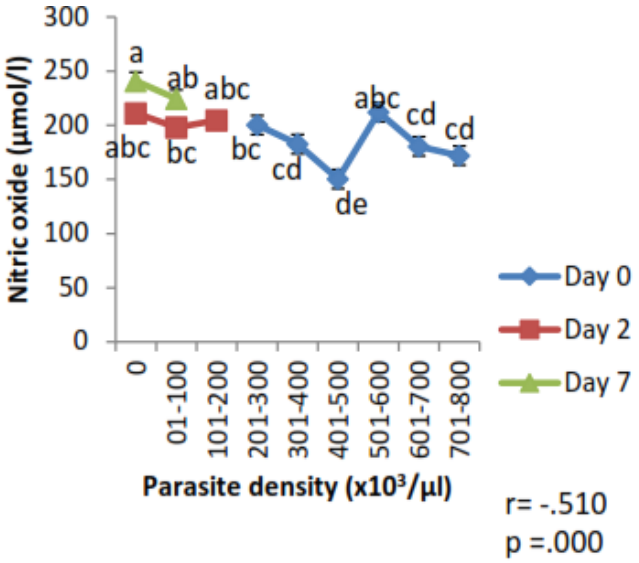

Figure 3:
Response of serum nitric oxide to change in parasite density in children with severe malaria before, during and after treatment with artesunate/artemether-lumefantrine combination therapy.
Values are means ± S.E.M of 100 children; Day 0 - before treatment, Day 2 - 48 hours of treatment; Day 7 - 48 hours after treatment. Values with different superscripts are significantly different (p<0.05).

R**esponses of some renal function indices in serum to change in parasite density in children with severe malaria before, during and after treatment***:* The result revealed that serum urea concentration showed multiphasic non-specific variations with significant rise (p<0.05) in parasite density from 201 to 800 x10^3 parasites/µl before treatment (Fig. 4A). Serum creatinine level was significantly increased (p<0.05) with significant rise (p<0.05) in parasite density from 301 to 500 x10^3 parasites/µl (mild to moderate hyperparasitaemia), which was not significantly changed (p>0.05) with further significant increase (p<0.05) in parasite densities from 601 - 800 x10^3 parasites/µl (marked hyperparasitaemia) day 0 (Fig. 4B). Serum creatinine and urea levels decreased significantly (p<0.05) with treatment and significantly correlated (r=.555; r= .552 respectively) with parasitaemia and disease severity (Fig. 4A-B).

Responses of some enzymic liver function indices in the serum of children with severe malaria to change in parasite density before, during and after treatment
There was significant increase (p<0.05) in serum ALT activity with significant rise (p<0.05) in parasite density from 301 to 800 x10^3parasites/µl to those days 2 and 7 (Fig. 5A). There was significant increase (p<0.05) in serum AST activity with increase in parasite densities from 301 to 600 x10^3 parasites/µl compared to those of day 7 and from 601 – 800 x10^3 parasites/µl compared to those of day 2 (Fig. 5B). There was no significant change (p>0.05) in ALP activity with significant increase (p<0.05) in parasite density from 201 to 800 x10^3 parasites/µl on day 0 compared those of days 2 and 7 (Fig. 5C). Serum ALT and AST activities correlated with parasitaemia and disease severity (r = .774; r = .661 respectively) (Figs. 5A-B), but serum ALP activity was not significantly changed (p>0.05) during and after treatment and showed a non-significant negative correlation (r = -.042; p>0.05) with parasitaemia and malaria severity (Fig. 5C).

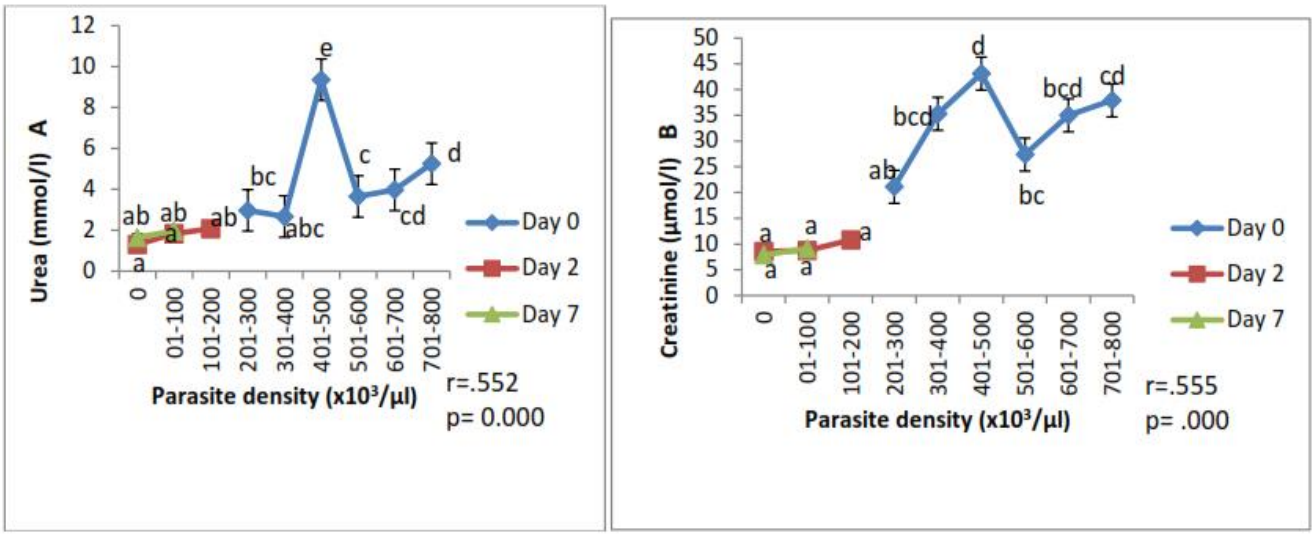

Figure 4:
Responses of selected kidney function indices in the serum to change in parasite density in children with severe malaria before, during and after treatment with artesunate/artemether-lumefantrine combination therapy. A – Urea concentration; B – Creatinine concentration; Values are means ± S.E.M of 100 children; Day 0 - before treatment, Day 2 - 48 hours of treatment; Day 7 - 48 hours after treatment. Values with different superscripts for each parameter are significantly different (p<0.05).

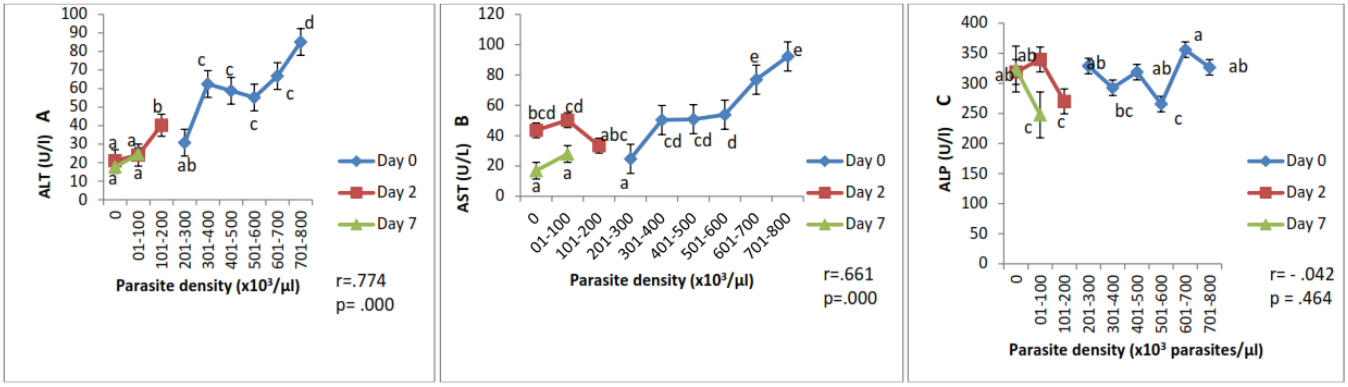

Figure 5:
Responses of selected enzymic liver function indices to change in parasite density in children with severe malaria before, during and after treatment with artesunate/artemether-lumefantrine combination therapy.
Values are means ± S.E.M of 100 children; Day 0 - before treatment, Day 2 - 48 hours of treatment; Day 7 - 48 hours after treatment. Values with different superscripts are significantly different ($p<0.05$).

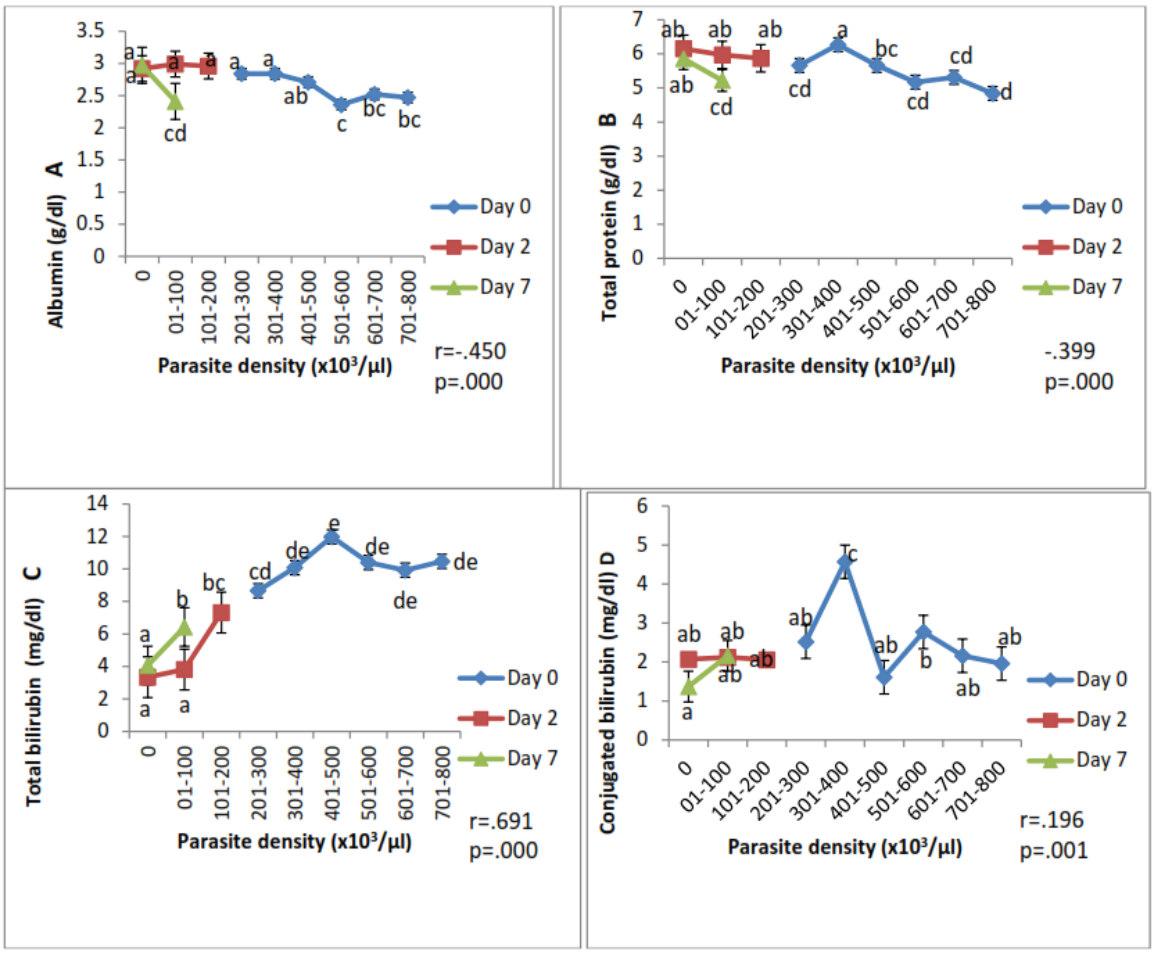

Figure 6:
Responses of selected non-enzymic liver function indices to change in parasite density in children with severe malaria before, during and after treatment with artesunate/artemether-lumefantrine combination therapy.
Values are means ± S.E.M of 100 children; Day 0 - before treatment, Day 2 - 48 hours of treatment; Day 7 - 48 hours after treatment. Values with different superscripts are significantly different ($p<0.05$);
A - Serum albumin concentration; B – Serum total protein concentration (TB); C – Serum total bilirubin concentration; and D - Serum conjugated bilirubin concentration (CB)

Responses of some serum non-enzymic markers of liver function to change in parasite density in children with severe malaria before, during and after treatment

There was no significant change ($p>0.05$) in serum albumin level with significant rise ($p<0.05$) in parasite density from 201 to 400 $x10^3$ parasites/µl (mild to moderate hyperparasitaemia) on day 0 compared to those of days 2 and 7, but serum total protein concentration was not significantly changed ($p>0.05$) at all parasite densities on day 0 compared to those of day 7 (Fig. 6). Serum albumin and total protein levels were reduced significantly ($p<0.05$) on day 0 compared to those of day 2 at parasite densities of 501 – 800 $x10^3$ parasites/µl) (Figs 6A and B). They correlated negatively with parasitaemia and disease severity ($r= -.450$; $r= -.399$ respectively; $p<0.05$) (Figs. 6A and B).

There was significant increase ($p<0.05$) in total bilirubin level (TB) with significant increase ($p<0.05$) in parasite density from 301 to 800 $x10^3$ parasites/µl on day 0 compared to those of days 2 and 7 (Fig. 6C). Serum conjugated bilirubin concentration (CB) was significantly increased ($p<0.05$) at parasite density of 301 to 400 $x10^3$ parasites/µl (moderate hyperparasitaemia), with no significant change ($p>0.05$) at other parasite densities compared to those of days 2 and 7. Serum TB and CB correlated with parasitaemia and disease severity ($r=.691$; $r=.196$ respectively; $p<0.05$) (Figs. 6C and D).

DISCUSSION

The clinical presentations in children with severe malaria include anaemia, jaundice, respiratory distress and prostration (Okoli *et al*., 2017). Various physiological mechanisms are responsible for these, such as inflammation, multi-organ complications etc. In this study, the possible modulation of the markers of inflammation, liver and kidney functions in children with severe malaria treated with artesunate/artemether-lumefantrine combination therapy was evaluated, with the aim of getting more reliable markers for monitoring disease severity and recovery during treatment. The treatment ameliorated the adverse effects which hyperparasitaemia and the concomitant increased inflammatory and immune responses would have exerted on various organs, which in turn could have led to more advanced complications and death.

Host's defensive capacity affects the outcome of severe malaria (Ayres and Schneider, 2008). Tolerance, host's defensive strategy, does not affect the pathogen burden or density but reduces tissue damage and other pathological effects of disease caused by the pathogen or immune response (Medzhitov *et al*., 2012). Host expression of tolerance as a means of preventing symptomatic or severe malaria even in the presence of the pathogen has been reported (Boutlis *et al*., 2006; Medzhitov *et al*., 2012). However, the mechanisms underlying host expression of tolerance in severe *P. falciparum* malaria as a means of limiting the damaging effects of increasing parasite densities on host tissues or organs has not been fully elucidated.

Severe malaria alters haematological indices (Bidaki and Dalimi, 2003). White blood cells are known to be involved in immune response (Pilger *et al*., 2011). From this study, the pattern of regulation of white blood cell indices did not connote them as markers for tolerance. This is in line with already established fact that white blood cells are not involved in expression of tolerance (Boutlis *et al*., 2006; Medzhitov *et al*., 2012). Total white blood cell, neutrophil, monocyte and eosinophil counts exhibited weak positive correlation with parasitaemia. Thus, they may not be good markers for disease severity and monitoring recovery during treatment because their values before, during and after treatment were not significantly different.

Modulatory effects of *Plasmodium* infection on the concentrations of pro- and anti-inflammatory markers in the serum have been well documented (**Nmorsi *et al*., 2010;** *Prakash et al., 2006*). The results of this study, revealed that the serum levels of TNF-α, IFN-γ, IL-12, IL-4, and CRP correlated positively with parasite density and disease severity, NO correlated negatively and IL-10 showed a non-significant negative correlation. However, serum TNF-α, IFN-γ, IL-12, CRP and NO levels may not be good markers for disease severity because they were not significantly changed at higher parasite densities, possibly due to self-protective feedback control (Ejezie and Ezedinachi, 1992). The serum IL-4 levels of the categories of parasite densities used in this study were significantly different from each other, suggesting that serum IL-4 level may be a good marker for determining disease severity. Serum TNF-α, IFN-γ, IL-12 and NO levels may not be good markers for monitoring recovery because they were not significantly changed before, during and after treatment. However, serum IL-4 and CRP levels may be effective markers for monitoring recovery from the disease because they were significantly reduced during and after treatment compared to their values before treatment.

Elevated serum TNF-α, IL-12 and IFN-γ levels have been reported severally in severe malaria (Nmorsi *et al*., 2010; Prakash *et al*., 2006). TNF-*α* and IFN-γ are T1 proinflammatory cytokines (Nmorsi *et al*., 2010**;** *Prakash et al., 2006*). IL-12 is also pro-inflammatory cytokine, required for the production of protective immunoglobulin IgG2a (Su and Stevenson, 2002). From this study, elevated serum TNF-*α*, IFN-γ and IL-12 levels showed a self-protective feedback control (Ejezie and Ezedinachi, 1992) before treatment in response to increasing parasite density (Dubey *et al*., 1999; Tiago *et al*., 2011). Tiago *et al*. (2011) found that the expression of cytokine genes, which is related to serum levels of cytokines, can be modulated by the number of parasites. This curtails damage on tissues or organs due to overproduction of these pro-inflammatory cytokines (Ayres and Schneider, 2008; Medzhitov *et al*., 2012). This suggests that TNF-*α,* IFN-γ and IL-12 are markers for tolerance (Ayres and Schneider, 2008; Medzhitov *et al*., 2012), which is expressed in these children with severe *falciparum* malaria in this malaria endemic region. Expression of tolerance via a self-protective control of elevated serum TNF-α could be due to inhibition of soluble TNF-α receptor, as it has been reported that at high TNF-α concentrations, the soluble TNF-α receptors compete with TNF-α for binding with cellular

receptors particularly membrane-bound TNF-α (Opal *et al*., 1991), thereby inhibiting TNF-α bioactivity; this, by implication, results in negative feedback control of serum TNF-α (Overbeek and Veringa 1991; Ayres and Schneider, 2008). The mechanism for the increased serum IFN-γ level in this immunological response could be related to the finding that Th1 effector cells can exert negative feedback control to regulate themselves (do Rosario, 2012). Also, the roles of pro-inflammatory cytokines such as TNF-α and IFN-γ and the anti-inflammatory cytokine, IL-4, in iron delocalization pathway lead to malaria anaemia (Garcia, 2010; Heinrich *et al*., 2003) and reduced availability of iron to the parasite which reduces the parasite pathogenecity and confers tolerance to malaria (Gozzelino *et al*., 2012).

Elevated serum IL-4 and CRP levels in severe malaria have been reported (Nmorsi *et al*., 2010**;** Streetz *et al*., 2003; Kulkarni *et al*., 2003). IL-4 is a regulator of the heavy chain class switch from IgM to IgG1, IgG3 and IgE in B cells (Curfs *et al*., 1990**;** Calissano *et al*. 2003). C-reactive protein is a peitrophic acute phase protein synthesised by hepatocytes (Tilg *et al*., 1993; Streetz *et al*., 2003). In this study, elevated serum IL-4 and CRP levels showed a self-protective feedback control (Ejezie and Ezedinachi, 1992) before treatment in response to increasing parasite density (Dubey *et al*., 1999; Ejezie and Ezedinachi, 1992), suggesting tolerance. This corroborates earlier report by Medina *et al*. (2011) that serum levels of IL-4 may increase or decrease significantly over the course of infection. Moreover, Gennaro (2000) reported that cytokines can suppress their own effect by feedback inhibition. Thus, IL-4 and CRP are markers for tolerance (Ayres and Schneider, 2008; Medzhitov *et al*., 2012).

IL-10 is an anti-inflammatory Th2-type cytokine (Tiago *et al*., 2011). Decreased serum IL-10 level has been shown to have a non-significant or weak negative correlation with parasite density (Tiago *et al*., (2011). In this study, it was observed that the decreased serum IL-10 level did not express a self-protective feedback control (Ejezie and Ezedinachi, 1992) with increasing parasite density (Dubey *et al*., 1999) because there was no significant change in its concentration at various parasite densities. Thus, IL-10 is not a marker for tolerance. This suggests that the mechanisms of tolerance for the disease expressed by these children did not involve reduction in the serum level of this anti-inflammatory cytokine (Ejezie and Ezedinachi, 1992).

Decreased serum NO level has been reported to exist in patients with severe malaria (Weiberg *et al*., 2008). NO is a final effector molecule; it is a highly diffusible, lipid-soluble, free radical that has been identified to mediate in malaria tolerance (Anstey *et al*., 1996; Jeney *et al*., 2014). In this study, the reduced serum NO level suggests a self-protective feed-back control (Ejezie and Ezedinachi, 1992) with increasing parasite density before treatment. This suggests that one of the mechanisms of tolerance for the disease in these children involves reduction of serum NO level thereby limiting the damaging effect on organs (Ayres and Schneider, 2008; Medzhitov *et al*., 2012). This finding corroborates earlier reports by Chung *et al*. (1980) and Stefano and Magazine (2001) that NO may be a self-regulatory molecule. In addition, suppressed NO synthesis and overproduction of TNF-α have been associated with cerebral malaria (Weiberg *et al*. 2008; Grau *et al*., 1989). However, the fact that none of our subjects in this study had cerebral malaria (Okoli *et al*., 2017) despite decreased serum NO and increased TNF-α levels further suggest the conferment of tolerance; otherwise, this possibly, could have escalated to cerebral malaria and, possibly, death (Ayres and Schneider, 2008; Medzhitov *et al*., 2012). Severe malaria is associated with dysregulated inflammatory and immune responses which can result in liver damage (Gozzelino and Soares, 2011), causing alteration in the serum levels or activities of liver function markers (Kochar *et al*., 2003; Nautiyal *et al*., 2005). Elevated serum activities of ALT and AST and serum concentrations of total bilirubin and conjugated bilirubin concentrations are associated with severe malaria (Kochar *et al*., 2003; Nautiyal *et al*., 2005), which indicate liver injury, because, the invasion of hepatocytes by parasites causes compromise of the cell membrane integrity (the marker of which is ALP activity) and conjugating ability of the liver (Adegbesan *et al*., 2014; Ki-Soo, 2013). Hepatocytes contain characteristic enzymes including ALP, ALT and AST which are released into the blood only when these cells are damaged (Ki-Soo, 2013). The results revealed that serum ALP activity was not significantly changed during and after treatment compared to the value before treatment suggesting that it may not be useful for monitoring disease severity neither for monitoring recovery during and after treatment. AST and ALT activities positively correlated with parasite density, though they were not significantly changed over a range of parasite densities. This suggests that they may not be effective markers for disease severity. However, serum ALT activity was significantly reduced during and after treatment compared to the value before treatment, suggesting that it may be a good marker for monitoring recovery during treatment.

From this study, elevated serum ALP, ALT and AST activities did not show any self-protective feedback modulation with increasing parasite density, suggesting that maintenance of integrity of hepatocyte membrane is not one of the mechanisms of conferring tolerance in these children (Ayres and Schneider, 2008; Medzhitov *et al*., 2012). This finding corroborates the reports of Wunderlich *et al*. (2014) who stated that blood stage malaria causes injury to the liver evidenced with elevated ALP, ALT and AST activities and that there are liver injuries mainly produced by malaria-induced overreactions of the liver-inherent immune system. However, our findings differ from that of *Vlahopoulos et al. (2015) who reported* that normal tissue integrity is preserved by feedback interactions between diverse cell types mediated by adhesion molecules and secreted cytokines.

Decreased serum levels of albumin and total protein, which are markers of liver synthetic ability (Ki-Soo, 2013), are associated with severe malaria (Ki-Soo, 2013). In this study, decreased serum albumin and total protein levels showed a self-protective feedback control with increasing parasite density and increased inflammatory and immune responses. This suggests that one of the mechanisms involved in conferment of tolerance was by reduction in the synthetic ability of the liver (Ayres and Schneider, 2008; Medzhitov *et al*., 2012). In this study, serum albumin and total protein concentrations weakly correlated negatively with parasitaemia but were not significantly changed before, during and after

treatment, suggesting that these parameters may not be good markers of disease severity neither for monitoring recovery during treatment.

Also, severe malaria is associated with elevated serum total bilirubin and conjugated bilirubin (a marker of liver conjugating ability) levels (Adegbesan *et al*., 2014; Ki-Soo Kang, 2013). In this study, elevated serum total bilirubin level and a latter reduction in serum conjugated bilirubin level showed a self-protective feedback control with increasing parasite density and increased inflammatory responses before treatment. This implies that tolerance was conferred by increasing conjugating and secretory activities of liver cells (Ayres and Schneider, 2008; Medzhitov *et al*., 2012; Medeiros *et al*., 2013; Brugat *et al*., 2014). This also suggests that elevated bilirubin clinically presented as jaundice by the children (Okoli *et al*., 2017) was because of excessive haemolysis that resulted in hepatomegaly due to liver overload (Mackowiak, 2000) and not necessarily because of severe liver damage as liver conjugating ability was relatively preserved (Ayres and Schneider, 2008; Medzhitov *et al*., 2012). This agrees with the report of Wunderlich *et al*. (2014) who reported that the liver-inherent immune system has the capacity to generate tolerance. The results revealed that serum total bilirubin and conjugated bilirubin concentrations correlated positively with parasitaemia; however, they may not be effective markers of disease severity because they were not significantly changed with increasing parasite density. Also, only the total bilirubin concentration was significantly reduced during and after treatment compared to the values before treatment, suggesting that the total bilirubin concentration may be good marker for monitoring recovery during treatment. However, serum conjugated bilirubin level may not be a good marker for monitoring disease severity because there was generally no significant change in its values before, during and after treatment. The restoration of these liver functions and resolution of the serum levels of these markers after treatment corroborates earlier report of Sumanta *et al*. (2014) who stated that the liver efficiently restores function after damage induced during malarial infection once the parasites are cleared from the blood. Moreover, it has been reported that the liver also possesses the capability for fast regeneration after damage and this capability also involves T cell-mediation via cytokine repair mechanisms of the liver-inherent immune system (Mastelic *et al*., 2012).

Elevated serum urea, a marker of renal function is associated with severe malaria (Ekeanyanwu and Akpoilih, 2010). In the present study, the pattern of regulation of elevated serum urea in children with severe malaria did not show a self-protective feedback control (Ejezie and Ezedinachi, 1992). On the other hand, elevated serum creatinine, a more reliable marker for renal function (Gowda *et al*., 2010), showed a self-protective feedback control (Ejezie and Ezedinachi, 1992) with increasing parasite density. This suggests that maintenance of glomerular filtration in the kidney despite increasing parasite density and increased inflammatory response (Ayres and Schneider, 2008; Medzhitov *et al*., 2012) may be one of the mechanisms of conferring tolerance. These findings corroborate our earlier report that only few children with severe malaria exhibited dysuria or oliguria (Okoli *et al*., 2017) without any severe renal damage. Ramosa *et al* (2019) have also reported the establishment of tolerance to malaria through a tissue damage-control mechanism that operates specifically in renal proximal tubule epithelial cells (RPTEC). As labile heme (which has the potential of causing membrane damage) accumulates in plasma and urine during the blood stage of *Plasmodium* infection, it is detoxified in RPTEC by heme oxygenase-1 and ferritin H chain, thereby preventing the development of acute kidney injury. The restoration of renal functions and resolution of the serum levels of the markers after treatment with antimalarial drugs (without any renal disease therapy) still confirmed that there was no severe renal damage. The results of this study established a weak positive correlation between serum urea and creatinine concentrations and parasite density, with no significant change at higher parasite densities. This suggests that they may not be good indicators of disease severity. However, there was significant reduction in these parameters during and after the treatment compared to the values before treatment, especially at higher parasite densities. This suggests that serum urea and creatinine concentrations may be good markers for monitoring recovery during treatment.

In conclusion, the results of this study suggests that serum TNF-α, IFN-γ, IL-12, IL-4, CRP and NO are immuno-protective markers for tolerance, indicating that the immune system of children in this malaria endemic region expresses tolerance even in severe *falciparum* malaria. This prevented fatal liver and kidney damage with increasing parasite density, thereby preventing possible mortality before treatment and resulting in 100% recovery in children after treatment. The results also suggest that the effective markers of monitoring recovery during treatment are serum IL-4, CRP, total bilirubin, urea, creatinine levels and ALT activity and only serum IL-4 level may be used for determining disease severity. Thus, serum IL-4 level may be used as an effective marker for disease tolerance, disease severity and recovery from the disease during treatment.

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