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Assessment of merozoite surface protein-1 antibodies and anaemia severity in various treatment stages of a nutrananosphere artemisinin-bioflavonoid antimalarial therapy

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Abstract

Artemisinin-based combination therapy (ACT) effectively treats uncomplicated malaria, but treatment failures and resistance occur in some regions. This study is to assess the merozoite surface protein antibodies and anemia development in an artemisinin-bioflavonoid antimalaria. Seventy-seven individuals, comprising forty-two adults and thirty-five children diagnosed with malaria, were recruited from a primary health care facility in Osun state, Nigeria. Following treatment with the TriAntiMal^{TM} NutraNanosphere, blood samples were collected on days 0, 3, 7, and 16 (D0, D3, D7, and D16) and analyzed for malaria parasite density, anti-MSP-1, and anaemia indicators; Hb, HCT, MCV, MCH and MCHC using thick and thin film examination by WHO-certified microscopists; ELISA and Sysmex K2ZIN autoanalyser respectively. Statistical analyses were conducted using SPSS version 20, with a p-value ≤0.05 considered significant. Anti-MSP-1 levels declined significantly over time; malaria parasite density values decreased from day 0 (103.04±39.04) to day 3 (93.77±36.98), 7 (81.46±30.4), and 16 (67.72±34.53) respectively. Participants with severe anaemia with various anti-MSP-1 concentration decreased from 14.3% (5 subjects) to 2.8% (1 subject) in children and 7.1% (3 subjects) to 2.1% (1 subject) in adults respectively by day 3. Their hematological parameters improved significantly by days 7 and 16 from anaemic state seen in some subjects to normal condition. The decline in MSP-1 antibodies, reduction in parasite density, and improved hematological parameters indicate TriAntiMal^{TM} efficacy. The potential link between MSP-1 antibodies and anaemia severity underscores the complex nature of the host immune response in malaria pathogenesis.

Keywords: Artemisinin-Bioflavonoid, Merozoite Surface Protein, Antimalarial

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INTRODUCTION

Malaria is a major cause of human morbidity and mortality in Africa, it is responsible for fifteen percent (15%) of clinical illnesses in the tropical regions of the continent (Igbeneghu *et al*., 2013; WHO, 2023), however, it varies greatly in endemicity across the continent with consequent variation in levels of immunity and age-specific patterns of disease (Snow *et al*., 1997). Most malaria cases and deaths occur in sub-Saharan Africa (Talapko *et al*., 2019) with about 619,000 deaths in 2021 and an estimate of 90 % malaria deaths in sub- Saharan Africa (WHO, 2023). Four African countries were observed to account for more than half of all malaria deaths worldwide with Nigeria having the highest population in this category at 31.3% followed closely by the Democratic Republic of the Congo (12.6%), United Republic of Tanzania (4.1%) and Niger Republic (3.9%) (WHO, 2023).

Invasion of human red blood cells (RBCs) by malaria parasites is an intricate process which requires a number of distinct ligand-receptor interactions at the merozoite-erythrocyte interface (Michael *et al*., 2015). Merozoite surface protein-1 is a dominant antigen with the most abundant protein on the surface of merozoites which is essential for parasite survival and red cell invasion (Michael *et al*., 2015; Xu *et al*., 2021) and has long been considered the best candidate to mediate initial contact to the host erythrocyte (Goel *et al*., 2003). This property suggested its importance as a malaria vaccine candidate (Punath *et al*., 2021). It is approximately 190-kDa glycosylphosphatidylinositol (GPI)-anchored precursor protein and processed into four major subunits (MSP-1_{83}, MSP-1_{30}, MSP-1_{38}, and MSP-1_{42}) by parasite subtilisin prior to invasion (Pachebat *et al*., 2007; Collins *et al*., 2020; Tarr *et al*., 2020). Also, it could be involved in the initial recognition of the erythrocyte in a sialic acid-dependent way (Alves *et al*., 2023). During the final stages of erythrocyte invasion, $MSP1_{42}$ undergoes a second cleavage event called secondary processing and mediated by another parasite subtilisin (PfSUB2), generating MSP-1_{33} and MSP-1_{19} (Harris *et al*., 2005). However, MSP-1_{19} remains attached to the merozoite due to the GPI anchor and are taken into the erythrocyte (Dluzewski *et al*.,2018). The antibodies to MSP-1 antigens have been researched to possess protective mechanism against disease severity and endemicity through reduction of parasitemia which makes it a vaccine candidate of choice (Stanistic, 2009; Punath *et al*., 2021).

In the combat against malaria, the World Health Organisation (WHO) recommended Artemisinin based combination therapy (ACT) for uncomplicated malarial treatment (WHO, 2018; Akanni *et al*., 2019). This has been effective in treatment, faster clearance of parasites and reduction of gametocyte development in the peripheral blood; however, some treatment failures coupled with resistance were recorded in some endemic regions with various ACTs (WHO,2018). TriAntmal™, a combination of Artemisinin and bioflavonoids, was developed to cure malaria and prevent re-infection through a 16- day one-time low dosage therapy by inhibiting artemisinin intestinal degradative enzymes and strengthening of blood vessels (Akanni *et al*., 2019; Thornwaite *et al*., 2019). The drug has been seen to proffer long term defence against malarial symptom reoccurrence through enhancement of the humoral, innate and innate defensin immunity against *P.falciparum* which has been a success for about a 14 year period in Haiti and was also seen in Nigeria (Akanni *et al*., 2019; Thornwaite *et al*., 2019). The drug was also under clinical trial in Rwanda with clearance of the parasite within one week among participants which is indicative of a successful outcome (Jejje, 2021). However, more studies are being undertaken on its role on other physiological functions of the body with significant emphasis on the haemopoietic system, liver functions etc among Nigerian populace. Although antibody responses against MSP-1 are seen to be associated with protection against clinical malaria and disease severity; these associations are known to vary across different endemic regions (Punath *et al*., 2021; Lapp *et al*., 2022; de Assi, 2023). Hence, there is the need to assess the level of MSP-1 antibodies with anaemia

development in malaria infected population on the artemisinin-bioflavonoid based treatment with focus on Osun state, Nigeria.

MATERIALS AND METHODS

Study site and subject selection

This community-based experimental clinical trial was carried out at a major Primary Health Centre in Osun state, European-Union Prime Project facility, Nigeria for a period of 12 months. At the inception of the study, a total of one hundred and three (103) subjects were recruited for the study however seventeen participants were non-compliant with drug administration and interval while nine participants discontinued due to administration of other undefined antimalaria therapies and personal traditional myths. So, a total of seventy-seven individuals diagnosed with malaria parasite infection on presentation completed the study. These participants enrolled are forty-two adults (16-42 years) comprising of 27 females and 15 males; and thirty-five children (2-15 years) comprising of 20 females and 15 males, respectively. Ethical approval was obtained from the UNIOSUN Health Research Committee (UNIOSUN HREC 2017/01B) with follow up by representatives of the committee while informed consents were obtained from all adult participants as well as the parent/guardian of the children. Individuals with underlining disorders, infectious diseases, anaemia of chronic diseases and those already on antimalarial therapy/self- treatment were excluded from the study.

Drug provision and administration

The TriAntiMal™ formulations were supplied by Dr Jerry T. Thornthwaite, Director of the Cancer Research Institute of West Tennessee. Each gel capsule TriAntiMal™ contains a proprietary blend of 50 mg antioxidant citrus bioflavonoids including synephrine, artemisinin, quercetin, curcuminoids, hesperetin, plus flavonoids and 50 mg artemisinin (97%) (patent pending) (Akanni *et al.,* 2021; Thornthwaite *et al.*, 2021). The NutraNanoSphere™ (NNS) TriAntiMal™ contained a proprietary blend of micellized artemisinin, bilberry and curcumin. The administration of TriAntiMal™ treatment for this study was done using the malaria medicine designate for a period of 16 days whereby the drug was administered from day 1 to day 16. The NNS TriAntiMal™ formulation was encapsulated in micelles of a natural origin with an average sphere diameter of 18.51 nm ± 7.45 SD. This formulation contained mixtures of micellized curcumin, bilberry and artemisinin. This formulation is completely miscible in baby's milk and is practically tasteless. The NNS do not require refrigeration, naturally are antibacterial, and are very stable, even at elevated temperatures (37˚C) for several days (Thornthwaite *et al.*, 2019). All treatments were directly administered at the clinic and patients observed for 30 minutes and doses re-administered when vomiting occurred.

Handling of adverse effects

Symptoms and signs that were not part of presenting features were taking as adverse effects. Though bilberry, other bioflavonoids, artemisinin and curcumin are known to be safe, adequate medical personnel were available to take care of any side effects. There were no noticeable adverse side effects of the drug observed during or post treatment.

Exclusion criteria

Unwillingness to take the malaria formulation for 16 consecutive days; concomitant infection, i.e., malaria infected patient that has any other infection; non-compliance and non-conformity with drug administration and duration; treatment with any anti-malaria in the next one week before presentation; acute severe complicated malaria e.g. vomiting frequently that requires the administration of intravenous fluid, convulsion, severe anaemia with PCV<18%, clinical evidence of pulmonary oedema, feature suggestive of renal failure, history of dark brown color urine which is suggestive of severe red blood cell haemolysis; hyperparasitemia with >105 parasites/µl; patient with temperature > 37.5˚C; hyperpyrexia with temperature ≥ 40˚C. Data were handled by the researchers and the names of each patient coded.

Sample collection and methods

Blood samples were collected at the days 0, 3, 7 and 16 of the drug administration from each participant consecutively thereby having pre-treatment (D0), post-treatment days (D3, D7 and D16 (after last dosage) samples. At each collection, exactly 4ml of whole blood was

obtained from each participant and equal volumes dispensed in EDTA anticoagulated and plain bottles. Serum was obtained from the plain bottles and refrigerated for 4±2°C for subsequent enzyme linked immunosorbent assay (ELISA). The EDTA bottles were used for analysis of haemoglobin (Hb), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) which was performed on Sysmex K2ZIN. Also, thick and thin blood film were made from the EDTA anticoagulated blood, stained using Giemsa staining techniques, then malaria parasite identification and quantification for malaria parasite density (MPD) were examined by WHO certified malaria microscopist to determine parasitemia and the parasite species.

Demonstration of parasite by thin blood film

A thin film was made by making a drop of blood on a grease free slide and with the aid of a spreader placed at an angle, the blood was spread to make the thin film having a tail, body and head. It was allowed to air dry and fixed with absolute methanol for two minutes and then stained with GIEMSA stain (1 in 10 dilution) for 10 minutes. Rinsed and examined with X40 and X100 Objectives to determine the parasite species (Yeka *et al*., 2015).

Demonstration of parasite by thick film

A thick smear of blood was made at the center of a grease-free slide in a circular motion and allowed to dry, prevented from flies and dust. It was flooded with GIEMSA stain (1in 10 dilution) for 10 minutes. Rinsed and examined with X40 and X100 Objectives to determine the percentage parasitemia (Yeka *et al*., 2015).

Estimating parasite density using WHO standard.

Parasite densities were determined with absolute WBC counts as a ratio of *P. falciparum* counts relative to 200 WBC in thick films. For *P. falciparum*, counts ≥ 100 parasites per thick smear high power field, parasite counts were confirmed in thin films (against 2,000 red blood cells) and recalculated with 200 WBC. At least 100 high power field were examined before thick films were described negative. Parasite density was calculated using the formula below.

$$\frac{\text{(No of parasites counted)}}{200\ \text{WBCs}} \times 8000$$

Where 8000 is the average range of normal total White blood cell count (WBC)

Sandwich enzyme linked immunosorbent assay (ELISA) for merozoite surface protein-1 (MSP-1) antibodies

All serum samples were tested for the presence of *Plasmodium* merozoite surface protein-1 antibodies by using a commercially available ELISA (Biorex, UK) reagent which detects *P. falciparum*- and *P. vivax*-specific IgG, IgM, and IgA by using a mixture of four recombinant antigens coated onto microtiter plates. The assay was performed as directed by the manufacturer. Serum or plasma was tested for immunoreactivity to antigen-coated polystyrene beads. Specimens were diluted 1:16 in diluent buffer (Tris-phosphate buffer [pH 7.8], comprising 20% goat serum, 10% calf serum, 0.2% Triton X-100, and sodium azide), and 0.010 ml was added to a well of a plastic test tray and then combined with an additional 0.20 ml of the same diluent buffer for a final sample dilution of 1:336. The recombinant protein-coated bead was added to the diluted sample and incubated at 37°C for 90 min with mixing. Beads were then washed with 11 to 14 ml of deionized water followed by the addition of 0.2 ml of peroxidase-labeled goat anti-human IgG (20 ng/ml) or anti-human IgM (250 ng/ml). Beads were incubated at 37°C for 30 min with mixing. Beads were washed with 11 to 14 ml of deionized water and then transferred into plastic tubes to which 0.3 ml of OPD substrate (0.3% *O*-phenylenediamine-2-HCl in citrate buffer containing 0.02% H_2O_2) was added and were incubated in the dark at room temperature for 30 min without mixing. Reactions were quenched by the addition of 1 ml of 1 N H_2SO_4, and the optical density (OD) at 450 nm was determined using 1 N H_2SO_4 as the blank. The OD is directly proportional to the amount of antibody bound to the bead (Muerhoff *et al*., 2010).

Statistical analysis

The data was summarized in tabular form as means ± standard deviation (mean±SD) for continuous variables and percentages for categorical variables. The mean difference

across various categories of subjects' variables were analyzed using Student "*t*" test. The association between anaemia parameters of study subjects and merozoite surface protein – 1 (MSP-1) were analyzed using Pearson's correlation and depicted graphically using scatter plot. All the analyses were done with Statistical Package Social Sciences (SPSS) software version 20 (IBM Incorporated). A p-value ≤0.05 were considered statistically significant.

RESULTS

The mean±SD age of the adult (16-42years) and children (2-15years) participants were 23.62±6.23 and 8.84±3.96 respectively while the mean±SD of all parameters across the pre- and post- treatment groups days 0,3,7 and 16 are represented in Table 1. From the table, the anti-MSP-1 is seen to be significantly declining as the days increases whereby the pretreatment day 0 has higher MSP-1 value than for other days ($p<0.05$). The MPD values were only seen in days 0 and 3 with no values observed in other days, this invariably shows a significantly ($p<0.05$) higher value in day 0 compared with day 3 and other non-valued days 7 and 16 within the adult and children participants. Among the children, the haemoglobin (Hb), haematocrit, mean corpuscular haemoglobin (MCH) and MCHC levels are significantly higher in days 7 and 16 ($p<0.05$), respectively, when compared with day 0.

In Table 2, majority of the participants (adult and children) had their anti-MSP-1 within 31-100 level followed closely by those with greater than 100 level while few of them had their antibodies less than 31. In the children participants, the anaemia indicator in day 0 displayed 14.3% with severe anaemia and within the 31-100 anti-MSP-1 value while 7.1% of the adults are severely anaemic concurrently with anti-MSP-1 greater than 100 ($p<0.05$). These severely anaemic children and adult participants were observed to reduce in number drastically to 2.8% and 2.3 %, respectively on day 3 with no participant with anaemia on days 7 and 16.

The relationships between malaria parasite density and merozoite surface protein-1 antibodies across days 0 and 3 were displayed in Figure 1 where weak negative relationships were seen in both days among the children (R^2=0.067 (Day 0) and R^2= 0.027(Day 3)) depicting a slightly reduced MPD level in an increased anti-MSP level. Whereas, a positive linear relationship was observed in day 3 among the adults (R^2= 0.034) implying an increased MPD in an increased anti-MSP state. Although it was observed that other days displayed straight linear relationships between the MPD and anti-MSP indicating no correlations exist in the parameters within those days (R^2= 0.000).

DISCUSSION

Antimalarial therapy by various drug regimen has undergone several evolutionary developments as medicines ranging from the past regimen such as quinoline drugs (quinine, mepacrine, chloroquine and mefloquine) and halofantrine (Tse *et al*., 2024); to present drugs listed on WHO essential drug list which include artemisinin and derivatives, amodiaquine, piperaquine, lumefantrine, proguanil and atovaquone etc (Tse *et al*., 2019; WHO, 2024). These drugs have varied mechanism of actions ranging from parasite reduction through haem generation replication protein reduction; inhibition of B-haematin formation, inhibition of haemozoin and several other parasitic replication inhibition mechanisms (Tse *et al*., 2019; Eastman and Fiddock, 2009). Due to toxicity and red cell destructive abilities of the past drugs as well as resistance observed in both past and present drug regimen despite combination therapies, there are developments of studies addressing the limitations in order to develop an highly effective drug devoid of toxicity and resistance.

Table 1: The mean ±SD of all parameters assessed among the adult and children's participants

*Statistical significance at p<0.05.

Key: Haemoglobin (Hb), Haematocrit (HCT), Mean corpuscular volume (MCV), Mean Corpuscular haemoglobin (MCH) and Mean Corposcular Haemaoglobin concentration (MCHC), Merozoite Surface Protein -1 (MSP-1) and Malaria Parasite Density (MPD)

	Adults (Mean ± SD)				Children (Mean ± SD)			
	Pre-treatment	Post-treatment			Pre-treatment	Post-treatment		
Parameters	Day 0	Day 3	Day 7	Day 16	Day 0	Day 3	Day 7	Day 16
Anti-MSP-1	103.04±39.04*	93.77±36.98*	81.46±30.4*	67.72±34.53*	93.93±39.72*	85.25±38.13*	72.06±31.46*	60.42±36.64*
MPD (uL^{-1})	6747.62±848.76*	84.29±42.98*	0*	0*	15764.63±5336.06*	141.47±71.1*	0*	0*
Hb (g/dl)	11.41±1.97	11.14±1.78	11.58±1.47	12.37±1.49	10.48±2.05*	10.42±1.94	11.08±1.72*	12.02±0.82*
HCT (l/l)	34.61±6.07	31.19±4.98	33.57±4.26	36.98±3.46	31.91±5.89*	32.04±5.79	33.12±4.88*	36.46±2.08*
MCV (fl)	83.59±9.19	83.44±8.70	84.30±6.42	84.39±6.07	80.83±7.05	81.03±6.62	84.24±2.39	87.49±1.86
MCH (pg)	28.88±2.92	28.43±2.93	28.84±1.99	28.57±2.39	26.31±3.19*	26.84±2.59	26.74±2.47*	28.20±1.96*
MCHC(g/dl)	33.86±2.88	34.36±4.58	34.37±3.23	33.80±2.72	30.94±3.32*	31.19±2.80	32.75±2.81*	33.24±2.23*

Table 2: Distribution of Anaemia Indicators across MSP-1 levels within the pre-treatment and post-treatment stages in participants

*Statistical significance at p<0.05.

	Adult Anti-MSP-1 (n=42) (Frequency/%)				**Children Anti-MSP-1 (n=35) (Frequency/%)**			
Day 0 (Pre-treatment)								
Anaemia Indicators	**<31**	**31-100**	**>100**	**p-value**	**<31**	**31-100**	**>100**	**p-value**
Anaemic	0	1(2.3)	3(7.1)		0	5(14.3)	1(2.9)	
Normal	0	23(54.8)	15(35.7)	0.001*	0	20(57.1)	9(25.7)	0.001*
Day 3 (Post-treatment)								
Anaemia Indicators	**<31**	**31-100**	**>100**	**p-value**	**<31**	**31-100**	**>100**	**p-value**
Anaemic	0	0	1(2.3)		0	1(2.8)	0	
Normal	0	24(57.1)	17(40.5)	0.001*	0	25(71.4)	9(25.7)	0.001*
Day 7 (Post-treatment)								
Anaemia Indicators	**≤30**	**31-100**	**>100**	**p-value**	**≤31**	**31-100**	**>100**	**p-value**
Anaemic	0	0	0		0	0	0	
Normal	1(2.4)	22(52.4)	19(45.2)	0.001*	2(5.7)	28(80.0)	5(14.3)	0.001*
Day 16 (Post-treatment)								
Anaemia Indicators	**≤30**	**31-100**	**>100**	**p-value**	**≤30**	**31-100**	**>100**	**p-value**
Anaemic	0	0	0		0	0	0	
Normal	1(2.4)	22(52.4)	19(45.2)	0.001*	5(14.3)	28(80)	2(5.7)	0.001*

Key: HCT, Haematocrit; MSP-1, Merozoite surface protein-1. **Anaemic**- HGB<10, HCT<30, MCV<70, MCH <21, MCHC<29; **Normal**- HGB>10, HCT-31- 45, MCV-70-95, MCH -21-30, MCHC-29-34

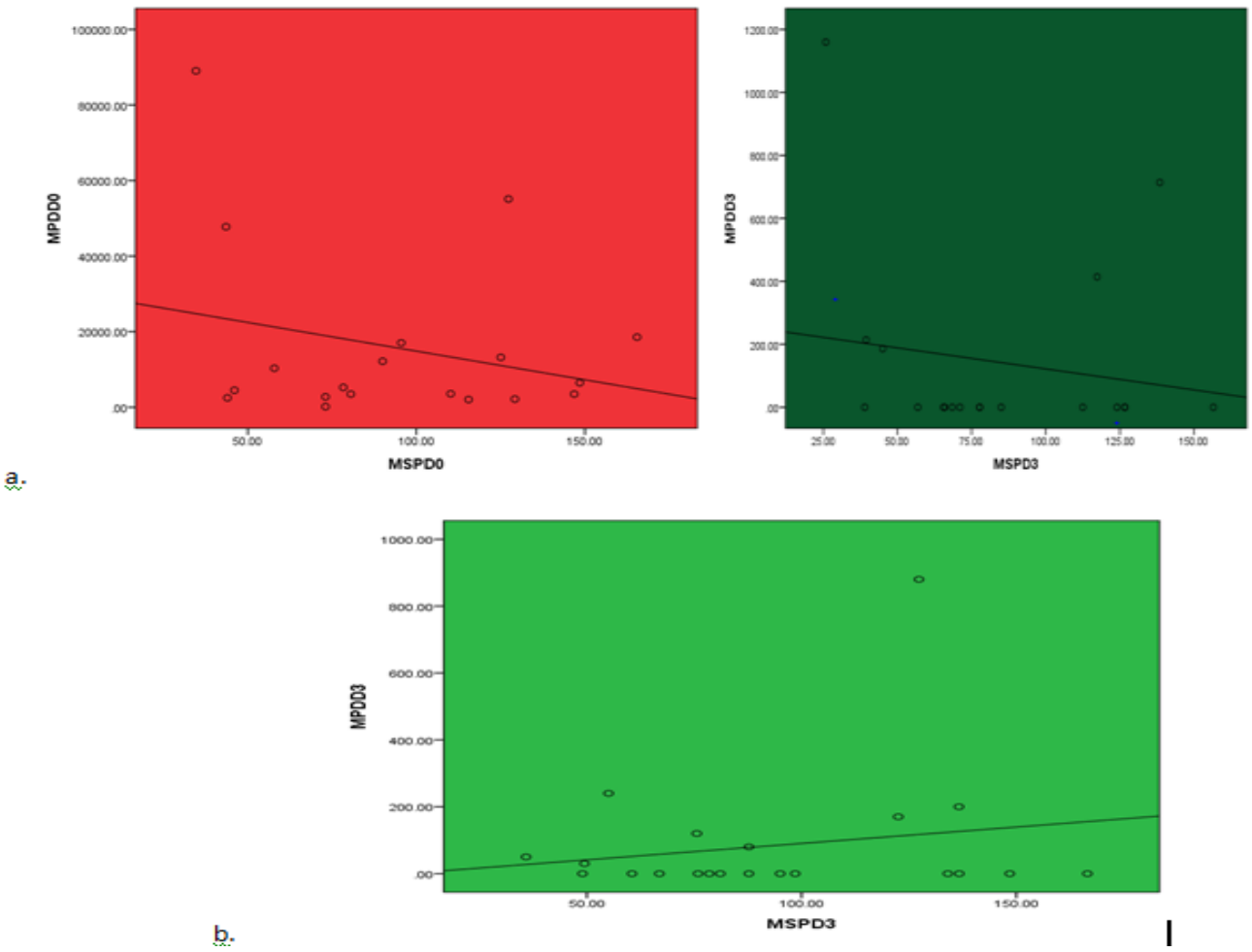

Figure 1. a. Relationship between Merozoite Surface Protein-1 and Malaria Parasite Density for pre-treatment and post-treatment days 0 and 3 in the children. b. Relationship between Merozoite Surface Protein-1 and Malaria Parasite Density for post-treatment day 3 in the adult participants.

Flavonoids are phytochemicals which are secondary metabolites found in many plants, fruits and vegetables and they possess several medicinal benefits which include anticancerous, anti-inflammatory, antioxidant, anti-viral activities as well as neuroprotective and cardio-protective benefits (Tse *et al*., 2019; Asad *et al*., 2020). They have been seen to be less toxic in application and efficacious when administered therefore the combination of flavonoids with artemisinin along with curcuminoids with selected antioxidants in TriAntiMal^{TM} when tested among Haiti and Nigerian malaria infected individuals was seen to be effective (Akanni *et al*., 2019; Thornwaite *et al*., 2019). It was so efficacious to the extent that parasitemia was cleared in their system; long-term immunity mechanisms were boosted in the humoral, cellular and innate imunity despite being exposed to malaria parasite re-infection (Thornwaite *et al*., 2019).

The results of this study shed light on the dynamics of Merozoite Surface Protein-1 (MSP-1) antibodies, malaria parasite density (MPD), and hematological parameters in individuals undergoing the NutraNanosphere Artemisinin-Bioflavonoid antimalarial therapy, specifically the TriAntiMal^{TM} treatment.

The findings from this study reveal a significant decline in anti-MSP-1 levels as the days of

treatment progress. This decline suggests a potential association between the TriAntiMalTM treatment and the modulation of the host immune response against the malaria parasite. The reduction in MSP-1 antibodies may be indicative of a successful therapeutic response due to the fact that the decline in the antibodies is directly interpreted as the decline in the MSP of the infected individual. This is possibly linked to the artemisinin's mechanism of action whereby it acts as a potent inhibitor of *P. falciparum* phosphatidylinositol-3-kinase (*Pf*PI3K) (Mbengue *et al*., 2015). Also, it was generally discovered that free radicals are generated to damage the proteins required for parasite survival when artemisinin is activated by the haem (Wang *et al*., 2015) hence the reduction in the protein antibodies responsible for the parasitic survival. Other mechanism of actions by arteminisin observed to play a role in the reduction of parasite load is in its relationship of haem with *Pf*ATP6 (Ca^{2+} transporter) (Shandilya *et al*., 2013).

The observed decline in anti-MSP-1 antibodies also aligns with previous research highlighting the importance of MSP-1 as a vaccine candidate (Punath *et al*., 2021) and its potential as a protective mechanism against disease severity and parasitemia. The TriAntiMalTM treatment appears to influence the host immune response, leading to a decrease in MSP-1 antibodies, which may contribute to the successful clearance of parasites and prevention of malarial symptom recurrence that was observed in this study.

Our study demonstrates a significant reduction in malaria parasite density (MPD) from day 0 to day 3 which is directly related with the decline in the MSP-1 antibodies. This decline in parasitemia aligns with the expected therapeutic outcome of artemisinin-based treatments, including the TriAntiMal™ therapy. The rapid clearance of parasites within the first few days of treatment is consistent with the effectiveness of artemisinin in targeting the asexual stage of the malaria parasite life cycle.

The substantial decrease in MPD is a positive indicator of treatment efficacy, suggesting that the TriAntiMal™ regimen effectively curtails the proliferation of *Plasmodium falciparum* in infected individuals. This efficacy being due to its artemisinin parasite clearance mechanisms coupled with the anti-malaria abilities of bioflavonoids content of the drug under study. These antimalarial roles include the *Plasmodium* organelle disruption role of curcumin (Chakrabarti *et al*., 2013; Sharfia *et al*., 2022); curcumin induced increase in reactive oxygen species thereby activating *PPARγ/Nrf2*, and upregulating CD36 expression in monocytes or macrophages in order to phagocytose parasite-infected erythrocytes (Mimche *et al*., 2012; Sharfia *et al*., 2022), as well as the role of quercetin in suppression of parasitaemia coupled with its mechanism in decreasing serum levels of TNF-α and IFN-γ whilst increasing of IL-10 and IL-4 levels and glycogen synthase kinase-3β (GSK3β) mediated cytokine-modulating effects (Ali *et al*., 2021)[33]. The absence of MPD values in later treatment days (days 7 and 16) further supports the sustained impact of the TriAntiMal's one- time low dose extended treatment with synergism of its natural components in preventing parasite resurgence.

The rationale for utilizing low-dose Triantimal™ for 16 days stems from the authors' expertise in cancer treatment. Oncologists are reconsidering traditional chemotherapy protocols and increasingly recognizing the advantages of administering lower doses more frequently, as opposed to conventional high-dose regimens (Finn, 2012; Akanni *et al.*, 2019). This approach has shown promising antiangiogenic effects, demonstrating significant therapeutic benefits with low-dose chemotherapy as seen with cyclophosphamide administration for anti-tumour activities (Madondo *et al*., 2015). This 16-day treatment is therefore projected to produce complete parasite clearance and seen in the study, IgM-IgG transition leading to continuous destruction of parasites and eventual long term immunity development (Akanni *et al.*, 2019)

An interesting observation in our study is the fluctuation in hematological parameters across different treatment days. Initially, haematological parameters were all seen to be reduced on day 0 with majority not being significantly different on day 3 which can be attributed to confluence of factors including anemia from chronic illness due to the *Plasmodium* infection; rapid hemolysis of red blood cells that are both parasitized and innocently non-parasitized; decreased and inadequate erythropoiesis with dyserythropoietic alterations which have been described in haematology of malaria by White (2018) and Barber *et al*. (2018). Anaemia in malaria is also

caused by splenic phagocytosis, pooling, and/or impaired red blood cell deformability resulting in a higher clearance rate from the circulation (Barber *et al*., 2018). The parasitic malaria within red blood cells consumes and breaks down more haemoglobin which was responsible for the low haemoglobin level observed on day 0 in this study coupled with the reduction in haematological parameters property of artemisinin as explained by Osonuga *et al*. (2012).

Among the children, we noted a significant increase in hemoglobin (Hb), hematocrit, and mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) levels on days 7 and 16 compared to day 0. These variations in hematological parameters may be attributed to the complex interplay between the host's immune response, the efficacy of the antimalarial treatment, and the pathophysiology of malaria. The increase in Hb, hematocrit, and MCH levels suggests a positive response to treatment, potentially indicating improved erythropoiesis and a reduction in anemia severity. This control of anaemia could be supported by the haemomodulatory role of the bioflavonoid content of the drug whereby cucurmininduces increase in transferrin receptor 1 (TfR1) level thereby activating the iron-regulatory proteins (IRPs) which all results reduction of hypochromic RBCs, and controlled the decreased levels of Hb, haematocrit, serum iron, ferritin, hepcidin, and transferrin saturation, as well as iron levels in the spleen and bone marrow whuch were observed in a similar (Jiao *et al*., 2006; Cotoraci *et al*., 2021). Quercetin is also observed to increase the expression of hepcidin, one of the main hormones involved in intestinal absorption of iron, which could involve the Nrf2 pathway (Cotoraci *et al*., 2021; Bayele *et al*., 2015) despite its chelating activities when preventing iron induced oxidative damage (Lesjak *et al*., 2019). A similar study on haemoglobin changes and risk of anaemia in Sub-Saharan Africa indicated that majority of patients experience a drop in Hb while on treatment with artemisinin containing combinations as early as day 1–2, followed by a linear increase through follow-up. The degree of the early Hb reduction they observed is however determined by pre-treatment parasitemia and parasite clearance rates (Zwang *et al.*, 2017).

Our study indicates a potential association between MSP-1 antibodies and anaemia severity. Specifically, severe anaemia in both children and adults was observed concurrently with elevated anti-MSP-1 levels on day 0. This finding suggests a relationship between the host immune response, MSP-1 antibodies, and the development of anaemia in malaria-infected individuals depicting severity with increase in the anti-MSP-1 as explained by Punnath and colleagues (2021) in a study were anti-MSP-1_{19} IgG1 and IgG3 were found to be increased in malaria infected patients with severe anaemia.

The subsequent decrease in severe anaemia cases on days 3, 7, and 16, coupled with the decline in anti-MSP-1 antibodies, implies a potential role of the TriAntiMal^{TM} treatment in mitigating anaemia associated with malaria. However, the intricate relationship between MSP-1 antibodies and anaemia requires further exploration to elucidate the underlying mechanisms and potential implications for treatment strategies.

The relationship suggests that higher levels of MSP-1 antibodies are associated with increased parasitemia at the beginning of treatment in the adults. Antigenic variation of malaria parasites plays important role in chronicity of infection (Schneider *et al*., 2021). In this study, we found high level of MPD antibodies at the time of infection and sharp reduction in the level of MPD as treatment with this new cocktail (TriAntiMalTM) of anti-malaria drug proceeded. The inability of the parasite to replicate in the presence of this agent and total eradication of the parasites with a corresponding induction of the necessary immune response through the increasing MSP antibodies against the malaria parasite suggest the efficacy of the drug in clearing the parasites. Infection with malarial parasites normally elicits wave of antibodies production which because of antigenic variation might make the antibodies not to be effective in destroying the parasites leading to chronic infection; hence the drug augments the destruction of parasites in the system. The subsequent reduction in MPD accompanied by a decline in anti-MSP-1 antibodies observed in the adult participants reinforce the observation that the host immune response (Punath *et al*., 2021), as reflected by MSP-1 antibodies, and the treatment of choice plays a pivotal role in controlling parasitemia. While there were display of weak negative and positive correlations on days 0 and 3 accordingly, the linear relationships

observed on other days indicate a potential temporal evolution in the interplay between MSP-1 antibodies and MPD during the course of treatment with drug being understudied. Further investigations into the dynamics of this relationship may offer insights into the host-parasite interaction and guide the development of targeted therapeutic interventions.

CONCLUSION

This study provides valuable insights into the intricate dynamics of MSP-1 antibodies, hematological parameters, and malaria parasite density during NutraNanosphere Artemisinin-Bioflavonoid antimalarial therapy. The observed decline in MSP-1 antibodies, coupled with a significant reduction in malaria parasite density and improvements in hematological parameters, suggests the efficacy of the TriAntiMalTM treatment in malaria-infected individuals. The potential association between MSP-1 antibodies and anaemia severity highlights the multifaceted nature of the host immune response in malaria pathogenesis. Further research is warranted to elucidate the mechanistic aspects of these interactions and explore the broader implications for malaria treatment strategies despite the development of vaccine strategies in elimination of malaria. Due to non-compliance and personal reasons such use of other drugs, non-conformity with drug administration interval, traditional myths etc., twenty-six subjects discontinued from participating in the study resulting in low number of participants.

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Conflict of Interest

The authors have no conflict of interest to declare.

Author Contribution

AOE, AOAT, JTT: Conceived and designed the experiment: AOE, BEO, OBB, SOC, AAA: Performed the experiments, analyzed and interpreted the data; AOE and BEO: Wrote the paper. AOE, AOAT, JTT: Supervised the study; AOE, AOAT, BEO: Revised the paper

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