Comparative Haematologic Parameters of Paediatric Uncomplicated *Plasmodium falciparum* Malaria in Children Treated with Artemether-Lumefantrine and Artesunate-amodiaquine in Jos, North-Central Nigeria.

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Abstract

Background: *Malaria is still threatening the lives of millions of children particularly the under five years living in malaria endemic countries of the world. Acute malarial episodes cause many pathophysiological changes in the haematological system of man including alterations in erythrocytes, leucocytes and thromobocytes in the peripheral blood. These changes occur before and after treatment with antimalarial drugs and could also be influenced by the type of antimalarial drugs used.*

Aim: *To determine and compare the changes in haematologic parameters before and after treatment of uncomplicated P. falciparum malaria with artemether-lumefantrine (AL) and artesunate-amodiaquine (AA) in under-five children using 28 day study protocol.*

Method: *Data on 111 children aged 6 to 60 months who were enrolled into a drug therapeutic efficacy testing (DTET) comparing the efficacy, safety and tolerability of AL (20/120mg) with AA (25mg/67.5mg or 50/135mg) in the treatment of uncomplicated falciparum malaria, were analyzed. This study was over a period of 10 weeks (27/06/201 - 16/11/2010) at the General Hospital Brakin Ladi, Plateau State, Nigeria. Inclusion criteria were: history of fever in the last 24hrs and /or measured axillary temperature 37.5°C, P. falciparum infection with parasitaemia ≥ 1000 to ≤ 250,000 parasites/µL, HIV seronegative status, and a written informed consent from parents/ guardians including readiness to comply with the follow-up visits by the parents. The children who met the inclusion criteria were randomized into the two treatment arms and their haematological parameters (haemoglobin (Hb) levels, platelet, neutrophil, lymphocyte counts, and total white cell count (WBC) measured.*

Results: *Of 649 subjects screened for parasitaemia in the study, 282 (43.5%) were febrile (temperature 37.5°C). Out of 649 subjects, 252 (38.8%) had parasitaemia. Only P. falciparum was identified. Parasite count varied from 1000 - 200,000 asexual forms/µL. The mean age (months) of study population in the AL and AA arms were 38.9 16.90 and 37.7 16.76 respectively, (p= 0.72). Thirty one (55.4%) and 25 (44.6%) were males and females in the AL study arm respectively while 32 (58.2%) and 23 (41.8%) were males and females respectively in the AA study arm, (p=0.77). The mean Packed cell volume (PCV) % pre-treatment (D0) rose from 32.14.7 to 35.7 7 in AL treatment arm and from 32. 5.5 to 35.14.4 in AA treatment arm at D28 post-treatment, respectively, p= 0.351. The mean total white cell counts (µ/L) (WBC) dropped from pre-treatment levels of 9.83.5 and 10.0 4.7 to 7.8 2.2 and 7.72.4, in the AL and AA treatment arms D28 post-treatment respectively, P= 0.523. The mean neutrophil subpopulations (%) also showed a progressive decline from pre-treatment (D0) levels of 36.912.5 to 32.88 in the AL treatment arm and from 37.517.0 to 35.0 10.6 in the AA treatment arm respectively, p=0.356. The mean monocytes counts (%) similarly dropped from pre-treatment levels of 11.14.8 to 8.63.4 in the AL treatment arm and from 10.7 4.2 to 8.2 3.7 in the AA treatment arm respectively, p= 0.404. Mean platelete counts also showed a decreasing trends from 545.3216.4 and 280.2151.8 to 268.2106.3 and 258.898.7 in the AL and AA treatment arms respectively at D28, post-treatment, p= 0.394. Compared to the PCV, WBC, neutrophil, monocytes and platelete subpopulations of cells, the mean lymphocyte counts demonstrated a progressive increase from nadir of 51.415.7 to 58.711.6 in the AL treatment arm and 52.016.6 to 56.611.4 in the AA treatment arm at D28 post-treatment respectively, p= 0.630.*

Conclusion: *Uncomplicated paediatric Plasmodium falciparum malaria induces transient alterations in haematologic parameters before and after antimalarial treatment in Jos, North Central Nigeria. Therefore, malaria infection should be considered as a differential diagnosis in febrile children with alterations in haematologic parameters.*

Key words*: Children, uncomplicated Plasmodium falciparum malaria, haematological parameters, ACTs.*

Background

Despite the substantial progress being made in the prevention and treatment of malaria over the past decades, malaria still threatens the lives of millions of children particularly the under-five sub-population in the Sub-Saharan Africa.[1-3] More than 40% of the world`s population (approximately 3 billion people) are exposed to malaria in 108 endemic countries of the world. The World Health Organization (WHO) estimated that 243 million cases occurred in 2008 globally. Out of these, approximately 863,000 deaths resulted. About 80% of the deaths occurred in children under five years of age living in sub-Saharan Africa. Most of the infections are attributable to *P. falciparum* mono-infection. Although mixed infections do occur, these are often unrecognized. [4,5]

Malaria episode causes many pathophysiological disturbances in its host haematologic system including alterations in erythrocytes, leucocytes and thromobocytes subpopulations in the peripheral blood. Haematological alterations may be induced by several other factors as reported 2004 by Wickramasinghe *et al*. Several clinical studies have examined the changes in the peripheral blood counts in uncomplicated malaria infection; few however, have examined and compared these haematologic alterations in children pre and post treatment with ACTs up to 28 days and how the ACTs affect these haematologic parameters during recovery.

The objective of the study therefore, is to determine and compare the natural history of haematologic parameters before and after treatment of uncomplicated *P. falciparum* malaria in under-five children treated with artemether-lumefantrine (AL) and artesunate-amodiaquine (AA).

Methodology

Study design

The present study was part of comparative drug therapeutic efficacy testing (DTET), safety and tolerability of artemether-lumefantrine (AL) and artesunate-amodiaquine (AA), both co-formulated combination therapies for the treatment of uncomplicated *P. falciparum* malaria based on adequate clinical and parasitological response (ACPR) using a 28-day follow-up protocol.

Sample size The sample size was calculated based on the WHO recommendation guidelines (2003) on sample size estimation on assessment and monitoring of antimalarial drugs therapeutic efficacy which accepts a precision of 5-10% at 95% confidence interval (CI) and a 15% absolute difference in failure rate. The expected treatment success rate is 100% for AL and 85% for AA on day 28.

Subjects The subjects consisted of consecutive children aged 6 to 60 months old with history of fever in the last 24hrs and /or measured axillary temperature 37.5°C, mono *P. falciparum* infection with parasitaemia $\geq$ 1000 to $\leq$ 250,000 parasites/µL who have given written informed consent, including readiness to comply with the follow-up visits by the child's parent (s) or other accompanying adult relative but excluded subjects with signs of severe malaria or other common childhood diseases associated with fever for example; otitis media, tonsillitis. Urinary tract infection was excluded by the use of combi-11 urinalysis strip test Kitts for presence of nitrite and leukocyte esterase) in a clean catch midstream urine specimen. Severe malnutrition defined by z score < -3 SD or bilateral oedema.[6] Patients with history of allergy to ACTs or refusal to give consent were also excluded. HIV infection was excluded by Abbott Determine HIV-1 and 2 Whole Blood Assay Kit (**Abbott Laboratory, USA**). Those who where positive were to be confirmed by Western Blotting principle using Qualicode ™ HIV-1and 2 Kitts.

Prior to screening, the subjects were randomized into two study arms to receive 6-dose regimen of artemether-lumefantrine (AL) versus 3-day course of daily amodiaquine-artesunate (AA). In each study arm, there were a total of 57 subjects.[7,8]

Following endorsement of written informed consent by parents or legal guardians of eligible study subjects, bio-data, anthropometric measures were documented. Two milliliters of blood specimen were taken from the left forearm to determine their parasitaemia, haematologic parameters (total leucocyte counts, lymphocyte, monocyte, neutrophil counts, haematocrit and platelet counts). Parasite polymerase chain reaction (PCR) for genotyping to distinguish between re-infection and recrudescence were also undertaken. Treatment and post-treatment outcomes using a 28-day study protocol was used.

Blood sampling Arterialized (Finger prick) blood films for malaria parasites was obtained, subjected to Giemsa stain, and the parasites were counted. Two milliliters of blood samples for biochemistry and haematology were taken using a vacutainer

specimen container. Biochemical parameters estimated included Creatinine, Alanine and Aspartate transaminases and total Bilirubin. These were estimated using **Cobas C111** auto-analyzer. While haematological parameters were estimated using **Minray 200** Auto-analyzer included total WBC count with differential white cell count, Platelet counts, Haematocrit. PCV was determined using **Hawksley Micro-haematocrit centrifuge** of heparinized capillary tubes spun for five minutes at 1500rpm. Blood spot on filter paper for parasite genotyping was obtained on Day 0 for all subjects then on any subsequent day patient was found to have parasitaemia.

Urinalysis A clean catch mid-stream urine sample was collected into a sterile container. A **Combin-11** reagent urinalysis test strips were used to determine presence of nitrite and leucocyte esterase which are markers for urinary tract infection (UTI).

HIV screening Following pretest counseling and endorsement of consent, consecutive patients were screened for HIV infection using Abbott Determine HIV-1 and 2 Whole Blood Assay Kit (**Abbott Laboratory, USA**). Those who where positive were to be confirmed by Western Blotting principle using Qualicode™ HIV-1and 2 Kitts.

Parasite counts: (10-15 minutes**)** At screening prior to enrollment, thick and thin blood films were examined. A second, Giemsa-stained thick film was examined with a binocular light microscope with an oil immersion objective lens (X100) to quantify the parasitaemia. Parasitaemia was measured by counting the number of asexual parasites against a number of leukocytes in the thick blood film, based on a putative count of 8000 leukocytes per microlitre of blood. The number of asexual parasites was counted against 200 leukocytes using a hand tally counter. The number of parasite per microlitre of blood was calculated by using the formula:

$$\text{Parasite Density (parasite } \mu l^{-1}) = \frac{\text{Number of asexual parasites x WBC count}}{\text{Number of leukocytes counted (200)}}$$

If *P. falciparum* gametocytes were encountered in the blood film, a gametocyte count was also performed against 8000 leukocytes (WHO / MAL / 82.988).

Gametocyte Density was then calculated with the formula:

$$\text{(Gametocyte } \mu l^{-1}) = \frac{\text{Number of gametocytes x WBC count}}{\text{Number of leukocytes counted (200)}}$$

This stage of the study lasted for about 45 60 minutes.

Parasite genotyping and Polymerase chain reaction (PCR) results

Polymorphic markers; Merozoites Surface Protein-1(MSP-1), MSP-2 and Glutamate-Rich Protein (GLURP) in *P. falciparum* isolates were used to examine the complexity of parasite populations and to discriminate between recrudescent and re-infections in the study participants.

Matched sample pairs collected before and after treatment from patients who failed AL or AA treatment were successfully analyzed at all the three loci *MSP-1*, *MSP-2* and *GLURP*. Genotyping of these samples showed that the allelic families of MSP-1, MSP-2 and GLURP were often represented in parasite DNA derived from a single patient, indicating a polyclonal infection. The isolates were those of *Plasmodium falciparum* mono-infection.

Antimalarial chemotherapy Enrollees were given weigh-dependent study medications artemether-lumefantrine (20/120mg) or artesunate-amodiaquine (25mg/67.5mg or 50mg/135mg) according to the manufacturer's instructions. Clinical, parasitological and haematological evaluations were carried out on Days 0, 7 and 28. The results were entered into case record forms (CRF) and subsequently transferred into computer using software SPSS versions 15.0 and 17.0, 2011, South Africa, and then analyzed at the end of the study.

Data management All patients' specific details and laboratory parameters were recorded in a case record form (CRF). The information was then entered into the computer using SPSS software versions 15.0 and 17.0, 2011, Illinois Chicago, USA. All patients' codes were double entered serially to avoid transcriptional errors and were only analyzed at the end of the study. Frequency tables and percentages were used to determine the means of all parameters used. Means were used to get the difference in averages between the two drugs (AL and AA) according to all parameter used. Student`s t-test was used to calculate the significant difference between the two drugs. Standard deviation and standard error were also calculated. Levene`s test for equality of variances was used to get the F and P-

values. All tests of significance were two-tailed. P-value < 0.05 was taken to indicate significant difference.

Site of the study This study was conducted in General hospital Barkin-Ladi, Plateau State. It is located 53 km south of Jos, the state capital. The LGA occupies a landmass of 1312.50 km^2 with a population of 175, 267 (2006 National Census). It is bounded by four LGAs: Bokkos LGA to the south, Mangu LGA to the east, Jos-south LGA to the north and Riyom LGA to the west.[9,10]

Ethical consideration Ethical/approval was received by the Institutional Health Research Ethic Committee (IHREC) of the Jos University Teaching Hospital, Jos.

Time of the study The study was conducted between September and December, 2010.

Results

The mean age (months) of study population in the AL and AA arms were 38.9 16.90 and 37.7 16.76 respectively, (p = 0.72). Thirty one (55.4%) and 25 (44.6%) were males and females in the AL study arm respectively while 32 (58.2%) and 23 (41.8%) were males and females respectively in the AA study arm, (p = 0.77). Figure 1 below shows the study profile.

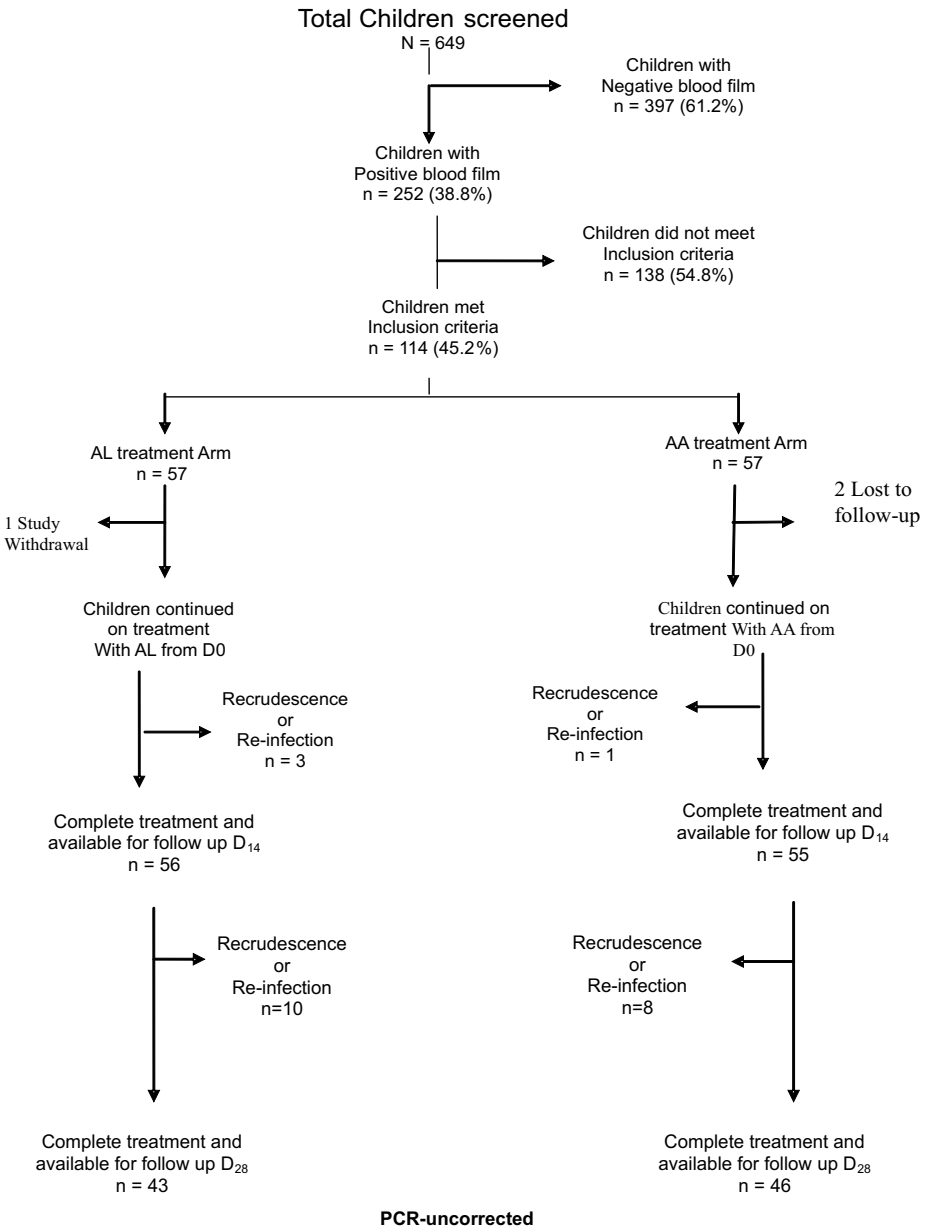

Figure 1**:** Study profile of Patients studied.

The baseline presenting symptoms were compared with the study medications in the two treatment arms; there was no significant difference, Table 1.

1: **Baseline Presenting Symptoms of Patients studied.**

Baseline symptoms	All subjects N=111(%)	Subjects on AL treatment arm n=56 (%)	Subjects on AA treatment arm n=55 (%)	X^2	P – value
Abdominal pain	27(24.3)	17(30.4)	10(18.2)	2.235	0.135
Vomiting	23(20.7)	10(17.9)	13(23.6)	0.564	0.453
Cough	22(19.8)	12(21.4)	10(18.2)	0.841	0.668
Diarrhoea	18(16.2)	9(16.4)	9(16.4)	0.002	0.967
Headaches	9(8.1)	6(10.7)	3(5.5)	1.030	0.310
Poor appetite	4(3.6)	2(3.6)	2(3.6)	0.000	0.985
Mean axillary Temp (°C)	37.8 0.14	37.8 0.98	37.9 1.12	0.865	0.684
<37.5°C	31(27.9)	16 (28.6)	15 (27.3)		
≥37.5°C	80(72.1)	39 (69.8)	41(74.5)		
Hepatomegaly	14(12.6)	8(57.1)	8(57.1)	0.370	0.543
Splenomegaly	20(18.0)	10(50.50)	10(50.50)	0.002	0.965
Spleen Rate(%)	20 (18.0)	9 (50.0)	11 (50.0)	0.002	0.660

*Temp = Temperature

Pre-enrollment parasite count (Day 0)

When subjects were grouped according to range-specific parasitaemia and according to study arms (AL versus AA), there was no statistical difference in the range of parasitaemia and the number of study participants in different ranges, Table 2.

Table 2**: Day 0 Parasite Counts of Patients studied**

Laboratory Feature	All subjects N=111	AL Treatment arm n = 56(%)	AA Treatment arm n = 55(%)	X^2	P-value
Mean Parasite Density at D0(µl)	27,884.2 ± 23,797.0	27.697.6± 26,025.6	27,884.2 ± 23,797.0	0.49	0.92
1000 - <5000	13(11.7)	7(6.3)	6(5.4)	0.07	0.79
5000 - <50000	84(75.7)	42(37.8)	42(37.8)	0.03	0.87
50000 - <100000	11(9.9)	5(4.5)	6(5.4)	0.12	0.73
100000 - <200000	3(2.7)	2(1.8)	1(0.9)	0.32	0.57
>200000	0(0.0)	0(0.0)	0(0.0)		

µl = microlitre, D0 = Day zero,

The haematologiccharacteristics of patients studied is shown in Figure 3 below:

Table 3**: Haematologic characteristics of Subjects on D_o, D_7, and D_{28}**

Parameter	D_o		D_7		D_{28}		t-test	p-value
	AL	AA	AL	AA	AL	AA		
PCV (%)	32.1 4.7	32.5 5.5	33.0 4.8	31.7 3.9	35.7 4.3	35.1 4.4	-0.936	0.351
<30 (%)	12(21.0)	16(29.1)						
30 (%)	44(78.6)	39(70.1)						
Total WBC (/µL)	9.8 3.5	10.0 4.7	8.3 2.5	9.2 3.2	7.8 2.2	7.7 2.4	0.641	0.523
WBC Differential (%)								
L	51.4 15.7	52.0 16.6	55.3 15.2	54.4 12.7	58.7 11.6	56.6 11.4	0.483	0.630
N	36.9 12.5	37.5 17.0	33.8 12.7	36.2 11.8	32.8 7.5	35.0 10.6	0.928	0.356
M	11.1 4.8	10.7 4.2	10.3 7.9	9.7 3.3	8.6 3.4	8.2 .3.7	-0.839	0.404
Platelet (/mm^3)	545.3 216 4.3	280.2 151.8	277.1 110. 9	293.7 127 .6	268.2 106.3	258.8 98.7	-0.857	0.394

DISCUSSION

Anaemia is a common and important complication of acute uncomplicated *P. falciparum* malaria both in asymptomatic and symptomatic children with acute febrile episode.[11] One quarters of the study participants had mild anaemia at enrollment. The difference in the two treatment arms however, did not reach statistical significant levels. Progressive increase in packed cell volume was noticed following initiation and completion of effective antimalaria chemotherapy to patients' normal values. Anaemia associated with malaria has a multi-factorial aetiology. The peripheral blood film is that of normocytic normochromic picture.[12]

The pathogenesis of human *P. falciparum* malarial anaemia involves the destruction and decreased production of red blood cells (RBCs), both occur through several mechanisms: Intravascular haemolysis that accompanies the cyclical pattern of erythrocytes rupture by merozoites accounts for a significant proportion of red blood cell loss. [12]

Phagocytosis of infected and uninfected cells contributes to erythrocyte loss. Malaria antigens transported to the infected erythrocytes surface may lead to opsonization by circulating antimalarial antibodies and subsequent removal of erythrocytes by reticuloendothelial cells that express Fc- receptors.[12]

Uninfected red blood cells may also develop abnormally rigid membranes in the malaria-infected patients and be similarly susceptible to elimination. This pathophysiology may underlie the progressive decrease in red blood cells that occurs after drug-mediated clearance of malaria parasites (drug attributable anaemia). Finally, there may be increased pooling and removal of erythrocytes (and other cellular elements of the blood) in malaria patients with splenomegaly and hypersplensm.[13,14]

Underproduction of red blood cells may be explained by inflammatory cytokines, tumour necrosis factor-a (TNF-a) and interleukin-10 (IL-10).[15, 16] TNF-a plays a role in T-helper type 1-like immune responses. It has been implicated in several biological actions during acute malaria, including stimulation of nitric oxide production, enhancement of the products of other cytokines e.g. IL-6. [12, 17, 18] TNF-a is down regulated by IL-10, an anti-inflammatory cytokine that plays a role in T-helper type 2-like immune responses. Their levels are frequently elevated. They suppress erythropoiesis by inhibiting optimal bone marrow responses to erythropoietin or impairing the growth of early erythroid progenitor-burst and colony forming cells. It has been proposed that the balance between IL-10 and TNF-a may modulate the severity of malarial anaemia in children.[12, 17-20] No study participant required blood transfusion for anaemia.

No study participant recorded a total white blood cell count equal to or below 2500/mm^3 which is the lower limit of normal in the study hospital even though amodiaquine was reported in 2004 to cause leucopenia after long time use as a prophylactic agent. [21] In the present study, the exposure to amodiaquine was transient. Compared to pre-treatment total WBC counts, there was steady increase in WBC following effective treatment and clearance of parasitaemia from the peripheral blood. The difference in the AL and AA treatment arms was not statistically significant. The elevated mean levels of total white blood cell (WBC), neutrophils and monocytes are not completely understood. It may be explained by the fact that, they are the first responders to an infectious attack and are potent biological sensors of infection. They detect molecular patterns on the surfaces of pathogens, transmitting signals into the cell to activate pro-inflammatory and anti-inflammatory cytokine and chemokine gene expression. [22] While the elevated levels and subsequent decline in the monocytes subpopulation were similar in both treatment arms, same was not observed with the neutrophils and total WBC, even though the differences did not reach statistical significant levels. The total WBC, monocyte and neutrophil levels returned to normal between the second and the third weeks following effective therapy and clearance of malaria parasites from the peripheral blood. This is consistent with recovery from paediatric uncomplicated malaria and also agrees with the findings of other authors in Zanzibar, Tanzania, [23] Accra, Ghana,[24] Ibadan, Nigeria. [25]

There was marked initial decrease in mean lymphocyte counts in AL and AA treatment arms at enrolment. This initial decrease was more on the AL treatment arm. The difference was not significant. These lymphocyte levels steadily returned to normal by the fourth week following antimalarial chemotherapy and parasites clearance from the peripheral blood. The gradual normalization of mean lymphocyte counts suggests that the initial decrease may reflect sequestration into deep lymphoid organs rather than their death.[26]

Mild thrombocytosis on D0 was noted in the AL treatment arm compared with the normal counts in the AA treatment arm. The difference was not significant. In both treatment arms, the platelet counts returned to normal by the second week following antimalarial chemotherapy. These levels remained normal 4 weeks post treatment with ACTs. The observation in our study compares favorably with findings reported by Soogarun *at al* (2004) in Bankok, Thailand but contrasts sharply with 50-70% [12] and 73.6% [27] of thrombocythopenias reported by authors elsewhere. No case of thrombocytopenia was recorded during this study. The normal range in the study hospital was 150 x 10^3 - 140 x 10^3/µl. Therefore, peripheral blood film for malaria parasites should be included as a differential for malaria in children under the age of five presenting with febrile episodes.

In conclusion, uncomplicated paediatric *Plasmodium falciparum* malaria induces transient

alterations in haematologic parameters in Jos, North Central Nigeria. Our findings agreed with the literatures. Therefore, malaria infection should be considered as a differential diagnosis in febrile children with alterations in haematologic parameters. This should be supported by parasitologic diagnosis with light microscopy.

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