JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Variation in neutrophil levels and artemisinin-based combination therapy efficacy in West-Africa

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Abstract

Introduction: Polymorphonuclear neutrophils (PMN) are involved in pathogen clearance by phagocytosis. However, the role of PMNs in the efficacy of artemisinin-based combination therapy (ACT) is poorly understood.

Methodology: In a prospective longitudinal in vivo study, neutrophil rates were compared with malaria carriage after treatment with different ACTs: Artemether - lumefantrine (AL), Artesunate - amodiaquine (ASAQ), Dihydroartemisinin - piperaquine (DP) or Pyronaridine artesunate (PA). The study cases were classified as having neutropenia, normal neutrophil levels or neutrophilia depending on the level of neutrophils in the blood. This study included 3148 patients and was analyzed using R.

Results: On day 7, only four patients in the neutropenia group and treated with AL had a malaria positive blood smear based on microscopy. On day 28, the rate of recurrent parasitemia in the AL arm was significantly higher in neutropenia patients (50.9%) than in patients with normal rates of neutrophils (43.1%) or in those with neutrophilia (6.0%) ($p < 0.001$). In ASAQ arm, the rate of recurrent *Plasmodium falciparum* parasitemia was 58.8% in the neutropenia group versus 29.4% in patients with normal rates of neutrophils and 11.8% in patients with neutrophilia ($p < 0.001$). No patient treated with DP with normal neutrophil counts or neutrophilia was carrying malaria parasites on day 28. Among the 15 patients with parasitemia on day 28 in the PA arm, 11 (73.33%) had neutropenia while 4 (26.67%) had a normal neutrophil count ($p < 0.001$).

Conclusions: Patients with neutropenia had higher rates of recurrent *P. falciparum* parasitemia after ACT.

Key words: malaria; neutrophils; artemisinin-based combination therapy; West Africa.

J Infect Dev Ctries 2023; 17(9):1337-1345. doi:10.3855/jidc.17089

(Received 10 July 2022 – Accepted 04 March 2023)

Introduction

Polymorphonuclear neutrophils (PMN) are the most important component of the white blood cells (WBC). In healthy individuals PMN play an important role in the host's immune response against bacterial, fungal and malarial parasites (*Plasmodium* spp.) infections because of their capacity to phagocytose pathogens [1,2]. In addition, they contribute to the recruitment of both nonspecific and specific immune effector cells [3]. WBC and neutrophils modulate the balance between humoral and cell-mediated immunity by producing cytokines [4,5], thereby contributing to the promotion of T helper cells (i.e. TH1 or TH2) response [6]. Neutrophils are engaged in a complex back-and-forth with immune and endothelial cells that bridges innate and adaptive immunity.

In some individuals, neutrophil levels may be lower than normal (neutropenia), which can be acute, congenital or cyclic. Neutropenia, defined as an absolute decrease in the number of neutrophils circulating in the blood [7], increases the risk of infection, in particular from pyogenic and enteric bacteria. In addition, the risk of fungal infections increases further when neutropenia is severe and

prolonged [8]. Acute neutropenia, the resolvable short-term drop in absolute neutrophil counts due to an infection or lesion, is much more common than congenital or cyclic neutropenia [9]. Neutropenia is known to be quite common in populations of African origin [10,11] and a genetic deletion of the Duffy antigen receptor for chemokines (DARC-null genotype) is likely to be a major determinant for neutropenia [11]. In West Africa, over 90% of the population is reported to be Duffy antigen negative [12].

Neutrophils representing ~60% of circulating leukocytes are rapidly solicited at sites of infection or tissue injury [13]. Furthermore, neutrophils have a longevity of ~7h, increasing upon activation to 2-4 days. Therefore, the large pool of neutrophils in the peripheral blood and tissues is continuously renewed from bone marrow precursors [14–16]. They can control mycobacterial infection by phagocytosis of pathogens at the site of infection [17]. Neutrophils could act with similar activities against malaria infection. Neutrophils are known to phagocytose infected red blood cell in vivo [18]. It has been shown that neutrophils can phagocytose merozoites and occasionally trophozoites in bone marrow [19]. Neutrophils can also clear pathogens by respiratory burst called reactive oxygen species (ROS). Malaria parasites growth inhibition by ROS occurs during the intra-erythrocytic development stage [20]. Furthermore, it has been demonstrated in vitro that neutrophils from children infected with *Plasmodium* are more efficient in inhibiting parasite growth than neutrophils from uninfected subjects [21]. Besides the asexual forms of the malaria parasite, neutrophils can phagocytose extracellular gametocytes, while, intra-erythrocytic gametocytes are not very susceptible to neutrophils [22].

Artemisinin-based combination therapy (ACT) is recommended by the World Health Organization (WHO) for almost two decades as the first-line treatment of uncomplicated falciparum malaria [23]. However, the efficacy of ACTs is an ongoing concern due to the emergence of parasite resistance to artemisinins [24]. Furthermore, the interaction between neutrophils and ACTs is largely understudied. In a previous analysis, using data from 7 randomized trials conducted in 9 countries assessing the efficacy of artesunate-amodiaquine, amodiaquine mono-therapy, artesunate mono-therapy, artemether-lumefantrine, artesunate and sulphadoxine-pyrimethamine, and dihydroartemisinin, it was found that neutropenia was frequently recorded as an adverse event at a rate of 11% [25].

Although they act as the first line of innate immunity, the role of neutrophils in the immune response against malaria infections is not sufficiently studied. In particular, there are no extensive studies on their role in parasite clearance [26]. Moreover, since populations of African origin are generally neutropenic [27], one of the concerns is to understand the involvement of neutrophil levels variation in the efficacy of ACTs.

The aim of the present study was to explore the influence of neutrophil levels on the in vivo efficacy of four frequently used artemisinin-based combinations: artemether lumefantrine (AL), artesunate amodiaquine (ASAQ), dihydroartemisinin piperaquine (DP) and pyronaridine artesunate (PA).

Methodology

Study sites and study participants

This is a secondary data analysis of the WANECAM study [28], which was a clinical trial involving 5360 volunteers from West-Africa assessing efficacy and safety of three ACTs. The original WANECAM study (PACTR201105000286876) was a randomized, multicenter, open-label, longitudinal clinical study carried out across seven study sites in three countries: Burkina Faso, Guinea, and Mali [28]. Briefly, study cases were enrolled and followed up over two years for consecutive malaria episodes between October 24, 2011, and February 1, 2016. Patients were treated either with AL, ASAQ, DP or PA at recommended doses. Patients, irrespective of their gender (age ≥ 6 months and body weight ≥ 5 kg), were eligible for enrolment in the study if they had acute uncomplicated microscopically confirmed *Plasmodium* sp. malaria with a parasite density < 200 000 parasites per µL blood, and with fever or a history of fever within 24 hours and hemoglobin (Hb) concentration > 5 g/dL [28]. Before enrolment, all adult study cases provided informed consent after being informed on the aims, benefits and constraints of the study in their own language. For children (< 18 years), informed consent was obtained from parents or legal guardians after the child's assent. Ethical review and clearance for this project was provided by the respective ethical review boards.

Neutrophils status during malaria episode

Blood samples were collected from patients from an alcohol-cleaned peripheral vein into sterile venous blood collection tubes with ethylene diamine tetra

acetic acid (EDTA). Blood counts, including neutrophils, lymphocytes, WBC and Hb were measured immediately before enrolment using ABX Pentra 60 – HORIBA (Montpellier, France) [29]. Blood count measurements were also performed on days 3, 7 and 28 after treatment. Neutrophils status was defined on the basis of the following cut-offs [30]: 1) neutropenia: patients who had a neutrophil count below 40% of WBC; 2) normal: neutrophils level between 40% and 60%; 3) neutrophilia: neutrophil count above 60%.

Drug efficacy

During the first two days after inclusion, finger-prick blood was taken every 12 hours to make thick and thin smears to determine the presence and number of malaria parasites. Subsequently, study cases were seen on days 3, 7, 14, 21, 28, 35, 42 and on days of recurrent illness for clinical examination and finger-prick for thick and thin smears, which were stained with 10% Giemsa for 15 minutes. Parasites density was determined by double reading of the thick blood smear using an Olympus CX21 microscope (Tokyo, Japan). The discordance threshold was set at 50%. Discordant results were subjected to a third independent reading. Asexual parasites were counted against 200 leukocytes (in accordance with WHO standards [31]). If the number of parasites was less than 10 parasites per 200 leukocytes, the parasite count was extended to 500 leukocytes. Parasitaemia was estimated by reporting the number of parasites per microliter of blood based on the count of 8,000 leukocytes. Gametocytes were counted against 1000 WBCs. Non-polymerase chain reaction (PCR) corrected drug efficacy results were used in this study. Pre-treatment and days 3, 7 and 28 neutrophils status and parasitaemia carriage were used to evaluate drug efficacy.

Statistical analysis

Frequency of patients with neutropenia, normal neutrophil levels and neutrophilia were computed, and descriptive statistics were calculated (percentages, median and quartiles). Chi square or Fisher tests were used to compare proportions where appropriate. The threshold of 0.05 was used as statistical significance. Bar plots were used to assess the changes in gametocyte prevalence during follow-up after the administration of antimalarial drugs. Logistic regression analyses were performed to predict the effect of neutrophils status, treatment arms, lymphocytes levels, Hb levels, patients' gender, and age categories on the efficacy of the treatment. Using 'meta' R package (version 4.13-0), a forest plot was performed to investigate the association among socio-demographic parameters and treatment used on gametocytes reappearance on day 3 according to neutrophils variation.

Table 1. Baseline characteristics according to neutrophils status.

Baseline characteristics	Neutropenia N = 809; n (%)	Neutrophilia N = 1208; n (%)	Normal count N = 1131; n (%)	*p* value
Treatment arms				
AL (n = 639)	123 (19.2)	278 (43.5)	238 (37.2)	0.0006
ASAQ (n = 693)	188 (27.1)	262 (37.8)	243 (35.1)	
DP (n = 904)	249 (27.5)	351 (38.8)	304 (33.6)	
PA (n = 912)	249 (27.3)	317 (34.8)	346 (37.9)	
Age categories				
< 5 years (n = 818)	277 (33.9)	219 (26.8)	322 (39.4)	< 0.0001
5 to 14 years (n = 1920)	466 (24.3)	769 (40.1)	685 (35.7)	
> 14 years (n = 410)	66 (16.1)	220 (53.7)	124 (30.2)	
Patient gender				
Male (n = 1637)	427 (26.1)	625 (38.2)	585 (35.7)	0.876
Female (n = 1511)	382 (25.3)	583 (38.6)	546 (36.1)	
Countries				
Burkina Faso (n = 1456)	272 (18.7)	628 (43.1)	556 (38.2)	< 0.0001
Guinea (n = 769)	338 (44.0)	187 (24.3)	244 (31.7)	
Mali (n = 923)	199 (21.6)	393 (42.6)	331 (35.9)	
Gametocytemia	31 (31.0)	23 (23.0)	46 (46.0)	0.0052
Patients weight (kg): median (Q1, Q3)	20.0 (14.0, 29.0)	24.2 (17.3, 37.2)	21.1 (15.5, 31.0)	< 0.0001
Temperature (°C): median (Q1, Q3)	36.8 (36.5, 37.5)	38.2 (37.5, 38.9)	37.5 (36.8, 38.3)	< 0.0001
P. falciparum (/µL): median (Q1, Q3)	2200 (480, 16640)	30580 (10560, 64075)	15660 (1310, 48520)	< 0.0001
Haemoglobin (g/dL): median (Q1, Q3)	10.2 (9.1, 11.2)	11.1 (10.1, 12.0)	10.6 (9.6, 11.6)	< 0.0001
White blood cell: median (Q1, Q3)	6.6 (5.2, 8.9)	8.1 (6.3, 10.8)	7.0 (5.3, 9.0)	< 0.0001
Lymphocytes: median (Q1, Q3)	48.4 (42.7, 53.8)	15.6 (8.1, 20.4)	32.6 (27.2, 38.3)	< 0.0001

N: frequency; AL: artemether lumefantrine; ASAQ: artesunate amodiaquine; DP: dihydroartemisinin piperaquine; PA: pyronaridine artesunate; Q1: 25th percentile; Q3: 75th percentile.

The R software version 3.5.1 (with aod, agricolae, ggplot2, dplyr, plotly, Hmisc, corrr, GGally, meta, and Rmisc libraries) was used for the statistical analysis.

Results

Baseline general study characteristics

Data of 3148 patients were used for this secondary analysis from the total 5360 enrolled in the WANECAM study. Data from 2212 participants were not used because neutrophil counts and/or other relevant variables needed for this sub-study were not recorded. The study cases were distributed amongst the treatment arms as follows: 912 were treated with PA, 904 with DP, 693 with ASAQ and 639 with AL (Table 1). With respect to gender balance, 1637 (52.0%) patients were male and 1511 (48.0%) were female (p = 0.876). A total of 923 patients were enrolled in Mali, 1456 in Burkina Faso and 769 in Guinea. Children between 5 and 14 years (n = 1920) was the most frequently represented age group (60.9%) (Table 1).

Neutropenic patients were more prevalent in Guinea with 44% (338/769). However, in Burkina Faso and Mali, 18.7% (272/1456) and 21.6% (199/923) were recorded, respectively ($p < 0.0001$). The median and interquartile range (IQR) of patients' body weight were significantly lower in patients with neutropenia (20.0 kg, IQR, 14.0-29.0) compared to patients with neutrophilia 24.2 kg (IQR, 17.3 - 37.2) and normal neutrophil levels (21.1 kg, IQR, 15.5-31.0) (p = 0.0011). In contrast, patient's median temperature was higher in the neutrophilia group with 38.2 °C (IQR, 37.5-38.9) (Table 1) compared to patients with neutropenia (36.8 °C, IQR, 36.5-37.5) and normal neutrophil levels (37.5 °C, IQR, 36.8-38.3) ($p < 0.0001$) (Table 1).

There was also a significant difference in patients with neutropenia in the density of trophozoites per µL of blood at the baseline level. These patients had a significantly lower *P. falciparum* parasite count compared with the other groups ($p < 0.0001$) (table 1). However, compared to patients with neutropenia (31 out of 100 carrier, 31.0% of the sexual form of the parasite), *P. falciparum* gametocyte carriage was higher in the normal neutrophil group (46 out of 100, 46.0%) and lower in those with neutrophilia (23 out of 100, 23.0%) ($p < 0.0001$).

The median WBC count was significantly lower in patients with neutropenia 6.6 (IQR, 5.2-8.9) compared to subjects with neutrophilia 8.1 (IQR, 6.3-10.8) and those with normal neutrophil levels 7.0 (IQR, 5.3-9.0) ($p < 0.0001$) (Table 1).

ACTs efficacy according to neutrophil levels

The dynamics of malaria parasite carriage after treatment according to neutrophil status is presented in Table 2. Patients with neutropenia had at baseline the lowest parasite density compared to the other groups (Table 1). However, irrespective of the type of malaria drugs used in this study, patients who were considered to be neutropenic at enrolment had a significantly higher malaria parasite carriage frequency up to day 28 after treatment compared to cases with normal neutrophils level and subjects with neutrophilia at enrolment ($p < 0.0001$). However, microscopy revealed that at day 7 only patients in the neutropenia group, and treated with AL, had a positive blood smear (Table 2). Data from Day 28 showed that the chance of parasite recurrence is higher in patients with neutropenia regardless of the type of ACT used for treatment in this study (Table 2).

Table 2. Proportion of patients with parasitaemia on days 3, 7 and 28 during the first malaria episode.

Patients with parasitaemia	Neutropenia n (%)	Normal count n (%)	Neutrophilia n (%)	*p* value
Artemether lumefantrine				
Day 3	2 (100.00)	0 (0.00)	0 (0.00)	-
Day 7	4 (100.00)	0 (0.00)	0 (0.00)	-
Day 28	85 (50.90)	72 (43.11)	10 (5.99)	< 0.0001
Artesunate amodiaquine				
Day 3	1 (100.00)	0 (0.00)	0 (0.00)	-
Day 7	0 (0.00)	0 (0.00)	0 (0.00)	-
Day 28	20 (58.82)	10 (29.41)	4 (11.77)	< 0.0001
Dihydroartemisinin piperaquine				
Day 3	2 (100.00)	0 (0.00)	0 (0.00)	-
Day 7	0 (0.00)	0 (0.00)	0 (0.00)	-
Day 28	5 (100.00)	0 (0.00)	0 (0.00)	-
Pyronaridine artesunate				
Day 3	1 (100.00)	0 (0.00)	0 (0.00)	-
Day 7	0 (0.00)	0 (0.00)	0 (0.00)	-
Day 28	11 (73.33)	4 (26.67)	0 (0.00)	< 0.0001

Figure 1 shows that irrespective of the treatment arm, the pre-treatment prevalence of gametocytaemia was higher in patients with normal neutrophils level compared to neutropenia and neutrophilia groups ($p < 0.0001$). Three days after antimalarial drug administration, and regardless of the type of ACT used, the data show an increase in the prevalence of gametocytaemia in the neutropenic group while gametocyte clearance is observed in patients with normal neutrophil counts and those with neutrophilia (Figure 1). On day 28, after treatment, only patients with neutropenia and treated with ASAQ, DP and PA carried gametocytes (Figure 1). A forest plot shows that the ACT used (AL: OR = 0.80 [0.26; 2.46], ASAQ: OR = 1.73 [0.85; 3.52], DP: OR = 0.99 [0.47; 2.06], PA: OR = 0.97 [0.45; 2.06]) was not associated with a risk of post treatment day 3 gametocytaemia (Figure 2). This figure also shows that neither the country of residence (Burkina Faso: OR = 0.88 [0.46; 1.68], Guinea: OR = 0.29 [0.07; 1.19], Mali: OR = 1.51 [0.87; 2.61]), nor age category (< 5 years old: OR = 0.48 [0.09; 2.53], > 14 years old children: OR = 0.56 [0.24; 1.26]) were associated with a risk of gametocytaemia 3 days after treatment in patients with neutropenia compared to those without neutropenia.

Secondary neutropenia after ACTs administration

During the first malaria episode (Figure 3A) neutrophil levels on follow-up days 3, 7 and 28 were significantly lower ($p < 0.001$) compared to pre-treatment neutrophil levels irrespective of the antimalarial treatment received by the patients. This decrease in neutrophils levels was more pronounced on day 3 after treatment. All treatment arms taken together, the overall mean level of neutrophil was 52.7% before

Figure 2. Association among socio-demographic parameters and treatment used on gametocytes reappearance at Day 3 according to neutrophil variation.

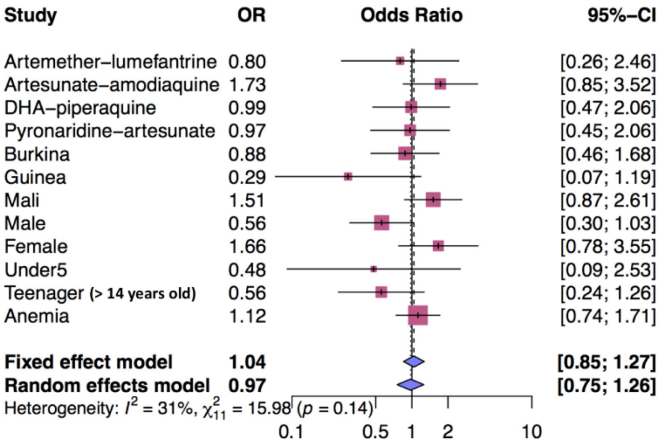
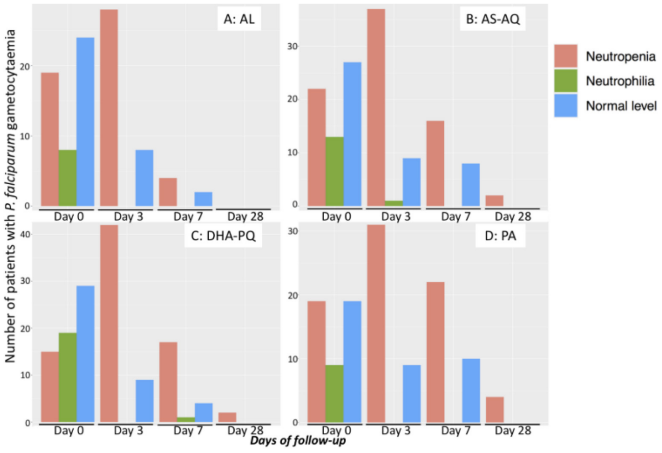


Figure 1. Number of patients with gametocytemia before treatment and on days 3, 7 and 28 during the first malaria episode.



AL: artemether lumefantrine; ASAQ: artesunate amodiaquine; DP: dihydroartemisinin piperaquine; PA: pyronaridine artesunate.

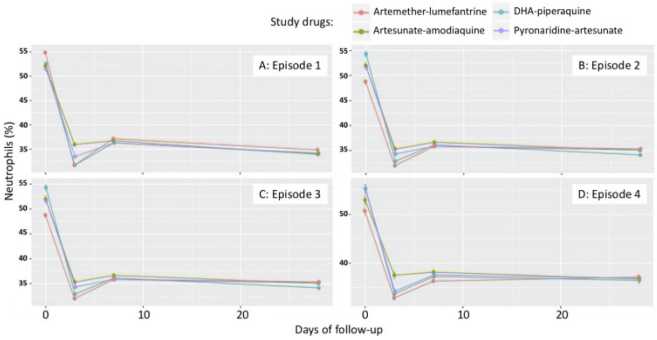
treatment and decreased to 33.4% on day 3 after treatment ($p < 0.0001$). Seven days after treatment, the level of neutrophils increased significantly ($p < 0.001$) regardless of treatment arm and subsequently decreased again significantly regardless of treatment arm from day 7 to day 28 ($p < 0.001$) (Figure 3).

During consecutive malaria episodes (episode 2: Figure 3B, episode 3: Figure 3C and episode 4: Figure 3D), the dynamics of neutrophil levels followed the same trend as in the first episode. Irrespective of the malaria episodes, three days after drug administration the neutrophil counts were significantly higher in the ASAQ treatment arm ($p < 0.0001$) (Figure 3).

Association between neutrophil count variations and reinfection parasitaemia after treatment

Logistic regression analysis (Table 3) shows that having neutrophilia seems to protect against the

Figure 3. Comparison of post treatment neutrophil levels means between treatment arms. DHA: dihydroartemisinin.



reappearance of malaria parasites on day 28 after treatment compared to patients with neutropenia, however the association is not statistically significant (OR = 0.87, 95% CI 0.48-1.58, $p = 0.6577$).

Other factors, i.e., treatment and age, were found to be associated with malaria parasite reappearance during post treatment follow-up (Table 3). Compared to AL treatment arm, ASAQ (OR = 0.64, 95% CI 0.43-0.93, $p = 0.0223$), DP (OR = 0.10, 95% CI 0.05-0.18, $p < 0.0001$) and PA (OR = 0.23, 95% CI 0.14-0.37, $p < 0.0001$) showed a protective effect against malaria parasite reappearance. Compared to under 5 years old children, those older than 14 years of age (OR = 0.22, 95% CI 0.10-0.44, $p < 0.0001$) were protected against malaria parasite reappearance on day 28 of follow-up.

Neither the change in lymphocyte count nor the change in hemoglobin count was associated with post treatment day 28 parasitaemia.

Discussion

This study showed that there was an association between neutrophil levels and parasitaemia in patients. Patients who were neutropenic before treatment had lower baseline parasitaemia, a higher rate of recurrent parasitaemia and higher rate of gametocyte carriage by day 28 post-ACT compared to patients with normal or high neutrophil levels. These differences were found to be independent of the type of ACT used in this study. The baseline *P. falciparum* parasite density was lower in patients with neutropenia. This is in line with previous finding showing that during an acute uncomplicated malaria episode the increase of neutrophils is positively associated with parasitaemia [32]. It has also been reported that in semi-immune travelers neutrophil counts increase with the severity of malaria infection compared to those with uncomplicated malaria [33]. An important finding from this study with regard to transmission potential of malaria is the fact that the prevalence of post treatment gametocytemia was higher in patients with neutropenia regardless of treatment arm. Neutrophils constituting the most important component of the leukocyte population [2] play an important role in malaria infection due to their phagocytic activity [1]. The role of neutrophils in the phagocytosis of extracellular gametes has been previously described [22], and this could explain the almost total absence of gametocytes in post treatment follow-up in subjects with neutrophilia. Another possible explanation is the faster clearance of asexual parasites in patients with neutropenia, which would leave very little time for differentiation into sexual stages [34].

Although they were already infected with *P. falciparum* before neutrophil levels assessment, approximately one third of the patients had neutropenia and these results support previous studies [10,11] indicating that neutropenia is quite common among African populations, where more than 90.0% of the African population are Duffy antigen negative [12] and that the genetic deletion of the Duffy antigen receptor is a major determinant for neutropenia [11].

Our analysis revealed secondary neutropenia three days after the administration of antimalarials, regardless of the treatment arm. It has been previously described that 70–90.0% of acute and severe neutropenia has been shown to be attributable to nonchemotherapy drugs [35]. Several medications including antimalarial drugs are described to be associated with the decrease of neutrophils levels [35,36]. A larger analysis including data from 7 clinical trials carried out in 9 countries comparing the changes in hematologic parameters of ASAQ to AQ mono-therapy, AS mono-therapy, AL, artesunate + sulfadoxine pyrimethamine (ASSP) and dihydroartemisinin (DHA), revealed 11.0% of post treatment neutropenia [25]. A study conducted in Ghana to compare the efficacy of ASAQ versus AL in children showed that 3, 7, 14 and 28 days post treatment

Table 3. Logistic regression model showing the effect of socio-demographic, baseline biological parameters and malaria treatment used on *Plasmodium ssp* reappearance on day 28

	OR	95% CI		*p* value
		Lower	Upper	
Neutrophils normal	1.11	0.71	1.75	0.6546
Neutrophilia	0.87	0.48	1.58	0.6577
ASAQ	0.64	0.43	0.93	0.0223
DP	0.10	0.05	0.18	< 0.0001
PA	0.23	0.14	0.37	< 0.0001
Lymphocytes	1.00	0.99	1.00	0.9630
Haemoglobin	0.99	0.99	1.00	0.3719
Gender Male	1.04	0.76	1.42	0.8164
Age: 5 to 14 years	0.73	0.51	1.07	0.1022
Older children	0.22	0.10	0.44	< 0.0001

ASAQ: artesunate amodiaquine; DP: dihydroartemisinin piperaquine; PA: pyronaridine artesunate; OR: odds ratio; CI: confidence interval.

neutrophil counts were significantly lower ($p < 0.01$) compared to day 0 counts in both treatment arms [37]. On the other hand, it is known that populations of African descent have a benign neutropenia [27]. This secondary neutropenia, apparently very important, could be a return to the known low neutrophil count in populations of African origin. Nevertheless, the neutrophil count 3 days after the start of treatment was lower than on day 7. Apart from the effect of drugs, neutrophils are known to be short-lived [16] although how they are eliminated remains debatable. It has been described that senescent neutrophils in the peripheral circulation upregulate CXCR4 expression, allowing them to return to the bone marrow for final disposal [38]. A study in an animal model found that the number of aged neutrophils oscillated over time. Their numbers increased during the day (when the mice were at rest) and disappeared in the evening, when the mice started their active phase [39]. We did not take into account the possibility of this time-dependent oscillation in the sampling of our patients.

The present study revealed that of all four drug combinations used in this analysis, AL is the least protective against parasites carriage during 28 days' post treatment follow-up in patients with normal neutrophil levels. Artemether with a half-life of two to three hours is rapidly eliminated from plasma whereas lumefantrine is eliminated more slowly [40]. The combination allows a rapid clearance of parasitaemia but does not prevent new infections. In DP combination, piperaquine is characterized by a slow absorption and long half-life, which might prevent new *Plasmodium* infections [41]. While the half-life of amodiaquine is about 5 hours [42,43], the combination ASAQ presented a protective effect in the multivariate model. Pyronaridine is described to have a mean half-life of 194.8 ± 47.8 hours [44].

Furthermore, the present research showed that compared to AL treatment arm, patients treated with ASAQ, DP or PA were less likely to carry gametocytes post treatment which is in line with previous studies. A meta-analysis of individual patient data demonstrated that the appearance of gametocytes in patients' blood was lowest after AL treatment and significantly higher after DP (adjusted hazard ratio (AHR), 2.03; 95 % CI, 1.24–3.32; $p = 0.005$ compared to AL) and ASAQ (AHR, 4.01; 95 % CI, 2.40–6.72; $p < 0.001$ compared to AL) [45]. A previous study conducted in Mali revealed that the day 3 gametocytes prevalence was lower in AL arm compared to ASAQ and ASSP arms [34]. This could be because, unlike AL, which is rapidly eliminated from the patient's blood, the other combinations used in this study would remain at sub-therapeutic levels for a longer period of time, promoting the production of gametocytes.

A limitation of the present study is the absence of parasitaemia data every six to eight hours after treatment. Such frequent measurements would have allowed accurate measurement of the parasite clearance time and the slope of the parasite clearance half-life using the worldwide antimalarial resistance network (WWARN) parasite clearance estimator [46] and to relate these to changes in patient neutrophil counts. In addition, the lack of data on Duffy antigen is a limitation in determining the proportion of benign ethnic neutropenia. [11]. However, the limitations did not prevent us from determining the impact of lower neutrophil counts on the efficacy of ACTs in West African children.

A major strength of this study is the participation of several countries that represent West Africa, which makes this study relevant for a large population in Africa. In addition, the large sample size allowed this study to have sufficient power to detect even small differences or effects. This study effectively compared the four major anti-malarial drugs in relation to neutropenia and parasite recurrence.

In conclusion, this study suggests that neutropenia could decrease the efficacy of ACTs in West Africa. The immunological mechanisms involved warrant further investigations.

Acknowledgements

We thank the field, laboratory, data management and study administration staff at each of the seven trial sites in Burkina Faso, Guinea and Mali whose combined efforts made this work possible. We express our special thanks and gratitude to WANECAM for providing us the data that enabled this analysis.

Author's contributions

Abdoulaye Djimde, Sodiomon B Sirima, Jean Bosco Ouedraogo, Abdoul Habib Beavogui and Issaka Sagara were responsible for the conception and design of the clinical trial on which this study was based. Moussa Djimde, Kassoum Kayentao, Issaka Sagara, Alassane Dicko, Petra F. Mens, Henk DFH Schallig and Abdoulaye Djimde conceived this analysis. Moussa Djimde did statistical analysis and wrote the first draft of the paper. All authors contributed to critical review and approved the final manuscript.

Funding

The main clinical trial [Phase IIIb/IV, randomized, open, parallel to 3 arms and multicenter comparative clinical study comparing the efficacy and tolerance in repeated treatment of

pyronaridine-artesunate and dihydroartemisinin-piperaquine with those of artesunate-amodiaquine and artemether-lumefantrine over a two-year period in children and adults with uncomplicated *Plasmodium* sp.] work was supported by the European and Developing Countries Clinical Trial Partnership (EDCTP), Medicines for Malaria Venture (MMV), the UK Medical Research Council, the Swedish International Development Cooperation Agency, German Ministry for Education and Research, University Claude Bernard (Lyon, France), University of Science, Techniques and Technologies of Bamako (Bamako, Mali), the Centre National de Recherche et de Formation sur le Paludisme (Burkina Faso), Institut de Recherche en Sciences de la Santé (Bobo-Dioulasso, Burkina Faso), and Centre National de Formation et de Recherche en Santé Rurale (Republic of Guinea).

This analysis was supported through the PYRAPREG project capacity building activities (PhD training). The PYRAPREG project is part of the EDCTP2 programme supported by the European Union (grant number RIA2017MC-2025-PYRAPREG).

Ethics approval and consent to participate

The West African Network for Antimalarial Drugs (WANECAM) protocol (IP_07_31060_002) was approved by local ethics committees for each site. All children included in this study provided assent before parents or legal guardians provided their signed informed consents.

Availability of data and material

The study is registered at the Pan African Clinical Trials Registry, number PACTR201105000286876. Registered 31 March 2011: https://pactr.samrc.ac.za/Search.aspx.

References

1. Kolaczkowska E, Kubes P (2013) Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 13: 159-175. doi: 10.1038/nri3399.
2. Hsieh MM, Everhart JE, Byrd-Holt DD, Tisdale JF, Rodgers GP. (2007) Prevalence of neutropenia in the US population: age, sex, smoking status, and ethnic differences. Ann Intern Med 146: 486-492. doi: 10.7326/0003-4819-146-7-200704030-00004.
3. Kondera-Anasz Z, Mielczarek-Palacz A, Switała J, Piasecka A (2004) Metabolic activity of peripheral blood neutrophils in women with ovarian tumours. Ginekol Pol 75: 609-614.
4. Jenne CN, Wong CHY, Zemp FJ, McDonald B, Rahman MM, Forsyth PA, McFadden G, Kubes P (2013) Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. Cell Host Microbe 13: 169-180. doi: 10.1016/j.chom.2013.01.005.
5. Vazquez MI, Catalan-Dibene J, Zlotnik A (2015) B cells responses and cytokine production are regulated by their immune microenvironment. Cytokine 74: 318-326. doi: 10.1016/j.cyto.2015.02.007.
6. Kaiko GE, Horvat JC, Beagley KW, Hansbro PM (2008) Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology 123: 326-338. doi: 10.1111/j.1365-2567.2007.02719.x.
7. Bajaj L, Hambidge S, Nyquist AC, Kerby G (2011) Berman's Pediatric Decision Making (Fifth Edition). Philadelphia: Elsevier 616 p.
8. Wood AJJ, Pizzo PA (1993) Management of fever in patients with cancer and treatment-induced neutropenia. N Engl J Med 328: 1323-1332. doi: 10.1056/NEJM199305063281808.
9. Donadieu J, Fenneteau O, Beaupain B, Mahlaoui N, Chantelot CB (2011) Congenital neutropenia: diagnosis, molecular bases and patient management. Orphanet J Rare Dis 6: 26. doi: 10.1186/1750-1172-6-26.
10. Haddy TB, Rana SR, Castro O (1999) Benign ethnic neutropenia: what is a normal absolute neutrophil count? J Lab Clin 133: 15-22. doi: 10.1053/lc.1999.v133.a94931.
11. Thobakgale CF, Ndung'U T (2014) Neutrophil counts in persons of African origin. Curr. Opin. Hematol 21: 50-57. doi: 10.1097/MOH.0000000000000007.
12. Miller LH, Mason SJ, Clyde DF, McGinniss MH (1976) The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med 295: 302-304. doi: 10.1056/NEJM197608052950602.
13. Evgenyi B. Eruslanov, Irina V. Lyadova, Tatiana K. Kondratieva, Konstantin B. Majorov, Ilya V. Scheglov, Marianna O. Orlova, Apt AS (2005) Neutrophil responses to *Mycobacterium tuberculosis* infection in genetically susceptible and resistant mice. Infect Immun 73: 1744-1753. doi: 10.1128/IAI.73.3.1744-1753.2005.
14. Laskay T, van Zandbergen G SW (2008) Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. Immunology 213: 183-191. doi: 10.1016/j.imbio.2007.11.010.
15. Korbel DS, Schneider BE, Schaible UE (2008) Innate immunity in tuberculosis: myths and truth. Microb Infect 10: 995-1004. doi: 10.1016/j.micinf.2008.07.039.
16. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER (2010) Neutrophil kinetics in health and disease. Trends Immunol 31: 318-324. doi: 10.1016/j.it.2010.05.006.
17. Yang CT, Cambier CJ, Davis JM, Hall CJ, Crosier PS, Ramakrishnan L (2012) Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. Cell Host Microbe 12: 301-312. doi: 10.1016/j.chom.2012.07.009.
18. Sun T, Chakrabarti C (1987) Schizonts, merozoites, and phagocytosis in falciparum malaria. Ann Clin Lab Sci 15: 465-469.
19. Wickramasinghe SN, Phillips RE, Looareesuwan S, Warrell DA, Hughes M (1987) The bone marrow in human cerebral malaria: parasite sequestration within sinusoids. Br J Haematol 66: 295-306. doi: 10.1111/j.1365-2141.1987.tb06913.x.
20. Kharazmi A, Jepsen S (1984) Enhanced inhibition of in vitro multiplication of *Plasmodium falciparum* by stimulated human polymorphonuclear leucocytes. Clin Exp Immunol 57: 287-292.
21. Brown J, Smalley ME (1981) Inhibition of the in vitro growth of *Plasmodium falciparum* by human polymorphonuclear neutrophil leucocytes. Clin Exp Immunol 46: 106-109.
22. Healer J, Graszynski A, Riley E (1999) Phagocytosis does not play a major role in naturally acquired transmission-blocking immunity to *Plasmodium falciparum* malaria. Infect Immun 67: 2334-2339. doi: 10.1128/IAI.67.5.2334-2339.1999.
23. World Health Organisation (2012) World Malaria Report. Available: http//www.who.int/malaria/publications/world_malaria_report_2012/en/. Assessed: 17 March 2017.

24. Siddiqui FA, Liang X, Cui L (2021) *Plasmodium falciparum* resistance to ACTs: emergence, mechanisms, and outlook. Int J Parasitol Drugs Drug Resist 16: 102-118. doi: 10.1016/j.ijpddr.2021.05.007.
25. Zwang J, Ndiaye JL, Djimdé A, Dorsey G, Mårtensson A, Karema C, Olliaro P (2012) Comparing changes in haematologic parameters occurring in patients included in randomized controlled trials of artesunate-amodiaquine vs single and combination treatments of uncomplicated falciparum in sub-Saharan Africa. Malar J 11: 25. doi: 10.1186/1475-2875-11-25.
26. Aitken EH, Alemu A, Rogerson SJ (2018) Neutrophils and malaria. Front. Immunol 9: 3005. doi: 10.3389/fimmu.2018.03005.
27. Thobakgale CF, Ndung'U T (2014) Neutrophil counts in persons of African origin. Curr Opin Hematol 21: 50-57. doi: 10.1097/MOH.0000000000000007.
28. The West African Network for Clinical Trials of Antimalarial Drugs (WANECAM) (2018) Pyronaridine-artesunate or dihydroartemisinin-piperaquine versus current first-line therapies for repeated treatment of uncomplicated malaria: a randomised, multicentre, open-label, longitudinal, controlled, phase 3b/4 trial. Lancet 391: 1378-1390.
29. HORIBA (nd) ABX Pentra 60. Available: https://www.horiba.com/fra/medical/products/hematology/bench-top/pentra-60/. Accessed: 06 September 2023.
30. Berliner N (2011) Leukocytosis and leukopenia. Goldman's Cecil Medicine: Twenty Fourth Edition. Philadelphia: Elsevier 1098 p. doi: 10.1016/B978-1-4377-1604-7.00170-6.
31. World Health Organisation (2016) Malaria microscopy quality assurance manual - Version 2. Available: https://www.who.int/docs/default-source/documents/publications/gmp/malaria-microscopy-quality-assurance-manual.pdf. Accessed: 06 September 2023.
32. Olliaro P, Djimdé A, Dorsey G, Karema C, Mårtensson A, Ndiaye JL, Sirima SB, Vaillant M, Zwang J (2011) Hematologic parameters in pediatric uncomplicated *Plasmodium falciparum* malaria in sub-Saharan Africa. Am J Trop Med Hyg 85: 619-625. doi: 10.4269/ajtmh.2011.11-0154.
33. Berens-Riha N, Kroidl I, Schunk M, Alberer M, Beissner M, Pritsch M, Kroidl A, Fröschl G, Hanus I, Bretzel G, von Sonnenburg F, Nothdurft HD, Löscher T, Herbinger KH (2014) Evidence for significant influence of host immunity on changes in differential blood count during malaria. Malar J 13: 155. doi: 10.1186/1475-2875-13-155.
34. Ouologuem DT, Kone CO, Fofana B, Sidibe B, Togo AH, Dembele D, Toure S, Koumare S, Toure O, Sagara I, Toure A, Dao A, Doumbo OK, Djimde AA (2018) Differential infectivity of gametocytes after artemisinin-based combination therapy of uncomplicated falciparum malaria. Afr J Lab Med 7: 784. doi: 10.4102/ajlm.v7i2.784.
35. Andersohn F, Konzen C, Garbe E (2007) Systematic review: agranulocytosis induced by nonchemotherapy drugs. Ann. Intern. Med 146: 657-665. doi: 10.7326/0003-4819-146-9-200705010-00009.
36. Kaufman DW, Kelly JP, Issaragrisil S, Laporte JR, Anderson T, Levy M, Shapiro S, Young NS (2006) Relative incidence of agranulocytosis and aplastic anemia. Am J Hematol 81: 65-67. doi: 10.1002/ajh.20489.
37. Adjei GO, Kurtzhals JAL, Rodrigues OP, Alifrangis M, Hoegberg LCG, Kitcher ED, Badoe EV, Lamptey R, Goka BQ (2008) Amodiaquine-artesunate vs artemether-lumefantrine for uncomplicated malaria in Ghanaian children: a randomized efficacy and safety trial with one year follow-up. Malar J 7: 127. doi: 10.1186/1475-2875-7-127.
38. Martin C, Burdon PCE, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM (2003) Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. Immunity 19: 583-593. doi: 10.1016/S1074-7613(03)00263-2.
39. Casanova-Acebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chèvre R, A-González N, Kunisaki Y, Zhang D, van Rooijen N, Silberstein LE, Weber C, Nagasawa T, Frenette PS, Castrillo A, Hidalgo A (2013) Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell 153: 1025. doi: 10.1016/j.cell.2013.04.040.
40. Novartis (2018) Drug Regulatory Affairs Coartem®/Riamet® Prescribing Information - Version 2.1. Available: https://rwandafda.gov.rw/wp-content/uploads/2023/02/COARTEM%C2%AE20%20mg-120%20mg%20Dispersible%20tablets-Artemether20mg-lumefantrine120mg.pdf . Accessed: 06 September 2023.
41. D'Alessandro U (2010) Dihydroartemisinin-piperaquine: an update. In: Parasitology, Institute Tropical Medicine, Antwerp, Flanders. Belgium. Available: https://www.dndi.org/wp-content/uploads/2010/08/dalessandro.pdf. Accessed: 29 April 2020.
42. Winstanley P, Edwards G, Orme M, Breckenridge A (1987) The disposition of amodiaquine in man after oral administration. Br J Clin Pharmacol 23: 1-7. doi: 10.1111/j.1365-2125.1987.tb03002.x.
43. Krishna S, White NJ (1996) Pharmacokinetics of quinine, chloroquine and amodiaquine: clinical implications. Clin Pharmacokinet 30: 263-299. doi: 10.2165/00003088-199630040-00002.
44. Ramanathan S, Karupiah S, Nair NK, Olliaro PL, Navaratnam V, Wernsdorfer WH, Mansor SM (2005) A new and simple solid-phase extraction method for LC determination of pyronaridine in human plasma. J Chromatogr B Anal Technol Biomed Life Sci 824: 45-50. doi: 10.1016/j.jchromb.2005.06.034.
45. WWARN Gametocyte Study Group (2016) Gametocyte carriage in uncomplicated *Plasmodium falciparum* malaria following treatment with artemisinin combination therapy: a systematic review and meta-analysis of individual patient data. BMC Med 14: 79. doi: 10.1186/s12916-016-0621-7.
46. Flegg JA, Guérin PJ, Nosten F, Ashley EA, Phyo AP, Dondorp AM, Fairhurst RM, Socheat D, Borrmann S, Björkman A, Mårtensson A, Mayxay M, Newton PN, Bethell D, Se Y, Noedl H, Diakite M, Djimde AA, Hien TT, White NJ, Stepniewska K (2013) Optimal sampling designs for estimation of *Plasmodium falciparum* clearance rates in patients treated with artemisinin derivatives. Malar J 12: 411. doi: 10.1186/1475-2875-12-411.

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Conflict of interests: No conflict of interests is declared.