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Therapeutic Efficacy and Safety of Artemether-Lumefantrine Plus a Single Low-Dose Primaquine for The Treatment of Uncomplicated Plasmodium Falciparum Malaria at Maksegnit Health Center, Northwest Ethiopia

Tadesse, Misganaw

BAHIR DAR UNIVERSITY
COLLEGE OF MEDICINE AND HEALTH SCIENCES
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THERAPEUTIC EFFICACY AND SAFETY OF ARTEMETHER LUMEFANTRINE PLUS A SINGLE-LOW-DOSE PRIMAQUINE FOR THE TREATMENT OF UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA AT MAKSEGNIT HEALTH CENTER, NORTHWEST ETHIOPIA

BY: TADESSE MISGANAW (BSc, MSc CANDIDATE)

A THESIS SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE, SCHOOL OF HEALTH SCIENCES, COLLEGE OF MEDICINE AND HEALTH SCIENCES, BAHIR DAR UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL PARASITOLOGY AND VECTOR CONTROL.

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By: Tadesse Misganaw (Bsc, Msc Candidate)

ADVISORS: Mrs. WOYNESHET GELAYE (PhD. FELLOW., ASSISTANT. PROF.)
Dr. TADESSE HAILU (PhD., ASSOCIATE. PROF.)
Dr. FITSUM GIRMA (PhD)

A THESIS SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE, SCHOOL OF HEALTH SCIENCES, COLLEGE OF MEDICINE AND HEALTH SCIENCES, BAHIR DAR UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL PARASITOLOGY AND VECTOR CONTROL.

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DEPARTMENT OF MEDICAL LABORATORY SCIENCE

Advisors' Approval of Thesis for Defense

I hereby certify that I have supervised, read, and evaluated this thesis titled **"T**herapeutic Efficacy and Safety of Artemether-Lumefantrine Plus a Single Low-Dose Primaquine for the Treatment of Uncomplicated *Plasmodium falciparum* Malaria at Maksegnit Health Center, Northwest Ethiopia**"** by Tadesse Misganaw, prepared under my guidance. I recommended the thesis be submitted for oral defense (mock viva and viva voce).

Advisors' Name	Signature	Date
1. Mrs. Woyneshet Gelaye	……	……
2. Dr. Tadesse Hailu	…….	…….
3. Dr. Fitsum Girma	…….	…….

DECLARATION

I, the undersigned, hereby declare that this thesis, entitled **"Therapeutic Efficacy and Safety of Artemether-Lumefantrine Plus a Single-Low-dose Primaquine for the Treatment of Uncomplicated *Plasmodium falciparum* Malaria at Maksegnit Health Center, Northwest Ethiopia"** is my original work, has never been submitted for a degree, diploma, or other qualification anywhere, and all the resources and materials used for the thesis have been fully acknowledged.

Name: **Tadesse Misganaw**

Signature: _____

Date: _____

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ABBREVIATIONS AND ACRONYMS

ACPR:	Adequate Clinical and Parasitological Response
ACTs:	Artemisinin-based Combination Therapies
AHRI:	Armauer Hansen Research Institute
AL:	Artemether-Lumefantrine
DBS:	Dried Blood Spot
DNA:	Deoxyribose Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic acid
ETF:	Early Treatment Failure
FMoH:	Federal Ministry of Health
G6PD:	Glucose-6-phosphate dehydrogenase
Hb:	Hemoglobin
K-M	Kaplan Meier
LCF:	Late Clinical Failure
LPF:	Late Parasitological Failure
MSP-2:	Merozoite Surface Protein-2
PP	Per Protocol
PCR:	Polymerase Chain Reaction
PI:	Principal Investigator
SLD-PQ:	Single Low Dose Primaquine
WBC:	White Blood Cell
WHO:	World Health Organization

ABSTRACT

Background: *Plasmodium* species are obligate intracellular protozoan parasites that cause

malaria, which is a major public health problem. One of the most important malaria control strategies in endemic areas is the treatment of uncomplicated *Plasmodium falciparum* infections with artemether-lumefantrine plus a single low-dose primaquine. However, drug resistance to uncomplicated *Plasmodium falciparum* infection remains a threat to effective malaria control programs worldwide. Furthermore, the periodic assessments of the efficacy of artemether-lumefantrine plus single low-dose primaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria and its adverse effects are poorly addressed in Ethiopia.

Objective: This study aimed to assess the therapeutic efficacy and safety of artemether-lumefantrine plus a single low-dose primaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria.

Methods: An *in vivo* prospective single-arm study was conducted at Maksegnit Health Center from February to May 2022. Eighty-eight uncomplicated *Plasmodium falciparum* malaria cases whose age ≥18 years were enrolled in the study. A standard dose of artemether-lumefantrine twice daily for three days along with a single low-dose primaquine (0.25 mg/kg) was given, and clinical and parasitological outcomes were assessed for 42 days. Capillary blood was collected for parasitological identification, quantification, genotyping, and hemoglobin determination. Thick and thin blood films were prepared, stained with Giemsa, and examined microscopically. The nested polymerase chain reaction method was used to detect and genotype recurrent *Plasmodium falciparum* cases. Hemoglobin level was measured by the HemoCue HB 301 Sweden spectrophotometer on days 0, 14, 28, and 42. Data entry and analysis were performed using the World Health Organization-designed Excel spreadsheet and SPSS version 25 software. All comparisons were performed at a 95% CI and a significant level of <0.05.

Result: Among the 88 enrolled patients, 85 patients completed a 42-day follow-up. The per-protocol polymerase chain reaction uncorrected and corrected cure rates were 94.1% (95% CI: 86.8–98.1%) and 96.4% (95% CI: 89.8–99.2%), respectively. Artemether lumefantrine plus a single low dose of primaquine cleared parasites and fevers quickly, with only one participant having parasitemia on day 3 and no febrile cases on day 2. On day three, gametocytes were completely cleared. The mean hemoglobin on days 0, 14, 28, and 42 was 14.2±2.07 g/dl, 13.96±1.47 g/dl, 14.4±1.32 g/dl, and 14.65±1.35 g/dl, respectively. There was no statistically significant difference in mean hemoglobin among the follow-up days (0, 14, 28, and 42). Generally, adverse events were mild to moderate, but no severe adverse events were recorded.

Therefore, Artemether lumefantrine plus single-dose primaquine should continue as a first-line drug to treat uncomplicated *Plasmodium falciparum* malaria in the study area.

Conclusion: Artemether-lumefantrine plus single-low-dose primaquine was efficacious and safe in the study area for the treatment of uncomplicated *Plasmodium falciparum* malaria.

Key words: Efficacy, Adverse events, Artemether-lumefantrine, Primaquine, *Plasmodium falciparum*, Ethiopia

1. INTRODUCTION

1.1. Background

Plasmodium species are obligate intracellular protozoan parasites that cause malaria, which is a major public health problem and potentially life-threatening disease. Malaria is still a major concern in tropical and subtropical regions of the world. *Plasmodium (P) falciparum, P. vivax, P. ovale, P. malaria,* and *P. knowlesi* are the five *Plasmodium* species that are known to cause malaria in humans (WHO, 2011). *Plasmodium falciparum* causes the most severe forms of malaria infection around the globe and is the most prevalent species in Africa (Ferri, 2009; WHO, 2010).

The World Health Organization (WHO) reports indicated that estimated 247 million cases of malaria documented globally by 2021 (WHO, 2022). Of which, the African region accounted for 96% of all malaria cases, followed by the Southeast Asia region (2%); likewise, there were an estimated 619,000 deaths due to malaria around the globe. Of which 96% occurred in the African region (WHO, 2022). In addition to its health burden, malaria has also placed a heavy economic burden on the globe. Total funding for malaria control and elimination reached an estimated US$3.3 billion in 2020, and contributions from governments of endemic countries cost US$ 1.1 billion, representing 33% of total funding (WHO, 2022).

In Ethiopia, around 75% of the landmass is estimated to be malarious, and 68% of the country's population is at risk of the disease (FMoH, 1999; Adhanom *et al*., 2006). The overall prevalence of malaria in healthcare-related febrile study participants in Ethiopia was 13.61% among adults (Kendie *et al*., 2021). According to subgroup analysis based on the different categories of malaria cases, the prevalence of malaria in symptomatic and asymptomatic individuals was 15.34% and 11.99%, respectively (Kendie *et al*., 2021). *Plasmodium falciparum* accounts for 60%–70% of malaria cases in Ethiopia (FMoH, 2017); a systematic review and meta-analysis study also showed the pooled prevalence of *P. falciparum* in Ethiopia was 25.8% (Deress and Girma, 2019). Similar to this, a regional subgroup study revealed that the Southern Nations, Nationalities, and Peoples' Region had the greatest malaria prevalence (16.17%), followed by Oromia Regional State (13.11%) and Amhara Regional State (12.41%) (Kendie *et al*., 2021). In

the Gondar Zuria district, the overall malaria count for 2019 was 5893, compared to 31,550 for 2020 and 33,248 for 2021 (Ewnetu and Lemma, 2022).

The lifecycle of *Plasmodium* in a human host begins with the bite of a female *Anopheles* mosquito infected with sporozoites during a blood meal. Parasites circulate in the blood after inoculation, and those that reach the liver go through one cycle of development. The parasites then infect and multiply inside red blood cells, causing the typical signs and symptoms such as fever, headache, and vomiting to appear between 10 and 15 days after the mosquito bite (Antia *et al*., 2007). Malaria can be fatal if not treated because it disrupts the blood supply to vital organs (Anderson *et al*., 2003). Anemia, impaired consciousness, respiratory distress, hypoglycemia, and jaundice are common features of complicated malaria (Schellenberg *et al*., 1999).

Based on clinical signs and symptoms, malaria can be diagnosed clinically, and in the laboratory, *Plasmodium* parasites can be diagnosed by microscopy, parasite antigens through rapid diagnostic tests, or deoxyribose nucleic acid (DNA) via polymerase chain reaction (PCR) (Wilson, 2012). Microscopic examination is the gold standard for detecting and identifying malaria parasites (Chiodini, 2014).

Artemisinin combination therapy (ACT) is recommended globally for the management of uncomplicated *P. falciparum* malaria (Bhatt *et al*., 2015; WHO, 2015). Since 2004, one of the ACTs, artemether-lumefantrine (AL), has been used as a first-line treatment in Ethiopia (FMoH, 2004), and currently, single low-dose primaquine (SLD-PQ) 0.25 mg/kg is added to standard AL treatment (FMoH, 2018). If AL is not available or a patient is hypersensitive to this drug, oral quinine is recommended as a second treatment option. For severe *P. falciparum* malaria, the first-line treatment is intravenously (IV) or intramuscularly (IM) artesunate, IM artemether (as an alternative), or IV quinine infusion (if artesunate or artemether are not available) or IM quinine (if artesunate is not available) (FMoH, 2018).

Artemether is derived from the Chinese herb sweet wormwood (*Artemisia annua*) and has a half-life of about 1 hour. Artemether's antimalarial effects are derived from interfering with parasite transport proteins, impairing parasite mitochondrial function, preventing parasite angiogenesis, and modifying the host immune response after it is converted into its active metabolite, dihydroartemisinin (Golenser *et al*., 2006). Lumefantrine is an aryl-amino alcohol (Aweeka and

German, 2008) that prevents the detoxification of haem, such that toxic haem and free radicals induce parasite death (Kokwaro *et al*., 2007). It has a long elimination half-life of 3-6 days. Absorption is highly dependent on fat coadministration, and the predominant metabolic enzyme for both artemether and lumefantrine drugs is Cytochrome P450 3A (White *et al*., 1999). The most common adverse events associated with AL are gastrointestinal (vomiting and diarrhea) and hematologic (anemia and eosinophilia) (Falade *et al*., 2005).

Primaquine is an 8-aminoquinoline antimalarial drug; its oxidation, principally by Cytochrome P2D6 (CYP2D6), generates hydroxyl metabolites, whose oxidation to quinoneimine generates hydrogen peroxide (H_2O_2). Quinoneimines in turn are substrates for Cytochrome P450 Reductase, thus leading to H_2O_2 accumulation, which can exert anti-parasitic activity directly through oxidation of protein sulfhydryl groups and damage to iron and iron-sulfur containing proteins or the generation of superoxide and hydroxyl radicals (Camarda *et al*., 2019). Primaquine is highly active against mature gametocyte stages of *P. falciparum* malaria, and its elimination time is 3.5-8 hours (Lawpoolsri *et al*., 2009). Importantly, PQ, when used for radical cure, can cause life-threatening hemolysis in patients who are glucose-6-phosphate dehydrogenase (G6PD) deficient, but SLD-PQ for *P. falciparum* gametocidal effect is very tolerable even in G6PD deficient patients (Mwaiswelo *et al*., 2016; Tine *et al*., 2017).

Most antimalarials, including artemisinin, have incomplete activity against gametocytes (Kumar & Zheng, 1990; Price & Nosten, 1993). The transmission-reducing potential of the combination of ACT with the *P. falciparum* gametocytocidal drug, PQ, was a key factor in adopting SLD-PQ administration as the first-line *P. falciparum* treatment in Ethiopia (FMoH, 2018). Potent gametocytocidal drugs could help prevent the spread of antimalarial drug resistance (Azazy, 2014).

Resistance of *Plasmodium* species to artemisinin has been reported from eastern and southern Asian countries, which threatens malaria control and elimination efforts worldwide (Noedl *et al*., 2008; Ménard *et al*., 2016). The WHO advises routine monitoring of anti-malarial drug efficacy at least every two years in malaria-endemic countries to ensure good performance and detect the emergence of resistance to anti-malarial drugs, especially those used as first-line and second-line treatment (WHO, 2009).

1.2. Literature review

The burden of malaria is a significant public health issue, and programs to eradicate it are being hampered, particularly in developing countries, by the low efficacy of antimalarial medications. Therapeutic efficacy studies employed the clinical and parasitological efficacy of antimalarial drugs on *plasmodium* infections through defined follow-up criteria. It can be determined using parasitemia counts in blood films and by clinical assessments of malarial signs and symptoms. To decide on therapeutic efficacy studies, complete clearance of parasitemia and complete relief of the clinical signs and symptoms of the disease are criteria. Treatment failure rate or cure rate can be used to express the outcomes of therapeutic efficacy studies. The presence or absence of parasitemia and clinical signs and symptoms of malaria following the completion of a full course of treatment is the basis for the determination of efficacy study treatment outcomes.

A single-labeled clinical trial was conducted in Yombo, Bagamoyo district, Tanzania, between July and November 2014, to assess the safety and efficacy of AL plus SLD-PQ in uncomplicated *P. falciparum* malaria in 220 patients, 110 of whom were assigned to the AL + PQ arm and 110 to the AL-only treatment arm. Parasite clearance by microscopy was fast, but PCR-detectable parasitemia on day 3 was at 28.4% and 26.9% in patients treated with AL + PQ and AL, respectively. On day 28, the PCR-adjusted adequate clinical and parasitological response (ACPR) in AL + PQ was 100 % and for AL it was 99 %, and the re-infection rate was 4.7% and 4.8% in AL + PQ and AL arms, respectively (Mwaiswelo *et al*., 2016).

Additionally, between July 2017 and March 2018, another drug efficacy study was conducted in the Bagamoyo district of Tanzania on a total of 280 patients who were infected with uncomplicated *P. falciparum.* A standard 3-day treatment with AL (the control) was compared to an extended 6-day treatment with SLD-PQ (the intervention); in a randomized, controlled, parallel-group study, 141 patients were grouped in the control group and 139 in the intervention arm, of whom 121 completed 42 days of follow-up in each arm. There was no difference in the proportion of PCR positivity across the arms at day 5 (61.5% in AL+SLD-PQ vs. 66.4% in AL) or day 7 (55.0% in AL vs. 52.2% in AL+SLD-PQ). Day 42 microscopy determined that adjusted cure rates were 97.4% in the control arm and 98.3% in the intervention arm. Microscopy determined crude recurrent parasitemia during follow-up was 17.4% and 11.6% in the control

and intervention arms, respectively, and it took 34 days and 42 days in the respective arms for 90% of the patients to remain without recurrent parasitemia. Lumefantrine exposure was significantly higher in the intervention arm from D3 to D42, but cardiac, biochemical, and hematological safety was high and similar in both arms (Mhamilawa *et al*., 2020b).

Furthermore, in 2017, an open-label randomized trial was undertaken in adult patients presenting with malaria, which was conducted at the Deggo Health Post in Pikine, Dakar, Senegal. About 274 patients with ages > 18 years and *P. falciparum* malaria were randomized to receive 1 of 3 ACTs with or without PQ (0.25 mg/kg), 139 received an ACT alone, and 135 received an ACT plus PQ. The mean reduction in hemoglobin (Hb) on day 7 was similar in each group; there was a difference of -0.04 g/dL between the ACT + PQ group and the ACT alone group, but the effect of PQ differed according to G6PD status. In G6PD-deficient patients, the drop in Hb was 0.63 g/dL greater in those who received PQ than in those who received an ACT alone. In G6PD-normal patients, the reduction in Hb was 0.22 g/dL less in those who received PQ. One G6PD normal patient who received PQ developed moderately severe anemia (Hb <5g/dL). Dark urine was more frequent in patients who received PQ. Primaquine was associated with a 73% reduction in gametocyte carriage (Tine *et al*., 2017).

Additionally, a randomized, controlled, open-label trial was investigated in South Africa from 2017–2018 among 217 patients by adding SLD-PQ on day 3 to standard AL treatment for uncomplicated *falciparum* malaria. Efficacy, safety, and tolerability of AL and SLD-PQ treatments were assessed on days 3, 7, 14, 28, and 42. Lumefantrine concentrations were assayed from DBS samples collected on day 7. From the 217 patients screened, 166 were enrolled, with 140 randomly assigned on day 3, and 70 were allocated to each study arm (SLD-PQ and no SLD-PQ). No gametocytes were detected by either microscopy or PCR in any of the follow-up samples collected after randomization on day 3, precluding assessment of SLD-PQ efficacy. Day 7 AL concentrations and the number and nature of adverse events were similar between study arms; only one serious adverse event occurred (renal impairment in the no SLD-PQ arm). The PCR-corrected ACPR was 100% in AL, with only one re-infection found among the 128 patients who completed a 42-day follow-up (Raman *et al.*, 2019).

Moreover, from December 2011 to March 2013, a randomized, double-blind, placebo-controlled trial with four parallel groups was conducted in Jinja district, eastern Uganda. Four hundred sixty-eight patients with uncomplicated *P. falciparum* malaria and normal G6PD enzyme function were randomly allocated to receive AL, 119 participants were randomly grouped to receive AL combined with a placebo or with 0·1 mg/kg (116), 0·4 mg/kg (116), or 0·75 mg/kg (117) PQ base. The mean duration of gametocyte carriage was 6·6 days in the 0·75 mg/kg reference group, 6·3 days in the 0·4 mg/kg PQ group, 8·0 days in the 0·1 mg/kg PQ group, and 12·4 days in the placebo group. No children showed evidence of treatment-related hemolysis, and the mean maximum decrease in hemoglobin concentration was not associated with the dose of PQ received—it did not differ significantly compared with the placebo 10·7 g/L in the 0·1 mg/kg (11·4 g/L), 0·4 mg/kg (11·3 g/L), or 0·75 mg/kg (12·7 g/L) in the PQ groups (Eziefula *et al*., 2014).

Furthermore, in 2013, the Enfranz Health Center conducted a one-arm prospective evaluation of the clinical and parasitological response to first-line treatment for uncomplicated *P. falciparum* malaria in 80 patients. Patients were treated with a 3-day course of AL, and clinical and parasitological parameters were monitored over a 28-day follow-up. The PCR-corrected cure rate was 95.0% (95% CI: 87.0–98.4%), and there were two ETFs, one LCF, and three LPFs. Two of the LPFs were classified as reinfections by PCR. Seventy-three-point-seven-five percent of patients cleared parasitemia by day 1, 91.25% cleared by day 2, 95% of patients had cleared their parasitemia by day 3, and 75%, 91.25%, and 96.25% of patients had cleared their fever by days 1, 2, and 3, respectively. Gametocytes were completely cleared in all patients by day 7 (Getnet *et al*., 2015).

Also, from October 2014 to January 2015, another one-arm, prospective 28-day *in vivo* therapeutic efficacy study was undertaken in 91 uncomplicated *P. falciparum* malaria patients administered with a standard six-dose regimen of AL. Eighty study subjects completed the follow-up, and based on the per-protocol analysis, the unadjusted cure rate of AL in the study area was 98.8% (95% confidence interval: 93.3%–100%)of AL. Eighty study subjects completed the follow-up, and based on the per-protocol analysis, the unadjusted cure rate of AL in the study area was 98.8% (95% CI: 93.3%–100%). Eighty study subjects completed the follow-up, and based on the per-protocol analysis, the unadjusted cure rate of AL in the study area was 98.8%

(95% confidence interval: 93.3%–100%). The recurrence of one *P. falciparum* case was detected on day 28, with a late parasitological failure rate of 1.2%. No early treatment failures occurred. Complete parasite and fever clearance were observed on day 3. Gametocyte carriage was 4.4% at enrollment and cleared on day 21. Although the difference is statistically not significant, a slight increase in the level of mean Hb from baseline to day 28 was observed (Wudneh *et al*., 2016)

Furthermore, from October 28, 2014, and January 9, 2015, in Ethiopia, a single-arm prospective 28-day follow-up involving 92 patients was conducted in Setit Humera, Northwest Amhara, after administering a standard dose of AL twice a day for three consecutive days. The PCR uncorrected cure rate was 98.8%, with 100% of the study participants clearing parasitemia and fever on D3-PCR. Gametocyte carriage was reduced from 7% on D0 to 1% on D3, and complete clearance was achieved on D14. On D28, the mean hemoglobin concentration was significantly higher than on D14. There was no incidence of a serious adverse event in the study population (Teklemariam *et al*., 2017).

Besides, from April 2015 to February 2016, a prospective study was conducted at Koladiba Health Center in the Dembia district to determine the therapeutic efficacy and safety of AL for the treatment of uncomplicated *P. falciparum* mono-infection. A total of 80 patients enrolled in the AL efficacy study, and 75 patients completed the 28-day follow-up. None of the participants reported major adverse events. No Early treatment failure (ETF) or late clinical failure (LCF) was observed during the study, but there were six (8.0%) late parasitological failures (LPFs). The uncorrected per-protocol cure rate of AL was 92.0 (95% CI: 85.7–98.3). Treatment with AL cleared parasitemia and fever in 95% of the patients by day 3 (Deressa *et al*, 2017).

Additionally, a one-arm longitudinal study with uncomplicated *P. falciparum* malaria was conducted at Shecha Health Centre in Arba Minch town, Southern Ethiopia, from March 2021 to January 2022. Patients received a six-dose course of AL over three days, and they were then observed for 28 days with clinical and laboratory tests. Eighty-eight study subjects were recruited, and 69 of them had an adequate clinical and parasitological response. Two LPFs were observed, and one of them was identified by PCR as a recrudescence. The ACPR after PCR correction was 98.6% (92.3–100, 95% CI). The parasitemia cleared completely on day 2, and the fever cleared on day 3, demonstrating that AL had a rapid parasite and fever clearance with no

complications. Gametocytes were completely cleared by day three. No serious adverse events were reported throughout the 28-day follow-up (Gubae *et al*., 2023).

1.3. Statement of the problem

Selective application of vector control, early diagnosis, effective and prompt treatment, and forecasting of epidemics have resulted in a steady decline in the burden of malaria disease. However, malaria still poses a significant threat to public health.

The emergence of drug-resistant *Plasmodium* parasites has called into question the efficacy of anti-malarial drugs. As chemotherapy remains the mainstay, surveillance of therapeutic effectiveness over time becomes an essential component of malaria control and elimination efforts. The WHO recommends ACTs such as AL as first-line treatments for uncomplicated *P. falciparum* malaria in all malaria-endemic countries (WHO, 2021). However, *P. falciparum's* resistance to artemisinin has been confirmed in western Cambodia and Thailand (Noedl *et al*., 2008). This resistance has spread from the Thai–Cambodia border to the Greater Mekong region, up to the border of Myanmar and India (Dondorp *et al*., 2009; Ashley *et al*., 2014; Takala-Harrison *et al*., 2015; Tun *et al*., 2015). This resistance has the potential to spread to other parts of the world, including Ethiopia, and thus the efficacy of these drugs may slowly deteriorate over time.

The efficacy and safety of AL have been well-studied in Ethiopia (Tseha, 2011; Getnet *et al.*, 2015, Mohammed *et al*., 2016; Deressa *et al.*, 2017). However, there is a lack of studies on the efficacy and safety of AL plus SLD-PQ for the treatment of uncomplicated *P. falciparum* malaria in the country. Studies showed that AL can kill immature stages (I-IV) of *P. falciparum* gametocytes and significantly reduce gametocyte carriage (Premji, 2009; Makanga, 2014; Abamecha *et al*., 2020), and the effect of SLD-PQ in gametocidal effect is well known but their efficacy when administered together not well studied in Ethiopia, so this study aimed to evaluate the overall efficacy of AL plus SLD-PQ in the treatment of uncomplicated *P. falciparum* malaria in Ethiopia.

1.4. Significance of the Study

Treating *Plasmodium* parasites using appropriate antimalarial drugs that have good quality, efficacy, and safety is important to alleviate morbidity and mortality and also minimize the rate of transmission. Moreover, frequent administration of antimalarials in the treatment of malaria raises concerns about drugs that may lead to resistance to parasites. As a result, evaluating the therapeutic status of different antimalarial drugs could be crucial to providing evidence to replace the existing drug, which is widely used in a malaria treatment program, if it is found to be better.

Evaluating the efficacy of AL plus SLD-PQ thereby provided information for stakeholders and policymakers, for evidence-based treatment strategies and policies. It also provided information for clinicians for better treatment and management of uncomplicated *P. falciparum* malaria. The finding can also be used as baseline data for researchers to conduct further studies on the efficacy and safety of AL plus SLD-PQ in the treatment of uncomplicated *P. falciparum* malaria.

2. OBJECTIVES

2.1. General objective

To assess the therapeutic efficacy and safety of AL plus SLD-PQ for the treatment of uncomplicated *P. falciparum* infections at Maksegnit Health Center, Northwest Ethiopia from February to May 2022.

2.2. Specific Objectives

- To determine the cure rate of AL + SLD-PQ treatment in patients with uncomplicated *P. falciparum* malaria.
- To assess the possible incidences of adverse drug reactions of AL + SLD-PQ treatment in patients with uncomplicated *P. falciparum* malaria.
- To determine hemoglobin level in the blood before and after AL + SLD-PQ treatment in patients with uncomplicated *P. falciparum* malaria.

3. MATERIAL AND METHODS

3.1. Study Area

This study was conducted at Maksegnit Health Center, which is located in Central Gondar Zone, Amhara Regional State, 695 km, 137 km, and 40 km away from Addis Ababa, Bahir Dar, and Gondar town, Ethiopia, respectively. The area has the following latitude, longitude, and altitude: 12.3–13.8° N, 35.3-35.7° E, and 2,220 meters above sea level respectively. Maksegnit Health Center is the only health institute, providing healthcare services to the Maksegnit town community. The average temperature ranges from 15 °C to 31 °C with two distinct rainy seasons (June to August peak rainfall season and March to May minor rainy season). According to the Amhara Regional Health Bureau report, Maksegnit is known for a recurring outbreak of malaria cases after the main rainy season (Source: Gondar Zuria Woreda Health Office) (Figure 1).

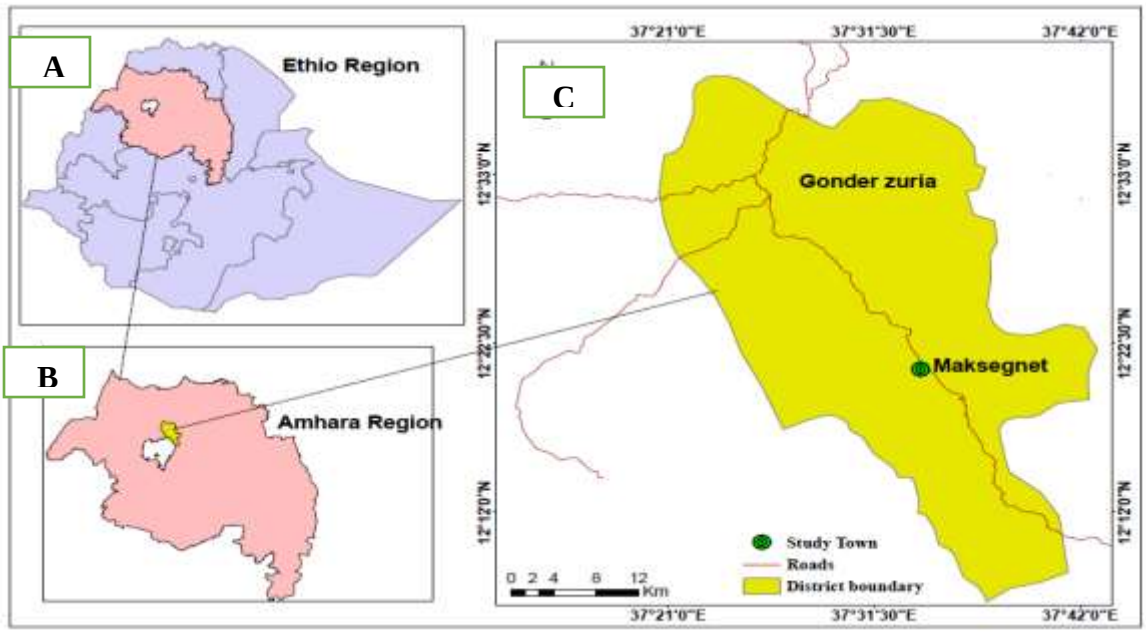

Figure 1. Study area map, Ethiopian map (Image **A**); Amhara Region map (Image **B**); Gondar Zuria district and Maksegnit town map (Image **C**).

3.2. Study design and period

An *in-vivo* prospective single-arm evaluation of the clinical, parasitological, and hematological responses of AL plus SLD-PQ for the treatment of uncomplicated *P. falciparum* malaria. This study was conducted from February to May 2022.

3.3. Source population

All malaria-suspected individuals attending Maksegnit Health Center during the study period

3.4. Study population

Patients with confirmed uncomplicated *P. falciparum* malaria mono-infection attending Maksegnit health center during the study period

3.5. Study Participants

All uncomplicated *P. falciparum* mono-infected patients whose age ≥18, came to Maksegnit Health Center during the data collection period, fulfilled the inclusion criteria, and gave consent to participate were included in the study.

3.6. Sample size determination

The study was conducted based on the revised WHO recommendation for the assessment and monitoring of antimalarial drug efficacy (WHO, 2009). According to the WHO protocol for estimating the population proportion, the sample size (n) is determined using the single population proportion formula, whereby treatment failure of AL is assumed to be 5% with a confidence level of 95% and a precision around the estimate of 5%. Accordingly, the following formula is used to calculate the number of study subjects: where "P" stands for the expected treatment failure, "d" for the margin of error (precision), and "Z" for the Z statistic at a 95% confidence interval. For a level of confidence of 95%, the Z value is 1.96. With a 20% increase to allow a loss to follow-up and withdrawals during the 42-day follow-up period, the sample size can be calculated by substituting the given values into the above formula.

$n = \frac{1.96^2}{0.05^2} * 0.05(1 - 0.05) = 1536.64 * 0.05 * 0.95 = 72.9904 + (0.2 * 72.9904) = 87.58848 \approx$ 88 study subjects.

3.7. Sampling technique

The health center was selected purposefully, and a convenient sampling technique was used to recruit study participants until the sample size was reached.

3.8. Study variables

3.8.1. Dependent variable

Therapeutic efficacy and safety of AL plus SLD-PQ

3.8.2. Independent variables

Independent variables include: age, sex, baseline clinical signs and symptoms, baseline hemoglobin level, baseline parasite density, and baseline axillary temperature

3.9. Inclusion and Exclusion criteria

3.9.1. Inclusion criteria

The study was carried out following the WHO's 2009 revised protocol for therapeutic efficacy studies on antimalaria drugs (WHO, 2009). The following selection criteria were run on patients who attended the health center with malaria signs and symptoms.

1. Patients living within the health center catchment area
2. Both sexes ≥18 years of age
3. Non-pregnant or non-breast-feeding women
4. Slide-confirmed with *falciparum* infection and parasitemia of 1000–100,000 asexual parasites/μl
5. Axillary temperature ≥37.5 ºC or history of fever during the previous 24 hours
6. Ability to swallow oral medication

3.9.2. Exclusion criteria

1. General danger signs or symptoms of severe malaria (Annex III)

2. Severe anemia, defined as Hb <5 g/dl
3. The presence of febrile conditions caused by diseases other than malaria (e.g., measles, acute lower respiratory tract infection, severe diarrhea with dehydration)
4. Known serious or chronic medical conditions (e.g., cardiac, renal, hepatic diseases, sickle cell disease, HIV/AIDS)
5. Presence of severe malnutrition (BMI 16.5 kg/m^2)
6. History of hypersensitivity to AL and PQ
7. Taking regular medication, which may interfere with AL and PQ (Annex IV)

3.10. Lost to follow-up

Loss to follow-up occurs when, despite all reasonable efforts, an enrolled patient did not attend the scheduled visits and cannot be found within the allowed time interval. No treatment outcome was assigned to these patients. Every effort was made to schedule a follow-up visit for patients who failed to return to the study site, especially after the administration of the study drug. If these patients cannot be found, they are classified as lost to follow-up and censored or excluded from the analysis.

3.11. Patient discontinuation or protocol violation

Study participants who meet any of the following criteria were classified as the following:

- ✓ **Enrolment violation:**

When screened patients fulfilled the following criteria, they were excluded from enrollment.

- ➢ Severe malaria on day zero
- ➢ Erroneous inclusion of a patient who did not meet the inclusion criteria.

- ✓ **Withdrawal of consent**- a patient might withdraw consent at any time, without prejudice for further follow-up or treatment at the study site.
- ✓ **Failure to complete treatment**, due to:
  - ➢ persistent vomiting during the treatment. A patient who vomits the study medication twice was withdrawn from the study and given a rescue treatment.
  - ➢ Failure to attend the scheduled visits during the first 3 days; or
  - ➢ Serious adverse events necessitate termination of treatment before the full course is completed. A patient was discontinued from the study due to an AE of adequate nature or

intensity. In this case, information on the AE and symptomatic treatment given must be recorded on a CRF. If the AE was serious, it was investigated well by the study team.

Voluntary protocol violation

- Self- or third-party administration of the antimalarial drug (or antibiotics with antimalarial activity) (Annex IV)

Involuntary protocol violation

- Occurrence during follow-up of concomitant disease that would interfere with a clear classification of the treatment outcome;
- Detection of a mono-infection with another malaria species during follow-up and
- Misclassification of a patient due to a laboratory error (parasitemia), leads to the administration of rescue treatment.

3.12. Treatment and dosing procedure

3.12.1. Study medication and dosing procedure

The correct drug dose was determined according to the national malaria treatment guidelines of Ethiopia (FMoH, 2018) (Annex V). Accordingly, enrolled patients were treated with the standard AL (20 mg/120 mg) (manufacturer: IPCA Laboratories Ltd., batch: HWE 110621, expiration date: 11/2023) and SLD-PQ (0.25 mg/kg) (manufacturer: Remedica Ltd., Cyprus, lot number: 92154, expiration date: 01/2025). Primaquine was administered once or stat after enrollment, and AL was given twice a day for three consecutive days with a milk biscuit to ensure good absorption in the health center with direct supervision, specifically the initial and each morning dose, and the night dose was taken by the patient himself or herself after deep advice by qualified clinicians.

On follow-up days 1-3, patients were asked again if the drug was taken appropriately the previous night. Any patient who vomited during the observation period was re-treated with the same dose of medicine and monitored for another 30 minutes. If the patient vomits again, he or she is withdrawn and offered rescue therapy.

3.12.2. Concomitant treatment and medication

Paracetamol with a standard dose of 10 mg per kilogram was given to all patients with axillary temperature ≥37.5°C, every 6 hours until the symptoms subsided (WHO, 2009). Patients who encountered illnesses other than malaria during a 42-day follow-up received standard care and treatment in the clinic free of charge. Routine use of non-study medications with antimalarial activity (Annex IV) was avoided. The patient with treatment failure received oral quinine at 10 mg/kg three times a day for seven days, per national antimalarial treatment guidelines (FMoH, 2018).

3.13. Baseline Evaluation

Potential participants were further evaluated for compliance with the remaining inclusion criteria. Baseline physical, and clinical examinations, with particular attention to any danger signs or symptoms associated with severe malaria, were thoroughly assessed by a clinician (Annex III). History and demographic data were taken; axillary temperature and body weight were measured. Patients who meet the inclusion criteria at this stage were assigned a patient identification number and referred to the laboratory again for further laboratory investigation and sample collection. Before the patient enrolled and was treated with an antimalarial drug, a finger-prick blood sample was collected for repeated thick and thin blood smears. Dried blood spots (DBS), containing three drops of 20 µL each on filter paper, were also prepared for all participants on the day of enrollment. Hemoglobin was also determined to rule out severe anemia (Hb <5g/dl) for exclusion and to compare with days 14, 28, and 42 of follow-up.

3.14. Study endpoints

Study endpoints are the classifications assigned to a patient. Valid study endpoints include treatment failure (early treatment failure, late clinical failure, and late parasitological failure), completion of the follow-up period without treatment failure (adequate clinical and parasitological response), loss to follow-up, or withdrawal from the study, including protocol violation.

Results were classified into primary and secondary outcomes:

The primary outcome: was the day 42 overall efficacy of AL plus SLD-PQ or cure rate expressed as the PCR uncorrected and corrected cumulative success rate (or the cumulative failure rate) and the proportion of adequate clinical and parasitological response (or the proportion of early treatment failure, late clinical failure, or late parasitological failure). The primary outcome was analyzed using per-protocol and the Kaplan-Meier survival estimator analysis methods.

The secondary outcomes: Parasite clearance rate; the proportion of patients with a negative blood smear on Day 1, Day 2, and Day 3. Fever clearance rate: Proportion of patients without fever (axillary temperature <37.5^{o}c) on day 1, day 2, and day 3. Hematological recovery: Change in mean blood Hb concentration from day 0 to day 14, day 28, and day 42. At all times, the well-being of the patient took priority over his or her continuation in the study (WHO, 2009).

3.15. Classification of Treatment outcomes

Treatment outcomes were classified based on an assessment of the parasitological and clinical outcomes of antimalarial treatment according to the latest WHO guidelines. Thus, all patients were classified as having one of the following:

Early treatment failure: Danger signs or severe malaria on day 1, 2, or 3, in the presence of parasitemia; parasitemia on day 2 higher than on day 0, irrespective of axillary temperature; parasitemia on day 3 with axillary temperature ≥ 37.5 °C; and parasitemia on day 3 ≥ 25% of count on day 0.

Late clinical failure: Danger signs or severe malaria in the presence of parasitemia on any day between day 4 and day 42 in patients who did not previously meet any of the criteria of early treatment failure; and presence of parasitemia on any day between day 4 and day 42 with axillary temperature ≥ 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure.

Late parasitological failure: Presence of parasitemia on any day between day 7 and day 42 with axillary temperature: < 37.5^{o}c in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response: The absence of parasitemia on day 42, irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure, or late parasitological failure.

3.16. Enrolment and Follow-up protocol

The health center clinicians, laboratory professionals, and principal investigator at the site of enrolment checked:

1. Clinical malaria and make a microscopic confirmation from a finger prick
    a) Febrile illnesses other than malaria; including but not limited to pneumonia, otitis media, tonsillitis, measles, chicken pox, abscesses, and COVID-19
    b) Severe disease/ presence of any danger signs, as indicated in the exclusion criteria. NB: Volunteers found to have any of these conditions were treated as per the appropriate national guidelines, and if deemed necessary were sent to the next referral level in the health system.
    c) For volunteering, for women of childbearing age (15-49 years) (WHO, 2006) or who are menstruating; a urine pregnancy test was conducted and if tested positive they were excluded.
    d) Hemoglobin measurement (Hemocue™ Angelholm, Sweden) was carried out and those with Hb<5 g/dL were excluded.
    e) A blood glucose test was done by glucometer (Gold AQ Plus brand) and those with 40mg/dl were excluded
    f) If the volunteer fulfills the inclusion criteria
        I. The screening case reporting forms (CRFs) were completed.
        II. Their appointment schedule was clearly explained, and a follow-up card with a personal identification number was provided (Annex II)
2. Those enrolled, confirmed *P. falciparum* cases, had a finger prick blood sample (250 µL) collected in EDTA-coated microtainer tubes collected on days 0 and follow-up dates assessed for the following:
    a) A thin smear was used to verify parasite species and to conduct a formal parasite count
    b) Hemoglobin measurement (Hemocue™ Angelholm, Sweden) was carried out and that hemoglobin was recorded.
    c) DBS samples prepared for molecular identification and genotyping
3. Follow-up clinical reassessments were made on days 1, 2, 3, 7, 14, 21, 28, 35, 42, and any unscheduled date (Annex II). Participants should also return to the health facility if they have

chronic fatigue, abdominal pain, nausea and vomiting, and/or notice a change in skin and/or eye color.

4. If participants did not appear for scheduled follow-up, the study team attempted to reach them at home. For follow-up visits on days 1 and 2, while the study drugs are being administered, the study staff attempted to reach the patient by phone and by communicating with health extension workers at the site in addition to the home visit on that day. After day 2, those who failed to return on their scheduled day but return one day early or one day late may still be included in the analysis. After day 2, an attempt was made to reach them on the day of their missed visit and again the next day. After day 3, if failed to return on their scheduled day but return one day early or one day late may still be included in the analysis.

3.17. Clinical evaluation

Clinical assessment was done on enrolment or day 0 and the scheduled visit dates: were 1, 2, 3, 7, 14, 21, 28, 35, and 42.

3.17.1. Physical Examination

A standard physical examination was performed at baseline (day 0 before dosing) and on days 1, 2, 3, 7, 14, 21, 28, 42, and any unscheduled date. A complete medical history, including prior and concomitant medication, demographic information, and contact details were recorded at baseline.

3.17.2. Body weight

Body weight was recorded on day 0 to the nearest kilogram scales. The reliability of the scales was verified before the study begins and checked at regular intervals. Patients were not wearing; excessive clothing while being weighed. The screening weight was used to satisfy inclusion or exclusion criteria as well as to calculate the dose (number of tablets) to be administered.

3.17.3. Body temperature

Axillary temperature was measured at baseline and on follow-up days (1, 2, 3, 7, 14, 21, 28, 42, and any unscheduled date). The temperature was measured with a digital thermometer that has a

precision of 0.1°C. In case the result was less than 36 °C the measurement was repeated. The same route was used throughout the study. The quality of the temperature-taking technique and the thermometer was assessed regularly. A thermometer was tested in a water bath of known temperature before the study begins.

3.18. Data Collection Methods

3.18.1. Questionnaire data

Structured questionnaires were used to collect socio-demographic data, clinical signs, and symptoms, information related to the drug administered, etc. Patient screening and clinical record forms were used during enrollment and follow-up days (Annexes VIII–X). Trained health professionals at Maksegnit Health were engaged in questionnaire-based data collection in addition to the principal investigator.

3.18.2. Laboratory Procedures

Blood Sample collection: At baseline or day 0 and follow-up days (1, 2, 3, 7, 14, 21, 28, 35, 42, and any unscheduled date) 250 µL, of capillary blood was collected using the finger prick method and labeled anonymously (study number and day of follow-up). Thick and thin blood films were stained with Giemsa stain and examined microscopically to identify the parasite species, stage, and density. Capillary blood collected in an EDTA-coated microtainer tube was also used for the measurement of the level of hemoglobin at 0, 14, 28, and 42 follow-up days. Dried blood spots, containing three drops of 20µL each on filter paper (Tadesse, 2021), were also prepared on the day of enrollment and the day of treatment failure to differentiate recurrent malaria as re-infection and recrudescence from collected capillary blood.

Microscopic Blood Film Examination: Thick and thin blood smears were prepared on the same slide for the detection of parasites at all visits during the 42-day follow-up period. Two smears were prepared per participant; the first was stained immediately with 10% Giemsa for 10 minutes for initial screening and examined by light microscopy immediately. On day 0, to rapidly confirm adherence to the lowest parasite density considered for enrollment, initial screening of patients was made in 10% Giemsa-stained thick film after counting at least one parasite for every

6 White Blood cells (WBCs), which corresponds to approximately 1000 parasites/µL (WHO, 2009). The principal investigator (PI) and other lab staff examined the slides, and the parasite density was determined per the WHO revised protocol (WHO, 2009). The second slide was stained slowly with 3% Giemsa for 45 minutes and examined later to provide a definitive parasite count.

The parasite density per µL blood was calculated as follows.

$$\text{Parasite density (per µl)} = \frac{Number\ of\ parasites\ counted\ X\ 8000}{Number\ of\ leukocytes\ counted\ (approximately\ 200)}$$

Thin blood smears were examined to determine parasite species. Parasite densities were calculated from thick blood smears by counting the number of asexual parasites against 200 WBC. When the count < 10 parasites/200 WBC, at least 500 WBC were counted. A thick blood smear was declared negative when no parasite was detected in 100 high-power fields. Gametocytes were detected and counted in the thick film against 1000 WBCs. A thick blood smear was declared gametocyte negative when no parasite was detected in 1000 WBCs (WHO, 2009).

Molecular Tests:

DNA Extraction: DBS, containing three drops of 20µl each on filter paper, were prepared on the day of enrollment and the day of treatment failure to extract genomic DNA using 6 mm punches after an overnight treatment in Proteinase K (Qiagen) and Tissue Lysis Buffer (Roche Applied Sciences) at 56 ^{o}C using commercial DNA extraction kits by strictly following the manufacturer's protocol (Tadesse, 2021) (Annex XIII).

Genotyping of recurrent *P. falciparum* cases: The *P. falciparum* Merozoite Surface Protein-2 (*msp*-2) gene was amplified by multiplex primary and nested PCR (nPCR) using allele-specific primers as described elsewhere (Foley *et al*., 1992). Allelic variants of *msp-2* (FC27 and 3D7) were discovered using allelic family-specific nPCR. Amplicons were loaded on 2% agarose gel containing GelRed™ (Biotium, Inc. Hayward, California, USA), separated by electrophoresis, and then visualized under ultraviolet-transillumination (Gel Doc™ (Bio-Rad, Hercules, California, USA). The fluorescent dye (which indicates the allelic family) and the size, were used to distinguish genotypes. In the case of parasitemia detected on or after day 7, PCR

genotyping was performed on paired whole blood to differentiate between recrudescence and reinfection for all treatment failures (Annex XIII).

The WHO protocol for genotyping *P. falciparum* polymorphic gene *msp-2* was followed (Felger and Snounou, 2008; WHO, 2008). Based on the degree of allelic matching, recurrent parasites can be classified into four categories: If, (i) all alleles in the baseline and recurrent parasite samples are identical; alleles were considered the same if molecular weights were within 20 bp for *msp-2* (Mwaiswelo *et al*., 2022) (ii) some alleles are missing in the recurrent parasites; (iii) recurrent parasites contain alleles identical to those at baseline plus additional/new ones not seen at baseline; and (iv) alleles in the baseline and recurrent parasite samples are different. It is widely assumed that categories (i-iii) represent recrudescent infections and categories (iv) represent new infections (Mugittu *et al*., 2006); Juliano *et al*., 2010). Molecular testing was conducted at the AHRI laboratory in Addis Ababa, Ethiopia.

Measurement of blood Hemoglobin level: Hemoglobin concentrations were measured using a portable spectrophotometer (HemoCue®, Angelholm, Sweden) on days 0, 14, 28, and 42 from the finger-prick capillary blood sample. Hemoglobin was measured to observe improvements after treatment and classify the level of anemia. Anemia was defined according to the WHO classification (Hb=10-12.9 g/dl) as mildly anemic; 8-10.9 g/dl moderately anemic; Hb<5 g/dl was considered as severe anemia) (WHO, 2009; WHO, 2011).

3.19. Safety Assessment

Safety was assessed by recording the nature and incidence of adverse events and serious adverse events. An adverse event was defined as a sign and symptom of abnormal laboratory findings not presented at enrollment but occurring during follow-up, or presented at day 0 and increased in intensity during follow-up despite clearance of parasitemia. Serious adverse events were defined as any adverse experience that resulted in death, a life-threatening experience, participant hospitalization, persistent or significant disability or incapacity, or specific medical or surgical intervention to prevent a serious outcome (WHO, 2009). Clinicians evaluated adverse events through clinical and physical examinations as well as by direct questioning. At all times, all demographic and clinical information was recorded on a standard record form.

3.20. Data quality

Appropriate training was given to data collectors to help the PI during data collection time, and their progress was monitored by the PI early in the trial to assess the quality of enrollment and documentation practices. The questionnaire and all necessary materials were checked before the actual data collection. Blood film examination and hemoglobin test were performed on the same day of collection to maintain the reliability of the study findings, and 10% of slides were randomly selected for quality control and re-examined by other laboratory personnel who were blind to previous results. Every discordant result was checked by the principal investigator to see if there was a real discordant and for molecular tests, the AHRI laboratory team including the principal investigator discuss and arrived at the solution if there were any non-conformity. Samples that were not examined at the field health center were stored per standard and transported as soon as possible to the AHRI laboratory. The quality of the reagents was checked by running known positive and negative samples, checking the expiration date and lot number, preparing reagents with an exact ratio from stock, and storing the reagents under proper storage conditions.

Auditors from AHRI and the principal investigator were adapting the common audit checklist and incorporating local requirements as well. The systems used to assure the quality of every aspect of the study were focused on subject protection and reliability. Generally, the data quality was assured during the pre-analytical (sample collection, reagent preparation, etc. and analytical (smear microscopy, Hb, PCR, etc.), and post-analytical (record completeness, and result interpretation, etc.) phases.

3.21. Data analysis and interpretation

First, every piece of data was entered into Redcap data collection software, then the data were cleaned and validated, then analyzed using SPSS version 25 and the WHO drug efficacy tools. The per-protocol (PP) analyses excluded patients who were withdrawn or lost to follow-up from the study and the Kaplan-Meier (K-M) survival analysis included all enrolled patients in the analysis until the last day before drop-out or upon reaching an outcome. In addition to voluntary withdrawals or treatment non-compliance, patients were considered for the analysis as withdrawn if the PCR results are unclassifiable.

- ✓ The final analysis included:
    - A description of the patients included in the study.
    - The proportion of patients lost to follow-up or withdrawn, and a list of reasons for withdrawal.
    - The proportion of adverse events and serious adverse events in all the patients included in the study
    - The proportion of ETF, LCF, LPF, and ACPR in the *P. falciparum* at day 42 with 95% confidence intervals with and without PCR correction.
    - Frequency of severe malaria, anemia, and hospitalizations
    - Asexual parasite and gametocyte clearance rates: proportion of patients with negative thick blood smears on days 1, 2, 3.
    - Gametocyte carriage/clearance rates: proportion of patients with gametocytes on follow-up days
    - Hematological recovery: Paired sample t-test was used to compare mean Hb level, between Day 0 and day 14, day 0 and day 28, day 0 and day 42, day 14 and day 28, day 14 and day 42, day 28 and day 42.
    - Prevalence of mild and moderate anemia was assessed at day 0, day 14, day 28, and day 42.
    - All comparisons were performed at 95% CI and a significant level of 0.05.

3.22. Ethical consideration

Ethical clearances were obtained from the ethical review board of the College of Medicine and Health Sciences, Bahir Dar University, the Institutional Review Board (IRB) (protocol number **383/2022**), and the AHRI/ALERT ethics review committee (protocol number **PO/23/20**). In addition, a permission letter was collected from Amhara Public Health Institute, Central Gondar Zonal Health Department, Gondar Zuria Woreda Health Office, and Maksegnit Health Center to perform data collection.

The investigator explained the study to each potential subject verbally, provided all the information (purpose, procedures, risks, benefits, alternatives to participation, etc.), and gave time for any queries. The potential subject was provided with a written informed consent form

and sufficient time was given to read it or have been read for them. Once an individual had all his/her questions answered and agreed to participate in the study informed consent was taken before inclusion in the study. Confidentiality of information and freedom to withdraw from the study at any time were guaranteed. In addition, study subjects were reimbursed for transportation costs during the 42-day follow-up period by the International Center for AIDS Care and Treatment Programmes/Columbia University (ICAP).

4. RESULTS

4.1. Characteristics of the study participants

4.1.1. Socio-Demographic Characteristics

A total of 4800 suspected malaria patients were screened, and 63% of them were men. About 10.5% (504/4800) of the participants were found to be slide-positive for malaria, of which 69% (348/504) of the cases were attributed to *P. falciparum.* Seventy-one-point-three percent (71.3%) (248/348) of the *P. falciparum* mono-infection cases did not meet the inclusion criteria, and 3.5% (12/348) refused to be included in the study.

The study enrolled a total of 88 consenting patients who met the inclusion criteria. Of the total 88 participants, 60 (68.2%) were male and 28 (31.8%) were female. The median age of the patients was 28 years, ranging from 18 to 63 years. About 11.4% (10/88) of the participants had access to an insecticide-treated bed net (ITN), with a proper bed net utilization rate of 80% (8/10), and none of the study participants had used indoor residual spraying (IRS) in the previous 12 months. Three participants (3.4%) were lost to follow-up on days 21, 28, and 42. As a result, the total number of patients per protocol was 85 (Figure 2).

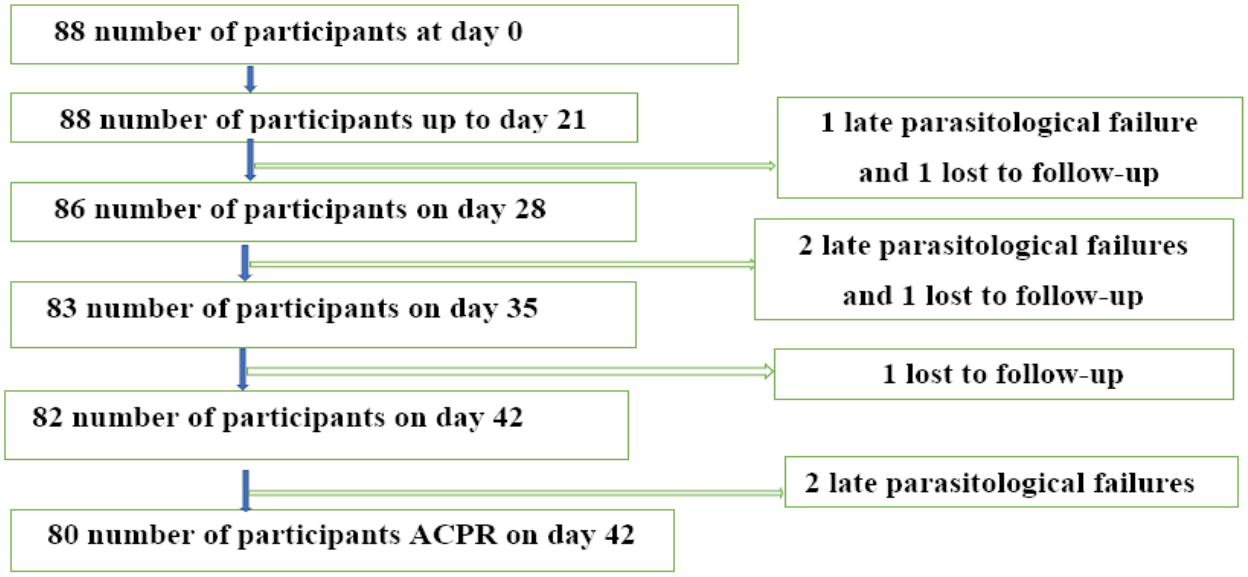

Figure 2. Study participants' flow chart during the 42-day follow-up period at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

4.1.2. Baseline Clinical Characteristics

From the total 88 enrolled participants, 51 patients had ≥ 37.5 ^{o}C axillary temperature at the time of enrollment with a mean ±SD of 38.4 ^{o}C (±0.7). The remaining 37 patients had <37.5^{o}C axillary temperature during enrollment, but they were recruited since they had a history of fever within the last 24 hours, according to the WHO 2009 protocol. Headache, fever, and body aches were the major clinical signs and symptoms reported on day 0, of which headache was the most common (93.2%). The patients' mean weight and height at day 0, were 52.1 kg and 165.8 cm, respectively. The geometric mean of the asexual-blood-stage parasite load at baseline was 6300 /µL. The number of patients who had gametocytaemia at baseline was eight (9.1%). The study participants' baseline hemoglobin level was 14.2±2.07 g/dl (Table 1).

Table 1. Baseline clinical characteristics of the study participants at Maksegnit Health Center, northwest Ethiopia, from February to May 2022

Variables	Value
Mean Body weight (Range) in kg	52.1 (39-67)
Geometric mean Parasite count (Range) per µl	6300 (1000-76850)
Gametocyte carriage, n (%)	9.1% (8/88)

Axillary temperature in ^{o}C, mean (±SD)	38.4 (±0.7)
Hg (g/dl), mean (±SD)	14.2 (±2.07)
Number of patients who had household ITNs	11.4% (10/88)
Proper bed net utilization rate	80% (8/10)
The number of study participants who did not access the IRS in the last 12 months	100%

4.2. The cure rate of AL plus SLD-PQ

The PP-PCR-uncorrected cure rate of AL plus SLD-PQ among study participants was 94.1% (80/85) (95% CI: 86.8–98.1); while the PP-PCR-corrected cure rate was 96.4% (80/83) (95% CI: 89.8–99.2). Five LPFs were observed (one case on day 21, two cases on day 28, and the rest on day 42), so the PCR-uncorrected failure rate was 5.9% (5/85) (95% CI: 1.9–13.2); however, after PCR correction, three of the LPFs were confirmed as recrudescent cases, resulting in a failure rate of 3.6% (3/83) (95% CI: 0.8–10.2) (Table 2).

Table 2. Summary of treatment outcomes among *P. falciparum* malaria patients who were treated with AL plus SLD-PQ at Maksegnit Health Center, Northwest Ethiopia, 2022

Treatment outcomes	**n (%)**
ETF	0 (0.0%)
LCF	0 (0.0%)
LPF without PCR correction	5.9% (5/85) (95% CI 1.9-13.2)
LPF with PCR correction	3.6% (3/83) (95% CI 0.8-10.2)
PP PCR-uncorrected cure rate	94.1% (80/85) (95% CI 86.8 – 98.1)
PP PCR-corrected cure rate	96.4% (80/83) (95% CI 89.8-99.2)
K-M PCR-uncorrected cure rate	94.2% (95% CI: 86.7–97.6)
K-M PCR-corrected cure rate	96.5% (95% CI: 89.5–98.9)

ACPR = Adequate clinical and parasitological response, ETF = Early treatment failure, LCF = Late clinical failure, LPF = Late parasitological failure, n = number, PP = per protocol

Based on PCR-uncorrected K-M survival estimates, the cumulative incidence of AL plus SLD-PQ success without PCR correction was 94.2% (95% CI: 86.7–97.6) and with PCR correction, 96.5% (95% CI: 89.5–98.9). The cumulative incidence of AL plus SLD-PQ failure without and with PCR correction was 5.8% (95% CI: 2.4–13.3) and 3.5% (95% CI: 1.1–10.5), respectively (Figures 3 and 4).

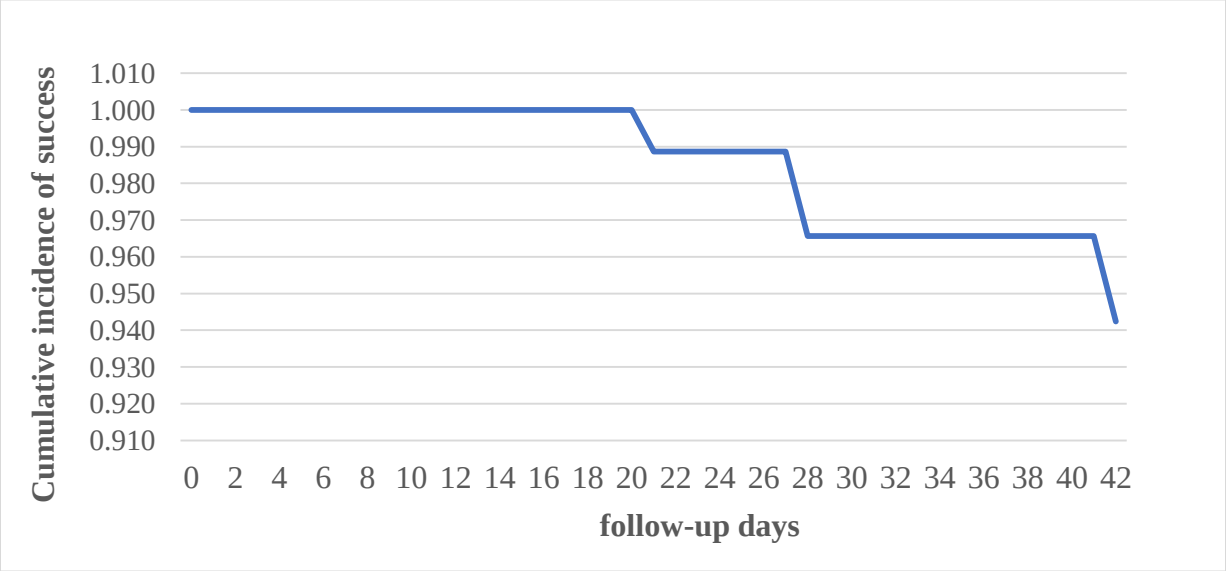

Figure 3. PCR-uncorrected Kaplan–Meier survival curve following AL plus SLD–PQ treatment of uncomplicated *P. falciparum* malaria patients at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

Figure 4. PCR uncorrected K-M survival curve following AL plus SLD-PQ treatment of uncomplicated *P. falciparum* malaria patients at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

4.3. Asexual Parasite clearance

The geometric mean parasitemia of study participants on day 0 or before drug administration was 6300 (1000- 76850)/µL. Fifty-one point-one percent (51.1%) (45/88), 91% (80/88), and 99% (87/88) of patients cleared their parasitemia on days 1, 2, and 3, respectively. The median parasitemia declined from 7,340 on day 0 to 660 on day 1, 330 on day 2, 120 on day 3, and 0 afterward (Figure 5).

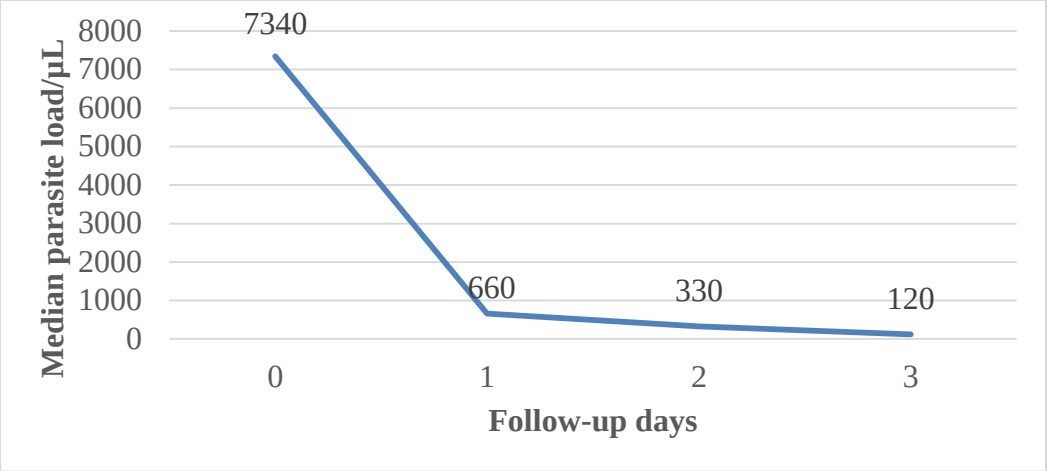

Figure 5. Graphic presentation of median parasite density from baseline to the three follow-up days at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

4.4. Gametocyte Carriage

Gametocytes were found in 9.1% (8/88) of the study participants' blood. On day 1, 62.5% (5/8) of participants cleared their gametocytaemia, and on day two, 75% (6/8) cleared it. On day 3, 100% of patients cleared gametocytaemia. The gametocyte carriage declined from 8 on day 0 to 3 on day 1, 2 on day 2, and 0 on day 3 (Figure 6).

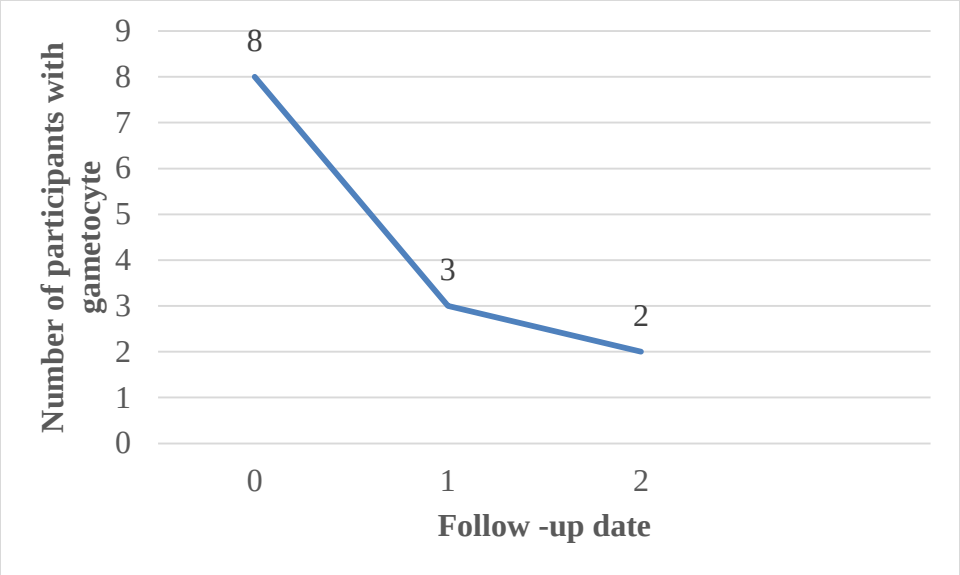

Figure 6. The number of participants who have microscopically detected gametocytes on each follow-up day at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022.

4.5. Fever clearance

Participants were enrolled if they had an axillary temperature of ≥37.5 °C and a self-reported history of fever within the last 24 hours, even if they may have had <37.5 °C. However, to determine the fever clearance rate across follow-up days, we only looked at patients who had an axillary temperature of ≥37.5 °C at baseline. Fifty-one patients had an axillary temperature of ≥37.5°C, with mean axillary temperature of 38.4°C (±0.7). Only three patients 5.9% (3/51) had an axillary temperature of ≥37.5 °C on day one, meaning 94.1% (48/51) were fever-free on day 1. On day 2, all of the study participants' fevers had subsided, with a fever clearance rate of 100%, and no febrile cases were detected afterward. The mean axillary temperature of participants declined from 38.4^{o}c on day 0 (38.4^{o}c) to, 35.8% on day 1, 35.5% on day 2, and 35.4^{o}c on day 3 (Figure 7).

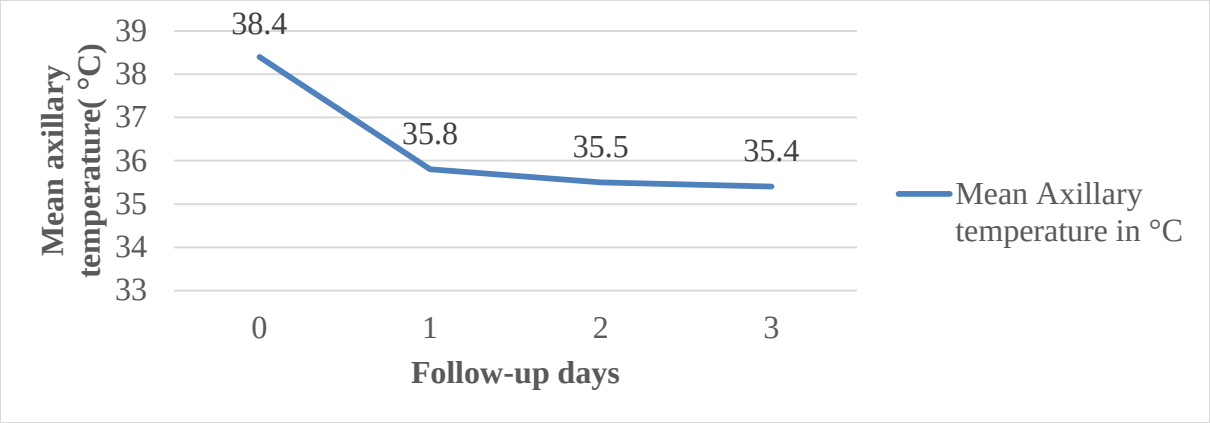

Figure 7. The Mean axillary temperature on the first three follow-up days, at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

4.6. Recurrent malaria

Five study participants developed recurrent parasitemia between days 21 and 42 follow-up days. All treatment failures observed were fever free on the day of presentation but three of them had a history of fever on the previous day. All received quinine (2^{nd}-line treatment) at the Health Center since the cases were uncomplicated *P. falciparum* malaria. Patients 2, 3, and 4 are classified as recrudescent because they share at least one common allele within 20 base pair differences. Two of the recurrent parasites were different parasite strains from those detected on day 0, so the results were classified as re-infection (Figure 8 and Table 5).

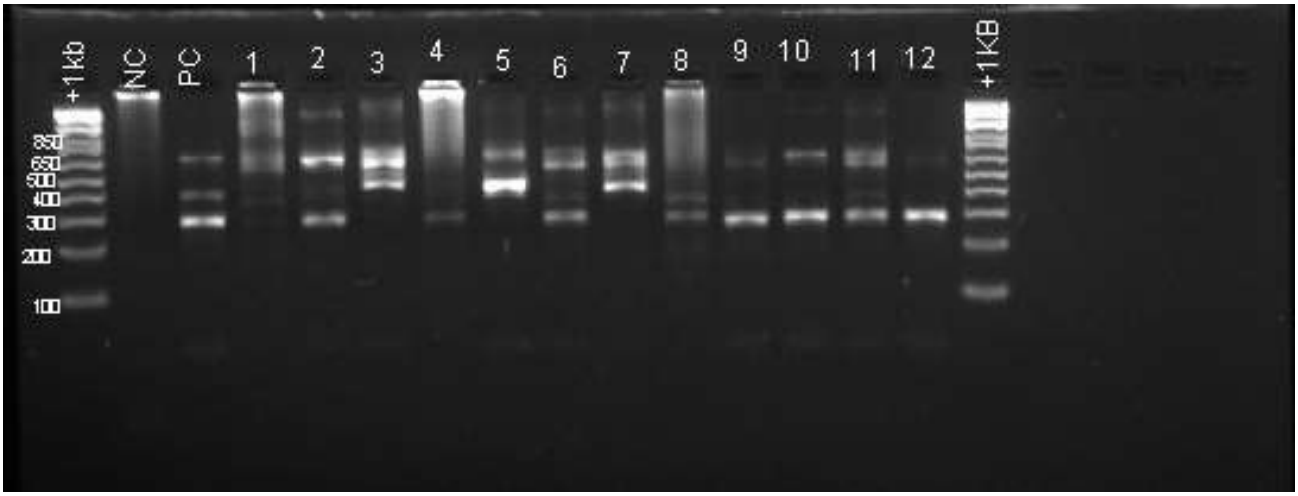

Figure 8. *msp2* gene PCR products run on a 2% agarose gel on recurrent cases of uncomplicated *P. falciparum* malaria at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

Footnote: The numbers in the bracket are day 0 and treatment failures, (1, 7), (2, 8), (3, 9), (4, 10), (5, 11), respectively, and NC is a negative control, PC is a positive control (NF54), and (6, 12) are other *P. falciparum* day 0 and treatment failure positive samples used as an additional positive control. PCR bands lie between 300 and 700 bps.

Table 3. Amplicon sizing (bp) of msp-2 for patients presenting recurrent *P. falciparum* infection on day 0 and the day of recurrence, at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

Patients with treatment failure	***msp-2* (bp) on Day 0**	***msp-2* (bp) on the day of recurrence**
Patient 1	400, 660	500, 680
Patient 2	300, 450, 650	300, 400
Patient 3	490, 650	300, 660
Patient 4	300	300, 700
Patient 5	490, 700	300, 400, 660

Footnote: *msp-2* = Merozoite surface protein-2, bp = Base pair

4.7. Hemoglobin Determination

The mean Hb level showed a slight decline from day 0 (14.2 ±2.07) to day 14 (13.96 ±1.47 g/dl), but not a statistically significant difference (P = 0.068). On days 28 and 42, the mean Hb slightly increased to 14.356 ± 1.32 g/dl (p = 0.756) and 14.65 ±1.35 g/dl (P= 0.135) even though there was no statistically significant difference between them (Figure 9).

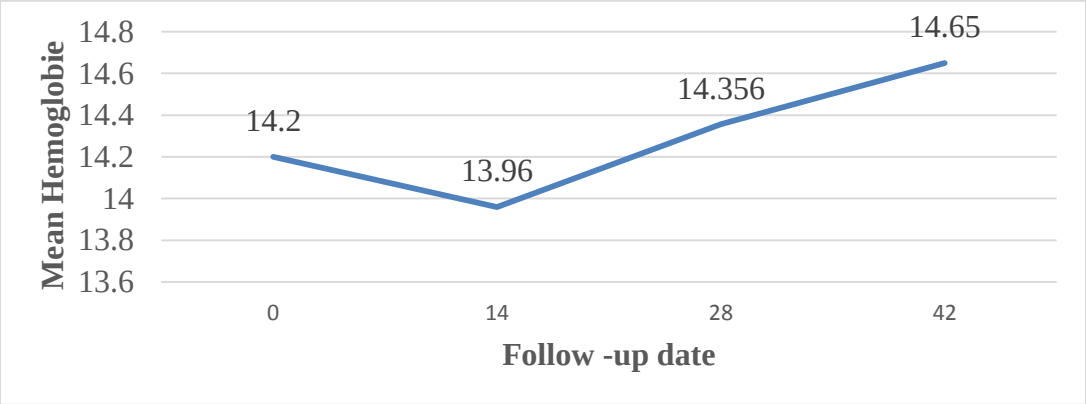

Figure 9. The Mean Hb (g/dl) of the study participants at day 0 and after AL plus SLD-PQ treatment of uncomplicated *P. falciparum* malaria, at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

On the day of recruitment (day 0), about 19% and 4.5% of the participants were mildly and moderately anemic, respectively. The number of mildly and moderately anemic participants decreased on day 14 to 10% and 2.3%, respectively. On 28 and 42 follow-up days, the proportion of individuals with mild to moderate anemia markedly decreased (Table 6).

Table 4. Mean hemoglobin concentration (g/dl) of the study population during follow-up days at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

Variables			Follow up days			
			D0, n=88	**D14, n=88**	**D28, n=86**	**D42, n=82**
Anemia status	**Mild, n (%) (11-12.9 g/dl)**	**Male**	9 (10%)	2 (2.3%)	2 (2.3%)	0
		Female	8 (9%)	7 (8%)	5 (5.8%)	6 (7.3%)
		Total	17 (19%)	9 (10%)	7 (8%)	6 (7.3%)
	Moderate, n (%) (8-10.9 g/dl)	**Male**	4 (4.5%)	2 (2.3%)	1 (1.2%)	1 (1.2%)
		Female	0	0	0	0
		Total	4 (4.5%)	2 (2.3%)	1 (1.2%)	0
	Total		21 (23.5%)	11 (12.6%)	8 (9.3%)	7 (8.5%)

4.8. Clinical Sign Symptoms, and Probable Adverse Events

At baseline headache, fever, and body aches were the most frequently encountered signs and symptoms, accounting for 93.2%, 67%, and 48.9%, respectively. Adverse events observed following AL plus SLD-PQ were diarrhea (2.3%) and mouth lesions (3.4%). The number of patients with abdominal pain and cough also showed an increment from the baseline (6.8% vs. 12.5% and 8% vs. 12.0%, respectively) (Table 7). However, most clinical sign symptoms and adverse events declined on day 7 and thereafter. No serious drug adverse events were observed.

Table 5. Common malaria clinical signs and symptoms and probable drug-related adverse events observed before and following AL plus SLD-PQ treatment at Maksegnit Health Center, Northwest Ethiopia, from February to May 2022

Clinical signs and symptoms	Day 0	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Fever, n (%)	59 (67%)	1 (1.1%)	0	0	0	0	0	1 (1.1%)	0	0
Headache, n (%)	82 (93.2%)	40 (45.5%)	21 (23.9%)	18 (20.5%)	7 (8%)	4 (4.5%)	3 (3.4%)	5 (5.7%)	4 (4.5%)	4 (4.5%)
Nausea, n (%)	25 (28.4%)	9 (10.2%)	2 (2.3%)	0	0	0	0	1 (1.1%)	0	0
Abdominal pain, n (%)	7 (6.8%)	11 (12.5%)	3 (3.4%)	4 (4.5%)	2 (2.3%)	1 (1.1%)	1 (1.1%)	2 (2.3%)	0	1 (1.1%)
Diarrhea, n (%)	0 (0%)	2 (2.3%)	1 (1.1%)	0	0	1 (1.1%)	0	0	0	0
Cough, n (%)	7 (8%)	11 (12.5%)	5 (5.5%)	3 (3.4%)	2 (2.3%)	3 (3.4%)	1 (1.1%)	0	0	0
Weakness, n (%)	36 (40.9%)	25 (28.4%)	12 (13.6%)	8 (9.1%)	5 (5.7%)	0	2 (2.3%)	2 (2.3)	0	0
Body ache, n (%)	43 (48.9%)	21 (23.9%)	4 (4.5%)	3 (3.4%)	3 (3.4%)	0	3 (3.4%)	1 (1.1)	2 (2.3%)	1 (1.1%)
Itching, n (%)	2 (2.3%)	1 (1.1%)	0	0	1 (1.1%)	0	0	0	0	0
Rash, n (%)	1 (1.1%)	0	0	0	0	0	0	0	0	0
Eye discharge	1 (1.1%)	1 (1.1%)	0	0	0	0	0	0	0	0
Mouth lesion	0%	3 (3.4%)	5 (5.5%)	3 (3.4%)	1 (1.1%)	0	0	0	0	0
Dark urine, n (%)	15 (17%)	5 (5.5%)	1 (1.1%)	0	0	0	0	0	0	0

5. DISCUSSIONS

Uncomplicated *P. falciparum* malaria is an important public health disease that needs prevention and control. In this study, the PCR-uncorrected and PCR-corrected per-protocol ACPRs were 94.1% and 96.4%, respectively. The high therapeutic efficacy of AL plus SLD-PQ for the treatment of uncomplicated *P. falciparum* malaria in the study area met the WHO recommendation of at least 90% cure rates for a given antimalarial drug to be efficacious (WHO, 2009). The observed cure rate of AL plus SLD-PQ in this study is comparable to previous studies as reported in Tanzania 100% (Mwaiswelo *et al*., 2016), and 98.3% in Tanzania also (Mhamilawa *et al*., 2020a), and South Africa 100% (Raman *et al*., 2019).

The overall efficacy of AL plus SLD-PQ in our study was comparable to the AL-only efficacy reported in Ethiopia: 96% PCR-corrected ACPR in Southwest Ethiopia (Abamecha *et al*., 2020) and 98.8% PCR-uncorrected ACPR in Northwest Ethiopia (Teklemariam *et al*., 2017). The efficacy of AL plus SLD-PQ in the current study was also comparable to AL-only efficacy reported in Tanzania and Myanmar: 93.3% PCR, corrected ACPR in mainland Tanzania (Ishengoma *et al*., 2019), and 100% ACPR in Western Myanmar (Wu *et al*., 2020). Studies indicate there might be an antagonistic effect if different drugs are administered together (Barnes *et al*., 2007; Dooley *et al*., 2008). Contrary to some expectations, the overall high efficacy of AL plus SLD-PQ in this study might indicate the absence of drug-drug interactions that affect metabolism and efficacy.

Apart from inherent parasite susceptibilities, factors such as host nutritional and immune status, and the pharmacokinetics, and pharmacodynamics of the drug may influence the therapeutic efficacy of a drug (WHO, 2009). These and other factors may lead to treatment failure, demonstrating the low efficacy of an otherwise effective drug. Simultaneously, immune-mediated clearance of resistant parasites may result in the exaggerated efficacy of less effective antimalarial drugs. In this study, however, the effect of such potentially confounding factors was left undetermined (WHO, 2009).

In this study, the absence of ETF and low parasitological failure (3 LPFs) might indicate low suspected artemisinin resistance and the high therapeutic efficacy of AL plus SLD-PQ. Generally, failures in treatment may occur due to insufficient drug levels or parasite resistance to

the drug. Because of artemisinin's short half-life, plasma drug concentrations do not rise above the minimum inhibitory concentration for periods long enough to kill all parasites (White., 1997). Moreover, the asexual blood stage *P. falciparum* parasites may become temporarily dormant and survive the therapeutic concentration of artemisinin derivatives (Hoshen *et al*., 2000). The development of *P. falciparum* ring-stage parasites is rapidly interrupted after exposure to artemisinin derivatives. However, these parasites recover and resume normal growth (Siddiqui *et al*., 2021).

Parasite clearance rates can be influenced by drug blood concentration profiles, host defense mechanisms, initial parasitemia, concurrent infection, and pharmacodynamic properties. Controlling for such potentially confounding factors is therefore critical to identifying temporal changes in parasite clearance caused by reduced antimalarial drug susceptibility. The ability of artemether to rapidly metabolize to its active ingredient, dihydroartemisinin, and be absorbed into the bloodstream results in rapid parasite clearance. The rate of elimination of artemether is also rapid, with a half-life ranging from 0.86 to 5.16 hours, while its partner drug, lumefantrine, is a slow-acting drug with a longer half-life of 32.7 to 275 hours (WHO, 2015). Even though only one patient had parasitemia on day 3, this could be due to the patient's immune status, parasite susceptibility to antimalarial drugs, and partner drug efficacy, which lags parasite clearance.

In our study, 98.8% of patients cleared parasitemia on day 3, which is consistent with AL alone efficacy studies in Ethiopia (Kanche *et al*., 2016; Wudneh *et al*., 2016; Deressa *et al*., 2017; Seid *et al*., 2017; Teklemariam *et al*., 2017; Abamecha *et al*., 2020). The high parasite clearance rates in this study could be explained by the rapid action of artemether to clear parasite biomass, leading to rapid resolution of clinical manifestations. A study conducted in the Great Mekong sub-region has found a link between AL resistance and a slower parasite clearance rate (Noedl *et al*., 2008; Tun *et al*., 2015).

The baseline mean axillary temperature was 38.3±0.7 ^{o}C, and all study participants were considered febrile, but only 58% had a fever during enrollment. The fever clearance rate was rapid, with all participants becoming fever-free on day 2. The rapid fever clearance capacity of AL was also reported in previous studies conducted in Ethiopia (Teklemariam *et al*., 2017;

Tseha, 2019; Abamecha *et al*., 2020). The rapid action of artemether to clear parasite biomass, possibly resulting in rapid resolution of clinical manifestations, could explain the high fever clearance rates in this study, and other evidence also supported this (Muhindo *et al*., 2014; WHO, 2022a).

The results of this study revealed that AL plus SLD-PQ have a strong gametocytocidal effect. It reduced gametocyte carriage from 9% on day 0 to 3.4% on day 1 and 2.3% on day 2, before clearing completely on day 3. This finding is consistent with findings from other studies in Tanzania (Mwaiswelo *et al*., 2016). In Senegal, AL administered with SLD-PQ on day 0 showed a statistically significant higher mean effect on gametocyte prevalence and density in the AL plus SLD-PQ arm with zero prevalence and density on day 14 (Tine *et al*., 2017). Similarly, Gonçalves and his colleagues found gametocyte density over time was lower in AL with SLD-PQ than without (Gonçalves *et al*., 2016).

Primaquine at a single low dose is a potent gametocidal drug, irrespective of G6PD deficiency (White *et al*., 2012; Graves *et al*., 2012). Artemether-lumefantrine can reduce gametocyte carriage and disrupt transmission because it acts on the immature stages of *P. falciparum* gametocytes (Ippolito *et al*., 2017). The rapid decline in gametocytes and absence of gametocytes after day 2 in our study might therefore be attributable to the combined gametocidal effects of AL plus SLD-PQ. Our findings, in line with an individual meta-analysis reviewed by Kasia Stepniewska and his colleagues, showed that the rate of decline in gametocyte carriage was faster when PQ was combined with AL, and the addition of 0.25 mg/kg PQ was associated with near-complete prevention of transmission to mosquitoes (Stepniewska *et al*., 2022).

The mean Hb level at baseline (14.3±2.07 g/dl) was slightly higher than on day 14 (13.96±1.47g/dl), but it slightly increased on the remaining follow-up days (days 28 (14.34±1.32 g/dl) and 42 (14.65 ±1.35 g/dl), even though the difference in mean Hb level across follow-up days was insignificant. Following parasite clearance, Hb was expected to rise or remain unchanged. Other studies conducted in Ethiopia and elsewhere revealed a slight to significant increase after treatment (Hwang *et al*., 2011; Shayo *et al*., 2014; Getnet *et al*., 2015). If there was a decrease in Hb levels after drug administration, this could be explained in part by the fact that a transient and clinically moderate but significant decrease in Hb occurs after artemisinin

derivative and 0.25 mg/kg single dose primaquine treatment initiation due to hemolysis of parasitized and non-parasitized red cells. The slight increase in mean Hb from day 14 to day 42 in this study is also consistent with another study (Wudneh *et al*., 2016; Teklemariam *et al*., 2017). It suggests that AL plus SLD-PQ therapy may not have a late, clinically relevant deleterious effect on Hb.

Delayed hemolysis (1–3 weeks after treatment) with artemisinin derivatives has been described by Rehman and colleagues (Rehman *et al*., 2014). Artemether-containing drugs are effective in their rapid killing of ring-stage parasites, and most of the killed ring-stage parasites are cleared rapidly by the spleen, whereby the dead parasite is removed from the erythrocyte (Arguin, 2014). These infected erythrocytes are returned to circulation, but they have a reduced lifespan of about 7–15 days. The delayed destruction of the infected erythrocytes corresponds with the time course of post-treatment delayed anemia. Rehman and colleagues reviewed the evidence of delayed anemia occurring in patients from malaria-endemic regions of Southeast Asia and Africa. Rehman and his colleague's review indicated that late hemolysis leading to a significant decrease in hemoglobin was associated with all artemisinin derivatives and all routes of administration (Rehman *et al*., 2014).

Adverse events observed following AL plus SLD-PQ were diarrhea and mouth lesions since these symptoms did not exist at baseline. The number of patients with abdominal pain and cough also showed an increment from the baseline; their persistence after the recovery of other malaria symptoms observed in this study makes them drug-related adverse events. However, most clinical symptoms and adverse events declined on day 7 and thereafter. No serious drug adverse events were observed.

6. LIMITATIONS OF THE STUDY

Although Maksegnit town has a moderate malaria transmission area, which necessitates the recruitment of both pediatrics and adults, this study only evaluated the efficacy and safety of AL plus SLD-PQ in adults.

7. CONCLUSION

AL plus SLD-PQ has a high therapeutic efficacy with rapid fever and parasite clearance and low gametocyte carriage rates for uncomplicated *P. falciparum* malaria. There were no serious drug adverse events observed, demonstrating the drug's high safety.

8. RECOMMENDATION

Based on our findings, we recommend:

- Clinicians in the study area continue administering AL plus SLD-PQ for the treatment of uncomplicated *falciparum* malaria.
- Policymakers in the field monitor the AL plus SLD-PQ drug storage conditions and the appropriate drug administration system in the health center on a regular basis, because any problem in these conditions can lead to drug resistance.
- Policymakers should once again encourage researchers to carry out a similar study in a wide geographic area with a large sample size in different malaria endemic areas.
- Researchers in the field should evaluate the efficacy and safety of PQ in adults and children by comparing AL plus SLD-PQ with AL-only.
- Further studies should be conducted by researchers on the susceptibility of *falciparum* isolates in vitro to (AL plus SLD-PQ), the polymorphism of molecular markers for AL and PQ, and the drug plasma concentration to rule out subtherapeutic dosing as the cause of the treatment failure.

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ANNEXES

Annex I. Summary of activities by day of study

Day 0:

Screening

- Clinical assessment including measurement of body weight and height — referral in case of severe malaria/danger signs
- Measurement of axillary temperature
- Parasitological assessment
- Informed consent
- Pregnancy test, if indicated
- Hemoglobin

Enrollment

- Treatment, the first dose

Day 1:

- Clinical assessment — referral in case of severe malaria/danger signs
- Measurement of axillary temperature
- Treatment, second dose, or alternative treatment in case of early treatment failure

Day 2:

- Clinical assessment — referral in case of severe malaria/danger signs
- Measurement of axillary temperature
- Blood sampling for blood smears
- Cell pellet for molecular test
- Blood sampling for RNA protect
- Treatment, third dose, or alternative treatment in case of early treatment failure

Day 3, Day 7, Day 14, Day 21, Day 28, Day 35, and Day 42:

- Clinical assessment — referral in case of severe malaria/danger signs
- Measurement of axillary temperature
- Blood sampling for blood smears (day 3, 7, 14, 21, 28, 35, 42, and day of failure)
- Blood sampling for qPCR (day 0 and day of failure)

- An alternative treatment in case of treatment failure
- Hemoglobin (Day 0, Day 14, Day 28, and Day 42)

Any other day:

- Clinical assessment — referral in case of severe malaria/danger signs
- Measurement of axillary temperature
- Blood sampling for blood smears and Whole blood for asexual parasite identification parasite genotyping to differentiate recurrent cases as recrudescence and re-infection
- Hemoglobin, if indicated clinically
- An alternative treatment in case of treatment failure

Annex II: Schedule of follow-up activities

STUDY VISIT DAYS	D0	D1	D2	D3	D7	D 14	D 21	D 28	D 35	D 42	Any other day
PROCEDURES											
Clinical assessment	X	X	X	X	X	X	X	X	X	X	X
Temperature	X	X	X	X	X	X	X	X	X	X	X
Blood slide for parasites count	X	X	X	X	X	X	X	X	X	X	X
Hemoglobin	X					X		X		X	X
Whole blood for	X	X	X	X	X	X	X	X	X	X	X
Drug adverse event monitoring	X	X	X	X	X	X	X	X	X	X	X
TREATMENT											
AL drug to be given	X	X	X								(X)
Primaquine	X										

Annex III: Symptoms of severe malaria

A severe manifestation of *P. falciparum* malaria in adults: **Clinical manifestations**: - prostration, impaired consciousness, respiratory distress (metabolic acidosis), multiple convulsions, circulatory collapse, pulmonary edema, abnormal bleeding, jaundice, hemoglobinuria.

Laboratory findings: Severe anaemia (haemoglobin <5 g/dl), hypoglycemia (blood glucose<2.2mmol/lor40mg/dl),

Annex IV: Medications with an antimalarial activity that shouldn't be used during the study period

- Antibiotics: tetracycline*, doxycycline, erythromycin, azithromycin, clindamycin, rifampicin, trimethoprim;
- Amodiaquine
- Artemisinin and its derivatives (artemether, artesunate, dihydroartemisinin)
- Atovaquone *Chloroquine *Chlorproguanil
- Dapsone *Halofantrine * Lumefantrine
- Mefloquine *Naphthoquinone *Pentamidine
- Piperaquine *Primaquine *Proguanil
- Pyrimethamine * Pyronaridine * Quinidine
- Quinine *Sulfadoxine *Sulfalene
- Sulfamethoxazole *Tetracycline eye ointments can be used.

Annex V: Drug dosing chart

All patients weighed to determine the accurate weight-based dose for all drugs.

Artemether-lumefantrine was administered twice daily for three days as tablets containing 20 mg of artemether plus 120 mg of lumefantrine in a fixed dose combination at a dosage.

Weight (kg)	**Day 1**		**Day 2**		**Day 3**	
	Morning	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**
> 35	4	4	4	4	4	4

Primaquine: 7.5 mg base tablet. Medication is given as 0.25 mg/kg stat

Body Weight (kg)	**A single dose of PQ (mg base)**

5 to 10	Weight-based using extemporaneous preparation of crushing the tablets as outlined by Sanofi
10 to < 25	3.75
25 to <50	7.5
50 to 100	15

Annex VI: Consent forms (English version)

Information sheet for participants

Introduction

This consent form is written to address a research subject. The Federal Ministry of Health (FMOH), Armauer Hansen Research Institute, Bahir Dar university in Ethiopia, United States Agency for International Development, U.S. Centers for Disease Control and Prevention, and ICAP at Columbia University invite you to be in this study.

Voluntary Participation (Do I have to be in this study)?

Taking part in this study is your choice. You can decide not to take part in or stop being in the study at any time. Your choice will not affect your treatment for malaria.

What is the purpose of the study?

The purpose of this study is to find out how well the drugs for malaria are working in Ethiopia.

How long would you need me?

You will come to the clinic nine times over the next 42 days. On day1,2,3 you will come consecutively. However, on days 7,14,21,28,35, and 42 you will be expected to come weekly. But if you do not appear on the scheduled follow-up, our study team will visit you at home. You decide whether you will be in this study. You can stop the study at any time without any problem.

Participation Duration: 42 days

Anticipated Number of Subjects:

What do you want me to do if I decide to be in study?

If you agree that you will be in this study, a study staff person will contact you. For women of reproductive age, we will conduct a pregnancy test since pregnant women can't be included in the study. The staff person will take blood from your finger (less than ¼ of a teaspoon) or your arm vein (one teaspoon of blood) and ask some questions each time you come. We will tell you what kind of malaria you have.

- If you have a certain kind of malaria (which doctors call *P. falciparum*), you will get either of the World Health Organization recommended drugs for treatment for falciparum malaria, artemether-lumefantrine (Coartem) plus primaquine, you will need to come to the clinic to swallow the tablets every day for the first three days, and you may take some tablets at home. We will observe you for about an hour after you take the medicine in the clinic to make sure you do not vomit.

We will ask you to return to the clinic on days 1, 2, 3, 7, 14, 21, 28, 35, and 42 after the treatment starts so we can assess you and make sure that malaria has been cured and does not return. We will give you a visit card so you know what days to come.

At every visit, we will do a finger prick blood test for malaria. A test to check for malaria in the blood will be done today and at all follow-up visits. At each visit, a study staff person will receive you and ask some questions. If you do not come to the clinic, someone from the clinic will come to your home to check on you.

If you have a severe case of malaria, this will lead to admission to the hospital. There, strong drugs and other care will be given based on standards in Ethiopia.

Are there any risks to me if I decide to be in study?

There is no specific risk associated with your participation in the study as three of the drugs you will be taking are the same drugs and amounts you would normally take even if you don't participate in the study.

If something more serious happens, such as fever, convulsions, becoming very sleepy or cannot be woken up, a lot of vomiting, being unable to drink or eat, having a painful rash or mouth sores or red eyes, chest pain or difficulty breathing or very fast breathing, dark-brown or reddish discoloration of urine, you should come to the clinic where you will get treatment or be sent to the hospital. You can come back anytime even if not on a scheduled day.

Rarely, do more severe side effects can occur. If you have severe side effects, like persistent vomiting, dark-brown or reddish discoloration of urine, severe rash, or dizziness, we will stop the drug. We will also advise that you be treated with different anti-malaria drugs in the future.

We expect that these drugs will work well in most people. But if malaria is not cured or if it comes back, we will provide treatment with other drugs called artesunate or quinine. This is the standard treatment in Ethiopia for this situation. This treatment should cure malaria.

There may be other risks of taking part in this study that we don't know about. If we learn about other risks, we will let you know what they are so that you can decide whether or not you want to continue to be in the study. If you need any medical treatment or need a hospital stay during this study, you will not pay for it.

Are there any benefits from being in the study?

You may not benefit from this study financially. You will not pay for anything during the 42 days. There will be someone here to see you every day. You may come for a visit at any time if ill, even at night or on weekends, or in-between visits. This study will also help Ethiopia learn if the standard drugs, alternative drugs, and the current rapid diagnostic tests work best in this region. This may help you or someone you know in the future. If you decide that you do not want to take part in this study, treatment for malaria will be provided.

Confidentiality

Any information collected during this study that can identify you by name will be kept confidential. We will do everything we can to keep your data secure, however, complete confidentiality cannot be promised. Despite all of our efforts, unanticipated problems, such as a stolen computer may occur, although it is highly unlikely.

Your clinical and laboratory information and blood samples will be assigned a code number and separated from your name or any other information that could identify you. The research file that links your name to the code number will be kept in both a locked file cabinet (hard copy) and a password-protected computer and only the investigator and study staff will have access to the file.

The following individuals and/or agencies will be able to look at and copy your research records:

- The investigator, study staff, Columbia University staff, and medical professionals who may be evaluating the study or providing services for the study
- Authorities from Columbia University Institutional Review Board ('IRB')

- Authorities from Bahir Dar university
- Authorities from Armauer Hanson Research Institute IRB
- The Office of Human Research Protections ('OHRP')

Compensation (Do I get compensation for participating in the study?)

There is no special incentive you would get by participating in the study. However, you will receive 100 Ethiopian Birr for each visit for ground transportation costs.

Alternative Procedures (Would I still get proper care if I don't participate in the study?)

This study intends to assess how the drugs currently in use and an alternative drug would work for malaria treatment in Ethiopia. However, if you choose not to take part in this research, you would still get the standard treatment for malaria in Ethiopia which is expected to cure you of malaria.

Consent for future research

We are asking people in the study if the study team can use their blood samples for future projects. These projects will help find new ways to treat or find malaria. If you say yes, we will keep your blood sample. We will label your blood sample with a number.

The blood sample will be kept in Ethiopia. After the study is over, we will not be able to connect you with your sample. If you do not want us to keep your blood sample, you can still be in the study.

If you decide to participate in the study, we would require you to initial next to your chosen action regarding the use of your blood sample for future research. Please initial one of the following options:

1) I opt-in the use of my blood sample for future research

2) I opt out of the use of my blood sample for future research

Who should I call if I have any questions?

If you have questions or concerns about taking part in this study, or you have any questions later about the study or would like to withdraw from the study, please contact Mr. Tadesse Misganaw at 0923042602, Mrs. Woyenishet Gelaye (0913105751), Dr. Tadesse Hailu (0912332655) and Dr. Fitsum Girma (0912627540) at Bahir Dar University and AHRI, and Dr. Bereket Alemayehu at 0988 14 3843 (ICAP) or the secretariat of the Ethics Review Committee at AHRI (+251-921562909).

If you have any questions about your rights as a research participant, or if you have a concern about this study, you may contact the following offices below.

1. Bahir Dar university, College of Medicine and Health Sciences (at +251582264162, P.O.box: 79)
2. In Ethiopia: Mrs. Hiwot Solomon at 0910100255 at the Federal Ministry of Health in Ethiopia.
3. In the United States of America: Human Research Protection Office, Institutional Review Board Columbia University Medical Center

154 Haven Avenue, 1st Floor, New York, NY 10032Telephone: (212) 305-5883; email: irboffice@columbia.ed

Consent form

After reading the information sheets or listening to someone reading, I agreed to give samples through all scheduled visits come on every scheduled visit, take drugs, be visited at home, and store samples for future studies. Therefore, I agreed to participate in the study by understanding all the study procedures and my rights.

Signatures

Participant Signature Lines

Study Participant

Print Name: _____ Signature: _____ Date: _____

Research Signature Lines

Person Obtaining Consent

Print Name: _____ Signature: _____ Date: _____

Witness Signature Lines

Witness

Print Name: _____ Signature: _____ Date: _____

Annex VII: የተሳታፊዎች መረጃ መስጫና የስምምነት ፎርም(በአማርኛ)

የጥናቱ ተሳታፊ የመረጃ ፎርም

ለተሳታፊዎች መረጃ

የጥናቱ ርዕስ፡ የኩአርትምና የፐሪማኩይን መዳኒቶች ፈዋሽነትና የሚያመጡት የጎንዮሽ ችግር ውስብስብ ባልሆነ የፐላስሞዲየም ፋልሲፋረም ወባ ህመም ጥናትና ምርምር በማከሰኝት ጤና ጣቢያ

መግቢያ

በዚህ በሚደረገው ምርምር ውስጥ እንዲሳተፉ የኢፍድሪ ጤና ሚኒስቴር፣አርማውር ሃንሰን የምርምር ኢንስቲትዩት እና ባህርዳር ዩኒቨርሲቲ ህክምናና ጤና ሳይንስ ኮሌጅ እና በሽታን መከላከልና መቆጣጠር ባለስልጣን ጋብዘዋል፡፡

በፈቃደኝነት ላይ የተመሰረተ ተሳትፎ

እርሶ በጥናቱ እንዳይሳተፉ ወይም እንዲያቋርጥ መወሰንዎ እርሶ ሊያገኙ የሚችሉት የጤና አገልግሎት ላይ ምንም ተፅእኖ አይኖረውም።እርሶ ጥናቱን ማቋረጥ ከፈለጉ በማንኛውም ጊዜ ማቋረጥ ይችላሉ።

የጥናቱ ዓላማ

የዚህ ጥናት ዋና አላማ የጸረ-ወባ መዳኒቶች ምንያህል ውጤታማ እንደሆኑ መፈተሽ ይሆናል፡፡

ጥናቱን የሚወስደው ጊዜ

እርሶ ይህን ጥናት ለመሳተፍ በመጀመሪያ ለአርባ ሁለት ቀን የሚቆይና ዘጠኝ ያህል ጊዜ መመላለስን የሚጠይቅ ይሆናል፡፡በቀን 1፣2፣3 በተከታታይ ወደ ክሊኒክ መምጣት ይጠበቅቦታል ከዛ በኋላ በቀን 7፣14፣21፣28፣35 እና 42 በየሳምቱ እንዲመጡ ይጠየቃሉ፡፡በከትትል ወቅት ድንገት መምጣት ባይችሉ የኛ ጥናት ቡድን ቤት ድረስ በመምጣት ይጎበኞታል፡፡በዚህ ጥናት ለመሳተፍ ከወሰኑ መሳተፍ ይችላሉ ጥናቱ በሙሉ ፈቃደኝነት ላይ የተመሰረተ ነው፡፡በማንኛውም ጊዜ ከጥናቱ ለመውጣት ከፈለጉ መውጣት ይችላሉ፡፡

ለጥናቱ የሚያስፈልጉ ተሳታፊዎች

በአጠቃላይ 88 የወባ ተዋሲያን በደማቸው የተገኘባቸው ሰዎች በጥናቱ ይሳተፋሉ፡፡

በጥናቱ ለመሳተፍ ከወሰንኩኝ ከኔ የሚጠበቀው ምንድነው?

እርሶ በጥናቱ ለመሳተፍ የእርስዎን ፍቃደኝነት እንጠይቃለን።ለዚህም እንዲረዳን የተለያዩ ምርመራዎችን ለማድረግ የሚረዳ አንድአራተኛ(1/4ኛ)የሻይ ማንኪያ የሚሆን የደም ናሙና ከጣት ላይ ይወሰዳል፡፡ሁሌ ለክትትል ሲመጡ ከወባ ጋር የተያያዘ አንዳንድ ጥያቄዎች ይጠየቃሉ፡፡በደሞ ውስጥ የወባ ተዋሲ ከተገኘና በደም ውስጥ የተገኘው ፕላስሞዲየም ፈልሲፓረም ከሆነ አርተሜሲኒን ሉፋትሪን ከፕሪማከውን ጋር ሊሰጦ ይችላል፡፡መዳኒቶቹን ለመውሰድ ለተከታታይ ሶስት ቀን ወደ ክሊኒክ መምጣት ይኖርበታል፡፡ይህም የሚሆነ.መዳኒት ጋር ተያይዞ የሚመጣ ማስመለስ ካለ ለመከታተል ነው፡፡ማታ የሚወሰድ መዳኒት ካለ ወደ ቤት ይዘው ይሄዳሉ፡፡መጀመሪያ ቀን ላይ የደምና ሙና ከሰጡ በኋላ ለተከታታይ ቀናቶች በሚኖሩ የክትትል ቀናት (1, 2, 3, 7, 14, 21, 28, 35, 42) ተመሳሳይ መጠን ያለው ደም ከጣት ላይ ይወሰዳል፡፡ሁሌም ለክትትል ሲመጡ ከወባ ጋር የተያያዘ ጥያቄ ይጠየቃሉ፡፡ነገርግን እርሶ በቀጠሮቀን መምጣት ካልቻሉ መምጣት ያልቻሉበትን ምክንያት የጥናቱ ባለሙያዎች ቤትድረስ በመምጣት ይጠይቆታል፡፡ሌላው በክትትል ወቅት የተወሳሰበ የወባ አይነት ከተገኘቦት ሆስፒታል ተኛተው እንዲከታተሉና አስፈላጊው ህክምና እንዲደረግሎት ይደረጋል፡፡የጥናቱን ሂደት በመረዳት ላይ የተመረኮዘ ተሳትፎ እንዲያደርጉ በጥናቱ ለመሳተፍ ፍቃድዎን እንዲገልጹልን እንጠይቃለን፡፡

የምቾት መጓደልና ተጋላጭነት

በዚህ ጥናት በመሳተፍዎ ምንም የተለየ ጉዳት አይደርስቦትም ምክንያቱም መድሃኒቶቹ በፌደራል ጤና ጥበቃ ሚኒስተር ፍቃድ የተሰጣቸው እና በመደበኛነት ለወባ በሽታ ህክምና በኢትዮጲያ እየተጠቀምናቸው ያሉ ናቸው፡፡

መዳኒቶቹ ብዙም የጎላ የጎንዮሽ ጉዳት ባይኖራቸውም አንዳንድ ጊዜ እንደከፍተኛ ትኩሳት፣እራስ መሳት፣እንቅልፍ እንቅልፈ ማለት ወይም መደበት፣በተደጋጋሚ ማስመለስ፣መብላትና መጠጣት አለመቻል፣በሰውነት ላይ ከፍተኛ ህመም ያለው ሽፍታ መታየት ወይም የአይን መቅላት እና የአፍ መቁሰል፣የደረት ህመም ወይም ለመተንፈስ መቸገር ወይም ቶሎ ቶሎ መተንፈስ እና

የሽንት ቀለም መቀየር ሲኖር ቀጠሮ ሳይጠብቁ በአስቸኳይ ወደ ህክምና ቦታ ይምጡ::መዳኒቱን እንዲያቆሙ ይደረግና በምትኩም ሌላ መዳኒት እንዲወስዱ ይደረጋል::

ጠቀሜታ

እርሶ በጥናቱ በመሳተፎ የተለየ ጥቅም ባይኖርም በክትትል ጊዜ ውስጥ ከወባ ጋር የተያያዘ ነፃ የህክምና አገልግሎት ያገኛሉ።የተለየ አትኩሮት የሚፈልጉ ሁኔታዎች ከተከሰቱ በጤና ተቋም ህክምና እንዲያገኙ ይደረጋል።በተጨማሪ ከክትትል ቀን ውጪ እነኳን የተለየ የህመም ስሜት ከተሰማዎ በማንኛውም ጊዜ ወደ ህክምና ተቋም መምጣት ይችላሉ::ይህ ጥናት እየተጠቀምናቸው የነበሩ የወባበሽታ መዳኒቶችና አዲሱ መዳኒት እንዲሁም ፈጣን የወባ በሽታ የመመርመሪያ ዘዴ ምንያህል ውጤታማ እነደሆኑ ሊነግረን ይችላል::በዚህ ጥናት መሳተፍ ባይፈልጉ አንኳን ህክምናውን ያገኛሉ::

ሚስጥርን ስለመጠበቅ

ማንኛውም ከእርሶ መሳትፍ ጋር የሚገናኙ መረጃዎች ለዚህ ጥናት ጠቀሜታ ብቻ ይውላሉ።የእርሶም ሆነ የእርሶ ስም ለናሙና መለያነት ወይም በማንኛውም የዚህ ጥናት ውጤት ሪፖርት ላይ አንጠቀምም።በጥናቱ መጀመሪያ ለተሳታፊዎች የመለያ የሚስጥር ቁጥር ይሰጣል፤ይህም ለናሙናዎችና በጥናቱ በጥቅም ላይ ለሚውሉ ቅጾች መለያነት ይጠቅማል።ማንኛውም ከዚህ ጥናት በተዛመደ የሚገኝ መረጃ በሚስጥር ይያዛል፤መረጃዎችም በቁልፍ ተቆልፎባቸው ይቀመጣሉ።ይህን መረጃ መጠቀም የሚችሉት ከጥናቱ ጋር ህጋዊ ሰውነት ተቋማት ማለትም

- የጥናቱ ዋና ተመራማሪዎች፣
- የጥናቱ አባላት፣
- ባህር ዳር ዩኒቨርሲቲ
- የአርማወር ሃንሰን የጥናት ኢንስቲትዩት የስነ-ምግባር ኮሚቴ
- የኮሎምቢያ ዩንቨርስቲ የስነ-ምግባር ኮሚቴ
- የሰው ልጆች የምርምር ደህንነት ጥበቃ ቢሮ

በጥናቱ ላይ በመሳተፍ የሚገኝ ክፍያ

በዚህ ጥናት በመሳተፍ የሚገኝ የተለየ ጥቅም ባይኖርም በየቀጠሮ ቀን ላወጡት የትራንስፖርት ማካካሻ የሚሆን አንድመቶ(100) የኢትዮጲያ ብር ይከፈላል::

ናሙናዎችን የጥናቱ ጊዜ እስከ ሚያልቅ ስለማስቀመጥ

ከእርሶ የሚገኘውን ናሙና የጥናቱ ጊዜ እስከ ሚያልቅ ማስቀመጥ እንድንችል ፈቃድዎትን እንጠይቃለን። እኛ አሁን ለመመለስ የምንሞክረውን ጥያቄ ለመመለስ የሚያስችሉ አዳዲስ ብልሃቶች ሊገኙ ይችላሉ።

ስምን ከናሙናዎች የጥናቱ ሚስጥር ቁጥር የሚያገናኙ መረጃዎችን እናስወግዳለን። ናሞናዎቹን በአገር ውስጥ ቤተሙከራ ሊቀመጡ ይችላሉ:: ናሙናዎችን የምንጠቀማቸው የወባጸረ-መዳኒቶች ላይ ያላቸውን አቅም ከመፈተሽ ጋር በተዛመደና ከጥናቱ ጋር ለተያያዘ መረጃዎችን ለማግኘት ብቻ ይሆናል። ተጨማሪ ጥናት አስፈላጊ ከሆነ የስነ-ምግባር ኮሚቴ ይሁንታን የሚጠየቅበት ይሆናል።

እርሶ በዚህ ጥናት ለመሳተፍ ፈቃደኛ መሆኖን አለመሆኖን የሚቀጥሉት ምርጫዎች ያመላክቱ::

1.አዎ 2. አይደለሁም

ጥያቄዎችን የመጠየቅና ከጥናቱ አቋርጦ የመውጣት ነጻነት

ከጥናቱ ጋር በተያያዘ ጥያቄ ካላችሁ፤ የጥናቱን ዋና ተመራማሪ ፍጹም ግርማ ከአርማወርሀንሰን የምርምርተቋም,ፖስት ሳጥን ቁጥር 1005, አዲስአበባ, ኢትዮጵያ ስልክቁጥር፡-0912627540) ወይም +251 923042602 ታደሰ ምስጋናውን ከ ባህር ዳር ዩኒቨርሲቲ መጠየቅ ይችላሉ፡፡ስለጥናቱ እና ከጥናቱ ጋር ያልተገናኘ ገለልተኛ ወገንን ማማከር ካስፈለጋችሁ እንደ ጥናቱ ተሳታፊነታችሁ የአርማዉር ሃንሰን ምርምር ተቋም/የአለርትየስነ ምግባር ኮሚቴ ጸሃፊን በዚህ ስልክ ቁጥር ማግኘት ይችላሉ (251-21562909)።.በተጨማሪም ልጅዎ በዚህ ጥናት በመሳተፉ ስለልጅዎ ሙበት ለመጠየቅ ወይም ሰለዚህ ጥናት መጠየቅ ወይም ማወቅ የምትፈልጉት ነገር ካለ የሚከተሉን አካላት ማናገር ይችላሉ

1.ወ/ሮ ህይወት ሰለሞን በኢትዮጲያ ፌደራል ጤናሚኒስቴር ስልክቁ 0910100255 ወይም 2. የሰው ልጆች የምርምር ደህንነት ጥበቃ ቢሮ በአሜሪካ፣በኮሎቢያ ዩኒቨርስቲ የህክምና ማዕከል የምርምር ስነምግባር ኮሚቴ የስልክ ቁ (212) 305-5883 ወይምኢሜል፡irboffice@columbia.edu

የስምምነት ቅጽ

ስለጥናቱ አንብቤ ወይም ተነግሮኝ፤ጥያቄ የመጠየቅ እድል ተሰቶኝና ጥያቄዎቼ ተመልሶልኝ፤በጥናቱ ውስጥ ለመሳተፍ እና በጥናቱ መጀመሪያ ቀንና ተከታታይ ባሉ መደበኛ የቀጠሮ ቀናት ማለትም በቀን 1፣2፣3፣7፣14፣21፣28፣35 እና 42 አንድ አራተኛ የሻይ ማንኪያ ደም ለመስጠት፣ በክትትል ቀጠሮዬ ሁሉ ለመምጣት፣የተሰጠኝን መዳኒት ባግባቡ መውሰድ፣በቀጠሮዬ ቀን መምጣት ባልችል ቤቴ መተው እነዲያዩኝ እና ከኔ የሚወሰደው የደምናሙና ተመሳሳይ ለሆነ ጥናት እንዲውል በማለት በዚህ ጥናት ለመሳተፍ በፈቃደኝነት ተስማምቻለሁ፡፡

የጥናት ተሳትፎ ፍቃድ

ስም----------

ፊርማ ----------

ቀን ----------

በተመራማሪው / ስምምነትን በሚቀበል ሰው/ የተሰጠ መግለጫ

የመረጃ ቅጹን ለተሳታፊው በትክክል አምብቢያለሁ እናም ጥናቱ ምን እንደሚያካትት መረዳታቸውን አረጋግጫለሁ፡፡ተሳታፊው ስለ ጥናቱ ጥያቄዎችን ሊጠይቅ ይችላል፡፡እናም የተጠየቁት ጥያቄዎችን ሁሉ ችሎታዬ በሚፈቅደዉ መጠን መልሻለሁ፡፡ስምምነቱ በነፃነትና እና በፍቃደኝነት የተሰጠ ሲሆን የዚህ ቅጽ ኮፒ ለተሳታፊ ተሰጥቷል፡፡

ስምምነቱን የሚወስደው ሰው መጠሪያ እና የአባት ስም----------

ፊርማ----------

ቀን----------

ምስክር

መጠሪያ እና የአባት ስም ----------

ፊርማ ----------

ቀን ----------

Annex VIII: Patient Screening Form

Participant name /Identification number: _____ Health facility: _____ Name of provider: _____	Study no: ---------- -- Card No: _____ Date: __ / __ / __ __

Demographic data
Date of birth____/____/_____Sex: ☐Male ☐ Female: Age: _____
Weight(kg)----------Height(cm)----------
Have you ever had a serious or chronic medical illness? ☐ Yes ☐ No **(If yes, the subject is not eligible.) If yes,** what illnesses? ☐ heart disease ☐ kidney disease ☐ liver disease ☐ HIV/AIDS ☐ other ___ Have you ever had an allergy or sensitivity to artemether-lumefantrine and primaquine? ☐ Yes ☐ No **(If yes, the subject is not eligible.)** Have you ever had hemolysis or severe anemia? ☐ Yes ☐ No **(If yes, the subject is not eligible.)**
Pretreatment temperature
History of fever in previous 24 hr.? Yes ☐ No ☐, Temperature (axillary): ____/_____/. _____^{O}C
Thick and thin blood smear for parasite estimation
Are *P. falciparum* asexual parasites present? ☐ Yes ☐ No If yes, parasite density (parasites/µL): ☐☐☐☐ if <500/ul not eligible Are species other than *P. falciparum* present? ☐ Yes ☐ No ***If the patient has mixed infections including p. vivax, ovale or malariae, they are not eligible for the study.*** ***Estimated parasite count: _____ (if not in range, treat the patient as per the guideline and send home)***
Study recruitment
Are you living within the catchment areas of Maksegnit Health Center? ☐ Yes ☐ No

Do you stay in this area for the next 6 weeks? ☐Yes ☐ No ***If yes, refer the patient to the study team.*** ***In Screening Log, record the names of all patients screened and record whether the patient accepted the study referral.***

Annex IX. Case screening form

Study code_____Age: __ __ in year Mobile phone #: _____ ☐ N/A Date of visit: __ __ / __ __ / __ __ __ __ GC Health center: _____	Will you live in this area for at least 6 weeks? ☐Yes ☐No **If no, the subject is not eligible.** **(If the home is more than 20 km from the health center the subject is not eligible.**
Thick and thin blood smears for parasite confirmation (from finger stick #1)	
Lab diagnosis of *P. falciparum* mono-infection: ☐ Yes☐ No **(If mixed infection, the subject is not eligible.)** *P. falciparum* parasitemia 1000–100,000 asexual forms/µl ☐ Yes☐ No **(If no, the subject is not eligible.)**	
Weight and temperature	
Weight: ☐☐☐. ☐ kg Height: ☐☐☐m Age: _____ in year Temperature (axillary): _____/__˚C History of fever in previous 48 h? ☐Yes ☐No	
Demographic data/past medical history	
Sex: ☐ Male ☐Female, **if female 18-49 years:** Are you pregnant? ☐ Yes☐ No ☐ Not sure **(If yes, the subject is not eligible.)** Are you breastfeeding? ☐ Yes ☐ No **(If yes, the subject is not eligible.)** Have you ever had a serious or chronic medical illness? ☐ Yes ☐ No **(If yes, the subject is not eligible.)** **If yes,** what illnesses? ☐ heart disease ☐ kidney disease ☐ liver disease ☐ HIV/AIDS ☐ other _____ Have you ever had an allergy or sensitivity to artemether-lumefantrine and primaquine? ☐ Yes ☐ No **(If yes, the subject is not eligible.)** Have you ever had hemolysis or severe anemia? ☐ Yes ☐ No **(If yes, the subject is not eligible.)**	

Symptom assessment
Seizures? ☐ Yes ☐ No, Vomiting everything? ☐ Yes ☐ No, Lethargy? ☐ Yes ☐ No, Inability to sit/stand? ☐ Yes ☐ No, Not eating anything? ☐ Yes ☐ No, Discolored urine? ☐ Yes ☐ No
Inclusion criteria
Slide-confirmed mono-infection with *P. falciparum* ☐ Yes ☐ No, Age ≥ 18 years ☐ Yes ☐ No, Permanent resident within 20km of the enrolling health facility ☐ Yes ☐ No, Axillary temperature ≥ 37.5˚C or history of fever during the previous 48 hours ☐ Yes ☐ No, can swallow medication ☐ Yes ☐ No Able and willing to comply with the study protocol for study duration ☐ Yes ☐ No **(If the subject answers NO to any of the above questions, the subject is not eligible.)**

Malaria prevention data (HO)					
Does the subject's household own a bed net? ☐Yes ☐ No Did the patient sleep under a bed net last night? ☐ Yes ☐ No Was the subject's home sprayed with insecticide in the past 12 months? ☐Yes ☐No ☐Not sure					
Baseline symptom data (HO)					
Presence of danger signs or signs of severe or complicated malaria? ☐Yes ☐No					
Recent medication (HO)					
Report all meds, including natural remedies and homeopathic medicines, taken within the previous 14 days.					
Have you taken any antimalarial medication in the past 14 days? ☐ Yes ☐No Have you taken any other medication in the past 14 days? ☐ Yes ☐ No If yes, give the names and dates of taking the medication. ***Complete either the stop date or the 'ongoing' box.***					
Generic medicine name	Dates	Ongoing (Yes =☐	Total daily dose and unit	Route of administration	Indication for use
	Start: Stop:	☐			
Physical exam (HO)					

Axillary Temperature (^{0} C):----- Pulse (heartbeats/min): ----Respiratory rate (breaths/min):----		
Organ system	Normal?	If abnormal, describe the finding
HEENT (MMM, evidence of OM)	☐ Yes ☐ No	
Cardiorespiratory	☐ Yes ☐No	
Signs of severe malnutrition (wasting, pedal edema)	☐ Yes ☐ No	
Petechial rash or jaundice	☐Yes ☐ No	
Mental status	☐ Yes ☐ No	
Other	☐ Yes ☐ No	
Did the exam reveal any febrile conditions caused by other diseases OR evidence of severe malnutrition? ☐ Yes ☐No **(If yes, the subject is not eligible.)**		
Medication administration (nurse)		
Send subject to nurse for medication administration. Subject weight: -----kg ***Tick the box with the correct medication dose below.***		
☐**AL: Twice a day (BID)**	☐**PQ: (7.5 mg tablets)**	
☐Weight 25-34 kg: 3 tabs twice a day ☐Weight >35 kg: 4 tabs twice a day		
Does the subject have a temperature >38 ^{o}C?☐ Yes ☐No, *if yes, give paracetamol now and two additional doses to take home.*		

Antimalarial drug(s)	Time of dose (hh: min)	Number of tablets	Subject vomited?	Time of vomiting (hh: min)
Artemether –lumefantrine			☐ Yes ☐ No	
Primaquine			☐Yes ☐ No	
Other med (s) administered in the study clinic (i.e., redose vomited antimalarial)				
Other medicines				

			☐ Yes ☐ No	
			☐ Yes ☐ No	

Medication is given to go	
Name(s) of antimalarial drug(s)	Number of tablets
Artemether-lumefantrine *(if in AL-PQ arm)*	
Other meds (i.e., paracetamol): _____	

End-of-visit checklist	
☐*Consent form completed (HO)*	☐*Visit calendar completed (nurse)*
☐D*ay 0 CRF completed (HO)*	☐*Appointment card was given (nurse)*
☐*Lab request form completed/reviewed (HO)*	☐*Drug administered (nurse)*
☐*Symptom log completed (HO)*	☐*Travel reimbursement is given (nurse)*
☐*Enrolment log book completed (nurse)*	

Study staff name: _____ Signature: _____

Annex X. Case Record Form

Pin……No of tablets …...Name……

Follow-up day	0	1	2	3	7	14	21	28	35	42	Unscheduled day
Date											
Success of treatment *											
Axillary Temperature											
Asexual Parasite count											
Gametocyte count											
Hemoglobin											
Sign and Symptom or possible Adverse event **											
Concomitant treatment											
Reason for withdrawal											
Remarks											

Completed by initials											

*1. Observed by the health professional and successfully took medication

**1) Headache 2) Vomiting 3) fever 4) Abdominal pain 5) Body ache 6) Mouth ulcer 7) Cough 8) Dark urine 9) Mouth lesion 10) Itching 11) Rash 12) Diarrhea 13) Weakness 14) Eye discharge 15) Other, specify

Annex XI. Laboratory Request Form

Laboratory request form (Form 3) Study Laboratory
1.Client details: Study ID: _____Card No_____ Date of visit: __ / __ / __, Age----------Sex---
Place label in lab log book. Write all results in the spaces below and the laboratory logbook
1. Follow-up visit
☐ Day 0 ☐Day 1 ☐Day 2 ☐ Day3
☐ Day 7 ☐ Day 14 ☐Day 21 ☐Day 28
☐Day35 ☐ Day 42 ☐ Unscheduled Day
3. Laboratory tests
I. Hg (g/dl)
II. Blood film (p/µl) ☐
III. ☐ HCG ☐Pos ☐Neg N/A
IV. Collecting whole blood by EDTA microtainer test tube for molecular tests ☐ Yes ☐ No
V. Please re-check species by rapid test (only on day 0). ☐ *P. falciparum* ☐ *P. vivax* ☐ Negative
PCR result for recurrent parasitemia cases: ☐ Yes New infection ☐ Yes Recrudescence

Annex XI: መረጃ መሰብሰቢያ መጠይቅ (በአማርኛ)

ተ.ቁ	ጥያቄዎች	መልስ
1	እድሜ	_____ዓመት
2	ጾታ	1. ወንድ 2. ሴት (ታካሚው ሴት ከሆኑ ወደ ተራ ቁጥር 2 ይሂዱ)
3	ነፍሰ-ጡር ነዎት፣ ያጠባሉ	1. አዎ 2. የለም
4	የምትኖሩበት ቀበሌ፣ንጥ	ቀበሌ_____ንጥ_____
5.	የትንኝ መከላከያ አጎበር ይጠቀማሉ?	1. አዎ (መልስው አዎ ከሆነ ወደ ተራ

		ቁጥር 6 ይሂዱ) 2. የለም
6.	ባለፈው ሌሊት ከመኝታው አንበር ዘርግተዋል	1. አዎ 2. የለም
7.	ባለፉት 12 ወራት ቤትዎ ፀረ-ተባይ መድሃኒት ተረጭቶል	1. አዎ 2. የለም
8.	ከዚህ በፊት በሃኪም የተረጋገጠ እንደ እንኩላሊት፣ጉበት፣ልብ፣ኤችአይቪና የመሳሰሉት ህመም አለበወት ተብለዋል	1.አዎ 2. የለም
9.	ለወባ መድሃኒት (ለኩአርትምና ፕሪማኩይን አለርጅ ነወት)	1.አዎ 2. የለም
10.	የሚኖሩበት ቤት ከማክሰኝት ጤና ጣቢያ 20ኪ.ሜ ርቀት ውስጥ ነው የሚገኝ	1.አዎ (መልስወ አወ ከሆነ ወደ ተራ ቁጥር 11 ይሂዱ) 2. የለም
11.	በሚቀጥሉት 6 ወራት አሁን ከሚኖሩት ቦታ ይቆያሉ	1.አዎ (መልስወ አወ ከሆነ ወደ ተራ ቁጥር 11 ይሂዱ) 2. የለም
12.	ለሚቀጥሉት 6 ሳምንታት አሁን ከሚኖሩበት ቦታ ይቆያሉ	1.አዎ 2. የለም
12.	በሃኪም የተረጋገጠ ከፍተኛ የሆነ የደም ማነስ አለበወት	1.አዎ 2. የለም
13	ባለፉት 14ቀናት የወባ መድሃኒት ወስደዋል	1.አዎ 2. የለም
14.	ባለፉት 14 ቀናት ከወባ መድሃኒት ውጭ ሌላ መድሃኒት ወስደዋል መልስወ	1.አዎ 2. የለም
15	በወሰዱት የወባ መድሃኒት ምክንያት ሰውነተወት ላይ ያዩት የተለየ ችግር አለ	1.አዎ 2. የለም

Annex XII. Patient follow-up card

Patient follow-up card

Patient Identification number…….

Name…….

Scheduled visit day

Day	0	1	2	3	7	14	21	28	35	42	unscheduled
Appointment date											

Clinician name and sign……...

Annex XIII. Laboratory procedures

A. Procedural steps of collection of blood by EDTA-coated microtainer tube

1. Wash your hands and the participants thoroughly with soap and water; rinse and dry well.
2. Put on gloves.
3. Assemble equipment.
4. Clean the puncture area with an alcohol swab and allow it to air dry.
5. Warm and/or massage the finger to increase circulation before puncturing the skin.
6. Grasp the participant's finger between your thumb and index finger, with the palm of the participant's hand facing up.
7. Examine the participant's fingers to identify the best location for the finger stick:
8. Position the participant (lying down or sitting) and hyperextend the participant's arm.
9. Massage the finger to increase blood flow by gently squeezing from hand to fingertip 5-6 times.
10. Clean the fingertip using an alcohol wipe and dry it with a piece of gauze to obtain a blood drop at the puncture site.
11. Firmly press the lancet to make a skin puncture just off the center of the finger pad. The puncture must be made perpendicular to the ridges of the fingerprint to prevent blood from running along the ridges.
12. Wipe away the first drop of blood with a sterile gauze or cotton ball. The first drop of blood tends to contain excess tissue fluid.
13. Remove the cap of microtainer tube and place the cap aside in a clean location
14. Place the microtainer tube horizontally or at a slight incline and hold the finger down over the collection vent of the microtainer tube. A well-beaded drop of blood should form at the collection site and allow falling into the tube.
15. Fill the blood collection tube as quickly as possible to keep the specimen from clotting in the tube before it is mixed. **Tap the collection tube** with your finger while collecting the specimen. Draw time should be under 2 minutes. Avoid under-filling or overfilling the collection tube. Fill the tube according to the manufacturer's stated fill volume. Whole blood collection tubes contain only enough anticoagulant for the stated fill volume.

16. Immediately after collection, thoroughly mix the blood collection tube to prevent clotting. Be sure that the blood comes in contact with the entire inner surface of the blood collection tube so all the anticoagulant is mixed with the blood.
17. Once there is an adequate amount of blood in the microtainer tube, apply a cotton ball to the puncture site until the bleeding stops.
18. Mix the tube by turning it end over end, for 8-10 complete inversion
19. Properly label all the sample types (sample from a single participant should be labeled with the same code)

B. Procedures of Thick and thin blood films preparation

1. Label the frosted end of the glass slide with the participant's details, and document it in record form
2. Wearing protective latex gloves, select the third finger from the thumb of the non-dominant hand.
3. Hold the participant's hand, palm facing upwards, and clean the selected finger with a piece of cotton soaked lightly in 70% ethanol. Make sure the finger is warm by applying gentle massage if required. Let the alcohol dry from the finger.
4. Using a new, sterile contact-activated lancet and quick-rolling action; puncture the center of the ball of the finger.
5. Apply gentle pressure to the finger.
6. Wipe the first drop of blood off with dry cotton, making sure that no cotton strands remain on the finger that might stick to the blood.
7. Working quickly and handling the slides only by the edges, collect blood by applying gentle pressure to the finger and touching the slide to the blood; collect a single small drop of blood on the middle of the slide for the thin film.
8. Apply further gentle pressure to express more blood, and collect two or three drops on the slide about 1 cm from the drop intended for the thin film.
9. Wipe the remaining blood from the finger with clean, dry cotton.
10. Using a micropipette, place 6µL and 2µL of blood collected using EDTA-coated microtainer tubes for thick and thin smear preparation respectively.
11. Do not pause between applying and spreading the drops. Prepare the blood films with the slide lying on a flat surface.

12. To prepare the thin film, place the edge of a clean "spreader" slide at 45^0 in front of the blood drop intended for the thin film.
13. Slowly pull the "spreader" back until it touches the drop of blood and the blood spreads along the edge of the "spreader".
14. Rapidly push the "spreader" forwards (away from the center) in a smooth, continuous motion, until the spreader leaves a "feathery" end for the thin film.
15. With the corner of the same "spreader" used for making the thin film, make the thick film by swirling the three drops of blood together forming a circle of about 1 cm in diameter size.
16. After preparing the thin and thick blood films, allow them to dry in air in a horizontal position on a slide tray.
17. After both the thin and thick films are completely dried, fix the thin film by dipping it in absolute (100%) methanol for a few seconds and then letting the slide air dry completely before staining. Since, both thin and thick films will be made on the same slide, fix only the thin film carefully. The thick film should not be fixed. Dry the thin film at an acute angle, with the film side of the slide facing up and the thin film downwards. This protects the thick film from being fixed by methanol fumes and run-off.
18. After drying the films completely Giemsa staining will be done (separate SOP)

C **Procedure for DNA Extraction from DBS by using Qiagen DNA Extraction Kit**

Reagents
- ATL buffer
- AL buffer
- Ethanol (96-100%)
- Proteinase K
- AW1
- AW2
- AE buffer
- 70% Alcohol (disinfecting)

Supplies
- Cylinder gas
- Tips (200µl, 1000µl)

- Bunsen burner
- Pipettes (200µl, 1000µl)
- Match
- A bowel to discard tips
- Plaster to fix Bunsen burner on the bench
- Vortex
- Puncher (6mm)
- Falcon tube (mix the ATL & Proteinase K)
- Forceps
- Water Bath

- Paper (to cool down puncher)
- Heat Block
- Autoclaved eppendorf tubes
- Centrifuge
- A bowel to soak punchers and forceps o Collection tubes (ependroff)
- Spin column
- Disposable glove

NOTE:
- Equilibrate Buffer AE to room temperature, for elution in step 9.
- Prepare a 56°C incubator oven (heat block) for use in steps 2 and 4.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to instructions.
- If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 70°C.
- Use of a multichannel pipet is recommended.
- All centrifugation steps are carried out at room temperature.

1. Place 1 punched-out circles from a dried blood spot into a 1.5 ml micro centrifuge tube and add 200µl of mixture of ATL buffer and proteinase k. (Before the beginning of this step prepares a mixture of ATL buffer and proteinase k in a new falcon tube. The proportion

will be 190 µl ATL and 10 µl of proteinase k that will be multiplied by the sample number. **Note: The addition of proteinase K is essential)**

2. Incubated at 56°C and 450 rpm overnight in a heat block. (Alternative incubation at 56°C and 1000rpm for 2 hours and kept in water-bath over night at the same T0)
3. Briefly centrifuge to remove drops from inside the lid then squeeze the DBS disc and remove it. (Use 1000 µl pipette tip for squeezing)
4. Add 200µl of buffer AL to the sample. Mix thoroughly by vortexing and incubate 56°C for 10 minutes. Briefly, centrifuge to remove drops from inside the lid. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly. The precipitate will dissolve during incubation but it does not interfere with the QIAamp procedure.
5. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. Briefly, centrifuge to remove drops from inside the lid. It is essential that the sample and ethanol are mixed thoroughly.
6. Carefully apply the mixture (approximately 600 µl) from step-5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at centrifuged at 6000rcf for 1min. (Place the QIAamp Mini spin column in a clean 2ml collection tube (provided), and discard the tube containing the filtrate).
7. Carefully open the QIAamp Mini spin column and add 500µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000rcf for 1 min. (Place the QIAamp Mini spin column in a clean 2ml collection tube (provided) and discard the collection tube containing the filtrate.)
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at 20,000 rcf for 3 min. (Place the QIAamp Mini spin column in a new 2 ml Eppendorf tube (not provided) and discard the old collection tube with the filtrate).
9. Carefully open the QIAamp Mini spin column and add 50 µl Buffer AE. Incubate at room temperature (15–25°C) for 1minute, and then centrifuge at 6000 rcf for 1 min. (Note: Repeating the last step to get better volume of yield)

D. Procedure for malaria detection using Nested PCR

Principle: -Nested PCR is the most ultrasensitive method for the detection of *Plasmodium* parasites. DNA can be extracted through different extraction methods. After preparation of the PCR master mix, aliquots of the DNA sample will be mixed with it and run in the thermocycler according to the procedure. Nested PCR uses two sequential sets of primers. The first primer set binds to sequences outside the target DNA, as expected in standard PCR, but it may also bind to other areas of the template. The second primer set binds to sequences in the target DNA that are within the portion amplified by the first set (that is, the primers are nested). Thus, the second set of primers will bind and amplify target DNA within the products of the first reaction. The first one is for the detection of the *plasmodium* parasite at the generic level. The second one is for detection at the species level. Finally, the second PCR product will be run on gel electrophoresis for the detection of PCR bands specific to the malaria species and it will be documented using UV imaging and a recording device attached to the computer for analysis

Equipment

- ✓ Biosafety Cabinet
- ✓ Thermocycler
- ✓ Centrifuge
- ✓ Pipettes of different volumes (10, 20, 50, 1000µl)

Materials

- ✓ PCR tubes/plates
- ✓ Pipet tips (10, 200, 1000 µl)
- ✓ Cryo tubes for master mix preparation
- ✓ Gloves
- ✓ M-tork
- ✓ Racks for holding the PCR tubes

Reagents

- ✓ 70% Ethanol
- ✓ DNA away Solution

- ✓ PCR master mix reagents (PCR grade water, PCR buffer, Forward primer, Reverse primer, Mg Solution, dNTPs, Taq polymerase)

Procedure

1. Before starting to work in the master mix room, put on the safety hood and the light.
2. Clean the working area (safety hood) first using 70% ethanol and wiping it with M-tork, and then with DNA away.
3. Take out the master mix reagents from the fridge except for Taq polymerase and leave them to thaw for some time.
4. Taq polymerase can be withdrawn from the fridge after the other components are mixed.
5. Prepare the PCR master mix in the following sequential manner.
6. Add the required volume of PCR-grade water into 2ml of cryotube.
7. Then add PCR buffer, then add Mg solution, then add dNTPs, then add the forward and reverse primers, respectively.
8. Finally, add Taq polymerase.
9. Label PCR tubes or plates according to your sample numbers.
10. Return the reagents to the refrigerator once the master mix is complete.
11. For the Nested-1 (N1) PCR reaction, dispense 20µl of the master mix into each PCR tube or plate.
12. Then add 5uL of the DNA template to make the total PCR reaction volume in a single PCR tube 25µl is going to be added in a separate room.
13. For Nested-2 (N2) PCR reactions, prepare a master mix similar to the previous preparation.
14. Take 2µl of N1 PCR product and mix it with 23µl of master mix to make 25µl of N2 reaction.
15. Two genus-specific primers, rPLU6 and rPLU5, are used for the first cycle of amplification (N1).
16. For N2 (species-specific primers), *Plasmodium falciparum* rFAL and rFAL2 primers are used.
17. Finally, clean the working area and take the prepared PCR master mix aliquots and DNA samples to the sample room.

18. The safety hood in the sample room must be cleaned with 70% ethanol and M-tork.

19. Dispense the DNA template sample into the prepared master mix aliquots according to their sample number.

20. Then, after finishing in the sample room, take the prepared master mix and DNA template sample to the PCR room.

21. Then centrifuge it using a mini-centrifuge to make sure the mixtures are mixed evenly.

22. Program the PCR machine (thermocycler) as follows.

- For **Nested-1 (N1) PCR:**
  - ✓ Initial denaturation at 95ºc for 10 minutes,
  - ✓ Denaturation at 95ºc for 1 minute for 35 cycles.
  - ✓ annealing at 58 ºc for 1 minute
  - ✓ Extension at 72ºc for 1.5 minutes
  - ✓ and a final extension at 72ºc for 10 minutes.
- For **Nested-2 (N2) PCR**:
  - ✓ Initial denaturation at 95ºc for 10 minutes,
  - ✓ denaturation at 95ºc for 1 minute for 30 cycles,
  - ✓ annealing at 58 ºc for 1 minute
  - ✓ extension at 72ºc for 1 minute
  - ✓ And final extension at 72ºc for 10 minutes.
    - Place PCR tubes or PCR plates in the PCR machine.
    - Run the PCR machine.