JIMMA UNIVERSITY

THERAPEUTIC EFFICACY OF ARTEMETHER-LUMEFANTRINE FOR THE TREATMENT OF UNCOMPLICATED FALCIPARUM MALARIA AND GENOTYPING OF *PLASMODIUM FALCIPARUM MSP-1* AND *MSP-2* GENES IN ETHIOPIA

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This Ph.D. dissertation; entitled "Therapeutic efficacy of artemether-lumefantrine for treatment of uncomplicated falciparum malaria and genotyping of *Plasmodium falciparum msp-1* and *msp-2* genes in Ethiopia" has been submitted for examination with my approval as **Candidate's Promoter (supervisor).**
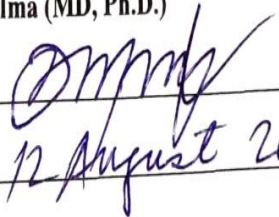
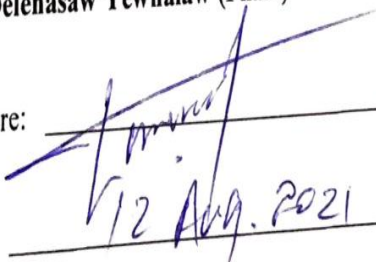**Main Promotor:**

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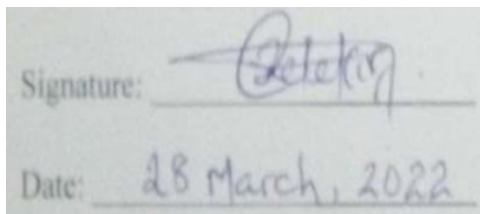
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Dedicated to my father Abamecha Abafogi and my mother Fatima Ababillo

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LIST OF ABBREVIATIONS ACRONYM

ACPR	Adequate clinical and parasitological response
ACT	Artemisinin-based combination therapy
ADRs	Adverse drug reactions
AE	Adverse events
AL	Artemether–lumefantrine
DHP	Dihydroartemisinin–piperaquine
ETF	Early treatment failure
He	Heterozygosity index
LCF	Late clinical failure
LPF	Late parasitological failure
MOI	Multiplicity of infection
MSP	Merozoite surface protein
NOS	Newcastle Ottawa Scale
PCR	Polymerase chain reaction
PFMDR1	*Plasmodium falciparum* multidrug resistance protein 1
PICOS	Participants, population, Intervention, comparator(s), outcome(s), study design
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
RBC	Red blood cell
RCT	Randomized controlled trials
ROBINS-I	Risk of Bias in Non-Randomized Studies of Interventions
SAE	Serious adverse events
TF	Treatment failure
WHO	World Health Organization

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ABSTRACT

The introduction of artemisinin-based combination therapy (ACT) substantially reduced malaria-related mortality and morbidity during the past two decade. However, there is limited information on ACT effectiveness in routine health care when treatment is not monitored. Malaria patients infected by multiple parasite strains have been shown to be high risk of treatment failure. Genetically distinct malaria parasites in natural population have an extremely high rate of recombination during sexual stage in mosquito gut during zygote formation, resulting in gene variations of *P. falciparum*. Because of this variation, the conformation of anti malarial drug targets is altered and then renders the parasite drug resistant which hinders the outcome of malaria treatment. Hence, broad understanding of the genetic variations of the parasite population can contribute to the definition of control measures including an appropriate anti-malarial treatment

The first section of this PhD study is a prospective study of the clinical and parasitological efficacy of artemether-lumefantrine (AL) to directly observed therapy for uncomplicated *P. falciparum* malaria according to WHO revised protocol for malaria drug therapeutic efficacy study. Real-time polymerase chain reaction (PCR) and nested PCR reaction methods were used to quantify and genotype *P. falciparum*. Of the 80 study participants enrolled, 75 completed the follow-up at day-28 with ACPR. For per protocol (PP) analysis, PCR-uncorrected and -corrected cure rate of AL among the study participants was 94.7% (95% CI 87.1–98.5) and 96% (95% CI 88.8–99.2), respectively. For intention to treat (ITT) analysis, the cure rate was 90% (95% CI 88.8–99.2). Based on Kaplan–Meier survival estimate, the cumulative incidence of failure rate of AL was 3.8% (95% CI 1.3–11.4). Only three participants 3.8% (95% CI 0.8–10.6) of the 80 enrolled participants were found to be positive on day-3. The day three positive participants were followed up to day 28 and did not correspond to treatment failures observed during follow-up. Only 7.5% (6/80) of the participants were gametocyte positive on enrollment and gametocytaemia was absent on day-2 following treatment with AL. The findings of this study advocate for the continuous use of AL as first-line therapy for uncomplicated malaria in Ethiopia. However, the threat of spreading or de novo development of artemisinin resistance, comprehensive and 'regular surveillance of ACT partner drugs needs be conducted' to not only

ensure early detection of resistance to *P. falciparum* but also guarantee informed decisions by policy makers on matters of malaria treatment.

The second section of this PhD study assesses the genetic polymorphism and multiplicity of *P. falciparum* infection from clinical samples using the *msp-1* and *msp-2* genes. Of 80 qPCR-positive samples analysed for polymorphisms on *msp-1* and *msp-2* genes, the efficiency of *msp-1* and *msp-2* gene amplification reactions with family-specific primers were 95 % and 98.8%, respectively. Allelic variation of 90% (72/80) for *msp-1* and 86.2% (69/80) for *msp-2* were observed. K1 was the predominant *msp-1* allelic family detected in 20.8% (15/72) of the samples followed by MAD20 and RO33. Within *msp-2*, allelic family FC27 showed a higher frequency (26.1%) compared to IC/3D7 (15.9%). Ten different alleles were observed in *msp-1* with 6allelesforK1, 3 alleles for MAD20 and1 allele forRO33. In *msp-2*, 19 individual alleles were detected with 10 alleles for FC27 and 9 alleles for 3D7. Eighty percent (80%) of isolates had multiple genotypes and the overall mean multiplicity of infection was 3.2 (95% CI: 2.87-3.46). The heterozygosity indices were 0.43 and 0.85 for *msp-1* and *msp-2*, respectively. There was no significant association between multiplicity of infection and age or parasite density. Thus, this information will serve as a baseline molecular evidence for further research on areas having similar malaria epidemiology to make the control and elimination efforts of malaria effective.

The third section of this PhD study is a systematic review and meta-analysis aimed to synthesize the available evidence on the efficacy of AL for the management of uncomplicated falciparum malaria in Ethiopia. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed. Relevant published studies were searched from the databases (PubMed, Google Scholar and Clinical trial registry) on published AL therapeutic efficacy studies conducted in Ethiopia from 2004 to 2020. The retrieved studies were assessed for quality using the modified Newcastle Ottawa Scale for observational studies and modified Jadad scale for interventional studies. Risk of bias was also assessed by using ROBINS-I tool. OpenMeta-Analyst software was used for the statistical analysis. The review protocol is registered in PROSPERO, number CRD42020201859. Fifteen studies (1523 participants) were included in the final analysis. The overall PCR-uncorrected pooled proportion of treatment success of artemether-lumefantrine therapy for uncomplicated falciparum malaria was 98.4%

(95%CI: 97.6-99.1). A random-effects model was used because of considerable heterogeneity (χ^2=20.48, *df*(14), *P*=0.011 and I^2=31.65). PCR-corrected pooled proportion of treatment success of artemether-lumefantrine therapywas 98.7% (95% CI 97.7–99.6).A random-effects model was used (χ^2=7.37, *df*(6), *P*=0.287 and I^2=18.69). Most studies included in the present review achieved a rapid reduction of fevers and parasitaemia between D0 and D3 of assessment. Adverse events were mostly mild and only two cases were reported as serious, but were not directly attributed to the drug. The present meta-analysis suggests that AL therapy is efficacious and safe in treating uncomplicated falciparum malaria in Ethiopia. However, owing to the high risk of bias in the included studies, strong conclusions cannot be drawn. Further high-quality RCTs assessing anti-malarial efficacy and safety should be performed to demonstrates strong evidence of changes in parasite sensitivity to AL in Ethiopia.

In summary, this PhD thesis focused on evaluating the efficacy of AL for the management of uncomplicated falciparum malaria in Ethiopia. Also, examined the genetic variation of *P. falciparum msp-1* and *msp-2* genes that can be used to assess intensity of parasite transmission and identify potential deficiencies in malaria control programmes, which provides vital information to evaluating malaria elimination efforts; strong recommendation from studies such as this PhD project, provide quality evidence that can be used to support national malaria control decision making for optimal impact in further reducing malaria transmission in the region.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Global malaria burden

Malaria continues to be the most important parasitic disease worldwide, despite a wide implementation of control and elimination measures through the international and national malaria control programs [1]. In the last two decades an historic progress against malaria has made, saving an estimated 7.6 million lives, preventing over 1.5 billion new malaria infections. In 2019, World Health Organization (WHO) estimated 229 million malaria cases and 409,000 deaths, mostly among children under the age of five in 87 malaria-endemic countries. Global malaria case incidence and death rates reduced by 29% and 60%, respectively, between 2000 and 2019, though the incidence and death rates have seen a slowing annual rate of decline in recent years. The case incidence (i.e. cases per 1000 population at risk) reduced from 80 in 2000 to 58 in 2015 and 57 in 2019 globally. Between 2000 and 2015, global malaria case incidence declined by 27%, and between 2015 and 2019 it declined by less than 2%, reflecting a slowing of the rate of decline since 2015 and the number of cases and deaths worldwide has plateaued, in stark contrast to the rapid decline seen over the previous decade [1].

As in past years, the African Region shouldered more than 90% of the overall disease burden. Since 2000, the region has reduced its malaria death toll by 44%, from an estimated 680 000 to 384 000 annually. However, progress has slowed in recent years, particularly in countries with a high burden of the disease [1, 2]. Nearly 99% of all estimated cases reported in the WHO African Region were caused by *Plasmodium falciparum* (Except in horn of Africa), and accounted for about 94% of all malaria cases and deaths globally in 2019[1]. *Plasmodium vivax*, the second biggest contributor to disease burden, is the most prevalent species, giving rise to cases within 95 countries across the globe, more common in the Americas and Asia & the Pacific [3]. However, the four remaining species, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri*, *Plasmodium malariae*, and the zoonotic parasite, *Plasmodium knowlesi*, still contribute a considerable disease burden, and must not be ignored in the strive towards malaria eradication[1,4].

Apart from illness and deaths, malaria has paused a profound economic effect [5]. The direct costs of malaria prevention and treatment are estimated to be over 12 billion USD per year.

However, this figure does not take into account the overall loss of capital a nation experiences as a result of illness and death reducing the workforce and, more indirectly, interrupting access to education. The ensuing poverty, however, in turn directly impacts public health, as individuals and entire nations are less able to afford nutritious food, quality housing, and accessible healthcare [5].

WHO's Global technical strategy (GTS) for malaria 2016-2030, endorsed by the World Health Assembly in May 2015, and the Roll Back Malaria (RBM) Partnership's *Action and Investment to defeat Malaria* (*AIM*) have embraced the goal of a "world free of malaria" and is designed to guide and support all malaria-affected countries as they work to reduce and eliminate the human suffering caused by the world's deadliest mosquito-borne disease [1, 2]. The strategy sets ambitious targets aimed at dramatically lowering the global malaria burden over a 15-year period, with milestones at each five year mark to track progress towards the targets and milestones of the GTS (a reduction in malaria case incidence and mortality rate of at least 40% by 2020, 75% by 2025 and 90% by 2030 from a 2015 baseline). The GTS is fully aligned with Sustainable Development Goals (SDGs); ending malaria is vital for achieving the SDGs beyond health, including those related to education, poverty eradication and gender equality [1, 2, 6]. It is synergistic. Since 2017, WHO has supported a group of 21 malaria-eliminating countries through a special initiative called the "E-2020" [7]. This report charts their progress towards a common goal: eliminating malaria within the 2020 timeline. According to this report, eight E-2020 member countries had successfully reported zero indigenous cases of malaria: Algeria, Belize, Cabo Verde, China, El Salvador, the Islamic Republic of Iran, Malaysia and Paraguay. However, the achievement of most of the GTS targets is currently off track. Of the 92 countries that were malaria endemic globally in 2015, 31 (34%) were estimated to be on track for the GTS morbidity milestone for 2020, having achieved 40% or more reduction in case incidence or reported zero malaria cases [1]. Analysis of the trends by region shows that the WHO African Region is off track for both the malaria morbidity and mortality 2020 GTS milestones, by 37% and 25%, respectively. Only Botswana, Cabo Verde, Ethiopia, the Gambia, Ghana, Namibia and South Africa are on track to achieve the GTS 2020 target of a 40% reduction in malaria case incidence, and Algeria has already been certified malaria free [1]. Botswana, Cabo Verde, Eswatini, and Sao Tome and Principe reported zero malaria deaths in 2019 and were projected to maintain this in 2020. Ethiopia and Namibia were estimated to have achieved a reduction in

mortality rate of more than 40% [1]. The progression from control to elimination status is hindered for many endemic nations due to several parasitological challenges. Firstly, resistance to front line antimalarials, including artemisinin and partner drugs, continues to rise [8, 9, 10]. Secondly, asymptomatic carriers and zoonotic sources create permanent reservoirs of infective parasites [11, 12]. Finally, hypnozoites, dormant forms of the parasite, cause relapsing *P. vivax* and *P. ovale* cases [13, 14].

To reignite the pace of progress and to get back on track to meet the GTS milestones, WHO and the RBM partnership to end malaria catalysed the "High Burden to High Impact" (HBHI) response, launched in 2018. HBHI builds on the principle that no one should die from a disease that is preventable and treatable. It is led by 11 countries, ten in sub-Saharan Africa (Burkina Faso, Cameroon, Democratic Republic of the Congo, Ghana, Mali, Mozambique, Niger, Nigeria, Uganda and United Republic of Tanzania) and India that, together, accounted for approximately 70% of the world's malaria burden in 2017[1]. Over the last two years, HBHI countries have implemented activities across four response elements: (i) Political will to reduce malaria deaths; (ii) Strategic information to drive impact, (iii) Better guidance, policies and strategies, and (iv) A coordinated national malaria response. While it is too early to measure the impact of the HBHI approach, the report shows that in the first year: The total number of cases in the 11 HBHI countries increased slightly from 155 million in 2018 to 156 million in 2019. Between 2018 and 2019, cases in India were reduced by 1.2 million and in Mali by 800 000. Over the same time frame, there was an increase in cases in Nigeria (2.4 million) and in the Democratic Republic of the Congo (1.2 million). Deaths were reduced in the 11 countries from 263 000 in 2018 to 226 000 in 2019[1].

The slow progress over the recent years as incidence has plateaued [1] and uneven global progress against malaria in recent years can be attributed to a variety of factors including, inadequate funding, emergence and spread of drug-resistant parasites and insecticide-resistant mosquitoes, suboptimal rapid diagnostic tests (RDTs), lack of universal access to malaria prevention and treatment and the lack of a highly effective vaccine, increasing population movement, and rising cases in the highest burden countries[1].

Generally, the future of malaria control is critically impacted by external factors, including population growth, migration, poverty, inequity, complex emergencies and climate change,

combined with weak health systems and biological threats, such as insecticide and drug resistance. Reduction in effective intervention coverage carries a high risk of rebounds and epidemics. Investment in core epidemiological and entomological capacity in countries is critical to identifying needs and deploying interventions. Acceleration of progress will require optimization of strategies and innovations both in delivery of available interventions and in new tools and approaches, as well as increased financial investment [1, 6].

1.1.1   Malaria burden in Ethiopia

Ethiopia has made remarkable progress in decreasing the prevalence and burden of malaria through public health measures taken in the last two decades [15]. However, malaria remains one of the public health problems with a high level of mortality and disability adjusted life years (DALYs) [16]. The economic burden of malaria is substantial in Ethiopia, leading to catastrophic costs for rural households [17]. The lost productive personnel due to malaria illness, school absenteeism, direct and indirect costs are the major economic burdens of malaria in Ethiopia [18]. The seasonal transmission of malaria in Ethiopia has complicated the burden of malaria because the peak malaria transmission season and the major planting and harvesting period coincide. Malaria is also among the diseases that classified as major killers of under five children [19]. Accordingly, malaria stands to be one of the top priority programmes in the national health and overall socioeconomic development agenda. Malaria prevention, control and elimination have been given due attention by the government and its partners. Moreover, the country launched a malaria elimination programme, which in turn demonstrated the government's commitment in the fight against the disease [15].

In the past five years, mortality and morbidity from malaria has declined dramatically. Between 2015 and 2019, malaria deaths dropped from 3.6 to 0.3 per 100,000 populations at risk, and malaria case incidence dropped from 5.2 million in 2015 to less than 1 million in 2019[20]. In 2017/18, a malaria elimination program was launched, with activities carried out in 239-targeted districts. The country has started a sub-national elimination program to comprehensively interrupt local transmission of the disease by 2030[20].

According to the recent malaria program review (MPR) 2020 report, malaria control initiatives have been on track [15, 21]. Between 2016 and 2019 the mortality due to malaria has declined by 67% from 0.9/100,000 population to 0.3/100,000 population at risk. Similarly, the annual

parasite incidence (API) has declined by 37% from 19/1000 population to 12/1000 population between 2016 and 2019. The number of confirmed malaria cases has reduced by 47% between 2016 and 2019. This reduction is in line with the NMSP target that aims at reducing malaria cases by 40% by the end of 2020 from baseline of 2016[15]. Accordingly, Ethiopia achieved the 2020 GTS milestones of reducing malaria incidence and deaths by 40% compared to 2015. However, high-level resistance of malaria vectors to insecticides, sub-optimal usage of interventions by target communities, complacency in maintaining the momentum, delay in implementing the national case management guidelines, and shortage of complete and timely data for evidence-based decision-making are remaining challenges that need close attention [15].

Following considerable successes in the control of malaria in the last two decades, the Ethiopian Health Sector Transformation Plan Two (HSTP-II) aims for a reduction in mortality rate from 0.3/100,000 population at risk to 0.2 in 2024/2025, and a reduction in incidence of malaria from 28 per 1,000 population at risk to 8 per 1,000 from a 2019 baseline [20].

The NMSP's top priority is building a robust, timely, and effective surveillance and response system. Such surveillance system will actively detect and respond to outbreaks, test, treat, and track individual cases, and investigate cases or foci with appropriate mitigation actions and monitor overall progress in implementation of planned activities. Additionally, the NMSP strives for significant malaria burden reductions in high and moderate transmission settings, eliminate the disease in low transmission districts and prevent reintroduction of malaria into districts reporting zero indigenous malaria cases. The proposed goals from 2021-2025 were reducing malaria morbidity and mortality by 50 percent from baseline of 2020 and achieve zero indigenous malaria in districts with annual parasite incidence of less than 10 malaria cases and prevent reintroduction of malaria in districts reporting zero indigenous malaria cases by 2025. In general, this strategy focuses on national malaria elimination programme, addresses the recommendations of the 2020 malaria programme review and responds to the major implementation gaps identified so far [15].

Despite the achievement, unstable malaria transmission patterns with other contributing factors such as a large and mobile population, heterogeneous transmission, and the presence of both *P. falciparum* and *Plasmodium vivax* malaria parasites make an estimated 52% of the population are at risk of contracting malaria[15]. Compared to other endemic countries in Sub-Saharan

Africa, malaria prevalence in Ethiopia is relatively low, but a few high-burden areas in the western lowlands remain a major threat to the elimination efforts in other districts. The heterogeneous transmission in Ethiopia is largely due to variation in elevation. Elevations below 2,000 meters have the highest transmission potential and contain the majority of the population [15, 22].

1.2 The malaria parasite: Origins and types of human-infective Plasmodium species

Of the five human *Plasmodium* species, *P. falciparum* belongs to subgenus *Laverania*, whereas all the others belong to subgenus *Plasmodium* [23]. *Plasmodium* species have remarkable genetic flexibility which lets them adapt to alterations in the environment, giving them the potential to quickly develop resistance to therapeutics such as antimalarials and to change host specificity. The origins of the human-specific Plasmodium species have been the subject of much debate. One hypothesis, popular until about 10 years ago, suggested that humans and chimpanzees acquired *P. falciparum*-like infections from their common ancestor and that these parasites had coevolved with their respective host species for millions of years [23, 24, 25]. In contrast*, P. vivax* was believed to have emerged in Southeast Asia following the transmission of a macaque parasite to humans [23, 24, 25]. However, the discovery of parasites closely related to *P. falciparum* and *P. vivax* in wild-living chimpanzees (Pan troglodytes), bonobos (Pan paniscus), and western gorillas (Gorilla gorilla) has contradicted these theories, indicating that both human pathogens emerged much more recently from parasites infecting African apes [23, 26, 27]. Although the origins of *P. malariae, P. ovale curtisi, and P. ovale walliker*i have not yet been fully deciphered, all of these human parasites also have closely related chimpanzee, bonobo, and/or gorilla counterparts [23, 28, 29, 30]. Though, many questions remain concerning the biology and zoonotic potential of the *P. falciparum* and *P. vivax*-like parasites infecting apes, comparative genomics, coupled with functional parasite and vector studies, are likely to yield new insights into ape *Plasmodium* transmission and pathogenesis that are relevant to the success of malaria control and elimination efforts of human malaria.

To date, over 250 species of *Plasmodium* have been formally described, and each species infects a certain range of hosts. *Plasmodium* species that naturally infect humans and cause malaria in large areas of the world are limited to five, i.e. *P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi* [4, 31]. *P. ovale* has been identified to exist in dimorphism of classical and variant

types i.e., *P. ovale curtisi* and *P. ovale wallikeri* respectively. All of *Plasmodium* species have variable severity and geographical distribution and the relative prevalence of these different species in endemic areas has been changing with the ongoing malaria control efforts.

1.2.1 *Plasmodium knowlesi*

Unlike the other species pathogenic to humans, the primary hosts of *P. knowlesi* are the long-tailed and pig-tailed macaques and can also cause severe human malaria if not treated early [32]. The majority, if not all, human *P. knowlesi* infections are thought to arise from an *Anopheles* mosquito first biting an infected macaque and then transmitting parasites to the human host. This phenomenon means *P. knowlesi* infections are geographically restricted by the requirement for overlapping human, macaque, and vector populations, resulting in transmission being limited to South-east Asia [33]. Practically speaking, this also means that risk factors for contracting *P. knowlesi* include working in and around forested areas, farmlands, and plantations, where both macaques and mosquito vectors are present[12, 34]. Importantly, since transmission between macaques cannot be curtailed with normal control measures, such as treatment with drugs, or using bed-nets, macaques remain a constant source of infection to humans within a given region [12]. Therefore, the development of a vaccine may be the only way to prevent *P. knowlesi* infections without also impacting macaque populations.

A potential reason for the emergence and rise in *P. knowlesi* infections is continuous deforestation, which ultimately brings human and macaque/vector populations into closer contact [35]. It has recently been shown to be a significant cause of zoonotic human malaria in that region, particularly in Malaysia where it accounts for up to 87% of human malaria cases [36, 37].The recent increases in cases and their severity may also be indicative of changes in host pathogen interactions and the emergence of some human-human transmission [38, 39]. There is also evidence that the current increasing risk from *P. knowlesi* may be partly due to an indirect consequence of successfully controlling other human malaria parasites [38, 39]. Recently, *P. vivax* antibodies targeting several erythrocyte invasion antigens demonstrated cross-inhibitory action against *P. knowlesi* parasites in culture. Thus the spread and intensity of *P. knowlesi* infections may be curbed by cross-immunity to *P. vivax* [38, 39].

1.2.2 *Plasmodium malariae*

P. malariae is often reported as a benign malaria parasite [42], distributed over most of the malaria endemic area and is the only human malaria species that has a quartian (72 hours) cycle [43]. If untreated, *P. malariae* often causes a long-lasting, chronic infection which often remains latent and can in some cases probably last a lifetime. Infections with *P. malariae* can in chronically infected patients cause serious complications such as nephrotic syndrome [44]. In Africa mixed infections with *P. falciparum* and *P. ovale* are common, and all three species can even occur simultaneously. *P. malariae* preferably invades RBCs older than 100 days making parasite densities seldom exceed 1% [42, 44].

1.2.3 *Plasmodium ovale*

P. ovale is found mostly in Africa (especially West Africa) and on the islands of the western Pacific. It is biologically and morphologically very similar to *P. vivax*. However, and in contrast to *P. vivax*, it can infect individuals who are negative for the Duffy blood group. Genetic studies have shown that *P. ovale* actually comprises two non-recombining species that are sympatric in Africa and Asia and these are morphologically identical [45]. Mixed infections with *P. falciparum* are common. Like *P. vivax, P. ovale* has long been considered to have a dormant hypnozoite stage that can persist in the liver and cause relapses, but Richter et al. have questioned whether such a stage actually exists for *P. ovale* [14]. *P. ovale* also has a tertian cycle. Both *P. vivax* and *P. ovale* preferably invades young RBC, i.e., reticulocytes, which make parasite densities therefore seldom exceed 1%.

1.2.4 *Plasmodium vivax*

P. vivax which is geographically more widespread than *P. falciparum* and responsible for about half of all malariarelated morbidity outside of Africa [14]. *P. vivax* is found mostly in Asia, Latin America, and in some parts of East Africa. It has been believed that *P. vivax* was virtually absent in Africa, because the absence of erythrocyte receptors for *P. vivax* (Duffy antigen) in most African population, tendering protection against the infection [14]. There is however growing evidence that the protection conferred to Duffy-negative individuals is not 100% effective, and the relative prevalence of *P. vivax* is increasing across Africa, especially in countries at the horn of Africa, such as Ethiopia, Djibouti, Eritrea, Somalia, Sudan and South Sudan [14, 46, 47]. This makes it more urgent to understand the true prevalence of *P. vivax* in

Africa to be able to eliminate malaria, especially since *P. vivax* requires different treatment and elimination strategies due to the hypnozoite stage (dormant parasite stage in the liver).

1.2.5 *Plasmodium falciparum*

P. falciparum parasite belongs to the lineage Laverania subgenus and has been suggested to be of more recent origin compared to other malaria species due to the low level of polymorphism within the *P. falciparum* genome [29, 30, 48]. However, other researchers have found that the *P. falciparum* parasites co-evolved with its human host [49, 50] and recent reports have shown that *P. falciparum* can infect both monkeys and gorillas [23, 26, 27, 28, 29, 50]. This may complicate concerns regarding the feasibility to eliminate malaria from areas where these reservoirs are maintained.

The most malignant form of malaria is caused by this species. *P falciparum* is able to infect RBCs of all ages, resulting in high levels of parasitemia because each schizonts can harbor up to 32 merozoites that are able to infect RBCs of all ages [52]. This enables the infection to become hyper parasitemic with parasite densities of more than 5% causing massive lysis of RBCs and subsequent anemia, a common cause of severe malaria in children. *P. falciparum* has a tertian (48 hour) cycle even though fever paroxysms generally do not show a distinct periodicity.

Maturing stages of *P. falciparum* are expressing cyto-adherent proteins, forming knobs on the RBC surface. The *P. falciparum* Erythrocyte Membrane Protein1 (PfEMP1), encoded by the *var* gene family, plays a major role in cytoadherence [53]. The knobs make the infected cells "sticky", binding uninfected RBCs to their surface forming rosettes [54, 55]. These proteins also mediate binding of the infected RBC to the endothelial of the deep vessels, known as sequestration. This prevents the infected RBCs from being cleared from the circulation by the spleen [56]. The sequestered parasites clog in the vessels hampering the blood circulation. When this occurs in the brain it can result in cerebral malaria [57], a complication that stands for a large proportion of malaria deaths. Since *P. falciparum* malaria claims hundreds of thousands lives annually in sub-Saharan Africa, and is prevalent in Ethiopia, this PhD thesis focuses on *P. falciparum* hereafter.

1.3 Malaria life cycle

1.3.1 The hidden malarial life cycle

Recent studies reported that large numbers of malaria parasites hiding in the human spleen where they actively multiply in a previously unrecognized life cycle [58, 59]. Until now, it was thought that once malaria parasites reached the blood stream, they circulated and multiplied only in the blood. However, researchers recently found that in chronic malaria, concentrations of parasites were hundreds to thousands of times higher in the spleen than found in the circulating blood and indicated that some people with large numbers of parasites hiding in the spleen do not have parasites detectable in the blood [58, 59]. Kho et al. [58] examined spleens from people in Papua, Indonesia, undergoing spleen removal following road accidents. This study found that the patients generally had no symptoms of malaria before the accident, but 95 percent of patients had large numbers of live parasites hiding in the spleen and suggested the importance of redefining the malaria life-cycle.

Accumulation of parasites in the spleen was found with both major Plasmodium species causing malaria, but was particularly apparent in *P. vivax*, where over 98 percent of all the parasites in the body were hiding in the spleen [59]. The study also found that the human spleen traps large numbers of young red blood cells, called reticulocytes, which are the only type of red cell that *P. vivax* can infect. This makes the spleen a highly favorable location in which the vivax malaria parasites can multiply. While the spleen does filter out and destroy some parasites, the researcher now show it also provides a shelter for long-term persistence of parasites. These findings emphasized that persistent infection of the spleen has major clinical and public health implications, including a significant contribution to anemia [58]. This is another factor limiting the success of malaria elimination programs relying on mass testing of blood and only treating those with detectable infection.

1.3.2 The *Plasmodium falciparum* life cycle

Malaria parasites pose a challenge to vaccine production because of the different stages of its life cycle which involves two hosts [60, 61]. The primary host of malaria is the female *Anopheles* mosquitoes and the secondary host the human being, all *Plasmodium* species shares a similar life cycle. The size and genetic complexity of the parasite mean that each infection presents thousands of antigens (proteins) to the human immune system. The parasite

also changes through several life stages even while in the human host, presenting different antigens at different stages of its life cycle [31, 61]. Understanding which of these can be a useful target for vaccine development has been complicated. In addition, the parasite has developed a series of strategies that allow it to confuse, hide, and misdirect the human immune system [62].

The complex life cycle of the *Plasmodium* parasite begins when a female *Anopheles* mosquito injects parasites in the form of sporozoites into the dermis of a vertebrate host [Figure 1]. Sporozoites travel through the blood stream to the liver, where they multiply asexually to form liver schizonts. In some species, a small fraction of the parasites remains dormant in the liver as hypnozoites. Once mature, liver schizonts release merozoites back into the blood stream. The merozoites infect red blood cells (RBCs), mature into ring stage parasites, progress to trophozoites and eventually become blood schizonts that release new merozoites into the blood. This asexual multiplication of blood stage parasites, also referred to as the intra-erythrocytic developmental cycle, leads to 10 to 30-fold increase in parasite numbers every 24-72 h (every 24h (*P. knowlesi*), 48h (*P. falciparum, P. ovale, P. vivax*) or 72h (*P. malariae*) hours.), and is responsible for all clinical symptoms[31, 60]. A small number of these asexual parasites differentiate into male and female gametocytes, the sexual forms of *Plasmodium*, which are taken up by a mosquito during a blood meal. The gametocytes are activated once exposed to the specific environment of the mosquito midgut lumen, and the male and the female gametocytes differentiate to produce microgametes and macrogametes, respectively. The microgamete fertilises the macrogamete to produce a zygote, the only developmental stage of the parasite that has a diploid genome [60]. Genetic crossing experiments with gametocytes of two clones of *P. falciparum* with different allelic variants demonstrated that recombination can occur in zygotes. Soon the zygote undergoes meiosis and differentiates into a motile form, the ookinete that now contains four haploid genomes in its nucleus. The ookinete penetrates the wall of the mosquito midgut and forms an oocyst on the outer side. In the oocyst, several rounds of mitosis take place, and numerous sporozoites are produced by sporogony. When the oocyst matures, it ruptures, and sporozoites released into the haemolymph migrate to the salivary glands, where they acquire the ability to infect human cells when released into the body of a vertebrate host during a blood meal. Human-infecting Plasmodium species complete this second part of the life cycle (gametocytes to sporozoites ready to infect the next person) in around 10-18 days [31, 60].
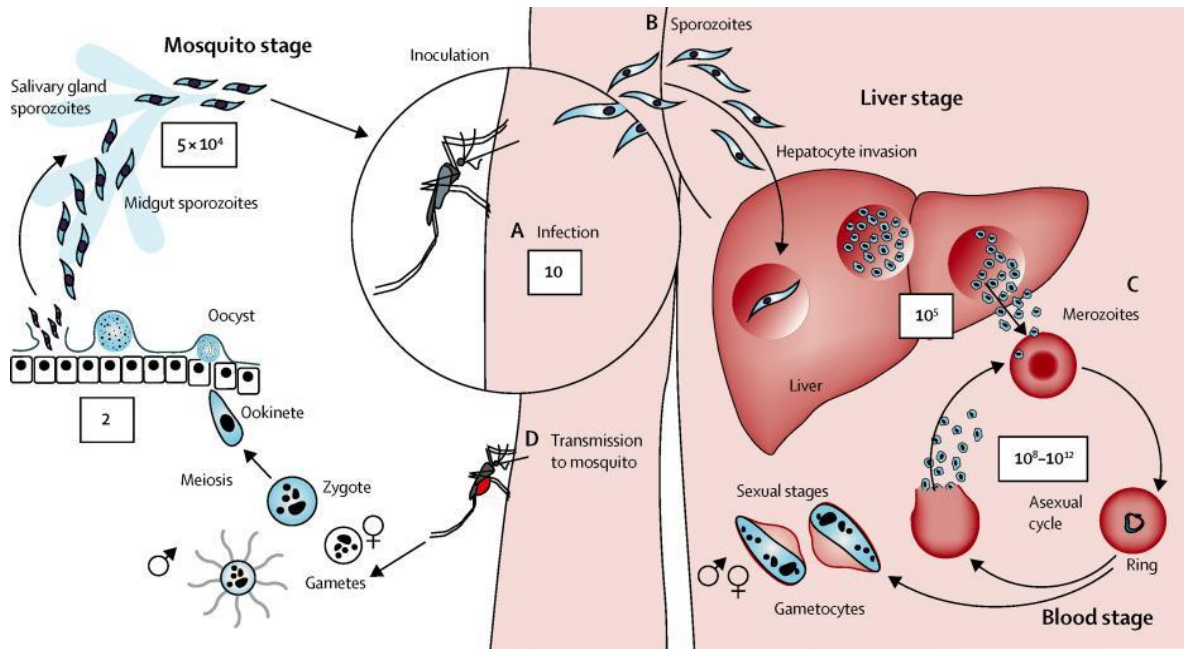
Figure 1. Life cycle of *P. falciparum* (Source: White et al. [31])

1.2 Malaria transmission and endemicity

The epidemiology of malaria is dependent on the environmental tropism, breeding activity and biting habits of its *Anopheles* vectors [63]. Several physiological, behavioral, and ecological characteristics determine how effective various *Anopheles* species are as vectors of malaria [63]. Besides, more than 45 species of *Anopheles species* have been identified worldwide as being capable of transmitting malaria to humans and only three, *Anopheles gambiae, Anopheles arabiensis* and *Anopheles funestus* are responsible for majority of the transmission [62, 63].

Depending on geographical location, some species are more prevalent and efficient vectors than others [63, 64]. In sub-Saharan Africa, the *Anopheles gambiae* and *Anopheles funestus* are the most dominant vectors [64, 65]. Each species may have differing breeding requirements, life spans, feeding habits, and vulnerability to environmental stressors or insecticides [63, 64, 65]. In contrast to the endemic African mosquitoes, the Asian malaria vector *Anopheles stephensi* is one of the few anopheline species found in central urban locations [66].The mosquito vectors classified based on their preference to feed on human (anthropophily) versus animals (zoophily), when they prefer to feed (night vs day), and whether they feed and rest more indoors or outdoors.

The female mosquito bites humans to get proteins from the blood, which are required for egg production [64, 65].

External factors playing important roles in the vector's transmission potential include: (a).Rainfall patterns and optimal ambient mean temperatures of about 26°C (minimum 17°C and maximum 35°C) that allows the parasite to develop inside the mosquito and be transmitted[67] (b).Presence of swampy areas where the mosquito can mate and complete all their four stages in the lifecycle, i.e. egg, larva, pupa and imago; and (c).Survival of mosquito for more than 10-21 days for the parasite to complete its cycle inside it. All these factors are essential to take into account in vector control interventions. However, there are cases, albeit rare, where transmission is independent of the mosquito vector, such as from pregnant mother to foetus/child (vertical transmission) or through transfusion of infected blood to an infection-free person or through contaminated syringes among intravenous drug users [68, 69]. Taken together, these factors explain why malaria endemicity can vary so widely within relatively small geographic areas. The long lifespan and strong human-biting habit of the African vector species is the main reason why approximately 90% of the world's malaria cases are in Africa [70].

The clinical epidemiology of *P. falciparum* malaria in any human population is the product of a complex interplay between the factors of age, acquired immunity and the level of endemicity of *P. falciparum* infection in the area where a population resides [71, 72, 73]. Individuals in endemic areas of *P. falciparum* transmission gained acquired immunity to the infection slowly over long period of time, probably years but such immunity may never result in sterile immunity. This means that an individual having acquired immunity to malaria can lose it depending on his exposure to the infection in endemic setting. It is however the belief of many that artificial immunity in the form of a vaccine may be an antidote to these challenges of acquired immunity but Polymorphism of the surface molecules of infected RBCs is thwarting the effort of developing an effective vaccine [73]. In areas of high transmission, immunity develops in an age-dependent manner where children under five years of age are at highest risk of disease, and clinical manifestations among adults are rare; while in areas of low/unstable transmission, immunity is not acquired and therefore all age groups are at risk. Individuals living in malaria-endemic setting develop premunition against malaria. Premunition is partial (non-sterile) immunity that a person develops when living in malaria-endemic region after exposure to

malaria infections and asymptomatic parasitemia is common in adults living in endemic regions [71]. In children older than 5 years of age, premunition protects against severe malaria. With continued exposure as the children become adults, it protects them against clinical disease and they become asymptomatic reservoirs of the parasite [72]. These asymptomatic reservoirs are very important in pre-elimination and elimination setting, as they pose a challenge in clearing the last source of local transmission. This immunity however develops slowly, may take 15–20 years of exposure with at least 5 infective bites per year, and it rapidly declines when an individual is no longer exposed to infections. After a period as short as one year of no exposure, an individual may no longer be protected by premonition [72, 73, 74].

Malaria endemicity classification can be done using various measures such as parasite rate (proportion of persons with laboratory-confirmed malaria infection), or spleen rate (the prevalence of enlarged spleen) or entomological inoculation rate (EIR) (number of infective mosquito bites per person per year). In most malaria-endemic countries, data collection and management are suboptimal due to deficiencies in health systems, making it rather difficult to classify endemicity in these regions correctly [75]. Traditionally, depending on parasite rate or spleen rate prevalence, classification can be in the following groups: *Hyperendemic regions* where transmission is high and parasite rate/spleen rate is >50% for *P. falciparum* among children 2-9 years old. In holoendemic regions parasite rates/spleen rate in this age group are >75%. In these regions, almost all individuals get infected during early childhood and infancy, and premunition is high. *Mesoendemic regions* where transmission is considered moderate, parasite rate/spleen rate is 11–50% for *P. falciparum* among children 2-9 years old. Age groups with the highest prevalence in these regions are children and adolescents [76]. *Hypoendemic regions* where transmission is considered low, parasite rate is ≤10% for *P. falciparum* among children 2-9 years old. In this region, premunition is low, and the prevalence of malaria infection and disease does not vary among age groups [76].

EIR on the other hand, measures the risk of infection over a transmission season depending on the number of infectious bites an individual is exposed to. It is a useful parameter when assessing interventions that focus on reducing human-vector contact. Using EIR, endemic areas can be classified as stable or unstable transmission regions [77]. In stable transmission regions, the case incidence of infections is rather steady from year to year unless there is an effective intervention

or unusual changes in the environment that alter the prevalence. It correlates with ongoing human exposure to bites of infectious mosquito throughout the year and subsequently higher morbidity and mortality. The EIR can be as high as 1000 infective bites per person per year in very high transmission setting and the individual who survive the exposure, develop premunition immunity. These are considered hyper- and holo- endemic regions (high transmission) which is more common in sub-Saharan Africa [77].

In unstable transmission regions, there are considerable differences in case incidence patterns from year to year and the EIR can be <5 or even <1 infective bite per person per year. In unstable transmission regions, the population has very low immunity and are vulnerable to epidemics. It is crucial, therefore to prevent case reintroductions in areas there is very little or no malaria. These are considered hypo- and meso- endemic regions (low transmission), this is common in tropical Southeast Asia, Central Asia and Latin America [77]. Malaria epidemics can occur with devastating consequences.

In Ethiopia, the diverse ecology of the country supports a wide range of transmission intensities, ranging from low-hypo-endemic transmission in the highland fringe areas and semi-arid regions to high endemic perennial transmission in the low land regions and valley floors. Generally, malaria transmission in Ethiopia occurs in areas located at altitudes below 2,000m above sea level, which is the target area for antimalarial interventions. However, some studies indicated that malaria infection was detected in areas beyond this cut-off [78, 79]. Ecological modification for agricultural activities like extensive deforestation in higher altitudes of Ethiopia in the last three decades [80] which is complexed with anomalous weather conditions that might have favored occasional malaria transmission. An estimated 52% of the population is at risk of malaria infection. Altitude, climate, and proximity of settlement villages to bodies of water such as streams and rivers are the most important determinants of malaria transmission risk [81]. In most parts of the country, transmission is seasonal, major transmission being from September to mid-December, following the main rainy season (June-August), and minor transmission season during March-May. As a result, malaria transmission pattern in Ethiopia is seasonal and unstable [82] often characterized by highly focal and large-scale cyclic epidemics [83, 84].Transmission of malaria in highland fringe and semi-arid areas of the country is found to be sharply decreasing over recent years, which is believed that the scale of interventions made in the country since

2005 has attributed for the decrease observed over the decade. Despite the reduction in overall incidence of malaria, malaria transmission has expanded to highland areas due to recent temperature warming in these highlands. An increase in the daily minimum temperature of 0.4°C per decade has been recorded in the highlands of Ethiopia [85].

Federal Ministry of Health (FMOH) updated the country's malaria risk strata based upon malaria annual parasite incidence (API) per 1,000 population (per the WHO recommendation) plus altitude and expert opinions, new malaria stratification developed. In addition, estimation of population at risk of malaria has done using elevation as a parameter. Accordingly, a total of 20,831 Kebeles and 154,000 enumeration areas (EAs) used for this purpose. The current stratification that assumed the level of malaria burden into account ensures suitability for different strategic objectives and will guide implementation of appropriate interventions across different strata. A malaria risk map from this analysis is showing areas with malaria transmission risk by API classified as High ($\geq$50 cases/1,000 population/year), moderate ($\geq$10 &<50), Low (>5 &<10), very low (>0 &<=5), and Malaria-Free (~0). Areas with the highest malaria transmission risk as stratified by district API appear to be largely in the lowlands and midlands of the western border with South Sudan and Sudan. Many densely populated highland areas were newly classified as malaria-free (API=0), including the capital city of Addis Ababa. Based on the current stratification, the proportion of the population at risk of malaria is about 52.1% percent with 68 (4.8%) districts having year-round high transmission [15].

1.4 Host- malaria parasite interaction

Host-parasite interactions are the main forces driving the evolution of both malaria parasites and their hosts [86]. Genome Polymorphism and gene function have co-evolved shaping the current genomes of malaria parasites and their hosts. Application of antimalarial drugs, deployment of vaccines, modulation of host immunity, and the development in mosquito vector control measures all have the potential to alter parasite populations including genome Polymorphism and virulence [86]. Proper monitoring of parasite populations in the context of drug, vaccine, and vector control programs is necessary to minimize unintended negative impacts on parasite population dynamics and disease severity [86].

Malaria parasites trigger an immune response the moment when they enter a host. To survive in this hostile environment, the parasite displays a range of strategies to evade host killing

mechanisms, including variations in antigen epitopes targeted by host immune machinery and interference or suppression of specific arms of the host immune response [86]. One of the consequences of these host-parasite interactions is increased genetic Polymorphism at genes encoding proteins under host immune selection [Figure 2A], leading to genetic signatures of diversifying selection in the parasite genome. Some highly polymorphic genes include those encoding proteins such as the apical membrane antigen 1 (AMA1), merozoite surface protein 1 and 2 (MSP1 and MSP2) along with the glutamate-rich protein (GLURP) and circumsporozoite protein (CSP). These highly polymorphic genes as well as genome-wide polymorphisms such as single nucleotide polymorphisms (SNPs) and microsatellites (MSs) have been used for genotyping parasite strains, tracking parasite migration and disease outbreak, studying parasite molecular evolution, and evaluating host immune response and vaccine efficacy and/or determining the impact and progress of malaria intervention [86]. In addition to highly polymorphic antigen genes, there are many polymorphic gene families in the *P. falciparum* genomes such as the *var* genes, *Plasmodium* interspersed repeat (*pir*) multi-gene family, which include repetitive interspersed (*ri)* and subtelomeric variant open reading frame (*stevor*) [86]. Indeed, large multigene families are present in many *Apicomplexa* parasites [86]. These gene families evolve at high rates and play critical roles in antigenic variation and immune evasion. The *var* genes encode *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins [86]. Switches in expression among an estimated 60 *var* genes in every single parasite have been shown to correlate with variation in antigenic determinants that bind to different host receptors such as ICAM-1, CD36, EPCR, and other receptors. The binding of PfEMP1 proteins to host receptors is responsible for cytoadherence of infected erythrocytes (iRBCs) and the pathogenicity of severe malaria, particularly cerebral malaria [86]. The *P. falciparum* parasite evades host immunity via mutually exclusive expression (e.g., only one PfEMP1 is expressed on the surface of an iRBC at a time) of the highly diverse *var* family representing an almost unlimited gene pool at the parasite population level [86].

Whereas host immunity is one of the major factors driving parasite evolution, malaria parasites and many other pathogens also have tremendous impacts on the adaption and evolution of their hosts [86]. A protective effect against malaria infection has been associated with genetic disorders involving the RBC, such as cytoskeleton disorders, surface antigen gene mutations, enzymatic machinery deficiencies, or hemoglobin alterations [86]. Genetic factors like RBC

polymorphism causing sickle-cell trait (carriers of Hb-S) and thalassemia have proven to have protective effect against *P. falciparum* malaria. Also glucose-6-phosfate dehydrogenase (G6PD) deficiency and Duffy blood group negativity have proven to have a protective effect against *P. falciparum* and *P. vivax* infection, respectively. These factors are primarily present in areas endemic for malaria. Malaria selection has played a major role in the distribution of all these polymorphisms [87, 88, 89]. On the contrary, patients who are homozygous for the sickle gene are more vulnerable to severe outcomes since malaria complicates the sickle cell anaemia [90].

A partially protective vaccine may selectively remove a specific parasite strain from the population [Figure 2B] [86]. Malaria parasite populations in endemic regions consist of a large number of strains that express different alleles of vaccine target proteins. Due to technical limitation, malaria vaccines are usually designed based on sequences from one to a limited number of alleles of a vaccine target. It is has been shown that anti-malaria immunity is mostly strain-specific [86], and a vaccine based on a limited number of alleles of a target may select for parasite populations with alternative alleles. Indeed, vaccination with pneumococcal conjugate vaccines (PCVs) changed the pneumococcal populations in children, resulting in statistically significant shift from vaccine-type population to non-vaccine type populations [86]. Similarly, using the rodent malaria model *P. chabaudi* and recombinant AMA-1 antigen, it was shown that mono-allelic immunization increased the frequency of heterologous clones in mixed clone infections [86]. Moreover, analysis of parasite genotypes collected from the RTS, S phase III trial showed that the RTS, S vaccine had greater activity against malaria parasites with the matched PfCSP allele than against those with mismatched alleles [86, 92]. Therefore, vaccination with an allele of the target antigen will likely change target allele proportion in parasite populations, and large-scale vaccination may lead to vaccine mediated depletion of specific alleles targeted by the vaccine [Figure 2B]. The impacts of this type of selection on parasite populations, including the possibility of selecting more virulent parasite strains, remain largely unknown and required further investigations [86]. Interestingly, a vaccine may also alter parasite expression of variant antigen genes. Vaccination of 10 African volunteers with the PfSPZ vaccine showed parasites from individuals with intermediate antibody levels expressed only few *var* gene variants, whereas those with low antibody levels expressed a broad spectrum of multiple, predominantly subtelomeric *var* genes coding PfEMP1 binding to endothelial protein C receptor (EPCR) that is associated with severe childhood malaria [93]. Therefore,

vaccination certainly can impact parasite population dynamics, genetic Polymorphism, and possibly virulence. RTS, S/AS01 (RTS, S) is the first and, to date, the only *P. falciparum* vaccine to reach the stage of an ongoing large-scale post-licensure pilot implementation programme in Ghana, Kenya, and Malawi, where the vaccine has been administered to hundreds of thousands of young children. Among children who received 4 doses in large-scale clinical trials, the vaccine gives a 35.9% protection even with multiple booster doses [70, 86]. This has mostly been attributed to the genetic Polymorphism of the parasite, which also affects drug efficacy [94, 95, 96]. Therefore, it is important to determine the genetic Polymorphism of antigens that are vaccine targets in different transmission settings to assist the development of effective malaria vaccines and monitor current interventions [94, 95, 96]. Besides, knowledge of the genetic structure of malaria parasites is also essential to predict what important phenotypes in relation to the novel antigenic variants or drug resistant strains originated and spread in the population [96].

In addition to host immune response, antimalarial drugs have played a significant role in shaping parasite populations. Drug treatment will reduce population Polymorphism by removing the drug sensitive parasites and selecting for one or a few drug resistant parasite clones that may then spread to many endemic regions, leading drug selective sweeps. Some examples of drug selective sweep include mutations in *P. falciparum* chloroquine resistance transporter (PfCRT) and in dihydrofolate reductase-thymidylate synthase and dihydropteroate synthase (PfDHFR-TS and PfDHPS) conferring resistances to chloroquine (CQ) and pyrimethamine/sulfadoxine (PS), respectively [86, 97]. Under drug pressure, a small number of parasites with resistant mutations will survive, whereas parasites without the mutations are killed by the drugs. With large-scale drug use, parasites with resistant mutations will spread, replacing parasites sensitive to the drugs. Indeed, worldwide CQ and PS selective sweeps have been reported [86, 97, 99], which can greatly reduce population Polymorphism in many endemic areas generating population bottlenecks. Drug selective sweeps are important factors in shaping the current worldwide parasite populations and may have contributed to Malaria's Eve hypothesis debate [101]. The sweeps of parasites derived from one or a few mutants conferring resistance to a drug will result in a relatively homogeneous parasite population [Figure 2C], as was seen in the Brazilian Amazon after wide spread use of CQ [99]. A relatively homogeneous parasite population may be advantageous for vaccine development [86].

Parasites passing through mosquitoes may generate new progeny through genetic recombination and chromosomal re-assortment, reset gene expression profile particularly those related to parasite survival, and select for clones that are better adapted to specific mosquito species [Figure 2D]. Mosquito vectors can shape parasite populations in many ways. First, genetic changes such as chromosome re-assortment and crossover will occur inside a mosquito and generate new parasite strains if a patient is infected with two or more variant strains, which will increase parasite population Polymorphism. A high multiplicity of infection is frequently observed in patients in regions with high transmission intensity [86, 101]. The genotyping of *Plasmodium falciparum* parasites has been shown to be a useful tool for exploring genetic Polymorphism (i.e., the complexity and size of the parasite populations) and multiplicity of infection (MOI), i.e., the number of clones per sample, which is generally considered to be strongly correlated with transmission intensity [102, 103, 104, 105]. Indeed, parasite genetic Polymorphism and MOI are high in areas with high rates of malaria transmission, whereas they tend to be markedly lower in regions implementing effective malaria control strategies [104, 106]. Second, parasite transmission through mosquitoes can also re-program gene expression profiles. For example, changes in composition and frequency of *var* gene transcripts were observed between cultured *P. falciparum* parasites used to infect mosquitoes and the parasites recovered from infected volunteers after mosquito bites, suggesting re-programing *var* gene expression profile [107].
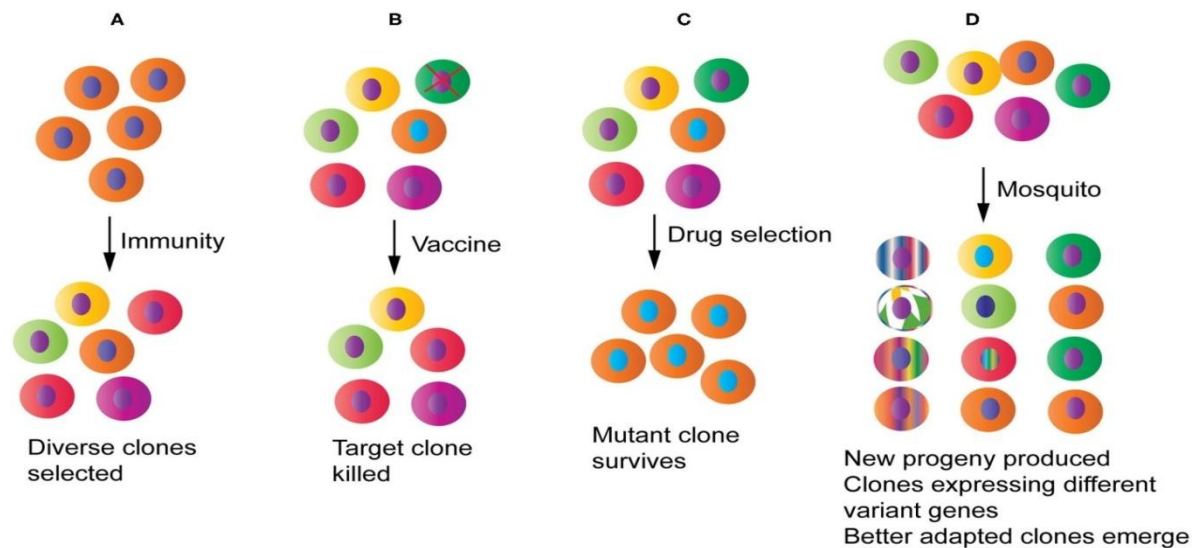
Figure 2. Potential impacts of host-parasite interactions and human intervention measures on parasite populations and evolution (Sourec: Su XZ et al. [86])

1.5 Clinical manifestations of malaria

Malaria infection presents with fever, as a constitutional symptom that could also be present in other viral or bacterial infections. Depending on the severity of the disease, malaria infection can be classified as uncomplicated or severe malaria. Uncomplicated malaria is more common during the early stage of the disease, and in individuals who have premunition. Features of uncomplicated malaria include fever, headache, cough, generalized body weakness, nausea and vomiting, muscle pain, enlarged spleen, and mild anaemia. Severe malaria can develop rapidly from uncomplicated malaria if not treated especially in pregnant women, malnourished children, elderly with co-morbidities, individuals without spleen or whose spleen has compromised function and immunocompromised individuals [108]. The tendency to develop severe malaria in *P. falciparum* is contributed by the promiscuous nature of the merozoites when it comes to infecting RBCs. Daughter merozoites can infect both young and matured RBCs during asexual replication, and the high number of merozoites per schizonts (up to 32) enables the infection to become rapidly hyperparasitaemic[31]. Clinical features of severe falciparum malaria include hyperparasitaemia (>10% of RBCs irrespective of endemicity) severe anaemia (haemoglobin <5 g/dL or haematocrit <15% mostly in young children), hypoglycaemia (blood glucose <2.2 mM (<40 mg/dL), altered consciousness that range from seizures to unarousable coma, respiratory

distress, metabolic acidosis (plasma bicarbonate <15mmol/L), acute kidney injury, pulmonary oedema, and jaundice [31, 109]..

1.6 Diagnosis *Plasmodium falciparum* malaria

Malaria case management and treatment outcomes depend on *timely accurate diagnosis* and *receiving efficacious medication*. Clinical diagnosis of malaria in endemic setting is traditionally based on fever or history of fever in the past 24 hours. With the declining prevalence of malaria globally, availability of improved and affordable diagnostic tools and threats of drug resistance development, WHO recommends that all suspected cases of malaria require laboratory confirmation before treatment [109]. This is due to the low specificity of clinical diagnosis that leads to unnecessary prescription of antimalarial drugs and missing other causes of febrile illness. WHO recommends antimalarial drugs to be given only to patients with laboratory-confirmed malaria [109]. There are different tools used in malaria diagnosis with respective advantages and limitations depending on the setting.

1.6.1   Light microscopy

Light microscopy remains the gold standard of malaria diagnosis and follow-up to assessment of treatment outcome [109, 110]. It involves visually inspecting Giemsa stained parasites through a microscope at 1000X magnification. Giemsa solution is the classical stain used in malaria microscopy. Peripheral blood, generally from a finger-prick, is collected on a glass slide as a thin or thick smear. The thin smear is fixed with alcohol before staining to maintain the integrity of RBC, which allows identifying of parasite species inside the RBC by comparing their different morphologies. In the thick smear, RBCs undergo haemolysis, and the ring-stage parasites are free for easy counting against WBCs. Parasite quantification from thick smear is done by counting against 500 or 200 WBCs. Each µL of blood is estimated to contain 8000 WBCs, meaning when the number of parasites counted per 500 or 200 WBC is multiplied by a factor of 16 or 40 respectively, one gets the parasite density per µL of blood [110]. This is expressed mathematically as: (parasites counted/number of WBC counted) x 8000 = p/µL blood An alternative method to quantify parasites in microscopy is by estimating the percentage of infected RBCs in a thin blood smear and density is reported as percent of the RBCs that are infected[111].

Advantages of microscopy include its low cost per test, ability to quantify parasites especially during diagnosis and follow-up when evaluating the effect of treatment in reducing parasite

density and efficacy outcome of treatment. It is a well-established method that is useful even in a limited resource setting without electricity. In a tropical setting, it is possible to detect other blood borne parasitic infections and with experienced technician may be able to determined anaemia or neutrophilia. However, its performance is highly dependent on the technician; it is labour-intensive, can take up to one hour to complete, and the multiple steps from smear collection, fixing, staining and storage can lead to variable results[110]. In a field setting, the limit of parasite detection is 50-100 p/μL, meanwhile with expert microscopist in an optimal setting the limit can be as low as 5-10 p/μL [112, 113].

1.6.2   Rapid diagnostic test (mRDT)

The malaria rapid diagnostic test (mRDT) is a field-friendly diagnostic tool that has had a significant contribution to improved case management and reducing malaria morbidity and mortality in sub-Saharan Africa since its introduction [113]. mRDTs are based on immuno-chromatographic detection of parasite antigens from peripheral blood of a febrile individual [112].

They are mainstay of routine malaria diagnosis in Africa, since it takes between 15 and 25 minutes to get results depending on the type of mRDT, and has very few steps, that can be done even by community healthcare workers with minimal training. WHO recommendations of widescale use of mRDTs has led to subsidised costs and easy access of mRDT at health care centres [110, 111]. Moreover, mRDT can be a valuable source of parasite DNA for molecular analysis [114].

Disadvantages of mRDT include; inability to quantify parasites, inability to distinguish between sexual and asexual parasites and they are not suitable for follow-up of treatment outcome since they remain positive up to 35 days after treatment[115]. Three types of antigens have been employed in this method, *Plasmodium* histidine-rich protein (HRP) 2 (pHRP-2), *Plasmodium* lactate dehydrogenase (pLDH) and *Plasmodium* aldolase. pHRP-2 is specific to *P. falciparum*, while pLDH and *Plasmodium* aldolase are found in all species [116]. More than 90% of commercially available RDTs target pHRP-2 [117]. False negatives are becoming more common due to parasites with pHRP-2 gene deletion and prozone phenomenon in patients with high parasitemia or antigen overload as it indirectly detects the presence of *antigen* [117]. The limit of detection for mRDT depending on the antigen detected is estimated to be comparable to

microscopy between 50-200p/µL. However, ultrasensitive mRDTs that have a detection limit up to 10 fold lower than current mRDTs are available, and are being explored for sensitivity and potential deployment for routine patient care in malaria endemic countries [118, 119, 120].

Globally, 2.7 billion rapid diagnostic tests (RDTs) for malaria were sold by manufacturers in 2010-2019, with nearly 80% of these sales being to sub-Saharan African countries. In the same period, national malaria programmes (NMPs) distributed 1.9 billion RDTs - 84% in sub-Saharan Africa. In 2019, 348 million RDTs were sold by manufacturers and 267 million distributed by NMPs. RDT sales and distributions in 2019 were lower than those reported in 2018, by 63 million and 24 million, respectively, with most decreases being in sub-Saharan Africa[1].

1.6.3 Nucleic acid amplification-based tests

These are molecular methods for malaria diagnosis, and currently they are not used in endemic setting as part of routine diagnosis [121, 122]. However, they offer opportunity to quantify parasite densities as low as <1 p/µL through detection of parasite DNA or RNA, making them ideal for research purposes due to the high sensitivity. In community-based patient screening in areas with unstable malaria transmission, these molecular methods can provide robust parasite detection and quantification results [123, 124]. In therapeutic efficacy studies molecular methods play a central role in distinguishing recrudescence from reinfection by genotyping, and can be used to detect drug resistance mutations and to determine complexity of infections (COI) in terms of number of different infecting clones in a patient[199, 199].

Several molecular methods exist, but all have similar steps they follow when used. The parasite DNA or RNA has to be extracted first, then amplified then detected and/or quantified. Main differences in these various methods are in terms of infrastructure required, samples preparation processes and the time it takes, detection limits, convenience of use and cost [123]. All these factors affect the efficiency. DNA extractions methods can use both whole blood or dried blood spots (DBS) on filter papers. Commonly available extraction methods are: Boil and spin method, chelex-100 beads-based method and colum-based extraction method. The nucleic acid amplification-based tests used in this PhD thesis were: Conventional polymerase chain reaction (PCR), Nested-PCR and real-time quantitative PCRs (qPCR).

1.6.4 Genotyping of *Plasmodium falciparum* parasites

In the past two decades advances in the development of molecular genotyping techniques have made it possible to distinguish individual parasite clones within a host and numerous molecular epidemiological studies have increased our understanding of the parasite population structure in endemic countries. A hallmark of *Plasmodium* infections in highly endemic areas is the presence of concurrently infecting parasite clones within a host. The molecular markers most often used to discriminate *P. falciparum* clones are the genes of merozoite surface proteins 1 and 2 (*msp1* and *msp2*) which are both highly polymorphic. *Msp2* alleles can be grouped into 2 allelic families (3D7 and FC27) according to a dimorphic non-repetitive region flanking a highly polymorphic domain of tandem repeats [125]. The size polymorphism of *msp2* is generated by differences in copy number and length of the repeat units. The *msp1* gene falls in three distinct allelic families (K1, MAD20 or RO33). Similar to *msp2*, the central repeat regions of MAD20-type and K1-type alleles give rise to size polymorphism and are flanked by family-specific sequences. The RO33 sequence does not contain any repeats [126]. Several genotyping techniques have been devised for studying genetic Polymorphism in these two genes encoding surface antigens. All assays are based on PCR amplification of the central polymorphic region. Two commonly applied methods are: (i) sizing of PCR fragments on agarose gels. Identification of the allelic family is achieved by either use of family-specific primers [127] or by hybridization with family-specific probes [127], and (ii) restriction digestion of the amplified PCR products [129, 130]. A more recent genotyping approach for *msp2* is based on capillary electrophoresis of fluorescently labeled family-specific PCR fragments [131]. The major advantage of this technique lies in the accuracy of discriminating distinct parasite clones, even in complex mixtures, and in facilitating high throughput genotyping. Genotyping studies have shown that *P. falciparum* infected individuals from endemic areas generally harbour multi-clonal infections ($\geq$ 2 clones). Multiplicity of infection (MOI) describes the number of parasite genotypes simultaneously infecting one host. MOI does not only vary by transmission intensity [131, 132] but also by age and parasite density. In this PhD thesis the *msp1* and *msp2* markers were used for genotyping of *P. falciparum* malarial parasites.

1.7 *Plasmodium falciparum* malaria treatment

According to WHO, the severity of disease determines the focus during malaria case management [76, 109]. For uncomplicated malaria, the objective is to prevent potential progression to severe malaria by clearing the parasite from the body and achieve cure. In severe malaria, the aim is to keep the patient alive, limit potential complications associated with severe disease and prevent recrudescence of infection. Preventing emergence and spread drug resistance together with blocking transmission to other people through mosquitoes is at the core of public health interest [76, 109].

Historically, the *P. falciparum* parasite has developed resistance against all known antimalarials; hence treatment strategies need to involve monitoring of parasites sensitivity to the drugs [133]. This resistance development has led to some antimalarials like CQ to be removed from routine care of *P. falciparum* malaria in Africa and sulfadoxine and pyrimethamine use is limited for use only as intermittent presumptive treatment for pregnant women, and quinine is reserved as second-line treatment for severe falciparum malariain Africa [76, 109]. With the recent outbreak of corona virus disease (COVID-19), CQ and hydroxychloroquine is being explored as a viable option for treatment of COVID-19 [134, 135], this requires further evaluation on its potential impact to *P. falciparum* parasites, especially when introduced to malaria endemic regions. The WHO recommends the use of artemisinin-based combination therapies (ACT) to achieve parasitological cure and prevent drug resistance [76, 109]. With artemisinin as a backbone, drugs such as lumefantrine, amodiaquine, piperaquine and mefloquine can be used efficaciously even in areas where they cannot achieve required cure rate as monotherapy [136].

1.8 Role of artemisinin-based combination therapies

1.8.1   Artemisinins

Artemisinins are currently widely known and researched antimalarial drugs that originate from the extract of the sweet wormwood plant - Qinghao (*Artemisia annua*), which has been used in Chinese traditional medicine for over 2000 years [137]. The discovery of artemisinin as the active ingredient in 1972 revolutionised malaria case management as an alternative to the already failing quinolines[138], and triggered increased interest in research around artemisinin derivatives and structurally similar drugs for use beyond malaria treatment (i.e. schistosomiasis, toxoplasmosis and cancer) [139, 140, 141]. Artemisinin has been used as a monotherapy against

malaria for more than 30 years in the region of Western Cambodia and other parts of the worlds at varying formulations and dosing, until the WHO banned artemisinin monotherapy use in 2007 [141]. Artemisinin has significantly contributed to the recent decline in global malaria burden; in the 2000-2015 period, more than 22% (of 663 million) of the reduction in malaria mortality was linked to ACTs [1, 141]. Its importance in global health was highlighted when Professor You You Tu received the 2015 Nobel Prize in Physiology or Medicine "for her discoveries concerning a novel therapy against Malaria", i.e. artemisinin [141]. The potency of artemisinin and its derivatives such as artemether, dihydroartemisinin, and artesunate is very high against all erythrocytic cycle asexual stages of *P. falciparum* with preference to the young ring stages[144], so much that it reduces the parasite biomass by 100 to 10000 folds per each asexual cycle (after 48 hours). It also kills young gametocytes, hence playing a role in reducing malaria transmission blocking [145]. The proposed mechanisms by which artemisinins kill the parasites are quite broad and are still being studied, but they generally fall under two categories: 1) Damaging parasite proteins such as transport proteins through haem activated endoperoxide activity and 2) Inhibition of proteasome activity (parasite's cellular repair mechanisms) leading to accumulation of damaged/unfolded proteins and stress-induced death[146, 147,148, 149, 150]. The safety profile of artemisinin and its derivatives in humans is remarkable and the drug is well tolerated [136, 151]. Some animal studies show concerning evidence on neurotoxicity, foetal abnormalities and death in early pregnancy, but in human pregnancies there is no demonstrable impact [152, 153, 154, 155]. However, the WHO does not recommend the use of artemisinins in first trimester pregnancy [76, 109]. There is conflicting evidence when it comes to demonstrating neurotoxicity of artemisinins in humans, manifesting as hearing loss in general (ototoxicity). Some researchers argue that there is association between oral artemisinin with ototoxicity based on their works, while subsequent studies with other researchers fail to demonstrate the same [157, 158, 159, 160, 161, 162, 163]. This is also in light of excellent safety profiles over decades of use of artemisinin derivatives to treat millions of patients around endemic countries [164]. It is a matter of importance to examine this safety aspect further especially if patients are exposed to higher doses of artemisinins. WHO recommends use of artemisinins is for both severe malaria and uncomplicated malaria. Severe malaria is treated with parenteral artesunate injection or artemisinin-based rectal suppositories for children <6 years only as pre referral treatment (10 mg/Kg) [76, 109]. Recommended parenteral dose is 2.4 mg/Kg for adults or 3 mg/Kg for

children with less than 20 Kg, given at 0 hours, 12 hours and 24 hours. After this dose, patients continue with oral ACT for three days as it is for uncomplicated malaria within 8-12 hours from the artesunate injection [76, 109]. Combining artemisinins with a longer half partner drug is believed to play a protective role from development of resistance for both artemisinin and partner drug, and reduces the likelihood of treatment failure. The complimentary pharmacokinetics of ACTs makes them the best drug against malaria [145, 164].

Artemisinin-based combination therapies Since WHO recommended ACTs in the guidelines of treatment of uncomplicated malaria, multiple studies have been done to assess the therapeutic efficacy of the combinations. ACTs that have been recommended by WHO for general use are fixed dose combination that includes artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, dihydroartemisinin-piperaquine and artesunate + sulfadoxine–pyrimethamine. There are many other ACT being assessed in the pipeline, and several that have been registered recently such as artesunate pyronaridine, arterolane-piperaquine, artemisininpiperaquine base and artemisinin-naphthoquinone. However due to limited evidence of safety and efficacy they are not yet recommended for general use, despite being used in some countries[76, 109].

For the commonly used ACT in Africa, artemether-lumefantrine and artesunate-amodiaquine, the efficacy is excellent and the safety profile is acceptable [164, 165, 166, 167, 168, 169]. Artemether-lumefantrine is the ACT used in this PhD thesis

1.8.2   Artemether-lumefantrine

Artemether-lumefantrine (20/120 mg) has been approved by the WHO as a fixed dose combination developed jointly by Novartis Pharma and the Academy Medical Sciences, Beijing, China [162]. Artemether is a highly lipophilic semi-synthetic derivative of artemisinin. It takes about 2 hours to be fully absorbed, and about the same time after absorption, to be hydrated to its active ingredient dihydro-artemisinin, where about 76% of it is bound to albumin. Artemether is short acting, with a terminal elimination half-life of 1-3 hours [170]. Lumefantrine shares structural similarities with drugs like halofantrine, quinine and mefloquine from the same aryl-amino alcohol group. It is also lipophilic and takes 8-10 hours to reach peak plasma concentrations with longer terminal elimination half-life of about 4-6 days. Almost 99% of plasma lumefantrine is protein bound [170, 171]. Lumefantrine's proposed mechanism of action

is by inhibiting the formation of hemozoin. It binds to hemin and leads to accumulation of haem that is toxic to the parasite and other free radicals leading to parasites' death [172]. Both artemether and lumefantrine are metabolized by cytochrome P450 3A4 (CYP3A4) [172, 173]. Artemether is more potent in the reduction of parasite biomass while lumefantrine ensures parasitological cure and prevents recrudescence [145]. Current duration of treatment is three days with a total of six doses given at 0, 8, 24, 36, 48 and 60 hours. The dose is given by weight i.e. between 5 Kg and <15 Kg get one tablet, 15 Kg to <25 Kg get two tablets and between 25 Kg and <35 Kg get three tablets, for >35 Kg it is four tablets [76, 109]. In different parts of the world, including sub-Saharan Africa where clinical trials have been conducted to test for the efficacy of the six dose regimen of artemether-lumefantrine for 3 days in treatment of uncomplicated malaria, it has achieved PCR-adjusted cure rate of >95% [167, 168, 169, 172, 164, 165, 166, 167, 168, 169, 170]. Safety profile and tolerability of the currently recommended six-dose regimen for artemether-lumefantrine (20/120 mg) tablets has also been demonstrated to have favourable outcomes across different age groups and sex. Most importantly artemether-lumefantrine can be used safely in treatment of uncomplicated malaria for pregnant women in second and third trimester [151].

1.8.3 Artesunate-amodiaquine

This is a WHO prequalified ACT for treatment of uncomplicated falciparum malaria. It is available in fixed dose combination, loose combination and as dispersible fixed dose combination for [76, 109]. Standard dosage is 4mg/Kg/day for artesunate and 10mg/Kg/day for amodiaquine given once a day for three days. Different from artemether, artesunate is a hydrophilic semi-synthetic artemisinin derivative which is rapidly absorbed orally, reaching peak plasma concentration after around 90 minutes. Similar to artemether, artesunate is also converted to dihydroartemisinin which is the active metabolite that is rapidly eliminated with a terminal elimination half-life of around 45 minutes [180, 181]. Amodiaquine is a quinoline belonging to the same group with CQ, since its synthesis in the 1940; it has been used extensively as monotherapy for treatment of uncomplicated malaria. Amodiaquine has schizonticidal activity through interfering with hemozoin formation through complexation with haem after accumulating in parasites' food vacuole. It is absorbed rapidly and converted to desethylamodiaquine, reaching peak plasma concentration after about 4 hours. It has a terminal elimination half-life of 3-12 days in African children with uncomplicated malaria [181].

1.8.4   Primaquine

Primaquine is an 8-aminoquinoline antimalarial drug which has been in use for more than 60 years for radical cure by clearing the dormant liver stage of *P. vivax* and *P. ovale* malaria (hypnozoites) [187]. The drug has also been used as a single dose treatment against *P. falciparum* gametocytes as a means to control and eliminate malaria in some parts of the world and also to control chloroquine resistance [184, 185, 186]. Almost all antimalarials can kill gametocytes of other human malaria parasites and developing *P. falciparum* gametocytes, but only primaquine and methylene blue can kill mature *P. falciparum* gametocytes [187]. When combined with ACT, primaquine rapidly shortens gametocyte carriage duration [188, 189]. Primaquine clears mature gametocytes rapidly, but unlike artemisinin derivatives it does not prevent gametocyte development [188] hence the two drugs act synergistically. Primaquine is absorbed rapidly and peak concentrations are reached in approximately 2 hours. It has a half-life of 6 hours and it is metabolized in the liver. The metabolically inert principle metabolite (carboxy-primaquine) reaches peak concentrations within 6 hours of administration. However, the active metabolite and the exact mechanism of action of primaquine has not yet been identified [189]. The kinetics of primaquine are affected by malaria (acute infection reduces oral clearance of primaquine), by food (increase primaquine bio-availability); or by other antimalarials (quinine induces a higher area under the curve (AUC) of the carboxy metabolite) [189]. In combination with schizonticidal drugs, primaquine at 0.75 mg/Kg has been used to reduce malaria transmission and control the spread of chloroquine resistance. However, the scaled-up use of the primaquine for malaria control has been hampered by the dose dependent haemolytic anaemia which the drug induces particularly in individuals with G6PD deficiency [189]. To address the issue of G6PD deficiency, Tafenoquine, a single-dose 8-aminoquinoline that has recently been registered for the radical cure of *P. vivax*[190] and this drug was safe, well tolerated, and highly effective in preventing *P. vivax* and multidrug-resistant *P. falciparum* malaria in Thai[191].

1.9 Assessment of anti-malarial drug efficacy

Efficacious malaria case management is severely impeded by resistance to antimalarial drugs, leading to an increase in malaria cases and deaths. When poorly monitored and poorly contained, resistance can spread across the world, or start locally as it was with chloroquine resistance in the 1970's [147, 192]. The historical experience we have with chloroquine resistance when it

reached Africa and caused an increase in malaria mortality and morbidity, teaches us to be even more proactive in monitoring the drug resistance to artemisinin and ACT, since it is the only option that we currently have for first-line management of malaria[193, 194, 195]. The WHO recommends a combination of *in vivo* therapeutic efficacy studies*,* and *in vitro* experiments, genotyping studies for molecular markers of resistance as complementing tools for drug resistance surveillance.

The main focus of this PhD thesis is *in vivo* therapeutic efficacy study and this will be discussed further in the next sub-section. However, in the following paragraph the *in vitro* experiments, genotyping studies for molecular markers of resistance will be described in brief.

In vitro tests are experiments that use laboratory parasite cultures to assess the capacity of a drug to inhibit normal growth of parasite (from trophozoite to schizonts) using different known concentrations. For example, ring stage assays are used to test the sensitivity of *P. falciparum* rings to artemisinin [196]. *In vitro* experiments have a good control of desired parasite's drug exposure and have flexibility of conducting parallel sensitivity analysis with different drugs and manipulating experimental conditions [197]. However, *in vitro* tests need highly skilled personnel, expensive equipment, long-time to results and are labour intensive. Also, the absence of influence of host factors that may be important in activation of pro drugs like proguanil can limits the utility of *in vitro* tests. The results of *in vitro* tests lack influence of immunity and interpersonal pharmacokinetic variabilities that are representative of the real population and depend on animal models for simulation [197]. This makes it hard to interpret objectively any correlations observed between *in vivo* and *in vitro* results.

1.9.1 *In vivo* **therapeutic efficacy studies**

In vivo studies are WHO standardized therapeutic efficacy studies (TES) for antimalarial drug efficacy. These TES have undergone several iterations of standardization to ensure comparability of results across different endemic regions while providing national malaria control programs and researchers with minimum essential data to inform national policy changes regarding malaria treatment regimen [198]. Since 1996 when the first standard protocol for high transmission regions was made, adjustment have been made to accommodate medium and low transmission regions in the new 2009 standard guidelines. In the latest document on methods for surveillance of antimalarial drug efficacy of 2009, WHO has developed and incorporated robust tools ranging

from study protocol templates to data collection tools and data entry programs/templates. The data entry programs are also embedded with formulae for data analysis which allows standardised analysis with the recommended Kaplan-Meier analysis [198]. The usual per protocol analysis can also be used in parallel for treatment outcomes. These tools are flexible enough to be customised for local needs by national malaria control programs while maintaining important standard features common to all TES.

The 2009 TES guidelines involves enrolment of patients under 5 years of age in high transmission setting and all patients over 6 months of age in areas of low-to-moderate transmission, with uncomplicated mono-infection falciparum malaria (microscopy confirmed). They receive standard treatment for malaria and undergo repeated assessment for clinical and parasitological outcome during follow-up period of 28 or 42 days depending on the antimalaria used. The drugs capacity to kill all parasites and resolve patients' symptoms determines the outcome of the treatment and ensuring that there is no recurrent parasitaemia during follow-up. Treatment outcomes are classified into four categories which are applicable to all levels of malaria transmission: early treatment failure (ETF), late clinical failure (LCF), and late parasitological failure (LPF), adequate clinical and parasitological response (ACPR) [198].

The outcome of these efficacy studies are, however, influenced not only by the true susceptibility of the parasite to the test drug but also several factors such as immune status of study participants, the individual drug bioavailability, as well as an often complex interpretation of PCR results[198].

The WHO and Medicine form Malaria Venture recommends use of merozoite surface proteins 1 and 2 (*msp*-1 and *msp*-2) and glutamate-rich protein (glurp) as molecular markers for primary endpoint analysis during TES. The following properties make them best suited as candidates capable of distinguishing *P. falciparum* parasite sub-populations. They have intragenic repeats that vary in length and copy number which makes them highly polymorphic markers in terms of both size and sequence (except for glurp where allelic differentiation is based on size alone). They are single copy genes located on different chromosomes hence they are unlinked [199]. They have been extensively used in many studies and gave useful results, this allow for comparison during interpretation of data [199].

The recommended samples for molecular genotyping are day 0 samples that were collected before start of antimalarial treatment and samples at the first occurrence of asexual parasitaemia by microscopy at or after day 7. These samples are paired and analysed for family specific allele of the makers. New infection is when all the alleles in parasites from the post treatment samples are different from those at day 0 for one or more loci tested. Recrudescence is when at least one allele at each locus is common to both paired samples [199]. The genotyping should be done sequentially, starting with either msp2 or glurp as they have highest discrimination power, then the last maker should be *msp1* [199].

Classical definition of antimalarial drug resistance according to WHO definition is; when parasites are still able to survive and/or propagate in presence of medicine administered and absorbed in recommended therapeutic doses or higher but within the tolerance level of the subject. It is important that the active form of the drug reaches the parasite or infected RBC for the duration needed for it to kill the parasite [76, 109].

Occurrence of parasite resistance to antimalarial drugs is a result of natural selection, where the drugs exert pressure on the parasite population for survival. It is a gradual process, involving a series of alterations in the parasite's genome to develop tolerance to the drug exerting selection pressure. The tolerant parasites have genomic alterations that decrease their susceptibility to the drugs, but they still die at therapeutic concentrations. Those genomic alterations can take a form of SNPs or amplification of gene copy numbers. This can result into changes in the drug target site, or increase mitigation of toxicity damages caused by the drug, or modification of transporter pumps that efflux the drugs to reduce intra-parasitic concentrations, or develop ability to alter the active components of the drug or a combination of any of those drug resistance mechanisms[200, 201, 202, 203, 204, 205]. Development and spread of resistance are a function of parasite factors, host factors, the drug itself, vectors and the environment. For instance, in high transmission setting, tolerant parasites that can survive sub-therapeutic concentration of the drug, are selected when reinfecting during the post-treatment prophylaxis until clinical treatment failure becomes apparent [205, 206].

1.10 *Plasmodium falciparum* drug resistance

1.10.1 Artemisinin resistance

In 2009 the first report of emerging artemisinin resistance in *P. falciparum* malaria was published from Southeast Asia-Cambodia (Pailin), where also chloroquine and sulfadoxine-pyrimethamine resistance was first documented [207]. The *P. falciparum* resistance to artemisinin does not well fit into the WHO definition of drug resistance, since it is phenotypically characterized by delayed parasite clearance times following ACT treatment hence it represents partial resistance. Patients with infections that demonstrate delay in clearance, eventually clear the parasites by the long-acting partner drug or longer treatment duration with artesunate. Microscopy based *P. falciparum* positivity rate on day 3 after initiation of ACT treatment is considered an important determinant, and if the day-3 positivity rate exceeds 10% this is considered an alert for artemisinin resistance [208]. The molecular basis for artemisinin resistance in Southeast Asia has been linked to SNPs in the *pfk13* gene. Kelch13 encodes a 726 amino acid protein containing a BTB/POZ domain and a C-terminal 6-blade propeller domain [209, 210]. The PfKelch13 is believed to be important in the regulation of protein quality control [133]. There is a growing list of SNPs in the Kelch13 propeller domain, that are considered markers associated with both *in vivo* and *ex vivo* artemisinin resistance. These markers are categorised as validated or candidate marker. To qualify as a validates maker, the SNP has to be correlated with delayed clearance phenotype in *in vivo* clinical studies, and be correlated with reduced *in vitro* drug sensitivity (e.g., ring-stage assay – RSA0-3h) using fresh isolates, or reduced *in vitro* sensitivity resulting from the insertion of the SNP in transfection studies. If the marker is only associated with the delayed clearance phenotype but not correlated with resistance in *in vitro* studies, it remains as a candidate marker. Some of the validated markers according to the WHO 2018 status report include F446I, N458Y, P553L, R561H, M4761, Y493H, R539T, I543T and C580Y. Other candidate markers are P441L, G538V, G449A, V568G, C469F, P574L, A481V, F673I, P527H, A675V and N537I [209, 210]. When taken together, the slow clearing phenotype and the identified *pfk13* mutations, the definition of artemisinin resistance becomes refined to two definitions:

1. Suspected partial artemisinin resistance is defined as:
   - ≥5% of patients harbour parasite with *pfk13* resistance-associated mutations; or

- ≥10% of patients with persistent parasitaemia by microscopy on day after treatment with ACT or artesunate monotherapy; or
- ≥10% of patients with a parasite clearance half-life of ≥5 hours after treatment with ACT or artesunate monotherapy.

2. Confirmed partial artemisinin resistance is defined as ≥5% of patients carrying *pfk13* resistance associated mutations, all of whom have been found, after treatment with ACT or artesunate monotherapy, to have either persistent parasitaemia by microscopy on day 3, or a parasite clearance half-life of ≥5 hours[209].

The parasite clearance time can be influenced by other confounding factors such as splenectomy, haemoglobin abnormalities and reduced immunity. Moreover, the proportion of patients who are parasitaemic after 3 days of treatment can be influenced by baseline parasitaemia, immunity of the patients, variability in skills of microscopist and time of assessment [209].

1.10.1.1 *Spread of artemisinin resistance*

Since the early reports of confirmed artemisinin resistance in Western Cambodia, Thailand, Vietnam, Eastern Myanmar and Northern Cambodia, it has been spreading reaching Central Myanmar, Southern Laos and North-eastern Cambodia, Bangladesh and spreading further west reaching Eastern India [211]. In addition to the widespread of *pfk13* mutations in the Greater Mekong Subregion, there are recent reports of independent origins of *pfk13* mutations detected at a prevalence of more than 5% in Guyana, Papua New Guinea and Rwanda [212, 213, 213]. Being faced by the threat of spreading drug resistance from Southeast Asia, and the potential of locally arising resistance as evidenced by other reports of *pfk13* polymorphisms in Africa [215, 216, 217, 218, 219, 220], developing strategies to protect the therapeutic efficacy of ACTs in Africa cannot be more urgent.

1.10.2 Lumefantrine resistance

Lumefantrine tolerance/resistance has been linked to SNPs in *pfmdr1* at positions N86Y, Y184F and D1246Y, and in *pfcrt* at position K76T and *pfmrp1* at positions *I876V SNP* [211, 213]. Interestingly, lumefantrine selects for *pfmdr*1 N86, 184F, D1246 and *pfcrt* K76, the chloroquine sensitive genotypes. Another genetic alteration previously linked to lumefantrine resistance in Southeast Asia is increased *pfmdr1* copy numbers [221]. Importantly, to date no clear evidence of *pfmdr1* copy number variation has been observed in EastAfrica. The development of

tolerance/resistance against lumefantrine, and other long acting partner drugs in ACT, has been suggested to start through post treatment selection among recurrent infections of less sensitive *P. falciparum* parasites, as reinfecting lumefantrine tolerant parasites are able to survive the exposure of sub-therapeutic blood levels of lumefantrine after treatment [211].

1.11   Malaria case management in Ethiopia

The introduction of AL in Ethiopia was secondary to *P. falciparum* drug resistance that developed to chloroquine and led to change of regimen in 1998 to sulfadoxine-pyrimethamine as an interim solution, which lasted only six years because of resistance. Chloroquine (CQ)-resistant *P. falciparum* became a major public health threat in the early 1990s in Ethiopia [220]. By the late 1990s, 86-88% treatment failure rates with CQ were being reported, which prompted change of first-line treatment to sulphadoxine-pyrimethamine (SP) in 1998 [223, 224]. In 2003, a nation-wide study evaluating SP efficacy showed 36% and 72% treatment failure rates with 14-day and 28-day follow-up, respectively [225]. Following a large-scale malaria epidemic that ravaged Ethiopia in 2003 [83] and the concomitant recognition of wide-spread resistance to SP [225], the Federal Ministry of Health (FMOH) adopted artemether lumefantrine (AL) for first-line treatment of uncomplicated *P. falciparum* malaria in Ethiopia [226].

According to Ethiopian NMCP, artemether-lumefantrine with single low-dose primaquine should be used to treat *P. falciparum* infections, whereas chloroquine combined with radical cure primaquine should be used to treat *P. vivax* cases without prior G6PD testing. Oral quinine remains the treatment of choice for uncomplicated *P. falciparum* for pregnant women during the first trimester of pregnancy, and as second-line for treatment failures. Rectal artesunate should be available at rural health posts for pre-referral treatment for children less than six years of age, and parenteral artesunate or artemether (alternate) should be available at health centers and hospitals for the treatment of severe malaria [227]. Widespread drug resistance to chloroquine and sulfadoxine-pyrimethamine was estimated to increase morbidity and mortality of malaria by 2 to 11 folds in endemic regions including Africa, this made it urgent to change to ACT as early as possible [228].

1.11.1 Experiences with artemether-lumefantrine since 2004 in Ethiopia

Since the roll out of ACT, artemether-lumefantrine from 2004 in Ethiopia, as first-line treatment of uncomplicated malaria, the therapeutic efficacy monitoring results from different malarious

parts of the Country with varied transmission intensity demonstrate that the AL in use in Ethiopia are still highly efficacious (>90%)against falciparum malaria, in accordance to the WHO parameters [198], while there is minor variation between the efficacies of AL by study settings[229, 230, 231, 232, 233, 234]. Over the years, administration of artemether-lumefantrine has been safe in the Ethiopia population, with no reports of new adverse events that were not previously identified, most of which are mild and often indistinguishable from the disease pathology [229, 230, 231, 232, 233, 234]. Experience has shown that Ethiopia has trained and deployed over 30,000 community health extension workers to manage malaria at the community-level health posts. Two HEWs are assigned for every 5,000 people, and they are fully integrated with the health system as part of the regular workforce [235, 236]. The impact of this large-scale deployment of AL was demonstrated in Tigray Regional State, where a two year study of community-based deployment of AL significantly lowered risk of malaria-specific mortality by 37% [237]. However, there are challenges with regards to prescribing practices such as prescribing antimalarial drugs to patients with negative test results and insufficient adherence to use of AL [239].Low health service utilization and inadequate diagnosis and treatment of malaria could be attributed to the following: (i) There is inadequate follow-up and supportive supervision of HEWs generally, and particularly for malaria prevention and control [238, 239]. As a consequence, accurate diagnosis with RDTs and recognition of the clinical symptoms of malaria is often not adequate; and (ii) Supportive supervision to strengthen disease management and diagnosis with RDTs by both HEWs and health facilities has not been well coordinated. This contributes to inappropriate malaria case management and may increase the risk of resistance to artemether-lumefantrine. Despite the known fact that for lumefantrine to be adequately absorbed it requires to be taken with a fatty snack or milk, data from a review in Africa including Ethiopia demonstrated that normal African diet has adequate fat content for sufficient absorption of artemether-lumefantrine for efficacious treatment outcome [145, 181].

1.11.2 Status of artemether-lumefantrine resistance in Ethiopia

Measurement and reporting of parasite clearance on day-3 after treatment with ACT is particularly important, as this is one of the first signals of emergence of parasite tolerance/resistance to artemisinin [240]. In Ethiopia, several studies showed day-3 parasitaemic cases of between 3.8% - 5.7%, after treatment with AL [230, 231, 241, 242, 243]. A recent publication from Ethiopia report the presence of a unique kelch 13 mutation (R622I) marker of

artemisinin resistance from northern of Ethiopia (Gondar)[215]. Moreover, recent data from Rwanda reports presence of similar locally arising *pfk13* mutation (R561H) at the rate of up to 20% among three TES sites, the highest documentation Africa to date [216]. Despite the documented rapid microscopy determined parasite clearance by day 3 after treatment with artemether-lumefantrine in Ethiopia. Taken together, the presence of a a unique kelch 13 mutation (R622I) marker of artemisinin resistance, and persistent day-3 positivity warrants further evaluation as to what their role may be in treatment outcome and continued transmission.

1.12   Significance of the study

Monitoring antimalarial drug efficacy and resistance is important for the early detection of resistance which in turn enables timely action to prevent its spread and limit the impact on global health. WHO recommends artemisinin-based combination therapies (ACTs) as first and second-line treatment for uncomplicated malaria caused by *P. falciparum* [198]. Maintaining the efficacy of ACTs for the management of malaria is a global health priority. However, artemisinin-resistant *P. falciparum* strains have emerged and spread in the Greater Mekong Subregion (GMS). More worrying, artemisinin resistance has been recently reported and confirmed in Rwanda, Uganda and elsewhere in East Africa. Furthermore, the development of resistance to the partner drugs used in the ACTs continues to pose a challenge in the treatment of malaria

To respond to malaria drug resistance, we need systems that:

1) Can detect changes in how well the recommended treatment is working

2) That can implement changes in policy when needed

Therapeutic efficacy studies (TES) are the gold standard for monitoring drug efficacy to inform treatment policy. Many factors can contribute to treatment failure, including incorrect dosage, poor patient compliance, poor drug quality, and drug interactions and resistance. Most of these factors are addressed in therapeutic efficacy studies. WHO recommended regular surveillance to monitor the performance of antimalarial drugs in use in all malaria endemic Countries. However, the efficacy of artemether-lumefantrine (AL) for treatment of uncomplicated *P. falciparum* malaria in south-western Ethiopia is poorly documented. Meta-analysis of multiple TES provides

more reliable indications of drug efficacy than studying individual TES separately. In this context, the study presented here aimed to investigate the therapeutic efficacy of AL for the treatment of uncomplicated *P. falciparum* malaria in Ethiopia

Malaria patients infected by multiple parasite strains have been shown to be high risk of treatment failure. Genetically distinct malaria parasites in natural population have an extremely high rate of recombination during sexual stage in mosquito gut during zygote formation, resulting in gene diversity of *P. falciparum*. Because of this variation, the conformation of ant malarial drug targets is altered and then renders the parasite drug resistant which hinders the outcome of malaria treatment. Hence, broad understanding of the genetic variation of the parasite population can contribute to the definition of control measures including an appropriate anti-malarial treatment

Genetic variation and multiplicity of infection (MOI) in *P. falciparum* populations can be used to describe the resilience and spatial distribution of the parasite in the midst of intensified intervention efforts. Genetic diversity and multiplicity of infection (MOI) are strongly correlated with transmission intensity. Indeed, parasite genetic diversity and MOI are high in areas where high rate of malaria transmission whereas they tend to be markedly lower in regions implementing effective malaria control strategies. Hence, MOI, the number of different parasite genotypes co-existing within a particular infection, has been suggested as a useful malaria metric describing transmission dynamics (malaria epidemiology)

In addition, the development of an effective vaccine is being hampered by genetic variation**.** understanding the extent and dynamics genetic variation in vaccine antigens (e.g *msp*) of all parasite strains is needed to guide rational vaccine design and to interpret the results of vaccine efficacy trials conducted in malaria endemic areas. E.g. RTS, S has greater activity against malaria parasite with matched circumsporozoite protein than mismatched parasite strains. The present study's findings provide an overview of the genetic variability of *P. falciparum* in symptomatic infection. These data can provide valuable information in the development of blood stage vaccine that could reduce the symptomatic disease

Hence, accurate malaria metrics are needed to complement the assessment of malaria transmission dynamics in a context of multiple control interventions. *msp-1* and *msp-2* gene

characterization are the most widely used techniques for assessing the genetic variation and MOI of *P. falciparum*. In this context, the study presented here aimed to provide new data concerning the genetic variation and multiplicity of *P. falciparum* populations in Chewaka district, Ethiopia

CHAPTER II: AIMS OF THE STUDY

2.1 General Objective

The overall aim of this thesis was to evaluate the therapeutic efficacy of artemether-lumefantrine for treatment of uncomplicated falciparum malaria and to analyze *Plasmodium falciparum msp-1* and *msp-2* genes in Ethiopia

2.2 Specific Objectives

- To assess the therapeutic efficacy of artemether-lumefantrine for treatment of uncomplicated falciparum malaria in Chewaka district, Ethiopia.
- To determine genetic polymorphisms and multiplicity of infection in *P. falciparum* parasite isolates by using antigenic polymorphic markers *msp1* and *msp2*
- To synthesize the available evidence on the efficacy of artemether-lumefantrine for the management of uncomplicated falciparum malaria in Ethiopia.

CHAPTER III: GENERAL METHODS

3.1 Study setting

The study site was Chewaka district, which is located in Buno Bedelle zone, Oromia Region of Ethiopia. The district covers a total area of 618.7 km^2 and it is about 552 km Southwest of Addis Ababa. Geographically, it lies between 8° 43′ 30″ N and 9° 5′ 30″ N latitude and 35° 58′ 0″ E to 36° 14′ 30″ E longitude (Figure 3). It was recognized as one administrative *woreda* of Oromia regional state since population resettlement in the area [244]. A diverse topographic condition which consists of undulating terrain, gentle sloping lowlands, gorges and small rounded hills characterizes the study site. Its altitude ranges between 1130 and 2053 m above mean sea level (Figure 3). The district lies in moist Woina Dega (cool sub-humid) and Kolla (warm semi-arid) agro-ecological zones and experiences both high temperatures and rainfall. It attains the maximum rain in the summer season (June, July and August) and small rainy season occurs from February to April. The dry season covers the remaining months of the year. The mean annual temperatures of the district range from 19.8 to 28.5 °C and the average annual rainfall varies between 800 and 1200 mm^3 [245]. Study section I and II of this PhD thesis was based on samples and data collected from Chewaka district and the third study was a systematic review and meta-analysis that reviewed the implementation of *in vivo* efficacy testing in Ethiopia after deployment of AL in order to monitor the efficacy of AL for the treatment of uncomplicated *P. falciparum* malaria
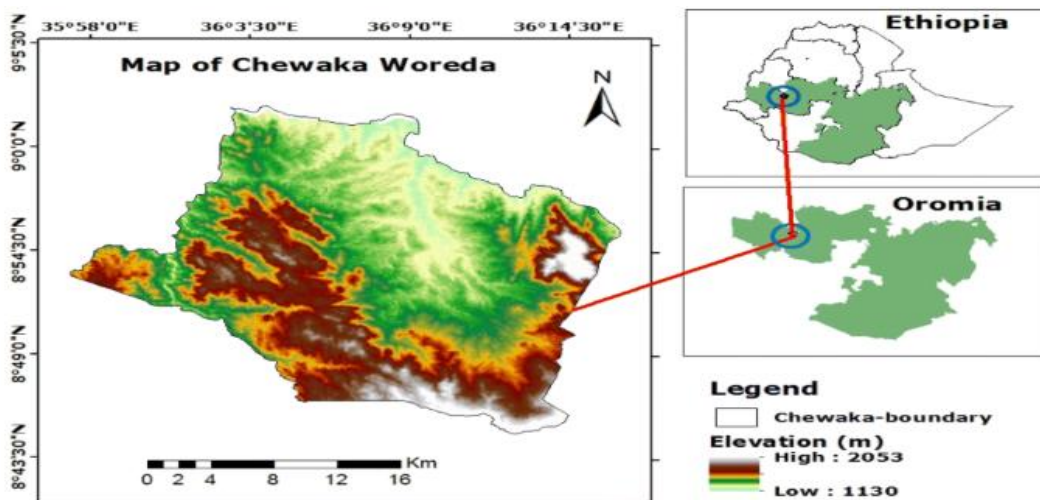
Figure 3. Map of the study area [Adopted from Abera et al. [244]]

3.2 Study specific methodologies

Detailed description of objectives and methodologies of specific studies are described in methods section of respective papers, however the general methods are summarized in Table 1

Table 1. Study specific objectives and methodologies

Study	Objectives	Study design	Study population	Study duration	Sampling method	Sample size	Major outcome variable	Analysis model
I	To assess the therapeutic efficacy of artemether-lumefantrine for treatment of uncomplicated falciparum malaria	*In vivo* therapeutic efficacy study	Patients aged ≥ 6 months of age who visited Chewaka HC with sign of uncomplicated malaria	Samples were collected between September and December 2017	Single population proportion	80	*Cure rate: Day-28 ETF,LCF,LPF, ACPR *Parasite clearance times * Fever clearance times	*Standard WHO Protocol for surveillance of anti-malarial drug efficacy: *Proportion of ACPR * Kaplan–Meier survival analysis
II	To determine genetic diversity and multiplicity of *P. falciparum* infection from clinical samples using the *msp-1* and *msp-2* genes	NA	Parasite population collected during evaluation of TES	2017-2020	NA	80	* Genetic diversity of *msp1* and *msp2*, Multiplicity of infection(MOI)	*Descriptive analysis *Mean MOI *Proportion *Spearman's rank correlation coefficients
III	To synthesize the available evidence on the efficacy of artemether-lumefantrine for treatment of uncomplicated falciparum malaria	Systematic review and Meta-Analysis (PICOS strategy)	Published artemether lumefantrine therapeutic efficacy studies conducted in Ethiopia from 2004 to 2020	The date of the last search was 30th October 2020	All randomized controlled trials (RCTs), non-randomized single-arm intervention studies (with or without a control group) and prospective cohort studies conducted between 2004-2020	N/A	*Pooled treatment success of AL(Cure rate) [Pooled ACPR]	Random effect meta-analysis model : Cochran Q and I^2 statistics

3.3 Study design

This study consisted of two study designs (Table 1). The first study section is a one-arm, prospective, evaluation of the clinical and parasitological, responses to directly observed treatment with AL among patients with uncomplicated *P. falciparum* malaria. The third study section is a systematic review and meta-analysis aimed to synthesize the available evidence on the efficacy of artemether–lumefantrine for the management of uncomplicated falciparum malaria in Ethiopia.

3.4 Genotyping for PCR adjusted cure rates

Patients with recurrent parasitaemia by microscopy were selected for genotyping. Nested PCR amplification targeting the unique sequence of 18 srRNA gene was held by using specific primer pairs for molecular detection of *P. falciparum* from the isolates. Timepoints of genotyped samples were enrolment (day 0) and day of recurrent parasitaemia. The analysis was conducted in a stepwise manner, as recommended by WHO [198, 199]. *msp-2* was chosen as the first marker as it is considered the most divisive marker, followed by msp-1. Analysis of each marker was conducted by nested PCR according to previously established lab protocols, as adapted from Snounou et al., 1999 [90]. Once *msp-1* and *msp-2* gene markers were complete, each patient was categorized into a final classification based on the following criteria as shown in the flow chart Figure 4. Recrudescent was when the two markers returned a recrudescent result, reinfection when one of the markers tested returned a reinfection result, negative when the two markers returned a negative result and unknown if the two or one markers returned either an unknown or recrudescent result.

3.5 Ethical considerations

For study section I and II, the study protocol was approved by the Ethics Review Board of Jimma University. Written informed consent from adult participants and parents/guardians of the study children and assent from children aged less than 18 years was obtained for all participants. If apatient, parent or guardian was illiterate, an impartial witness was used. For study section III, the PRISMA guideline recommendations were used and strictly followed to carry out this systematic review and meta-analysis. Ethical approval is not recommended and was not needed since it is a systematic review and meta-analysis.

Genotyping results with *msp-2* marker

No result → Continue with *msp-1* marker

Recrudescence → Continue with *msp-1* marker

New infection → Final result = new infection

No result → If no result with any marker, perform species PCR and re-check blood slide → Final result= failure, excluded, or censored according to study protocol

Recrudescence → Final result = Recrudescence

New infection → Final result = new infection

Figure 4. Flowchart to illustrate the process followed to determine final treatment outcome of microscopy recurrent samples

CHAPTER IV: THERAPEUTIC EFFICACY OF ARTEMETHER-LUMEFANTRINE FOR UNCOMPLICATED *PLASMODIUM FALCIPARUM* CASE MANAGEMENT

(Adopted from: Abamecha et al.2020)

Therapeutic efficacy of artemether-lumefantrine in the treatment of uncomplicated *Plasmodium falciparum* malaria in Chewaka District, Ethiopia

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Abstract

Background: The efficacy of artemether-lumefantrine (AL) for treatment of uncomplicated *Plasmodium falciparum* malaria in south-western Ethiopia is poorly documented. Regular monitoring of drug efficacy is an important tool for supporting national treatment policies and practice. This study investigated the therapeutic efficacy of AL for the treatment of *Plasmodium falciparum* malaria in Ethiopia.

Methods: The study was a one arm, prospective, evaluation of the clinical and parasitological, responses to directly observed treatment with AL among participants 6 months and older with uncomplicated *P. falciparum* malaria. Real-time polymerase chain reaction (PCR) and nested PCR reaction methods were used to quantify and genotype *P. falciparum*. A modified protocol based on the World Health Organization 2009 recommendations for the surveillance of antimalarial drug efficacy was used for the study with primary outcomes, clinical and parasitological cure rates at day-28. Secondary outcomes assessed included patterns of fever and

parasite clearance. Cure rate on day-28 was assessed by intention to treat (ITT) and per protocol (PP) analysis. Parasite genotyping was also performed at baseline and at the time of recurrence of parasitaemia to differentiate between recrudescence and new infection.

Results: Of the 80 study participants enrolled, 75 completed the follow-up at day-28 with ACPR. For per protocol (PP) analysis, PCR-uncorrected and -corrected cure rate of AL among the study participants was 94.7% (95% CI 87.1–98.5) and 96% (95% CI 88.8–99.2), respectively. For intention to treat (ITT) analysis, the cure rate was 90% (95% CI 88.8–99.2). Based on Kaplan–Meier survival estimate, the cumulative incidence of failure rate of AL was 3.8% (95% CI 1.3–11.4). Only three participants 3.8% (95% CI 0.8–10.6) of the 80 enrolled participants were found to be positive on day-3. The day three positive participants were followed up to day 28 and did not correspond to treatment failures observed during follow-up. Only 7.5% (6/80) of the participants were gametocyte positive on enrollment and gametocytaemia was absent on day-2 following treatment with AL.

Conclusions: The therapeutic efficacy of AL is considerably high (above 90%). AL remained highly efficacious in the treatment of uncomplicated malaria in the study area resulted in rapid fever and parasite clearance as well as low gametocyte carriage rates despite the use of this combination for more than 15 years.

Keywords*: Therapeutic efficacy, Arthemeter-lumfantrine, Uncomplicated malaria, Ethiopia

4.1 Background

Malaria, caused by infection with *Plasmodium* protozoan parasites, threatens over half the world's population [246]. Despite concerted efforts, which have considerably reduced the burden of mortality and morbidity in recent years, malaria remains a major public health threat [247, 248]. In 2017, over 219 million cases and 435,000 deaths were reported [247]. Approximately 92% of the cases and 93% of the deaths were from sub-Saharan Africa [247]. Between 2000 and 2015, the widespread adoption of artemisinin-based combination therapy (ACT), the increased use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) against the *Anopheles* mosquito vector, decreased the global number of malaria deaths by an estimated 37% [249]. Recently, these fragile gains are, jeopardized by the emergence and spread of drugresistance in the parasite and insecticide resistance in the mosquito vector [250].

Ethiopia is also one of the many malaria epidemic-prone countries in Africa [251]. The trends in malaria over the past five years have also shown a decline in malaria cases and reduced epidemics [252].In 2014/2015, Ethiopia reported 2,174,707 malaria cases and 662 reported malaria deaths among all age groups which is 98% reduction compared to 41,000 estimated deaths in 2006 [252, 253]. The key interventions which have been contributing to such significant decline includes: introduction of prompt and effective treatment with artemisinin-based combinationsto treat uncomplicated *P. falciparum* malaria, the distribution of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS); and to a lesser extent environmental management [252, 253, 254]. Following this, Ethiopia has also set a goal to eliminate the disease by 2030 [255, 256].

ACT is the first-line treatment for uncomplicated *P. falciparum* malaria and has been instrumental in reducing malaria burden [76, 258]. As yet the majority of endemic prone areas have little or no drug resistance to ACT, and itshigh efficacy (~95%) at clearing parasitaemia has been extensively demonstrated from clinical trials[259, 260]. However, there is limited information on ACT effectiveness in routine health care when treatment is not monitored.

Resistance of *Plasmodium* species to artemisinin has been reported from eastern and southern Asian countries which threatens malaria control and elimination efforts worldwide [261, 262, 263]. For the purpose of ensuring good performance and detection of emergence of resistance of

anti-malarial drugs, especially those used as a first-line and second-line treatment in a country, the World Health Organization (WHO) recommends regular monitoring of their efficacy at least every two years in malaria-endemic countries [198].

Early diagnosis and prompt treatment is one of the main strategies in malaria prevention and control and it is also the key to reducing morbidity and preventing mortality in Ethiopia [251]. According to the President's malaria initiative Ethiopia malaria operational plan fiscal year 2018, 60-70% of the total projected numbers of malaria cases are due to *P. falciparum* and to be treated with AL from year 2017-2019 [252]. The emergence and spread of both artemisinin and partner drug resistance threatens the efficacy of ACT and subsequently undermines the treatment of uncomplicated falciparum malaria, which is to eliminate all parasites from the body and prevent progression to severe disease [76, 263]. It is, therefore, necessary to generate continuous data on the therapeutic efficacy of first-line ACT to ensure real-time evidence-based review of national treatment policies as and when necessary. Since the introduction of ACT in Ethiopia in 2004, there have been few studies on therapeutic efficacy of AL [232, 233, 234]. This paper presents data on the therapeutic efficacy of AL for the treatment of uncomplicated falciparum malaria, the prevalence of day three parasitaemia, which has previously been used as a surrogate for artemisinin (partial) resistance and patterns of fever and parasite clearance.

4.2 Methods

4.2.1   Study setting and period

The study was conducted in Ilu Harar Health Center, Chewka district, Buno Bedele Zone, Southwest Ethiopia during September-December 2017.Chewaka district is located in Buno Bedele zone, Oromia regional state about 570 km southwest of Addis Ababa. The district has 26 administrative *kebeles* (villages) and has an altitude ranging from 1,600-2,000 above sea levels. As in most other areas of the country, malaria transmission in Chewaka follows rainy seasons, with transmission peaking in the months between September and December and between April and May.The main malaria control strategies in the district include, IRS,LLINs and malaria case management using ACTs [251].

4.2.2 Study design and participants

This was a prospective study of the clinical and parasitological efficacy of AL to directly observed therapy for uncomplicated *P. falciparum*malaria according to WHO revised protocol for malaria drug therapeutic efficacy study [198].

Inclusion criteria: Febrile patients (axillary temperature ≥37.5 0C) or having history of fever within the previous 24 hours, who fulfilled WHO revised protocol for malaria drug therapeutic efficacy study [198] and signed an informed consent were included inthe study. Briefly, all participants over 6 months of age and body weight > 5 kg, and microscopically confirmed *P. falciparum* mono-infection with asexual parasitaemia of 1000-100,000 parasites/μl of blood, non-pregnant or non-breast-feeding women, permanently living within the health centrecatchment area (5-10 km radius) during the study period were recruited.

Exclusion criteria: Evidence of mixed of mixed or mono-infection with Plasmodium species other than *P. falciparum*, haemoglobin (Hb) level ≤5.0 g/dl, AL intake within the previous 2 weeks, inability to take oral medication or continuous vomiting, known hypersensitivity to AL, severe malaria or other danger signs, severe malnutrition, febrile conditions due to diseases other than malaria (e.g. measles, acute lower respiratory tract infection, severe diarrhoea with dehydration) or other known underlying chronic or severe diseases (e.g. cardiac, renal and hepatic diseases, human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome(AIDS) cases); and regular medication which may interfere with AL pharmacokinetics.

4.2.3 Treatment and follow-up

AL [Batch: DYI476065; Mfg: 03, 2016; Exp: 02, 2018] which was manufactured by Ipca Laboratories Ltd (Plot №: 255/1; Athal, Silvassa 396 230 (D & NH), India) was provided by the Ethiopian FMoH through WHO support. Drug dosage was determined according to the revised WHO weight-based guideline [76]. Briefly, participants were treated with the standard six-dose regimen of AL given twice daily for three consecutive days under direct observation of a study nurse/public health officer and administered with a milk biscuit to ensure good absorption. The participants were then observed for 30 minutes to ascertain retention of the drug. Participants who vomited during the observation period were re-dosed with the same drug and observed for an additional 30 minutes. Participants with repeated vomiting were withdrawn from the study and treated as severe malaria according to national standard treatment guidelines [198].

On Day 0 (enrollment day), participants who were successfully treated with the first dose of AL were given an appointment card bearing patient name and identification code and next scheduled visit date, and the evening dose to be administered at home by health extension workers. Participants were then advised to come back for treatment the next two days (Day-1, Day-2). Scheduled follow-up visits were on Day-3, Day-7, Day-14, Day-21, and Day-28. There were unscheduled visits as well when a participant felt sick.Microscopy was performed during each subsequent visit to determine infection status, species, and parasite density.

4.2.4 Haemoglobin measurement

Finger-pick blood sample was used to measure haemoglobin using a portable spectrophotometer (Haemocue).

4.2.5 Parasitological Assessment

4.2.5.1 Microscopic analysis

Thick and thin blood films were collected from each patient at screening. Blood films were also obtained on days 1, 2, 3, 7, 14, 21, 28 and any other day, if the patient returned due to some complaints spontaneously [198]. Giemsa working solution with buffering PH of 7.2 was used to stain the smears. Double-slide blood smears were prepared; one stained rapidly with 10% Giemsa for 10 to 15 minutes to screen for recruitment, and the next stained with standard 3% Giemsa for 30 to 45 min as recommended elsewhere [198]. The blood smears were examined by two microscopists blinded to each other results at a magnification of 1000× to examine parasite positivity,to identify parasite species and determine parasite density. Asexual and sexual stage of the parasite was determined from Giemsa-stained thick blood smears and enumerated against the number of parasites per 200 white blood cells on day 0, based on an assumed density of 8000 white blood cells per µl of blood. A blood smear was declared negative after examination of 1000 white blood cells [264, 265]. Discrepant results in terms of parasite positivity, species or density (by > 25%), a third blinded, independent microscopist re-examined the blood slides. For parasite species and positivity, two concordant results were considered the final result, while for parasite density, the average of the two closest estimates of parasitaemia was considered final.

4.2.6 Molecular analysis

4.2.6.1 Screening of Plasmodium genus with qPCR

Primerdesign[TM]Genesig standard kit for Plasmodium spp. (all species) genomes was assayed for the *in vitro* quantification of all *Plasmodium* species genomes by targeting the 18S ribosomal RNA (18S) gene according to the protocol of Primerdesign[TM] Ltd [266]. Each reaction was performed in duplicate and the cycle threshold number (Ct) was determined as their mean. A sample was considered positive if the fluorescent signal was detected in at least one replicate; conversely, if no signal was detected within 40 cycles, a reaction was considered negative.

4.2.6.1 Species-specific qPCR

Plasmodium falciparum genome was analysed for the in vitro quantification of *P. falciparum* genomes by targeting the *plasmepsin 4* gene according to the protocol of Primerdesign[TM] Ltd [267].

4.2.6.1 Molecular genotyping of *msp-1* and *msp-2*

Dried blood spots were obtained for PCR analysis at enrolment (day 0) and on follow-up days 7, 14, 21, and 28. PCR genotyping was performed on paired dried blood spots in the case of parasitaemia detected on or after day-7 to distinguish between recrudescence and re-infection for all treatment failures. PCR genotyping of *P. falciparum* polymorphic genes *msp1* and *msp2* was performed as per WHO protocol [268, 269, 270]. The results were classified as recrudescence if the recurrent parasites were of the same parasite strain as those on days 0 or as a new infection if they were a different strain.

4.2.7 Treatment outcome classification

Treatment outcomes were classified based on parasitological and clinical outcomes as recommended by the WHO [198]. Efficacy was evaluated using microscopy and qPCR in conjunction with clinical signs and symptoms. Parasite genotyping was also performed at baseline and at the time of recurrence of parasitaemia to differentiate between recrudescence and new infection. Therapeutic responses on day 28 were classified as adequate clinical and parasitological response (ACPR), or treatment failure (TF); designated as early treatment failure (ETF), late clinical failure (LCF), or late parasitological failure (LPF). The primary outcome measure was ACPR, corrected for reinfection using PCR genotyping from the day of reemergence of parasitaemia based on per protocol method and Kaplan–Meier analysis. A

secondary treatment outcome was parasite clearance during the first three day of follow-up and patterns of fever (i.e. temperature $\geq 37.5\ ^{o}C$).

4.2.8   Data management

Data entry and analysis was done by using the WHO designed Excel spreadsheet [271] and SPSS version 20 for windows. Cure rate on day-28 was assessed by intention to treat (ITT) and per protocol (PP) analysis. The PP Kaplan Meir was used to analyse the primary therapeutic outcomes. Briefly, PCR-uncorrected per protocol analysis excluded participants lost to follow-up and with-drawn whilst Kaplan-Meier analysis censored last day of follow-up for such participants. PCR-corrected per protocol analysis excluded participants lost to follow-up, with-drawn, with falciparum re-infection, and undetermined PCR whilst Kaplan–Meier analysis censored last day of follow-up for those lost to follow-up as well as those withdrawn or with falciparum re-infection. Study participants with undetermined PCR were also excluded in the Kaplan-Meier analysis [198]. Secondary treatment outcomes analyzed were parasite clearance during the first three days of follow-up, patterns of fever (i.e. temperature $\geq 37.5\ ^{o}C$) and gametocyte clearance. Chi-square and Fisher's exact tests were used to compare proportions whilst Student's t-test was used to compare means and $p < 0.05$ was considered significant during the analysis.

4.3   Results

4.3.1   Study participant enrolment and demographic characteristics

A total of 282 febrile participants were screened and of these, 80 participants were included in this study. The rest who were not fulfilling the WHO revised protocol for malaria drug therapeutic efficacy study [198] criteria were excluded from the study. Of the 80 participants, four participants (5%) were lost to follow up on days seven and twenty-one and one participant with LCF was classified as reinfection on day fourteen and was excluded from per protocol analysis. Thus, 75 participants were successfully followed up during the course of the study (Figure 5).

```
282 participants assessed for
            eligibility
```

```
202 ineligible

    -   79 other Plasmodium spp. infection
    -   65 mixed Plasmodium spp. infection
    -   8 declined to provide consent and assent
    -   26 had low parasite count ( < 1000
        parasite/μL)
    -   4 severe malaria(Confirmed)
    -   2 severe malaria(presumed on screening)
    -   8 breast feeding
    -   10 pregnant
```

```
80 Enrolled
```

```
    -   4 participants Lost to follow-up (LFU) (
        One patient at Day7, 3 patients at day 21)
    -   1 LCF withdrawn with reinfection at day
                            14
```

```
75 Participants completed
  therapeutic efficacy study
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Figure 5. Study participant flow chart

The age, axillary temperature, Hb level, body weight, parasitaemia and gametocyte carriage of the study participants are summarized in Table 2. The majority (71.2%) of the study participants were males. The age group 5-15 years represented most participants 50 (62.5%) followed by the group >15 years of age 26 (32.5%). The mean age of the study population was 20.96 (range 3-60

years). Geometric mean parasitaemia at baseline was 12374.31 (95%CI: 3699-14744) parasite/µl among all study participants.

Table 2. Demographic characteristics of study participants in the evaluation of therapeutic efficacy of artemether-lumefantrine in the treatment of uncomplicated *Plasmodium falciparum* malaria in Chewaka district, Ethiopia

Patient characteristics	Age category		
	< 15 years (n=30)	>=15 years (n=50)	Total (n= 80)
Mean age(or range)	9.27(3-14)	27.98(15-60)	20.96(3-60)
Gender			
Male n(%)	20(66.7)	37(74.0)	57(71.2)
Female n(%)	10(33.3)	13(26.0)	33(28.8)
Temperature in ^{o}C, mean(SD)	37.68(0.58)	37.79(0.81)	37.75(0.73)
Weight(Kg), mean(SD)	32.77 (19.01)	63.62(18.43)	52.05(23.86)
Hemoglobin (g/dl), mean(SD)	11.37(3.21)	11.82(3.15)	11.65(3.16)
Parasitaemia(per µl), geometric mean (range)	12301.31(3699-14292)	12418.31(9539-14744)	12374.31(3699-14744)
Gametocyte carriage, n(%)	2(6.7)	5(10.0)	7(8.8)

Key: SD: standard deviation,   Kg: kilogram, Hb: hemoglobin, n: number, °C: degree centigrade; g/dl: gram/deciliter

4.3.2 Primary outcomes

Four treatment failures, three LPF (one case at day-14 and two cases at day-21) and one LCF case at day-14 were observed, giving PCR-uncorrected failure rate of 5.3% (4/76) (95%CI: 0.8-18.2). By PCR correction, only the LCF was confirmed as a reinfection case with a failure rate of 4 %(95CI:0.8-11.2). There was seen no early treatment failure (ETF). For per protocol (PP)

analysis, PCR-uncorrected cure rate of AL among the study participants was 94.7% (95%CI: 87.1-98.5) and the PCR corrected cure rate was 96% (95%CI: 88.8- 99.2). For intention to treat (ITT) analysis, the cure rate was 90% (95%CI: 88.8-99.2) (Table 3). Based on PCR-corrected Kaplan-Meier survival estimate, the cumulative incidence of failure rate of AL among study participants was 3.8 %(95%CI: 1.3-11.4) and the cumulative incidence of success rate of AL among study participants was 96.2 %(95%CI: 88.6-98.7) (Figure 6)
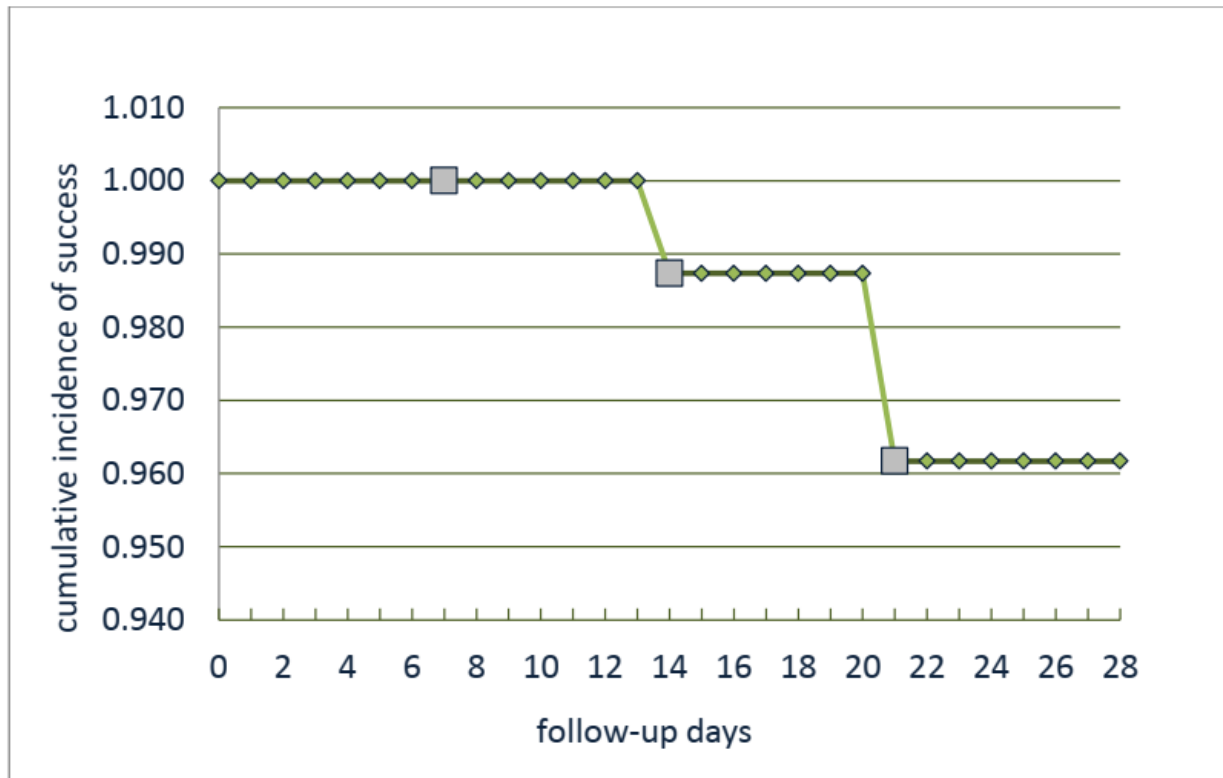
Key: ☐ Stands for censored (lost to follow-up and re-infection)

Figure 6. Kaplan-Meier Survival Curve with PCR corrected

Table 3. Results of therapeutic efficacy of artemether-lumefantrine in the treatment of uncomplicated *P. falciparum* malaria in Chewaka district, Ethiopia

Treatment outcome	n (%)
ETF	0(0.0)
LCF	1(1.3)[#]
LPF	3(4.0)[*]
ACPR	72(94.7)
PP PCR-uncorrected cure rate(95% CI)	72/76;94.7(87.1-98.5)
PP PCR-corrected cure rate(95% CI)	72/75;96.0(88.8-99.2)
ITT cure rate(95% CI)	72/80;90.0(83.3-96.7)

Key: ETF: Early treatment failure, LCF: Late clinical failure, LPF: Late parasitological failure, ACPR: Adequeate clinical and parasitological response, WTH: Withdrawal, LFU:Lost to follow-up, n.: number, PP: per protocol analysis; ITT: intention to treatanalysis, LFU: loss to follow up

Note: * stands for recrudescence and[#]stands for new infection.

4.3.3 Secondary outcomes

Parasite clearance: Based on qPCR quantitative parasite assessment, parasitaemia detection rate was 38.8% (31/80) on day-1, and declined to 18.8% (15/80) on day-2 and 3.8% (3/80) on day-3. Accordingly, parasite clearance rate was high that 61.2% of the participants cleared parasitaemia on day-1, 81.2% on day-2 and 96.2% on day-3 (Table 4). In total, only three participants (3.8%) of the 80 study participants were found to be positive on day-3. The day-3 positive participants were followed up to day 28 and had ACPR. Parasite clearance rate was compared between age groups taking day-2 mean parasitaemia as comparison variable; day-2 mean parasitaemia was significantly higher ($p<0.05$) in age group less than 15 years (702.4±1390.46) compared to age group greater than 15 years (408.3±1041.09) implying that study participants age greater than 15 years cleared the parasite faster than study participants age less than 15 years.

Fever clearance: Febrile individuals, with ≥37.5°C axillary temperature, accounted for 73.8% (59/80) at the day of recruitment and decreased to 47.8% (38/80) on day 1, 13.8% (11/80) on day-2 and 2.5% (2/80) on days-3. Accordingly, fever clearance rate was 87.2% (69/80) on day-2,

97.5% (74/80) on day-3 (Table 4 and Figure 7) and no febrile case was detected onwards except the three recrudescence and the one reinfection cases.

	Day 0	Day 1	Day 2	Day 3	Day 7
Parasitemia Detected (%)	100	38.8	18.8	3.8	0
Fever Cases (≥37.5°C) (%)	73.8	47.5	13.8	2.5	0
Gametocytes Carriage (%)	8.8	5	3.8	0	0
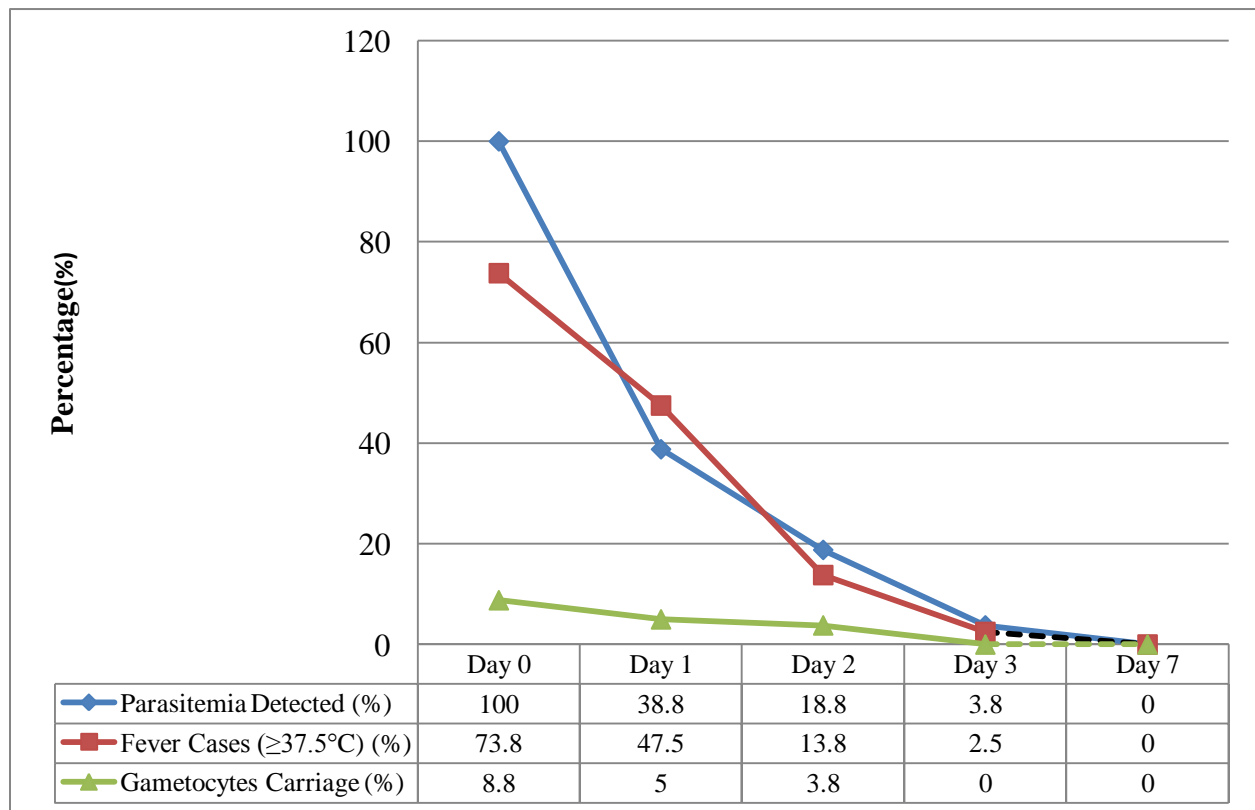
Figure 7. Parasitaemia, fever and gametocyte clearance rate during treatment and follow-up period in Chewaka district, Ethiopia

Gametocytaemia clearance: based on microscopic analysis, only 8.8% (7/80) gametocyte carriers, among all study participants, were detected at enrolment: Of these, 6.2% (2/30) detected in <15 years and 10% (5/50) was detected in ≥15 years. Of the day-0 gametocyte carriers detected, threecases on day-1 and fourcases on day-2 were cleared giving gametocytaemia clearance rate of 42.9 % (3/7) and/or 57.1 % (4/7) respectively. The proportion of gametocyte carriage per total study participants was declined from 8.8% (7/80) on day 0 to 5% (4/80) on day 1, 3.8 % (3/80) on day 2 and totally disappeared on day 3 (Table 4 and Figure 7). After initiation of the treatment nonew gametocyte carrier was observed.

Table 4 Parasite, fever and gametocyte clearance rate in study participants during treatment and follow-up period in Chewaka district, Ethiopia

Variable	Age Category	Follow-up days			
		D0 (Baseline)	D1 (24hr)	D2 (48hr)	D3 (72hr)
Parasitemia detected, n (%)	<15 yrs.	30(100)	12(40.0)	9(30.0)	0(0.0)
	≥15 yrs.	50(100)	19(38.0)	7(14.0)	3(6.0)
	Total	80(100)	31(38.8)	16(20.0)	3(3.8)
Fever cases (≥37.5°C), n (%)	<15 yrs.	24(80)	17(56.7)	7(23.3)	0(0.0)
	≥15 yrs.	35(70)	21(42.0)	4(8.0)	2(6.7)
	Total	59(73.8)	38(47.5)	11(13.8)	2(2.5)
Gametocytes carriage, n (%)	<15 yrs.	2(6.7)	0(0.0)	0(0.0)	0(0.0)
	≥15 yrs.	5(10.0)	4(8.0)	3(6.0)	0(0.0)
	Total	7(8.8)	4(5.0)	3(3.8)	0(0.0)

4.3.4 Clinical cases with recrudescence

Three study participants experiencing recrudescent parasitaemia between 14-21 days after treatment start. All attended their treatment at health centers for uncomplicated *P. falciparum* malaria and had an adequate initial treatment response and were parasite-negative by microscopy and qPCR analysis latest on the second day after initiated AL treatment of admission. Each dose was administered by a ward nurse and medical officer who documented intake in the medical record, and compliance according to documentation was 100%. The participants were 21-51 years old with body weight 54-70 kg.

4.3.5 Adverse Drug Reactions

Information on AL related side effects was collected through self-reporting and recorded in the case reporting forms. No serious adverse events were reported throughout the 28 days follow up period. Overall, 80% of all study participants reported no side effects and 20% reported one or

two side effects. The most reported adverse reactions in this study were headache (8.8%), vomiting (3.4%), Shortness of breath (2.5%), cough (1.3%), diarrhea (1.3%) and joint/muscle pain (2.5%). Most of these probable AEs disappeared with the clearance of parasitaemia except cough. Cough persisted for some time beyond parasite clearance.

4.4 Discussion

In this study the PCR-corrected cure rate 96% (95%CI; 88.8-99.2), which showed the high therapeutic efficacy of AL since its introduction for the treatment of uncomplicated falciparum malaria in the study area, meeting the WHO recommendation that cure rates for falciparum malaria should be at least 90% [198]. The observed PCR corrected cure rate in this study is comparable with what was documented in other parts of Ethiopia by Nega *et al*., Mekonnen *et al*., and Getnet *et al*. in which PCR-corrected ACPR of 97.8% , 98.8% and 95% respectively, were observed after 28 days follow following treatment with AL [232, 233, 234]. The observed high AL cure rate is comparable with other findings in other parts of Africa in which PCR-corrected ACPR of 95% in Ghana by Abuaku *et al.* and 99.3% in Tanzania by Ishengoma*et al*. were demonstrated [169, 272]. Most of these results are well within the confidence intervals of this study and minor differences may be attributable to host nutritional and immune status, initial parasitaemia level, pharmacokinetics and pharmacodynamics may influence the therapeutic efficacy of a drug apart from inherent parasite susceptibility [273].

Early treatment failure was not observed in this study, whereas three LPF were 3.9 %(95CI:0.8-11.1) in PCR uncorrected and 4 %(95CI:0.8-11.2) treatment failure was observed in PCR corrected data. Several studies[149, 171, 274, 275] in which therapeutic efficacy tests were combined with sampling of plasma or whole blood for drug concentration measurements at various times during follow-up have shown that cured patients have higher drug concentrations than those in whom treatment failed. There are two possible explanations for the latter finding. First, failures are associated with inadequate drug concentrations rather than resistance-this could be the case in our findings of treatment failure of 4%; secondly, when drug resistance emerges, there is a higher likelihood that a resistant strain will emerge if the drug is present at a suboptimal concentration.

According to the Kaplan Meier PP survival analysis in the current study, the PCR-corrected AL failure rate was 3.8% (95%CI; 1.3-11.4) and the cumulative incidence of success rate of AL

among study participants was 96.2 %(95%CI: 88.6-98.7) (Figure 6). The AL treatment failure rates observed is below the WHO threshold of 10%, and, therefore, suggests that lumefantrine was not failing as partner drug in AL use in the study area, with no change in the national treatment policy [198]. Inadequate drug absorption resulting in suboptimal serum drug concentrations can cause treatment failure. Artemether is rapidly absorbed and eliminated (half-life of a few hours), whereas lumefantrine was variably absorbed and more slowly eliminated [76]. Lumefantrine is a lipophilic compound with erratic bioavailability unless administered with a small fatty meal [171], and for this reason, guidelines recommend administration of AL with a fatty meal such as milk or a small biscuit. In the case of our study, we were unable to confirm adequate serum concentrations of lumefantrine; however, all participants received a complete course of treatment and all the doses were supervised in the health centreand administered with a milk biscuit to ensure good absorption. Nevertheless, considerable inter-individual variation exists in lumefantrine exposure, and these participants may have had relatively low concentrations.

Day-3 parasitaemia may be a poor predictor of patient outcomes on day 28 because the supplemental drug may still clear the infection. However, determining the presence of day 3 parasitaemia has been suggested as a surrogate for assessing artemisinin resistance e.g. in mobile populations [276]. In the present study, the parasitaemia on day 3 following treatment with AL was only 3.8% (95%CI; 0.8-10.6) on day-3 and Day-3 parasitaemia did not correspond to failures observed during follow-up. This may indicate absence of resistant strains of P. falciparum to artemisinin in study area. This is in line with the WHO 2009 anti-malarial protocol, that if 10% of the study participants have peripheral parasitaemia on day 3, it is an indicator of emergence of artemisinin resistance to Plasmodium species [198, 276]. The overall rate of day-3 positivity observed in this study are consistent with the 3-5% background rate of day-3 positivity that might be expected in the absence of resistance to artemisinin, but also the 3-10% range which in the past has been seen as appropriate window for initiating containment activities [277].

Episodes of recurrent parasitaemia following treatment may be due to recrudescence of the initial infection, reflecting failure of the drug to clear the infection; or, they may be due to new infections that occurred during the follow-up period [279]. In areas of high endemicity recurrent

infections are common although PCR analysis of *msp1* and *msp2* gene markers estimated that three cases were recrudescence and a single case of re-infections are observed in this study. The recrudescent parasitaemia resolved quickly after initiated re-treatment in all cases.

AL showed rapid parasite and fever clearance during the first three days of controlled supervised follow-up period. Over all prevalence of parasite and fever declined by 96.2% and 97.5 on day 3 respectively. Gametocytaemia was absent on day 3 following treatment with AL. These findings suggest that AL remains effective in rapidly clearing asexual parasites and fever as well as reducing gametocyte carriage rates in study Ethiopia [232, 233, 234]. The high parasite and fever clearance rates could be explained by the fast act of artemether to clear parasite biomass leading to rapid resolution of clinical manifestations [76, 279].

This study also showed that AL had a safety profile comparable to previous studies and was well tolerated with minimal adverse events. Studies conducted in other African countries [280, 281, 282] reported similar safety profiles of AL when used for the treatment of uncomplicated falciparum malaria. A high number of cases reporting cough at the study site could be attributed to weather conditions, which were relatively cold and rainy at the time of the study.

The relative bioavailability of artemether and lumefantrine increases by 2-3 times and 16 times, respectively, when administered after a high-fat meal [283].The limitation of this study is the lack of pharmacokinetic data to better explain the recrudescence observed. The cure rates for AL may therefore be higher than the rates observed in this study.

4.5   Conclusions

The findings of this study showed that the therapeutic efficacy of AL is considerably high (above 90%). AL remains highly efficacious in the treatment of uncomplicated malaria in the study area achieving rapid fever and parasite clearance as well as low gametocyte carriage rates despite the use of this combination for more than 15 years. Day-three parasitaemia warrants a close monitoring of the efficacy of AL in the future and should be continued in order to generate evidence to support national malaria treatment policy and practice.

CHAPTER V: GENETIC POLYMORPHISM *PLASMODIUM FALCIPARUM* PARASITES

(Adopted from: Abamecha et al. 2020)

Malaria Journal

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Genetic diversity and genotype multiplicity of *Plasmodium falciparum* infection in patients with uncomplicated malaria in Chewaka district, Ethiopia

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Abstract

Background: Genetic Polymorphism in *Plasmodium falciparum* poses a major threat to malaria control and elimination interventions. Characterization of the genetic Polymorphism of *P. falciparum* strains can be used to assess intensity of parasite transmission and identify potential deficiencies in malaria control programmes, which provides vital information to evaluating malaria elimination efforts. This study investigated the *P. falciparum* genetic Polymorphism and genotype multiplicity of infection in parasite isolates from cases with uncomplicated *P. falciparum* malaria in Southwest Ethiopia.

Methods: A total of 80 *P. falciparum* microscopy and qPCR positive blood samples were collected from study participants aged six months to sixty years, who visited the health facilities during study evaluating the efficacy of artemether-lumefantrine from September-December, 2017. Polymorphic regions of the *msp-1* and *msp-2* were genotyped by nested polymerase chain reactions (nPCR) followed by gel electrophoresis for fragment analysis.

Results: Of 80 qPCR-positive samples analysed for polymorphisms on *msp-1* and *msp-2* genes, the efficiency of *msp-1* and *msp-2* gene amplification reactions with family-specific primers were 95 % and 98.8%, respectively. Allelic variation of 90% (72/80) for *msp-1* and 86.2% (69/80) for *msp-2* were observed. K1 was the predominant *msp-1* allelic family detected in 20.8% (15/72) of the samples followed by MAD20 and RO33. Within *msp-2*, allelic family FC27 showed a higher frequency (26.1%) compared to IC/3D7 (15.9%). Ten different alleles were observed in *msp-1* with 6allelesforK1, 3 alleles for MAD20 and1 allele forRO33. In *msp-2*, 19 individual alleles were detected with 10 alleles for FC27 and 9 alleles for 3D7. Eighty percent (80%) of isolates had multiple genotypes and the overall mean multiplicity of infection was 3.2 (95% CI: 2.87- 3.46). The heterozygosity indices were 0.43 and 0.85 for *msp-1* and *msp-2*, respectively. There was no significant association between multiplicity of infection and age or parasite density.

Conclusions: The study revealed high levels of genetic Polymorphism and mixed-strain infections of *P. falciparum* populations in Chewaka district, Ethiopia, suggesting that both endemicity level and malaria transmission remain high and that strengthened control efforts are needed in Ethiopia.

Keywords: Genetic Polymorphism, Multiplicity of infection (MOI), *Plasmodium falciparum,* Ethiopia

5.1 Background

The intensification of malaria control interventions has resulted in its global decline, but it remains a significant public health burden across several malaria-endemic countries [247]. The 2018 global malaria report revealed that the incidence rate of malaria declined by 18% from 2010 to 2017, in the same period, the estimated number of cases dropped from 239 million to 219 million, and the number of deaths from 607,000 to 435,000 [247, 283, 284]. In Ethiopia, the trends in malaria over the past five years have also shown a decline in malaria cases and fewer epidemics [285, 286]. In 2014/2015, Ethiopia reported 2,174,707 malaria cases and 662 reported malaria deaths among all age groups which is a 98% reduction compared to 41,000 estimated deaths in 2006 [285285, 286]. Between June 2016 and July 2017, the Ethiopian Health Management Information System (HMIS) reported a total of 1,755,748 malaria cases and 356 deaths due to malaria [285]. The key interventions which have been contributing to such significant decline includes: introduction of prompt and effective treatment withartemisinin-based combination therapy (ACT), the distribution and promotion of the use of long-lasting insecticidal nets (LLINs), nationwide coverage of indoor residual spraying (IRS), and environmental management [285, 286]. Ethiopia adopted artemether-lumefantrine (AL) in 2004as first-line for the treatment of uncomplicated falciparum malaria, LLINs coverage has been scaled up in Ethiopia since 2005, resulting in over 64 million nets distributed by 2014. IRS, includingpermethrin, bendiocarb propoxur and deltamethrin, pirimiphos-methyl has been used between 2014 - 2020.Although these control measures have resulted in a substantial decrease in malaria infections in Ethiopia, malaria is still endemic, with populations in some areas remaining at high risk of infection. Ethiopia has set a goal to eliminate the disease by 2030 using these interventions [285, 287].

Genetically-distinct malaria parasites in natural populations have an extremely high rate of genetic recombination during the sexual stages in a mosquito host, often resulting in multiple strains being transmitted simultaneously [289]. This Polymorphism hampers development of effective vaccine as it limits the efficacy of protective immunity (i.e., antibody-mediated parasite inhibition) [290]. Highly endemic malaria settings are prone to infections containing multiple *P. falciparum* strains, primarily due to repeated exposure to mosquitoes infected with multiple parasite strains [291]. This genetic Polymorphism of the parasite is one of the main factors responsible for the slow acquisition (several years) of immunity against malaria. Thus,

individuals would have to encounter a broad range of circulating parasite populations before they develop an effective anti-malarial immunity [292].

Genetic Polymorphism and multiplicity of *P. falciparum* infections are essential parasite indices that could determine the potential impact on the selection of drug-resistant parasites. Although many polymorphic antigens have been described in several stages of the parasite life cycle, merozoite surface protein 1 and 2 (*msp-1* and *msp-2*) seem to be the most appropriate to distinguish parasite populations [95, 293, 294]. This markers are particularly useful in determining the multiplicity of infection (MOI), a measure of the effectiveness of intervention programmes and also *msp-1* and *msp-2* typing are widely used in anti-malarial drug efficacy trials to distinguishing recrudescent parasites from new infections [295, 296, 297]. Study reports by Jelinek *et al.* [298] and Meyer *et al.* [299] showed that increased genetic Polymorphism of circulating malaria parasites in a population increases the potential for the selection of drug resistance.

Declining malaria transmission as a result of scaling-up interventions has been shown to affect the parasite population genetics pattern and population structure of *P. falciparum* [104, 105, 300]. The scale-up interventions, such as the usage of insecticide-treated bed nets, indoor residual spraying [104, 301] and the introduction of new anti-malarial drug regimens [102, 103, 105, 302, 303, 304, 305] to control and treat malaria have been shown to cause the genetic drift and decrease the level of allelic Polymorphism(*He*) and MOI. However, this does not occurred in all settings [105, 104]. In addition, the genetic Polymorphism and population structure studies can be used to monitor the effects of any malaria scale-up interventions, such as the impact of malaria control and elimination programs [306]. Hence, accurate assessment of the parasite's genetic Polymorphism across malaria endemic regions could help plan or develop new control and elimination strategies. The MOI, which identifies the number of clones within a particular infection, can serve as a measure of the level of malaria transmission as well as identify hotspots [127, 307]. Malaria parasite Polymorphism is distinct in different individuals, populations, transmission settings and seasons within endemic zones and changes with variations in parasite prevalence [307], and has been suggested to be constantly changing [309, 310, 311, 312]. Parasite populations even respond to specific interventions, such as rapid diagnostic tests, human host immune pressure and mosquito vector [313, 314, 315]. The identification of hotspots is

important in understanding the epidemiology of *P. falciparum* infections for informed interventions to be implemented [307, 316]. The effect of malaria control interventions on the *P. falciparum* population structure in Ethiopia could not be assessed due to the lack of genetic data and systematic genetic surveillance study. Chewaka district in Southwest Ethiopia experiences frequent epidemic outbreaks of malaria. Parasite genetic Polymorphism and multiplicity of infection studies have also been found to be important in the surveillance of strains circulating in a particular transmission area especially in Southwest Ethiopia because there was so limited information available on the genetic structures of *P. falciparum* [317, 318, 319]. This study was aimed at characterizing the genetic Polymorphism and allele frequencies of *msp-1* and *msp-2* genes of *P. falciparum* isolates from uncomplicated malaria patients in Chewaka district, Southwest Ethiopia.

5.2 Methods

5.2.1 Study setting

The study was conducted in Ilu-Harar Health Centre, Chewka district, Buno Bedele Zone, Southwest Ethiopia during September-December 2017. Chewaka district is located in Buno Bedele zone, Oromia regional state, Ethiopia about 570 kilometres southwest of Addis Ababa. It is situated in lowland areas of Dhidhesa valley, which lies below 1500m above sea level. The district has 26 administrative *kebeles* (villages). As in most other areas, malaria transmission in Chewaka follows rainy seasons, with transmission peaking in the months between September and December and between April and May. The main malaria control strategy in the district includes long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS) and malaria case management with ACT [285, 287]. In 2017, the FMOH updated the country's malaria risk strata based on malaria annual parasite incidence (API), calculated from micro-plan data from more than 800 districts,classifying areas with malaria transmission risk by API as high (≥ 100 cases/1,000 population/year), moderate (≥ 5 and <100), low (>0 and <5), and malaria-free (~ 0). Chewaka district was classified as mesoendemic/moderate transmission setting [286].

5.2.2 Study population and blood sample collection

A total of 80 *P. falciparum* infected blood spots were collected during a therapeutic efficacy study of artemether-lumefantrine (Coartem®), between September and December 2017. The PCR analysis of *msp1* and *msp2 gene* markers showed that three cases were recrudescence and a

single case of re-infections was observed in the study. The observed recrudescent parasitemia was between 14-21 days after treatment start. However, the recrudescent parasitemia resolved quickly after initiated re-treatment in all cases with the same regimen (unpublished data). The participants were aged between six months and sixty years, were residents within Chewaka area, and had presented to the local health centre. Febrile patients with axillary temperatures ≥37.5°C, positive for asexual *P. falciparum* mono-infection giving written consent were included in the study. Children aged less than six month, pregnant women and individuals suffering from any other diseases were excluded.

After consent was obtained, the blood samples were obtained by finger prick and malaria infection was diagnosed using microscopy and qPCR. Whenever a participant tested positive for asexual *P. falciparum* mono-infection, approximately 50µl of whole blood was spotted onto filter paper (Whatman® 927 mm) and air dried. The blood spots were individually placed into plasticbags with desiccant and transported to the Jimma University Clinical Trial Unit (JU-CTU) and stored at -20 °C for a maximum of three months prior to further analysis.

5.2.3 Extraction of parasite DNA

Genomic DNA was extracted from whole blood using proteinase K-base method (GE Healthcare Illustra Blood Genomic Prep Mini Spin Kit) according to the manufacturer's instructions for qPCR species identification and parasite density determination. For nested PCR, the DNA was extracted from stored dried blood spots collected on enrollment (Day-0) and on any day after day 3 were deemed to have recurrent parasitaemia using Pure Link^{TM} Genomic DNA mini Kit (Invitrogen, USA) according to the manufacturer's instructions. DNA was checked for purity and quantity using Nanodrop spectrophotometer (ND 1,000), and stored at -20 °C until used for PCR amplification and detection.

5.2.4 Quantitative PCR (qPCR) screening for *Plasmodium falciparum*

Primer design genesig (Bio-Rad Laboratories,Inc. Germany) standard Kit for *Plasmodium* spp. genomes was analysed for the *in vitro* quantification of all *Plasmodium* spp.genomes by targeting the 18S ribosomal RNA (18S) gene according to the protocol of Primer design^{TM} Ltd [320].Species specific *Plasmodium falciparum* genome was analysed for *in vitro* quantification of *P. falciparum* genomes by targeting the *plasmepsin 4* gene according to the protocol of Primerdesign^{TM} Ltd [320]. Each reaction was performed in duplicate and the cycle threshold

number (Ct) was determined as their mean. A sample was considered positive if the fluorescent signal was detected in at least one replicate; conversely, if no signal was detected within 40 cycles, a reaction was considered negative.

5.2.5 Genotyping of *Plasmodium falciparum* isolates

Genotyping of *P. falciparum* isolates was carried out by Nested PCR amplification of the two highly polymorphic regions of *msp-1* (block2) and *msp-2* (block3) genes as reported previously [320, 321]. Primer sequences (Additional file 1: Table S1 and Additional file 2: Table S2) and cycling parameters used for amplification of the three allelic families of *msp-1*(K1, MAD20 and RO33) and two allelic families of *msp-2* (FC27 and 3D7) have been reported elsewhere [322, 323]. Briefly, in the initial amplification, primer pairs corresponding to conserved sequences within the polymorphic regions of each gene were included in separate reactions. The product generated in the initial amplification was used as a template in five separate nested PCR reactions. In the nested reaction, separate primer pairs targeted the respective allelic types of *msp-1*(K1, MAD20 and RO33) and *msp-2* (IC/3D7 and FC27), with an amplification mixture containing 250nM of each primer, 2mM of $MgCl_2$ and 125µM of each dNTPs and 0.4 units Taq DNA polymerase (MyTaq^{TM} DNA Polymerase, Bioline). The cycling conditions in the thermocycler (TECHNE, GENIUS), for initial *msp-1* and *msp-2* PCR were as follows: 5 min at 95 °C, followed by 25 cycles for 1 min at 94 °C, 2 min at 58 °C and 2 min at 72 °C and final extension of 5 min at 72 °C. For msp-1 and msp-2 nested PCR, conditions were as follows: 5 min at 95 °C, followed by 30 cycles for 1 min at 95 °C, 2 min at 61 °C and 2 min at 72 °C and final extension of 5 min at 72 °C [322]. The allelic specific positive control 3D7 and DNA free negative controls were included in each set of reactions [321]. Fragment analysis of *msp-1* and *msp-2* amplified products were then performed through electrophoresis on 2% and 3% ethidium bromide-stained agarose gel, respectively, and after migration, the DNA fragments were visualized by UV trans- illumination. A standard curve is then drawn by measuring the distances traveled (in cm) from the well, of the bands of the size marker, according to the mathematical function: f (x) = y; where f (x) = the actual distance traveled by the band on the gel; y = log10 (bp). The size of an unknown strip is then determined by plotting the distance travelled on the x-axis, then projection on the coordinate axis to determine the size in base pairs. For individual samples, alleles were identified according to band size (Additional file 3: Figure S3 and Additional file 4: Figure S4). This study assessed the frequency of the occurrence of each allele

in the population. The studycategorized clones into molecular weight groups differing by 20 bp for clear discrimination from other clones and elimination of errors that would result from estimating the molecular weight on agarose-gels.

5.2.6 Data analysis

The *msp-1* and *msp-2* allele frequencies were expressed as the proportion of samples containing an allelic family compared to the total number of samples that gene was detected in isolates. The detection of one *msp-1* and *msp-2* allele was considered as one parasite genotype. The multiplicity of infection (MOI) was defined as the minimum number of *P. falciparum* genotypes per infected subject and estimated by dividing the number of amplified PCR fragments reflecting the parasite genotypes by the number of positive samples in the same marker [323]. The size of polymorphism in each allelic family was analysed; assuming that one band represented one amplified PCR fragment derived from a single copy of *P. falciparum msp-1*or *msp-2* genes. Alleles in each family were considered the same if fragment sizes were within 20bp interval [296].

Spearman's rank correlation coefficients were calculated to assess association between multiplicity of infection (MOI) and geometric mean parasite density and age. The heterozygosity index (He), which represents the probability of being infected by two parasites with different alleles at a given locus, was calculated by using the Genetic Analysis in Excel toolkit (GenAIEx) [324]. Briefly, the allelic Polymorphism (*He*) for each antigenic markers was calculated based on the allele frequencies, using the formula: He= [n/ (n-1)] [(1-Σpi^2)], where n is the number of isolates sampled and pi is the allele frequency at a given locus. Allelic Polymorphism has a potential range from 0 (no allele Polymorphism) to 1 (all sampled alleles are different) [325]. Student's test was used to compare MOI. The chi-square test or Fisher's exact test was used for proportion comparisons. The *p* value < 0.05 was chosen as threshold significance for the various statistical tests. All statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

5.3 Results

5.3.1 Demographic and parasitological data

Of the 80 patients enrolled 57 (71.2%) were males, mean (±SD) age of participants was 20.96 (±13.6) years. Participants had asexual parasitaemia ranging from 3,699   to 14, 744 parasites/µL with a geometric mean of 12,513parasites/µl (95% CI 12,167-12,859). The parasite DNA from the 80 *P. falciparum* samples was analysed for *msp-1* and *msp-2* genes. The estimated frequency of *msp-1* and *msp-2* gene amplification reactions with family-specific primers was 90% (72/80) and 86.3% (69/80), respectively.

5.3.2 Allelic polymorphism of *P. falciparum msp-1* and *msp-2* genes

Polymorphism analysis was assessed in 80 *P. falciparum* isolates within the allelic families of *msp-1* and *msp-2* with a total of 253 distinct fragments detected. The *msp-1* gene analysis showed 63, 50, 31 fragments belonged to K1 (43.75% of overall detected *msp-1* alleles), MAD20 (34.72%) and RO33 (21.5%) allelic families noted, respectively. The *msp-2* gene analysis showed 58, 51 fragments belonged to FC27 (53.2% of overall detected *msp-2* alleles) and IC/3D7 (46.8%) allelic families noted respectively.

The proportion of K1, MAD20 and RO33 types were 20.8, 4.2, and 4.2%, respectively. The remaining 70.8% (51/72) were polyclonal infections. Among polyclonal infections carrying two allelic types, the frequency of samples with K1/MAD20, K1/RO33, and MAD20/RO33 was 31.9, 5.6, and 5.6%, respectively. Infections with all three allelic types were detected in 29.2% of cases (Table 5).

Table 5. Genotyping of *P. falciparum msp-1* polymorphic region block-2 in malaria patients from Chewaka district, Ethiopia

msp-1, N=72	Frequency (%)	Allele size (bp)	No of alleles	Overall MOI
K1	15(20.8)	130-300	6	2.0
MAD20	3(4.2)	180-220	3	
RO33	3(4.2)	150	1	
K1+MAD20	23(31.9)			
K1+RO33	4(5.6)			
MAD20+RO33	3(4.2)			
K1+MAD20+RO33	21(29.2)			

Key: MOI; multiplicity of infection

Allele genotyping demonstrated the highly polymorphic nature (i.e. more alleles) of *P. falciparum* in Chewaka isolates with respect to *msp-1* and *msp-2* (Additional file 3: Figure S3 and Additional file 4: Figure S4). A total of 29 individual with *msp* alleles were identified (10 for *msp-1* and 19 for *msp-2*). Among *msp-1* isolates, six K1 (130-300 bp), three MAD20 (180-220bp) and one RO33 (150bp) allelic families were noted.

In *msp-2*, a total of 19 different alleles were identified (Table 6), of which ten alleles belonged to FC27and nine alleles belonged to IC/3D7. Allele sizes ranged from (260 to 540 bp) for FC27 and (170 to 450 bp) for IC/3D7 allelic families. The frequency of samples with only FC27 and IC/3D7 were 26.1 % (18/69) and 15.9 % (11/69), respectively. Forty of the isolates (58%) carried both *msp-2* allelic families.

5.3.3 Genotype multiplicity of *P. falciparum* infection

Of the 80 positive samples, 64 (80%) harboured more than one parasite genotype identified by the presence of two or more alleles of one or both genes. with the overall mean MOI i.e., parasite clones per sample was 3.2 (95% CI: 2.87- 3.46). When considering *msp-1* and *msp-2* genes separately, the MOI was 2.0 (95% CI: 1.82-2.18) and 1.6 (95% CI: 1.46- 1.70), respectively, while 51/72(70.9%) and 40/66 (58%) of isolates contained multi-clonal infection at least with 2 clones, respectively. The heterozygosity index, which represents the probability of being infected by two parasites with different alleles at a given locus, was 0.43 for *msp-1*and 0.85 for *msp-2* loci. No significant correlation between multiplicity of infection and parasite density of patients (Spearman rank correlation = 0.094; *p*= 0.409) or multiplicity of infection and age (Spearman rank correlation = 0.072; *p*= 0.528). According to age, and parasite density the MOI was similar between individuals of different age and parasite density with-out significant difference (Table 7).

Table 6. Genotype of *P. falciparum* msp-2 polymorphic region block region block 3 in malaria patients from Chewaka district, Ethiopia

msp-2,N=69	Frequency (%)	Allele size(bp)	No of alleles	Overall MOI
FC27	18(26.1)	260-540	10	1.6
IC/3D7	11(15.9)	170-450	9	
FC27+IC/3D7	40(58.0)			

Key: MOI, multiplicity of infection

Table 7. MOI according age and parasite densityin malaria patients from Chewaka district, Ethiopia

	MOI		
Age	*msp1*	*msp2*	*msp-1+msp-2*
< 5 years	1.75	1.67	3.0
5-15 years	2.00	1.61	3.19
≥15 years	2.02	1.56	3.16
P Value	*p> 0.05*	*p> 0.05*	*p> 0.05*
Parasite density			
<1000	2.50	1.25	3.75
≥10000	1.97	1.60	3.13
P Value	*p> 0.05*	*p> 0.05*	*p> 0.05*

5.4 Discussion

The genetic Polymorphism of *P. falciparum* parasites impacts malaria transmission and malaria control strategies [326].Genetic structures and population genetics studies of *P. falciparum* may hold the key for effective disease surveillance and control programmes, especially in Southwest Ethiopia as so far there is very limited information available on the genetic structures of *P. falciparum*. As the country moves towards malaria elimination, understanding the genetic Polymorphism and population structure of the malaria parasite populations in hotspots is crucial to guide monitoring and evaluation of malaria control strategies and anti-malarial interventions. The present study provides a detailed assessment of genetic Polymorphism and multiplicity of infection of *P. falciparum* parasites from Chewaka district, Southwest Ethiopia.

In this study, allele-specific PCR typing of *the msp-1* and *msp-2* loci showed considerably diverse and extensive allelic polymorphisms in *P. falciparum* populations in the analysed samples. However, the number of alleles may have been underestimated due to the limitations of the technique used. Indeed, the numbers of alleles (bands) detected may be underestimated due to sensitivity of the PCR technique used as minor fragments (<50 bp) cannot be detected on the agarose gel and also similar sized fragments may be classified as identical leading to a false impression of similarity. Within allele families, alleles of the same size may have different amino acids motifs [324, 325], which emphasizes the importance of sequencing in future studies to confirm Polymorphism and extensive allelic polymorphisms in the *P. falciparum*. A total of 10 and 15 different alleles for *msp-1* and *msp-2*, respectively, were obtained from the parasite isolates in Chewaka district, Ethiopia. This genetic Polymorphism was consistent with the Polymorphism found in Kolla-Shele area, Southwest Ethiopia (*msp-1*: 11; *msp-2*: 12) in 2015 [317], in Northwest Ethiopia (*msp-1*: 12; *msp-2*: 22) in 2018 [318], and Brazzaville in the Republic of Congo (*msp-1*: 15; *msp-2*: 20) in 2018 [327]. In contrast, a higher Polymorphism (*msp-1*: 26; *msp-2*: 25) was found in Bioko Island, Equatorial Guinea in 2018, even though this area has comparable malaria endemicity patterns [326]. K1 was the predominant allelic family for *msp-1* as also demonstrated in previous studies in Africa, including Southwest Ethiopia [317], Brazzaville, Republic of Congo [296] and Gabon [328]. However, in studies conducted in Northern Ethiopia [319], Central Sudan [95] and Bioko Island, Equatorial Guinea [326] the MAD-20 allele was found to be predominant.

In this study, the RO33 family showed no polymorphism with only a single allele (160bp). This is similar to findings in Congo [296]. Allele typing of *msp-2* showed that FC27 was the predominant allelic family as also demonstrated in previous reports from Benin [328] and Central Sudan [95], but in contrast with previous studies in Ethiopia [317] and Brazzaville, Republic of Congo [296]. A variation in the prevalence of alleles between different studies likely reflects the differences in sample population. Thus, it is important to conduct studies that include adequate sample size as well as sampling at different time point within the same region to assess and compare the genetic profile of parasites circulating in endemic areas in an attempt to avoid intra and inter individual variation in the number of parasite genotypes detected in the different episodes of malaria. Besides, methodological differences may also affect the comparability of results. Hence, further investigations with more powerful techniques such as capillary electrophoresis and DNA sequencing are needed to better characterize the malaria parasites in the country.

Multiplicity of infection (MOI), i.e. the number of different *P. falciparum* strains co-infecting a single host, has been shown to be a common feature in most malaria-endemic areas and was reported to vary with age, parasite density, immune status, epidemiological settings and transmission intensity [132, 330, 331, 332]. In this study, 80% of the isolates harboured more than one parasite genotype identified by the presence of two or more alleles of one or both genes with the overall mean MOI being 3.2 (95% CI: 2.87- 3.46). The overall MOI value reported in this study was higher than previously reported studies, including Ethiopia (MOI: 1.8 - 2.6) between 2015-2018 [317, 318, 319], Brazzaville, Republic of Congo (MOI: 2.2) [296] in 2011 and Bobo-Dioulasso, Burkina Faso (MOI: 1.95) [333]. In contrast to study reported in Bioko Island, Equatorial Guinea (MOI: 5.51) [326] in 2018 and Gabon (MOI: 4.0) [334] in 2018. The difference in MOI can be explained by the differences in intensity of malaria transmission seasons. In this study, samples were collected during the major malaria transmission season of September to December, when malaria transmission is very intense. All year round (seasonal) studies covering major and minor transmission seasons are needed to better understand genetic profiles in this area including a sense on seasonal variations.

The results of this study show that age has no association on multiplicity of infectionsimilar to other studies [317, 319, 323], but in contrast with reports from Brazzaville, Republic of Congo

[296] and Central Sudan [95]. Previous studies regarding the variation of MOI over age have suggested that the influence of age on the multiplicity of infection is highly affected by endemicity of malaria [132, 329, 330, 331]. This is probably a reflection of the development of anti-parasite specific immunity [127]. Thus, in holo- or hyperendemic areas, immunity develops faster and at younger age than in areas with less intense transmission [335]. Studies have shown an age-dependent MOI in a village with intense perennial malaria transmission but not in areas where malaria is mesoendemic [132, 323]. Similarly, in this study reported that no significant relation between MOI and the parasite count, similar to reports from previous studies in Ethiopia [317, 319], but in contrast with reports fromBioko Island, Equatorial Guinea [329]. This may have been due to the small number of isolates analysed.

High transmission regions like those in many African countries are commonly characterized by *P. falciparum* populations that are genetically diverse. Antigenic marker genotyping carried out in African regions like Burkina Faso, Sao Tome, Malawi, Uganda and Tanzania have identified *P. falciparum* populations with alleles occurring at a frequency below 10 percent with a very high *He* level of 0.78 to 0.99 [297]. This study indicated that the genetic Polymorphism values were higher based on heterozygosity index for *msp-2* (He=0.85), than for *msp-1* (He=0.43), suggesting a large genotype Polymorphism within the *msp-2* locus, which was higher than previously reported from Northwest Ethiopia (*msp-2*: *He* 0.62) in 2018 [319]. Djibouti, a neighbouring country to Ethiopia, an initially moderate level of genetic Polymorphism declined over an 11-year period to the point that the expected heterozygosity reached zero in 2009 consistent with very low Polymorphism [306].

Despite the lack of entomological data from Chewaka district, the number of clones co-infecting a single host can be used as an indicator of the level of malaria transmission or the level of host acquired immunity [330, 336, 337]. Besides, transmission intensity can also be affected by other factors, such as vector biting behaviour and endemicity [336]. Inferring high transmission intensity from the presence of multi-clonal infections alone has additional limitations including estimates of MOI varying by genotyping method, potential impact from sampling frequency and a non-linear relationship between MOI and transmission intensity [336]. Despite these limitations, infections with multiple clones observed in this study, combined with evidence of high genetic Polymorphism may indicate high transmission intensity in the study area.

5.5 Limitations of the study

The limitation of the present study is the small number of isolates analysed, which were collected during a therapeutic efficacy study of artemether-lumefantrine (Coartem®) in the region. In this study, the association between the dominant allelic families and the manifestation of the disease was not examined because all samples were collected from uncomplicated malaria patients. Thus, the relationship between malaria severity or clinical symptom and genetic Polymorphism could not be addressed in the present study. The collection of samples throughout the year (not just in high transmission season) would potentially give a better understanding of the true Polymorphism in the region. Despite these limitations, the data from the present study has confirmed the high genetic Polymorphism profile and mixed-strain infections of *P. falciparum* populations in Chewaka district, Ethiopia, potentially reflecting both the endemicity level as well as the fact that malaria transmission remains high in Southwest, Ethiopia.

5.6 Conclusions

The high level of polyclonal infections with *P. falciparum* parasites harbouring multiple genotypes and also infections with high MOI in this study indicate the extensive genetic Polymorphism and complexity of *P. falciparum* infection in the region. More effort is needed to control malaria transmission and prevent the emergence of resistance alleles in the study area.

CHAPTER VI: MONITORING OF EFFICACY AND SAFETY OF ARTEMETHER-LUMEFANTRINE FOR TREATMENT OF UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN ETHIOPIA: A SYSTEMATIC REVIEW AND META-ANALYSIS OF THE EVIDENCE

(Adopted from: Abamecha et al. 2021)

RESEARCH   **Open Access**

Efficacy and safety of artemether–lumefantrine for treatment of uncomplicated *Plasmodium falciparum* malaria in Ethiopia: a systematic review and meta-analysis

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Abstract

Background: Regular monitoring of anti-malarial drug efficacy is vital for establishing rational malaria treatment guidelines and ensuring adequate treatment outcomes. This study aimed to synthesize the available evidence on the efficacy of artemether-lumefantrine (AL) for the management of uncomplicated falciparum malaria in Ethiopia.

Methods: The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed. Relevant published studies were searched from the databases (PubMed, Google Scholar and Clinical trial registry) on published artemether-lumefantrine therapeutic efficacy studies conducted in Ethiopia from 2004 to 2020. The retrieved studies were assessed for quality using the modified Newcastle Ottawa Scale for observational studies and modified Jadad scale for interventional studies.Risk of bias was also assessed by using ROBINS-I tool. OpenMeta-Analyst software was used for the statistical analysis. The review protocol is registered in PROSPERO, number CRD42020201859.

Results: Fifteen studies (1523 participants) were included in the final analysis. The overall PCR-uncorrected pooled proportion of treatment success of artemether-lumefantrine therapy for uncomplicated falciparum malaria was 98.4% (95%CI: 97.6-99.1). A random-effects model was used because of considerable heterogeneity (χ^2=20.48, df(14), P=0.011 and I^2=31.65). PCR-corrected pooled proportion of treatment success of artemether-lumefantrine therapywas 98.7% (95% CI 97.7–99.6).A random-effects model was used (χ^2=7.37, df(6), P=0.287 and I^2=18.69). Most studies included in the present review achieved a rapid reduction of fevers and parasitaemia between D0 and D3 of assessment. Adverse events were mostly mild and only two cases were reported as serious, but were not directly attributed to the drug.

Conclusion: The present meta-analysis suggests that artemether-lumefantrine therapy is efficacious and safe in treating uncomplicated falciparum malaria in Ethiopia. However, owing to the high risk of bias in the included studies, strong conclusions cannot be drawn. Further high-quality RCTs assessing anti-malarial efficacy and safety should be performed to demonstrates strong evidence onchanges in parasite sensitivity to AL in Ethiopia.

Keywords Therapeutic efficacy, Artemether-lumefantrine, *Plasmodium falciparum,* Systematic review, Ethiopia

6.1 Background

Malaria is one of the leading health problems in Ethiopia. Approximately 60% of the total populations in Ethiopia live in malaria-endemic area. Due to the unstable nature of malaria transmission in the country, major malaria epidemics had been one of the serious public health emergencies. Sixty percent of malaria infections in Ethiopia are due to *Plasmodium falciparum* and 40% of infections are due to *Plasmodium vivax* [15, 22].

Resistance of *P. falciparum* to the traditional anti-malarial drugs (such as chloroquine, sulfadoxine-pyrimethamine, amodiaquine, and mefloquine) is a growing problem and is thought to have contributed to increased malaria mortality in recent years [338]. Chloroquine resistance has now been documented in all regions except Central America and the Caribbean. There is high-level resistance to sulfadoxine-pyrimethamine throughout South East Asia and increasingly in Africa, including Ethiopia, and mefloquine resistance is common in the border areas of Cambodia, Myanmar, and Thailand [338, 339].

To combat the spread of resistance, the World Health Organization (WHO) now recommends that *P. falciparum* malaria should always be treated using a combination of two drugs that act at different biochemical sites within the parasite [338]. If a parasite mutation producing drug resistance arises spontaneously during treatment, the parasite should then be killed by the partner drug, thus reducing or delaying the development of resistance and increasing the useful lifetime of the individual drugs [340, 341]. The current drug combinations all include a short-acting artemisinin derivative (such as artesunate, artemether, or dihydroartemisinin), partnered with a longer-acting drug in combinations known as 'Artemisinin-based combination therapy' (ACT).In Ethiopia, the use of artemether-lumefantrine (20/120 mg) as the first-line treatment for uncomplicated falciparum malaria has been started in 2004 [251].

The potency of artemisinin and its derivatives such as artemether, dihydroartemisinin, and artesunate is very high against all erythrocytic cycle asexual stages of *P. falciparum* with preference to the young ring stages[144]*,* so much that it reduces the parasite biomass by 100 to 10000 folds per each asexual blood stage cycle (after 48 hours). It also kills young gametocytes, hence playing a role in reducing malaria transmission [145]. The proposed mechanisms by which artemisinins kill the parasites are quite broad and are still being studied, but they generally fall

under two categories: 1) Damaging parasite proteins, such as transport proteins through haem activated endoperoxide activity and 2) Inhibition of proteasome activity (parasite's cellular repair mechanisms) leading to accumulation of damaged/unfolded proteins and stress-induced death [146, 147,148, 149, 150].

Due to the risk of the emergence and spread of anti-malarial drug resistance, World Health Organization (WHO) recommends regular monitoring of anti-malarial drug efficacy at least every two years in malaria-endemic countries [198]. In Ethiopia, the Federal ministry of Health (FMOH), in collaboration with its partners, including President's Malaria Initiative (PMI), research institutions, universities, WHO country office and Global fund, have been conducting regular therapeutic efficacy studies (TESs). The efforts of the FMOH to ensure regular TESs have also been complemented by TESs conducted by independent researchers [227, 251].

A meta-analysis of AL efficacy studies in Ethiopia was also carried out in 2017, but had several limitations including failure to assess risk of bias and missed studies [342, 343]. Hence, this study aimed to synthesize the available evidence, including new studies and studies that were missed in the previous meta-analysis, on the efficacy of AL for the management of uncomplicated falciparum malaria in Ethiopia.

6.2 Methods:

6.2.1 Study protocol registration

The present study adhered to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guideline [344]. The completed PRISMA checklist is available in Additional file 1.The review protocol was registered in a repository of systematic review protocols prior to starting the research (PROSPERO, protocol number CRD42020201859) [345].

6.2.2 Searching strategies

The searching strategy was performed using approaches that enhance methodological transparency and improve the reproducibility of the results and evidence synthesis. In this sense, the search strategy was elaborated and implemented prior to study selection, according to the PRISMA checklist as guidance [344]. Additionally, using the Population, Intervention, Comparison, Outcome and Study design (PICOS) strategy [346, 347].The following major databases were searched: PubMed, Google Scholar, and ClinicalTrials.gov databases. In order to

reflect contemporary practice, a search of the literature from the last 16 years (January 2004 to October 2020) was performed. The starting year (i.e., 2004) was purposely chosen because that was the year when Ethiopia adopted use of AL for treating uncomplicated falciparum malaria [348]. The date of the last search was 30[th] October 2020.

The search terms were developed in line with the Medical Subject Headings (MeSH) thesaurus using a combination of the big ideas (or "key terms") which derived from the research question. The domains of the search terms were: "efficacy", "therapeutic efficacy", "artemether-lumefantrine", "Coartem", "*Plasmodium falciparum* malaria", "falciparum malaria", "antimalarial drug", and "Ethiopia". This study combined terms using the Boolean operator "OR" and "AND" accordingly [349]. Search was limited to studies published in English language until October 2020. Full search strategy for the databases is provided in Additional file 2. Two reviewers (AbAb, and WA) reviewed the search results independently to identify relevant studies. Also, the bibliographic software EndNote X5 citation manager (Thomson Reuters, New York, USA) was used to store, organize and manage all the references and ensure a systematic and comprehensive search.

6.2.3 Selection criteria

Eligible studies included randomized controlled trials (RCTs), non-randomized single-arm intervention studies (with or without a control group) and prospective cohort studies. This study intended to only include studies with a comparator or control group, but because of the varying quality of papers retrieved, the study methodology deviated from the original methodologic plan and included any study describing patients given a treatment of interest (i.e. AL), which advise a 28-day follow-up to capture cure rate, even if no specific control group was available. All the non-primary literature, retrospective studies, case reports and *in vitro* experiments were excluded.

A summary of the participants, interventions, comparators and outcomes considered, as well as the type of studies included according to PICOS criteria[346, 347], which is provided in Table 8. The primary objective of this review was the efficacy of AL measured as treatment success at day 28 (or adequate clinical and parasitological response (ACPR). ACPR is defined by the WHO as the "absence of parasitaemia on day 28 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" [198]. This is also consistent with previous Cochrane Reviews. The secondary endpoints were fever clearance, parasite clearance, and the frequency of adverse drug reactions (ADRs). ADRs were defined as 'signs and symptoms that first occurred or became more severe after treatment was started' or 'as a sign, symptom, or abnormal laboratory value not present on day 0, but which occurred during follow up, or was present on day 0 but became worse during follow up'. Serious adverse events were defined according to International Conference on Harmonization (ICH) guidelines. Studies included in this review are shown in Table 9.

Table 8. PICOS strategy and eligibility criteria

PICOS Strategy	Inclusion criteria	Exclusion Criteria
P:Population	Participants residing in Ethiopia and having uncomplicated falciparum malaria, irrespective of gender and age group were considered. Microscopy of the peripheral blood smear samples detected mono-infection with a *P. falciparum* parasite count of 1000 -100,000/µl.	
I:Intervention	Studies using fixed dose compound tablets artemether-lumefantrine (20/120 mg) were included. All participants must have received a standard six-dose regimen of AL over three days and were followed up for 28 days.	
C:Comparison	Standard treatment, no treatment or , not applicable	
O: Outcome	The primary objective of this review was the efficacy of AL measured as treatment success at day 28 (or adequate clinical and parasitological response (ACPR)). The secondary outcomes were measured based on the parasite clearance time and fever clearance time and the occurrence of adverse events (AEs).	Studies that do not report any treatment success (cure rates) of AL at day-28 as primary outcome

| **S: Study Design** | Randomized clinical trials (RCTs), nonrandomized single-arm intervention studies (with or without a control group) and prospective cohort studies that reported the therapeutic efficacy of AL for the treatment of uncomplicated falciparum malaria in Ethiopia | All the non-primary literature, retrospective studies, case reports and animal or *in vitro* experiments were excluded |

6.2.4 Data extraction and management

Initial screening of studies was based on the information contained in their titles and abstracts and was conducted by two independent investigators. When the reviewers disagreed, the article was re-evaluated and, if the disagreement persisted, a third reviewer made a final decision. Full-paper screening was conducted by the same independent investigators.

Data were extracted using a case record form (CRF), including four domains: (1) identification of the study (article title; journal title; authors name; country of the study; language, publication year and study setting); (2) methodological characteristics (study design; stated length of follow-up; sample size; gender; age; intervention details; literature quality assessment characteristics; statistical analyses); (3) main findings(treatment success rates; parasite clearance; fever clearance; adverse events) and (4) conclusions. If the outcome data in the original article were unclear, the corresponding author was contacted via email for clarification. A bibliographic software EndNote X5 citation manager (Thomson Reuters, New York, USA) was used to store, organize and manage all the references and ensure a systematic and comprehensive search.

6.2.5 Methodological quality assessment

Two review authors independently assessed the methodological quality of the selected studies by using methodological quality assessment forms and the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions [346, 347].Any disagreements between the two review authors were resolved through discussion. Quality assessment was undertaken using the Newcastle Ottawa Scale (NOS) for observational studies [350] and modified Jadad scale for interventional studies [351].NOS assess the quality under three major headings, namely, selection of the studies (representativeness and the exposure assessment/control selection), comparability (adjustment for main/additional confounders), and outcome/exposure (adequacy of outcome measured, exposure measured *vs.* self-report)(Additional file 3).The modified Jadad

scale included eight items: randomization, blinding, withdrawals, dropouts, inclusion/exclusion criteria, adverse effects and statistical analysis. The reviewers independently assessed the quality of the methodology of included studies (Additional file 4).This study also assessed using Risk of Bias in Non-Randomized Studies of Interventions (ROBINS-I) assessment tool for non-randomized intervention and cohort studies. Studies were ranked as low, moderate, serious, or critical risk of bias in seven domains [352].

6.2.6   Statistical analysis

OpenMeta Analyst software for Windows [353, 354] was used to perform the meta-analyses. The heterogeneity of the included studies was evaluated using the Cochran Q and I^2 statistics. The random effects model was used as standard in the determination of heterogeneity between studies [355].The I^2 values were expressed in percentages. Heterogeneity was classified as low, moderate and high, with upper limits of 0-25%, 25-50% and >50% for I^2, respectively [356, 357]. The method of random effects model was used to combine the included studies.

6.3 Results

6.3.1   Literature search results

A total of 1043 studies were retrieved from the database and manual searching. Among these, 724duplicated studies were excluded. From the remaining 319 articles, 303 of them were excluded after evaluation of their title and abstract confirming non relevance to this study. One paper [225] was excluded following full text review as data collection for the study was conducted before official adoption of AL in Ethiopia. Finally, a total of 15 papers met the eligibility criteria and were included in this systematic review and meta-analysis (Figure 8).

6.3.2   Characteristics of the included studies

The summary characteristics of the included studies are shown in Table 9. From 15 eligible studies a total 1523 participants were included. Seven of the studies were interventional [229, 230, 231, 232, 233, 234, 280], and the other eight studies were observational study [241, 242, 243, 358, 359, 360, 361, 362]. No RCTs had been completed at the time of review. These studies were conducted in different malarious parts of the country with varied transmission intensity (Figure 9). Most (10/15, 66.7%) of the studies included patients who were ≥ 6 months of age (Table 9).Treatment outcomes in all studies were assessed using clinical and parasitological

criteria according to WHO guidelines [198].In majority of the studies(86.7%), treatment compliance was assured by supervised administration of the study drug under direct observation on days 1, 2 and 3, i.e. the morning doses were directly observed over 3 days, while the evening doses were given to patients for intake at home by health extension workers. The endpoint was day 28 in all studies [198]. RoB assessment is shown for all studies in Table 12.

1041 Records identified through database searching (PubMed, Google scholar and Clinical trial registry)

2 Additional studies identified from gray literature

319 studies screened (titles and abstracts) after duplicates (724) were removed

303 studies excluded during screening
- 123 No relevant artemether-lumfantrine efficacy related outcomes
- 1 full text articles not available
- 161 study were done outside Ethiopia
- 2 review article
- 16 Non-relevant to the review

16 articles reviewed (full text)

1 full articles was excluded after full text review

- The study data was collected before the official adoption of AL, before 2004

15 studies included in final synthesis (N=1523)

Figure 8. PRISMA flow diagram showing study selection process, 2020

Distribution of artemether-lumefantrine therapeutic efficacy and safety study sites, Ethiopia(2004 - 2020)
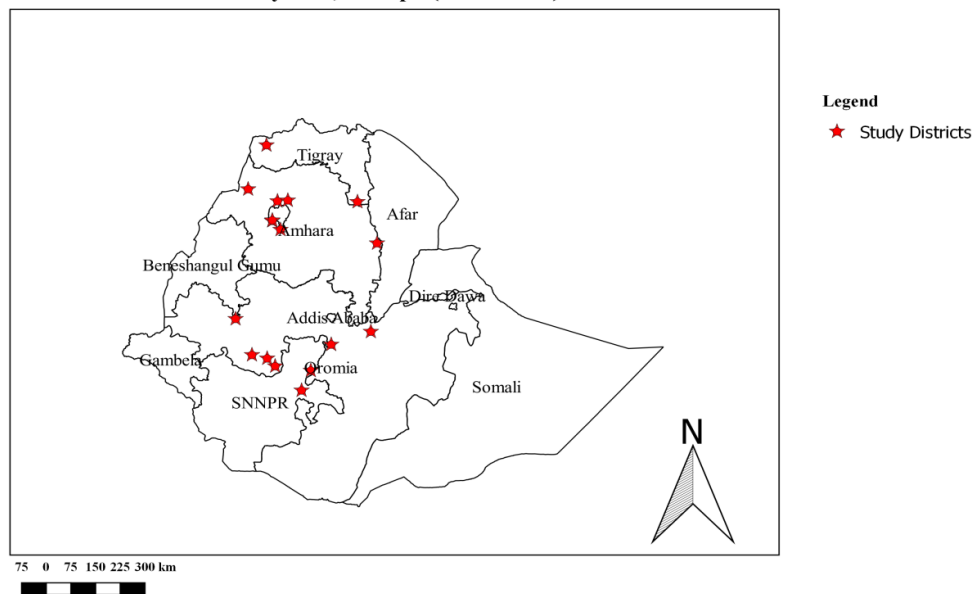
Figure 9. Distribution of artemether-lumefantrine efficacy and safety study sites in Ethiopia from 2004-2020.

Table 9. Summary characteristics of included studies on the efficacy and safety of artemether-lumefantrine for treatment of uncomplicated *P. falciparum* malaria in Ethiopia from 2004-2020(N=1523)

Study [Ref.No]	Study Settings	Study design	Study duration (Months)	Inclusion for age	Transmission level	Patient Enrolled (N)[a]	Patient available (n)[b]	Mean Hg	Pf-GMPD	Length of follow up (days)	Super-vision
Abamecha et al. 2020[230]	Ilu-Harar Health Center, Chewakadistrct, Ethiopia	One‑arm, prospective study	September-December 2017	Above 6 months of age	Moderate	80	76	11.7	12374.3	28	Partial
Teklemariam et al. 2017[358]	SetitHumera, Northwest Ethiopia	Single-arm prospective study	October 28, 2014 and January 9, 2015	≥ 6 months of age	High	92	79	13.2	27798.0	28	Partial
Deressa et al. 2017[242]	Kola Diba Health Center (KHC) in the Dembia district, Northwest Ethiopia	Prospective cohort study	April 2015 to February 2016	Above 6 months of age	High	80	75	n/a	8377.8	28	Partial
Nega et al. 2016[234]	Metehara Health Centre, Eastern Ethiopia	Open-label single-arm study	October 2014 to January 2015	≥ 6 months of age	Low-moderate	91	85	12.4	11509.6	28	Partial
Wudneh et al. 2016[359]	Gendewuha (Metema) Health Center, Northwest Ethiopia	One-arm open-label study	October 2014 to January 2015	Above 6 months of age	Moderate	91	81	13.7	13441.6	28	Partial
Kanche et al. 2016 [243]	Baddessa Health Center,Wolaita Zone, Southern Ethiopia Wolaita Zone, Southern Ethiopia	One-arm prospective study	February - March 2015	> 5 years old	Moderate	86	88	10.8	4238.8	28	Partial
Mekonnen et al. 2015[232]	Omo Nada health center in southwestern Ethiopia	Prospective cohort study	August-December 2011	Above 6 months of age	Moderate	88	86	11.6	8404.0	28	Partial

Study	Location	Study type	Time period	Age	Transmission	Enrolled[a]	Analysis[b]	Hg	Pf-GMPD	Days	Supervision
Ebstie et al. 2015[241]	Bahir Dar district, Northwest Ethiopia	Prospective observational cohort study	March and July 2012	> 5 years old	Moderate	93	89	10.8	8675.3	28	Partial
Getnet et al. 2015[233]	Enfranze Health Center, Northwest Ethiopia	One-arm, prospective study	January and May 2013	Above 6 months of age	Moderate	134	130	12.3	7898.0	28	Partial
Mulu et al. 2015[360]	Kemisie Health Center, Northeast Ethiopia	One-arm prospective study	September, 2012 to May, 2013	Above 6 months of age	Moderate	80	80	NR	10454.0	28	NR
Eshetu et al. 2012[229]	Agaro Health Centre, Jimma Health Centre, Serbo Health Centre, and Asendabo Health Centre	Open-label, single arm *study*	November 2008 and January 2009 and between August and December 2009	> 1 year	Moderate	348	315	NR	9720.0	28/42	non-supervised
Kinfu et al. 2012[361]	Tumuga health center Alamatadistrict,Tigrai regional state, North Ethiopia	Prospective cohort study	August–November 2009	Above 6 months of age	Moderate	66	60	N/R	20672.0	28	Partial
Hwang et al. 2011[231]	Bishoftu Malaria Clinic and Bulbula Health Center, Oromia Regional State, Ethiopia	Open-label, single arm *study*	October and November 2009	Above 6 months of age	Moderate	73	71	12.6	16374.0	28/42	Partial
Assefa et al.2010[280]	Serbo Health Center, Kersa District, Southwest, Ethiopia	Prospective cohort study	November 2007 and January 2008	N/R	Moderate	119	112	12.2	22660.0	28	Partial
Kefyalew et al. 2009[362]	AlabaKulito Health Center, Southern Ethiopia	Prospective cohort study	October - December 2007	> 1 year	Low - moderate	102	102	11.4	8264.3	28	Partial

Key: N/R: Not reported; TES: Therapeutic efficacy study   Hg: Hemoglobin; Pf-GMPD: *Plasmodium falciparum* geometric mean parasite density of asexual parasites per microlitre of blood

[a]*P.falciparum* patients enrolled in study as per manuscript, [b]Patients available for analysis from study

6.3.3 Treatment outcome

The overall PCR-uncorrected pooled proportion estimate of *treatment success* of AL therapy for uncomplicated falciparum malaria was 98.4% (95%CI: 97.6-99.1). A random-effects model was used because of substantial heterogeneity (χ^2=20.48, *df* (14), *P*=0.011 and I^2=31.65; Figure 10). PCR-corrected pooled proportion of *treatment success* of AL therapy was 98.7% (95% CI 97.7–99.6). A random-effects model was used (χ^2=7.37, *df* (6), *P*=0.287 and I^2=18.69; Figure 11).

The proportion of recurrence infection was ranging from 1%-5.6% at 28-day follow-up period after treatment with AL. The proportion of recurrence infection was ranging from 4.6- 6.7% at 42-day follow-up period after treatment with AL.

The PCR-corrected cure rates of AL therapy ranged from 95.0 to 99.4% in per-protocol analysis and 88.8 to 97.4% in intention-to-treat analysis. The percentage of ACPR and the 95% CI are presented in Table 10. The highest cure rate 99.4 %(95% CI: 97.4-100.0) was reported by study conducted in Jimma Zone, Southwest Ethiopia in 2012 [229], and97.4%(95% CI: 93.9-100) reported by study conducted in Bishoftu Malaria Clinic and Bulbula Health Center, Oromia Regional State, Ethiopia 2011[231].

6.3.4 Fever and parasite clearance rate

Among the five partially supervised efficacy studies that reported fever clearance, more than 75% of the patients cleared fever by day 1 post-treatment with AL [232, 233, 234, 242, 280, 361].Some authors did not measure fever clearance on subsequent days post drug administration and only choose day-3 for this clinical measurement [241, 280]. Among the fifteen studies that reported parasite clearance, five studies showed day-3 parasitaemic cases of 5.7%, 5.1%, 5%, 3.9% and 3.8% [230, 233, 241, 242, 243]. Table 11 shows the overall progress of fever and parasite clearance in the first three days of AL treatment.

Studies	Estimate (95% C.I.)	n/N
Abamecha A et al. 2020	0.947 (0.897, 0.998)	72/76
Teklemariam M et al. 2017	0.987 (0.963, 1.000)	78/79
Deressa T et al. 2017	0.920 (0.859, 0.981)	69/75
Nega D et al. 2016	0.976 (0.944, 1.000)	83/85
Wudneh F et al. 2016	0.988 (0.964, 1.000)	80/81
Kanche ZZ 2016	0.994 (0.978, 1.000)	86/86
Mekonnen SK et al. 2015	0.944 (0.896, 0.992)	84/89
Ebstie YA et al. 2015	0.985 (0.963, 1.000)	128/130
Getnet G et al. 2015	0.925 (0.867, 0.983)	74/80
Mulu A et al. 2015	0.967 (0.921, 1.000)	58/60
Eshetu T et al. 2012	0.990 (0.980, 1.000)	312/315
Kinfu G et al. 2012	0.972 (0.933, 1.000)	69/71
Hwang J et al. 2011	0.991 (0.974, 1.000)	111/112
Assefa A et. al 2010	0.963 (0.923, 1.000)	79/82
Kefyalew T et al. 2009	0.995 (0.982, 1.000)	102/102
Overall (I^2=31.65 % , P=0.116)	0.984 (0.976, 0.991)	1485/1523

Figure 10. PCR-uncorrected treatment success of artemether-lumefantrine therapy using a random effect model
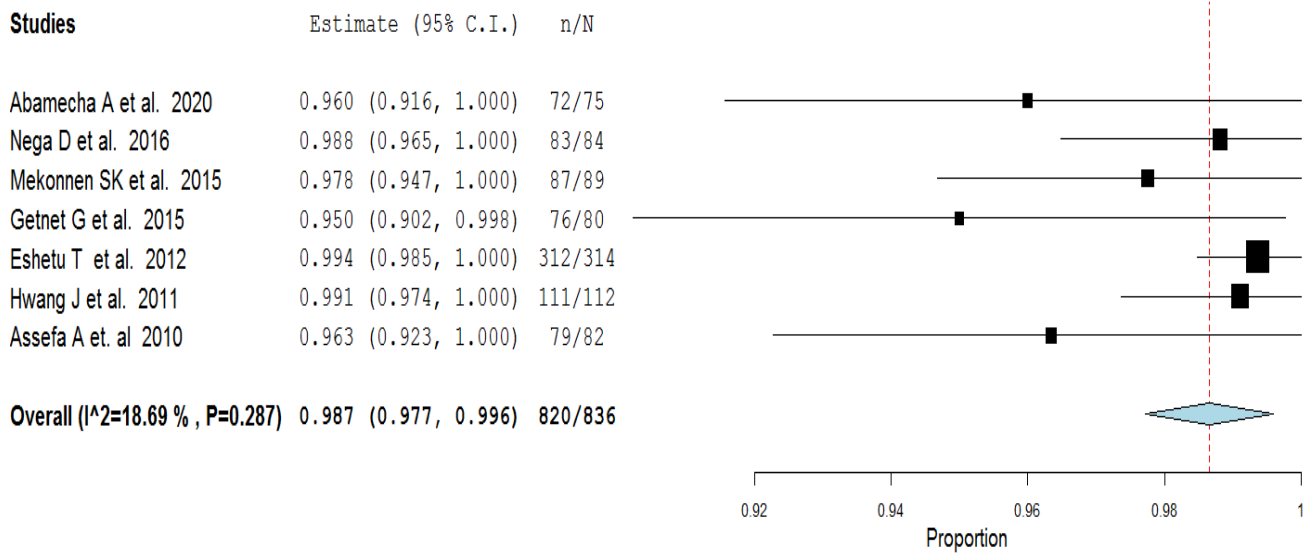
Figure 11. PCR-corrected treatment success of artemether-lumefantrine therapy a random effect model

Table 10. Treatment Outcome of AL Therapy reported in efficacy studies in Ethiopia

Study	PP PCR-corrected percentage cure rate (95% CI), day-28	ITT PCR-corrected percentage cure rate (95% CI), day-28
Abamecha A et al. 2020	96.0(91.6-100)	94.9(90.1-99.8)
Nega D et al. 2016	98.8(96.5-100)	92.2(86.7-97.8)
Mekonnen SK *et al.* 2015	97.8(94.7-99.8)	96.7(93.0-100)
Getnet G et al. 2015	95.0(90.2 -100)	97.4(93.9-100)
Eshetu T et al. 2012	99.4(97.4-100)	89.9(86.7-93.1)
Hwang J et al. 2011	99.1(91.6-100)	94.1(89.9-98.3)
Assefa A et al.2010	96.3(92.3-100)	88.8(82.2-95.3)

6.3.5   Safety outcomes

The current meta-analysis showed that 80% of the included studies reported ADRs to AL which were observed in 36.1%, (550/1523) patients.All of the ADRs were mild and resolved spontaneously. Two SAE were observed (Additional file 5).

6.3.6   Methodological quality assessment

Eight observational studies [241, 242, 243, 358, 359, 360, 361, 362] were assessed with the Newcastle Ottawa Scale (NOS) [350] with satisfactory qualities with a value score of 5 (Additional file 3) and while the remaining seven interventional studies [229, 230, 231, 232, 233, 234, 280] were assessed using the modified Jadad scale [351] with high qualities with a value score of 4 (Additional file 4).All or most of the included studies had a 'serious' or 'critical' risk of bias due to confounding because most were single-arm studies (Table 12).

Table 11. Fever and parasite clearance reported in efficacy studies in Ethiopia (2004- 2020)

Study	Patient Enrolled (N)	Patient available	Patient Included	Fever clearance (%)			Parasite clearance (%)			Supervised
				D1	D2	D3	D1	D2	D3	
Abamecha et al. 2020	80	76	72	52.5	87.2	97.5	61.2	81.2	96.2	Partial
Teklemariam et al. 2017	92	79	78	80.0	97.8	100.0	33.0	84.4	100.0	Partial
Deressa et al. 2017	80	75	69	62.5	93.7	97.5	67.5	85.0	95.0	Partial
Nega et al. 2016	91	85	83	78.7	94.3	97.7	69.7	95.5	100.0	Partial
Wudneh et al. 2016	91	81	80	69.6	97.8	100.0	23.6	91.0	100.0	Partial
Kanche et al. 2016	88	86	86	N/R	59.1	93.2	N/R	72.2	94.3	Partial
Mekonnen et al. 2015	93	89	84	88.1	94.4	100.0	88.8	96.6	100.0	Partial
Ebstie et al. 2015	134	130	128	NR	NR	87.9	NR	85.9	96.1	Partial
Getnet et al. 2015	80	80	74	75.0	91.3	96.2	73.8	91.3	94.9	Partial
Mulu et al. 2015	66	60	58	89.4	98.5	100.0	84.8	93.9	100.0	NR
Eshetu et al. 2012	348	315	312	NR	96.7	99.1	NR	98.2	99.4	non-supervised
Kinfu et al. 2012	73	71	69	NR	NR	100.0	NR	100.0	100.0	Partial
Hwang et al. 2011	119	112	111	65.2	90.5	93.0	NR	93.1	99.1	Partial
Assefa et al.2010	90	82	79	NR	NR	100	98	NR	100.0	Partial
Kefyalew et al. 2009	102	102	102	44.1	82.4	93.1	NR	NR	NR	Partial

Table 12. Quality assessment by 'Risk Of Bias in Non-randomized Studies-of Interventions (ROBIN-I)' for Non-randomized and cohort studies

Study[Ref.No]	Study design	Reason for risk of bias (RoB) determination							Overall RoB
		Confounding	Selection of participants	Classification of interventions	Deviations from intended interventions	Missing outcome data	Outcome measure ments	Selection of results reported	
Abamecha et al. 2020[230]	One arm, prospective study	Serious	Low	Moderate	Moderate	Low	Low	Moderate	Serious
Teklemariam et al. 2017[358]	Single-arm prospective study	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Deressa et al. 2017[242]	Prospective cohort study	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Nega et al. 2016[234]	Open-label single-arm study	Serious	Low	Moderate	Moderate	Low	Low	Moderate	Serious
Wudneh et al. 2016[359]	one-arm open-label study	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Kanche et al. 2016 [243]	One-arm prospective study	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Mekonnen et al. 2015[232]	In-vivo therapeutic efficacy study	Serious	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Ebstie et al.	Prospective	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical

2015[241]	observational cohort study								
Getnet et al. 2015[233]	One arm, prospective study	Serious	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Mulu et al. 2015[360]	One-arm prospective study	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Eshetu et al. 2012[229]	open-label, single-arm study	Serious	Low	Moderate	Moderate	Low	Low	Moderate	Serious
Kinfu et al. 2012[361]	Prospective cohort study	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Hwang et al. 2011[231]	single arm, open label study	Serious	Low	Moderate	Moderate	Low	Low	Moderate	Serious
Assefa et al.2010[280]	Prospective cohort study	Serious	Low	Moderate	Moderate	Critical	Low	Moderate	Critical
Kefyalew et al. 2009[362]	Prospective cohort study	Critical	Low	Moderate	Moderate	Critical	Low	Moderate	Critical

6.4 Discussion

The present study found high treatment success of AL therapy in the treatment of uncomplicated falciparum malaria in Ethiopia despite its use for more than 16 years. Besides, AL was generally a safe treatment. Previous meta-analysis in 2017 revealed similarly high efficacies of AL [342, 343]. This result is also consistent with neighboring Sudan, a high treatment success rate (98%) of malaria treatment was recently reported in a meta-analysis that included 20 studies with a total of 4070 patients [363].The treatment success of 98.7% (95% CI 97.7-99.6) found in this study suggests that, in accordance with WHO parameters [198], AL is still effective as first-line drug for uncomplicated malaria treatment in Ethiopia, but warrants regular monitoring.

There is a concern about the limited post-treatment prophylactic effects of AL in high transmission areas [198]. In this study, the proportion of recurrence infection ranging from 1% to 5.6% at 28-day follow-up period after treatment with AL. From the included studies, two studies [229, 231] also had 42-day follow up period, and the proportion of recurrence infection were relatively high (ranging from 4.6-6.7%). The study results showed that most recurrent parasitaemia occur after day 28 and this emphasizes the need for follow-up periods of at least 42 days. High recurrent parasitaemia rate in children ≤5 years (9.4%) was observed, which suggest that the partner drug may not provide prolonged protection despite high therapeutic efficacy [174].This observation has also been reported in Democratic Republic of Congo which showed high level of resistance to lumefantrine [364]. In most of the studies, a great majority of the recurrent infections were due to re-infections when assessed with a step-wise PCR genotyping protocol. This signifies that the drugs are still efficacious and the high rates of re-infections could only be attributed to high malaria transmission. In terms of clinical practice, the high re-infection rates are of great concern among clinicians. Clinicians should be clearly guided on what to expect and how to handle such cases with recurrent infections within a period of three to eight weeks post-treatment. The observed high re-infection rates after AL treatment underscores the importance of providing anti-malarial drug with a longer period of protection against re-infection, such as DHA-piperaquine [365] and integrating treatment with non-therapeutic prevention and control measures (insecticide-treated bed nets, indoor residual spraying and other vector control measures) to effectively prevent recurrent infections [247, 366]. Besides, it is also important to use transmission-blocking drugs (e.g. use of primaquine) (gametocytocidal) in low transmission areas.

Most studies included in the present review achieved a rapid reduction of fevers and parasitaemia between D0 and D3 of assessment. A previous aggregate study on the clinical predictors of early parasitological response to ACT in African patients with uncomplicated falciparum malaria confirmed the rapid decrease ofparasite positivity rate from 59.7% (95% CI: 54.5–64.9) on day 1 to 6.7% (95% CI: 4.8–8.7) on day 2and 0.9% (95% CI: 0.5–-1.2) on day 3 [367].

In resource-limited settings, the day-3 parasite-positive rate can be used as a proxy measure of delayed parasite clearance [240]. In the present review, few studies showed day-3 parasitaemic cases (3.8% - 5.7%) after treatment with AL [230, 233, 241, 242, 243].However, most of the studies reviewed in this article were based on 24-hour sampling, which is not the recommended method for assessing parasite clearance and detection of tolerance/resistance to artemisinins.

Regarding safety of AL for treatment of uncomplicated malaria, mild adverse events (a headache, cough, fever, diarrhoea, vomiting, perioral ulcer, anorexia, abdominal pain, dizziness and nausea, weakness/fatigue and others) were mostly reported in the eligible studies. Besides, almost all were resolved soon after completion of the treatment except cough [230, 280, 358]. Similar mild adverse events have been associated with AL; the most common being headache, fever, vomiting followed by gastrointestinal disturbances [363, 368].The observed rate of 36.1%, (550/1523)ADRs was comparable with the rate reported in the previous review in Ethiopia where 269 of 633 patients had ADRs, with a pooled event rate of 41.2% [343].

From the included studies, one study reported serious adverse events (SAE) in two infants [229]. These infants had SAE on the day of presentation (day-0) with high parasitaemia (>95,000/μL), no signs of severe malaria were noticed at admission and did not tolerate oral treatment. After re-dosing and repeated vomiting, the infants were referred to the ward for intravenous treatment; one died the same day. The cause of death was not established and its possible association with AL treatment could not be ascertained.

6.5 Limitation of the review

This review provided an overall country-specific performance of AL after the wide-scale deployment, since 2004 as first-line anti-malarials for treating uncomplicated *P. falciparum* malaria in Ethiopia. The main limitation of this work was the lack of a control group in the included studies that severely limits the ability to draw a firm conclusion regarding the efficacy

of an intervention. Moreover, there are insufficient number of therapeutic efficacy studies (TESs) studies with high-quality and more rigorous design. This may be due to the fact that TESs and long-term follow-up of patients require logistics and incur high cost in low and middle income countries, limiting regular implementation of clinical evaluation within the country. The current study howeveris the first most comprehensive effort at highlighting the levels of implementation of TESs in Ethiopia and provides an overall country-specific performance of AL after their wide-scale deployment since 2004 as first-line anti-malarials for treating uncomplicated *P. falciparum* malaria in the country.

6.6 Conclusions

The present meta-analysis provides some evidence to support that AL therapy is efficacious and safe in treating uncomplicated falciparum malaria in Ethiopia. However, owing to the risk of bias in the included studies, strong conclusions cannot be drawn. Further high-quality randomized controlled trials are warranted to substantiate the efficacy and safety of AL, to detect future changes in parasite sensitivity to AL in Ethiopia.

CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSION

6.1 Efficacy and safety of artemether-lumefantrine

In paper I it was concluded that artemether-lumefantrine is a highly efficacious and well tolerated treatment for patients with uncomplicated falciparum malaria in Chewaka district, Ethiopia after more than 16 years' use. However, it is important to consider whether using only the markers *msp-1* and *msp-2* might have contributed to this very low recrudescence rate. It is true that the markers recommended by WHO are *msp-1*, *msp-2* and *glurp* [198, 270], but these markers should be genotyped sequentially, from the higher to the lowest discriminatory power. Once the analysis of one marker has shown a new infection, the analysis should be stopped (Figure 4). If no evidence of new infection is detected with the first markers, the second marker should be analysed. If no new infection is detected, then the third marker should be used. This would mean that the result could be given with the genotyping of a single marker and the low rate of recrudescence in this study cannot be attributed to the use of only two markers. Furthermore, several studies [229, 233, 329,] in which three markers were used also found very low recrudescence rates. Findings in this study are consistent with other therapeutic efficacy studies with AL conducted both in the past in Ethiopia [232, 233, 234] and elsewhere in sub-Saharan African countries (SSA) in which the PCR corrected ACPR ranged from 95 to 100 % [167,169 329].

The absence of ETF during treatment with AL in this study and in several previous studies conducted in Ethiopia [229, 231, 234, 280] highlights the drug's efficacy and is emphasized by the rapid rate (48 h) of parasite clearance. These findings are similar to those previously reported in studies from several other countries [167, 329]. AL clears parasites quickly as a result of the rapidly absorbed, fast-acting artemisinin component.

A total of three cases of recrudescent parasitaemia were observed from day 14 onwards, giving a rate of treatment failure of 4% (95% CI 0.8–11.2) in the study population. However, the recrudescent parasitaemia resolved quickly after initiated re-treatment in all cases with the same regimen. Several studies [149, 171, 274, 275] in which therapeutic efficacy tests were combined with sampling of plasma or whole blood for drug concentration measurements at various times during follow-up have shown that cured patients have higher drug concentrations than those in whom treatment failed. There are two possible explanations for the latter finding. First, failures

are associated with inadequate drug concentrations rather than resistance, this could be the case in our findings of treatment failure; secondly, when drug resistance emerges, there is a higher likelihood that a resistant strain will emerge if the drug is present at a suboptimal concentration. Hence, proper absorption of the drug must be ensured and monitored through controlled nutritional interventions so that the parasites are not exposed to suboptimal and tolerable level of plasma lumefantrine concentration.

In the present study, the parasitaemia on day 3 following treatment with AL was only 3.8% (95% CI 0.8–10.6) and day-3 parasitaemia did not correspond to failures observed during follow-up. The overall rate of day-3 positivity observed in this study are consistent with the 3-5% background rate of day-3 positivity that might be expected in the absence of resistance to artemisinin, but also the 3-10% range which in the past has been seen as appropriate window for initiating containment activities [276, 277]. Hence, such results indicate a requirement for regular *in vitro* monitoring of the efficacy of lumefantrine on plasmodial strains in countries where the AL combination is used as first-line treatment.

This study also showed that AL had a safety profile comparable to previous studies and was well tolerated with minimal adverse events. Studies conducted in other African countries [281, 282] reported similar safety profiles of AL when used for the treatment of uncomplicated falciparum malaria. A high number of cases reporting cough at the study site could be attributed to weather conditions, which were relatively cold and rainy at the time of the study.

6.2 Genetic Polymorphism and genotype multiplicity of *P. falciparum* infection

In Ethiopia, even though enormous efforts have been made at national and local levels to control and eventually eliminate malaria, limited molecular data exists on genetic polymorphism of *P. falciparum,* the most predominant and virulent malaria parasite in the region. Paper II aimed to assess repeat length polymorphism and genetic Polymorphism of *P. falciparum* isolates from Ethiopia using the *msp-1* and *msp-2* genes. In addition, the multiplicity of infection and heterozygosity, both of which reflect the transmission intensity as affected by intervention were evaluated.

Size polymorphism of *msp-1* and *msp-2* allelic variant identified in the present study is consistent with the Polymorphism found in Kolla-Shele area, Southwest Ethiopia (*msp-1*: 11; *msp-2*: 12) in

2015 [317], in Northwest Ethiopia (*msp- 1*: 12; *msp-2*: 22) in 2018 [318], and Brazzaville in the Republic of Congo (*msp-1*: 15; *msp-2*: 20) in 2018 [327]. And less diverse than from Bioko Island, Equatorial Guinea (*msp-1*: 26; *msp-2*: 25) [326] and Bobo-Dioulasso of Burkina Faso[333]. The major factor that may account for such variation could be; the scope of study sites covered and local malaria transmission patterns might have contributed. Gel analysis of the present study revealed that; from 72 *msp-1* amplicon 21(30.5%) were monoclonal infection, whereas the remaining 51(70.9%) were poly-allelic type, with 31.9% for (K1 + MAD20), 5.6% for (K1 +RO33), 4.2% for (MAD20+RO33), and 29.2% were K1 + MAD20 + RO33 type. The proportion of monoclonal infection was 20.8% K1, 4.2% MAD20 and 4.2% RO33 (Table 5). From 69 *msp-2* amplicon 29(42%) were monoclonal infection, with 26.1% for FC27 and 15.9% for IC/3D7 whereas the remaining 40(58%) were poly-allelic type (Table 6). This finding is consistent with the report from previous studies in Africa, including Southwest Ethiopia [317], Brazzaville, Republic of Congo [88], and Gabon [91] of the three *msp-1* gene allelic families K1 was the predominant allelic type. However, this finding differ from the report from studies conducted in Northern Ethiopia [318] and Bioko Island, Equatorial Guinea [326], where MAD-20 allele type was the most prevalent allelic family. Allele typing of *msp-2* showed that FC27 was the predominant allelic family as also demonstrated in previous reports from Benin [329] and Central Sudan [95], but in contrast with previous studies in Ethiopia [317] and Brazzaville, Republic of Congo [88]. Although the deriving forces for such variation needs further investigation; the difference in micro-ecological factors and the local transmission intensity [199, 336], could play a significant role. Moreover, evolutionary process like genetic drift resulting uneven reproduction of the parasite lineages, types and rate of mutations, inbreeding, and the contribution of allelic variants in reproductive success are some of the factors that might have contributed for such variation[369].

High genetic Polymorphism is an indicator of the intensity of transmission [370], and potential challenges in malaria control programs [318]. Studies have shown that malaria reduction as the result of intensified control efforts is accompanied by reduced genetic Polymorphism of the parasite populations [371]. Widespread use of specific antimalarial drugs can also alter the genetic Polymorphism because of selective pressure on specific parasite strains. In this study, we found that, 80% of the isolates having multiple genotype infection (i.e more than one parasite genotype identified by the presence of two or more alleles of one or both genes); almost similar

frequency (83%) to that in the Republic of Congo [296], while 59-76% of the sample population harboured multiple genotypes, in previously reported studies between 2015 and 2018 in Ethiopia [317, 318, 319]. This shows that malaria transmission in our study area exhibits extensive genetic Polymorphism. This could be due to the gap in ongoing intensified scale up of interventions, differences in local epidemiology, demographic and environmental conditions that might have resulted in observed higher genetic Polymorphism pattern in Chewaka district.

Based on heterozygosity, which measures the level of genetic Polymorphism at polymorphic loci, this study indicate that the genetic Polymorphism values were higher based on heterozygosity index for *msp-2* (He=0.85), than for *msp-1* (He=0.43), suggesting a large genotype Polymorphism within the *msp-2* locus, which was higher than previously reported from Northwest Ethiopia (*msp-2*: *He* 0.62) in 2018 [319]. In contrast, declining rates of Polymorphism of alleles (heterozygosity) in *P.falciparum* are associated with decreasing transmission rates [306]. The current study area has high genetic Polymorphism in parasite populations with high local transmission and thus it requires increased attention with malaria control programs

Multiplicity of infection (MOI), index which is related to the number of clones per infection and usually associated with the level of malaria transmission [336, 372, 373]. The overall MOI value reported in this study was higher than previously reported studies, including Ethiopia (MOI: 1.8–2.6) between 2015 and 2018 [317, 318, 319], Brazzaville, Republic of Congo (MOI: 2.2) [296] in 2011 and Bobo-Dioulasso, Burkina Faso (MOI: 1.95) [333]. In contrast to study reported in Bioko Island, Equatorial Guinea (MOI: 5.51) [326] in 2018 and Gabon (MOI: 4.0) [334] in 2018. The difference in MOI can be explained by the differences in intensity of malaria transmission seasons. This observation needs caution as Ethiopia enter malaria elimination phase and as such, low MOI levels were expected. This could have several implications for the malaria control programme in Ethiopia: firstly, as NMCP target a more focal control, parasite could be circulating and transmission going on in other not-targetted areas, subsequently, this high MOI observed and if neglected could lead to extensive parasite recombination and hence further diverse falciparum strains that could pose problems in employing the conventional control methods (use of artemisinin combination therapy). Besides, caution is needed when interpreting such results as samples were collected during the major malaria transmission season of September to December, when malaria transmission is very intense. All year round (seasonal)

studies covering major and minor transmission seasons are needed to better understand genetic profiles in this area including a sense on seasonal variations.

Several studies reported conflicting results in which the MOI correlates with ages and parasite density [95, 132] but others studies failed to demonstrate this correlation[317, 318]. In the present study we found that, no significant correlation existed between MOI of *P. falciparum* with age and parasite density (Table 7). Previous studies regarding the variation of MOI over age have suggested that the influence of age on the multiplicity of infection is highly affected by endemicity of malaria [132, 329, 330, 331]. This is probably a reflection of the development of anti-parasite specific immunity [127]. Thus, in holo- or hyperendemic areas, immunity develops faster and at younger age than in areas with less intense transmission [335]. Studies have shown an age-dependent MOI in a village with intense perennial malaria transmission but not in areas where malaria is mesoendemic [132, 323]. Similarly, in this study reported that no significant relation between MOI and the parasite count, similar to reports from previous studies in Ethiopia [317, 318], but in contrast with reports from Bioko Island, Equatorial Guinea [329]. This may have been due to the small number of isolates analysed.

6.3 *P. falciparum* malaria treatment success in Ethiopia

In the absence of a full understanding of the antimalarial drug efficacy trend, the use of antimalarial drug efficacy data to inform antimalarial drug policy and guideline development would be challenging. In paper III therefore, aimed to provide significant information on antimalarial drug efficacy monitoring studies conducted in Ethiopia. Accordingly, the present study found high treatment success of AL therapy in the treatment of uncomplicated falciparum malaria in Ethiopia despite its use for more than 16 years. Besides, AL was generally a safe treatment. Previous meta-analysis in 2017 revealed similarly high efficacies of AL [342, 343]. This result is also consistent with neighbouring Sudan; a high treatment success rate (98%) of malaria treatment was recently reported in a meta-analysis that included 20 studies with a total of 4070 patients [363]. The treatment success of 98.7% (95%CI 97.7- 99.6) found in this study suggests that, in accordance with WHO parameters [198], AL is still effective as first-line drug for uncomplicated malaria treatment in Ethiopia, but warrants regular monitoring.

6.4 Strength and limitation

In study I, the use of qPCR for identification and quantification of parasite and, use validated genetic markers to identify recurrent infections are major strengths of this study. However, the lack of pharmacokinetic data to better explains the recrudescence observed.

In study II, use of validated genetic marker for Polymorphism and allelic frequency are major strengths of this study. However, this study acknowledges several limitations such as the inadequate amount of sample size, difficulties to precisely estimate the allelic frequencies and genetic Polymorphism due lower discriminatory power assay (agarose gel electrophoresis) compared to other assays (e.g. capillary electrophoresis). Alleles with short differences in length (less than 10 bp) might not be clearly distinguished.

In study III, the study employed an approach that allows reviewing and document findings of antimalarial efficacy studies conducted in Ethiopia and to assess their contribution to inform policy. The methodological approach and the data collection method used were appropriate for the study. However, the main limitation of this work was the lack of a control group in the included studies that severely limits the ability to draw a firm conclusion regarding the efficacy of an intervention. Moreover, there are insufficient number of therapeutic efficacy studies (TESs) studies with high-quality and more rigorous design. This may be due to the fact that TESs and long-term follow-up of patients require logistics and incur high cost in low and middle income countries, limiting regular implementation of clinical evaluation within the country.

6.5 Conclusions

These are the overall conclusions from this PhD thesis:

❖ The therapeutic efficacy of artemether-lumefantrine is considerably high (above 90%) despite the use of this combination for more than 15 years

❖ AL remains effective in rapidly clearing asexual parasites and fever as well as reducing gametocyte carriage rates

❖ Genetic polymorphisms and multiplicity of *P. falciparum* infections among symptomatic patients reveal a high level of multi-clonal infections and high genetic diversity of parasites circulating in the study area.

6.6 Recommendation

These are the overall recommendations from this PhD thesis:

❖ Artemether-lumefantrine remains highly efficacious as first-line drug for uncomplicated falciparum malaria treatment in Ethiopia
❖ Day-3 parasitaemia warrants a close monitoring of the efficacy of AL to detect changing patterns of parasite susceptibility and make timely revisions to national policies
❖ Periodic malaria molecular genetic surveillance is therefore recommended as a fundamental tool for monitoring changes in parasite population diversity and clonality over time, and for malaria control intervention programs' effectiveness

6.7 Future perspectives

❖ Further study should be done on *P. falciparum* malaria drug resistance markers to supplement and substantiate the current efficacy of AL in different populations and epidemiological regions
❖ This study also, warrants further *P. falciparum* gene diversity investigation in a wider population and also in asymptomatic individuals in order to have an inclusive picture of the parasite diversity

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ANNEXES

Annex I. Declaration

Letter for declaration (Dissertation work)

I, the under signed, declared that this Ph.D. dissertation entitled "Therapeutic efficacy of artemether-lumefantrine for treatment of uncomplicated falciparum malaria and genotyping of *Plasmodium falciparum msp-1* and *msp-2* genes in Ethiopia" is my bona fide original work, has never been presented in this or any other university, and that all the resources and materials used for the thesis, have been fully acknowledged.

Name: **Abdulhakim Abamecha Abafogi**

Signature: _____

Date: _____

Place: **Jimma, Ethiopia**

Main Promoter

Dr. Alemseged Abdissa (Ph.D.)

Signature: _____

Date: _____ 09 Avg 2021

Co-promotors:

Dr. Daniel Yilma (MD, Ph.D.)

Signature: _____

Date: _____ 12 August 2021

Prof. Delenasaw Yewhalaw (Ph.D.)

Signature: _____

Date: _____ 12 Avg. 2021

Annex II. Curriculum Vitae of PhD Candidate

PERSONAL INFORMATION

Full Name: Abdulhakim Abamecha Abafogi

Date of Birth: 14 September, 1987

Place of Birth: Mendera Kochi Kebele, Jimma Zone, Oromia Region, Ethiopia

Gender: Male

Marital Status: Married

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EDUCATION

PhD Candidate in Tropical and Infectious Diseases at School of Medical Laboratory Sciences, Institute of Health, Jimma University, Jimma, Ethiopia(December 2015 to present)

MSc in Medical Microbiology (Distinction), from School of Medical Laboratory Science, , Institute of Health, Jimma University, Jimma, Ethiopia,(2010/11- 2013).

PROFESSIONAL EXPERIENCE

Assistant Professor of Medical Microbiology**:** September 14, 2017 to present**,** College of Health Sciences, Mettu University, Mettu Ethiopia

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ADMINISTRATIVE EXPERIANCES

- **V/Dean**, Health Science Faculty, Mettu University, Mettu, Ethiopia, for 1 year.
- **Head,** Department of Biomedical, Mettu University University, Mettu, Ethiopia 2 years.
- **V/Chair,** University Research and Publications Committee, Mettu University University, Mettu Ethiopia for 1 year.
- **Coordinator,** Continuous Education Program, Mettu University Gambella Campus, Gambella, Ethiopia for 1 year.

International mobility and short-term trainings

Duration	Title of the training and type of award	Place
From August 04,2019 to 31 August 2019	Enhancing Academic Qualifications in Teaching and Research in Public Health(Certificate)	Heidelberg University, **Germany**
October 01,2018 to April 30, 2019	Advanced Molecular Biology techniques(Certificate)	Moulay Ismail University **Morocco**
From 8th to 12th May 2017	Advanced Level of Molecular Diagnosis of Malaria Using qPCR	Medical University of **Vienna, Vienna, Austria**
15th to 16th May 2017	Certificate of Participation on "Good Clinical Practice" from FHI360 organized by Clinical Trial Research Collaboration between Medicine for Malaria Venture (MMV), Switzerland and Jimma University	Jimma University, Jimma, Ethiopia
From June 19-30,2017	Certificate of Completion on "Basic Bioinformatics, Techniques Molecular Biology, and LAMP" at Institute Ethiopian Biotechnology and Institutes of Biotechnology, Addis Ababa University; in Collaboration with University of Calgry, Canada	Addis Ababa University, Addis Ababa, Ethiopia
From May 26-27,2017	Certificate of Training on "G6PD Analysis, Manual Reticulocyte and WBC Counting" at Jimma University; in Collaboration with Medical University of Vienna, Austria	Jimma University, Jimma, Ethiopia
From 8th to 12th May 2017	Certificate of Training on "Microscopy Training" from Swiss Tropical and Public Health Institute organized by Clinical Trial Research Collaboration between Medicine for Malaria Venture (MMV), Switzerland and Jimma University	Jimma University, Jimma, Ethiopia
From: Round I: February 22-28, 2016 and Round II: from April 11-18, 2016).	Certificate of Training on "Evaluation Research capacity building (Part I & II)" from centre for International Health Monitoring and Evaluation, Jimma University in Collaboration with TDR/WHO	Jimma University, Jimma, Ethiopia
From January 1, 017 to February 30, 2017	Certificate of Training on "GIS for Health Researcher" from University of Washington by Online Distance Learning for two Months	Distance learning

AWARDS

- **DAAD PhD In-Country/In-Region Scholarship** 2017, Government of Germany.

- **DAAD Summer School Award 2019,** Government of Germany.

- **MOUNAF Credit seeking PhD mobility**, 2018, Moulay Ismail University, Mekness, Morocco

- **TWAS Fellowship for Postgraduate research Scholarship** 2019, The World Academy of Sciences _Declined

- **HAWK Short term research visit Scholarship, Govornment of Germany_** Declined

PUBLICATIONS

1. **Abamecha A,** Yilma D, Addisu W, El-Abid H, Ibenthal A, Noedl H, Yewhalaw D, Moumni M, Abdissa A. Therapeutic efficacy of artemether-lumefantrine in the treatment of uncomplicated Plasmodium falciparum malaria in Chewaka District, Ethiopia. Malar J. 2020 Jul 10;19(1):240. doi: 10.1186/s12936-020-03307-4. PMID: 32650784; PMCID: PMC7350688.

2. **Abamecha A,** El-Abid H, Yilma D, Addisu W, Ibenthal A, Bayih AG, Noedl H, Yewhalaw D, Moumni M, Abdissa A. Genetic Polymorphism and genotype multiplicity of Plasmodium falciparum infection in patients with uncomplicated malaria in Chewaka district, Ethiopia. Malar J. 2020 Jun 8;19(1):203. doi: 10.1186/s12936-020-03278-6. PMID: 32513191; PMCID: PMC7281928.

3. **Abamecha A,** Yilma D, Adissu W, Yewhalaw D, Abdissa A. Efficacy and safety of artemether-lumefantrine for treatment of uncomplicated Plasmodium falciparum malaria in Ethiopia: a systematic review and meta-analysis. Malar J. 2021 May 6;20(1):213. doi: 10.1186/s12936-021-03745-8. PMID: 33957925; PMCID: PMC8101141.

4. Yeshanew S, Tadege M, **Abamecha A.** Prevalence and Associated Factors of Intestinal Parasitic Infections among Food Handlers in Mettu Town, Southwest Ethiopia. J Trop Med. 2021 Feb 18;2021:6669734. doi: 10.1155/2021/6669734. PMID: 33679993; PMCID: PMC7910069.

5. **Abdulhakim Abamecha**, Beyene Wondafrash and Alemseged Abdissa**.** Antimicrobial Resistance of Enterococci Isolated from intestinal tracts of hospitalized patients admitted at Jimma University Specialized Hospital, Southwest Ethiopia. *BMC Res Notes, vol 8, no.213, 2015.* DOI 10.1186/s 13104-05-1200-2

6. **Abdulhakim Abamecha**, Getahun Assebe, Belay Tafa, Beyene Wondafrash. Prevalence of Thermophilic Campylobacter and their Antimicrobial Resistance Profile in Food Animals in Lare District, Nuer Zone, Gambella, Ethiopia. J. Drug Res Dev 1(2) (2015)

7. **Abdulhakim Abamecha**, Alemayehu. Shiferaw, Ashenafi Kassaye. Assessment of Post Abortion Contraceptive Intention and Associated Factors Among Abortion Clients In Gambella Health Facilities, Gambella Town, South West Ethiopia. *International Journal of Medical Science and Clinical Invention* 3 (8) (2016). https://valleyinternational.net/index.php/ijmsci/article/view/539.

8. Adamu Kenea, Dereje Oljira, Bikila Tesfa, and **Abdulhakim Abamecha**: Assessment of Protection at Birth of Tetanus Toxoid Immunization and Associated Risk Factors in Ilu Aba Bora Zone Southwest, Ethiopia. Int J Vaccine Immunizat 2016, 2(3).

REFERENCE

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Annex III. Definition of severe falciparum malaria[1]

Severe manifestation of *P.falciparum malaria* in adults and children

Clinical manifestations

- prostration;
- impaired consciousness;
- respiratory distress (metabolic acidosis);
- multiple convulsions;
- circulatory collapse;
- pulmonary oedema (radiological);
- abnormal bleeding;
- jaundice;
- haemoglobinurea.

Laboratory findings

- severe anaemia (haemoglobin < 5 g/dl, haematocrit < 15%);
- hypoglycaemia (blood glucose < 2.2 mmol/l or 40 mg/dl);
- acidosis (plasma bicarbonate < 15 mmol/l);
- hyperlactataemia(venous lactic acid > 5 mmol/l);
- hyperparasitaemia (> 4% in non-immune patients);
- renal impairment (serum creatinine above normal range for age).

Classification of severe malaria in children

Group 1: children at increased risk for death

- prostration;
- Respiratory distress.

Group 2: children at risk for clinical deterioration

- haemoglobin < 5 g/dl, haematocrit < 15%;
- two or more convulsions within 24 h.

Group 3: children with persistent vomiting

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[1]World Health Organization.Severe falciparum malaria.*Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2000, 94(Suppl. 1):1–90.

Annex IV. Medications (with antimalarial activity) that should not be used during the study period in addition to the study drug(s)

- chloroquine, amodiaquine;
- quinine, quinidine;
- mefloquine, halofantrine, lumefantrine;
- artemisinin and its derivatives (artemether, arteether, artesunate, dihydroartemisinin);
- proguanil, chlorproguanil, pyrimethamine;
- sulfadoxine, sulfalene, sulfamethoxazole, dapsone;
- primaquine (for *P. vivax*)
- atovaquone;
- antibiotics: tetracycline*, doxycycline, erythromycin, azythromycin, clindamycin, rifampicin, trimethoprim;
- Pentamidine.

* Tetracycline eye ointments can be used.

Major side-effects of Artemether-lumefantrine

Abdominal pain, asthenia, cough, diarrhoea, dizziness, fever, headache, joint and muscle pain, loss of appetite, rush, nausea, vomiting.

Annex V. Dosing chart of artemether-lumefantrine (coartem; novartis) tablets

Weight	Age	Day 1		Day 2		Day 3	
		Morning	Evening	Morning	Evening	Morning	Evening
5–14	3mo–2yrs	1	1	1	1	1	1
15–24	3–7 yrs	2	2	2	2	2	2
25–34	8–10 yrs	3	3	3	3	3	3
> 35	10+ yrs	4	4	4	4	4	4

Annex VI. Classification of treatment outcomes

Early treatment failure

- danger signs or severe malaria on day 1, 2 or 3 in the presence of parasitaemia;
- parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature;
- parasitaemia on day 3 with axillary temperature $\geq$ 37.5 ºC;
- parasitaemia on day 3 $\geq$ 25% of count on day 0.

Late treatment failure

Late clinical failure

- danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 in patients who did not previously meet any of the criteria of early treatment failure;
- presence of parasitaemia on any day between day 4 and day 28 with axillary temperature $\geq$ 37.5 ºC or history of fever in patients who did not previously meet any of the criteria of early treatment failure.

Late parasitological failure

- presence of parasitaemia on any day between day 7 and day 28 with axillary temperature < 37.5 ºC in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response

- Absence of parasitaemia on day 28, 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.

Annex VII. Schedule of follow up activities

	Day										
	0	**1**	**2**	**3**	**7**	**14**	**21**	**28**	**35**	**42**	**Any other**
Procedure											
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 parasite count	X	(X)	X	X	X	X	X	X	(X)	(X)	(X)
Urine sample	(X)										
Blood for:											
genotyping	X				X	X	X	X	(X)	(X)	X
haemoglobin or haematocrit	(X)					(X)		(X)		(X)	(X)
molecular markers	(X)				(X)	(X)	(X)	(X)	(X)	(X)	(X)
in vitro test	(X)										
antimalarial blood concentration	(X)				(X)			(X)		(X)	(X)
Treatment											
Medicine to be tested	X	(X)	(X)								
Rescue treatment		(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)

Parentheses denote conditional or optional activities. For example, treatment would be given on days 1 and 2 only for 3-day dosing. On day 1, the patient should be examined for parasitaemia if he or she has any danger signs or if parasite clearance needs to be calculated. Rescue treatment could be given on any day, provided that the patient meets the criteria for treatment failure. Extra days are any days other than regularly scheduled follow-up days when the patient returns to the facility because of recurrence of symptoms. On extra days, blood slides may be taken routinely or at the request of the clinical staff.

Day 0

Screening

- clinical assessment, including measurement of weight and height; referral in cases of severe malaria or danger signs;
- measurement of temperature;
- parasitological assessment;
- pregnancy test (if necessary);
- informed consentand assent.

Enrolment

- treatment, first dose;
- blood sampling for genotyping.

Optional

- urinary test to detect antimalarial drugs;
- haemoglobin/haematocrit;
- molecular markers of drug resistance;
- in vitro test;
- antimalarial drug blood concentration.

Day 1

- clinical assessment; referral in cases of severe malaria or danger signs;
- measurement of temperature;
- parasitological assessment in cases of severe malaria or danger signs or if parasite clearance needs to be calculated;
- treatment, second dose or alternative treatment in case of severe malaria.

Day 2

- clinical assessment; referral in cases of severe malaria or danger signs;
- measurement of axillary temperature;
- parasitological assessment;
- treatment, third dose or alternative treatment in case of early treatment failure.

Day 3, day 7, day 14, day 21, day 28, or any other day

- clinical assessment; referral in cases of severe malaria or danger signs;
- measurement of axillary temperature;
- parasitological assessment;
- alternative treatment in cases of treatment failure;
- pregnancy test at the end of follow-up (if necessary);
- blood sampling for genotyping to distinguish between recrudescence and reinfection in cases of treatment failure after day 7.

Optional (on or after day 7)

- haemoglobin/haematocrit;
- blood sampling for antimalarial blood concentration andmolecular markers for drug resistance.

Anne XIII. Case report forms

Case report form:follow-up day 0	
Demographic data	

Case report form:follow-up day 0	
Health centre name:	Study number:
Locality:	Patient study number:
District:	Date of visit: dd/mmm/yyyy
Province:	

Demographic data

Date of birth: dd/mmm/yyyy   or estimated age: in: ☐ months or ☐ years

Height (cm):   Weight (kg):   Height (cm):

If female, is the patient pregnant? ☐ Yes ☐ No ☐ Not sure **(If yes, patient is not eligible)**

Provide the date of the last menstrual period: dd/mmm/yyyy

Pre-treatment temperature

History of fever in previous 24 h? ☐ Yes ☐ No

Temperature: ºC ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for *P.falciparum* : quantitative parasite counts and qualitative gametocyte counts

Average number of asexual *P.falciparum* parasites/µl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No **(If yes, patient is not eligible)**

If yes which species ☐ *P. vivax* ☐ *P. ovale* ☐ *P. malariae*

Has blood sample for PCR been collected? ☐ Yes ☐ No

Urinary test for antimalarial drugs

Test used:	Test result: ☐ Positive ☐ Negative
Test used:	Test result: ☐ Positive ☐ Negative

Prior medication

All prior medication, including natural remedies and homeopathic medicines, taken within the previous 14 days should be reported in this section.

Has the patient taken any prior antimalarial medication? ☐ Yes ☐ No. If yes, please specify below. Either the date of stopping or the 'ongoing' box should be checked.

Medicine name (generic name)	Dates	Ongoing (Yes = ☒)	Total daily dose and unit	Route of administration	Indication for use
	Start: dd/mmm/yyyy Stop: dd/mmm/yyyy	☐			
	Start: dd/mmm/yyyy Stop: dd/mmm/yyyy	☐			

Case report form:follow-up day 0 (page 2)				
Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐No	
			☐Yes ☐No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Case report form: follow-up day 1
Study number:
Patient study number:
Date of visit : dd/mmm/yyyy

Clinical status
Presence of danger signs or signs of severe or complicated malaria? ☐Yes☐No
If yes, perform thick blood smear
Temperature: ℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts
Average number of asexual *P.falciparum* parasites/µl:
Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No
Were species other than *P.falciparum* present? ☐ Yes ☐ No
If yes which species☐ *P. vivax*☐ *P. ovale*☐ *P. malariae*

Adverse events
Presence of an adverse event? ☐ Yes ☐ No
If yes, name the adverse event:
Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions

Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Case report form:follow-up day 2

Study number:

Patient study number:

Date of visit : dd/mmm/yyyy

Clinical status

Presence of danger signs or signs of severe or complicated malaria? ☐ Yes ☐ No

Temperature:　　　℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts

Average number of asexual *P.falciparum* parasites/μl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No

If yes which species☐*P. vivax*☐ *P. ovale*☐ *P. malariae*

Adverse events

Presence of an adverse event? ☐ Yes ☐ No No

If yes, name the adverse event:

Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions

Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Case report form:follow-up day 3

Study number:

Patient study number:

Date of visit : dd/mmm/yyyy

Clinical status

Presence of danger signs or signs of severe or complicated malaria? ☐ Yes ☐ No

Temperature: ℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts

Average number of asexual *P.falciparum* parasites/μl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No

If yes which species ☐ *P. vivax* ☐ *P. ovale* ☐ *P. malariae*

Adverse events

Presence of an adverse event? ☐ Yes ☐ No No

If yes, name the adverse event:

Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions

Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Case report form: follow-up day 7

Study number:
Patient study number:
Date of visit : dd/mmm/yyyy

Clinical status

Presence of danger signs or signs of severe or complicated malaria? ☐ Yes ☐ No

History of fever within previous 24 h? ☐ Yes ☐ No

Temperature: ℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts

Average number of asexual *P.falciparum* parasites/µl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No

If yes which species☐*P. vivax*☐ *P. ovale*☐ *P. malariae*

Has a blood sample for PCR been collected? ☐ Yes ☐ No

Adverse events

Presence of an adverse event? ☐ Yes ☐ NoNo

If yes, name the adverse event:

Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions.

Medication administration				

Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Case report form: follow-up day 14

Study number:
Patient study number:
Date of visit : dd/mmm/yyyy

Clinical status

Presence of danger signs or signs of severe or complicated malaria? ☐ Yes ☐ No

History of fever within previous 24 h? ☐ Yes ☐ No

Temperature:　　　℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts

Average number of asexual *P.falciparum* parasites/µl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No

If yes which species☐*P. vivax*☐ *P. ovale*☐ *P. malariae*

Has a blood sample for PCR been collected? ☐ Yes ☐ No

Adverse events

Presence of an adverse event? ☐ Yes ☐ NoNo

If yes, name the adverse event:

Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions.

Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Case report form: follow-up day 21

Study number:

Patient study number:

Date of visit: dd/mmm/yyyy

Clinical status

Presence of danger signs or signs of severe or complicated malaria? ☐ Yes ☐ No

History of fever within previous 24 h? ☐ Yes ☐ No

Temperature: ℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts

Average number of asexual *P.falciparum* parasites/µl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No

If yes which species ☐ *P. vivax* ☐ *P. ovale* ☐ *P. malariae*

Has a blood sample for PCR been collected? ☐ Yes ☐ No

Adverse events

Presence of an adverse event? ☐ Yes ☐ NoNo

If yes, name the adverse event:

Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions.

Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Case report form: final day of follow-up (28)				

Study number:

Patient study number:

Date of visit: dd/mmm/yyyy

Clinical status				

Presence of danger signs or signs of severe or complicated malaria? ☐ Yes ☐ No

History of fever within previous 24 h? ☐ Yes ☐ No

Temperature: ℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts				

Average number of asexual *P.falciparum* parasites/μl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No

If yes which species ☐ *P. vivax* ☐ *P. ovale* ☐ *P. malariae*

Has a blood sample for PCR been collected? ☐ Yes ☐ No

Adverse events				

Presence of an adverse event? ☐ Yes ☐ NoNo

If yes, name the adverse event:

Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions.

Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Urinary analysis (pregnancy test for female patients)				
Patients with a positive pregnancy test must be followed up for 6–8 weeks after delivery				

Result of pregnancy test: ☐ Positive ☐ Negative   Date of test: dd/mmm/yyyy

If the patient is pregnant, follow-up of the pregnancy is required, including: clinical examination of the infant at birth and 6-8 weeks after birth. Please provide comments below. If needed fill in the serious adverse event report form:

Case report form: final day of follow-up (28) (page 2)
Overall assessment

Outcome:

☐ adequate clinical and parasitological response

☐ early treatment failure

☐ late clinical failure

☐ late parasitological failure

☐ lost to follow-up

☐ withdrawn (complete section below: Reason for withdrawal)

Outcome occurred on follow-up day: (e.g. 1, 2, 3, 7, 14, …)

PCR:

☐ *P.falciparum* recrudescence

☐ *P.falciparum* reinfection

☐ other species

☐ mixed with *P.falciparum* recrudescence

☐ mixed with *P.falciparum* reinfection

☐ unknown

PCR corrected results:

☐ adequate clinical and parasitological response

☐ early treatment failure

☐ late clinical failure

☐ late parasitological failure

☐ lost to follow-up
☐ withdrawn
Reason for withdrawal:
Other comments:

Case report form: day (any other day that is not part of regular follow-up)

Study number:

Patient study number:

Date of visit : dd/mmm/yyyy

Clinical status

Presence of danger signs or signs of severe or complicated malaria? ☐ Yes ☐ No

History of fever within previous 24 h? ☐ Yes ☐ No

Temperature: ℃ ☐ Axillary ☐ Tympanic ☐ Rectal ☐ Oral

Thick blood smears for estimation of *P.falciparum* parasite counts

Average number of asexual *P.falciparum* parasites/µl:

Presence of *P.falciparum* gametocytes? ☐ Yes ☐ No

Were species other than *P.falciparum* present? ☐ Yes ☐ No

If yes which species ☐ *P. vivax* ☐ *P. ovale* ☐ *P. malariae*

Has a blood sample for PCR been collected? ☐ Yes ☐ No

Adverse events

Presence of an adverse event? ☐ Yes ☐ NoNo

If yes, name the adverse event:

Is it a serious adverse event? ☐ Yes ☐ No. If yes, inform the sponsor and other relevant institutions.

Medication administration				
Name(s) of antimalarial drug(s)	Time of dose (hh:min)	Number of tablets	Did the patient vomit?	Time of vomiting (hh:min)
			☐ Yes ☐ No	
			☐ Yes ☐ No	
Name(s) of other medicine(s)				
			☐ Yes ☐ No	
			☐ Yes ☐ No	

Annex IX. Serious adverse event report form

Serious adverse event report form	
Health centre name:	Study number:
Locality:	Patient study number:
District:	Date of visit: dd/mmm/yyyy
Province:	Follow-up day:

Demographic data		
Date of birth: dd/mmm/yyyy	or estimated age:	in: ☐ months or ☐ years
Height (cm): Weight (kg): Height (cm):		
If female, is the patient pregnant? ☐ Yes ☐ No ☐ Not sure		
Provide the date of the last menstrual period: dd/mmm/yyyy		

Serious adverse event		
Type of event:	Severity	Relationship to the study drug
☐ Death	☐ Mild	☐ None
☐ Life-threatening	☐ Moderate	☐ Possible
☐ Hospitalization or prolongation of hospitalization	☐ Severe	☐ Probable
☐ Permanent disability	☐ Life-threatening	☐ Definite
☐ Congenital anomaly or birth defect		
Date of occurrence: dd/mmm/yyyy		
Describe the serious adverse event (include all relevant laboratory results):		
Describe how the reaction was treated:		

Serious adverse event report form (page 2)					

Comments (e.g. relevant medical history, drug allergies, previous exposure to similar drugs, other laboratory data, whether reaction abated after stopping the drug, whether reaction reappeared after reintroduction):

Outcome					

☐ Recovered completely

☐ Not yet recovered

☐Recovered with long-term consequences

If patient recovered, provide date of recovery: dd/mmm/yyyy

Medicines (list the **medicine suspected of causing** the serious adverse event as well as all **concomitant medicines**)

Brand name, batch number, manufacturer name (list suspected medicine first)	Daily dose	Route	Start date	End date	Indications for use
			dd/mmm/yyyy	dd/mmm/yyyy	
			dd/mmm/yyyy	dd/mmm/yyyy	
			dd/mmm/yyyy	dd/mmm/yyyy	
			dd/mmm/yyyy	dd/mmm/yyyy	
			dd/mmm/yyyy	dd/mmm/yyyy	

Reporting officer					

Name:

Qualification:

Address:

Phone:

Fax:

Email:

Signature: Date: dd/mmm/yyyy

Anne X. Analysis methods

End-point for day X (X = 28 or 42)	PCR-uncorrected results	
	Cumulative success or failure rate (Kaplan-Meier analysis)	Proportion (per-protocol analysis)
Adequate clinical and parasitological response on day X	Success	Success
Early treatment failure	Failure	Failure
Late clinical failure before day 7	Failure	Failure
Late clinical failure or late parasitological failure on or after day 7	Failure	Failure
Other species infection	Censored day of infection	Excluded from analysis
Lost to follow-up	Censored last day of follow-up according to timetable	Excluded from analysis
Withdrawal and protocol violation	Censored last day of follow-up according to timetable before withdrawal or protocol violation	Excluded from analysis

End-point for day X (X = 28 or 42)	PCR-corrected results	
	Cumulative success or failure rate (Kaplan-Meier analysis)	Proportion (per-protocol analysis)
Adequate clinical and parasitological response at day X	Success	Success
Early treatment failure	Failure	Failure
Late clinical failure before day 7	Failure	Failure
Late clinical failure or late parasitological failure on or after day 7		
• falciparum recrudescence*	Failure	Failure
• falciparum reinfection*	Censored day of reinfection	Excluded from analysis
• other species mixed with falciparum recrudescence	Failure	Failure
• other species mixed with falciparum reinfection	Censored day of reinfection	Excluded from analysis
• other species infection	Censored day of infection	Excluded from analysis
• undetermined or missing PCR	Excluded from analysis	Excluded from analysis
Lost to follow-up	Censored last day of follow-up according to timetable	Excluded from analysis
Withdrawal and protocol violation	Censored last day of follow-up according to timetable before protocol violation or withdrawal	Excluded from analysis

* WHO. *Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations*. Geneva, World Health Organization, 2008 (http://www.who.int/malaria/areas/drug_resistance/en/).

Anne XI. Information Sheet

1. Study title

Therapeutic efficacy of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria and genotyping of *Plasmodium falciparum msp-1* and *msp-2* genes in Ethiopia

2. Invitation paragraph:

I'm PhD student of Tropical and Infectious diseases at Jimma University. I'm doing my PhD research on malaria, which is common in Ethiopia. I'm going to give you information and invite you to be part of this research. Before you decide, you can talk to anyone you feel comfortable with about the research.

If there is anything that you do not understand, please ask me to stop as we go through the information and I will take time to explain. If you have questions latter, you can ask me, or another member of the research team.

3. What is the purpose of the study?

In this study we intend to assess how well Coartem® works to cure malaria and to characterize the genetic polymorphisms of *msp-1* and *msp-2* genes in *P.falciparum* parasites in Ethiopia. The information from this study should help national malaria control program managers to determine whether there is evidence of Coartem® treatment failure and whether we may need to find other medications to substitute for Coartem®.

4. Why I have been chosen?

We are inviting all malaria patients aged 6 months and over living in this area to take part in this study. If you agree, you will be treated with six doses of Coartem® given twice daily for 3 days. (This is the same treatment that you would receive if you decide not to volunteer for this study.) The morning dose will be given at the clinic supervised by study nurse/Public Health Officer and the night dose will be given to you by health extension workers.

5. Do I have to take part?

Your participation in this study is completely voluntary and you can refuse to participate or are free to withdraw from the study at any time. Refusal to participate will not result in loss of medical care provided or all the services you receive at this clinic will continue as usual. Even if you agree now but decide to change your mind and withdraw later, the services you receive at the clinic will continue.

6. What will be happen to me if I take part?

The study will take place over 28 days. During that time, you will be asked to come to the health facility on scheduled days 1, 2, 3, 7, 14, 21 and 28. You will also be asked to come to the clinic at any other time if you become sicker, develop new symptoms, or if you fail to get better. Transportation fees will be provided to you during each scheduled study follow-up visit. During each follow-up visit, we would like to obtain a finger prick blood samples from you by a qualified technician that would be used only for malaria diagnosis, to detect the presence of markers for malaria drug resistance, and to see the outcome of treatment. There is no serious risk in participating, but you may experience a small pain during finger pricking. The pain should disappear within 1 day.

7. What side effect of taking part?

The Coartem medicine can have some unwanted side-effects or some effects that we are not currently aware of; however, we will follow you closely and ensure proper medical treatment. If you take Coartem as directed, the course of your illness and possible side effects from Coartem should not be any different whether you volunteer for this study or not. The Coartem medicine may have some unexpected effects; however, we will follow you closely and keep track of these effects, if they arise. Patients showing deterioration in their clinical status will be immediately admitted to the clinic free of charge for appropriate treatment according to the national policy till they recover. A health care worker will be responsible for every study related medical decision of the patient throughout the study period.

8. What are the possible risks and benefits of taking part?

The risk of being participating in this study is very minimal, but only taking few minutes from your time. There would not be any direct payment for participating in this study. But the findings from this research may reveal important information for the local heath planners.

9. Will my taking part in this study be kept confidential?

If you decide to participate in this study, the information in your records is strictly confidential and your name will not be used in any report and any illnesses related to malaria or to the malaria treatment will be treated at no charge to you. There will be no information that will identify you in particular. Do you understand what has been said to you? If you have any questions you have the right to get proper explanation.

10. What will happen to the results of the research study?

The findings of the study will be general for the study community and will not reflect anything particular of individual persons or housing. The questionnaire will be coded to exclude showing names. No reference will be made in oral or written reports that could link participants to the research. The knowledge that we get from doing this research will be shared with others by publishing the results. Confidential information will not be shared. Do you understand what has been said to you? If you have any questions you have the right to get proper explanation.

11. Who is organizing and funding the research?

The research study is being organized and funded by Tropical and Infectious diseases Center, Jimma University Ethiopia.

12. Who has reviewed the study?

The proposal has been reviewed and approved by Jimma University Ethical Review Board, which are committees whose task it is to make sure that research participants are protected from harm.

13. Contact for further information

In any case if you need any information. You can contact in the following address.

Full Name	Mobile Phone	Adress
Abdulhakim Abamecha	+251 911 05 04 37	Jimma University
Dr. Alemseged Abdissa	+251 911 40 90 74	Jimma University
Dr. Daniel Yilma	+251 911 923553	Jimma University
Professor Delenesaw Yewahalaw	+251 917 80 43 52	Jimma University

Anne XII. Written Informed Consent

Please <u>tick box and sign below</u> if you agree with the following:

1. I confirm that I have read (or have had read to me) and understand the information sheet. I have had time to review this information, have had an opportunity to ask questions and had answers in terms I understand.

2. I understand that taking part is completely voluntary and that I am free to stop at any time even without providing any reason and without my normal medical care being affected.

3. I understand that sections of any of my medical notes may be looked at by representatives of Independent Ethics Committee/Institutional Review Board or from regulatory authorities. The purpose of this is to check that the research is being carried out correctly. I am willing to allow access to my medical notes and understand that strict confidentiality will be maintained.

4. I agree to the extra stored sample for malaria parasite genetic research in Overseas.

5. I agree to take part in this research project.

_____   _____   _____

Name of Participant   **Signature**   **Date and time (dd/mmm/yy; hr min)**

If illiterate:
I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

_____   _____   _____
Name of Witness   Signature   Date and time (dd/mmm/yy; hrmin)

Statement by the researcher/person taking consent:
I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands the research obligations. I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily. A copy of this form has been provided to the participant.

_____   _____   _____
Name of person taking consent   Signature   Date and time (dd/mmm/yy; hrmin)
1 copy for subject, 1 for Investigator, 1 for records of health facility File

Anne XIII. Written informed consent

I have been invited to have my child participate in a study of a medicine used to treat malaria.

I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to my child's participation in this study.

Print name of participant:

Print name of parent or guardian: _____

Signature of parent or guardian: _____

Date: _____

                dd/mmm/yyyy

Witness' signature: A witness' signature and the thumbprint of the participant's parent or guardian are required only if the parent or guardian is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant's parent or guardian and should have no connection with the study team.

I have witnessed the accurate reading of the consent form to the potential participant's parent or guardian, who has had the opportunity to ask questions. I confirm that the participant's parent or guardian has given consent freely.

Print name of witness: and thumbprint of parent or guardian:

Signature of witness: _____

Date: _____

              dd/mmm/yyyy

Investigator's signature:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant's parent or guardian, who has had the opportunity to ask questions. I confirm that the participant's parent or guardian has given consent freely.

Print name of investigator: _____

Signature of investigator: _____

Date: _____

              dd/mmm/yyyy

1 copy for subject, 1 for Investigator, 1 for records of health facility File
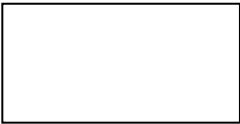
Anne XIV. Written informed assent

I have been invited to participate in a study of the efficacy of an antimalarial medicine.I have read this information (or had the information read to me), and I understand it. I have had my questions answered and know that I can ask questions later if I have them.I agree to take part in the study. _____ (initials) or I do not wish to take part in the study and I have not signed the assent below. _____ (initials)

Child's signature (only if the child assents):

Print name of child: _____

Signature of child: _____

Date: _____
dd/mmm/yyyy

Witness' signature:A witness' signature and the child's thumbprint are required only if the child is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.
I have witnessed the accurate reading of the assent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

and thumbprint of the child or minor:

Print name of witness: _____

Signature of witness: _____

Date: _____
dd/mmm/yyyy

Investigator's signature:
I have accurately read or witnessed the accurate reading of the assent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Print name of investigator: _____

Signature of investigator: _____

Date: _____
dd/mmm/yyyy

1 copy for subject, 1 for Investigator, 1 for records of health facility File

Anne XV. Consent statement for a pregnancy test

I have been invited to participate in a study on the medicine used to treat malaria. I have been asked to supply a specimen of urine at the first visit and at day 28 or on the day of withdrawal from the study, all of which will be used for pregnancy testing. I understand that the results of the tests will be kept fully confidential and anonymous. I understand that I must avoid becoming pregnant during the study because the medicine I will be taking would be dangerous for my child. I have discussed the different methods of birth control with my doctor, and I have been recommended use contraceptive method.I understand that if the test is positive, I will not be eligible to participate in this study.
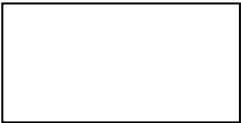
Participant's signature:

I accept to be tested. _____ (participant'sinitials) or

I do not want to be tested, and I have notsigned the consent form below. _____ (participant's initials)

Print name of participant: _____
Signature of participant: _____
Date: _____
              dd/mmm/yyyy

Witness' signature: A witness' signature and the thumbprint of the participant are required only if the participant is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participantand should have no connection with the study team.
I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Print name of witness: _____

Signature of witness: _____
Date: _____
              dd/mmm/yyyy

and thumbprint of the participant:

Investigator's signature:
I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Print name of investigator: _____
Signature of investigator: _____
Date: _____
              dd/mmm/yyyy

1 copy for subject, 1 for Investigator, 1 for records of health facility File

Annex XVI. Supplimentary file 1: Table S1: Primer sequence used for *msp-1*

Table 13:Primer sequence used for PCRs to screen and genotype samples collected in study of genotyping Polymorphism *of P. falciparum* parasites in Chewaka district, Ethiopia

PCR	Locus	Allele	Primer		Sequence (primer differences are shown in bold, underlined font)	Reference
			Forward	Reverse		
pPCR	*msp1*	N/A	✓		CTAGAAGCTTTAGAAGATGCAGTATTG	2
				✓	**C**TTAAATAGTATTCTAATTCAAGTGGATCA	
nPCR		K1	✓		**A**AATGAAGAAGAAATTACTACAAAAGGTGC	
				✓	**G**CTTGCATCAGCTGGAGGGCTTGCACCAGA	
		MAD20	✓		AAATGAAGGAACAAGTGGAACAGCTGTTAC	
				✓	ATCTGAAGGATTTGTACGTCTTGAATTACC	
		RO33	✓		TAAAGGATGGAGCAAATACTCAAGTTGTTG	
				✓	**CAAGTAATTTTGAACTCTATGTTTTAAATCAGCGTA** CATCTGAAGGATTTGCAGCACCTGGAGATC	

Annex XVII. Supplimentary file 2: Table S2: Primer sequence used for *msp-2*

Table S2:Primer sequence used for PCRs to screen and genotype samples collected in study of genotyping Polymorphism *of P. falciparum* parasites in Chewaka district, Ethiopia

PCR	Locus	Allele	Primer		Sequence (primer differences are shown in bold, underlined font)	Reference
			Forward	Reverse		
pPCR		N/A	✓		ATGAAGGTAATTAAAACATTGTCTATTATA	3
				✓	CTTTGTTACCATCGGTACATTCTT *ATATGGCAAAAGATAAAACAAGTGTTGCTG*	
nPCR	*msp2*	FC27	✓		**GCTTATAATATGAGTATAAGGAGAA** AATACTAAGAGTGTAGGTGCARATGCTCCA *GCAAATGAAGGTTCTAATACTAATAG*	4
				✓	TTTTATTTGGT**GCATTGCCAGAACTTGAA**C *GCTTTGGGTCCTTCTTCAGTTGATTC*	
		3D7/IC	✓		**GCTTATAATATGAGTATAAGGAGAA** AGAAGTATGGCAGAAAGTAAKCCTYCTACT *GCAGAAAGTAAGCCTTCTACTGGTGCT*	
				✓	**CTGAAGAGGTACTGGTAG** GATTGTAATTCGGGGGATTCAGTTTGTTCG *GATTTGTTTCGGCATTATTATGA*	

3 World Health Organization, 2007

4 Falk, Maire, Sama, Owusu-Agyei, Smith, & Beck, 2006) and (Zwetyenga, Rogier, Tall, Fontenille, Snounou, & Trape, 1998)

Annex XVIII. PRISMA Check list

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Abstract, Paragraph 1-4
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Introduction, paragraph 5-6
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Introduction, paragraph 6 & Table 1
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	Methods, paragraph 1
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Methods, Paragraph 4-5; & Table 1
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Methods, Paragraph 2
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Additional file 2: Table S2

Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Methods, Paragraph 4-6 & Table 1
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Methods, Paragraph 6-7
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	Methods, Paragraph 6-7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Methods, Paragraph 8
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	Methods, Paragraph 8
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	Methods, Paragraph 8

Annex XIX. Detailed search strategy for the different electronic databases

No.	Databases (Total 3)	Search Terms	Search results Total = 1041	Date of search
1	**PubMed**	(((((((Efficacy[MeSH Terms]) OR (Therapeutic efficacy[MeSH Terms])) AND (Artemether-lumefantrine[MeSH Terms])) OR (Coartem[MeSH Terms])) AND (Plasmodium falciparum malaria[MeSH Terms])) OR (falciparum malaria[MeSH Terms])) AND (Antimalarial drug[MeSH Terms])) AND (Ethiopia)	**44**	**15/09/2020**
2	**Google Scholar**	1. **With all of the words:** ("Therapeutic efficacy" AND "Artemether-lumefantrine" AND "Ethiopia") 2. **With at least one of the words:** ""Plasmodium falciparum" AND "falciparum malaria"	**545 + 451 = 996**	**15/09/2020**
3	**Clinical Trial.gov**	Condition or disease: Plasmodium falciparum, Ethiopia Other terms: artemether-lumefantrine	1	15/09/2020