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New strategies and tools for *Plasmodium falciparum* case management and surveillance in the era of imminent resistance to artemisinin-based combination therapy in Tanzania.

LWIDIKO E MHAMILAWA



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Abstract

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Artemether-lumefantrine has been an efficacious first line treatment for uncomplicated *Plasmodium falciparum* malaria in Tanzania since its introduction in 2006. Interest has developed in understanding the observation of high residual PCR determined positivity rates on day 3 after supervised artemether-lumefantrine treatment in the magnitude of almost 30% in previous assessments from 2015 in Bagamoyo district, Tanzania. Deep sequencing has recently been used to study these Bagamoyo parasites with delayed clearance, and the clearance times by PCR of some *P. falciparum* sub-populations were similar to artemisinin resistant parasites in Myanmar as assessed by microscopy, albeit lacking the described mutations in the Kelch13 propeller gene associated with artemisinin resistance. Moreover, molecular epidemiological studies from Bagamoyo, have shown temporal selection of lumefantrine associated genetic tolerance/resistance markers (*pfmdr1* - N86, 184F, D1246 and *pfprt* - K76) in the parasite population following wide scale use of artemether-lumefantrine but without signs of compromised treatment efficacy. On the other hand, traditional epidemiological studies have reported that imported malaria cases in Zanzibar from Tanzania mainland contribute to regressing the malaria elimination efforts in this pre-elimination part of the country.

This PhD project explored efficacy and safety of extending the artemether-lumefantrine regimen from standard 3 days to 6 days and adding single low dose primaquine (0.25mg/kg) as a new strategy that can be used in order to protect the therapeutic lifespan of artemether-lumefantrine. Also, whole-genome sequencing was used to study genomic epidemiology of *P. falciparum* population between Tanzania mainland and Zanzibar.

The results revealed that extended artemether-lumefantrine treatment did not have superior efficacy in the current context of artemether-lumefantrine sensitive *P. falciparum* parasites. However, the safety profile was excellent and similar to standard 3 days treatment. Parasite detection by molecular methods was 84% on day 3 after artemether-lumefantrine treatment. Meanwhile, significant decreases in the effective population sizes were inferred in both Tanzania mainland and Zanzibar parasite populations, that coincide with a period of decreasing malaria transmission in Tanzania. The parasite population from Tanzania mainland and Zanzibar were found to be connected, implying importation of cases from high transmission mainland to pre elimination regions of Zanzibar.

Utility of these results is during exploring options of alternative artemisinin-based combination therapy regimens to protect their therapeutic efficacy in an era of imminent artemisinin resistance in sub Saharan Africa. Moreover, the genomic epidemiological findings in this project may be of interest for malaria elimination programs, in the incorporation of molecular tools in future malaria elimination strategies and resistance surveillance, in the context of understanding importation of malaria from high to low transmission regions.

Keywords: Malaria, *Plasmodium falciparum*, artemether-lumefantrine, drug resistance, Tanzania

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If I have seen further, it is by standing on shoulders of giants.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Lwidiko E. Mhamilawa**, Berit Aydin-Schmidt, Bruno P. Mmbando, Billy Ngasala, and Ulrika Morris. (2019) Detection of *Plasmodium falciparum* by light microscopy, Loop Mediated Isothermal Amplification and PCR on day 3 after initiation of artemether-lumefantrine treatment for uncomplicated malaria in Bagamoyo district, Tanzania – a comparative trial. *American Journal of Tropical Medicine and Hygiene*
- II **Lwidiko E. Mhamilawa**, Billy Ngasala, Ulrika Morris, Eliford Ngaimisi Kitabi, Rory Barnes, Aung Paing Soe, Bruno P. Mmbando, Anders Björkman and Andreas Mårtensson (2020) Parasite clearance, cure rate, post-treatment prophylaxis and safety of standard 3-day versus an extended 6-day treatment of artemether-lumefantrine and a single low-dose primaquine for uncomplicated *Plasmodium falciparum* malaria in Bagamoyo district, Tanzania– a randomized controlled trial. Submitted manuscript
- III **Lwidiko E. Mhamilawa**, Sven Wikström, Billy Ngasala, Bruno P. Mmbando and Andreas Mårtensson. (2020) Electrocardiographic safety evaluation of a prolonged artemether-lumefantrine treatment in patients with uncomplicated *Plasmodium falciparum* malaria in Bagamoyo district, Tanzania. Submitted manuscript
- IV Andrew P. Morgan, Nicholas F. Brazeau, Billy Ngasala, **Lwidiko E. Mhamilawa**, Madeline Denton, Mwinyi Msellem, Ulrika Morris, Dayne L. Filer, Ozkan Aydemir, Jeffrey A. Bailey, Jonathan B. Parr, Andreas Mårtensson, Anders Bjorkman & Jonathan J. Juliano. (2020) *Falciparum* malaria from coastal Tanzania and Zanzibar remains highly connected despite effective control efforts on the archipelago. *Malaria Journal*.

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I have made primary contribution in the study design and field implementation during data/sample collection and analyses for studies included in this thesis. For **Paper I and II**, I collected samples, participated in all molecular and statistical analyses, prepared the figures and tables, and wrote a first draft of the manuscript and contributed to editing of the final version. For **Paper III**, I have a shared first authorship with Sven Wikström, whereby I performed the electrocardiograms from patients in the field, collected data related to the study and participated in the data analysis preparing the first draft of the manuscript to the final version. For **Paper IV**, I participated in field data collection and preparation of manuscript. The molecular works related to this paper were done at Professor Jonathan Juliano's laboratory at the UNC at Chapel Hill - High Throughput Sequencing Facility.

Contents

1	Introduction	11
1.1	Global malaria burden and Sustainable Development Goals	11
1.2	The malaria parasite	13
1.3	<i>Plasmodium falciparum</i> life cycle	14
1.4	Malaria transmission and epidemiology	17
1.4.1	Vector	17
1.4.2	Human host and immunity	17
1.4.3	Malaria endemicity classification	18
1.5	Clinical presentation and pathophysiology of <i>P. falciparum</i> malaria 19	
1.5.1	Clinical presentation	19
1.5.2	Pathophysiology	20
1.6	Diagnosis of <i>P. falciparum</i> malaria in Africa	21
1.6.1	Light microscopy	22
1.6.2	Rapid Diagnostic Tests	23
1.6.3	Nucleic acid amplification-based tests	23
1.7	<i>P. falciparum</i> malaria treatment	28
1.8	Role of artemisinin-based combination therapy in <i>P. falciparum</i> malaria case management	30
1.8.1	Artemisinins	30
1.8.2	Artemisinin-based combination therapies	31
1.8.3	Artemether-lumefantrine	32
1.8.4	Artesunate-amodiaquine	33
1.8.5	Cardiotoxicity concerns of quinolines	33
1.8.6	Primaquine	35
1.8.7	<i>P. falciparum</i> resistance to antimalarial drug	36
1.8.8	<i>In vitro</i> Tests	37
1.8.9	Molecular markers of antimalarial drug resistance	37
1.8.10	<i>In vivo P. falciparum</i> antimalarial therapeutic efficacy studies. 38	
1.8.11	PCR genotyping to distinguish recrudescence from reinfections in antimalarial drug trials	40
1.8.12	<i>P. falciparum</i> resistance to ACT	42
1.8.13	Malaria case management - experiences from Tanzania 45	

1.8.14	Malaria transmission dynamics in regions of pre-elimination - Zanzibar case study	49
2	Rationale and aims of the thesis	51
2.1	Rationale.....	51
3	Aims.....	52
4	Materials and methods.....	53
4.1	Study sites and population.....	53
4.1.1	Study Sites	53
4.1.2	General methodologies	55
4.2	Study specific methodologies.....	57
4.2.1	Electrocardiographic safety study III.....	57
4.2.2	Blood sampling and storage.....	57
4.2.3	DNA extraction.....	58
4.2.4	PCR genotyping.....	58
4.2.5	WGS	60
4.3	Ethical considerations.....	60
5	Results and discussion	62
5.1	Study I: Detection of <i>Plasmodium falciparum</i> on day 3	62
5.2	Study II: Efficacy of extended 6-day treatment of artemether-lumefantrine	63
5.3	Study III: Electrocardiographic safety of prolonged artemether-lumefantrine	65
5.4	Study IV. Genomic epidemiology of <i>P. falciparum</i> malaria from coastal Tanzania and Zanzibar	66
6	Conclusions	68
7	Personal reflections and future perspectives	69
8	Acknowledgements	70
9	References	73

Abbreviations

ACPR	Adequate clinical and parasitological response
ACT	Artemisinin-based combination therapies
AUC	Area under the curve
BRTU	Bagamoyo Research and Training Unit
<i>Bst</i>	<i>Bacillus stearothermophilus</i>
COI	Complexity of infection
COVID-19	Corona virus disease 2019
CQ	Cycles of quantification
CYP3A4	Cytochrome P450 3A4
Cyt b	Cytochrome b
DBS	Dried blood spots
DNA	Deoxyribonucleic acid
Δ QTc	Change in QTc interval
ECG	Electrocardiogram
EIR	Entomological inoculation rate
ETF	Early treatment failure
G6PD	Glucose-6-phosphate dehydrogenase
<i>glurp</i>	Glutamate-rich protein gene
hERG	<i>Human-ether-a-go-go-related gene</i>
HPLC-UV	High-performance liquid chromatography with a UV detection assay
ICH	International Conference on Harmonisation
IgE	Immunoglobulin E
IRS	Indoor residual spraying
ITN	Insecticide-treated nets
KAHRP	Knob-associated Histidine-rich Protein
LAMP	Loop mediated isothermal amplification
LCF	Late clinical failure
LPF	Late parasitological failure
mRDT	Malaria rapid diagnostic test
<i>msp-1</i>	Merozoite surface proteins 1 gene
<i>msp-2</i>	Merozoite surface proteins 2 gene

MUHAS	Muhimbili University of Health and Allied Sciences
p/μL	Parasite per microliter
PCR	Polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
<i>PfATP6</i>	<i>Sarco/endoplasmic reticulum Ca⁽²⁺⁾-ATPase orthologue of Plasmodium falciparum (pfATP6) gene</i>
<i>Pfcr1</i>	<i>P. falciparum chloroquine resistance transporter gene</i>
<i>PfEMP1</i>	<i>P. falciparum Erythrocyte Membrane Protein 1</i>
<i>Pfk13</i>	<i>P. falciparum kelch13 gene</i>
<i>Pfmdr1</i>	<i>P. falciparum multidrug resistance 1 gene</i>
<i>Pfmrp1</i>	<i>P. falciparum multi-resistance protein1 gene</i>
pH	Power of hydrogen
qPCR	Quantitative PCRs
QTc	Corrected QT interval
QTcB	Bazett's corrected QT interval
QTcF	Fridericia's corrected QT interval
RBCs	Red blood cells
RNA	Ribonucleic acid
SDG	Sustainable Development Goal
SNPs	Single nucleotide polymorphisms
sWGA	Selective whole genome amplification
TdP	Torsade de Pointes
TES	Therapeutic efficacy studies
TNF	Tumour Necrosis Factor
TORCA	Tandem Oligonucleotide Repeat Cascade Amplification
UNC	University of North Carolina
USA	United States of America
USD	United States Dollar
UV	Ultraviolet
WBC	White blood cells
WGS	Whole genome sequencing
WHO	World Health Organization

1 Introduction

1.1 Global malaria burden and Sustainable Development Goals

Malaria remains a significant public health problem globally despite substantial control efforts for the past 10–15 years, affecting mostly children and pregnant women. About 228 million cases and 405,000 deaths were reported globally in 2018 (1). Approximately 93% of the disease burden is in Africa (1). Despite countries like Ethiopia, India, Pakistan, and Rwanda reporting an impressive reduction in malaria cases, there is a worrying increase of more than 3.5 million cases in the ten highest burdened counties in Africa, and Tanzania is one of them (2,3). In 2017 alone, 61% (266,000) of malaria related deaths were of children under five years globally, and Africa accounted for 93% of these global deaths (2). During 2015-2018 period, the progress made towards malaria control for the past decade has stagnated; the number of cases per 1000 population at risk has remained at 57 after a decline from 75 cases per 1000 population in 2010. To reach the target set by the World Health Organization (WHO) - Global Technical Strategy of 40% reduction in case incidence globally by 2020, we needed to have been at 45 cases per 1000 population in 2018 (4). This puts all the impressive advances made over the last decade at risk. At the current trend, we are not on track to attain the 2030 goal of reducing malaria case incidence by 90% from the 2015 baseline unless we accelerate the efforts (4,5). In order to sustain the progress made, the WHO launched a new aggressive approach in 2018 known as “high burden to high impact” - a country-led response.

Moreover, due to the recent advancements in protecting children under five years old from malaria infection, a study by Griffin et al. predicts a changing age-burden of *P. falciparum* malaria disease in sub-Saharan Africa. Their prediction is based on a mathematical model that indicates children aged between 2 and 10 years have increased malaria incidence (6). This results in lost school days and subsequent poor performance in class hampering further progress in attaining Sustainable Development Goal (SDG) 4 on education.

SDG 3 targets to end malaria epidemic by 2030 among other infectious diseases. Current challenges in attaining this goal are interlinked with other SDGs in a variety of ways. For people living in poverty (SDG 1) the risk of diseases is higher, and deaths from lack of access to effective treatment or optimal vector control like insecticide-treated nets (ITN), cements the linkage

between malaria and poverty (7). Resistance to available interventions, developed by both the parasite and the vectors complicates this further. The increasing rate of urbanisation with poorly planned cities lead to an increase in slums residence. This is an issue addressed in SDG 11. The sub-optimal living environment in these urban settings, associated with poor sanitation, lack of adequate water (SDG 6) and low housing conditions, make it favourable for malaria transmission in the urban to continue (1). Moreover, the climate change poses a risk of changing malaria epidemiology by making it possible for malaria vectors to transmit malaria in places where they are currently unable to. The climate action addressed in SDG 13, plans to mitigate the effects of climate change, and subsequently mitigate the predicted increase in the range and intensity of malaria transmission

Globally, the required health expenditure for malaria is estimated to be 6.6 billion USD per year by 2020 (1). The investment in malaria control and elimination efforts has remained inadequate, with a total of 3.2 billion USD being invested globally in 2017 (2). It has dropped even further to 2.7 billion in 2018, resulting in inadequate access to essential malaria death averting tools and low levels of uptake of malaria control interventions (1).

The 1955 Global Malaria Eradication Program focused on interrupting malaria transmission in all endemic countries outside Africa. Despite its success in some countries that eliminated malaria and remained malaria free, after the funding collapsed in 1969, the program failed miserably and lead to a devastating resurgence in malaria cases, and deaths in countries that had weak malaria control programs. Vector insecticide resistance and parasite drug resistance played a major role in the failure (8–10). As history should be the best teacher, the current trends led WHO to call for countries and donor communities to increase financial commitment and re-strategize the approach with a focus in high burden countries to have a higher impact (3). Attaining SDG 17 on strengthening the global partnership for sustainable development will improve our chances of eliminating malaria. Investment in building robust health systems with drug resistance surveillance and proactive measures to protect the efficacy of available malaria control tools within high burden countries is critical to have a high impact on malaria control (5).

Eventually, achieving sustainable progress in reducing the global malaria burden requires a holistic view on SDG, and how it complements the bigger picture. Success in malaria control and elimination both enhances and is enhanced by the success in attaining other SDGs. It is synergistic.

1.2 The malaria parasite

Malaria is a vector borne infection, caused by a unicellular parasitic protozoan belonging to genus *Plasmodium*. An infectious female anopheline mosquito is the vector that transmits malaria to a human host through a bite when taking a blood meal. Malaria is thought to be older than humanity, with the genus *Plasmodium* to have evolved more than 130 million years ago, and some species adapted to infect humans more than 5 million years ago (11,12). Over 250 *Plasmodium* species exist, but five are commonly known to infect humans, i.e. *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (13). Recently, the *P. ovale* has been identified to exist in dimorphism of classical and variant types i.e., *Plasmodium ovale curtisi* and *Plasmodium ovale walikeri* respectively, making six plasmodium species that infect human (14–16). 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.

Deadly cases of malaria in sub-Saharan Africa and Southeast Asia are commonly caused by *P. falciparum* (1). However, *P. knowlesi* which was known to primarily infect long-tailed macaques, can also cause severe human malaria if not treated early, and can be fatal (17,18) *P. knowlesi* is prevalent throughout Southeast Asia where their natural hosts (macaque monkeys) are present (19). The other species cause less severe forms of malaria and have a wider distribution at lower prevalence. *P. vivax* is most common in South America but also found in Southeast Asia, and can become latent for months to years in the liver stage known as hypnozoites (13). 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 (20). 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 Djibouti, Eritrea, Ethiopia, Somalia, Sudan and South Sudan (20–22). 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). *P. malariae* and *P. ovale* (which can also form hypnozoites) occur throughout Africa, and in all regions endemic with malaria. As it is for *P. vivax*, the relative contribution of these milder forms of malaria in morbidity is increasing as the *P. falciparum* prevalence is driven down by effective interventions in endemic regions (23).

Since *P. falciparum* malaria claims hundreds of thousands lives annually in sub-Saharan Africa, and is prevalent in Tanzania, this PhD thesis focuses on *P. falciparum* only henceforth.

1.3 *Plasmodium falciparum* life cycle

The *P. falciparum* parasite has a complex life cycle that involves a sequence of sexual and asexual phases, occurring in the mosquito vector (definite host) and the human host (intermediate host) (Figure 1). The sexual phase begins when a female *Anopheles* mosquito takes a blood meal containing gametocytes (the sexual forms of the parasite) in red blood cells (RBCs) from an infected person. When the gametocytes reach the gut of the mosquito, they escape from the RBCs. The sudden lowering of temperature from that of a human body to mosquito, coupled with some activating factors inside the gut of the mosquito, trigger the gametocyte differentiation to male and female gametes. A violent ex-flagellation of the male gametocyte, produces eight flagellated male gametes (sperms) with haploid male nucleus that swim to fertilise female gametes to produce a diploid zygote. Then, the zygote develops to an invasive ookinete that migrates across the epithelial lining of the mosquito's midgut, settling in hemocoel as an oocyst. Depending on the ambient temperature, after 7–10 days, the first asexual phase (sporogony) begins. The oocysts undergo internal division to form thousands of slender sporozoites. Eventually, the oocyst bursts to release sporozoites in the hemocoel where they migrate to the salivary glands. Now the mosquito is infectious, and this takes about 10–21 days from the initial blood meal.

When the infectious mosquito feeds again, it inoculates the uninfected human host with sporozoites that are in the saliva. Each mosquito bite contains a small amount of sporozoites, about 125 (range 0-1300) in laboratory experiments. In the field setting, an infectious bite can have as low as 100 sporozoites (24). The injection of sporozoites is a vital life cycle stage for malaria control, since there is a low number of parasites to target. Various sporozoites vaccines have been developed to prevent establishment of infection albeit with limited efficacy (25). Within minutes to few hours, these motile sporozoites travel through the blood system to enter the liver and invade hepatocytes for the second asexual phase. Others that enter the lymph system may be cleared by the immune system from the nearest lymph node they invade as it has been demonstrated in rodent experiments (26).

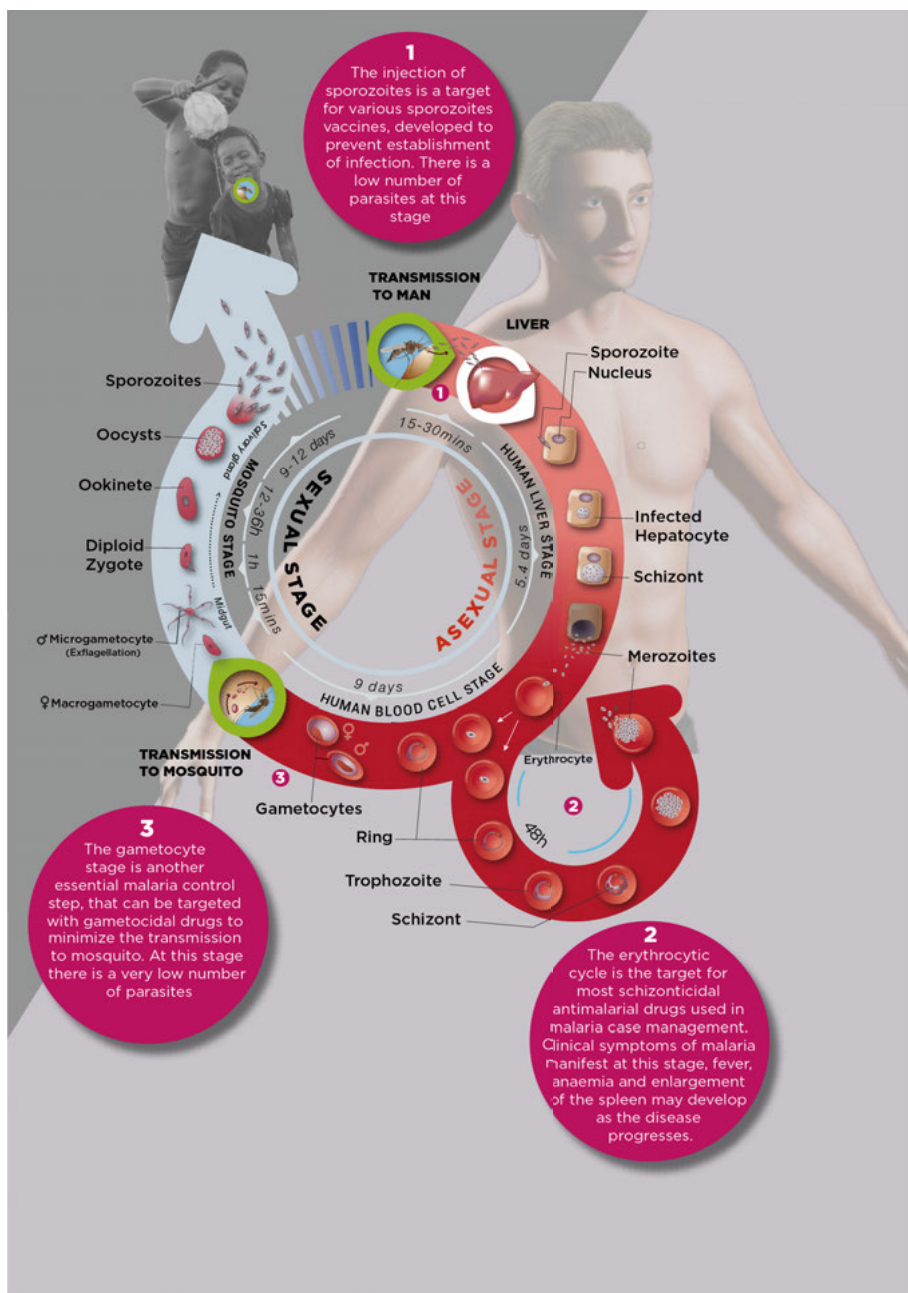


Figure 1: Illustration of the life cycle of the malaria parasite

The sporozoites in hepatocytes immediately start to feed by absorbing nutrients and become hepatic trophozoites and mature to schizonts. Asexual division of hepatic schizonts leads to multinucleated hepatic schizont, each with about 10,000–30,000 daughter merozoites during 5.5–8 days (13). The hepatic

schizont eventually bursts, releasing these merozoites into the hepatic capillaries, entering the circulation and within a few minutes of their release, they invade RBCs. The third asexual phase begins after merozoites invade RBCs, marking the beginning of the erythrocytic cycle. This phase is the clinically significant phase of the lifecycle.

During this phase, the parasites mature through different stages; begin as early trophozoites (ring stage) within the RBC, where it ingests and digests haemoglobin and accumulates the malaria pigment hemozoin. At this stage, they are most visible through microscopy from peripheral blood smears. Then develop into mature trophozoites, and finally becoming schizonts which rupture the RBC releasing between 6 and 30 daughter merozoites. The mature forms of the parasites are not visible in peripheral circulation because of a phenomenon known as sequestration that allows them to settle in deep tissues, contributing significantly to the pathophysiology of malaria. These merozoites infect new RBCs within 30–90 seconds, and the cycle repeats itself every 48 hours, clinically presenting as periodic fevers after 48 hours (tertian fevers). For *P. falciparum*, unlike other *Plasmodium* species, the periodicity of the tertian fevers is usually irregular, and lasts 6–8 hours (27). Rupturing of RBC by mature schizonts is accompanied by the release of pyrogens into the blood. This erythrocytic cycle produces the largest amount of parasites, where the parasite population can expand 6–20 times per each 48 hours cycle (13). It is the target for most antimalarial drugs used in malaria case management. Anaemia and enlargement of the spleen may develop as the disease progresses. Symptoms usually start approximately 6–8 days after merozoites emerge from the liver (13).

Only about 10% of the parasite population in the human host commits to becoming gametocytes. These are the sexual forms that survive longer in the human host circulation until when taken up by a feeding anopheline mosquito to continue the lifecycle. The gametocyte stage is another essential malaria control step, that can be targeted with gametocidal drugs, since there is a very low number of parasites. In membrane feeding studies, it has been demonstrated that an infection can be established in a mosquito, with gametocyte density as low as 1 parasite per microliter (p/μL) of blood (28). However, presence of both male and female gametocytes is important for successful infection in the mosquito, which is a subject of crucial consideration when using gametocidal drugs, since female gametocytes have demonstrated more susceptibility than male gametocytes (29). Higher gametocyte density has been linked to greater infection rates; however, it is not deterministic (30).

1.4 Malaria transmission and epidemiology

1.4.1 Vector

Transmission of *P. falciparum* to humans depends on the female *Anopheles* mosquito. It is considered a definitive host since the sexual cycle occurs in the mosquito. More than 450 species of *Anopheles* are known, but only about 10% of them (45 species) are essential for malaria transmission globally and only three, *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles funestus* are responsible for majority of the transmission (31).

Depending on geographical location, some species are more prevalent and efficient vectors than others. In sub-Saharan Africa, the *Anopheles gambiae* and *Anopheles funestus* are the most dominant vectors. Mosquito factors that influence their transmission potential include their preference to feed on human versus animals, when they prefer to feed (night vs day), and whether they feed and rest more indoors or outdoors (32,33).

External factors playing important roles in the vector's transmission potential include:

1. 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 (34)
2. 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
3. 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 (35–38).

1.4.2 Human host and immunity

It is believed that malaria parasites have provided high genetic selection pressure during human evolution. Polymorphisms in ABO blood groups, sickle cell disease (heterozygotes for the sickle gene), Glucose-6-phosphate dehydrogenase (G6PD) deficiency, and thalassemia, is evidence of how the human genome evolved as strategies to protect against continuous exposure to malaria (39–41). On the contrary, patients who are homozygous for the sickle

gene are more vulnerable to severe outcomes since malaria complicates the sickle cell anaemia (42).

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 (43). 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 (44). 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 wanes 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 premunition (44–46).

1.4.3 Malaria endemicity classification

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 sub-optimal due to deficiencies in health systems, making it rather difficult to classify endemicity in these regions correctly (47). 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 (48).
- 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 (48).

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 (49).

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 (49).

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 (49). Malaria epidemics can occur with devastating consequences.

1.5 Clinical presentation and pathophysiology of *P. falciparum* malaria

1.5.1 Clinical presentation

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, generalised 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 comorbidities, individuals without spleen or whose spleen has compromised function and immunocompromised individuals (50). 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 (13). 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 (13,48).

1.5.2 Pathophysiology

The pathophysiology of *P. falciparum* infection involves a complex interplay between host and parasite factors:

1.5.2.1 The inflammatory response (toxicity and cytokines)

Rupture of schizonts releases merozoites and glycolipid materials into circulation with properties similar to bacterial endotoxins. These materials together with IgE complexes of the malaria antigens mobilise pro-inflammatory cytokines such as Tumour Necrosis Factor (TNF), Interleukins and gamma interferons, subsequently leading to clinically observed fever, chills, headaches, shivering. In severe disease, these cytokines are associated with renal dysfunction, anaemia through suppression of erythropoiesis and hypoglycaemia through inhibition of gluconeogenesis (51).

The fevers caused by these cytokines is believed to be an outcome of the lipid peroxides released from white blood cells (WBC) and other cells as an immune response to kill the parasite. In patients with premunition, the release of cytokines is downregulated, leading to asymptomatic infections (51).

1.5.2.2 Microcirculation obstruction (cytoadherence, rosetting and sequestration)

Infected RBCs with mature *P. falciparum* tend to adhere to endothelium of microvasculature (cytoadherence), or to other uninfected RBCs (rosetting) (52). *P. falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1) (53), and Knob-associated Histidine-rich Protein (KAHRP) (54) are known to enable this pathogenic cytoadherence. As a result, sequestration of parasites in deep tissues occurs, and it starts after the first 24 hours of the 48 hours asexual cycle, rendering the mature forms of the falciparum parasite not visible in the peripheral circulation.

The infected RBCs sequestered in microvasculature cause occlusion of the vessels because of their rigidity and clumping. This obstruction is a significant contributor of injuries to various organs affected during the severe form of the disease.

1. Obstruction in the brain microvasculature compromises perfusion and contributes to the complications of cerebral malaria.
2. Obstruction in the kidney microvasculature leads to ischemia that causes acute tubular necrosis in kidney injury.
3. Metabolic acidosis from increased lactate production is contributed by microvasculature obstruction in tissues that lead to hypoxia and increased anaerobic glycolysis.
4. Placental sequestration leads to mobilisation of an inflammatory response to the placenta, compromising placental functions. The reduced perfusion contributes to foetal growth retardation and compromises maternal outcomes (51).

1.5.2.3 Anaemia

Haemolysis of parasitized RBCs during schizont ruptures is one of main causes of anaemia in malaria. However, the spleen can remove parasites from infected RBCs and returns the parasite free RBCs into circulation, a phenomenon known as pitting (55). Pitting is most notable after treatment with artemisinin. These recycled RBCs have a reduced lifespan, leading to delayed onset of anaemia. (56,57). In addition, the destruction of uninfected RBCs contributes to the anaemia. In an acute infection rigidity of RBCs increases, and it is thought to be due to oxidative damages, which consequently compromises the RBC membrane function and deformability (58).

Suppression of erythropoiesis by cytokines in the bone marrow during acute infection contributes to anaemia despite the presence of iron. Other cell lines in the bone marrow are usually not affected (51).

1.5.2.4 Hypoglycaemia

Hypoglycaemia most severely affects children and is believed to be multifactorial. The increase in metabolic demands of the body, consumption of glucose by parasitized RBC which is 35–70 times more than uninfected RBC (during infection, the parasite downregulates glucose consumption in uninfected RBC) (59). In addition, compromised hepatic gluconeogenesis and glycogenolysis contributes to the hypoglycaemia. Other literature suggests fasting plays a role in hypoglycaemia for severely ill patients (60).

1.6 Diagnosis of *P. falciparum* malaria in Africa

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 (48). 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 (48). 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 (48,61). 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 (62). This is expressed mathematically as:

$$(\text{parasites counted/number of WBC counted}) \times 8000 = \text{p}/\mu\text{L blood}$$

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 (63).

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 determine 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 (62). 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 (64).

1.6.2 Rapid Diagnostic Tests

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 (65). mRDTs are based on immuno-chromatographic detection of parasite antigens from peripheral blood of a febrile individual (64). 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 (48,62). Moreover, mRDT can be a valuable source of parasite DNA for molecular analysis (66).

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 (67). The limit of detection for mRDT depending on the antigen detected is estimated to be comparable to microscopy between 50-200 p/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 (68,69).

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 (70,71). 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 (72,73). 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 (74,75).

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 (73). 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:

1. Boil and spin method. This requires only a heat block and centrifuge machine, making it cheap, with simple and fast sample preparation methods. It is best for small volumes of blood. The DNA extracted is prone to amplification failure due to inhibitors, and is not stable for long term storage.
2. Chelex-100 beads-based method. Compared to boil and spin method, chelex based extraction produces improved quality of DNA from small blood volumes and short DNA fragments even from DBS. It is relatively cheaper but labour-intensive, and the extracted DNA cannot be stored long.
3. Colum-based extraction method. Compared to chelex, and boil and spin method, this produces highest quality DNA that is suitable for long term storage. It is however costly and labour-intensive.

The nucleic acid amplification-based tests used in this thesis were:

1. Polymerase chain reaction (PCR)
2. Loop mediated isothermal amplification (LAMP)
3. Whole genome sequencing (WGS)

1.6.3.1 PCR

PCR is the most commonly used nucleic acid amplification method, discovered in 1983 by Kary Mullis, who was awarded a Nobel prize in chemistry for this discovery. It is very sensitive with a detection limit between 0.002–30 p/μL depending on the assay used and the targeted genes during PCR (76–81). The method used for analysis in this thesis was nested PCR methods targeting *18S ribosomal(r)RNA* gene was developed by Snounou et al (82), and since then, several other methods of parasite detection have been published including probe based real-time quantitative PCRs (qPCR) developed by Kamau et al (77) and Rougemont et al (83), and nested PCR method for mitochondrial DNA targeting cytochrome b (*Cyt b*) published by Steenkeste et al (84). With the right set of species specific primers, PCR can detect all species of human malaria, enabling identification of mixed infections during antimalarial therapeutic efficacy studies, community screening studies to establish prevalence of different species in the community (85), and in vaccine studies (86). The robustness of PCR methods to use extracted DNA from DBS that are field friendly and do not need cold chain for storage, makes it possible to conduct analysis on large samples transported from high transmission areas.

The general working principle of PCR involves cyclic amplification of a specific targeted gene in a chain reaction fashion making millions of copies of the targeted DNA fragment that can be detected and quantified. For a PCR

reaction to occur, five core ingredients are needed, (i) parasite DNA with targeted sites (template to be copied); (ii) primers to initiate PCR reaction by flanking the targeted gene on both sides (forward and reverse primers); (iii) DNA bases (A,T,G and C) in order to build the new DNA strands; (iv) Enzyme to add in the DNA bases in the new strands during amplification (*Taq*-polymerase); (v) buffer solution to maintain the optimal pH for the reaction. With these ingredients, three main steps occur repetitively (20–40 cycles) at different temperatures enabling the exponential multiplication of the gene of interest:

1. **Denaturation:** DNA double helix is unwound and separated to become single stranded DNA by rapid heating of 94–95°C.
2. **Annealing:** The temperature for this reaction is cooled to 55–65°C to allow the forward and reverse primers to bind the DNA single strand (specific temperature is depended on the types of primers and their melting temperature).The *Taq*-polymerase then attaches to the primer-template DNA double strand, and adds complementary DNA bases making a copy of the template.
3. **Extension:** For the *Taq*-polymerase enzyme to work, temperature is raised to around 72°C, and the DNA template is extended, producing a duplicate of the original DNA double strand. The temperature is then increased for denaturation, and the cycle repeats.

If qPCR was used, computed generated quantification will be available in form of cycles of quantification (CQ), that is used to determine parasite density. Another method for detection of successful DNA amplification, is by gel electrophoresis or capillary electrophoresis. In gel electrophoresis, an agarose gel is prepared, stained with a dye to facilitate visualization of DNA (e.g. GelRed or SYBR Green), then immersed in an electrophoresis chamber filled with buffer. Then the PCR products are loaded on the gel wells together with ladder (a molecular weight standard for reference). When electric current is applied, the DNA products which are negatively charged migrate to positive electrode, lower molecular weight products travel further toward the positive electrode compared to heavier fragments. The distance travelled is visualised under ultraviolet (UV) light, and the size of target DNA fragments is compared with the reference standard (ladder).

1.6.3.2 LAMP

Notomi et al first published the LAMP technique in 2000 (87), demonstrating amplification of low-density DNA copies under isothermal conditions without the need of cycling between denaturation, annealing and extension. In 2006,

Poon et al described this method which could amplify up to 10^9 of DNA copies in under one hour (88).

This method uses three primer pairs to target four different DNA sequences in the genome of *P. falciparum*, providing high specificity. The *Bacillus stea-thermophilus* enzyme (*Bst*-DNA polymerase) used for this method, allows isothermal amplification occurring at 65°C, eliminating the need for expensive thermocyclers (87). The *Bst*-DNA polymerase is not prone to inhibition products that are common in simple boil and spin extraction, making it a field friendly molecular tool. The method used for DNA synthesis in LAMP is auto-cycling strand-displacement, which makes amplification highly efficient and very specific. In fact, because of this high efficiency, there is a high risk of contamination, making it of utmost importance to have a workflow where the tubes of amplified products are never opened (89–91). LAMP has been assessed for detection in studies that compared it with microscopy and with PCR, also in studies evaluating detection of low-density asymptomatic *P. falciparum* infection in the field using 18S ribosomal RNA and mitochondrial DNA as gene targets (88,89,92–94). LAMP has a detection limit of 2–5 p/μL, below which reproducibility is a challenge, but and has shown to have comparable sensitivity and specificity to PCR for detection of low density parasitaemia (95–97).

The Loopamp™ Malaria Pan/Pf Detection Kit (Eiken, Japan) has a set of tubes with reagent mixtures that are ready to use. They are vacuum-dries and stable at temperatures <30°C. This kit targets mitochondrial DNA. Detection of products of amplification in positive samples is visually under UV light, through fluorescence from quenched calcein that is in the reagent mixtures, or through a turbidimeter that measures the magnesium pyrophosphate precipitates (93,98).

1.6.3.2.1 Utility of LAMP

The cost per test for LAMP is comparable to cost for microscopy (<1\$) (99,100). This makes LAMP a feasible option as point of care in low endemic regions working toward elimination where there are asymptomatic parasitaemia and low densities that are missed by microscopy and mRDT (Figure 2) (101,102). Especially with the number of studies that demonstrate sensitivity of lamp being comparable to PCR in field settings. However, the utility of molecular tools such as LAMP in therapeutic efficacy studies to assess treatment outcomes needs to be evaluated. Limitations of LAMP include inability to provide parasite quantification without the use of special turbidimeter, and the current tool is unable to distinguish detection species other than *P. falciparum* and *P. vivax*, but can detect all species (103).

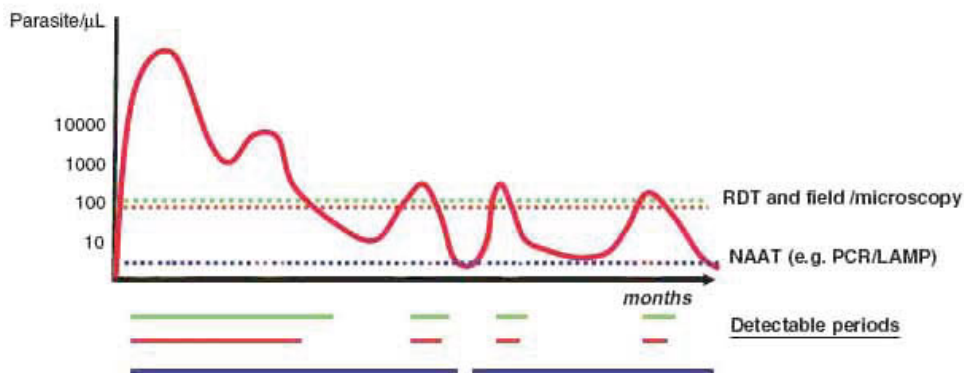


Figure 2: Diagnostic tools and their limits of parasite detection (The figure is adopted from a WHO power point document).

Other novel nucleic acid amplification-based tests such as the Tandem Oligonucleotide Repeat Cascade Amplification (TORCA) are under evaluation as more sensitive and field friendly tools for molecular detection of parasites (104).

1.6.3.3 Whole genome sequencing (WGS)

The improved DNA sequencing technologies, reduced cost and increased efficiency, has allowed scientist to sequence the entire genome of *P. falciparum* (around 5300 genes spread over 14 chromosomes) instead of amplifying only specific regions in genes of interest from the parasite genome as it is in conventional PCR (105,106). WGS avails scientists a unique opportunity to: evaluate and associate genomic changes such as single nucleotide polymorphisms (SNPs) with changes in parasites susceptibility to drugs, assess genomic relationship of parasite populations from various endemic areas, evaluate level of COI in parasite population genetic analysis within endemic regions (105).

However, clinical samples for WGS of parasite population are usually contaminated with human DNA, and this requires enriching of parasite genomic DNA before sequencing. Various techniques such as leucocyte depletion of clinical samples and selective whole genome amplification (sWGA) are among the low labour intensive and low-cost methods that can be used for enriching parasite DNA (107). Other techniques like short term *ex-vivo* cultures, RNA baits hybrid selection and single cell sequencing are expensive and relatively more labour intensive (108).

Enrichment of clinical samples (whole blood or DBS) with sWGA, requires use of primers that target nucleotide sequence motifs which are more common in parasite genomic DNA, but are rare in host genomic DNA (109). These primers are designed through a process that optimises their melting temperatures, binding frequency (for both human and target parasite genomic DNA), and dimerization ability (11,107,110). The designed primers are filtered

through algorithms that select most potent primer sets that will efficiently amplify parasite genomic DNA preferentially from a complex mixture with host DNA (110). The primers use *phi29* polymerase enzyme which is capable of generating large fragments and is ideal for multiple displacement of DNA strands during PCR (107). Prior to sequencing, the enriched parasite genomic DNA is prepared through creating sequencing libraries that have reference indices, sequencing primer binding sites and sequences complementary to oligonucleotides in the sequencing machine flow cell.

Currently there are several sequencing technologies since the foundation of sequencing was laid by Frederic Sanger who received a Nobel prize in chemistry in 1980 for the work (111). Each technology with varying advantages and limitations to consider including costs of running and equipment, length of sequence reads, throughput and mobility. The sequencing technology used in this thesis was Illumina sequencing by synthesis (112).

When the sequencing is complete, the sequencing reads from the sample libraries are compiled and compared with parasite genome sequences from publicly available databases using specialised software in order to identify variations, usually in the core (non-hypervariable) nuclear genome (113). The generated genomic data showing SNPs, insertions and deletions (indels) allow for identification of genetic diversity and differentiation also determination of ancestry when compared with existing isolated from around the globe (114). This genomic epidemiological information is vital when evaluating spatial malaria transmission and mapping. It complements traditional epidemiology especially in areas where malaria elimination is targeted or resistance is monitored (115,116).

1.7 *P. falciparum* malaria treatment

According to WHO, the severity of disease determines the focus during malaria case management (117). 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 (117).

There are different families of antimalarial drugs used for clearing parasites, that can be broadly categorised according to their targets of parasite stages in the human part of the life cycle. (i) Tissue schizonticides, which target the liver-stage parasites, acting as prophylaxis in preventing development in the liver or killing the dormant stages (hypnozoites) from *P. ovale* and *P. vivax*; (ii) Blood schizonticides, which target RBC trophozoites and schizonts

to reduce the parasite biomass that cause clinical malaria. Blood schizonticides are given for a duration long enough to cover more than two erythrocytic cycles of 48 hours are considered efficacious enough to eradicate the infection; (iii) Gametocides that target development of gametocytes, the sexual forms of the parasite in blood responsible for transmission to mosquitoes. It is common for parasite to be susceptible to one drug at multiple stages (118).

Based on structural similarities, antimalarial drugs can be categorised into (i) sesquiterpene lactones e.g. artemisinins that target all parasite stages; (ii) quinolines e.g. lumefantrine, amodiaquine, mefloquine, halofantrine, and chloroquine that are mainly blood schizonticides; quinine that also has some effect on stage III gametocyte, and primaquine that has both gametocidal and tissue schizonticidal effect; (iii) antifolates e.g. sulfadoxine and pyrimethamine that have both blood and liver schizonticidal effect (119) (Figure 3).

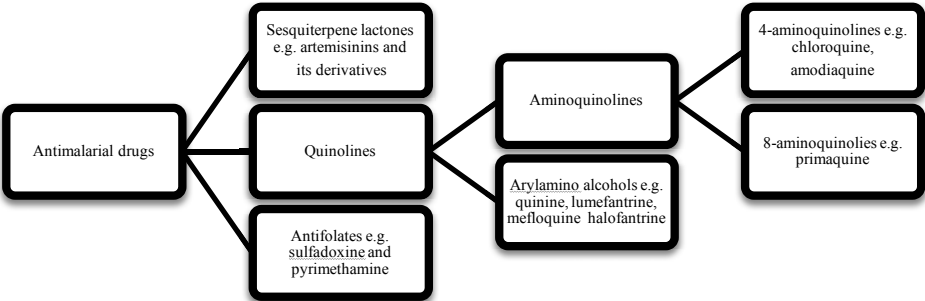


Figure 3: Antimalarial drug families

Depending on elimination half-life of the antimalarial drug and susceptibility of parasites, an efficacious treatment can provide protection known as post-treatment prophylaxis. This is the period whereby the residual drug in blood is in adequate concentration to prevent establishment of new infection. In areas where malaria endemicity is high, post treatment prophylaxis is critical to protect children against multiple infections during a transmission season, and pregnant women from developing clinical infection (55). However, this comes with a risk of resistance development, when the parasites are exposed to sub-therapeutic drug concentrations (120).

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 (119). This resistance development has led to some antimalarials like chloroquine 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 malaria

in Africa (48). With the recent outbreak of corona virus disease (COVID-19), chloroquine and hydroxychloroquine is being explored as a viable option for treatment of COVID-19 (121,122), 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 (48). With artemisinin as a backbone, drugs such as lumefantrine, amodiaquine, piperazine and mefloquine can be used efficaciously even in areas where they cannot achieve required cure rate as monotherapy (123).

1.8 Role of artemisinin-based combination therapy in *P. falciparum* malaria case management

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 (124). The discovery of artemisinin as the active ingredient in 1972 revolutionised malaria case management as an alternative to the already failing quinolines (125), and triggered increased interest in research around artemisinin derivatives and structurally similar drugs for use beyond malaria treatment (i.e. schistosomiasis, toxoplasmosis and cancer) (126–128). 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 world at varying formulations and dosing, until the WHO banned artemisinin monotherapy use in 2007 (129). 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 (1,128). 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 (128).

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 (130), 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 (131). 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 ac-

tivity and 2) Inhibition of proteasome activity (parasite's cellular repair mechanisms) leading to accumulation of damaged/unfolded proteins and stress-induced death (132–136).

The safety profile of artemisinin and its derivatives in humans is remarkable and the drug is well tolerated (123,137). Some animal studies show concerning evidence on neurotoxicity, foetal abnormalities and death in early pregnancy, but in human pregnancies there is no demonstrable impact (138–141). However, the WHO does not recommend the use of artemisinins in first trimester pregnancy (117). 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 (142–147). This is also in light of excellent safety profiles over decades of use of artemisinin derivatives to treat millions of patients around endemic countries (137). 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) (48). 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 (48).

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 (131,148).

1.8.2 Artemisinin-based combination therapies

Since WHO recommended ACTs in the guidelines of treatment of uncomplicated malaria, multiple studies have been done to assess the clinical efficacy of the combinations. ACTs that have been recommended by WHO for general use are fixed dose combination that include 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, artemisinin-piperaquine 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 (48).

For the commonly used ACT in Africa, artemether-lumefantrine and artesunate-amodiaquine, the efficacy is excellent and the safety profile is acceptable (149–155). Artemether-lumefantrine is the ACT used in this project.

1.8.3 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 (156).

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 (157).

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 (157,158). 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 (159). Both artemether and lumefantrine are metabolized by cytochrome P450 3A4 (CYP3A4) (159,160).

Artemether is more potent in the reduction of parasite biomass while lumefantrine ensures parasitological cure and prevents recrudescence (131). 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 (48). 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% (149–151,155,161–166). The current dose regimen is an extension of previously shorter two days regimen with four in doses that was associated with treatment failure (167).

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. Studies have shown that artemether-lumefantrine to be cardiac safe irrespective of structural similarities between lumefantrine and halofantrine that's known for prolongation of QTc interval (168). One recent study shows with only marginal prolongation in QTc interval that is clinically tolerable (169). It is not related to neurological or auditory deficits as demonstrated by many African studies, and it is not associated with any severe adverse effects during repeated

administration. Most importantly artemether-lumefantrine can be used safely in treatment of uncomplicated malaria for pregnant women in second and third trimester (137).

1.8.4 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 children (117). Standard dosage is 4 mg/Kg/day for artesunate and 10 mg/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 (170,171).

Amodiaquine is a quinoline belonging to the same group with chloroquine, 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 (172).

1.8.5 Cardiotoxicity concerns of quinolines

Quinolines have been correlated with cardiovascular effects such as prolongation of the time for depolarization and repolarization of ventricular heart muscles by influencing the flow of sodium, calcium and potassium ions in and out of the myocytes (173). The mechanism of prolongation is through blocking of the of the hERG potassium channel (named after the coding gene *human-ether-a-go-go-related gene*), leading to a delay in ventricular repolarization (174,175). This prolongation is an indicator of life-threatening cardiac events like Torsade de Pointes (TdP) which on rare occasions causes ventricular fibrillations and sudden cardiac death (176). Halofantrine, that was previously considered safe, it was banned in 1993 because reports of drug associated sudden cardiac deaths in both children and adults (177–179). Quinine is also known to have this prolongation effect but it is still recommended as treatment of severe malaria as second line after artesunate since its cardiotoxic effects are tolerable compared to its therapeutic effects (48).

1.8.5.1 Measuring electrocardiographic safety of antimalarial drugs

In an electrocardiogram (ECG) the QT interval is examined for prolongation when assessing for cardio-safety of antimalarials, since a prolonged QT interval correlates with the depolarization and repolarization of ventricular myocytes. The QT interval is also correlated with heart rate; it shortens in higher heart rate, and prolongs with slower heart rate. Hence the QT interval interpreted is corrected for heart rate using mathematical formulae to get a corrected QT interval (QTc). Several formulae exist for calculation of the QTc interval each with its limitation and advantages such as i.e. Bazett's (QTcB) (180), Fridericia's (QTcF) (181) and Framingham's (182).

The International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use in their 2014 guidelines on "The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs", recommends Fridericia's formula to be used for most QT studies (183). The reason being Bazett's formula is inferior when it comes to correcting for differences in heart rate among and within patients since it overcorrects the QT interval when the heart rate is high, and under-corrects during slower heart rates; this may overestimate the number of patients with dangerously prolonged QT intervals (183–185). In 2017 the WHO published a review on "Cardiotoxicity of antimalarials" where it was noted that, from a pooled data analysis Bazett's formula is performs well as a correction factor for sensitivity analysis in baseline QTc interval of patients with malaria, and Fridericia's formula performed well in healthy subjects (186). Overall QT interval values should be corrected by both Bazett's and Fridericia's and both analysis results be reported. Normal QTc intervals for ECG in males is between 360–440 ms and 370–460 ms in females. Cut-off values of >500 ms for QTc prolongation or >60 ms for change in QTc (Δ QTc) interval values from baseline are used to categorise supra-thresholds QTc intervals of clinical concern.

Recently an extensive systematic review and meta-analysis of factors affecting QT interval in malaria patients and healthy individuals was published, involving more than 10,452 individuals (93.6% had microscopy confirmed *P. falciparum* or *P. vivax* infection). It provided compelling evidence of the contribution of malaria disease severity, changes in heart rate and body temperature in affecting the QT interval. This brought to light the importance of taking into account disease process and other factors like age and sex when evaluating the effects of the antimalarial drugs (quinolines) or other important medications. By doing so, it may be possible to avoid unnecessary withdrawal of potent antimalarial drugs currently used in malaria case management, or unnecessary discontinuation of antimalarial drug development because of excessive attribution the QT prolonging effects of the drugs (187).

1.8.5.2 Artemether-lumefantrine and the QTc-interval

Lumefantrine is chemically and structurally similar to halofantrine, and because the historical cardiotoxic effects of halofantrine, studies of artemether-lumefantrine have often included ECG evaluations (188,189). A randomized double-blind cross over study in healthy males from 2002 compared the effects on the QTc-interval between artemether-lumefantrine and halofantrine. There was no significant prolongation of the QTc-interval in the artemether-lumefantrine group (190). Similar conclusion can be found in other studies that assessed safety and tolerability of artemether-lumefantrine (168,191). The United States Food and Drugs Administration's Centre for Drug Evaluation and research found a significant positive relationship between lumefantrine concentration and QTcF-time when tested in healthy adults. The participants were given a standard 6-dose regimen of artemether-lumefantrine over 3 days. A QTcF prolongation of 7.29 ms was seen 72 hours from start of treatment. However, there were no clinically significant effects identified (192). A study from 2000 compared the cardiac effects of treatment with artemether-lumefantrine given alone or in combination with mefloquine for standard therapy lengths. No correlation between QTc interval and the plasma drug concentration could be seen (193).

1.8.6 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) (194). 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 (195–197).

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 (197). When combined with ACT, primaquine rapidly shortens gametocyte carriage duration (198,199). Primaquine clears mature gametocytes rapidly, but unlike artemisinin derivatives it does not prevent gametocyte development (198) 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 (200). 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) (200).

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 (200).

1.8.7 *P. falciparum* resistance to antimalarial drug

Classical definition of antimalarial drug resistance according to WHO 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 (48).

Occurrence of parasite resistance to antimalarial drugs is a result of natural selection, where the drug exerts 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 (119,201–205). 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 re-infecting during the post-treatment prophylaxis until clinical treatment failure becomes apparent (120,205,206).

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 (125,207). 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 (208–210). The WHO recommends a combination of *in vitro* experiments, genotyping studies for molecular markers of

resistance and *in vivo* therapeutic efficacy studies as complementing tools for drug resistance surveillance.

1.8.8 *In vitro* tests

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 (211). *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 (212). 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 limit the utility of *in vitro* tests. The results of *in vitro* tests lack influence of immunity and inter-personal pharmacokinetic variabilities that are representative of the real population and depend on animal models for simulation (212). This makes it hard to interpret objectively any correlations observed between *in vivo* and *in vitro* results.

1.8.9 Molecular markers of antimalarial drug resistance

Among established molecular markers of resistance include SNPs in genes such as the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*), *P. falciparum* multi-resistance protein 1 (*pfmrp1*) and sarco/endoplasmic reticulum Ca^{2+} -ATPase orthologue of *P. falciparum* (*pfATP6*), which are also linked to artemisinin resistance, whereas other SNPs in the *pfmdr1*, *pfmrp1*, *P. falciparum* chloroquine resistance transporter gene (*pfcr*), and *P. falciparum* Na^+/H^+ exchanger-1 (*pfnh1*) are linked with resistance to quinolines like lumefantrine, amodiaquine, mefloquine, chloroquine and quinine (120,202,203,213–218). Genetic resistance markers in for example the *P. falciparum* *kelch13* (*pfk13*) gene, is currently playing an important role in artemisinin resistance surveillance. Having these markers is important especially for malaria endemic areas like sub-Saharan Africa, where they are useful as early warning sign of an imminent resistance. A limitation of these molecular markers is that they do not fully determine treatment outcome. The molecular markers observed in parasite population from one setting such as South East Asia, and are associated with resistance, does not necessarily mean that those same parasites when they move to Africa, will be fit enough to confer resistance. This is because parasites genetic background that supports survival of a genotype that is related to resistance in one setting, may be lacking in a setting that the resistant parasite may spread to. This lack of supportive genetic environment leads to high fitness cost for the resistant genotype, and it may fail to establish

(219). This cements the importance of monitoring molecular markers of resistance that may arise locally, independent of transmission.

1.8.10 *In vivo P. falciparum* antimalarial therapeutic efficacy studies

In vivo studies are WHO standardized clinical trials for antimalarial drug efficacy. These therapeutic efficacy studies (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 (220). 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 (220). 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:

1. Early treatment failure (ETF)

- 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 $\geq 37.5^{\circ}\text{C}$; and
- Parasitaemia on day 3 $\geq 25\%$ of count on day 0.

2. Late clinical failure (LCF)

- Danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure; and
- Presence of parasitaemia on any day between day 4 and day 28 (day 42) with axillary temperature ≥ 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure.

3. Late parasitological failure (LPF)

- Presence of parasitaemia on any day between day 7 and day 28 (day 42) with axillary temperature <37.5 °C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

4. Adequate clinical and parasitological response (ACPR)

- Absence of parasitaemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

To account for potential lost to follow-up, it is recommended to add 20% of the sample size calculated by classical statistical methods. In case of parasitological failure during follow-up, rescue treatment is currently recommended for all patients with parasites irrespective of level of endemicity. This is from evidence that 50–60% of patients with asymptomatic parasitaemia during follow-up develop clinical symptoms within 28 days (221).

In holoendemic or hyperendemic regions, recurrent parasitaemia is common during the 28 days or 42 days of follow-up, especially during transmission season where EIR is high. It is important to distinguish between new infection and parasites that were exposed to the drug but survived i.e. (reinfection vs recrudescence). Microscopy is unable to make this distinction; hence molecular genotyping is used based on polymerase chain reactions (PCR) methods (222). Genotyping to identify parasite population is now used as a vital tool in TES in order to get treatment outcomes that are adjusted by PCR. The recommended primary endpoint for TES in Africa is PCR adjusted cure rate.

1.8.10.1 Limitations of *in vivo* studies

These *in vivo* studies have limitations that may influence the amount of drug that the parasites are actually exposed to, such as interpersonal variability on how the drug is absorbed, metabolised and eliminated (diarrhoea/vomiting), patients' poor adherence to study protocols, wrong dose, counterfeit drugs,

and concomitant medication that may have drug interactions. High baseline parasitaemia can also influence the treatment outcome, presenting as delayed parasite clearance (223).

In areas with stable malaria transmission, influence of premunition on parasite clearance needs to be taken into account, because it varies depending on the age of recruited participants. Children with low immunity and gastrointestinal pathologies that may limit amount of drug absorbed will present with therapeutic failure. In addition, the treatment failure maybe be overestimated as the transmission decreases and patients lose their acquired immunity, or impending resistance could be missed in patients with strong premunition that clears remaining drug resistant parasites. All this may cause the observed treatment failure will not represent “true resistance” (223).

The clinical trials are resource intensive with estimated annual budget of 50,000\$ to 60,000\$ and in most endemic countries there is dependence to donor funds, which can affect the capacity to implement TES correctly (220). The need to ensure adherence to study protocol calls for training and regular supervision. Remoteness of study site can affect quality of supervision and subsequent data collected.

1.8.11 PCR genotyping to distinguish recrudescence from reinfections in antimalarial drug trials

The WHO and Medicine form Malaria Venture recommends use of merozoite surface proteins 1 and 2 (*msh-1* and *msh-2*) and glutamate-rich protein (*glurp*) as molecular markers for primary endpoint analysis during TES. The following properties makes 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 (75). They have been extensively used in many studies and gave useful results, this allow for comparison during interpretation of data (222).

The recommended samples for molecular genotyping are day 0 samples that were collected before start of antimalaria 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 (222).

The genotyping should be done sequentially, starting with either *msh2* or *glurp* as they have highest discrimination power, then the last maker should be *msh1* (Figure 4) (222).

Example for one marker gene:

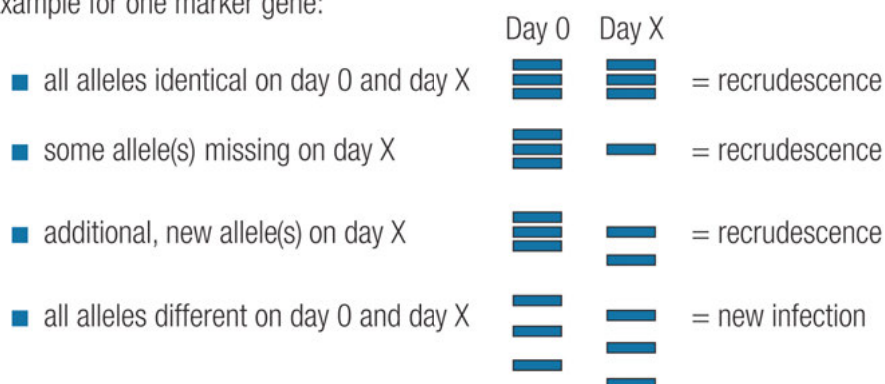


Figure 4. Illustration of different electrophoretic patterns on day of enrolment (Day 0) and day of parasite recurrence (Day X) during follow up in one genetic marker (222).

1.8.11.1 Limitation in *msp1*, *msp2* and *glurp* PCR genotyping

There are several limitation of PCR genotyping including:

1. Failing to amplify the minority clones which may be at low density in polyclonal infections (<10–20% of the parasite population) and subsequent increased amplification of abundant DNA especially with nested PCR. This is common in high or moderate transmission regions where the level of COI is high, corresponding to level of transmission (224–226);
2. Detecting gametocytes instead of asexual parasites since this genotyping method is not specific to parasite stage;
3. Failing to adequately discriminate new parasites from pre-treatment parasite in the case where they have same genotype, leading to classifying reinfection as recrudescence. This is common in areas with low COI such as low transmission areas where parasites genetic diversity is limited. To the contrary, in high transmission areas, reinfections tend to be classified as recrudescence due to high COI (227,228);
4. Dependence of product size polymorphism rather than sequence polymorphism is subject to intra-allelic family template competition during PCR e.g. due to the large product size of the *glurp* PCR, detecting size differences on an agarose gel proves difficult, and only slight variation in the migration distance of the product will result in an apparent size difference of above 20bp, thus indicating a reinfection (229,230). Acknowledging this challenge, the WHO recommends that the bands on agarose gel to be interpreted digitally

using software for image analysis or whenever digital image analysis is not available, paired samples run side by side on same gel should be analysed by two independent readers (222).

5. Parasite sequestration in deep tissues removes some parasite clones from peripheral circulation leading to missed detection of these genotypes. If they are resistant genotypes that recrudescence later, they will be classified as new infection rather than treatment failure (231).

Using capillary electrophoresis instead of agarose gels may improve the performance of the genotyping especially in areas of high transmission (232). Use of high-resolution melt assays (233), heteroduplex tracking assays (234) or microsatellites (235) are also alternative promising methods reducing the limitations identified here.

1.8.12 *P. falciparum* resistance to ACT

1.8.12.1 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 (236). 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 (237).

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 (215). The PfKelch13 is believed to be important in the regulation of protein quality control (119). 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 validated marker, 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, M476I, Y493H, R539T, I543T and C580Y. Other candidate markers are P441L, G538V, G449A, V568G, C469F, P574L, A481V, F673I, P527H, A675V and N537I (237,238).

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:

- $\geq 5\%$ of patients harbour parasite with *pfk13* resistance-associated mutations; or
- $\geq 10\%$ of patients with persistent parasitaemia by microscopy on day 3 after treatment with ACT or artesunate monotherapy; or
- $\geq 10\%$ 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 $\geq 5\%$ 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 (239).

1.8.12.1.1 Limitation of the definition of artemisinin resistance

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 (day 3 \neq 72 hours).

1.8.12.1.2 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 (240).

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 (241–243). However, in Rwanda, the presence of these mutations was not associated with treatment failure of artemether-lumefantrine, which is the of first-line treatment (1).

1.8.12.2 Partner drug lumefantrine resistance

Lumefantrine tolerance/resistance has been linked to SNPs in *pfmdr1* at positions N86Y, Y184F and D1246Y, and in *pfcr1* at position K76T and *pfmrp1* at positions *I876V* SNP (244,245). Interestingly, lumefantrine selects for *pfmdr1* N86, 184F, D1246 and *pfcr1* K76, the chloroquine sensitive genotypes. Another genetic alteration previously linked to lumefantrine resistance in South-east Asia is increased *pfmdr1* copy numbers (246). Importantly, to date no clear evidence of *pfmdr1* copy number variation has been observed in East-Africa.

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 (120).

1.8.12.3 Changing ACT treatment policy for *P. falciparum* resistance

Results of TES provide evidence that is used to guide change of treatment policy for ACT. The WHO recommends that the national malaria control programs in malaria endemic countries, conduct TES every two years to assess clinical and parasitological outcomes to ensure that the ACT recommended are still efficacious, and identify any changes. There are two important outcomes that are considered in order to change treatment policy:

- Proportion of patients with microscopy confirmed parasitaemia on day 3. If $\geq 10\%$ of the patients are parasitaemic on day 3, it is considered an alert that there is suspected artemisinin resistance. However, a change in treatment policy is not yet warranted. It depends on partner drug efficacy.
- Proportion of patients with treatment failure by day 28 or 42. If $\geq 10\%$ of patients have PCR adjusted recrudescence; it indicates that the partner drug is failing. However, before changing the ACT, WHO recommends provision of extra data to support interpretation of the results and remove doubts of potential misclassification of new infection as recrudescence. (i) Mean multiplicity of infection determined from at least 50 randomly chosen admission samples (day 0) for the respective site with the most discriminatory marker. This stands as a surrogate measure of transmission intensity. (ii) Allelic frequency of the dominant genotype serving as indicator for those with “true” new infections that were missed because they had the same genotype as the paired baseline sample. (iii) Presence of gametocytes on the day of failure (222,237).

If both day 3 microscopy positivity and treatment failure are $\geq 10\%$, that indicates failure of both artemisinin and partner drug, and a change of ACT has to be made or a non-ACT alternative should be considered (237).

1.8.13 Malaria case management - experiences from Tanzania

The introduction of ACT in Tanzania was secondary to *P. falciparum* drug resistance that developed to chloroquine and led to change of regimen in 2001 to sulfadoxine-pyrimethamine as an interim solution, which lasted only five years because of resistance (247). 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 (125).

1.8.13.1 Experiences with artemether-lumefantrine since 2006 in Tanzania

Since the roll out of artemether-lumefantrine from 2006 in mainland Tanzania, as first-line treatment of uncomplicated malaria, it has remained highly efficacious to date, with PCR corrected cure rate between 95.1% and 100% (153,155,248–250). Over the years, administration of artemether-lumefantrine has been safe in the Tanzanian 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 (131,153,155,250–252). Experience has shown that equipping community health worker with mRDT and artemether-lumefantrine can improve community level targeted malaria treatment with effective unsupervised home management of malaria (248,253). However, there are challenges with regards to prescribing practices and diagnosis, such as prescribing antimalarial drugs to patients with negative test results, insufficient adherence to use of artemether-lumefantrine, and mismatch in skills between microscopist in private and public health care centres (254–257).

This contribute to inappropriate malaria case management and may increase the risk of resistance to artemether-lumefantrine. Generic artemether-lumefantrine drugs present in private and public pharmacies in Tanzania, seem to have comparable efficacy to WHO pre-qualified innovator products, despite having higher odds of containing lower than acceptable active product ingredients for both artemisinin and the partner drug (258–260). The government's subsidizing of the artemether-lumefantrine cost, has made it more available to patients who are unable/unwilling to pay for the high costs unsubsidised drugs (261).

Population pharmacokinetics of Tanzanian children with uncomplicated malaria and their relationship to parasite clearance and treatment outcome have demonstrated comparable kinetics with the adult population. Similar to other models, in this population, the artemether component is responsible for

the initial rapid clearance of parasite biomass, and the lumefantrine component clears the remaining parasites (262). 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 Tanzania demonstrated that normal African diet has adequate fat content for sufficient absorption of artemether-lumefantrine for efficacious treatment outcome (262,263). In cases of recurrent parasitaemia after treatment, re-treatment with artemether-lumefantrine seem to be well tolerated and with excellent efficacy (161).

1.8.13.2 Status of artemether-lumefantrine resistance in Tanzania

The progress made over the past 10-15 years in malaria control in Tanzania in case management and reduction of transmission where national prevalence was reduced from 18.1% in 2008 to 7.3% in 2017 can be partly attributed to availability of efficacious artemether-lumefantrine among other interventions including vector control (264,265). This means that the malaria parasites have survived 10 years of ACT exposure, and may thus be particularly resistance prone. There are several unsettling observations such as (i) increased selection of parasite genotypes that are resistant to partner drugs and (ii) increased post treatment PCR positivity. These need to be evaluated further to avoid fall back of the progress.

The hypothesis that development of tolerance/resistance against lumefantrine, and other long acting partner drugs in ACT, starts through post-treatment selection among recurrent infections of less sensitive *P. falciparum* parasites can be demonstrated by the observed increased temporal selection of parasites containing markers of resistance to lumefantrine in Bagamoyo district since 2006 (120). The data suggest that re-infecting parasites carrying the *pfmdr1* N86/184F/D1246 haplotype were able to withstand 15-fold higher lumefantrine blood concentrations than those with the alternative haplotype (86Y/Y184/1246Y) (244). This is despite sustained high cure rate with artemether-lumefantrine for over 8 years (266), and may lead to a gradually shortened post-treatment prophylactic period, long before clinical treatment failures are apparent. This is why temporal surveillance of genetic antimalarial drug resistance markers of *P. falciparum* have been proposed as an early warning system of evolution of ACT tolerance/resistance.

Previous studies in Bagamoyo district have been able to demonstrate complex parasite population dynamics in polyclonal infections. More than 20 different clones (using *msp1* and 2) that were not detected before treatment, could be detected 24–48 hours after artemether-lumefantrine treatment, making it important to evaluate the utility of consecutive day sampling for genotyping (267,268). This poses a challenge in accurately interpreting treatment outcomes using currently recommended WHO genotyping guidelines. Moreover, high residual PCR determined positivity rate on day 3 after supervised artemether-lumefantrine treatment has been observed in varying prevalence from 2006 to 2014 in the magnitude of almost 44% as average of over the years,

with a maximum of 76% (269). This is despite the documented rapid microscopy determined parasite clearance by day 3 after treatment with artemether-lumefantrine in Bagamoyo district. Taken together, these parasite population dynamics, and persistent PCR positivity warrants further evaluation as to what their role may be in treatment outcome and continued transmission. In areas of low endemic transmission, sub-microscopic parasitaemia is determined to be responsible for up to 20% of malaria infection post ACT treatment (270). The day 3 sub-microscopic post treatment parasitaemia is also associated with recurrence of detectable parasitaemia on day 28 by microscopy, longer gametocyte carriage and higher transmission potential (271–273).

Further analysis of the persistent post treatment PCR positivity post treatment (which indicates a continued detection of parasites), was done using a deep sequencing approach (274) to characterise the clearance of different *P. falciparum* sub-populations in polyclonal infections. The clearance times by PCR of these sub-populations were similar to artemisinin resistant parasites as assessed by microscopy in Myanmar in absentia of any of the described mutations in the Kelch13 propeller domain associated with artemisinin resistance (275). A recent publication from Tanzania report the presence of a validated marker of artemisinin resistance R561H from Southeast of Tanzania (Kibiti), about four hours' drive from Bagamoyo (276). Also, another 24 *pfk13* non synonymous mutations were reported (in 7% of the samples) including A578S haplotype which has not been associated with artemisinin resistance. This is accompanied by almost 100% selection of chloroquine sensitive *P. falciparum* genotypes (203,276). 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 (277).

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 (243,278–280), developing strategies to protect the therapeutic efficacy of ACTs in Africa cannot be more urgent. Continued search for other markers of resistance is also a feasible endeavour, since the potential for locally arising resistance in Africa is high and reliance on only the existing markers may not be sufficient for monitoring (281).

1.8.13.3 Potential ways forward

WHO recommends several measures against *P. falciparum* drug resistance:

1. Addition of single low dose primaquine (0.25 mg/Kg) irrespective of G6PD status.

The addition of a single low-dose of primaquine to ACT is a recent WHO recommendation to be used without prior G6PD testing. It is considered safe

and not related to risk of serious haemolysis in G6PD deficient individuals, in areas threatened by artemisinin resistance and/or in low transmission areas (117,282). Since the new recommendations have been deployed, the single low-dose of primaquine has most often been administered together with the first ACT dose (283,284).

A study in Bagamoyo district in 2016 by Mwaiswelo et al evaluated safety and efficacy of the addition of a single low-dose primaquine (0.25 mg/Kg) to artemether-lumefantrine administered on the first day of artemether-lumefantrine treatment regardless of G6PD status versus the standard 3-day artemether-lumefantrine therapy. The addition of primaquine to artemether-lumefantrine was safe, and no severe haemolysis occurred in G6PD deficient patients (285) and did not compromise the efficacy of artemether-lumefantrine (286). However, preliminary data suggest that PCR determined gametocyte carriage was only transiently reduced by primaquine. A more recent study also from Bagamoyo district assessed single full-dose (0.75 mg/Kg) on G6PD normal individuals in a three-armed study. One arm given primaquine first day of artemether-lumefantrine seemed to reduce gametocyte carriage more than the arm with artemether-lumefantrine alone, or the arm given with the last dose (287). However, modelling data suggest that day 8 is the optimal day of administering primaquine to maximize the effect on gametocyte carriage/clearance since the initial key component for gametocyte reduction at treatment initiation is in fact associated with the parasite bio-mass reduction of ACT (288). This needs to be further investigated.

2. Extending treatment of ACT and alternating triple ACT

Extending the duration of artemether-lumefantrine treatment for uncomplicated *P. falciparum* malaria has been done before, from two days to three days to improve efficacy (150). Further extension of the dose further by prolonging the duration the same drug or sequential addition of a different ACT (triple ACT) is considered as a viable strategy. This will expose the infecting parasites to additional days of artemether/artesunate and increase the likelihood to eliminate parasites with signs of artemisinin tolerance. This strategy will afford the partner drugs more protection through killing the parasites tolerant to artemisinin (289). Alternating the partner drugs as in triple ACT, exposes the parasite to different drugs, minimising the likelihood of survival of parasite tolerant to all three antimalarial drugs. Extending artemisinin treatment has provided excellent cure rates even in areas with delayed parasite clearance times (290). Even though a majority of patients achieve adequate day 7 lumefantrine concentrations, there are well-defined sub-groups that presently are under-dosed with artemether-lumefantrine, i.e. young, malnourished children and pregnant women (during second and third trimester) (291,292). A recent study in Tanzania examined factors that influence day 7 lumefantrine concentration (a surrogate indicator for determining treatment outcome): they

identified that pregnancy, body weight and having CYP3A5 genotype affects the day 7 lumefantrine concentration and increasing the risk for treatment failure (293). These vulnerable groups with high risk of serious malaria disease and death may therefore particularly benefit from an extension of the proposed artemether-lumefantrine treatment duration. Few studies have been conducted using this approach (188,189) and further analysis should be done to evaluate safety and efficacy of extended treatment and triple ACT.

1.8.14 Malaria transmission dynamics in regions of pre-elimination - Zanzibar case study

The approach to malaria control differs depending on malaria endemicity of the region. The tools and strategies used in a high transmission setting are most useful for case management but tend to be insufficient in pre-elimination setting, where transmission is low and malaria case surveillance is most important (294).

In low transmission/ pre-elimination setting, local malaria transmission tends to be focal with certain areas serving as hotspots, maintaining infections during low and high transmission season (295). Most of the patients with parasitaemia are asymptomatic and harbour very low parasite densities, these can be easily missed by microscopy or mRDT. Also, importation of malaria cases is not uncommon in pre-elimination regions (294,296). Effective surveillance-response systems are critical in such settings to be able to achieve elimination or prevent outbreaks of malaria epidemics that have devastating morbidity and mortality since the population is often lacking acquired immunity for malaria (297).

Zanzibar, a semiautonomous part of The United Republic of Tanzania, has made significant improvement in malaria control since 2003 when malaria elimination strategies started being implemented (294). *P. falciparum* is the predominant species causing malaria in Zanzibar followed by *P. malariae* (298). By 2015, the community prevalence of *P. falciparum* by microscopy or mRDT had dropped by about 96% of the 2003 levels, and the total parasite burden had reduced by 1000 folds (99.9%) (294). This progress is attributed to successful vector control interventions, availability of diagnostic tools (mRDT), efficacious antimalarial drugs and community education which increased community positive reception of interventions (294,299,300). Since 2008 Zanzibar has in place surveillance systems such as reactive case detection, whereby when an index case with laboratory confirmed malaria presents to the health care facility will be treated with ACT and single low-dose primaquine for transmission blocking, then a follow-up visit at home is made to test and treat family members and neighbours (301). Evidence from studies in Zanzibar suggests that treating people within a radius of 300 m from the

mRDT positive individual, increases the coverage to reduce transmission (302).

Zanzibar adopted artesunate-amodiaquine as first line ACT treatment for uncomplicated malaria since 2003 and artemether-lumefantrine as second line and since then it has remained efficacious. Prevalence of *P. falciparum* resistance markers to amodiaquine *pfprt* 76T, *pfmdr1* 86Y, 184Y and 1246Y has been noted to decrease irrespective of its wide scale use since 2003 (303). Despite the underlying reason for this decrease being unclear, it has been argued that fitness cost and/or genetic dilution from imported infection from mainland could be a contributor (303,304). It should be noted that in Tanzania mainland the first line of treatment is artemether-lumefantrine; the long acting partners of the ACTs i.e. lumefantrine and amodiaquine exert opposite trends in selecting for *pfprt* and *pfmdr1* genotypes (305).

Despite the progress made thus far, the malaria prevalence has remained relatively stable at low level transmission since 2008 in Zanzibar (294). The reasons for this could be multifactorial, including missing of low-density parasites in asymptomatic or even febrile carriers (306) and continued importation of malaria from Tanzania mainland (296). More sensitive field friendly molecular diagnostic tools such as LAMP have been evaluated in Zanzibar, showing promising utility when it comes to diagnosing asymptomatic carriers of parasitaemia in community screening or as point-of-care diagnostic tool (89,307). Recently an alternative strategy of pooling samples for detection of *P. falciparum* using PCR during community screening was published. It increases throughput and reduce costs during reactive case detection, providing a clearer picture of community prevalence (72).

Evaluation of the efficacy of interventions such as reactive case detection has also shown to have limitations when it comes to timeliness of response by malaria surveillance officers despite adequate coverage of the intervention; and cases diagnosed in private health facilities are often not reported for follow-up (301). Mass drug administration, has also shown to have no significant impact on transmission in low transmission settings of sub Saharan Africa, bringing to question further the role of local transmission versus imported malaria in pre elimination setting like Zanzibar (308,309). Genomic epidemiology can provide a unique opportunity to deepen our understanding of malaria transmission dynamics in pre elimination setting, especially in the era of imminent resistance to ACT.

2 Rationale and aims of the thesis

2.1 Rationale

The situation in already high burden malaria endemic countries is threatened to get worse by the rapidly spreading resistance to the only drug combination (ACT) that has given us a fighting chance with malaria case management. While awaiting development of alternative, but hopefully equally efficacious antimalarial drugs, it is critical to be proactive and identify and scientifically evaluate new strategies to protect/prolong the therapeutic lifespan of ACT to be able to and provide evidence for policy recommendation. This should go in parallel with examining utility of new molecular tools that can be used for surveillance of resistant parasites. This PhD thesis focused on evaluating the efficacy and safety of extending the current artemether-lumefantrine regimen from standard 3 days to 6 days and adding single low dose primaquine (0.25 mg/Kg) as a new strategy that can feasibly be programmatically rolled out without delay in order to protect the therapeutic lifespan of ACTs. Also, we examined the utility of molecular tools in resistance surveillance and for improving our understanding of how importation may affect malaria elimination efforts in Zanzibar.

In order for the WHO and malaria endemic countries to successfully implement the “The high burden – high impact” strategy, strong recommendation from studies such as this PhD project, provide quality evidence that can influence policy changes towards malaria control are warranted.

3 Aims

- To compare the diagnostic accuracy of the LAMP (Loopamp™ MALARIA kit), and conventional microscopy with PCR as gold standard in parasite detection on day 3 after initiation of artemether-lumefantrine treatment.
- To compare the therapeutic efficacy and safety/tolerability of standard 3-day course versus 6-day course of artemether-lumefantrine in patients with uncomplicated *P. falciparum* malaria during high transmission season. Proportion of PCR positive *P. falciparum* on day 5 and 7 was primary outcome and, PCR adjusted cure rates determined parasite clearance and post treatment prophylaxis as secondary outcomes.
- To evaluate electrocardiographic changes (QTc interval) associated with prolonged artemether-lumefantrine treatment as compared with standard 3-day treatment.
- To examine genetic relationship of *P. falciparum* between higher transmission regions of mainland Tanzania to the lower transmission regions of the Zanzibar archipelago.

4 Materials and methods

4.1 Study sites and population

This thesis includes publications that are a result of one clinical trial and two cross-sectional surveys. The clinical trial was conducted in Bagamoyo district in the Coast Region, mainland Tanzania that provided data for studies I, II & III. The cross-sectional surveys that provided data for study IV are from Bagamoyo district and Zanzibar. All studies were conducted between 2016 and 2018.

4.1.1 Study Sites

4.1.1.1 Mainland Tanzania – Bagamoyo district (study I, II, III and IV)

Bagamoyo district (Figure 5) has an estimated population of about 3.1 million people, most of whom are subsistence farmers and fishermen (310). The district has six divisions, and 22 wards, comprising 97 villages with 67 health facilities, including one hospital, five health centres and 59 dispensaries. Forty-four of the dispensaries belong to government institutions, five to voluntary agencies, and ten are privately run (311). There were two study sites selected, Yombo and Fukayosi primary health care clinics, that serves a total population of about 8,000 and 25,000 people respectively. Both are within easy access to the referral hospital in Bagamoyo town.

Malaria transmission in Bagamoyo is moderate and occurs throughout the year with peaks related to the rainy seasons of March to May and November to December (265). After the introduction of artemether-lumefantrine as first-line treatment for uncomplicated malaria in 2006, together with insecticidal treated bed-net distribution campaigns, malaria morbidity and mortality declined significantly (312). *P. falciparum* is the predominant species responsible for >95% of diagnosed malaria cases and, *Anopheles gambiae* complex is the principal vector.

Participants for the clinical trial (studies I, II and III) were patients aged 1–65 years old. Participants for the cross-sectional studies (study IV) were 3 months and above. All presenting with microscopy confirmed uncomplicated *P. falciparum* malaria at the study sites. The clinical trial was registered at ClinicalTrials.gov (ID: NCT03241901) on July 27, 2017.

4.1.1.2 Study sites in Zanzibar (study IV)

Zanzibar is part of the United Republic of Tanzania, but it is a semi-autonomous region with two main islands; Unguja and Pemba, with populations of 900,000 and more than 400,000 people, respectively (313). Different from Tanzania mainland, Zanzibar adopted the use of ACT since 2003 and the first line is artesunate-amodiaquine. The predominant parasite species for malaria is *P. falciparum*. The outdoor biting *Anopheles arabiensis* is now a dominant vector after extensive use of indoor residual spraying (IRS), and long-lasting insecticide-treated nets (LLINs) between 2005 and 2010 replacing the indoor biting and resting *Anopheles gambiae* that was predominant (4,314). Malaria prevalence in Zanzibar has been <1% during the study period among children under 5 years old as measured by mRDT in household survey (312).

This cross-sectional study involved 14 "satellite sites" from Pemba Island and Unguja Island. All 14 satellite sites were primary health care facilities from three regions, i.e. at Micheweni (Pemba island) and Bububu and Uzini regions (Unguja Island) from April to October 2017. Patients presenting at the study sites were screened with mRDT, and microscopy confirmed uncomplicated *P. falciparum* malaria.

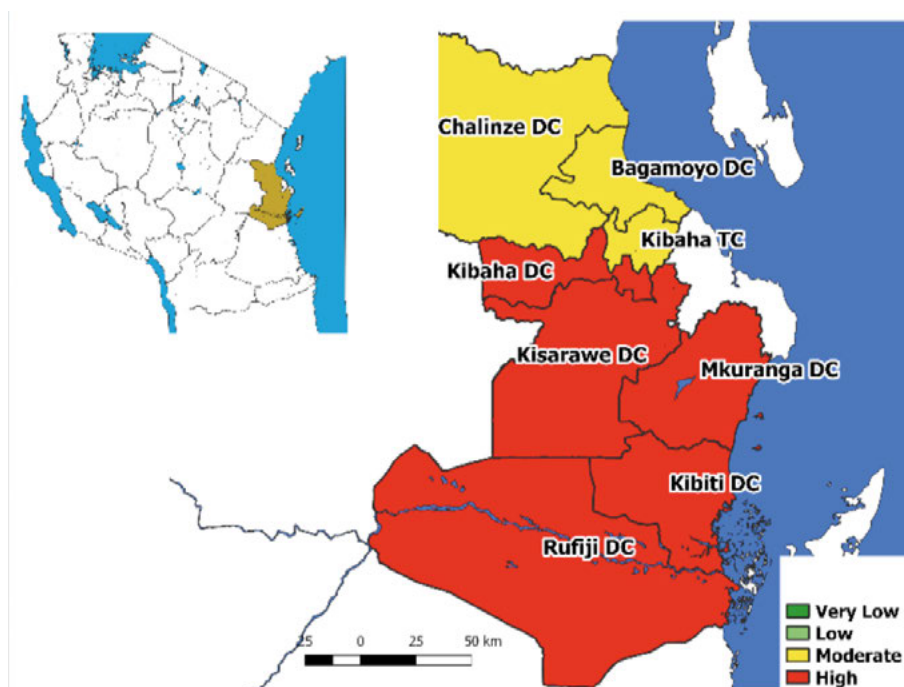


Figure 5. Map of coast region indicating transmission intensity of Bagamoyo district and neighbouring districts (265).

4.1.2 General methodologies

4.1.2.1 Clinical trial (studies I, II and III).

Data for studies I, II and III were a product of one randomised controlled, parallel-group, superiority clinical trial, where patients were randomly allocated to control and intervention treatment arms. Antimalarial drugs used were artemether-lumefantrine and primaquine. Treatments were given as directly observed oral therapy for all doses such that the control arm received (20/120 mg/Kg) twice a day as standard doses for three days and the intervention arm received extended six days treatment. Single low dose primaquine (0.25 mg/Kg) was given only in the extended treatment arm, with the last dose of artemether-lumefantrine. Clinical and laboratory evaluations were done on days 0, 1, 2, 3, 4, 5, 6, 7 then once weekly up to day 42 (Table 1). All adverse events were recorded during the 42 days of follow-up, and their relationship to the study drugs was assessed. Throughout the follow-up period, patients were encouraged to come back whenever they felt sick, even if it was not on their scheduled visit. For study II the primary outcome was evaluated at day 5 and day 7, comparing PCR positivity between the treatment arms.

Table 1. Schedule of follow-up visits for clinical trial

LAMP[†] - Loop-mediated isothermal amplification, *Artemether-lumefantrine (20/120 mg/Kg) was given twice a day for three days in control arm, and for six days in intervention arm. Primaquine** - Single low-dose (0.25mg/Kg) was given only in the intervention arm. (X) – Un-scheduled visits where study participants presented with clinical symptoms.

Procedure	D0	D1	D2	D3	D4	D5	D6	D7	D14	D21	D28	D35	D42	Other day
Inclusion/exclusion	X													
Informed consent	X													
Medical history	X													
Clinical assessment	X	X	X	X	X	X	X	X	X	X	X	X	X	(X)
Electrocardiograph	X					X								
Temperature	X	X	X	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	X	X	(X)
Urinalysis	X							X						
Venous blood for:														
Haemoglobin	X							X						(X)
Lumefantrine concentration	X	X	X	X	X	X	X	X	X	X	X			(X)
LAMP [†]	X			X										
Serum biochemistry	X							X						(X)
PCR filter paper	X	X	X	X	X	X	X	X	X	X	X	X	X	(X)
Treatment														
Artemether-lumefantrine*	X	X	X	X	X	X								
Primaquine**						X								

4.1.2.2 Cross sectional study IV

Clinical isolates that contributed data for this study represent a convenience sample from patients with uncomplicated malaria or asymptomatic infection, screened for enrolment in cross-sectional studies from Tanzania mainland and Zanzibar. The study from Tanzania mainland was an observational cohort study to determine the presence of slow-clearing ("resistant") parasites to artemether-lumefantrine among children with uncomplicated falciparum malaria infections in Bagamoyo district from 2016-2017. The Zanzibar samples were from cross-sectional survey of asymptomatic individuals and from screened patients for an *in vivo* efficacy study of artesunate-amodiaquine with

single low dose primaquine among paediatric population in Pemba and Unguja.

Isolates selected for subsequent analysis had no predetermined clinical or epidemiologic features. However, those with higher parasitaemia presented a higher likelihood of being successfully sequenced. Participants from Zanzibar were asked whether or not they travelled outside the island overnight within the last four months.

4.2 Study specific methodologies

Detailed description of objectives and methodologies of specific studies are described in methods section of respective papers, but briefly:

4.2.1 Electrocardiographic safety study III

For study III, electrocardiographic safety was evaluated by measuring the QT interval at day 5 and compared to the baseline values between the two arms. QT intervals were read both manually using the tangent method and automatically with the ECG machine. Bazett's (QTcB) and Fridericia's (QTcF) formulae were used for correction for heart rate to get the heart rate corrected QT intervals (QTc). Descriptive statistics were used to analyse differences between the arms and patients that had QTc prolongation >500 ms, or change in QTc interval (Δ QTc) >60 ms were reported.

4.2.2 Blood sampling and storage

For studies I, II, III and IV, thick and thin Giemsa-stained blood smears were collected at enrolment and follow up. Field experienced microscopist made the initial parasite count, and was confirmed by expert microscopist. Parasite density was reported as parasites/ μ L after multiplying the number of parasites counted by a factor of 40. Parasites were counted per 200 white blood cells on the thick film.

For study I, the primary outcome was assessed at day 3, comparing different diagnostic methods, i.e., microscopy, LAMP and PCR for parasite detection. Whole blood (30 μ L) for parasite detection on day 3 using LAMP, was collected in Eppendorf tubes containing extraction solution, (400 mM NaCl, 40 mM Tris, pH 6.5, and 0.4% SDS) and stored in -80°C until analysis.

For study II, an additional 2 mL of venous blood collected on day 0 and day 7 to assess haemoglobin concentration, liver and renal function tests at the Bagamoyo Research and Training Unit (BRTU).

For study I & II, PerkinElmer 226 filter papers (PerkinElmer, USA) were used to collect blood spots for detection and genotyping of parasites by PCR. After labelling the filter-papers and air-drying them in ambient temperature

for about 5 hours, individual DBS on filter papers were packed in plastic Zip-loc bags. They were stored in room temperature for a maximum of nine months before shipment to Karolinska Institutet, Sweden - Department of Microbiology, Tumour and Cell Biology for further analysis.

For study IV, samples used from Tanzania mainland were collected as whole blood in EDTA tube and were leukodepleted using Plasmodipur filter with Phosphate-buffered saline at the BRTU, and stored in -20°C until shipment to the University of North Carolina (UNC) at Chapel Hill, USA. Samples used from Zanzibar were collected as DBS on filter papers (3MM; Whatman) and stored in room temperature until shipment to UNC for sequencing and further analysis.

4.2.3 DNA extraction

Different methods for DNA extraction were employed depending on the study. In study I, for the LAMP analysis component, DNA was extracted by the boil and spin method with minor modifications from previously published protocols (103), followed by LAMP detection using LoopampTM Malaria Pan Detection Kit (Eiken, Japan) (87,93). In Study II, DNA extraction used the chelex®- 100 (Biorad Laboratory, USA) boiling method as previously described (315). In study IV, DNA from leukodepleted blood samples and DBS were extracted using QIAmp 96 DNA blood kits per the manufacturer protocol (Qiagen, Hilden, Germany).

4.2.4 PCR genotyping

4.2.4.1 Genotyping for PCR adjusted cure rates

Parasite genotyping for Study II was performed at the Karolinska Institutet, Sweden. Patients with recurrent parasitaemia by microscopy were selected for genotyping. Timepoints of genotyped samples were enrolment (day 0), 24 hours after the first dose (day 1) and day of recurrent parasitaemia. Two early time points (day 0 & day 1) were chosen, as opposed to just one, in anticipation of natural fluctuations in the density of each infecting clone, therefore maximising the chance of identifying all clones present in the initial infection.

The analysis was conducted in a stepwise manner, as recommended by WHO (222). *m*sp-2 was chosen as the first marker as it is considered the most divisive marker, followed by *m*sp-1, and finally *glurp*. Analysis of each marker was conducted by nested (semi-nested for *glurp*) PCR according to previously established lab protocols, as adapted from Snounou et al., 1999 (316).

Once all three gene markers were complete, each patient was categorised into a final classification based on the following criteria as shown in the flow chart (Figure 6. *Flowchart to illustrate the process followed to determine final*

treatment outcome of microscopy recurrent samples) (222). Recrudescence was when all three markers returned a recrudescence result, reinfection when one of the markers tested returned a reinfection result, negative when all three markers returned a negative result and unknown if all three or one markers returned either an unknown or recrudescence result.

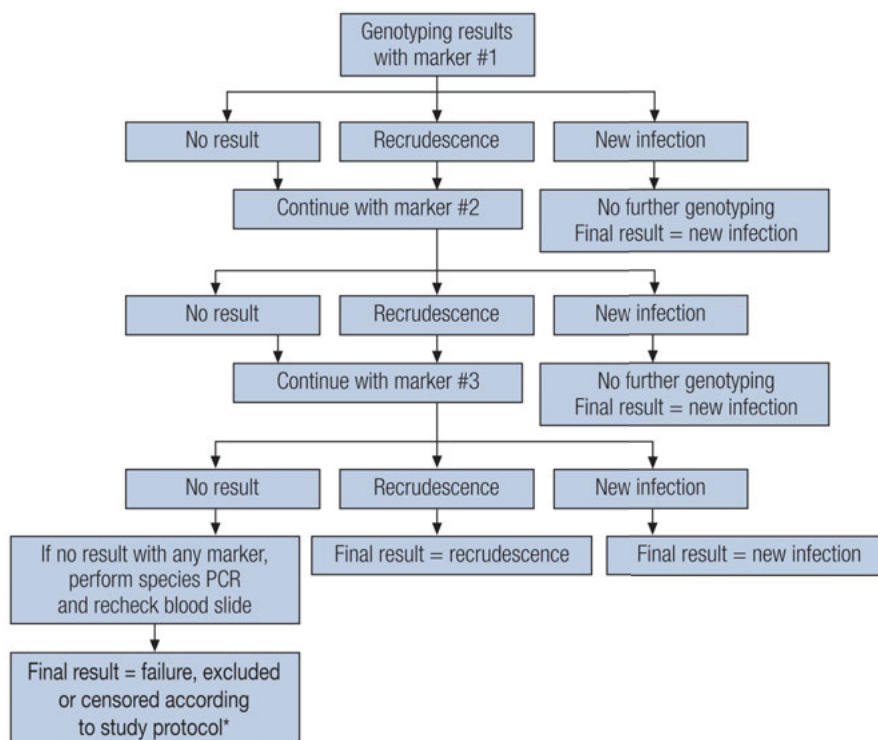


Figure 6. Flowchart to illustrate the process followed to determine final treatment outcome of microscopy recurrent samples *If another *Plasmodium* species is identified in the day X sample (in the absence of *P. falciparum*), a parasitaemia is regarded as being not a recrudescence but a new infection, and the patient will be considered as 'failure', 'excluded' or 'censored', according to the method of analysis dictated by the protocol (222).

4.2.4.2 Genotyping for resistance markers

For study II, genotyping for *pfm*dr1 N86Y SNP on samples of patients that had recurrent parasitaemia, and a subset of all patients that did not have recurrent parasitaemia was done using PCR-restriction fragment length polymorphism (PCR-RFLP) based methods as previously described (303,317)

Sequencing of the *pfk*13 propeller region covering the almost all six propeller domains (858 base-pairs wide) was done as previously described (318). 3D7 was used as a reference in the numbering of nucleotide and amino acid

positions for haplotype diversity in a subset of samples that were positive in the *pfk13* nested PCR.

For study IV, Whole Genome sequencing was conducted at Professor Jonathan Juliano's laboratory at the UNC at Chapel Hill - High Throughput Sequencing Facility. Preparation of sequencing DNA libraries from leukodepleted blood and DBS was done using previously established protocols (11,109,110). After the sequencing, the results were compared with publicly available sequencing data downloaded from public databases.

4.2.4.3 Determining plasma lumefantrine concentration

In study II, venous blood (3 mL) was collected from a subset of patients at randomly assigned sampling times for population pharmacokinetic. Heparinised tubes were used to collect the blood for plasma separation and storage in -80°C at BRTU. Lumefantrine concentration analysis was done at the University of Western Australia under Professor Timothy Davis's laboratory. Plasma lumefantrine concentration was evaluated using validated high-performance liquid chromatography with a UV detection assay (HPLC-UV) as previously described (319).

4.2.5 WGS

Sequencing for study IV was done at the University of North Carolina High Throughput Sequencing Facility. Prior to sequencing, the DNA extracted from Zanzibar DBS samples was enriched for *P. falciparum* DNA by sWGA as per published protocols, using custom primers and Probe_10 primer set previously described (109). Both sWGA enriched and leukodepleted DNA were acoustically sheared using Covaris E220 Instrument, then library preparation (barcoding) followed using Kappa Hyper library preps (Kappa Biosystems, Columbus, OH) as per manufacturer protocol. Sequencing was done with HiSeg 4000 Illumina using 2 x 150 chemistry.

The short read WGS data generated was processed for analysis with genome assembly software comparing with publicly available databases to identify sequence variations.

4.3 Ethical considerations

The clinical trial that is incorporated in this thesis was conducted according to the Good Clinical Practice and the Declaration of Helsinki. Ethical approval was sought for all the sub-studies in respective authorities. From Tanzania mainland, ethical approval was given from Muhimbili University of Health and Allied Sciences (MUHAS), and the National Institute of Medical Research. For Zanzibar, the approval came from Zanzibar Medical Research Eth-

ical Committee, from Sweden, the Regional Ethics Review Board in Stockholm, and from the USA, the University of North Carolina at Chapel Hill. These bodies are representative of the primary affiliations of researchers involved in these studies. Before enrolment, parents/guardians provided informed consent for their children to participate, and children above the age of seven years were involved in deciding to participate by signing the assent form.

5 Results and discussion

5.1 Study I: Detection of *Plasmodium falciparum* on day 3

This study was on parasite detection rate on day 3 after artemether-lumefantrine treatment using the conventional light microscopy and LAMP while using PCR as a reference standard. This study was conducted in order to (i) assess the proportion of patients with parasitaemia as assessed by microscopy on day 3 after treatment initiation as an *in vivo* study indicator for artemisinin resistance. (ii) compare the detection rate of light microscopy, LAMP and PCR in order to assess the utility of LAMP as a molecular tool for day 3 parasite detection in the scope of resistance surveillance (iii) to compare the proportion of patients with day 3 PCR positivity after artemether-lumefantrine treatment with day 3 PCR positivity in previous years (2006–2014) in Bagamoyo district.

This study evaluated patients aged 1–65 years, with uncomplicated *falciparum* malaria mono-infection enrolled as part of the main clinical trial, that had received a total of six doses of artemether-lumefantrine (20/120 mg) for 3 days. Samples that were available for microscopy analysis by day 3 were 265/280, and paired samples available for LAMP and PCR analysis were 256/280. Whole blood for LAMP analysis was collected in Eppendorf tubes containing buffer, and stored at -80°C until analysis. Samples for PCR were collected on filter paper as DBS.

Three key results/ messages derived from this study

- All the samples collected on day 3 were negative by microscopy. According to the current WHO guidelines regarding artemisinin resistance *in vivo* studies, these results indicate that currently, there is no resistance to artemisinin in Bagamoyo district. These results are in agreement with therapeutic efficacy studies conducted in the same area, and this is reassuring that the artemisinin component is still efficacious. Measures to protect against artemisinin resistance are warranted.
- *P. falciparum* day 3 positivity rates were 84.8% for LAMP and 84.4% for PCR, respectively. This parasite detection rate by molecular methods is the highest to be recorded in Bagamoyo district for the 2006–2017 period,

it could be a result of using more sensitive molecular tools. Patients that were PCR and/or LAMP positive on day 3 had higher baseline parasitaemia and body temperature compared to those who were PCR/LAMP negative, implying that PCR/LAMP positivity at day 3 was associated with baseline characteristics. The utility of results by molecular methods for resistance surveillance could not be answered by this study. Further analysis should be done on the day 3 positivity in relationship to clearance of different parasite clones and its association to treatment outcome, whether it reflects an increase in parasite tolerance and recrudescence before the day 3 prevalence by microscopy becomes apparent.

- LAMP sensitivity was 100% (95% CI, 96.1–100) and specificity 77.4% (95% CI, 58.9–90.4) when compared with PCR, as reference standard. LAMP had comparable diagnostic accuracy to PCR, and could potentially represent a field friendly tool for molecular detection of parasites.

5.2 Study II: Efficacy of extended 6-day treatment of artemether-lumefantrine

This was a randomized controlled, parallel group, superiority clinical trial of patients aged 1–65 years with microscopy confirmed uncomplicated *P. falciparum* malaria, enrolled in Bagamoyo district, Tanzania. A total of 280 patients were enrolled, 141 and 139 in the control and intervention arm, respectively. Standard 3-day treatment with artemether-lumefantrine given to the control arm was compared to extended 6-day treatment and single low-dose primaquine in the intervention arm. Follow-up was 42 days, where 121 patients completed follow-up from each arm.

The study evaluated parasite clearance, including proportion of PCR detectable *P. falciparum* on days 5 and 7 (primary endpoint), cure rate, post-treatment prophylaxis, safety and tolerability. Clinical, and laboratory assessments, including ECG were conducted during 42 days of follow-up. Blood samples were collected for parasite detection (by microscopy and PCR), molecular genotyping and pharmacokinetic analyses. Kaplan-Meier survival analyses were done for both parasite clearance and recurrence

Four key results/messages were retrieved from this study:

- There was no difference in proportion of PCR positivity between standard treatment and extended treatment across the arms at day 5 (80/130 (61.5%) vs 89/134 (66.4%), $p=0.44$), or day 7 (71/129 (55.0%) vs 70/134 (52.2%), $p=0.71$). This implies in the current study, doubling the arteme-

ther-lumefantrine duration does not have demonstrable impact on proportion PCR positivity. It remains to be elucidated whether the PCR positivity represents gametocytes or debris of parasite DNA or viable parasite minority clones that are tolerant/resistance to artemether-lumefantrine.

- Efficacy was excellent and similar in both treatment arms, day 42 microscopy determined cure rates (PCR adjusted) were 97.4% (100/103) and 98.3% (110/112), $p=0.65$, in the control and intervention arm, respectively. Moreover, the intervention arm demonstrated a relatively longer post treatment prophylaxis period where on average 90% of the patients were free from recurrent parasitaemia for 42 days in the intervention arm, and 34 days in the control arm, a difference of 8 days which was not statistically significant from the regression model. With longer follow-up periods the difference in post treatment prophylaxis could have been more apparent. Population pharmacokinetics analysis support that the patient in intervention arm had more drug exposure, and considering longer half-life of lumefantrine, it can explain the perceived difference in post-treatment prophylaxis and proportion of patients returning with recurrent parasitaemia in the control arm compared to intervention arm, 60% and 40% respectively.
- There were no SNPs associated with artemisinin resistance detected. Five samples were observed to have synonymous SNPs in the *pfk13* propeller region, of which; four had *pfk13* C469C, and one sample *pfk13* G545G. On the other hand, prevalence of molecular marker that is most strongly linked to lumefantrine resistance *pfmdr1* N86 was found to be high on baseline (day 0), 76/80 (95.0%) and among the microscopy determined recurrent infections the prevalence was 28/28 (100%) among samples with successful PCR. This indicates reduced potential of the artemisinin component to protect the partner drug from selecting tolerant parasites in the Bagamoyo district parasite population, leading to increased risk of subsequent artemisinin resistance and treatment failures.
- The extended 6-days treatment with single low-dose primaquine did not reveal any adverse events that were different from standard 3-days treatment of artemether-lumefantrine. Safety and tolerability were excellent and the adverse events reported were not perceived to be related with drug toxicity. There were no biochemical or haematological changes detected that were different between the treatment arms.

5.3 Study III: Electrocardiographic safety of prolonged artemether-lumefantrine

This was a sub-study from the parent clinical trial that examined in detail the cardio safety of prolonged 6-day course of artemether-lumefantrine because of history of cardiotoxicity among structurally similar quinoline antimalarials such as lumefantrine.

It evaluated the effect of extended artemether-lumefantrine treatment on the electrocardiographic QTc interval. QTc prolongation beyond 500 ms and change in QTc from baseline of >60 ms are considered thresholds for clinical concern. Also, it assessed the different formulae used to correct the QT intervals (Bazett's and Fridericia's formulae) for heart rate. A total of 195 patients had paired ECGs from baseline and day 5; 103 patients from the intervention arm and 92 patients from the control arm.

Two key results/messages were retrieved from this study:

- Extended treatment with artemether-lumefantrine did not reveal clinically relevant QTc prolonging effects despite increased exposure to lumefantrine between day 0 and day 5. No patient experienced QTc intervals >500 ms on day 5 by both formulae. There was a statistically significant difference in mean QTc interval between the two arms. The number of patients with QTc prolongation exceeding thresholds of clinical concern (Δ QTc >60 ms) was low and in line with previous findings from studies of the standard treatment course. These results indicate cardio safety of lumefantrine despite extended exposure. It is important to note that malaria disease pathology affects change in QTc interval due to changes in heart rate as the patient recovers, and body temperature goes back to normal. However, the statistically significant difference in mean QTc intervals between arms where the intervention arm had higher value warrants continued assessment of QTc interval in extended treatment.
- When examining for the overcorrection and under-correction with QTc formulae, the RR intervals were plotted in regression models against respective QTc intervals. With Bazett's formula, there was overcorrection of QTcB at high heart rate and under-correction at low heart rates. The slopes of regression lines were generally comparable with previous studies (32). With Fridericia's formula the inverse was observed, under-correction of QTcF at high heart rates, and overcorrection at low heart rates. This gives the impression that none of the two correction formulae is ideal in this population with high range in patient ages (20,21).

5.4 Study IV. Genomic epidemiology of *P. falciparum* malaria from coastal Tanzania and Zanzibar

This was a genomic epidemiology study conducted on samples from Bagamoyo district, in mainland Tanzania, and from Zanzibar, to evaluate the relationship of parasites from the mainland and the isles where there are continued elimination efforts (314). WGS was conducted on parasites population for genetic analysis of malaria transmission dynamics between Tanzania mainland and Zanzibar establishing the connectedness of these parasite species at genomic level. Zanzibar has been in a pre-elimination phase since 2008, and epidemiological studies have shown that imported cases contribute to the continued transmission. This study used modern molecular tools to assess this paradigm.

P. falciparum isolates from 106 subjects were analysed by WGS, 43 from Bagamoyo district, mainland Tanzania and 63 from Zanzibar, where 36 isolates (84%) from the mainland and 21 isolates (33%) from Zanzibar yielded sufficient data for analysis. Genomic data was compared with other publicly available data of isolates from Tanzania (68 isolates), Southeast Asia, South Asia, East and West Africa (179 isolates) to establish ancestry, level of relatedness, diversity and differentiation, and patterns of selection. The results demonstrate viability of genetic surveillance in assisting to design more efficacious interventions in malaria elimination efforts.

Four key results/messages were retrieved from this study:

- *Ancestry*: Principal component analysis (PCA) was performed to place the Tanzania mainland and Zanzibar isolates in the context of global genetic variation of *P. falciparum*. In the PCA, the isolates separated into South-east Asia cluster, east African cluster and the west African cluster. Tanzania mainland and Zanzibar isolates fell into the east African cluster. Further analysis of shared genetic variation among isolates from different clusters, showed that the Tanzania mainland and Zanzibar isolates shared mutually greater genetic affinity with previously published Tanzanian isolates followed by isolates from Malawi and Kenya. This provides further evidence that the Tanzanian mainland and Zanzibar samples share common ancestry with other east African parasites.
- *Level of parasite relatedness between mainland and isles*: Identity by descent analysis demonstrated that parasite populations between mainland Tanzania and the Zanzibar isles were almost indistinguishable. Long haplotypes (i.e. genome segments inherited unchanged from the same recent common ancestor) were identified as shared between the parasite populations in the order of 5 centimorgans. This indicates genetic exchanges between populations within 10-20 sexual generations. Moreover, a group of

mainland isolates were found to relate with a Zanzibar isolate at a half sibling level. This is suggestive of recently imported cases and serve as evidence of ongoing genetic exchange between mainland and the isles, secondary to human migration.

- *COI*; Polyclonal infections were observed in about half of the isolates from both Tanzania mainland and Zanzibar, with similar distribution in both populations. Also, these polyclonal infections had a median within-host relatedness that is expected for half siblings for both Tanzania mainland and Zanzibar isolates. These results are suggestive of co-transmission of related parasites in both parasite populations. This is in keeping with other evidence that even in high transmission setting, polyclonal infections are mainly due to co-transmission rather than superinfection.
- *Shrinking effective population size*; In keeping with the decreasing malaria transmission in Tanzania, effective parasite population size was found to have significantly decreased in both Tanzania mainland and Zanzibar isolates.

6 Conclusions

- The LAMP (LoopampTM MALARIA kit) demonstrated high diagnostic accuracy for parasite detection on day 3 after treatment with artemether-lumefantrine which is comparable to PCR. The utility of LAMP as a molecular tool in resistance surveillance need to be further elucidated.
- In the treatment of ACT sensitive *P. falciparum* uncomplicated malaria, extended 6-day artemether-lumefantrine treatment together with a single low-dose of primaquine was not superior with regards to efficacy, and was equally safe compared to standard 3-day artemether-lumefantrine in Tanzania.
- Electrocardiographic changes in QTc interval after extended artemether-lumefantrine treatment did not reveal clinically relevant QTc prolonging effects compared to standard treatment. However, significant QTcF prolongation and presence of patients with supra-threshold QTc values observed in the intervention arm underscore the importance of further monitoring of QTc parameters should the extended 6-day course be used for treatment of uncomplicated malaria. The monitoring analysis should take into account contribution of disease process in cardiac pathology.
- Genomic epidemiology study provided findings that suggest *P. falciparum* parasite populations from coastal Tanzania and Zanzibar are highly connected, and that importation plays an important role for malaria incidence on Zanzibar despite effective control efforts.

7 Personal reflections and future perspectives

It is my opinion that the focus of future research areas should include:

1. Further exploring the utility of molecular methods as tools for resistance surveillance, including exploring novel molecular markers of resistance that are novel in the sub Saharan Africa.
2. Evaluating the safety and efficacy of triple ACT as a viable option to combat the threat of artemisinin resistance in sub Saharan Africa in wait of the discovery of new drugs and drug combinations that are equally efficacious or better than ACTs. I am involved in coordinating a triple ACT trial in Bagamoyo district, planned for later this year.
3. Large scale genomic epidemiologic studies to better understand malaria transmission especially in malaria elimination settings.

In general, we need to take advantage of technological advancements to improve drug resistance surveillance, diagnosis and case management for malaria in regions with high burden of disease for sustained and improved high impact of malaria control strategies.

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