College of Medicine

Comparison of Malaria Parasite Clearance Times during Quinine and Artesunate Administration for Cerebral Malaria in Blantyre, Malawi

By

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DECLARATION

I, Alexuse Mustaph Saidi,, hereby declare that this thesis is a result of my work except where I have indicated. Some other activities in this thesis were done in collaboration with other groups.

Activity	**Responsibility**
Sample Collection	Collaborators
Sample processing and analysis	Shared
Data analysis	Shared
Thesis write up	Sole

The content material in this report has not been presented for any other award at the College of Medicine (CoM) or any other university for other degrees or qualifications.

Signature _____

Date March 2022

CERTIFICATE OF APPROVAL

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ABSTRACT

Malaria which is caused by *Plasmodium* species is one of the most important human parasitic diseases. In recent years, preceding 2014, the most severe form of the disease, cerebral malaria, was recommended by World Health Organization (WHO) to be treated with quinine, but due to its increased side effects over the recently discovered drug, artemisinin derivatives, WHO substituted quinine as first line treatment with artemisinin derivatives in combination with a long-acting drug such as lumefantrine, piperaquine, amodiaquine, mefloquine, pyronaridine or sulfadoxine-pyrimethamine. Delay in parasite clearance time in treated patients is the main characteristic of parasite resistance to a particular antimalarial drug. Malaria parasites have already shown resistance to the currently recommended artemisinin derivatives in South East Asia, a development that prompted WHO to recommend periodic monitoring of the drug's effectiveness in endemic countries. Malawian children admitted with cerebral malaria (CM) between 2010 and 2019 in a long-standing pathogenesis study at Blantyre's main referral hospital, Queen Elizabeth Central Hospital (QECH) were retrospectively sampled at admission and every six hours until two consecutive malaria smears were negative. This was done to monitor parasite clearance times across those years. Yearly average clearance time was compared to 2014, the year when artesunate replaced quinine as first-line therapy for CM in Malawian hospitals. Parasite clearance time was shown to be slower during the quinine era compared to the artesunate, an indication that artesunate is superior to quinine. There was no increase in clearance times from the onset of artesunate as first-line therapy, showing that the current recommended anti-malarial drug is still effective.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-based Combined Therapy
AIDS	Acquired Immunodeficiency Syndrome
BMP	Blantyre Malaria Project
CM	Cerebral Malaria
COMREC	College of Medicine Research Ethics Committee
COVID-19	Corona Virus Disease of 2019
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
HIV	Human Immunodeficiency Virus
ICEMR	International Centers of Excellence for Malaria Research
ITN	Insecticide-treated nets
MP	Malaria Pathogenesis
MoH	Ministry of Health
mRDT	malaria Rapid Diagnostic Test
*Pf*kelch13	*Plasmodium falciparum* kelch 13
PRW	Pediatric Research Ward
QECH	Queen Elizabeth Central Hospital

qHRP-2	quantitative Histidine Rich Protein-2
rbcs	Red blood cells
SP	Sulfadoxine-pyrimethamine
SSA	Sub-Saharan Africa
WHO	World Health Organization

CHAPTER ONE: INTRODUCTION AND BACKGROUND

1.1 Introduction

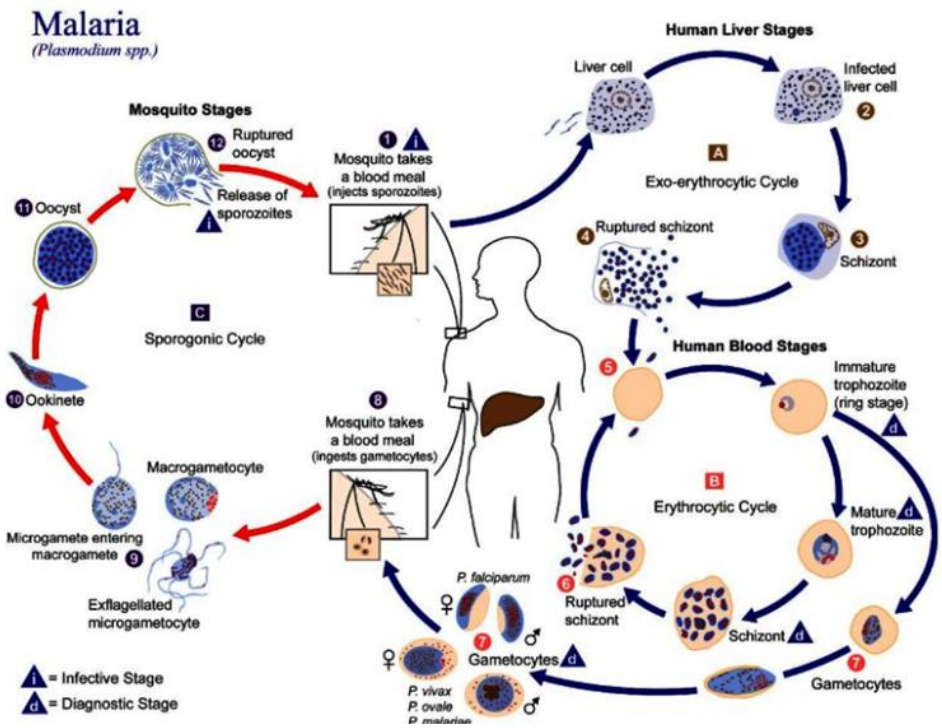
1.1.1 Malaria

Malaria is one of the deadly diseases that is caused by a protozoan parasite of the genus *Plasmodium* (1,2). The *Plasmodium* parasite spreads to humans through the bite of female *Anopheles* mosquitoes as they feed on human blood while nourishing their eggs (3).

1.1.2 Malaria life cycle

The malaria parasite life cycle involves two hosts: mosquitoes and humans (4). Female anopheles mosquitoes infected with *Plasmodium* sporozoites inoculate human beings with the parasites from their salivary glands. The sporozoites then travel through the circulatory system until they reach the liver cells, where they undergo exo-erythrocytic schizogony (asexual reproduction outside red blood cells (rbcs)). Merozoites are released from the rupture of these mature schizonts in the liver cells and get transported throughout the body through the circulatory system, where they undergo erythrocytic schizogony (asexual reproduction inside rbcs). The merozoites enter red blood cells and develop into trophozoites, and later either enter into the asexual cycle where trophozoites mature to schizonts that rupture to release merozoites again or they take the sexual cycle which involves the formation of male (microgametocytes) and female (macrogametocytes) (5). The rupture of infected rbcs and infection of other rbcs is responsible for clinical manifestations of the malaria disease (2). When female mosquitoes take a blood meal, they ingest circulating mature gametocytes. Once gametes are in the mosquitos' stomach, male gametocytes undergo exflagelation which allows them to enter into female gametocytes forming Zygotes. The Zygotes change to motile Ookinates that penetrate the mid-

gut wall of the mosquito and develop into Oocytes. These formed Oocytes (where sexual multiplication takes place) grow and rupture, thereby releasing sporozoites that find their way into mosquito salivary glands (5). See diagram below:



Ncbi.nml.nih.gov

Figure 1 : Malaria parasites life cycle (5)

1.1.3 Types of malaria parasites and distribution

There are several types of *Plasmodium* species that cause malaria, the most prominent ones are *Plasmodium ovale*, which is mainly found in West Africa, *Plasmodium malariae*, found across Africa, *Plasmodium falciparum*, which is the most common type and is found in Africa and other parts of the world and *Plasmodium vivax*, which is commonly found in South America and Asia,

and *Plasmodium knowlesi*, which is very rare and is only found in South Asia (6).See the world malaria distribution map below:

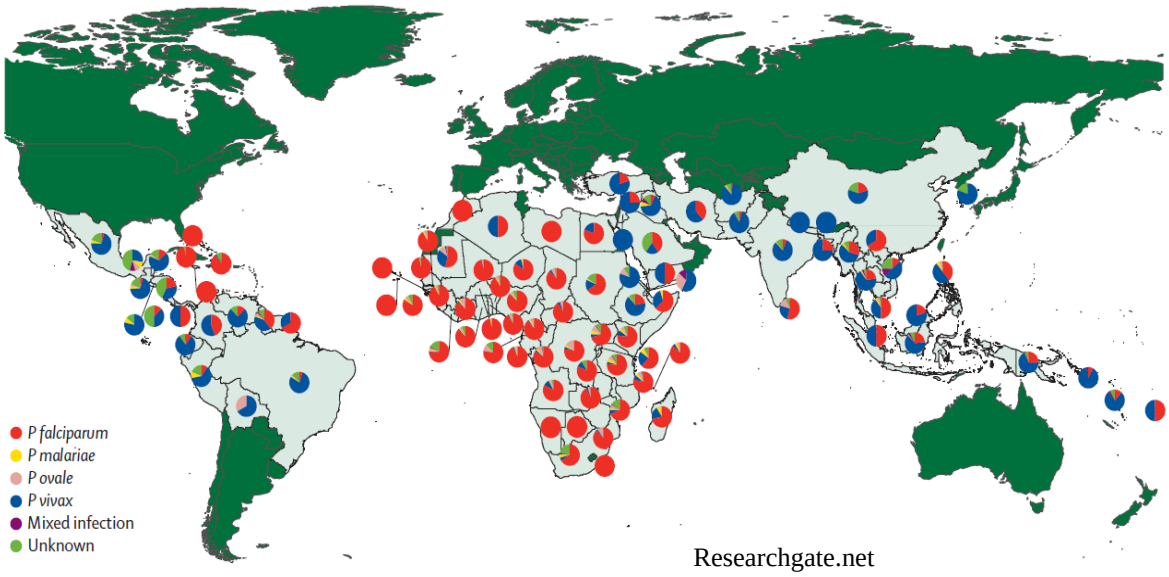
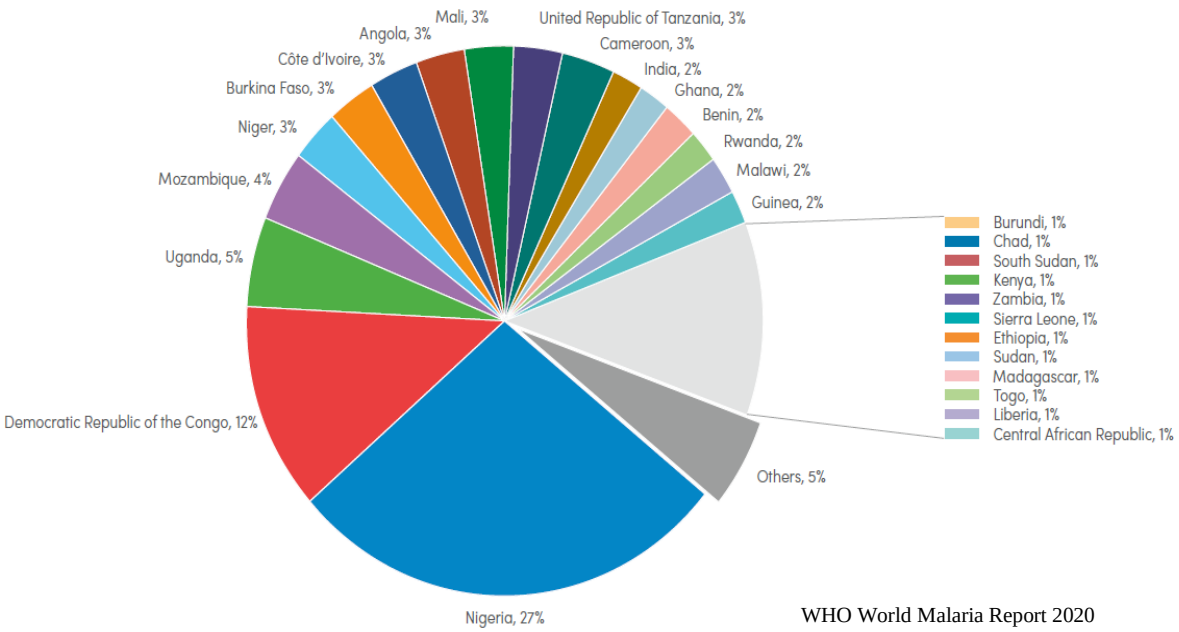


Figure 1.2: World malaria species distribution (7)

1.1.4 Malaria epidemiology globally

According to the 2020 World Health Organization (WHO) World malaria report, malaria is still a leading cause of hospital admissions and mortality. Sub-Saharan African (SSA) children under the age of 5 years and pregnant women are the populations at the most risk. The same 2020 WHO report indicates that globally, there were about 229 million reported cases in 87 endemic countries out of which 29 African countries counted for 95% malaria cases. Five African countries have contributed 51% of malaria cases globally (8). See figure below:



WHO World Malaria Report 2020

Figure 1.3: Malaria cases distribution by country

Incidence of malaria cases have reduced from 80 cases in every 1000 population at risk in 2000 to 57.5 cases in 2015 representing a 22.5% decline, then it slightly went further down to 56.8 cases per 1000 population at risk between 2015 and 2019, representing a 1.2% reduction (8). See figure 1.4.

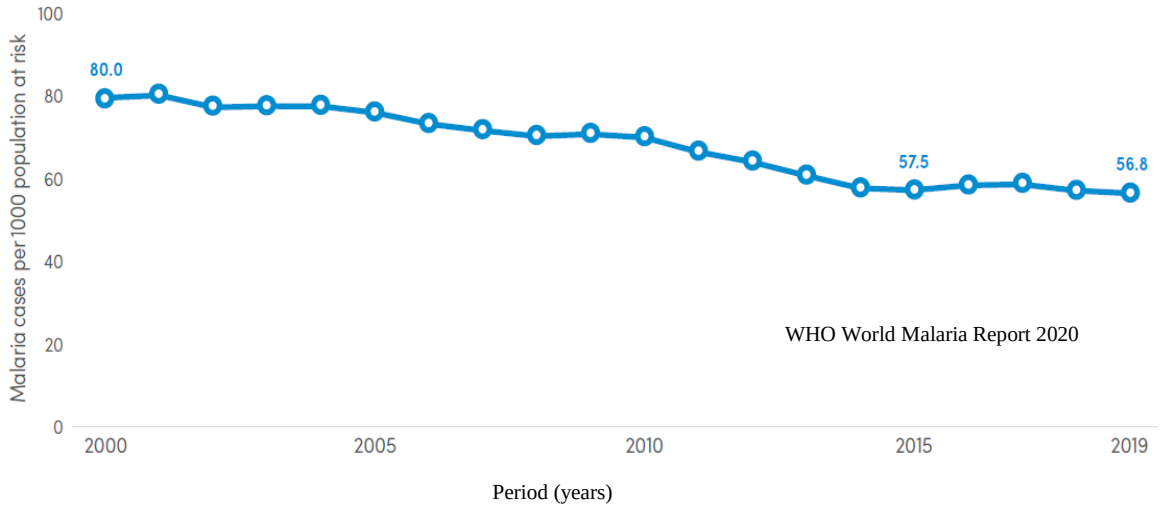


Figure 1.4: Global malaria cases incidence rate, these are cases /1000 population at risk

The malaria deaths per 100 000 populations at risk have also gone down from 25 in the year 2000 to 12 in 2015 and then slightly reduced to 10 in 2019. See figure below:

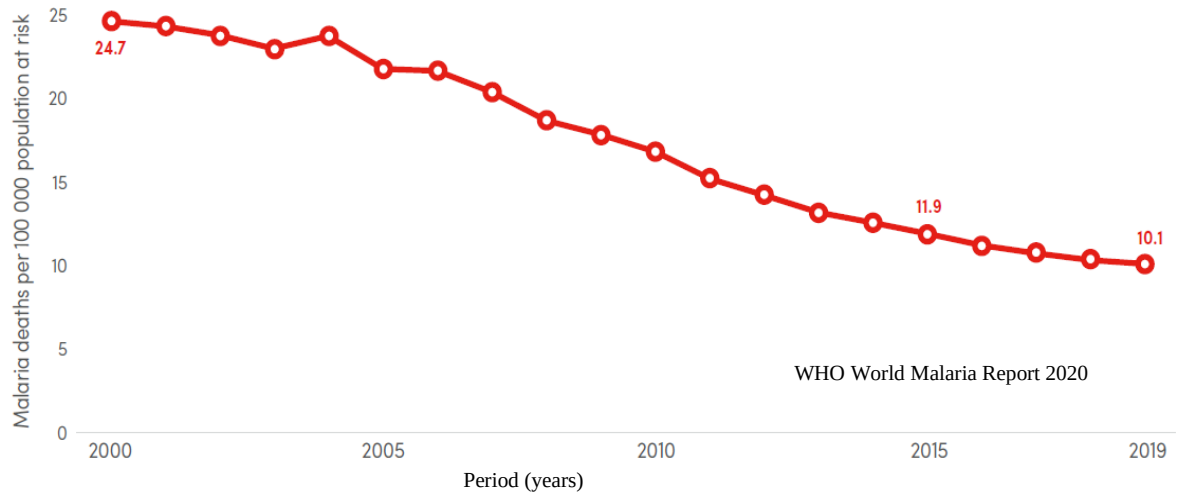
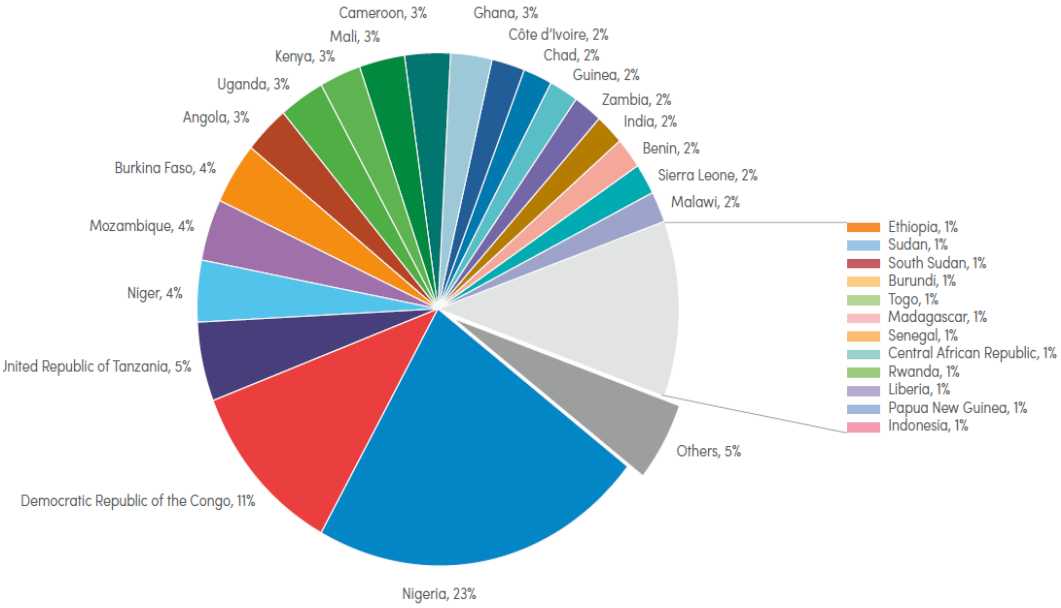


Figure 1.5: Global malaria mortality rate. These are deaths /100 000 population at risk

In 2019, about 95% of malaria deaths were accounted for in 32 countries globally and Nigeria contributed the highest. See figure



WHO World Malaria Report 2020

): World Health Organization.

Figure 1.6: Global malaria deaths by country

1.4.1 Malaria epidemiology in Malawi

Malawi is one of the 87 countries whose population is at high risk of malaria infection and is among the top 15 countries with high malaria prevalence in Africa. The country contributes about 7.4% of malaria cases in Eastern and Southern Africa (8). Malaria is amongst the most significant public health problems in Malawi accounting for 23% of all out-patient visits in all age groups (8). Transmission in most parts of the country is perennial and picks soon after the start of annual rains that occur between October to April. Areas that frequently experience hot weather as well as wet conditions and those leaving along low lying places have the highest recorded transmission; these are areas like the lakeshore, central, and Shire river valleys (9,10).

The most predominant malaria species in Malawi is *Plasmodium falciparum. Anopheles gambie s.s, arabiensis,* and funestus are the major vectors in Malawi (11). Every year, nearly 4 million people are diagnosed with malaria infection. The country accounts for 2% of malaria cases worldwide (8). Children under the age of 5 and pregnant women are the most population at high risk of being infected with malaria compared to other groups (12). Geography plays a significant role in malaria prevalence among children. This is evidenced by studies that suggest that malaria morbidity in children under the age of 5 is not evenly distributed across Malawi. According to 2017 RDT (Rapid Diagnostic Test) and microscopy data, child malaria prevalence in the central region is highest followed by the southern region and the northern region. Children in rural areas are more vulnerable to malaria infection compared to those in urban areas (13).

1.5 Cerebral malaria pathogenesis

There are three different clinical syndromes of severe malaria which include; severe malaria anemia, severe malaria with respiratory distress and cerebral malaria. The three types of severe malaria have distinct pathophysiology and their underlying etiology of each subtype is unclear (14). For example, the pathophysiology leading to difficulty in breathing may include pulmonary edema, fluid over load, lung injury, hyperventilation or pneumonia, whereas that leading to severe anemia includes destruction of red blood cells as well as a decreased production on new red cells in the bone marrow (14). Although the actual mechanism of CM is not clear, the most distinctive feature is the sequestration of parasitized red blood cells in organ capillaries including the brain which is mediated by cytoadherence of infected rbcs to endothelial cells (14,15). The sequestered rbcs block the capillaries thereby reducing the flow of blood to downstream organs. The infection may lead to brain swelling, a condition believed to cause death as indicated by

Magnetic resonance imaging (MRI) and presence of retinal changes in children (14). Swelling of the brain compresses the brain stem that contains the respiratory center causing stoppage to breathing (14).

1.1.6 Malaria prevention and control

Malaria prevention faces quite a number of challenges. The Malawian government is implementing comprehensive malaria prevention programs that target more than 85% of its population. The main focus in the prevention and control of malaria is on reduction of contact between mosquitos and humans, as well as the elimination of vector larvae through good environmental management systems, in order to clear vector breeding grounds (16,17).

Some of the main interventions are preventing the malaria vector mosquitoes from biting people by promoting the use of long-lasting Insecticide-treated nets (ITNs), an exercise which is programmed to be carried out every three years, indoor insecticide spraying where most endemic areas like along the lake show, shire valley, and all low land areas are sprayed with long-lasting insecticides especially towards the beginning of rain seasons which mostly starts in November (18), the exercise of giving pregnant women at least three doses of Intermittent Preventive Treatment of Malaria during pregnancy with Sulphadoxine-Pyrimethamine (IPTp-SP) (19), encouraging people to be putting mesh wire to screen their houses to avoid unnecessary mosquito entry into houses (20), spraying stagnant water bodies with insecticides to prevent malaria vector larvae multiplication (13,21).

To reinforce the linkage between health and the environment, integrated vector management is essential. Malaria can be controlled by adopting biological methods that can target and kill malaria vector larvae without using dangerous chemicals that can damage the ecosystem. This safe method can be achieved through the introduction of bacteria or fish that only destroy malaria vector larvae without generating the ecological impacts of chemical use (17,22).

Anopheles mosquitos behavior is characterized by high activity during the night, from dusk to dawn; therefore, the easiest way to reduce transmission is to stay indoors during the night or to apply mosquito repellent on exposed skin, or wear long-sleeved shirts, a hat, and long pants if one is outside and always sleep in long-lasting ITN (23).

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Case management which includes diagnostic testing to all patients presenting in hospitals with malaria signs and symptoms and prescribing the right anti-malarial drugs to all patients with positive malaria tests is the best practice to control the spread of malaria.

1.1.8 Malaria tests

There are several methods of testing malaria, the most commonly used and one that is rated as a gold standard for testing malaria is the microscopy method where a well-stained thick or thin smear is scanned under a microscope. Some of the limiting factors for this particular method include dependency on the experience of the technician and the quality of the reagents used for staining as well as that of the microscope equipment (24,25).

Another approved common method in use is the Rapid diagnostic test which relies on checking the presence of malaria antigen in the examined blood. As the name suggests, the method is quick but one remains positive for a little longer time after parasites microscopy clearance (26).

Malaria can also be detected serologically by the use of quantitative methods using ELISA analysis. The good thing about this method is that it measures the total body malaria pathogenesis including sequestered parasites, unlike microscopy which only looks at peripheral parasites. Some of the disadvantages include failure of the technique to be performed at a low-resource setting healthcare facility, requiring of high tech equipment, time-consuming and needs well-experienced personnel (27).

Polymerase Chain Reaction (PCR) is a modern method currently used in some laboratories to detect malaria parasites. This is one of the most reliable in terms of specificity/accuracy because the method uses specific primers for a specific gene of interest but just like the ELISA method, it requires very expensive equipment and well-trained personnel (28).

1.1.9 Malaria treatment

Clinical malaria can be classified into two forms: uncomplicated (mild) and complicated (severe) malaria (29). There are three types of severe malaria namely: severe malaria anemia, severe malaria with respiratory distress, and cerebral malaria (30). WHO defined cerebral malaria as Blantyre coma score of ≤2, *P.falciparum* parasitaemia on peripheral blood smear and no other known cause of coma (31)

According to the WHO 2018 World malaria report, the recommended antimalarial drug for mild malaria caused by *Plasmodium falciparum* is ACT (artemisinin-based combined therapy). ACT drugs are administered in the following combination: artemether with lumefantrine (LA), dihydroartemisinin with piperaquine, artesunate with either amodiaquine, mefloquine, pyronaridine, or sulfadoxine-pyrimethamine (32,33). On the other hand artesunate is administered on its own for cerebral malaria (either intravenously or intramuscularly) for 2-3 days, with the monotherapy followed by a three days oral course of an ACT when the patient starts to tolerate oral medication (34)

Quinine had been the main drug treatment of choice for severe malaria since its discovery from Cinchona Bark in the 1600s (35). WHO's regimen for quinine was a starting dose of 20mg/kg through intravenous infusion followed by 10mg/kg quinine sulfate or bisulfate every 12 hours to achieve the therapeutic plasma concentration (36). There has been a decrease in the use of quinine over recent years due to its toxicity, poor patient compliance, and implementation of newer and more effective drugs such as ACT (37).

Quinine had been a super sole drug for malaria treatment up until the 1940s the time when it was substituted with chloroquine, which showed higher efficacy then. It was around the 1950s when malaria parasites started to show some resistance characteristics to chloroquine. In the 1980s, quinine was brought back as a first-line treatment drug to malaria replacing chloroquine (35). Quinine was again set aside with the introduction of sulfadoxine-pyrimethamine (SP); a drug that also later showed reduced susceptibility to malaria. In 2014, treatment with artemisinin derivatives was introduced in Malawi, because of the excellent attributes artemisinin has shown over its predecessors, like high clinical efficacy, rapid parasite clearance time, and fast clinical recovery (38).

Recommendations to switch from quinine to artesunate were published in 2012 based on a large clinical trial conducted in Africa (26).

Malawi government recommended a dosage of 2.4 mg/kg of artesunate at admission and every 12 hours up to 24 hours, thereafter the patient is encouraged to take oral medication of LA if the patient can take oral medication. (39)

Apart from the usual ACT antimalarials, there are other drugs on the market currently approved for the treatment of malaria, these include; Atovaquone-proguanil, Quinine sulfate with either doxycycline, tetracycline or clindamycin, Chloroquine phosphate, Hydroxychloroquine, Primaquine phosphate, and Tafenoquine (33)

Recently, scientists have broken the barrier of the malaria vaccine by rolling out trials on a malaria vaccine, a very promising milestone that is expected to reduce malaria in children and hepatitis, including cases of severe malaria, related hospital admissions, and the need for blood transfusions (33). RTS,S is one of the vaccines which are on trial in the African countries of Malawi, Ghana, and Kenya to assess the protective benefits and safety of the vaccine in real-life settings (33).

1.1.10 Antimalarial drug resistance in *P.falciparum*

WHO defined antimalarial drug resistance as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject" (11). Resistance to antimalarial drugs is mainly preceded by progressive delays in parasite clearance times (measured in hours) after antimalarial therapy administration (40). There are three independent processes that contribute to malaria parasite clearance: Host-defense mechanism, effects of antimalarial drugs and malaria parasites sequestration (41)

Malaria infection can be controlled by innate host defense mechanisms which later may become specific humoral and cellular immunity which serve to reduce parasite infection to tolerated levels with few or no symptoms (41)

The majority of anti-malarial drugs have greatest activity against trophozoites, a malaria parasite stage which is metabolically active while artemisinin effectively kills the young ring stages of *P. falciparum* (41).

Sequestration in *P.falciparum* is a mechanism malaria parasites adapted in order to escape splenic filtration where malaria parasitized red blood cells are removed from peripheral circulation and then get destroyed through pitting (41,42)

Rapid malaria parasites clearance in peripheral blood minimizes chances of parasites development to sexual stages, male and female gametocytes which accelerate malaria transmission (41,43).

Several reasons accelerate antimalarial drug resistance development, some include: increased production of fake antimalarial drugs on the market which leads to inadequate drug exposure on parasites, high parasite mutation rate when parasites try to adapt to drug exposure, poor antimalarial quality, or poor drug strength which allows parasites to survive despite drug exposure, improper drug dosing, poor adherence to antimalarial treatment, poor antimalarial drug pharmacokinetic properties and lack of treatment compliance by patients (11)

Most antimalarial drug resistance emerge from South East Asia (SEA) region (44), Malawi was not spared from antimalarial drug resistance development scenarios. The country has been using quinine to treat both uncomplicated and severe malaria for decades up until the years the 1940s when malaria-endemic countries changed first-line treatment for uncomplicated malaria to chloroquine. In the 1950s malaria parasites started showing resistance to chloroquine in SEA and later spread to other parts of the world, Malawi inclusive, by the 1980s (35). The parasite again started showing resistance to chloroquine which prompted the Malawian government to substitute chloroquine with sulfadoxine-pyrimethamine (SP) in 1993 but later SP's effectiveness

to malaria parasites started to weaken. In 2007, the Malawi government replaced SP with ACT, artemether-lumefantrine (AL) for uncomplicated malaria (45).

Currently, there is no evidence of malaria parasites resistant to the current ACT in Malawi although WHO 2020 report showed a 19.5% treatment failure rate in one of the surveys conducted between the years 2010-2019, a finding which until now has not been confirmed by subsequent studies (46,47).

Malawi as one of the malaria-endemic countries in Sub-Saharan Africa (SSA) is encouraged to periodically do malaria resistance monitoring studies/surveys to contain and prevent spread to other parts of the world (34,48).

The investigators of this study decided to do this particular project because of the points outlined understudy justification.

1.1.11 Study Rationale

Quinine has been a readily available substitute drug to many antimalarials whenever the recommended drug in use at that particular time has started showing failure in treating the disease (49,50) hence the idea to do this project.

Malaria parasites have already developed resistance to both quinine and the currently recommended artemisinin derivatives in SEA (51), an indication that it can easily spread to other regions, therefore, the need to pursue the project.

So far, most studies conducted across Africa have not yet reported signs of resistance development to either quinine and ACT (52). This does not mean that the current drugs cannot slowly start to lose their effectiveness on malarial parasites, rather, it should be the right time to be alert in readiness for possible occurrence (48), there is a need to conduct the study.

The characterization of parasite resistance to chloroquine and SP was done after the resistance had already spread globally, so it was difficult to contain (52). To avoid a recurrence of such scenarios in other areas of the world, it is wise to monitor resistance development to artemisinins periodically; again it is the idea for the study.

AQUAMAT (Artesunate versus quinine in the treatment of severe falciparum malaria in African children) was an open-label, randomized trial conducted across Africa to confirm findings of the 2005 SEAQUAMAT (South East Asia Quinine Artesunate Malaria trial) conducted in SEA. The majority of subjects had uncomplicated malaria, with only a few complicated malaria cases, therefore, there was a need to compare the efficacy of intravenous artesunate specifically in patients with cerebral malaria (53,54).

The final reason that motivated this investigator is the WHO's recommendation of carrying out periodic resistance development checks to the current ACT in all malaria-endemic countries to contain and prevent spread to other parts of the world (34,48).

1.12 Hypothesis

From this background, we hypothesized that the effectiveness of quinine or artesunate would change with increased time of exposure compared to the period when the drugs were just being rolled out. Longer parasite clearance times in the peripheral blood would indicate a progression towards poor therapeutic response. In this study, we compared malaria parasites clearance times of the two drugs in question using previously collected data from a long-standing cerebral malaria pathogenesis study.

CHAPTER TWO: STUDY OBJECTIVES

2.1 Objectives

To compare malaria parasite clearance times during quinine and artesunate administration periods in children with cerebral malaria.

2.2 Specific Objectives

1. To monitor quinine and artesunate's effectiveness in treating *Plasmodium falciparum* throughout 2010-2013 and 2014-2019.
2. To relate parasite clearance to parasitic load (as measured by Histidine Rich Protein 2 (HRP2)).
3. To estimate parasite resistance development to quinine and artesunate.
4. To determine the proportion of patients who were still parasitaemic after a standard treatment course of quinine or artesunate

CHAPTER THREE: METHODS

3.1 Study Methodology

3.1.1 Ethics statement

The authority to undertake this project was granted by the College of Medicine Ethics Committee (COMREC) under reference number P.11/20/3207 while the MP Study, had the permission of doing the project by COMREC under two protocol approvals P09/16/2024 - *Treating brain swelling in Paediatric Cerebral Malaria* and P11/07/593 - *Clinicopathologic Magnetic Resonance and Electrophysiologic Correlates of Cerebral Malaria*.

The MP study collected samples only after signed written informed consent from either a parent or guardian of the patients and samples were de-identified with patient identification numbers. Those signed informed consents allowed the use of samples for further research.

3.1.2 Methods

3.1.2.1 MP study

a. Study area

MP study is being conducted at Queen Elizabeth Central Hospital (QECH) on children referred from health centers around Blantyre as well as completed cases from other districts in the southern region of Malawi.

b. Study population

The study is recruiting children from six months up to 14 years of age. Cerebral malaria is very rarely seen in children under the age of five months – perhaps because at this age children retain some anti-malarial immunity from placental antibodies transferred from their mothers.

c. Inclusion criteria

In this study, patients were included based on the following criteria; children aged between six months to fourteen years as well as those diagnosed with CM as defined by WHO and are retinopathy positive. Participants whose ages were above 14 years and tested negative for malaria using the mRDT (malaria Rapid Diagnostic Test) or thick smear were not included.

d. Sample collection and preparation

Anti-coagulated blood was collected at admission and used to prepare thin and thick smears. The remaining blood was spun for 30 minutes at 2000 revolutions per minute (rpm) at room temperature. Plasma aliquots were prepared and stored at -80 ^{o}C for qHRP-2 (quantitative Histidine Rich Protein 2) analysis using (Enzyme-Linked Immunosorbent Assay) ELISA method.

Thick and thin smears were being prepared every 6 hours up until two consecutive slides came out negative by microscopy.

Peripheral parasite density on missing results was determined by counting the number of parasites against 500 white blood cells (WBCs) with two tally counters on the thick smear under

the light microscope using a 100X magnification lens. Counting was switched to a corresponding thin-film slide if 100 parasites or more were observed in every field. On the thin film, infected red blood cells (RBCs) were counted against total RBCs up to 5000 total RBCs. Parasitemia was calculated using either RBC counts for thin films or WBC counts for thick smears determined on admission and analyzed on a Coulter A^C.T5diff AL (Beckman Coulter Life Sciences, Indianapolis, Indiana, USA).

Admission quantitative HRP-2 on missing data was determined on samples frozen at -80 ^{0}C. HRP-2 manufacturer's protocol was used with the modification of incubations being performed at 37 ºC (Cellabs, Brookvale, Australia). The plate was analyzed using an ELx800 reader at 450nm (BioTek Instruments, Winooski, Vermont, USA). Plasma HRP-2 concentrations were calculated by comparing the results from patient samples with a standard curve generated from the analysis of the recombinant stock. All results that fell outside the linear range were re-analyzed after adjustment of dilution factors.

e. Study design

From the 706 CM clients admitted between 2010 and 2019, 55 participants were left out because they either had no parasites or their parasitemia was less than 2 per microliter. This reduced the number to 651. The remainder was divided into two groups, those that received quinine between 2010 and 2013 and those treated with artesunate between 2014 till 2019. According to prior parasite clearance literature, malaria parasite clearance investigations is supposed to include only samples with parasitemia of at least 1000 parasites per microliter or higher. As a result, the final analysis did not include 68 clients from the quinine group and 118 participants from the

artesunate group. Therefore, the total number of subjects analyzed was reduced to 259 and 206 for quinine and artesunate groups respectively. Refer diagram 4.1

f. Sample size

The investigator used convenience method of sampling because this study depended much on the number of patients who presented to the clinic and consented to participate in the main study. The investigator included 324 clients from each arm based on the number of patients enrolled during the artesunate period because this was the time the study registered fewer participants compared to the quinine period.

Power statistics have shown that from this sample size we have managed to detect a 10% difference in parasite clearance time between the two arms with a power of 80% and at a confidence of 95%.

Generally, quinine has quite a lot of participants; therefore, the investigator chose 324 clients from 2019 going backward. These samples have been evaluated and matched with samples from the quinine era.

There is a likelihood that the final number of samples analyzed decreased because there are some clients who died before finishing the prescribed standard treatment course and there were excluded from the final analysis.

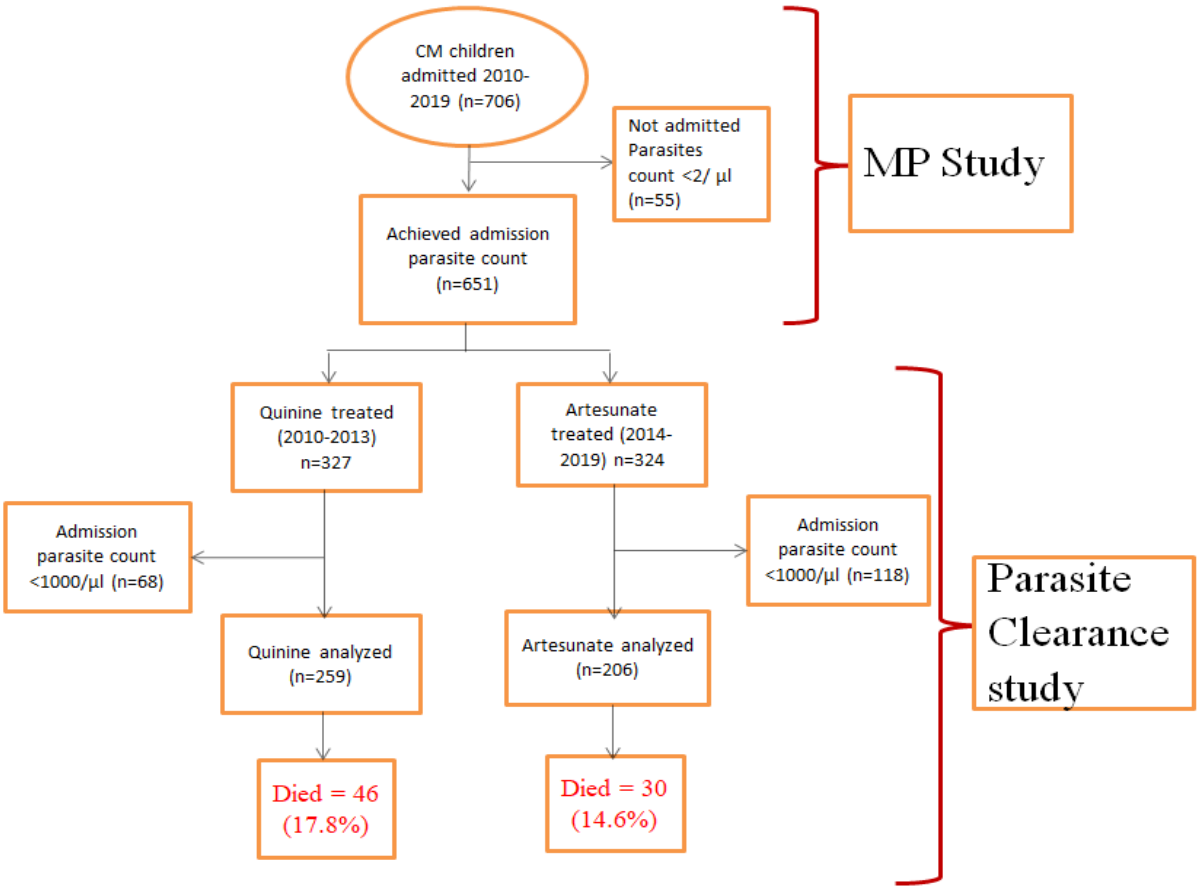

Figure 3.1: Study design

g. Data collection

Data was collected using ADR (Antimalarial Drug Resistance) form 01 and qHRP-2 concentration form 02, see appendix. Samples were arranged sequentially according to sample identification numbers, from the first sample in the year 2010 to the last sample in 2019. All the results that were missing were left with gaps in their respective data table cells.

The investigator got permission to "dig" into the archive and retrieve all slides and plasma samples whose data were not filled in the two forms.

These slides were already stained using the procedure described under MP study methodology, therefore the investigator did microscopy on all the thin and thick smears and quantified HRP-2 on plasma samples using the same MP study protocols.

h. Statistical analysis

Admission demographic, laboratory, and outcome characteristics between patients treated with quinine and artesunate were compared. Data summaries included means and standard deviations for continuous variables or counts and frequencies for categorical variables. T-tests were used to compare characteristics between quinine-treated and artesunate-treated patients for continuous characteristics, or chi-square tests for categorical variables. Peripheral parasite densities and qHRP2 levels were logarithmically transformed to stabilize the variance when making comparisons.

Parasite clearances and parasite count graphical representations were created to evaluate subject-level and cohort-level (natural logarithm scale, y-axis) over time in hours post-admission. To establish cohort-level summaries, linear interpolations to subject-level curves from discrete measures, and boxplot of parasite measures at each measurement time were used.

Two time-to-event analyses were performed. Unadjusted analyses comparing time-to-event curves over years included Kaplan-Meier curves of time to the first parasite count less than 1000 parasites per microliter by antimalarial administered, and by year within quinine-treated and artesunate-treated patients. A log-rank test was used to compare time-to-event curves by antimalarial (quinine vs. artesunate), and across years within quinine-treated and artesunate-

treated children. Microsoft Excel 2010 was used to arrange samples and analyze the relationship between parasite clearance and parasitic load measured in qHRP-2.

CHAPTER FOUR: RESULTS

4.1 Findings

Between January 2010 and June 2019, 706 children with CM were enrolled in the parent study (Figure 4.1). Fifty-five children were excluded as they had either no admission parasite density or their parasites counts were less than two parasites per microliter. Out of the 651 children remaining, 327 participants received quinine (admitted 2010-2013), and 324 received artesunate (2014-2019). There were again 186 children who were excluded from the final analysis because their parasites count did not reach the set parasite clearance threshold of ≥1000 parasites/microliter. Therefore, 259 and 206 participants for quinine and artesunate respectively, made to the final analysis (figure 3.1):

4.2 Participants demographics

More than 85% of participants in each arm presented to medical attention while already taken anti-malarial medication. Participants in the quinine era had higher parasite concentrations, increased HRP2 levels and showed more neurological problems at the time of discharge as compared to those who received artesunate (Table 4.1).

Table 4.1: Participants' clinical demographic outcome

	Antimalarial		
Demographic, clinical, outcome measure	Quinine-treated (2010-2013) (n=259)	Artesunate-treated (2014-2019) (n=206)	P value for difference
Age (months): mean, SD	50.9 (28.1)	49.9 (29.8)	0.711
Parasites/µl: mean (SD) Log (parasites): mean (SD)	260,872 (458,810) 11.48 (1.63)	187,612 (278,800) 10.84 (1.94)	0.045 <0.001
HRP2 (ng/ml): mean (SD) Log (HRP2): mean (SD)	7,909 (10,028) 7.30 (2.65)	5441 (10,693) 6.96 (2.23)	0.012 0.001
Participants who received antimalarial treatment before hospital admission (%)	222 (85.7)	180 (87.4)	0.70
Outcomes			0.732
	Died: N (%)	46 (17.8)	30 (14.6)
Number (%) with neurological sequelae at hospital discharge	21 (8.1)	16 (7.8)	

4.3 Quinine versus Artesunate parasites clearance

The two anti-malarials, quinine and artesunate, were compared in their ability to clearing malarial parasites in the peripheral blood. Parasite decay curves showed that on average children treated with artesunate had a shorter lag phase, a steeper decay, and a short tail, compared to children treated with quinine, see figure 4.2.

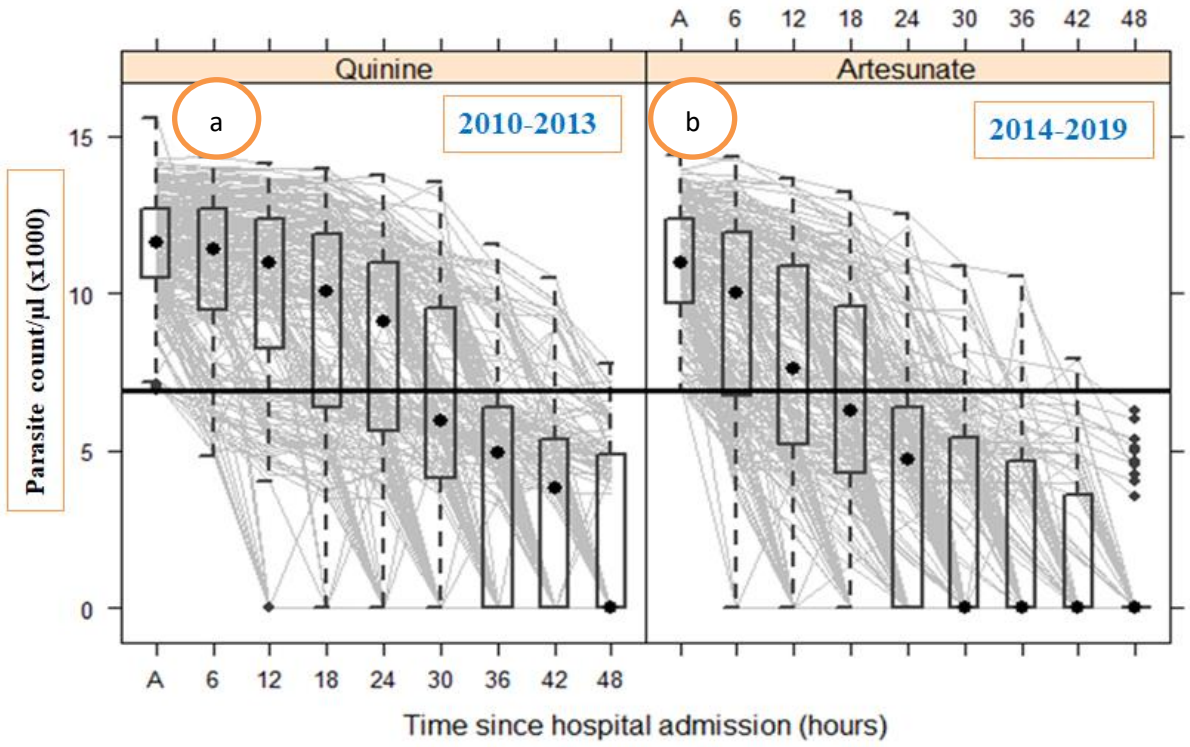

Figure 4.2: Parasite decay curves for children treated with quinine and artesunate. Graph "a" shows longer parasite clearance while "b" shows shorter parasites clearance

4.4 Quinine versus Artesunate over time

The figure below (figure 4.3) compares participants who were treated with quinine (light gray graph) and those who received artesunate (deep gray) over time.

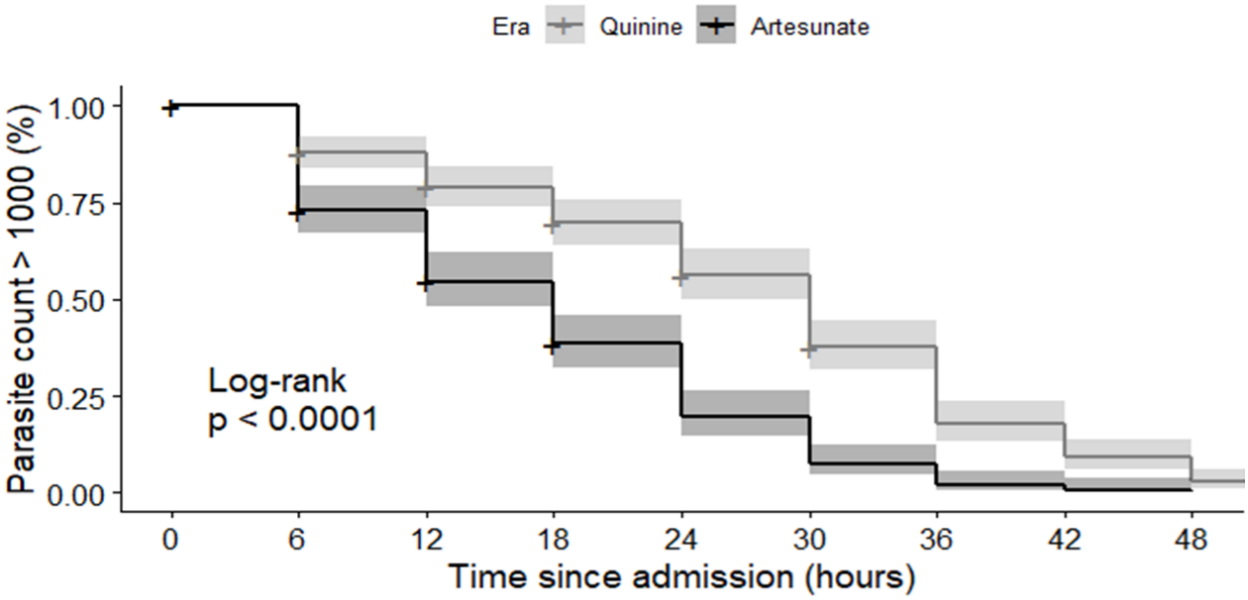

Figure 4.3: Kaplan-Meier curves showing parasite clearance in quinine (light gray) and artesunate (deep gray). The curves separate as early as 6 hours after admission and remain separated over time.

4.5 Artesunate treated participants' comparison

Clearance of parasites of all those who received artesunate were compared amongst themselves to see if there was any change over time as shown in figure 4.4 below. According to this figure, there is no year that shows a clear separation from 2014 (artesunate roll out year), indicating the lack of resistance development. Years 2015 through 2017 show earlier clearance than 2018 and 2019 but the differences are not statistically significant.

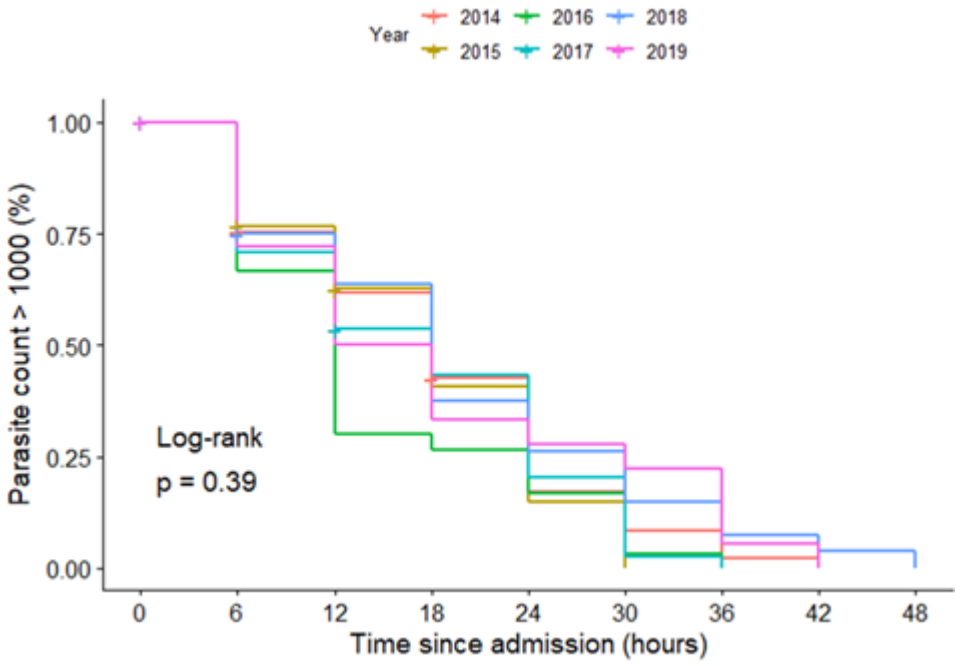

Figure 4.4: Comparison of parasite clearance for artesunate-treated participants from 2014 (artesunate roll out year) onwards.

4.6 Parasites concentration and clearance time

Parasites concentrations in the peripheral blood were compared with the time of parasite clearance to see if clearance time was dependent on the number of parasites in the body. See figures 4.5 and 4.6:

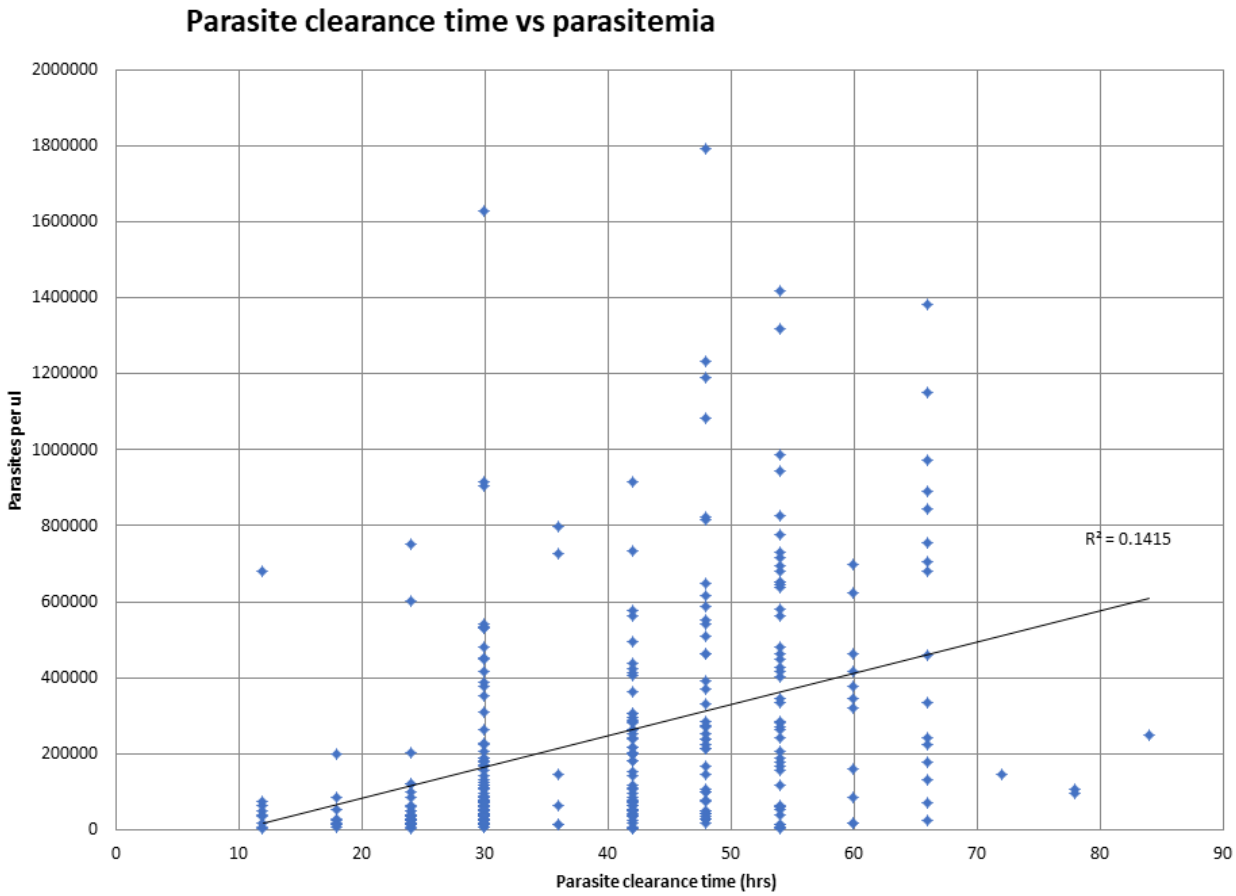

Figure 4.5: Clearance time in relation to peripheral blood parasite concentration

4.7 Total body parasitemic load versus clearance time

Total body parasites, including those sequestered in other organs, measured in HRP-2 were analyzed in order to see if there was any relationship with parasites clearance time.

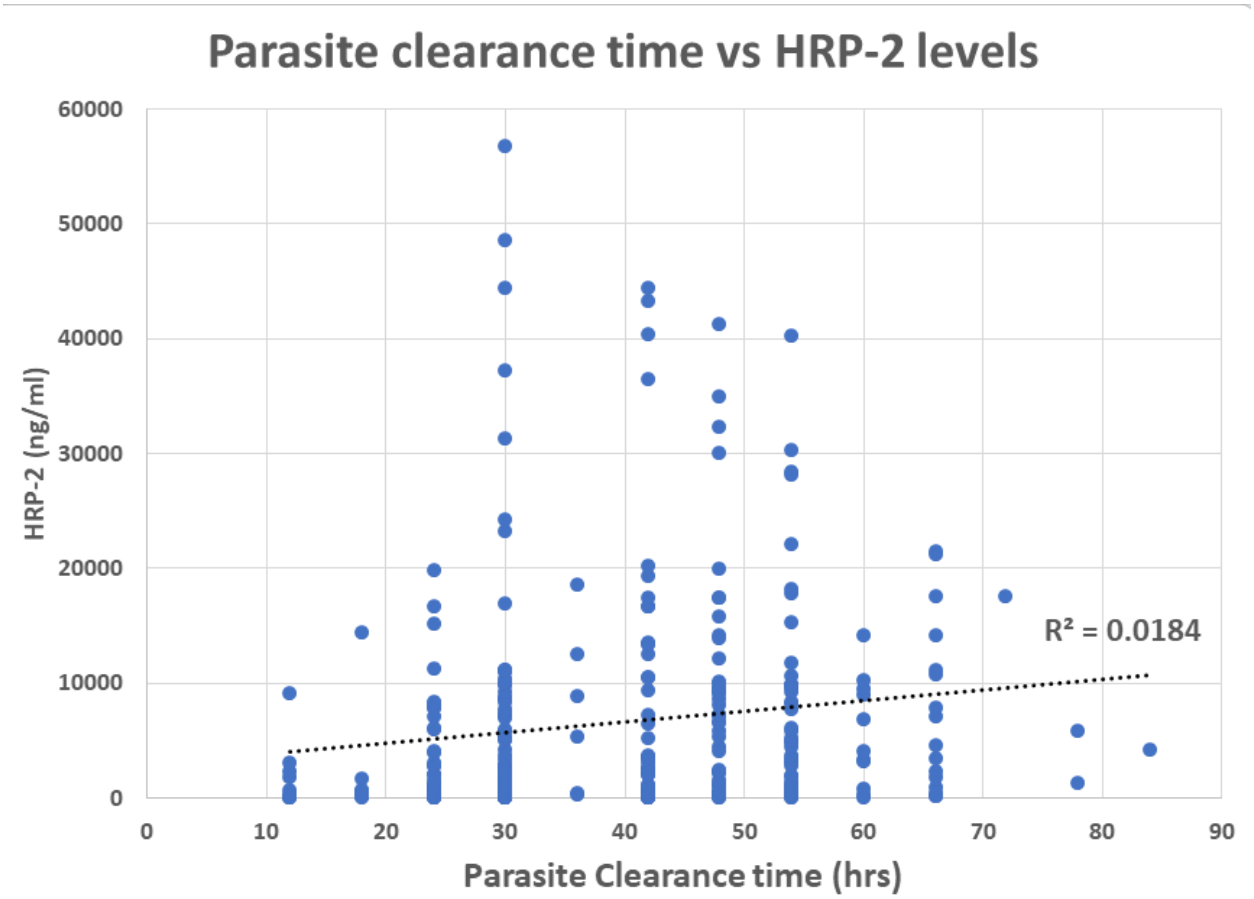

Figure 4.6: Clearance time in relation to total body parasitemic load (HRP-2 levels)

4.8 Hazard ratio analysis

This statistic evaluates the time taken to reach parasites density of < 1000 parasites /microlitre

Table 4.2: Results from Cox proportional hazard model analyses of time to parasite density less than 1000 per microliter, adjusting for baseline parasite density, year and antimalarial administered

Treatment Regimen	Year	Hazard Ratio (95% Confidence Interval)
Quinine	2010	0.34 (0.24, 0.48)
	2011	0.46 (0.32, 0.68)
	2012	0.29 (0.18, 0.47)
	2013	0.57 (0.36, 0.90)
Artesunate	2015	1.36 (0.86, 2.17)
	2016	1.20 (0.76, 1.90)
	2017	1.25 (0.82, 1.89)
	2018	0.85 (0.53, 1.36)
	2019	0.95 (0.55, 1.63)

Delayed clearance

No change in clearance

*All hazard ratio estimates are relative to the reference year 2014, the first year of artesunate use in Malawi.

4.9 Survival likelihood dependent on drug type

The likelihood of participants surviving the disease was tabulated as well to see chances of participants surviving dependent on the type of drug taken.

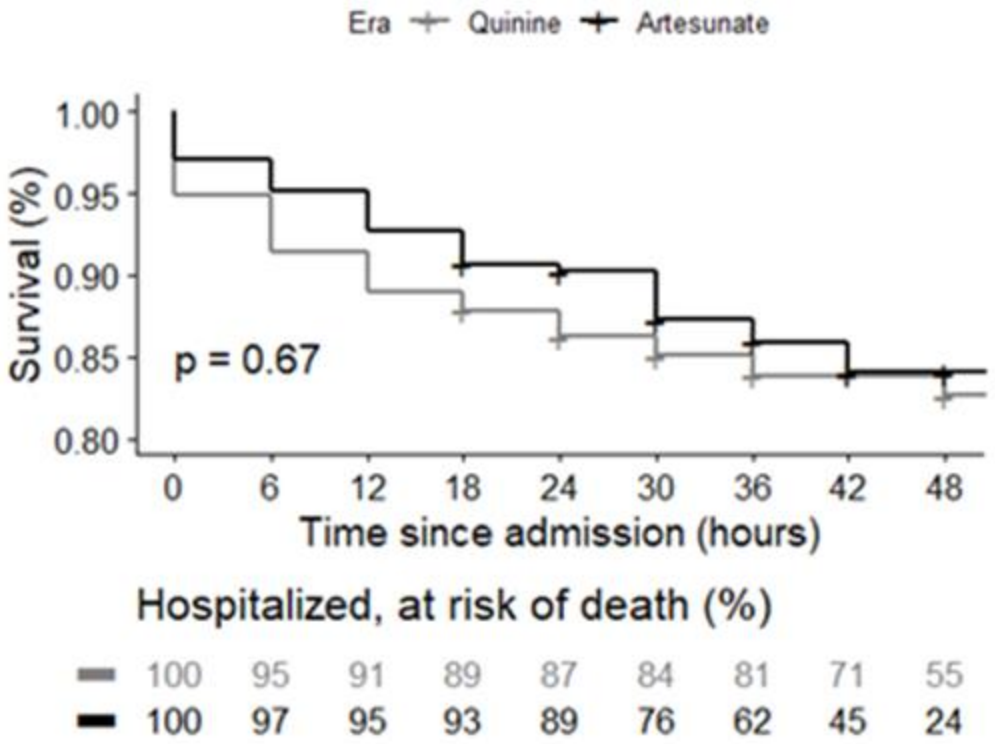

Figure 4.7: Likelihood of surviving when artesunate is used compared to quinine.

CHAPTER FIVE: DISCUSSION

5.1 Discussion

This study has shown that artesunate produces rapid clearance results. Clearance is starting as soon as the child has just been administered with intravenous artesunate thereby shortening the time to reach parasite concentrations below which a patient's symptoms will likely improve. This finding is in agreement with previously published study in Asia in 2017 where Nicholas J. White compared effectiveness of four anti-malarial drugs, quinine, Chloroquine, Artesunate and Cipargamin. In his finding, just at 12 hours after anti-malarial doses quinine group still had parasites above an average of 60 count (% baseline) while artesunate had only less than 10 parasite count. Just like this study, quinine group of Nicholas' work showed a longer lag phase while artesunate group demonstrated an instant decline in malaria density (41). However, it should be noted that most artesunate participants' parasitemia baseline was lower than the quinine group (figure 4.2). This is because of the tremendous work of various interventions done so far in malaria prevention and control towards eradication in Malawi (55,56). The proportions of participants presented to the clinic while they had already taken anti-malarials (85.7% for quinine and 87.4% for Artesunate) are so close yet artesunate group had low malaria parasites concentrations at admission than quinine group, suggesting that it is the increased efficacy of artemisinin derivatives that has led to the lower parasitemia concentrations in the artesunate era than in the quinine era.. Malaria parasites clearance is rapid when using artesunate than quinine as parasite decay and Kaplan-Meier curves have demonstrated in figure 4.1.

According to prior published work, one by Jennifer A. Flegg in her project of "Standardizing the measurements of parasite clearance in falciparum malaria", she demonstrated that using the parasite clearance estimator tool provide a consistent, reliable and accurate method to measure the lag phase as

well as malaria parasite clearance rate this tool could be used to detect early signs of emerging resistance to artemisinin derivatives (57). Post-treatment parasite density curves are supposed to have three characteristics:

1. A lag phase (time between intravenous antimalarials administration and beginning of the negative slope of parasite density curve)
2. An exponential decay (linear after logarithmic transformation)
3. A "tail" included most measurements with parasite densities of less than 1000 per microliter.

The above-elaborated features were noted when plotting population averages through time, for both quinine-treated and artesunate-treated patients (Figure 4.2), however, the hazard ratio revealed an increased risk from the year 2015 to 2017, no wonder the artesunate dosage was revised to 3.0 mg/kg from 2.4 mg/kg in 2017 (table 4.2). If the change in regimen action was not taken promptly and timely there could have been a high proportion of patients who could have been still parasitemic after the approved standard treatment course, a scenario which was timely avoided.

Parasite clearance was significantly slower in 2010 to 2013 (quinine period) compared to the artesunate period (2014 to 2019) (figure 4.2). This finding is covering this study's first objective of monitoring quinine and artesunate's effectiveness in treating *P.falciparum*. Although the number of participants in this study was much lower than some larger previous studies like the two famous Open-label randomized trials that changed the landscape of malaria treatment due to their overwhelming results in favor of artesunate, one conducted in four endemic countries in Asia which is popularly known as South East Asia Quinine Artesunate Malaria Trial (SEAQUAMAT) that enrolled a total of 1461 participants and a similar study conducted at eleven centers enrolling 5425 severe malaria African children in nine African countries popularly called African Quinine Artesunate

Malaria Trial (AQUAMAT). Both the SEAQUAMAT and AQUAMAT compared effectiveness of artesunate over quinine in treating malaria parasites. The results from both studies were in agreement in terms of artesunate superiority over quinine(53,54). This study demonstrates that children treated with artesunate achieved a post-treatment parasite count of less than 1000/µl more rapidly than those treated with quinine (log-rank test p-value < 0.05) (figure 4.3) while SEAQUAMAT and AQUAMAT got p-values of 0.0002 and 0.0022 respectively. Clearance did not significantly change in the years after 2014 as shown in results of the Kaplan-Meier curves plotted for the years 2014 and after, reflecting no change in clearance rates time across years after 2014 (Figure 4.3).

Results of relating parasite clearance to parasitic baseline load as measured by HRP-2 have been demonstrated in figures 4.5 as well as 4.6 where it has clearly shown that clearance is in no way dependent on either concentration of parasites in the blood (parasitemia) or the number of total body parasites present including those sequestered in other body organs like liver and bone marrow. This is observed after plotting the two variables on the regression curve. This is in agreement with other previous parasite clearance studies where three independent researchers; Smita Das in 2017, Ihn Kyung Jang and Natalie E. Hofman in 2019 have shown that clearance is independent of parasiteamia or HR-2 concentration (58–60).

Monitoring parasite clearance time is necessary to alert public health officials that parasite resistance is approaching. Progressive delays in parasite clearance time monitored in hours after antimalarial administration is a parasite resistance indicator (61). In 2010, the WHO observed a 19.5% treatment failure in Malawi during its periodic monitoring surveys (46), a finding which has not been confirmed by subsequent studies (62,63). Evidence of clinically significant resistance to artemisinins has not yet been found in SSA, though recent studies have shown development of *Pfkelch13* mutations, a malaria resistance candidate gene (64). The likely future development of

artemisinin-resistant *P. falciparum* in Africa is of big concern to clinicians and malaria researchers and may have devastating effects on populations living in endemic areas (65).

The advantage of this study over all other previous studies is that it is homogenous in a way that it involved specifically Malawian children with cerebral malaria, who were, recruited from the same catchment area, and of a similar age group, thereby minimizing the likelihood that age-dependent immunity varied across years or treatments received. Despite using data from a long-standing study, participants have been treated at the same unit, and samples were treated using the same sampling analysis procedure. The only prominent difference is the change of antimalarial type and dosage of which the investigators had no control.

5.1.1 Limitations

Just like any other project, this study had some limitations that included the self-pre-treatment practice of participants before coming to the hospital, which resulted in other clients not being included in the study because their parasite counts fell below the set threshold.

Patients presenting at clinical care units with different parasite stage compositions which could lead to different initial antimalarial susceptibility to specific parasite stages depending on parasite lifecycle (66) was another limiting factor that made this study a little heterogeneous, however, the good thing is that most in vivo parasite resistance studies can't have control over participant's body parasites multiplication.

Being a retrospective study, the investigators were limited to subjects who were already enrolled in previous studies and were not specifically recruited to answer this study's questions. As a result, there were unequal numbers of subjects in the master study who were either treated with artesunate and quinine.

The limited sample size number compromised mortality risk analysis. Other confounding factors like self-treatment, host immunity effects, and antimalarial drug resistance profile analysis of *Plasmodium falciparum* Kelch 13 (*Pf*K13) gene was not studied due to the nature of the study design.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Malaria parasite clearance times in children with CM did not show a marked difference between 2014 and 2019. This is an encouraging finding to clinicians and all public health officials in the region.

There was no difference after 48 hours in parasite clearance for both artesunate and quinine, however, the clearance threshold for the artesunate group reduced to as low as 30 hours an indication that artesunate is still the drug of choice.

6.2 Recommendations

There is a need for continued parasite resistance monitoring to mitigate possible parasite resistance development.

Future CM parasites resistance studies should include participants from endemic areas of the country like areas along the lakeshore as well as the lower shire to easily compare with results in low transmission areas like Blantyre.

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